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The Role and Expression of PKC\(\alpha\) in Breast Cancer

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Abstract

The role of the atypical PKC iota (PKCi) in breast cancer is unknown. In this study, the expression of PKCi in breast cancer tissue was investigated using tissue microarrays. PKCi was over-expressed in 70% of breast cancers. The expression and activation of PKCi was also elevated in a subset of breast cancer cell lines. Two cell lines with the highest activation and expression of PKCi, T47D and MCF7, have PIK3CA mutations E545K and H1047R respectively. To investigate the effects of PIK3CA mutations on PKCi, stable MCF10A cells expressing E545K and H1047R mutations were generated using retroviral transduction. Western blot analysis showed that these mutations are sufficient to increase PKCi expression and activation. To analyze the role of PKCi in breast cancer pathogenesis, siRNA targeting PKCi were used. MDAMB-231, T47D and MCF7 breast cancer cells treated with siRNA had decreased proliferation compared to control cells. MCF7 and T47D cells treated with siRNA to PKCi had morphological changes associated with senescence, increased SA-β-Gal activity and decreased BrdU incorporation. These results demonstrate that PKCi is overexpressed in a subset of breast cancers and can promote the growth of breast cancer cells by repressing premature senescence.
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List of Abbreviations

PKC\textsubscript{i}: Protein kinase C iota
aPKC: atypical protein kinase C
ER: Estrogen receptor
PR: Progesterone receptor
EGFR: Epidermal growth factor receptor
PI3K: phosphatidylinositol 3-kinase
RTK: receptor tyrosine kinase
PIP\textsubscript{2}: phosphatidylinositol-4,5-bisphosphate
PIP\textsubscript{3}: phosphatidylinositol-3,4,5-triphosphate
PDK1: phosphatidylinositol-3 dependent kinase
PTEN: phosphatase and tensin homolog
DCIS: ductal carcinoma in situ
IDC: invasive ductal carcinoma
PB1: Phox Bem 1
NSCLC: non-small cell lung cancer
CML: chronic myelogenous leukemia
SA-\textbeta-Gal: senescence associated \textbeta-galactosidase
SAHF: senescence associated heterochromatic foci
CDK: cyclin dependent kinase
ROS: reactive oxygen species
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Introduction

1.1 Breast Cancer

Breast Cancer is a complex and heterogeneous disease which varies in clinical behaviour and histology. There are several different subtypes which vary based on histopathology and receptor expression or amplification. Protein biomarkers are analyzed using immunohistochemistry to determine if the tumour is estrogen receptor (ER) or progesterone receptor (PR) positive or displays ERBB2 (HER2) overexpression. Breast cancers are graded on a scale of I to IV based on degree of malignancy; stage IV is recurrent, metastatic disease.

1.2 Treatment of Breast Cancer

Treatment options for breast cancer patients are generally based upon tumour-node-metastasis staging and other important factors such as histological grade, hormone receptor status and lymphovascular spread[1]. For in situ cancer, surgery followed by whole breast radiation is the standard treatment for most patients. In early stage breast cancer (stage I and II), patients will undergo additional adjuvant therapy such as chemotherapy, endocrine therapy or trastuzumab therapy. Standard chemotherapy drugs include anthracyclines such as doxorubicin and epirubicin and taxanes such as docetaxel and paclitaxel. Endocrine therapies include aromatase inhibitors or estrogen receptor modulators such as tamoxifen. These therapies prevent estrogen production or block estrogen, preventing stimulation of an estrogen-sensitive tumour[2]. In late stages of disease (III and IV), adjuvant therapy begins pre-operative and continues post-operative. Mastectomies may be necessary if cancer is extensive or multifocal in the breast. In recurrent disease, bisphosphonates and radiation
therapy may be used in palliative care to alleviate pain from bone metastases. While there have been many advances in breast cancer treatment with the arrival of targeted therapies such as trastuzumab, there is still a need to find new therapies. Many patients who initially respond to trastuzumab or other chemotherapies acquire secondary resistance and no longer respond to current therapies[3]. Additionally, approximately one third of ER positive patients do not respond to any type of endocrine therapy[4].

1.3 Molecular Etiology of Breast Cancer

Transformation of breast cells requires numerous cellular alterations in order for initiation and progression of breast carcinoma to proceed. It has been suggested that the heterogeneity seen in breast carcinoma may be due to transformation of different cells of origin in the breast[5]. The mammary epithelium mainly consists of distinct cell types: ductal luminal cells, alveolar luminal cells which produce milk, and an outer myoepithelial cell layer. In addition to fully differentiated cells, transformation could occur in mammary stem cells or committed progenitor cells. To evaluate the effects of different cells of origin on tumourigenesis, one study isolated two different mammary epithelial cell populations from tissue and transformed these cells using the exact same set of oncogenes[6]. When introduced into mice, these distinct set of cell lines produced tumours with significantly different histology, tumorigenicity and metastatic behaviour.

Hereditary breast carcinoma is an example of genomic instability involving germ-line BRCA-1 and BRCA-2 mutations[7, 8]. Approximately 0.12% of the general population are heterozygous for BRCA-1/2 mutations, but is much higher in certain groups such as Ashkenazi Jews where it is 1%[9, 10]. BRCA-1/2 encode proteins involved in DNA repair
mechanisms and in regulation of transcription[11]. Estrogen, a steroid hormone, can promote proliferation by acting as a transcription factor to activate target genes[12]. ERBB2 is a transmembrane receptor tyrosine kinase which upon ligand binding can form homodimers or heterodimers with other family members, such as EGFR, ERBB3 and ERBB4. The ERBB2 protein is overexpressed in approximately 25% of breast cancers due to gene amplification[13]. Oncogenic signalling by ERBB2 has been linked to disruption of epithelial organization, uncontrolled proliferation and protection from apoptosis[14].

1.4 PI3-kinase Pathway and PIK3CA mutations

Mutations or alterations in the phosphatidylinositol 3-kinase (PI3K) pathway occur frequently in breast cancer[15]. These alterations can include phosphatase and tensin homolog (PTEN) deletion or mutations in the PI3K gene[16, 17]. PI3-kinases are a family of lipid kinases which contain an 85 kDa regulatory subunit and a 110 kDa catalytic subunit and four isoforms have been previously identified (α, β, γ & δ)[18]. PI3-kinases are activated by receptor tyrosine kinases (RTKs) such as EGFR by RAS-dependent or RAS-independent pathways[19]. The p85 subunit inhibits the lipid kinase activity of the p110 subunit. Upon activation by RTKs, p85 binds to phosphorylation sites on RTKs via its SH2 domain. This binding alleviates inhibition of p110, allowing it to be recruited to the cell membrane, where it can phosphorylate phosphatidylinositol-4,5-bisphosphate (PIP₂) to phosphatidylinositol-3,4,5-triphosphate (PIP₃) as shown in Figure 1.1[20]. Pleckstrin homology domain-containing proteins such as phosphatidyl inositol-3 dependent kinase, PDK1, and Akt/PKB are activated by PIP₃ and initiate signalling cascades which promote cellular growth, motility, proliferation and survival[21, 22]. The phosphatase PTEN is a
negative regulator of this pathway and dephosphorylates PIP₃ to PIP₂. Aberrant activation of
the PI3K pathway can occur by different mechanisms. These mechanisms include deletion or
mutation of PTEN, amplification or mutation of the Akt gene, or mutation of PIK3CA, the
gene which encodes the p110 catalytic subunit[15, 23]. Somatic Akt mutations have recently
been discovered in breast, ovarian and colorectal tumours[24]. These mutations are
proposed to constitutively activate Akt by abrogating the requirement for phosphoinositides
to induce membrane recruitment[24].

PIK3CA is mapped to the 3q26 locus which is frequently amplified in cancers[25].
Mutations in PIK3CA have also been reported in breast, colon, brain, stomach, liver, lung
and ovary cancers[15, 17, 26, 27]. In breast cancer, the reported frequency of PIK3CA
mutation varies from 18% to 40%[17, 26-29]. More than 25 mutations have been identified
but the majority of these mutations lie in two hotspot regions: the central helical domain
encoded by exon 9 and the kinase domain encoded by exon 20. The most frequently
occurring mutations are E545K in the helical domain and H1047R in the kinase domain.
Both of these mutations render PI3-kinase constitutively active by different molecular
mechanisms. E545K mutations disrupt the inhibitory interaction between p85 and p110 but
still requires Ras binding for its transforming activity[30-32]. Using structural studies, it was
demonstrated the H1047R mutation likely increases lipid kinase activity in a Ras-
independent manner by allowing easier access to membrane bound PIP₂[32, 33].

PIK3CA mutations are proposed to occur early in breast carcinoma tumourigenesis.
In one study, PIK3CA mutations were present in in situ carcinomas as well as matched
invasive carcinomas[34]. In another study, PIK3CA mutations were found at approximately
equal frequency (30%) in cases of pure ductal carcinoma in situ (DCIS), DCIS adjacent to
IDC and IDC[35]. They found that invasive tumour areas and \textit{in situ} areas of the same tumour had identical \textit{PIK3CA} sequences in the majority of cases[35]. These results support the hypothesis that \textit{PIK3CA} mutations may play a role in the early stages of tumour initiation.

There is conflicting views in the literature as to whether \textit{PIK3CA} mutations are prognostic of poor clinical outcome or a prognostic advantage. Several studies have demonstrated these mutations are associated with a poor prognosis in breast cancer[36-39]. Other studies have indicated these mutations are associated with improved clinical outcome[40]. More studies with larger cohorts of patients may help find a conclusion to these conflicting observations. Several studies have introduced \textit{PIK3CA} mutations into untransformed cells or used tumour cell lines with these mutations to uncover downstream signalling events and phenotypic changes both \textit{in vitro} and \textit{in vivo}. Overexpression of these PI3K mutants in chicken embryo fibroblasts and NIH3T3 mouse fibroblasts caused increased \textit{in vitro} PI3K activity and transformation[41, 42].
**Figure 1.1 PI3K Pathway and PIK3CA mutations.**

A) Overview of PI3K signalling
The regulatory p85 subunit of PI3K inhibits the p110 catalytic subunit. Upon growth factor stimulation, p85 binds phosphorylation sites on RTKs via its SH2 domain. The p110 subunit is recruited to the membrane where it can phosphorylate PIP2 to PIP3. Downstream effectors are activated and initiate signalling cascades.

B) Domain structure of the PIK3CA catalytic subunit
The most common PIK3CA mutations occur in the helical domain and the kinase domain.
A) RTK

B) p85 binding

RAS binding

C2

Helical

Kinase

E545K

H1047R
In the context of breast cancer, Isakoff et al. have demonstrated that E545K and H1047R mutations induced multiple phenotypic changes in the nontransformed mammary epithelial cell line MCF10A[43]. These phenotypic changes included constitutive activation of Akt, anchorage-independent growth in soft agar, hyperproliferation and resistance to chemotherapy[43]. \textit{PIK3CA} mutations were shown to increase phosphorylation of the tumour suppressor GSK3β which is functionally inhibited when phosphorylated at serine 9[44]. Another study introduced E545K and H1047R mutations in MDA-MB-231 breast cancer cell lines and determined that both of these mutations caused increased cell motility \textit{in vitro}[45]. As these mutations have different mechanisms of activation, it is proposed that they may have different functional phenotypes. \textit{In vivo}, cells with E545K mutations had a smaller increase in tumour growth rate than cells with H1047R mutations and also had significantly more metastases in the lung[45].

PDK1 is also recruited to the cell membrane in PI3K signalling through its pleckstrin homology domain and can initiate signalling cascades through phosphorylation of Akt and other kinases[46]. In several breast cancer tumours with \textit{PIK3CA} mutations, there was increased expression and activation of PDK1[47]. This increased activity and expression was also seen in a panel of breast cancer cell lines[47]. This study also showed that cells with \textit{PIK3CA} mutations can signal independent of Akt through PDK1, suggesting that inhibition of other downstream effectors of the PI3K pathway could be effective therapeutic targets[47].
1.5 PKCi

PKCi is a member of the atypical PKC subclass of PKC isozymes. PKC i is ubiquitously expressed and knockout of this gene is embryonic lethal[48, 49]. Unlike other subclasses of PKCs, atypical PKCs (aPKCs) do not depend on diacylglycerol, calcium, or phosphatidylserine for catalytic activity[50, 51]. Atypical PKCs lack these binding motifs in the regulatory domain and instead have a Phox Bern 1 (PB1) protein-protein interaction domain. PB1 domains allow binding to other proteins with PB1 domains, such as the polarity protein Par6[50, 52]. PKCi is important for the establishment and maintenance of cell polarity in several epithelial cell types, including the breast epithelium, by forming a conserved polarity complexes with Par6 and GTPases such as Rac1 or Cdc42[53]. The activity of aPKCs can be regulated through phosphorylation by PDK1 and protein-protein interactions through its PB1 domain[54, 55].

In addition to its role in polarity, PKCi has been implicated in carcinogenesis and oncogenic signalling in brain[56], lung[57], colon[58] and ovarian carcinomas[59]. Transgenic mice expressing constitutively active PKCi exhibited a higher incidence of pre-neoplastic lesions and colon tumours than control mice[58]. Expression of kinase-inactive PKCi in non-small cell lung cancer (NSCLC) cells inhibits anchorage-independent growth and proliferation both in vitro and in vivo[57]. PKCi promotes several oncogenic signalling pathways that promote cell survival, tumour growth and invasion. However, the signalling mechanisms can vary with different tumour types. For example, PKCi can promote cell survival through three different pathways: phosphorylation of Bad proteins in NSCLC[60], activation of the NF-κB pathway in chronic myelogenous leukemia (CML) cells [61] or attenuation of p38 MAP kinase signalling in glioblastoma cells [56]. PKCi is also involved
in oncogenic Ras signalling in both colon and lung cancers, playing a critical role in Rasmediated transformed growth[57, 58]. One study demonstrated that PKC\textsubscript{t} activated a Rac1/Mek/Erk signalling axis that was required for transformed growth and invasion[57]. In NSCLC, PKC\textsubscript{t} has been shown to phosphorylate \( \mu \) and m-calpains which can promote migration and invasion[62].

Elevated PKC\textsubscript{t} expression has been frequently observed in ovarian and NSCLC tumours[59, 63, 64]. In ovarian tumours, PKC\textsubscript{t} expression is prognostic of poor clinical outcome and also correlates with tumour stage[59, 64]. This may suggest that PKC\textsubscript{t} contributes to tumour development and more aggressive disease. Amplification of the PKC\textsubscript{t} gene was one of the molecular mechanisms for the over-expression of PKC\textsubscript{t} in these tumour types[63, 64]. The PKC\textsubscript{t} gene resides on chromosome 3q26 and amplification of this area is a common chromosomal change in human cancers such as NSCLC, ovarian, esophagus, cervical and squamous cell carcinoma of the head and neck[65, 66]. Elevated PKC\textsubscript{t} expression has been seen in tumour types such as pancreatic cancers and colon cancers that do not harbour gene amplification, indicating that another mechanism must play a role in controlling expression[58, 67].

1.6 PKC\textsubscript{t} in Breast Cancer

There has been little previous research into the role and expression of PKC\textsubscript{t} in breast cancer. Only one study has looked at the expression of PKC\textsubscript{t} in breast cancer tissue samples[68]. The authors found that PKC\textsubscript{t} was overexpressed in 80% of the tumours studied and that there was a correlation between PKC\textsubscript{t} expression and pathologic type[68]. In another study, it was shown in breast cancer cell lines that ERBB2 activation disrupts the Par
complex of Par3, Par6 and PKC\(\text{\textit{\i}}\) and associates with Par6-PKC\(\text{\textit{\i}}\)[69]. This newly formed signalling complex is required for ErbB2 disruption of apical-basal polarity in mammary cells[69]. Finally, overexpression of Par6 in mammary epithelial cell lines caused an increase in proliferation but depended on interaction with PKC\(\text{\textit{\i}}\) and Cdc42[70]. In order to test whether the polarity complex of Par6/PKC\(\text{\textit{\i}}\)/Cdc42 is a viable therapeutic target, they used a stably expressing shRNA system to downregulate PKC\(\text{\textit{\i}}\) expression in MCF7 breast cancer cell lines. Downregulation of PKC\(\text{\textit{\i}}\) caused a decrease in proliferation after seven days[70].

1.7 Senescence and Cancer

Hayflick and Moorhead pioneered the concept that isolated primary somatic cells have a finite capacity to replicate[71, 72]. Replicative senescence refers to mitotic cells undergoing cell cycle arrest due to telomere degradation. Telomeres become shorter with each round of cell division due to incomplete DNA replication by DNA polymerases[73]. Premature senescence, which differs from replicative senescence, describes cells that undergo senescence independent of reaching a critical telomere length. Premature senescence can be induced by a number of different mechanisms, including DNA damage, oncogene over-expression, inactivation of tumour-suppressors, sustained stress signalling or chromatin perturbations[74-76].

Many studies have demonstrated the introduction of oncogenes causes premature senescence and have proposed that oncogene-induced senescence acts as a cellular defence mechanism to limit tumourigenesis[77, 78]. One of the most common examples in humans of in vivo senescence are naevi or ‘moles’, which frequently harbour pro-oncogenic
mutations in \textit{BRAF} or \textit{RAS} genes\cite{79}. In mouse models, introduction of K-Ras-V12, mutant 
\textit{BRAF} or \textit{PTEN} inactivation have all supported that \textit{senescence occurs in vivo}\cite{78, 80, 81}.
However as cancers do develop, there must be mechanisms for evasion of senescence. It has 
been proposed that cancers can evade senescence by accumulating more genetic mutations to 
circumvent senescence\cite{82}. Inactivation of several genes such as p53, Rb, CHK2, or ATM 
can disable several critical senescence pathways and supply a growth advantage\cite{83, 84}.
Oncogene-dependent reactive oxygen species (ROS) production which can trigger premature 
senescence, paradoxically may also provide an escape mechanism by promoting continuous 
DNA damage and accumulation of genetic mutations\cite{85}.

Triggering senescence in cancer cells may represent an alternate therapeutic strategy.
In one study, reactivation of p53 in hepatocarcinomas triggered senescence \textit{in vivo}\cite{86}.
Following induction of senescence, not only did tumours arrest in growth but actually 
regressed due to initiation of an anti-tumour inflammatory response. Upregulation of 
inflammatory cytokines and immune modulators has been observed in Ras-induced 
senescent cells\cite{87}. The fact that the innate immune system can be harnessed to attack 
senescent cells and facilitate tumour clearance provides another reason why triggering 
senescence could be attractive as a therapeutic option.

\textbf{1.8 Markers of Senescence}

When cells became senescent, they exhibit several characteristics other than 
irreversible growth arrest. Senescent cells undergo morphological changes such as an 
increase in cell size, cell flattening, and vacuolated and thin cytoplasm\cite{88}. Since 
senescence may result in genomic instability, binucleated or multinucleated cells can
One of the most widely used markers of senescence is senescence associate β-galactosidase (SA-β-Gal) activity. β-galactosidase is a hydrolase localized in the lysosomal compartment and cleaves β-D-galactosides. It has optimal activity around pH 4. The assay for SA-β-Gal involves detection of a chromogenic substance, 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-gal), which is cleaved by β-galactosidase to leave an insoluble compound. To distinguish normal β-galactosidase activity from SA-β-Gal, the assay is performed at pH 6. At this pH, β-galactosidase activity is not detected in quiescent or actively dividing cells but blue perinuclear staining can be seen in senescent cells. It was demonstrated that the increase in SA-β-Gal activity was due to an increase in the amount of enzyme which is thought to be associated with the increase in lysosome biogenesis in senescent cells[89].

Senescence-associated heterochromatic foci (SAHF) accumulate in interphase nuclei of senescent cells and are visualized using DAPI stain. These changes in chromatin structure exclude active transcription and co-localize with histone H3 methylation on lysine 9, which is a marker for heterochromatin[90]. SAHFs also prevent access of E2F to its target genes, helping to stabilize the cell cycle arrest[90]. Another molecular marker for senescence is the presence of DNA damage foci in the nucleus[91]. DNA damage foci can accumulate in oncogene-induced senescent cells or cells with dysfunctional telomeres[91, 92]. DNA damage foci are visualized using immunofluorescence microscopy and antibodies against phosphorylated histone H2AX and proteins involved in the DNA damage response such as 53BP1[91]. Finally, another characteristic of senescent cells in culture is that they are resistant to apoptosis[93].
1.9 Signalling pathways involved in induction of senescence

It has been demonstrated that several growth-regulatory proteins are either upregulated or have altered activation states with senescence. One of the major pathways for induction of senescence involves p53-dependent activation of the cyclin-dependent kinase (Cdk) 2 inhibitor p21 as shown in Figure 1.2[94]. At the onset of senescence, there is an increase in protein levels of p21 which results in cell-growth arrest in G1[95]. After senescence is established, levels of p21 drop off[96]. Another Cdk inhibitor, p16, increases and assumes the role of inhibiting cdk4 and cdk6[97]. Retinoblastoma protein (Rb) is an important cell cycle regulator and when it is hyperphosphorylated by cdk2, cdk4 and cdk6, it promotes cell cycle progression[97]. However, in senescent states it is hypophosphorylated because p21 and p16 inhibit the cdks[98].

Induction of senescence does not always require p53. The p38\textsuperscript{MAPK} pathway can also induce senescence via a Ras-Raf-Erk-p38MAPK signalling axis as shown in Figure 1.2[99]. This signalling cascade induces p21 and p16 upregulation in a p53 independent manner, resulting in cell cycle arrest[100]. The unfolded protein response (UPR) can also induce senescence in a p53- and p16-independent manner[101]. In HRAS- transformed melanocytes, there was rapid expansion of the endoplasmic reticulum and induction of ER stress chaperones leading to permanent cell cycle arrest. Recently, inactivation of the proto-oncogene Skp2, a E3-ubiquitin ligase, induced cellular senescence in combination with PTEN inactivation[102]. This senescence response was independent of p53 and p19\textsuperscript{Arf} but dependent on p27, p21 and Atf4 induction.
Figure 1.2. Classical Senescence Pathways.

Inhibition of cyclin dependent kinases (CDKs) by p16 or p21 results in Rb becoming hypophosphorylated, causing cell cycle arrest.
Hypothesis

Part I

*PIK3CA* mutations are a common mutation in breast cancer and PKC\(\text{\textalpha}\) is a known
downstream effector of the PI3K pathway. We propose that these mutations will increase
activation of PKC\(\text{\textalpha}\) in breast cancer.

Part II

PKC\(\text{\textalpha}\) is a known oncogene in other cancers and has previously been shown to promote cell
proliferation in other tumour types. We propose that PKC\(\text{\textalpha}\) functions to promote proliferation
in breast cancer by repressing the induction of premature senescence.
Specific Aims

Part 1

1. Determine the level of expression of PKC\textsubscript{t} in breast tumours and in a subset of breast cell lines.
   a) Use immunohistochemistry on breast cancer tissue microarrays to determine level of expression
   b) Compare protein levels and phosphorylation of PKC\textsubscript{t} using Western blotting in a subset of breast cell lines

2. Determine if PIK3CA mutations are sufficient to increase activation or expression of PKC\textsubscript{t}.
   a) Retrovirally transduce non-tumourigenic mammary epithelial cell lines with PIK3CA mutations and determine effects on PKC\textsubscript{t}

Part II

3. Use RNAi to knock down expression of PKC\textsubscript{t} and determine the effects on proliferation by
   a) Perform proliferation assays using trypan blue exclusion
   b) Perform flow cytometry to determine if any decrease in proliferation is due to apoptosis
   c) Investigate induction of senescence by assessing senescence markers in cells depleted of PKC\textsubscript{t}

4. Investigate methods to assess PKC\textsubscript{t} function in vivo
   a) Create inducible shRNA lentiviral systems targeting PKC\textsubscript{t}
   b) Create adenoviral shRNA systems targeting PKC\textsubscript{t}
Significance

While PKC\(\text{t}\) has been identified as an oncogene in other cancers, previously there has been little research into the expression or role of PKC\(\text{t}\) in breast cancer. Only one study has published immunohistochemistry data and they only looked at invasive ductal carcinoma[68]. Using tissue microarrays, we examined the expression of PKC\(\text{t}\) across a spectrum of breast disease from hyperplasia to metastatic carcinoma. We demonstrate that PKC\(\text{t}\) is overexpressed in breast cancer tissue and in vitro also show that PKC\(\text{t}\) is overexpressed in a subset of breast cancer cell lines. We propose that a novel mechanism for elevated PKC\(\text{t}\) both in vitro and in vivo is through PIK3CA mutations. In other tumour types such as glioma, it has been demonstrated that PKC\(\text{t}\) is required for proliferation[103]. We demonstrate that PKC\(\text{t}\) promotes proliferation in breast cancer cell lines, through a mechanism where PKC\(\text{t}\) prevents cells from undergoing premature senescence. This is a completely novel function for PKC\(\text{t}\) not only in breast cancer cells but in other tumour types as well.
Chapter 2

Materials and Methods

2.1 Reagents and Antibodies

PKC\textsubscript{c} mouse monoclonal and pPKC\textsubscript{c} rabbit polyclonal antibodies were purchased from BD Transduction Laboratories (Mississauga, ON, Canada) and Invitrogen (Carlsbad, CA, USA) respectively. The Akt goat polyclonal and pAKT (Ser473) rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology and Cell Signaling respectively. The p27 monoclonal mouse antibody was purchased from BD Biosciences. The mouse monoclonal actin antibody was purchased from Sigma-Aldrich Canada. All secondary antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA, USA). The PI3-kinase inhibitors Wortmannin and LY294002 were purchased from Sigma Aldrich and Cell Signaling respectively. The MEK inhibitor U0126 was purchased from Cell Signaling.

2.2 Cell Culture

MCF-10A, HTERT-HMEC, MDA-MB-231, MCF7, BT549, and T47D cell lines were all purchased from ATCC and cultured at 37°C and 5% CO\textsubscript{2}. MCF-10A and HTERT-HMEC cells were cultured in Mammary Epithelial Cell Basal Medium (MEBM, Lonza) with 100 units/ml penicillin, 100 µg/ml streptomycin and the following growth supplements: BPE, 2 ml; hEGF, 0.5 mL; hydrocortisone, 0.5 mL, 0.5 mL; GA-1000, 0.5 mL; insulin, 0.5 mL. MDA-MB-231 and MCF7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% (v/v) of a 3:1 mixture of donor bovine serum and fetal bovine serum. BT549 and T47D cell lines were cultured in RPMI-1640 media (Invitrogen) with 10% (v/v) of a 3:1 mixture of
donor bovine serum and fetal bovine serum, 100 units/mL penicillin and 100 µg/mL streptomycin.

2.3 Expression constructs
The JP1520 vector was purchased from Harvard Institute of Proteomics (Boston, MA, USA). JP1520/PIK3CA, JP1520/E545K and JP1520/H1047R constructs were created by Isakoff SJ et al[43]., and purchased from Addgene (Addgene plasmids 14570, 14571, 14572).

2.4 Retroviral Transductions
Replication-incompetent retroviruses were made using the three plasmid transient transfection systems as previously described[104]. Transduction of MCF-10A cells with JP1520, JP1520/PIK3CA, JP1520/E545K or JP1520/H1047R retrovirus were performed by incubating the cells with virus in the presence of 8 µg/ml Polybrene overnight. Cells were selected with 1µg/mL puromycin.

2.5 Immunohistochemistry
Slide preparation and staining
Tissue microarray slides were purchased from US Biomax Company (Maryland, USA). One slide from each array was stained using standard H&E staining method to assess the histology component of each core. TMA slides were cut from a formalin-fixed paraffin embedded block, and covered with an extra thin layer of paraffin to prevent oxidation. Slides were baked at 60°C until extra layer of paraffin is melted (30 minutes). Slides were deparaffinized and rehydrated in xylene and ethanol respectively. Antigen is retrieved using
sodium citrate pH 6 using a microwave. Endogenous peroxidase activity was quenched by incubation in 3% H₂O₂ in PBS for 15 min.

Primary mouse monoclonal PKCδ antibody was used at a dilution of 10 μg/ml and incubated on slides overnight at 4°C. Immunohistochemical detection was performed using the Envision Polymer Detection System (Dako, Mississauga, Canada), according to the manufacturer’s instructions. The staining scored from 0 to 3+ based on predominant cytoplasmic staining intensity, and % tumour cells demonstrating staining. Scoring was performed independently by two pathologists.

2.6 Western Blot Analysis

Cells were harvested in protein lysis buffer consisting of 100 mM Tris pH 6.8, 20 mM DTT, 4% SDS, 5% glycerol. Protein concentrations were determined using the BCA assay reagents (Pierce, Rockford, IL, USA). Reduced proteins were separated through 4–12% bis-tris polyacrylamide gels using an Xcell II min cell system (Invitrogen, Carlsbad, CA, USA). Proteins were transferred onto PVDF nylon membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) and stained with amido black prior to probing with the appropriate primary antibody. Proteins were detected using the HRP method and SuperSignal West Pico Chemiluminescent Substrate reagents (Pierce, Rockford, IL, USA). Proteins were visualized using the GeneGnome (Syngene, Frederick, MD, USA) or Alpha Innotech Fluorchem FC2 (Cell Biosciences, Santa Clara, CA, USA). Sequential probing of membranes was performed after stripping with the use of Western Blot Stripping Buffer (Pierce) for 20 min at room temperature. Quantification of western blots by densitometry was performed using
GeneGnome chemiluminescent system and Syngene software (Syngene) or Alpha Innotech Fluorchem system and Alphaview software (Alpha Innotech).

2. 7 RNAi

To minimize off-target effects, two different siRNA sequences were used to target PKC\(\alpha\). The RNAi target sequences used are as follows: PKC\(\alpha\) A 5’-GTGCATCAACTGCAAACTC-3’ and PKC\(\alpha\) C 5’-CTTCCTGAAGAACATGCCA-3’. The siGENOME Non-Targeting siRNA #2 (Dharmacon) sequences is proprietary and Dharmacon does not release the sequence. Briefly, cells were washed once with PBS and 400 \(\mu\)L of Opti-MEM I (Invitrogen, Carlsbad, CA, USA) was added to each well. 5 nM of siGENOME Non-Targeting siRNA #2, PKC\(\alpha\) A, PKC\(\alpha\) C (Dharmacon) RNAi was mixed with OptiMEM I to total 185 \(\mu\)L. For a mock transfection control, OptiMEM I was added instead of RNAi. 4 \(\mu\)L of oligofectamine (Invitrogen, Carlsbad, CA, USA) was mixed with 11 \(\mu\)L Opti-MEM I and allowed to incubate for 10 minutes. The RNAi mixture and oligofectamine mixture was mixed together and allowed to incubate for 20 minutes. For 6 well assays, the 200 \(\mu\)L mixture was added dropwise to each well. For 12 well assays, 100 \(\mu\)L of each mixture was added dropwise to each well. After 4 hours of incubation at 37°C and 5% CO\(_2\), 1 or 2 mL of media was added to each well. 48 hours later, media was aspirated and refreshed to remove oligofectamine and siRNA.

2.8 Proliferation assays

MDAMB231 or MCF7 cells were plated in duplicate at 10000 cells/well in a 12 well tissue culture plate. T47D cells were plated at 2000 cells/well and hTERT-HMECs were plated at
5000 cells/well. 24 hours later, RNAi was added to cells. Briefly, cells were washed once with PBS and 400 μL of Opti-MEM I was added to each well. 5 nM of sicontrol 2, PKCı A, PKCı C RNAi were prepared and added as previously described. To count cells, cells were washed once with PBS and trypsinized until cells lifted off. To stop the reaction, DMEM with 10% FBS was added to each well. 500μL was counted using trypan-blue exclusion by a Vi-Cell XR (Beckman Coulter, Mississauga, Ontario) cell counter. Cells were counted 48, 96 and 144 hours after RNAi addition.

2.9 SA-β-Gal Staining and Analysis
MCF10A, T47D and MCF7 cells were plated at 30,000 cells per well on gelatin-coated coverslips in each well of a 6 well dish. hTERT-HMECs were plated at 10,000 cells per well and a positive control cell line that expresses higher SA-β-Gal activity was also plated to ensure staining procedure was effective. 24 hrs later, 5 nM RNAi was added as previously described. Media was refreshed every 48 hours. 5 days after RNAi transfection, cells were fixed in 4% paraformaldehyde. SA-β-Gal stain was made fresh each experiment and contained the following: 40 mM citric acid/sodium phosphate pH 6, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, 2 mM MgCl2, 1mg/mL X-Gal and distilled water to make up the volume. Cells were stained 14-16 hours at 37°C. Stained coverslips were washed twice with PBS before being mounted onto slides using Dako Faramount Aqueous Mounting media (Dako). Slides were analyzed using a Zeiss Axioskop 2 microscope. The number of positive nuclei and the total number of cells were counted in three separate fields for each condition.
2.10 Morphology

Slides from previous SA-β-Gal experiments were used to analyze morphology. Slides were analyzed using a Nikon Eclipse TE 2000-U microscope using 20x magnification. Representative pictures were taken using a Coolpix MDC lens (Nikon) and Nikon 5400 digital camera.

2.11 Flow Cytometry

150,000 cells were plated in duplicate in 6 cm dishes. 24 hrs later, RNAi was added to cells. Media was refreshed after 48 hours. Media was collected from each plate and cells were trypsinized and collected as well. Cells were pelleted and washed by resuspending in PBS. Cells were again pelleted and resuspended in ice-cold 70% ethanol with vortexing. Cells were transferred to eppendorf tubes and stored at -20°C until staining with propidium iodide. To stain, cells were centrifuged at 2000 rpm for 5 minutes. The ethanol was aspirated and cells were resuspended in PBS. Cells were again centrifuged for 5 minutes at 2000 rpm and the PBS was aspirated. Cells were then resuspended in 1 mL of staining buffer (0.2% Triton X-100, 1mM EDTA in PBS) and incubated for 5 minutes on at 4°C. Cells were pelleted at 2000 rpm for 5 minutes and resuspended in 500 μL of staining buffer with 25 μg/mL propidium iodide (Sigma) and 40 μg/mL RNase A (Sigma). Cells were incubated for 1 hour at room temperature in the dark. Cells were run on a Coulter Epics XL flow cytometer (Beckman Coulter) and analyzed using FCS Express v.3 software or ModFit LT software (Verity Software House Inc, Sopsham, ME, USA).
2.12 Statistical analysis

Unless otherwise indicated, all values are represented as the average of three independent experiments performed in triplicate, with error bars indicating standard error of the mean. Statistical significance was determined by a two tailed Student's t-test. Values were considered significant when $P<0.05$.

2.13 Adenovirus Vectors and Preparation of Crude Adenovirus

Short hairpin for PKCI or GFP sequences with BamHI and EcoRI sites were annealed by heating a mixture of the two sequences to 90°C for two minutes and then letting solution cool slowly to room temperature. Then the cassette was cloned into pSuper plasmid.

**PKCI**

5' GAT CCC CGT GCA TCA ACT GCA AAC TCT TCA AGA GAG AGT TTG CAG TTG ATG CAC TTT TTG  
5' AAT TCA AAA AGT GCA TCA ACT GCA AAC TCT CTC TTG AAG AGT TTG CAG TTG ATG CAC GGG

**GFP**

5' GAT CCC CAA GCT GGA GTA CAA CTA CAT TCA AGA GAT GTA GTT GTA CTC CAG CTT TTT TTG  
5' AAT TCA AAA AAA GCT GGA GTA CAA CTA CAT CTC TTG AAT GTA GTT GTA CTC CAG CTT GGG

Once in pSuper, the short hairpin sequence and H1 RNA polymerase III promoter were excised from the vector using EcoRI and SalI. After gel purifying this fragment, it was cloned into the pDC311 Adenoviral shuttle vector. Clones were verified by sequencing. The pBHGloxAEl,3Cre vector and pDC311-shRNA plasmids were co-transfected in 293 cells and recombinant adenovirus vectors were rescued by Cre-mediated recombinations previously described by Ng and Graham[105]. Isolated plaques were picked by punching out agar plugs and transferred into PBS supplemented with 10% glycerol. 293 cells were treated with 200 μL of plaque pick and crude adenovirus was collected 3-4 days later once cells had undergone cytopathic effect.
2.14 Lentivirus vectors and Preparation of stable cell lines

Short hairpin for PKC\(\text{I}\) or GFP sequences with AgeI and EcoRI sites were annealed by heating a mixture of the two sequences to 90°C for two minutes and then letting solution cool slowly to room temperature. Then the cassette was cloned into pLKO-Tet-On (Novartis, Cambridge, MA). Clones were verified by sequencing.

\begin{align*}
PKC\text{I}A \\
5'&-CCGGGTGCATCAACTGCAAACTCCTCGAGGAGTTTGCAGTTGATGCACTTTTT-3' \\
5'&-AATTAAAAAGTCATCAACTGCAAACTCCTCGAGGAGTTTGCAGTTGATGCAC-3' \\
GFP \\
5'&-ACCGGAAGCTGGAGTACAACTACACTCGAGTGTAGTTGTACTCCAGCTTTTTTTATTTAATT-3' \\
5'&-TGGCCTTCGACCTCATGTTGATGTGAGCTCACATCAACATGAGGTCGAAAAAAATTAAG-3'
\end{align*}

To generate lentivirus, 293T cells were seeded at 4x106 cells in 10 cm plates. The next day, media was aspirated and replaced with 6 mL of DMEM with 10% FBS (no antibiotics). 36\(\mu\)L of Gene Juice was added to 600 \(\mu\)L OptiMEM I and incubated for at least 5 minutes. The following was mixed with OptiMEM I to total 50\(\mu\)L and added to Gene Juice and OptiMEM I mixture:

\begin{align*}
p\text{LKO-Tet-ON shRNA} & 4\mu\text{g} \\
p\text{LP1} & 3\mu\text{g} \\
p\text{LP2} & 3\mu\text{g} \\
p\text{LP/VSVG} & 2\mu\text{g}
\end{align*}

Mixture was incubated for 20 minutes before adding drop-wise to media. 24 hours later, media was changed. After 48 hours post-media change, supernatant was collected and
centrifuged at 3000 rpm for 15 minutes. Viral supernatants were filtered through 0.45µM filter and aliquoted and stored at -80°C.

2.15 BrdU Immunofluorescence

MCF7 and T47D cells were plated on gelatin-coated coverslips at 25,000 cells per well. Cells were treated with siRNA as previously described. Media was refreshed every 48 hours. Six days after siRNA treatment, BrdU (Sigma) was dissolved in ddH₂O and added to fresh media on cells at a concentration of 10 µM. After two hours, media was aspirated and cells were immediately fixed with 70% ethanol for five minutes. Ethanol was aspirated and cells were washed 3 x 5 minutes with PBS. 1.5 M HCl was added to cells and incubated for 30 minutes at room temperature. After HCl was aspirated, cells were washed 3 x 5 minutes with PBS. Cells were incubated with blocking buffer (5% normal goat serum in PBS) for one hour. BrdU mouse monoclonal antibody (Cell Signalling) was diluted 1/1400 in antibody buffer (1% BSA in PBS). BrdU antibody was put on cells and incubated overnight at 4°C. Cells were washed 3 x 5 minutes with PBS before incubating with AlexaFluor 555 goat anti-mouse secondary antibody at a dilution of 2µg/mL in antibody buffer. Cells were again washed and mounted on slides using Prolong Antifade with DAPI mounting media. Slides were sealed and visualized using Zeiss Axioplan 2 microscope. A minimum of 3 fields and 250 cells for each condition was used to analyze data.
Results

Part I: Expression of PKC\(\text{\i}\) in Breast Cancer

3.1 PKC\(\text{\i}\) is highly expressed in a subset of breast cancers \textit{in vivo}

In order to examine the expression profile of PKC\(\text{\i}\) in breast cancer tissue, we used three different tissue microarrays which contained cases of different histological type. A total of 244 cores from 146 cases were assessed and stained. However, 64 cores were not used in the final analysis due to the lack of epithelial cells on cores (7 cores), duplication of cores (48 cores), or low sample size (normal=4 cores, fibroadenosis=5 cores). Accordingly, 180 breast tissue samples were assessed, including 81 cases of invasive ductal carcinoma (IDC), 49 cases of lymph node metastases, 15 cases of lobular carcinoma, 11 cases of DCIS, 9 cases of other types of breast cancer and 15 cases of hyperplasia.

Weak PKC\(\text{\i}\) staining was detected in non-neoplastic breast tissue. There was no staining in the cytoplasm of luminal or myoepithelial cells which form the ducts. Predominant PKC\(\text{\i}\) staining was observed along the membrane separating myoepithelial cells and the stroma in the normal tissue as shown in Figure 3.1. However, after consultation with Dr. Islam (Department of Pathology, Ottawa Hospital) this staining was ruled a fixation artefact because the same line was seen on the hematoxylin and eosin control slide. Positive staining was mainly cytoplasmic with nuclear staining in some cases (Figure 3.1). No significant positive staining was seen in the stroma surrounding cancer cells, indicating that PKC\(\text{\i}\) staining is limited to the tumour tissue. Results of PKC\(\text{\i}\) expression in tumour tissues was based on scoring the intensity of
Figure 3.1 PKCl is overexpressed in breast cancer tissues

Immunohistochemistry was performed on three breast TMAs using a PKCl monoclonal antibody. In panels A-E are representative images of normal tissue, hyperplasia, DCIS, IDC and lymph node metastatic disease respectively.
cytoplasmic staining. The intensities of PKC\(\text{\textgamma}\) staining were scored as 0, 1+, 2+, or 3+ for each case. A score of 0 represented no staining, similar to the negative control, and 3+ represented the most positive staining we saw on the TMA, similar to a cell line positive control. Scores 2+ or higher were defined as positive for PKC\(\text{\textgamma}\) expression (Table 3.1).

Scoring profiles for cases of hyperplasia, DCIS, IDC and lymph node metastases are shown in Figure 3.2.

| Table 3.1 Immunohistochemical Cytoplasmic Scoring of PKC\(\text{\textgamma}\) expression |
|---------------------------------|--------|--------|--------------------|----------------|
|                                 | PKC\(\text{\textgamma}\) + | PKC\(\text{\textgamma}\) - | Total # of cases | % of PKC\(\text{\textgamma}\) + cases |
| Hyperplasia                     | 4      | 11     | 15                 | 27%            |
| DCIS                            | 9      | 2      | 11                 | 82%            |
| Lobular Carcinomas              | 6      | 9      | 15                 | 40%            |
| IDC                             | 54     | 27     | 81                 | 67%            |
| Lymph Node Metastases           | 37     | 12     | 49                 | 76%            |
| OTHER*                          | 6      | 3      | 9                  | 67%            |
| TOTAL of Cancers                | 112    | 53     | 165                | 68%            |

*OTHER includes 1 case of apocrine, 1 case of tubular, 2 cases of neuroendocrine cancer, 4 cases of comedo-type intraductal carcinoma with early infiltrate & 1 case of micropapillary carcinoma
Figure 3.2  PKC\(\tau\) expression profiles

Immunohistochemical scoring is plotted according to pathologic type and intensity of PKC\(\tau\) staining. 0 represents weak to no staining and 3+ represents strong PKC\(\tau\) staining.
3.2 PKC\(\varepsilon\) is not correlated with ER, PR or HER2 expression

We examined PKC\(\varepsilon\) expression and common clinicopathologic factors associated with breast cancer to determine if there was any correlation (Table 3.2). No significant correlation between HER2, ER, PR status and PKC\(\varepsilon\) expression was found in a subset of IDC samples (n=32).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PKC(\varepsilon) staining</th>
<th>PKC(\varepsilon) -</th>
<th>PKC(\varepsilon) +</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER2</td>
<td>Cytoplasmic</td>
<td>+</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>6</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td>Cytoplasmic</td>
<td>+</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>7</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td>Cytoplasmic</td>
<td>+</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>9</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

3.3 PKC\(\varepsilon\) is highly expressed in a subset of breast cancer cell lines

PKC\(\varepsilon\) phosphorylation (activation) and total protein expression were analyzed in a subset of breast cancer cell lines and non-tumourigenic mammary epithelial cell lines using Western blotting (Figure 3.3). PKC\(\varepsilon\) activation was measured by using a phosphospecific antibody to the Threonine 555 site. Phosphorylation of PKC\(\varepsilon\) at this site in the turn motif of the protein which stabilizes the kinase in an activated conformation[106]. Three out of four breast cancer cell lines had higher PKC\(\varepsilon\) activation and expression compared to non-tumourigenic mammary epithelial cell lines MCF10A and hTERT-HMEC. MCF7 and T47D had a 2.5 and 4-fold increase of PKC\(\varepsilon\) total protein levels respectively when compared to
Figure 3.3 PKC\(\zeta\) is highly expressed and activated in a subset of breast cancer cell lines

Activation of PKC\(\zeta\) and expression of PKC\(\zeta\) were analyzed in normal mammary epithelial cell lines (MCF10A and hTERT-HMEC) and breast cancer cell lines (MCF7, T47D, MDA MB231 and BT549) using Western blotting. Densitometry was used to analyze relative expression and activation levels. PKC\(\zeta\) expression was normalized to Actin levels for each cell line. To determine activation levels of each cell line, pPKC\(\zeta\) levels were normalized to total PKC\(\zeta\) levels.
Breast cancer cell lines

![Image of Western blots showing expression of pPKCt, PKCt, and Actin]

**Fold change in pPKCt expression**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>MCF10A</th>
<th>hTERT-HMEC</th>
<th>MCF7</th>
<th>T47D</th>
<th>MDA-MB 231</th>
<th>BT549</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF10A</td>
<td>1.0</td>
<td>1.0</td>
<td>2.0</td>
<td>3.0</td>
<td>4.0</td>
<td>1.0</td>
</tr>
<tr>
<td>hTERT-HMEC</td>
<td>1.0</td>
<td>1.0</td>
<td>1.5</td>
<td>2.5</td>
<td>3.0</td>
<td>1.0</td>
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<td>MCF7</td>
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<td>3.0</td>
<td>4.5</td>
<td>5.0</td>
<td>2.0</td>
</tr>
<tr>
<td>T47D</td>
<td>3.0</td>
<td>2.5</td>
<td>4.5</td>
<td>6.0</td>
<td>7.0</td>
<td>3.0</td>
</tr>
<tr>
<td>MDA-MB 231</td>
<td>4.0</td>
<td>3.0</td>
<td>5.0</td>
<td>6.5</td>
<td>8.0</td>
<td>4.0</td>
</tr>
<tr>
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<td>1.0</td>
<td>2.0</td>
<td>3.0</td>
<td>4.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**Fold change in pPKCt/PKCt ratio**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>MCF10A</th>
<th>hTERT-HMEC</th>
<th>MCF7</th>
<th>T47D</th>
<th>MDA-MB 231</th>
<th>BT549</th>
</tr>
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<td>2.0</td>
<td>3.0</td>
<td>4.0</td>
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<tr>
<td>hTERT-HMEC</td>
<td>1.0</td>
<td>1.0</td>
<td>1.5</td>
<td>2.5</td>
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<td>MCF7</td>
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<td>3.0</td>
<td>4.5</td>
<td>5.0</td>
<td>2.0</td>
</tr>
<tr>
<td>T47D</td>
<td>3.0</td>
<td>2.5</td>
<td>4.5</td>
<td>6.0</td>
<td>7.0</td>
<td>3.0</td>
</tr>
<tr>
<td>MDA-MB 231</td>
<td>4.0</td>
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<td>5.0</td>
<td>6.5</td>
<td>8.0</td>
<td>4.0</td>
</tr>
<tr>
<td>BT549</td>
<td>1.0</td>
<td>1.0</td>
<td>2.0</td>
<td>3.0</td>
<td>4.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>
MCF10A cells (Figure 3.3). MDA-MB 231 cells had approximately 2.5-fold high expression than normal cell lines. PKC\textsubscript{\theta} activity was normalized to PKC\textsubscript{\theta} protein levels for each cell line and compared in Figure 3.3. The pPKC\textsubscript{\theta}/PKC\textsubscript{\theta} ratio was highest in MCF7 and T47D cell lines, at approximately 3.5-fold higher. The other breast cancer cell lines, MDAMB231 and BT549, did not have higher pPKC\textsubscript{\theta}/PKC\textsubscript{\theta} ratios than the normal mammary cell lines.

### 3.4 Activating PIK3CA mutations increase PKC\textsubscript{\theta} expression and activation

High levels of activation and expression of PKC\textsubscript{\theta} were observed in MCF7 and T47D cells and each harbour PIK3CA mutations. MCF7 and T47D cells have E545K and H1047R mutations respectively, which have been verified in our lab (Jenn Hanson, unpublished data). We tested whether PIK3CA mutations are sufficient to increase PKC\textsubscript{\theta} activation and expression. PIK3CA mutants, E545K or H1047R, were retrovirally transduced into the non-tumourigenic mammary epithelial cell line MCF10A. MCF10A cells transduced with empty vector (JP1520) or wild-type PIK3CA were used as controls. Western Blot analyses of phospho-Akt/PKB and total Akt/PKB levels were verified that the activating mutations were functional (Figure 3.5). In the mutant E545K or H1047R expressing cell lines, phospho-Akt/PKB levels were higher than control cells which correspond to previous literature[43]. Total Akt levels were lower in PIK3CA mutant cell lines than control cells, making the increase in phospho-Akt levels even more pronounced. Western Blot analysis of PKC\textsubscript{\theta} in MCF10A cells expressing PIK3CA mutations (E545K or H1047R) had both increased expression and activation compared to control cells. PKC\textsubscript{\theta} expression was found to be 2 fold
higher in E545K cell lines and 3.5 fold higher in H1047R expressing cells when compared to empty vector control (Figure 3.4).

To confirm that these increases in PKC\(\varepsilon\) activation were due to the \textit{PIK3CA} mutations, two structurally independent PI3K inhibitors, Wortmannin and LY294-002 were used. Mutant cells treated with Wortmannin or LY294-002 for two hours had decreased Akt activation compared to vehicle treated cells, as would be expected with a PI3K inhibitor (Figure 3.5). PKC\(\varepsilon\) activation was also decreased when treated with PI3K inhibitors. This is consistent with the fact that PKC\(\varepsilon\) is a downstream effector in the PI3K pathway. There was no effect on PKC\(\varepsilon\) protein levels with the use of these inhibitors. Interestingly, these agents did not inhibit the low level of basal PKC\(\varepsilon\) activation in MCF10A cells expressing wild-type PI3K.
Figure 3.4 PIK3CA mutations increase expression and activation of PKC\textsubscript{\textalpha}.

Transduced cells and non-transfected control MCF10A were growth-factor starved (MEBM media with 100 units/ml penicillin, 100 \(\mu\)g/ml streptomycin and BPE, hydrocortisone, and GA-1000) for 24 hours before cell lysates were collected. Cell lysates were analyzed using Western blotting with the indicated antibodies. Three separate experiments were quantified using GeneGnome Syngene software. (* indicates \(p<0.05\)).
Figure 3.5 PI3K inhibitors affect PKC\(\varepsilon\) activation

Transduced MCF10A cells were growth factor starved (MEBM media with 100 units/ml penicillin, 100 μg/ml streptomycin and BPE, hydrocortisone, and GA-1000) for 24 hours prior to a 2 hour treatment with DMSO, Wortmannin (0.1μM) or LY294-002 (25μM). Total cell lysates were then collected and analyzed by Western blotting for expression and activation of Akt and PKC\(\varepsilon\).
Part II: PKCι promotes proliferation in breast cancer by preventing induction of premature senescence

3.5 Down-regulation of PKCι results in decreased proliferation in breast cancer cell lines

In order to determine if PKCι promotes proliferation in breast cancer lines, we used two different siRNA sequences to target PKCι and downregulate protein levels. We used non-targeting siRNA and mock transfected cells as controls in each experiment. Proliferation was measured by viable cell counts with trypan-blue exclusion days two, four and six post-siRNA transfection. T47D, MCF7 and MDAMB231 cell lines showed a decrease in viable cell number at day 6 (Figure 3.6). This decrease in viable cell number indicates a decrease in proliferation in all three cell lines.

3.6 Down-regulation of PKCι does not cause apoptosis

There was little change in the ratio of viable cells to total cells between any of the conditions in the proliferation assays, indicating no increase in apoptosis with siRNA treatment. To confirm that no apoptosis was occurring, flow cytometry was performed on cells treated with siRNA to PKCι. Propidium iodide staining, which measures DNA content, was used to determine the cell cycle profile of the MDAMB231 and MCF7 cell lines. DNA fragmentation and loss of nuclear DNA content are characteristic of cells undergoing apoptosis. Apoptotic cells are consequently hypodiploid and a sub G1 peak can be seen
Figure 3.6 Depletion of PKCζ inhibits proliferation in MCF7, T47D and MDAMB231 cell lines

Cells were plated in duplicate for each condition and treated with siRNA to PKCζ. Cells were then counted 48 hours, 96 hours and 144 hours using trypan-blue exclusion to determine cell viability and cell number. MCF7 cells are shown in A, MDAMB231 cells in B and T7D in C. These graphs are representative of three independent experiments.
using flow cytometry[107]. Cells were transfected with siRNA for 72 hours before fixing and staining the cells. As shown in Figure 3.7, there is no evidence of a sub G1 peak with treatment of siRNA. This indicates that down-regulation of PKCt does not induce apoptosis in these cell lines at the time point analyzed.

### 3.7 Knock down of PKCt in breast cancer cells causes induction of premature senescence

To determine if senescence was a factor in the decreased proliferation seen with down-regulation of PKCt, we assayed for SA-β-Gal activity. MCF7 and T47D cells were plated on gelatin-coated coverslips and treated with siRNA to PKCt. Five days later, cells were fixed with paraformaldehyde and incubated with X-gal at pH 6.0. Three different experiments were performed for each cell line. There was endogenous perinuclear SA-β-Gal activity in approximately 3% and 8% of mock transfected and control siRNA transfected MCF7 and T47D cells respectively (Figure 3.8). With siRNA to PKCt, we saw an increase in SA-β-Gal staining in both MCF7 and T47D cell lines to approximately 20 to 25% of total cells. This reproducible increase was significant when we compared levels to mock and control siRNA transfected cells. There were several morphological changes characteristic of senescence in cells treated with siRNA to PKCt. MCF7 and T47D cells are generally more round or cuboidal in shape and grow tightly packed in clusters. When PKCt was knocked down, there was a higher prevalence of SA-β-Gal positive cells which were larger and flattened out as shown in Figure 3.9. These cells were also less tightly packed.
Figure 3.7 Downregulation of PKC\(\text{\textalpha}\) does not induce apoptosis

MCF7 and MDAMB231 cells were treated with siRNA for three days. Media and cells were collected and pelleted. Cells were fixed and stained with propidium iodide for one hour before being analyzed using flow cytometry. Panel A represents mock transduced cells, B is si control siRNA, C is PKC\(\text{\textalpha}\) A siRNA and D is PKC\(\text{\textalpha}\) C siRNA transduced cells respectively. The red line on each panel represents where subG1 cells would be located.
Figure 3.8 Knockdown of PKC\(\alpha\) increases SA-\(\beta\)-Gal staining in MCF7 and T47D cell lines

MCF7 or T47D cells were plated on gelatin-coated coverslips and treated with siRNA to PKC\(\alpha\). Five days later cells were fixed with paraformaldehyde and stained with X-gal overnight at 37°C. Coverslips were then mounted on slides and viewed using bright field microscopy. Three random fields were used for each condition and total cells as well as the number of positive cells were counted for MCF7 (shown in A) and T47D cells (shown in B). These results are representative of three independent experiments. A Student’s two-tailed t-test was performed to determine significance. (* indicates p<0.05, ** indicates p<0.01)
Figure A shows a bar graph representing the percentage of SA-β-Gal positive cells for different conditions: mock, sicontrol 2, PKCα A, and PKCα C. The graph indicates higher positive cell percentages in treatments PKCα A and PKCα C compared to mock and sicontrol 2.

Figure B displays a similar bar graph illustrating the percentage of SA-β-Gal positive cells under the same conditions. This graph highlights the increased positive cell percentages in PKCα A and PKCα C compared to the control groups.
Figure 3.9 Knockdown of PKC\(\text{\textregistered}\) induces morphology changes characteristic of senescence

Cells were plated in gelatin-coated coverslips and treated with siRNA to PKC\(\text{\textregistered}\). Five days later, cells were fixed with paraformaldehyde and stained with X-gal overnight at 37°C. Coverslips were then mounted on slides and visualized using bright-field microscopy.
<table>
<thead>
<tr>
<th>T47D</th>
<th>MCF7</th>
<th>Mock</th>
<th>Sicontrol</th>
<th>PKC(_1)A</th>
<th>PKC(_1)C</th>
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Using immunofluorescence, MCF7 and T47D cells treated with siRNA were analyzed for incorporation of BrdU. Immunofluorescence staining was entirely nuclear and this antibody was specific as demonstrated in the no BrdU label controls (Appendix I). Six days after treatment with siRNA, there was a decrease in BrdU staining in cells treated with siRNA to PKCι (Figure 3.10). In both cell lines the percentage of BrdU positive cells in mock and control siRNA treated cells was approximately 40% and 30% in MCF7 and T47D cells respectively. When PKCι is depleted, the percentage drops to approximately 25% and 18% in MCF7 and T47D cells respectively. Cells with morphological changes typical of senescence showed no BrdU incorporation.

### 3.8 PKCι does not promote proliferation or induce premature senescence in non-tumourigenic mammary cell lines

To determine if PKCι has similar effects on proliferation in non-tumourigenic cell lines, we analyzed hTERT-HMEC cells. MCF10A cell lines grow slowly in culture, and the long doubling times of the cells do not allow any meaningful or measurable changes to occur during the length of the experiment. Depletion of PKCι with siRNA did not cause a decrease in proliferation in hTERT-HMEC cells as shown in Figure 3.11. There was no difference in viable cell number between control cells and cells treated with siRNA over six days. No increase in SA-β-Gal staining in hTERT-HMECs or MCF10A cells was seen with siRNA to PKCι as shown in Figure 3.11. Only MCF10A data is shown in Figure 3.11 because there was no positive staining counted in hTERT-HMEC cell lines. To ensure that the siRNA was indeed functional and still knocking down PKCι, we plated HTERT-HMEC cells and
Figure 3.10 Depletion of PKCı decreases BrdU incorporation in MCF7 and T47D cells

A. Representative pictures of MCF7 and T47D cells mock transfected or transfected with control siRNA or PKCı siRNA and stained for BrdU incorporation. Six days after transfection, cells were incubated with BrdU for 2 hours before fixation and immunostaining. Slides were mounted using mounting media containing DAPI (blue nuclei). Anti-BrdU antibodies and Alexa 555 conjugated goat anti-rabbit antibodies were used to immunostain for BrdU label (pink nuclei).

B. Quantification of BrdU

A minimum of three separate fields and 250 cells were quantified for each condition. The total amount of cells and the amount of BrdU-positive cells were counted for each field. These results are representative of three independent experiments. A Student’s two-tailed t-test was performed to determine significance. (* indicates p<0.05)
Figure 3.11 PKC\(\alpha\) does not promote proliferation in normal mammary breast cells by inducing senescence

MCF10A cells were plated on gelatin-coated coverslips and treated with 5 nM siRNA. Five days later, cells were fixed and stained with X-gal overnight at 37°C. Coverslips were mounted on slides and viewed using bright-field microscopy. Three fields were counted for each condition. For the proliferation assays, hTERT-HMEC cells were plated in duplicate for each condition and treated with siRNA to PKC\(\alpha\). Cells were then counted 48 hours, 96 hours and 144 hours using trypan-blue exclusion to determine cell viability and cell number.
MCF10A

% of SA-β-Gal Positive cells

0 5 10 15 20 25
Mock Sicontrol 2 PKC\_A PKC\_C

hTERT-HMEC

Table Cell Number (x 10^3)

0 0.1 0.2 0.3 0.4 0.5 0.6
Day 2 Day 4 Day 6

- Mock
- Sicontrol
- PKC\_A
- PKC\_C
collected protein at Day 2, Day 4 and Day 6 post-siRNA transfection. Even six days after siRNA transfection, we see knockdown of PKCγ (Figure 3.12). Similar knockdown in MCF7 cells is seen with siRNA treatment (Figure 3.12). This indicates that PKCγ is depleted in the normal mammary cell lines but not promoting proliferation as it is in breast cancer cell lines.

3.9 Knock down of PKCγ does not increase p27 levels

It has previously been demonstrated in our lab that PKCγ regulates cyclin dependent kinase inhibitor p27 levels in glioblastoma (M.Sc thesis, Jana Gilles). In glioblastoma cell lines depleted of PKCγ using siRNA, a strong increase in p27 levels was observed (M.Sc thesis, Jana Gilles). It was also demonstrated that inhibition of the MEK pathway using a MEK inhibitor such as U0126 in combination with knockdown of PKCγ caused a robust and synergistic increase in p27 levels (M.Sc thesis, Jana Gilles). In cell cycle progression, p27 can inhibit cyclin-dependent kinases and cause cell cycle arrest in G1. Inhibition of PI3K signalling has been shown to decrease proliferation and result in increased p27 levels both in glioblastoma and breast cancer[108, 109]. We hypothesized that knock down of PKCγ, thus attenuating a part of the PI3K cascade, may cause an increase in p27 levels and cell cycle arrest. However, MCF7 cells treated with siRNA to PKCγ did not show an increase in p27 after three days (Figure 3.13). We did observe an increase in p27 levels with MEK inhibition, using 20 μM U0126 (Figure 3.13). No additional increase in p27 levels was observed with the combination of PKCγ depletion and MEK inhibition. Therefore, PKCγ does not appear to regulate p27 levels in breast cancer.
Figure 3.12 PKCβ knockdown in MCF7 cells and hTERT-HMEC cell lines.

MCF7 and hTERT-HMEC cells were plated in six well dishes and treated with 5 nM siRNA (si control, PKCβ A, and PKCβ C). Cell lysates were collected at 48 hours, 96 hours and 144 hours post-transfection. Control lysates were collected at 96 hours. Cell lysates were analyzed using Western blotting to determine levels of PKCβ expression. GAPDH was used as a loading control.
MCF7

hTERT-HMECs

Mock Si control PKC\textsuperscript{\textalpha}A PKC\textsuperscript{\textbeta}C Day

2 4 6 2 4 6

PKC\textalpha
GAPDH

PKC\textbeta
GAPDH
Figure 3.13 Depletion of PKC\(\eta\) does not affect p27 levels in the absence or presence of MEK inhibition

MCF7 cells were plated at 50,000 cells per well in a six well dish. RNAi was added to cells as previously described. 48 hours after RNAi treatment, media was aspirated and refreshed. Cells were treated with 20 \(\mu\)M U0126 or an equal volume of DMSO as a control. 24 hours later, cell lysates were collected and analyzed by Western Blotting.
3.10 Knockdown of PKC\(\tau\) does not increase levels of \(\gamma\)H2AX

DNA damage, either telomeric or non-telomeric, is the currently best characterized inducer of senescence[84]. DNA damage checkpoints are activated by DNA double-strand breaks and can initiate senescence through activation of several important proteins such as p53, ATM, CHK2 and chromatin regulators such as phosphorylated histone H2AX (\(\gamma\)-H2AX) [82]. \(\gamma\)-H2AX is used as a marker of DNA-damage foci using Western blotting or by immunofluorescence. No induction in \(\gamma\)-H2AX was observed with PKC\(\tau\) knock down over six days in MCF7 cells (Figure 3.14). As a positive control, small-cell lung cancer cells (H69 cells) treated with the Hsp90 inhibitor geldanamycin was used. These cells have previously been shown to be senescent with geldanamycin treatment and also shown to exhibit an increase in \(\gamma\)-H2AX[110]

3.11 Systems to analyze PKC\(\tau\) function in vivo

Our lab has used transient RNAi knockdown or stably-expressing RNAi cell lines to analyze the effects of PKC\(\tau\) on oncogenic signalling or malignant behaviour. However, our stably-expressing RNAi cell lines have severe growth defects which tend to be selected out over time. Transient RNAi is effective for loss-of-function studies in vitro, but its short-lived effects and limitations in in vivo efficacy make it a less than ideal system. To look at PKC\(\tau\) in in vivo systems, we developed two viral delivery systems to express short hairpin RNA, adenovirus and lentivirus delivery. Adenoviruses are double-stranded DNA viruses that do not integrate into the host genome. Adenoviruses have been used to deliver short
Figure 3.14 PKC\(\alpha\) depletion does not increase levels of \(\gamma\)H2AX in MCF7 cells

We used MCF7 cell lysates, previously used to show PKC\(\alpha\) knockdown, to determine if \(\gamma\)H2AX levels increased. Lysates were analyzed by Western blotting and antibodies to \(\gamma\)H2AX and GAPDH.
GAPDH

γH2AX

Day 2
Day 4
Day 6

Control RNA
Mock

GA
H69

PKCα
PKCγ

hairpin RNA both in vitro and in vivo and can mediate a longer duration of knockdown compared to RNAi[111, 112]. The lentiviral system we have used is an inducible system which has all the components in one vector: tetracycline repressor protein, shRNA sequence and puromycin resistance[113]. Lentivirus can be generated, collected, and then used to infect cells of interest. After selection with puromycin, doxycycline (dox) can be used to initiate transcription of shRNA. In Figure 3.15, the adenoviral and lentiviral systems both show effective knockdown of PKC\(\text{\text{1}}\) by Western blotting. With the lentiviral system, after three days we see knockdown of PKC\(\text{\text{1}}\) in a dose-dependent manner with increasing amounts of dox. Using only 1 ng/mL of dox, we see a 60% reduction in protein levels and at 100 ng/mL of dox, we see approximately 80-90% knockdown. With the adenoviral system, we see knockdown of PKC\(\text{\text{1}}\) by approximately 60% with higher amounts of crude virus.
Figure 3.15 Lentiviral and Adenoviral Systems to express shRNA to PKC\(\text{I}\)

A. *Lentiviral System*. MDAMB231 cell lines were generated and tested with increasing amounts of Dox for 72 hours before cell lysates were collected. Three separate experiments were quantified to determine amount of knockdown.

B. *Adenoviral System*. MDAMB231 cell lines were treated with adenovirus for 1 hour in serum-free media before addition of full media. Cell lysates were collected after three days.
B.

**Fold Change in PKCl expression**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>GFP + 100 ng/mL Dox</td>
<td>1.1 ± 0.05</td>
</tr>
<tr>
<td>PKClA no Dox</td>
<td>1.0 ± 0.02</td>
</tr>
<tr>
<td>PKClA 1 ng/mL Dox</td>
<td>0.9 ± 0.03</td>
</tr>
<tr>
<td>PKClA 10 ng/mL Dox</td>
<td>0.8 ± 0.04</td>
</tr>
<tr>
<td>PKClA 50 ng/mL Dox</td>
<td>0.7 ± 0.05</td>
</tr>
<tr>
<td>PKClA 100 ng/mL Dox</td>
<td>0.6 ± 0.06</td>
</tr>
</tbody>
</table>

**MOI**

- 0
- 1
- 10
- 50
- 100
- 400

---

**PKCl**

**Erk**
Discussion

Breast cancer is a heterogeneous disease, varying both in biology and clinical behaviour. While there have been improvements in targeted therapies for breast cancer such as trastuzumab, there is still a need to understand complex cellular signalling networks to determine potential new targets. It is also critical to understand how potential targets contribute to tumour initiation and progression. It is well established that the PI3K pathway has a role in cancer progression by promoting proliferation and cell survival[114]. The high frequency of PI3K pathway alterations in cancer makes this pathway an attractive target for therapeutic strategies. Approximately 30% of breast cancers harbour PIK3CA mutations, making it one of the most frequent alterations in breast cancer[27]. There are several PI3K inhibitors currently in clinical trials but there are still several ongoing challenges with targeting PI3K. Several of these drugs are pan-PI3K inhibitors which target two or three PI3K isoforms. This presents a problem because proper immune system function relies on the p110δ and p110γ isoforms and targeting these isoforms results in toxicity[114]. Because of these problems with targeting PI3K directly, identifying key downstream molecules of the PI3K pathway could lead to more effective targets in tumours with PI3K activation.

PKCδ is a known downstream effector of the PI3K pathway. PKCδ has been implicated in promoting tumourigenesis in lung[57] and colon[58] cancers and is involved in oncogenic signalling in glioblastoma[56]. However, little is known about the role and expression of PKCδ in breast cancer. Only one study has previously looked at PKCδ expression in breast cancer tissue samples and they only investigated IDC samples[68]. It is important to further examine PKCδ expression not only to validate this result but also to determine if PKCδ is overexpressed across a spectrum of breast cancer tumour types.
In this study, non-neoplastic cases of normal tissue and hyperplasia as well as neoplastic cases of DCIS, IDC, lobular carcinoma and lymph node metastases were examined. Immunohistochemically, expression was found to be mainly cytoplasmic but nuclear staining was also detected (Figure 3.1). In cases of hyperplasia, which is non-cancerous abnormal proliferation, we found weak PKC\textsubscript{1} staining, indicating lower expression of the kinase in this tissue. However, in cases of breast cancer we found much stronger staining which indicates elevated levels of the kinase. We have found that PKC\textsubscript{1} is over-expressed in approximately 70\% of breast cancers (n=157, Table 3.1). This is comparable to the previous study which found PKC\textsubscript{1} over-expressed in 80\% of IDC cases (n=110) [68]. Over-expression of PKC\textsubscript{1} suggests that PKC\textsubscript{1} could be an important downstream effector in malignant breast cancers.

PKC\textsubscript{1} expression did not correlate with any established clinicopathologic factors such as HER2, PR or ER status (Table 3.2). This was also found in a similar study looking at IDCs and PKC\textsubscript{1} expression[68]. Elevated PKC\textsubscript{1} expression has been shown in several different types of tumours such as ovarian and NSCLC tumours[63, 64]. In colon cancer, it was demonstrated using immunohistochemistry, Western blotting and RT-PCR that elevated PKC\textsubscript{1} was present in colon tumours when compared to normal tissue[58]. In some tumour types, such as ovarian and lung, PKC\textsubscript{1} gene amplification was found[57, 64]. This has not been assessed in breast cancers and may also contribute to the upregulation of PKC\textsubscript{1} expression. However, over-expression of PKC\textsubscript{1} in other tumour types that do not have gene amplification has also been seen in colon cancer[58], CML[115] and pancreatic cancers[67]. Transcriptional and translational regulation of PKC\textsubscript{1} is still relatively uncharacterized in normal or transformed cells and may provide another mechanism for over-expression.
We demonstrated that a subset of breast cancer cell lines has increased PKC\(\alpha\) activation and expression when compared to non-tumourigenic mammary epithelial cell lines (Figure 3.3). MCF7 and T47D cells had an approximately 2.5 and 4-fold increase of PKC\(\alpha\) expression compared to MCF10A and hTERT-HMEC normal mammary epithelial cells. MDA-MB-231 cells had a 2.5-fold increase of PKC\(\alpha\) while the BT549 cells did not have increased expression of PKC\(\alpha\). The elevated expression of PKC\(\alpha\) in breast cancer cell lines mirrors the PKC\(\alpha\) expression pattern seen in the breast cancer tissue microarrays. When pPKC\(\alpha\) levels were normalized to PKC\(\alpha\) levels for each cell line, the subset of breast cancer cells displayed an increase in activation compared to non-tumourigenic cell lines. MCF7 and T47D cells had the highest activation of all cell lines with 3.5-fold increases respectively when compared to MCF10A cell lines. The data from the breast cancer cell lines suggest that the overexpression observed in the breast cancer tissue microarrays also corresponds with increased PKC\(\alpha\) activation. PKC\(\alpha\) activation was not evaluated in the tissue microarrays because there is inadequate preservation of phosphorylation status during fixation.

Interestingly, MCF7 and T47D cell lines both contain \textit{PIK3CA} activation mutations, E545K and H1047R respectively. We tested whether \textit{PIK3CA} mutations alone are sufficient to increase in PKC\(\alpha\) activation and expression \textit{in vitro}. Using retroviral transduction, we found that \textit{PIK3CA} mutations increased expression and activation of PKC\(\alpha\) in normal mammary epithelial cells (Figure 3.4). There was a 3.5-fold increase in protein expression in the H1047R cell line and a 2-fold increase in the E545K cell line. The differences in expression between the two mutations could be due to different mechanisms of activation of PI3-kinase and alternate downstream signalling. E545K mutations disrupt the inhibitory interaction between p85 and p110 but still requires Ras binding for its transforming
activity[30-32]. H1047R mutations increase lipid kinase activity in a Ras-independent manner by allowing easier access to membrane bound PIP$_2$[32, 33]. However, it is unknown how $PIK3CA$ mutations could affect transcription or translation of PKC$t$. We did not see an increase in PKC$t$ mRNA levels that could account for this increase in protein levels (D Parolin, J Paget, I Lorimer, unpublished data). This would point to a translational or post-translational mechanism. Increased activation of PKC$t$ by introduction of $PIK3CA$ mutations was expected because it is a known downstream effector of the PI3K pathway.

Pharmacologically, we have demonstrated that increases in activation and expression are due to increased PI3K activity (Figure 3.5). The inhibitors only decreased activation of PKC$t$ in mutant cell lines and not the low activity seen in wild-type PI3K cells. It has been demonstrated that cell lines and tumour xenografts which harbour mutations in $PIK3CA$ are more sensitive to PI3K-inhibitors, resulting in greater apoptosis or tumour regression[116]. Thus mutant $PIK3CA$ MCF10A cells could be more sensitive to Wortmannin and LY294-002 than wild-type PI3K cells. It has been demonstrated that cell lines and tumour xenografts which harbour mutations in $PIK3CA$ are more sensitive to PI3K-inhibitors, resulting in greater apoptosis or tumour regression[116]. Thus mutant $PIK3CA$ MCF10A cells could be more sensitive to Wortmannin and LY294-002 than wild-type PI3K cells. While not extensively studied, PI3K-independent activation of PKC$t$ has been proposed in one instance to act through a proline-rich tyrosine kinase-2 and phospholipase D signalling axis[117].

One mechanism for the over-expression of PKC$t$ seen in the breast cancer tissues is through $PIK3CA$ mutations. $PIK3CA$ mutations have been detected in DCIS and papillary lesions, indicating that these mutations can occur early in cancer development [34, 118].
were able to detect PKC\(\delta\) over-expression in DCIS and in cases of hyperplasia and this could be attributed to mutations in \textit{PIK3CA} at these early stages. In a cell model system for studying breast cancer progression, it was demonstrated that \textit{PIK3CA} mutations could be a critical genetic change required in formation of malignant breast tumours [119]. Perhaps as a direct consequence of these mutations, elevated PKC\(\delta\) activity also contributes to tumour progression.

We observe a 70\% overexpression of PKC\(\delta\), yet \textit{PIK3CA} mutations are only present in approximately 30\% of breast cancers[17, 26-29]. Therefore there must be alternate mechanisms to increase PKC\(\delta\) expression in breast tumourigenesis. ErBB2 overexpression occurs in 20 to 25\% of breast cancer and represents an upstream mechanism of activation of the PI3K-Akt pathway [120]. PTEN loss occurs in approximately 30-40\% of breast cancers and also constitutively activates the PI3K-Akt pathway[121-123]. These other alterations in the PI3K pathway may also cause elevated PKC\(\delta\) and provide an explanation why we see higher frequency of expression of PKC\(\delta\) in the tissue microarrays than would be expected for \textit{PIK3CA} mutations alone.

To investigate possible functions of PKC\(\delta\) in breast pathogenesis, we utilized siRNA to silence the expression of the kinase. In proliferation assays, we found that knocking down PKC\(\delta\) caused a decrease in proliferation (Figure 3.6). This difference in proliferation was seen in three separate breast cancer cell lines, MCF7, T47D and MDAMB231. In previous work investigating the effects of Par6 on proliferation in breast cancer, a group also targeted PKC\(\delta\) and observed a decrease in proliferation in MCF7 cells after seven days[70]. The involvement of PKC\(\delta\) in promoting proliferation has also previously been demonstrated in
glioma by two separate groups[103, 124]. They both found that targeting PKC\(\text{\(\text{\textregistered}\)}}\) transiently with siRNA or stably transduced shRNA expressing cell lines resulted in decreased proliferation. There was an approximate decrease in viable cell number of about 40-50% when compared to control cells. These results are in contrast to previous work in other cancers where PKC\(\text{\(\text{\textregistered}\)}}\) only affected anchorage-independent growth[63, 125]. This discrepancy could be due to cell-type specific functions of PKC\(\text{\(\text{\textregistered}\)}}\).

Apoptosis could cause a decrease in viable cell number in proliferation assays. While we did not see any change in viability in our proliferation assays, we confirmed that depletion of PKC\(\text{\(\text{\textregistered}\)}}\) did not induce apoptosis using flow cytometry (Figure 3.7). There was no increase in hypodiploid cells with knock down of PKC\(\text{\(\text{\textregistered}\)}}\) in MCF7 or MDAMB231 cells. These results are consistent with findings in intestinal epithelial cells, pancreatic carcinoma and NSCLC where depletion of PKC\(\text{\(\text{\textregistered}\)}}\) did not induce apoptosis [57, 58, 125]. It was also previously shown \textit{in vivo} that pharmaceutical PKC\(\text{\(\text{\textregistered}\)}}\) inhibition caused decreased proliferation of lung tumour cells without induction of apoptosis [126].

A decrease in cellular divisions can be caused by apoptosis or cell cycle arrest. As apoptosis is not a contributor, we explored whether senescence, an irreversible cell cycle arrest, could be contributing to decreased proliferation when breast cancer cells are treated with siRNA to PKC\(\text{\(\text{\textregistered}\)}}\). SA-\(\beta\)-Gal is the most commonly used and consistent marker of senescence and has been used to detect senescence both \textit{in vitro} as well as \textit{in vivo} in a variety of cancer settings[88, 127]. Mouse models of tumour cellular senescence including oncogene activation or tumour suppressor inactivation also display SA-\(\beta\)-Gal activity[127]. Two breast cancer cell lines, MCF7 and T47D, were tested to determine if there was an increase
in SA-β-Gal with knock down of PKCι (Figure 3.8). After five days of siRNA treatment, the percentage of MCF7 and T47D cells that were SA-β-Gal positive increased from approximately 5% to 20-25% of cells. This increase in SA-β-Gal was significant when compared to mock and control siRNA treated cells.

SA-β-Gal on its own is not a definitive marker of senescence. It has been demonstrated that senescence can occur without positive SA-β-Gal staining[128]. GLB1 is the gene encoding lysosomal β-D-galactosidase, the enzyme responsible for SA-β-Gal activity[128]. Using GLB1-deficient fibroblasts or fibroblasts depleted of GLB1 using siRNA, it was shown that replicative senescence still occurs without positive staining and does not require GLB1[128]. The increase in β-Galactosidase activity is not a cause of senescence but due to increased lysosomal mass or activity which has been shown in senescent cells[129-131]. In our lab, we have shown in small-cell lung cancer cells that premature senescence is induced in H69 cells by Hsp90 inhibition but these cells do not display SA-β-Gal activity[110]. There have also been instances of non-specific SA-β-Gal activity in cells[132]. For example, serum-starvation or confluent culture can induce SA-β-Gal activity in non-senescent fibroblast cells[132]. In our experiments, cells were always sub-confluent and media was refreshed every 48 hours to ensure an ample amount of growth factors were available.

In addition to SA-β-Gal staining, it is important to show other markers of senescence. We also saw several characteristic morphological changes occur with breast cancer cells depleted of PKCι. Cell enlargement and flattening out are distinguishing features of senescent cells and we see both of these changes in MCF7 and T47D cells treated with
siRNA to PKC1 (Figure 3.9). Normally tightly packed, these cells are spread out and much larger than control cells. It has been proposed that these morphological changes are due to redistribution of focal adhesion proteins and actin stress fibre formation both of which can be regulated by caveolin-1[133, 134]. To further support our data that PKC1 knockdown causes premature senescence, BrdU incorporation was analyzed to show that there is a decrease in actively proliferating cells when PKC1 is depleted (Figure 3.10). There was a decrease of approximately 10 to 15% in cells depleted of PKC1 when compared to mock or control siRNA transfected MCF7 and T47D cells. While analyzing the BrdU incorporation data, we found that cells displaying significant morphological changes were BrdU negative (Figure 3.10). In conclusion, we have demonstrated that cells depleted of PKC1 have morphological changes associated with senescence and these cells have higher SA-β-Gal activity and are negative for BrdU incorporation.

Other markers can also be examined to understand possible mechanisms of premature senescence. Senescence associated heterochromatin foci (SAHF) are a late-onset senescence marker that can be visualized using DAPI staining. Another marker widely used in the literature as a marker of both replicative and stress-induced senescence is phosphorylation of histone H2A.X (γH2AX). When we probed cell lysates with and without knockdown of PKC1, we did not see an induction of γH2AX (Figure 3.14). This indicates that there are most likely no DNA damage foci present with siRNA treatment in these cell lines. There are limitations to these senescence markers as well. In a paper by Denoyelle et al, they showed that SA-β-Gal positive cells were not always positive for morphological changes or SAHF and those cells with morphological changes were not always correlative of SA-β-Gal positivity[101]. There is also recent evidence that senescence can occur in the
absence of DNA damage, specifically by showing a lack of γ-H2AX upon induction of senescence [102].

To determine if PKC\(\iota\) depletion induces senescence in non-tumourigenic mammary cell lines, hTERT-HMEC and MCF10A cells were analyzed. There was no decrease in proliferation in the hTERT-HMEC cell line when treated with siRNA to PKC\(\iota\) (Figure 3.11). The MCF10A cell line was not analyzed for proliferation because the doubling time is too long to notice any appreciable differences in cell number using this assay. However, both MCF10A and hTERT-HMEC cell lines were analyzed for SA-β-Gal staining and there was no increase in SA-β-Gal staining with depletion of PKC\(\iota\) (Figure 3.11). Only data for the MCF10A cell lines is shown because there was no detectable SA-β-Gal activity in hTERT-HMECs either without or with PKC\(\iota\) depletion. The hTERT-HMEC cells are immortalized through exogenous transfection of human telomerase, to prevent replicative senescence. Premature senescence, as studied here, can occur independently of telomerase function [135]. We also ensured that PKC\(\iota\) was being depleted over the time course analyzed (Figure 3.12). While there was a depletion of PKC\(\iota\) protein levels, there was no effect on proliferation or SA-β-Gal activity. This suggests that the induction of premature senescence upon PKC\(\iota\) is a cancer-cell specific phenomenon.

During tumourigenesis, cancer cells need to overcome several barriers and control mechanisms that restrict uncontrolled proliferation and invasion. One of these barriers to tumour development is the tumour suppressive mechanism of senescence. Tumour cells are primed for induction of premature senescence because they have genetic instability, oncogenic mutations and highly express mitogenic factors such as E2F1 [136]. These factors
can all cause senescence in normal cells, suggesting that removing or altering some of the mechanisms that the tumour cell has set up to suppress senescence could be used to initiate senescence programs in tumour cells without affecting normal cells.

Our data show that PKC\(\alpha\) prevents the premature induction of senescence in breast cancer cells. While this is a completely novel function for PKC\(\alpha\), inhibition or depletion of PKC\(\alpha\) has been shown in other tumour types to inhibit proliferation[103, 124, 126]. This decrease in proliferation occurred without induction of apoptosis [124, 126]. While senescence was not tested in these instances, mechanisms for inhibition of proliferation were not provided. It is possible that senescence is also being induced in these tumour types. The mechanism by which decreased PKC\(\alpha\) causes senescence is unknown. Determining the molecular mechanism by which premature senescence is being induced with PKC\(\alpha\) knockdown will be difficult for a few reasons. Few downstream targets of PKC\(\alpha\) have been verified which makes identifying critical phosphorylation targets further down its signalling cascade more complicated. Several known mechanisms or markers of senescence occur in a time-dependent manner and thus careful examination of several timepoints will be needed. To narrow down some of the potential mechanisms, certain pathways can be ruled out by examination of critical effectors in those pathways.

It appears that the mechanism is p53 and p16-independent. MCF7 cells have homozygous deletion of the p16 locus and T47D cells have p16 promoter methylation, resulting in barely detectable protein expression [137, 138]. T47D cells also have mutant p53 [139]. The p53 and p16/Rb pathways are certainly important in the induction of premature senescence and there is substantial evidence that they play a role in oncogene-
induced senescence (reviewed in [88]). Alterations involving these signalling pathways are prevalent in most cancers, supporting the notion that both p53 and p16 are key mediators in initiating senescence programs[140]. However, there are also several instances of premature senescence that do not require p53 or p16. In response to chemotherapy and radiation, human lung carcinoma cells deficient in p53 and p16 can undergo senescence[141]. Our lab has also shown that Hsp90 inhibition can cause premature senescence in small-cell lung cancer H69 cells which lack Rb and are also p53-mutant[110]. In human fibroblasts oncogene-induced senescence can still occur even in the presence of a dominant-negative p53[90]. Finally, high levels of the tumour suppressor kinase Chk2 led to arrested proliferation and senescence in human lung carcinoma cells and p53-mutant breast carcinoma cells[142].

There is substantial literature demonstrating that p21 plays a major role in mediating p53 and p16-independent senescence. TGF-β induced senescence in hepatocellular carcinoma cells caused robust elevated expression of p21 and p15 which was critical for the senescence phenotype[143]. TGF-β induced senescence in vivo was also associated with a strong anti-tumour response and tumour regression[143]. Ras association domain family 1 isoform A (RASSF1A) is commonly down-regulated in cancer due to promoter hypermethylation[144]. Restoration of RASSF1A inhibits tumour cell growth and causes senescence both in vitro and in vivo[144]. Induction of senescence by RASSF1A restoration was associated with upregulation of p21 in a p53 and p16 independent manner[144]. It has also recently been shown that when the E3 ligase Skp2 is targeted, it causes cellular senescence in Arf-/- Skp2 -/- cells (Arf encodes the p16^{ink4a} locus in mice) as well as in p53 and PTEN null PC3 cells[102]. They found that loss of Skp2 triggered elevated levels of
p21, p27 and ATF4. Knockdown of these proteins reversed cellular senescence upon Skp2 inactivation.

Denoyelle et al., have shown that HRAS\textsuperscript{G12V} and BRAF-driven senescence in human melanocytes does not require p53 or p16\cite{101}. In fact, they demonstrated that HRAS-driven senescence is due to an expansion of the ER and induction of the UPR by increased expression of classical markers of ER stress such as XBP1, BiP (Grp78), CHOP, and ATF4\cite{101}. They confirmed that induction of the UPR was required for senescence by using dominant-negative proteins or shRNA against XBP1, ATF6 and ATF4 which reduced the amount of senescent cells. To determine if PKC\text{\textgamma} knockdown can initiate upregulation of the UPR, we can examine BiP, CHOP, or ATF4 protein levels following siRNA treatment. If these molecular markers indicate activation of ER stress pathways, different pathways of the UPR can be knocked out using shRNA to assess if the UPR is required for induction of senescence. For example, we could use shRNA against XBP1 or ATF4 after depletion of PKC\text{\textgamma}.

Finally, autophagy has been proposed as a necessary step for the establishment of oncogene-induced senescence. Autophagy is a complex cellular process responsible for turnover of proteins and organelles as well as a cell survival mechanism during nutrient starvation\cite{145}. The process is characterized by formation of specialized vesicles called autophagosomes which envelop proteins, aggregates and organelles. It has also been described as a tumour suppressor mechanism because it prevents accumulation of damaged proteins and organelles which could lead to oxidative stress\cite{146}. In a paper by Young et al., they examined H-Ras driven senescence in human fibroblasts and found that autophagic
activity was upregulated [147]. When autophagy was inhibited, this delayed senescence and the accumulation of senescence-associated secreted proteins[147]. To determine if autophagy plays a role in the induction of senescence upon PKCδ depletion, we can look for changes in protein levels of the autophagosome marker LC3 as well as the presence of autophagosomes using electron microscopy. Lastly, using chemical inhibitors of autophagy such as Bafilomycin A or Chloroquine or siRNA to key proteins such as Beclin1 would enable us to determine if autophagy is required for PKCδ depleted induction of senescence.

As described above, p21 is an important mediator in the induction of senescence. It has already been demonstrated that atypical PKCs phosphorylate the adjacent Ser146 phosphorylation site of p21 in vitro and in vivo[148]. Phosphorylation at Ser146 causes rapid degradation of p21, representing one mechanism to modulate p21 activity [148]. An adjacent phosphorylation site, at Thr-145 is also a part of consensus phosphorylation sequences for the AGC family of kinases (BXBXXS/T, where X is any amino acid and B is a basic residue) and could be phosphorylated by aPKCs. Phosphorylation at Thr-145 affects the cellular localization of p21[149]. Inhibition of cell cycle progression by p21 is strongly associated with localization in the nucleus[150]. It has been demonstrated in breast cancer cells that inhibition of the Akt pathway using dominant-negative Akt constructs or PI3K-inhibitors inhibits proliferation without induction of apoptosis[149]. Inhibition of the Akt pathway did not affect p21 expression but inhibited phosphorylation in the nuclear localization signal region of p21[149]. In the presence of constitutively active Akt, p21 was predominantly in the cytoplasm but inhibition of the Akt pathway caused nuclear translocation[149]. Mutation at the phosphorylation site (T145) abolished cytoplasmic localization of p21 and inhibited proliferation even in the presence of constitutively active
Akt[149]. PKC\(_\alpha\) is a downstream kinase in the Akt pathway and may mediate this nuclear translocation event. PKC\(_\alpha\) may function as an important kinase to phosphorylate either one of these sites to promote cell cycle progression. When PKC\(_\alpha\) is depleted, p21 could be stabilized or translocated to the nucleus if phosphorylated at Ser146 or Thr145 respectively. This would allow p21 to function as a cell cycle inhibitor and mediate the induction of senescence.

In order to test the *in vivo* significance of PKC\(_\alpha\) knockdown we needed to develop suitable systems to target PKC\(_\alpha\) that could be used in animal models. While there are small molecule PKC\(_\alpha\) inhibitors being developed, to date these are not effective or selective. Therefore we decided to develop systems which can express shRNA to PKC\(_\alpha\). There has been literature to suggest that shRNA produce fewer off-target effects than siRNA because shRNA are processed in the nucleus and therefore are subject to endogenous processing and regulatory mechanisms[151]. Lentiviruses can transduce non-dividing cells and dividing cells and have high transduction efficiency[152]. The doxycycline-inducible lentiviral system allows tighter control of when and how much of the shRNA is being expressed and a stable population of cancer cells can be selected out using puromycin. Xenograft tumours could be established in mice and then expression of shRNA to PKC\(_\alpha\) could be initiated by putting doxycycline in the drinking water or feed. This inducible system has been used extensively in the literature[153-155]. This system would enable study of whether PKC\(_\alpha\) affects tumour growth *in vivo* and if there is evidence of induction of senescence.

After producing lentivirus in 293T cells, we infected and selected MDAMB231 cell lines that stably expressed the vector (*Figure 3.15*). We have shown that the system is
tightly controlled because in the absence of dox, there is no activity of the shRNA. When we add dox to cell lines, we see induction of the shRNA with as little as 1 ng/mL of dox. There is a dose-dependent induction of the shRNA as we see the highest knockdown of PKC\(\iota\) with 100 ng/mL of dox. While not as effective as the lentiviral system, we also developed an adenoviral system to express shRNA to PKC\(\iota\). With the assistance of the McKay lab, we were able to prepare crude adenovirus. We infected MDAMB231 cells and saw approximately 60\% knockdown of PKC\(\iota\) after three days (Figure 3.15). While we did titer the crude virus to get a rough MOI, perhaps infection would improve if the virus was purified and concentrated. The lentiviral system will allow us to continue this study \textit{in vivo} but also be able to have consistent long-term expression of shRNA during \textit{in vitro} experiments. In addition, SA-\(\beta\)-Gal and BrdU are established markers of senescence \textit{in vivo} [reviewed in\cite{88, 156}] and would enable the \textit{in vitro} results in this study to be further assessed in xenograft models of breast cancer.

In conclusion, we have shown that PKC\(\iota\) is overexpressed in a subset of breast cancers and in breast cancer cell lines. \textit{PIK3CA} mutations are sufficient to increase both activation and expression of PKC\(\iota\) \textit{in vitro}. Inhibition of PKC\(\iota\) causes an induction of premature senescence in a cancer cell-specific manner. The role of PKC\(\iota\) in overcoming the tumour suppressive mechanism of senescence has not been previously described. PKC\(\iota\) is therefore a promising and novel therapeutic target in the treatment of a subset of breast cancers.
References


MCF7 and T47D cells were not treated with BrdU but still fixed and stained according to immunofluorescence protocol using both primary and secondary antibodies. There was no background staining anywhere on the slide.
Contributions of Collaborators

Dr. Manijeh Daneshmand performed immunohistochemical staining of all three breast TMAs. Dr. Shahrier Amin, Dr. Shahidul Islam and Dr. Manijeh Daneshmand evaluated TMA samples. Dr. Bruce McKay generously gave our lab the adenoviral vectors and Jeff Hamill prepared crude adenovirus preps from our constructs.
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