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The Role and Expression of PKC_1 in Breast Cancer

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The Role and Expression of PKCι in Breast Cancer

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Thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the degree of Masters of Science in Biochemistry

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Abstract

The role of the atypical PKC iota (PKCι) in breast cancer is unknown. In this study, the expression of PKCι in breast cancer tissue was investigated using tissue microarrays. PKCι was over-expressed in 70% of breast cancers. The expression and activation of PKCι was also elevated in a subset of breast cancer cell lines. Two cell lines with the highest activation and expression of PKCι, T47D and MCF7, have *PIK3CA* mutations E545K and H1047R respectively. To investigate the effects of *PIK3CA* mutations on PKCι, stable MCF10A cells expressing E545K and H1047R mutations were generated using retroviral transduction. Western blot analysis showed that these mutations are sufficient to increase PKCι expression and activation. To analyze the role of PKCι in breast cancer pathogenesis, siRNA targeting PKCι 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 PKCι had morphological changes associated with senescence, increased SA-β-Gal activity and decreased BrdU incorporation. These results demonstrate that PKCι 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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This thesis could not have been completed without the support and encouragement of many people. With his advice and insight, my supervisor Dr. Ian Lorimer has been instrumental in guiding me throughout this thesis. His ability to put a positive spin on any result has pushed me through some of the bumps throughout the past two years. Dr. Lorimer runs one of the most hardworking and enjoyable labs and I have to thank him for hiring so many fantastic people. Working in the Lorimer Lab has provided me with so many great memories and every day has been filled with laughter and music (and work!). I would like to thank the Lorimer Lab for all of their help, suggestions, and support over the past two years. In particular, I am grateful to Ian Restall for his enthusiasm about research, his wonderful idea about senescence, and for providing support and humour when I needed it the most. I would also like to thank Dr. Mitch Baldwin for his help in all things 'iota-related' and Dr. Manijeh Daneshmand for a crash-course on immunohistochemistry. Finally, I couldn't have completed this thesis without the love and support from my family and friends.

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List of Abbreviations

PKCι: 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_2: phosphatidylinositol-4,5-bisphosphate

PIP_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-β-Gal: senescence associated β-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 to recruited to the cell membrane, where it can phosphorylate phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-triphosphate (PIP_3) 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 PIP_3 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 PIP_3 to PIP_2. 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 PIP_2[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 ad IDC[35]. They found that invasive tumour areas and *in situ* areas of the same tumour had identical *PIK3CA* sequences in the majority of cases[35]. These results support the hypothesis that *PIK3CA* mutations may play a role in the early stages of tumour initiation.

There is conflicting views in the literature as to whether *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 *PIK3CA* mutations into untransformed cells or used tumour cell lines with these mutations to uncover downstream signalling events and phenotypic changes both *in vitro* and *in vivo*. Overexpression of these PI3K mutants in chicken embryo fibroblasts and NIH3T3 mouse fibroblasts caused increased *in vitro* PI3K activity and transformation[41, 42].

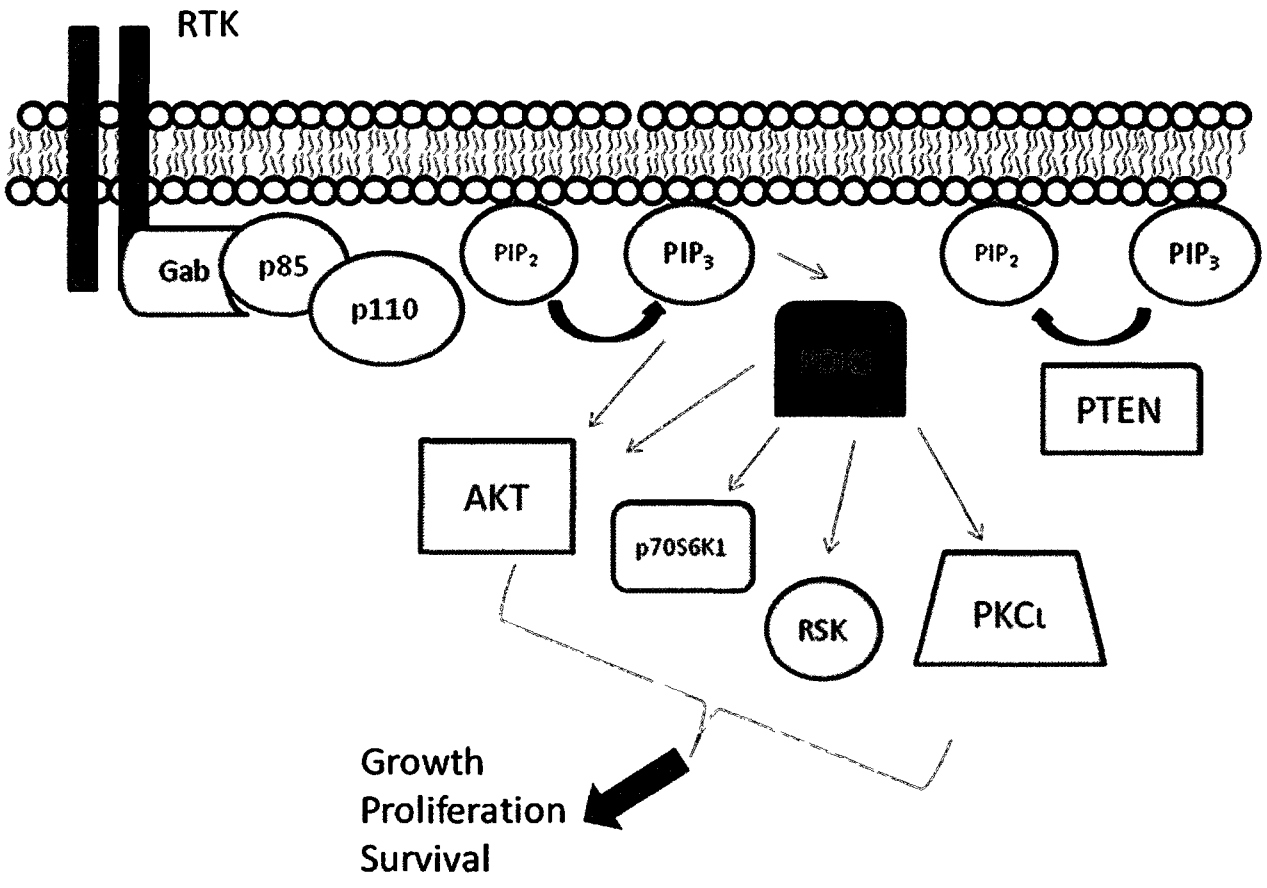
Figure 1.1 PI3K Pathway and *PIK3CA* mutations.

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.

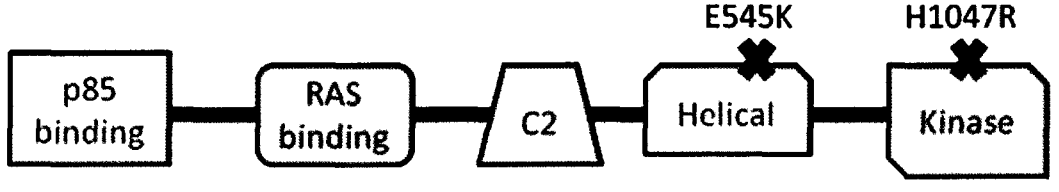
B) Domain structure of the *PIK3CA* catalytic subunit

The most common *PIK3CA* mutations occur in the helical domain and the kinase domain.

A)


RTK
Gab
p85
p110
PIP2
PIP3
PIP2
PIP3
PTEN
AKT
p70S6K1
RSK
PKCι
Growth
Proliferation
Survival

B)


E545K
H1047R
p85 binding
RAS binding
C2
Helical
Kinase

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]. *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 *in vitro*[45]. As these mutations have different mechanisms of activation, it is proposed that they may have different functional phenotypes. *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 *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 *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 PKCι

PKCι is a member of the atypical PKC subclass of PKC isozymes. PKC ι 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 Bem 1 (PB1) protein-protein interaction domain. PB1 domains allow binding to other proteins with PB1 domains, such as the polarity protein Par6[50, 52]. PKCι 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, PKCι has been implicated in carcinogenesis and oncogenic signalling in brain[56], lung[57], colon[58] and ovarian carcinomas[59]. Transgenic mice expressing constitutively active PKCι exhibited a higher incidence of pre-neoplastic lesions and colon tumours than control mice[58]. Expression of kinase-inactive PKCι in non-small cell lung cancer (NSCLC) cells inhibits anchorage-independent growth and proliferation both *in vitro* and in *vivo*[57]. PKCι 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, PKCι 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]. PKCι is also involved

in oncogenic Ras signalling in both colon and lung cancers, playing a critical role in Ras-mediated transformed growth[57, 58]. One study demonstrated that PKCι activated a Rac1/Mek/Erk signalling axis that was required for transformed growth and invasion[57]. In NSCLC, PKCι has been shown to phosphorylate μ and m-calpains which can promote migration and invasion[62].

Elevated PKCι expression has been frequently observed in ovarian and NSCLC tumours[59, 63, 64]. In ovarian tumours, PKCι expression is prognostic of poor clinical outcome and also correlates with tumour stage[59, 64]. This may suggest that PKCι contributes to tumour development and more aggressive disease. Amplification of the PKCι gene was one of the molecular mechanisms for the over-expression of PKCι in these tumour types[63, 64]. The PKCι 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 PKCι 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 PKCι in Breast Cancer

There has been little previous research into the role and expression of PKCι in breast cancer. Only one study has looked at the expression of PKCι, in breast cancer tissue samples[68]. The authors found that PKCι was overexpressed in 80% of the tumours studied and that there was a correlation between PKCι 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 PKCι and associates with Par6-PKCι[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 PKCι and Cdc42[70]. In order to test whether the polarity complex of Par6/PKCι/Cdc42 is a viable therapeutic target, they used a stably expressing shRNA system to downregulate PKCι expression in MCF7 breast cancer cell lines. Downregulation of PKCι 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 *BRAF* or *RAS* genes[79]. In mouse models, introduction of K-Ras-V12, mutant BRAF or PTEN inactivation have all supported that senescence occurs *in vivo*[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[82]. Inactivation of several genes such as p53, Rb, CHK2, or ATM can disable several critical senescence pathways and supply a growth advantage[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[85].

Triggering senescence in cancer cells may represent an alternate therapeutic strategy. In one study, reactivation of p53 in hepatocarcinomas triggered senescence *in vivo*[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[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.

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[88]. Since senescence may result in genomic instability, binucleated or multinucleated cells can

develop[88]. One of the most widely used markers of senescence is senescence associate β-galactosidase (SA-β-Gal) activity. B-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].

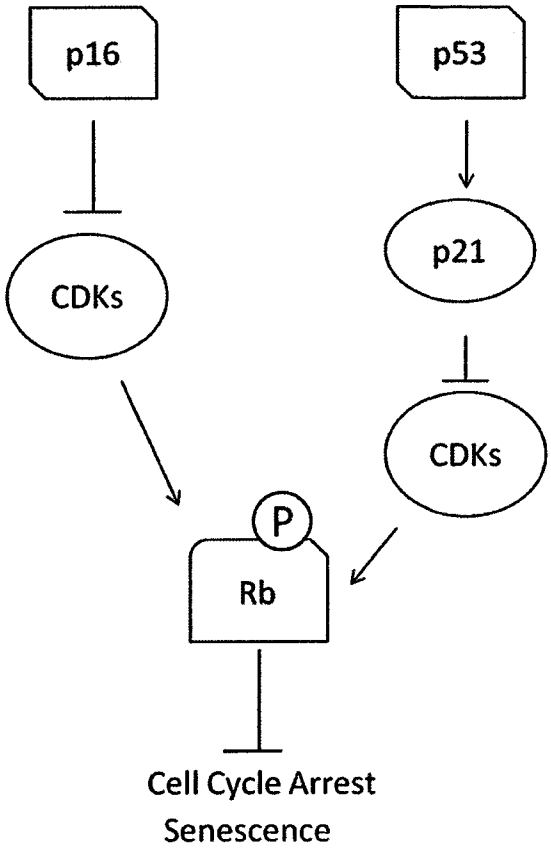
1.9 Signalling pathways involved in induction of senescence

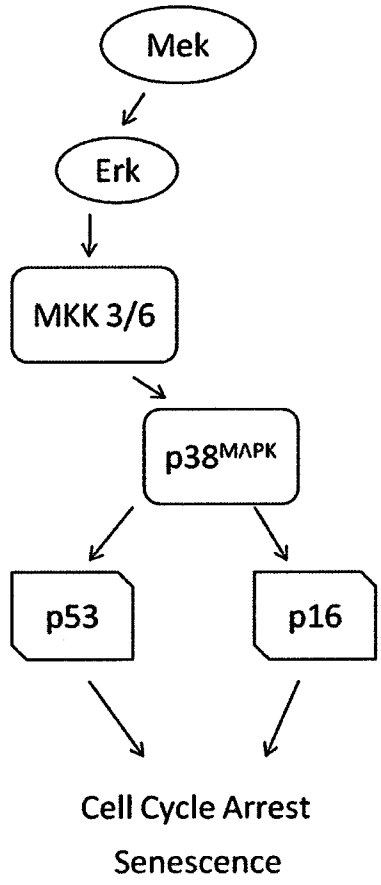
It has been demonstrated that several growth-regulatory proteins are either upregulated or have altered activation states with senescence. One of the major pathways for induction of senescence involves p53-dependent activation of the cyclin-dependent kinase (Cdk) 2 inhibitor p21 as shown in **Figure 1.2**[94]. At the onset of senescence, there is 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 $p38^{MAPK}$ 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 $p19^{Arf}$ 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.


p16
p53
CDKs
p21
CDKs
P
Rb
Cell Cycle Arrest
Senescence


Mek
Erk
MKK 3/6
p38MAPK
p53
p16
Cell Cycle Arrest
Senescence

Hypothesis

Part I

PIK3CA mutations are a common mutation in breast cancer and PKCι is a known downstream effector of the PI3K pathway. We propose that these mutations will increase activation of PKCι in breast cancer.

Part II

PKCι is a known oncogene in other cancers and has previously been shown to promote cell proliferation in other tumour types. We propose that PKCι 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 PKCι 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 PKCι using Western blotting in a subset of breast cell lines

2. Determine if *PIK3CA* mutations are sufficient to increase activation or expression of PKCι.

    a) Retrovirally transduce non-tumourigenic mammary epithelial cell lines with *PIK3CA* mutations and determine effects on PKCι

Part II

3. Use RNAi to knock down expression of PKCι 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 PKCι

4. Investigate methods to assess PKCι function *in vivo*

    a) Create inducible shRNA lentiviral systems targeting PKCι
    b) Create adenoviral shRNA systems targeting PKCι

Significance

While PKCι has been identified as an oncogene in other cancers, previously there has been little research into the expression or role of PKCι 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 PKCι across a spectrum of breast disease from hyperplasia to metastatic carcinoma. We demonstrate that PKCι is overexpressed in breast cancer tissue and *in vitro* also show that PKCι is overexpressed in a subset of breast cancer cell lines. We propose that a novel mechanism for elevated PKCι both *in vitro* and *in vivo* is through *PIK3CA* mutations. In other tumour types such as glioma, it has been demonstrated that PKCι is required for proliferation[103]. We demonstrate that PKCι promotes proliferation in breast cancer cell lines, through a mechanism where PKCι prevents cells from undergoing premature senescence. This is a completely novel function for PKCι not only in breast cancer cells but in other tumour types as well.

Chapter 2

Materials and Methods

2.1 Reagents and Antibodies

PKCι mouse monoclonal and pPKCι 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_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% H_2O_2 in PBS for 15 min.

Primary mouse monoclonal PKCι 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 PKCι. The RNAi target sequences used are as follows: PKCι A 5'-GTGCATCAACTGCAAACTC-3' and PKCι 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 μL of Opti-MEM I (Invitrogen, Carlsbad, CA, USA) was added to each well. 5 nM of siGENOME Non-Targeting siRNA #2, PKCι A, PKCι C (Dharmacon) RNAi was mixed with OptiMEM I to total 185 μL. For a mock transfection control, OptiMEM I was added instead of RNAi. 4 μL of oligofectamine (Invitrogen, Carlsbad, CA, USA) was mixed with 11 μL 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 μL mixture was added dropwise to each well. For 12 well assays, 100 μL 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 20000 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, PKCι A, PKCι 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 $MgCl_2$, 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 PKCι 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.

PKCι A
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 pBHGloxΔE1,3Cre 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 PKCι 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.

PKCι A
5'-CCGGGTGCATCAACTGCAAACTCCTCGAGGAGTTTGCAGTTGATGCACTTTTT-3'

5'- AATTAAAAAGTGCATCAACTGCAAACTCCTCGAGGAGTTTGCAGTTGATGCAC-3'

GFP

5'-ACCGGAAGCTGGAGTACAACTACACTCGAGTGTAGTTGTACTCCAGCTTTTTTTAATTC-3'

5'-TGGCCTTCGACCTCATGTTGATGTGAGCTCACATCAACATGAGGTCGAAAAAAATTAAG-3'

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). 36μL of Gene Juice was added to 600 μL OptiMEM I and incubated for at least 5 minutes. The following was mixed with OptiMEM I to total 50μL and added to Gene Juice and OptiMEM I mixture:

pLKO-Tet-ON shRNA	4μg
pLP1	3 μg
pLP2	3 μg
pLP/VSVG	2 μg

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 ddH_2O 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 PKCι in Breast Cancer

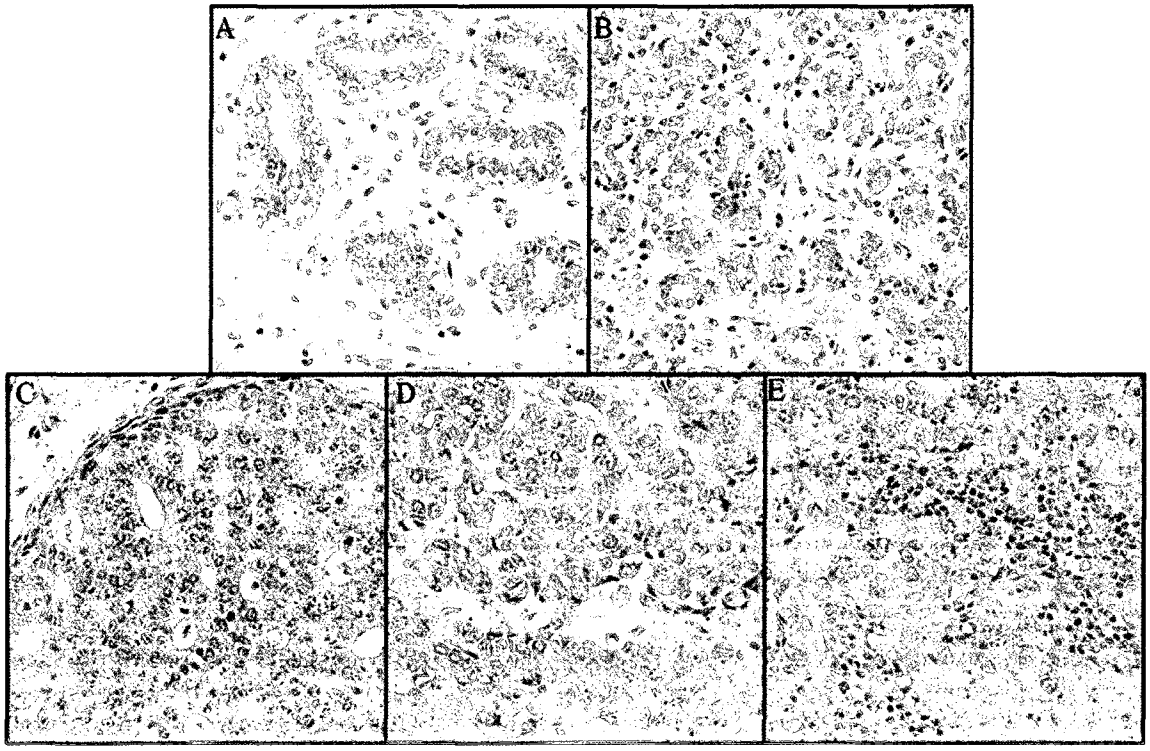
3.1 PKCι is highly expressed in a subset of breast cancers *in vivo*

In order to examine the expression profile of PKCι 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 PKCι 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 PKCι 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 PKCι staining is limited to the tumour tissue. Results of PKCι expression in tumour tissues was based on scoring the intensity of

Figure 3.1 PKCι is overexpressed in breast cancer tissues

Immunohistochemistry was performed on three breast TMAs using a PKCι monoclonal antibody. In panels A-E are representative images of normal tissue, hyperplasia, DCIS, IDC and lymph node metastatic disease respectively.


A
B
C
D
E

cytoplasmic staining. The intensities of PKCι 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 PKCι expression (**Table 3.1**). Scoring profiles for cases of hyperplasia, DCIS, IDC and lymph node metastases are shown in **Figure 3.2**.

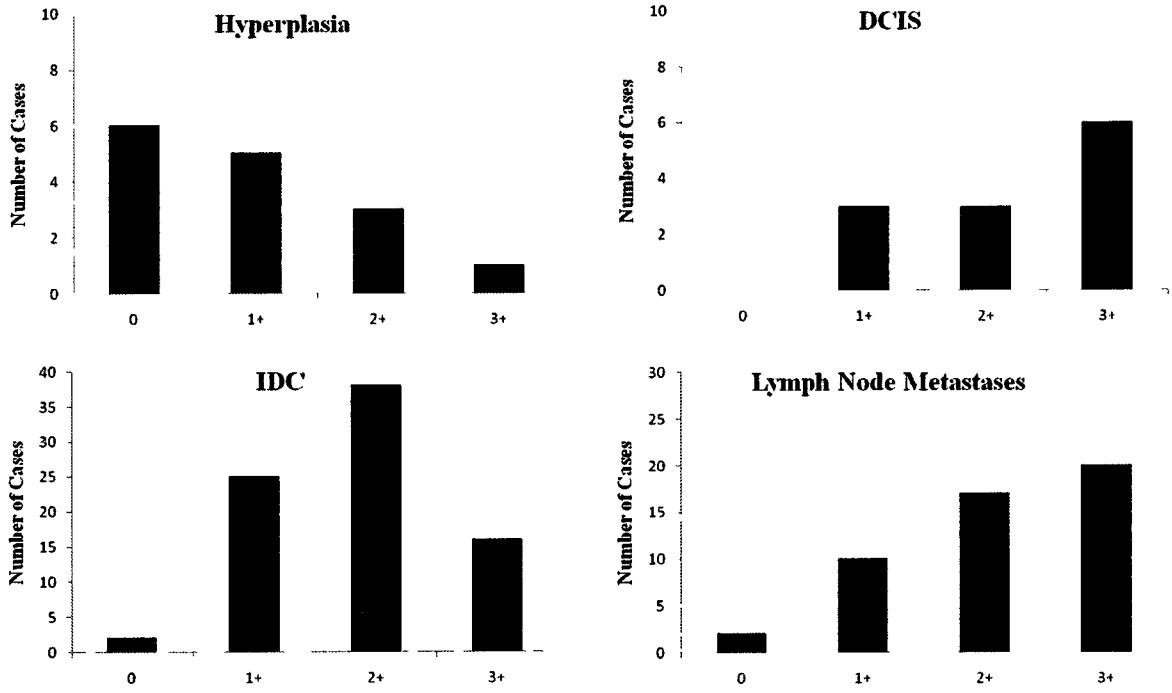
Table 3.1 Immunohistochemical Cytoplasmic Scoring of PKCι expression

	PKCι +	PKCι -	Total # of cases	% of PKCι + 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 PKCι expression profiles

Immunohistochemical scoring is plotted according to pathologic type and intensity of PKCι staining. 0 represents weak to no staining and 3+ represents strong PKCι staining.


Hyperplasia
Number of Cases
0
2
4
6
8
10
0
1+
2+
3+
DCIS
Number of Cases
0
2
4
6
8
10
0
1+
2+
3+
IDC
Number of Cases
0
5
10
15
20
25
30
35
40
0
1+
2+
3+
Lymph Node Metastases
Number of Cases
0
5
10
15
20
25
30
0
1+
2+
3+

3.2 PKCι is not correlated with ER, PR or HER2 expression

We examined PKCι 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 PKCι expression was found in a subset of IDC samples (n=32).

Table 3.2 Clinicopathological Factors and PKCι expression

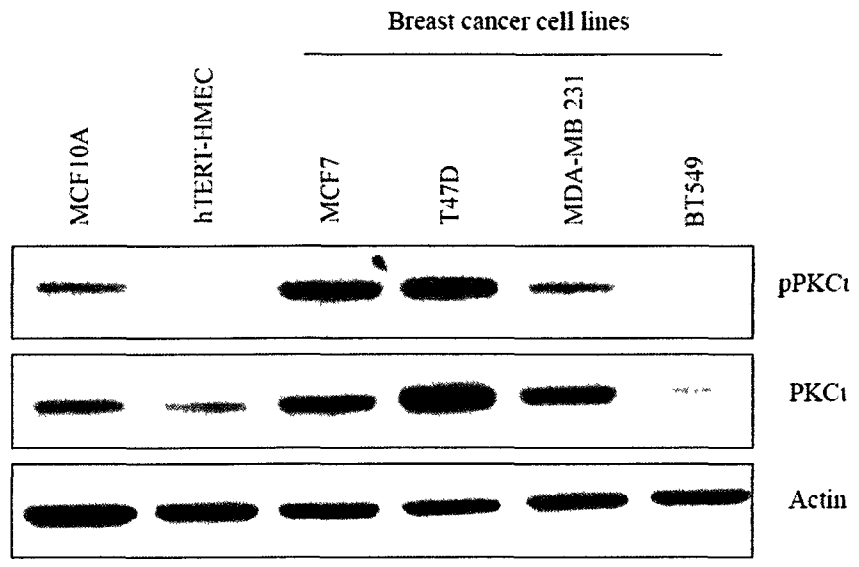
PKCι staining	Parameter		PKCι -	PKCι +	P value
Cytoplasmic	HER2	+	8	5	
		-	6	13	0.149
Cytoplasmic	ER	+	7	10	
		-	7	8	1
Cytoplasmic	PR	+	5	6	
		-	9	12	1

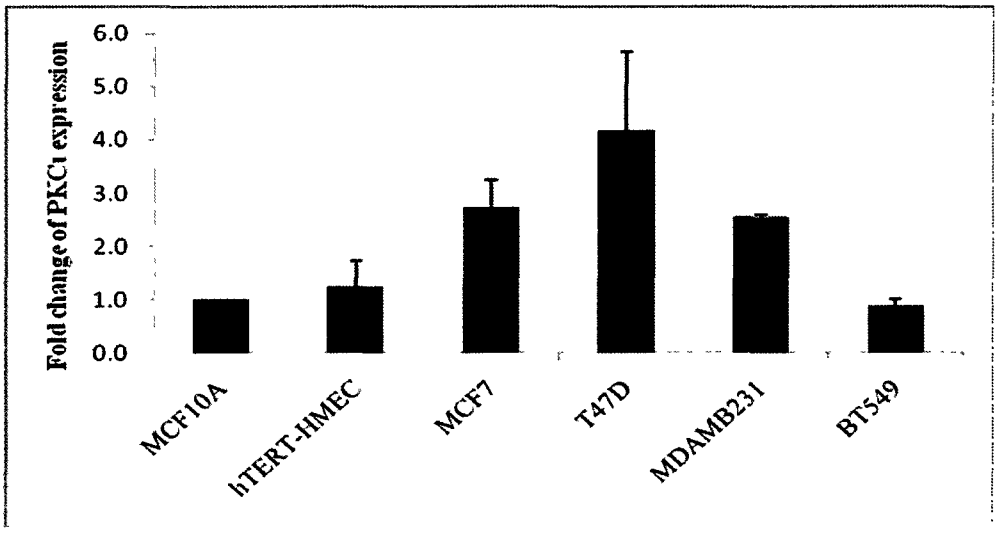
3.3 PKCι is highly expressed in a subset of breast cancer cell lines

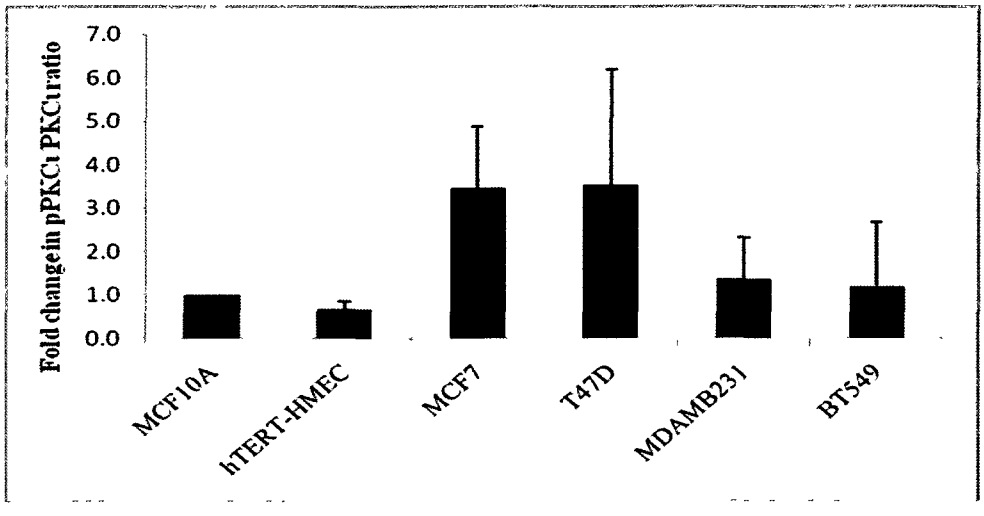
PKCι phosphorylation (activation) and total protein expression were analyzed in a subset of breast cancer cell lines and non-tumourigenic mammary epithelial cell lines using Western blotting (**Figure 3.3**). PKCι activation was measured by using a phosphospecific antibody to the Threonine 555 site. Phosphorylation of PKCι 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 PKCι 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 PKCι total protein levels respectively when compared to

Figure 3.3 PKCι is highly expressed and activated in a subset of breast cancer cell lines

Activation of PKCι and expression of PKCι were analyzed in normal mammary epithelial cell lines (MCF10A and hTERT-HMEC) and breast cancer cell lines (MCF7, T47D, MDAMB231 and BT549) using Western blotting. Densitometry was used to analyze relative expression and activation levels. PKCι expression was normalized to Actin levels for each cell line. To determine activation levels of each cell line, pPKCι levels were normalized to total PKCι levels.


Breast cancer cell lines
MCF10A
hTERT-HMEC
MCF7
T47D
MDA-MB 231
BT549
pPKCι
PKCι
Actin


Fold change of PKCι expression
6.0
5.0
4.0
3.0
2.0
1.0
0.0
MCF10A
hTERT-HMEC
MCF7
T47D
MDAMB231
BT549


Fold change in pPKCι PKCι ratio
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
MCF10A
hTERT-HMEC
MCF7
T47D
MDAMB231
BT549

MCF10A cells (**Figure 3.3**). MDA-MB 231 cells had approximately 2.5-fold high expression than normal cell lines. PKCι activity was normalized to PKCι protein levels for each cell line and compared in **Figure 3.3**. The pPKCι/PKCι 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 pPKCι/PKCι ratios than the normal mammary cell lines.

3.4 Activating *PIK3CA* mutations increase PKCι expression and activation

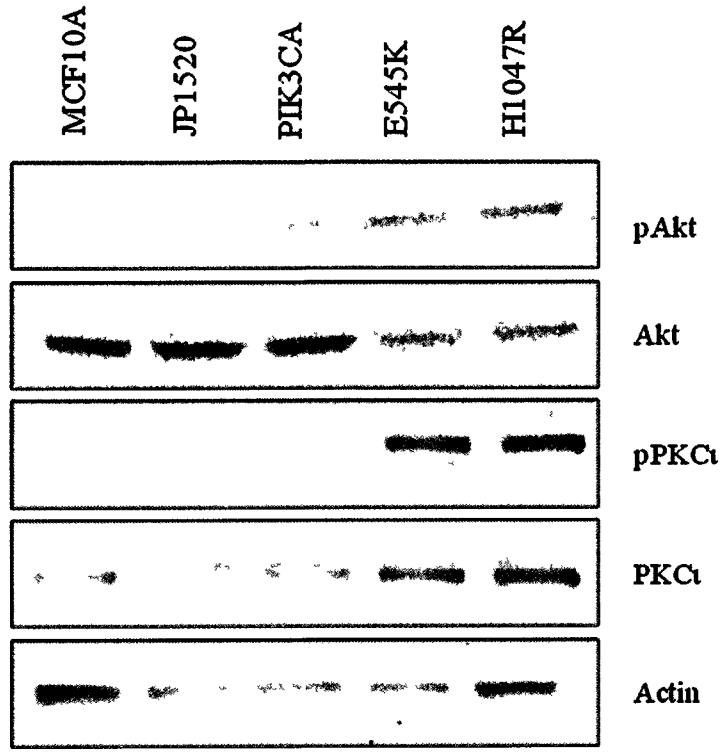
High levels of activation and expression of PKCι were observed in MCF7 and T47D cells and each harbour *PIK3CA* mutations. MCF7 and T47D cells have E545K and H1047R mutations respectively, which have been verified in our lab (Jenn Hanson, unpublished data). We tested whether *PIK3CA* mutations are sufficient to increase PKCι 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 PKCι in MCF10A cells expressing *PIK3CA* mutations (E545K or H1047R) had both increased expression and activation compared to control cells. PKCι 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 PKCι activation were due to the *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)**. PKCι activation was also decreased when treated with PI3K inhibitors. This is consistent with the fact that PKCι is a downstream effector in the PI3K pathway. There was no effect on PKCι protein levels with the use of these inhibitors. Interestingly, these agents did not inhibit the low level of basal PKCι activation in MCF10A cells expressing wild-type PI3K.

Figure 3.4 *PIK3CA* mutations increase expression and activation of PKCι

Transduced cells and non-transfected control MCF10A were growth-factor starved (MEBM media with 100 units/ml penicillin, 100 μg/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 $p<0.05$)


MCF10A
JP1520
PIK3CA
E545K
H1047R
pAkt
Akt
pPKCι
PKCι
Actin

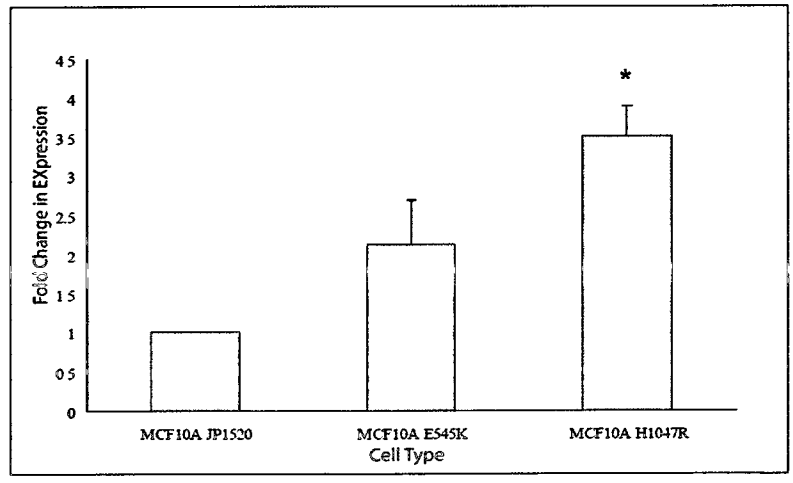
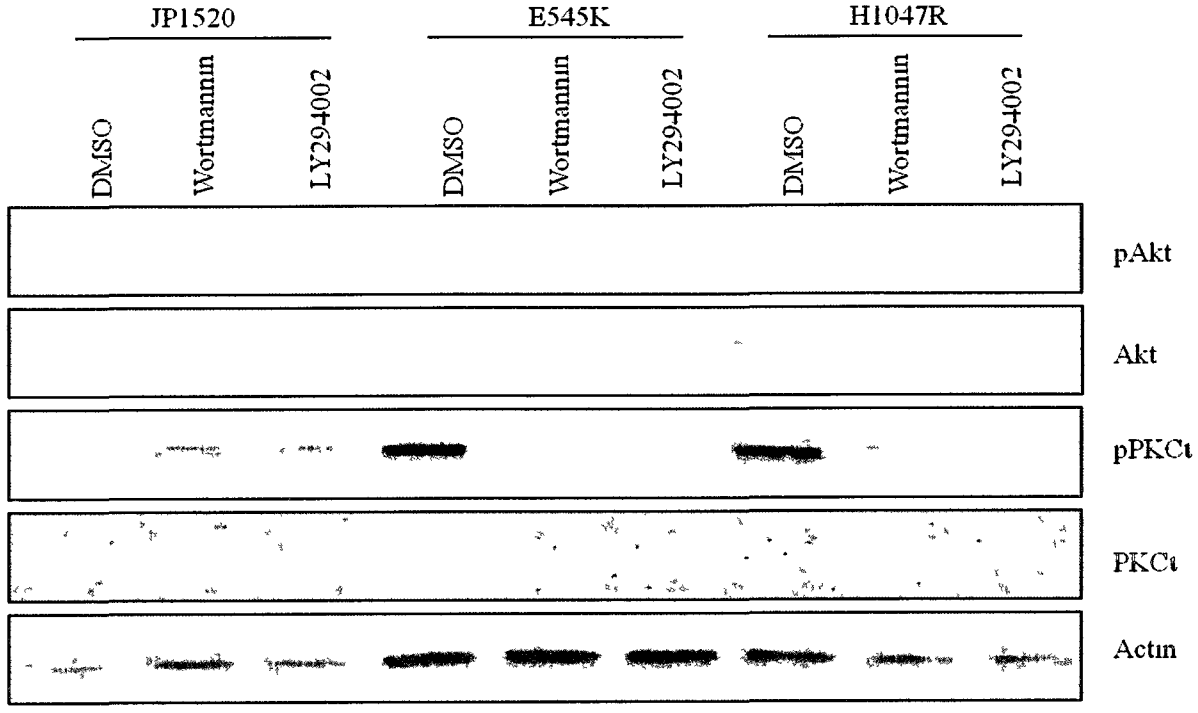

Fold Change in EXpression
45
4
35
3
25
2
15
1
05
0
*
MCF10A JP1520
MCF10A E545K
MCF10A H1047R
Cell Type

Figure 3.5 PI3K inhibitors affect PKCι 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 PKCι.


JP1520
E545K
H1047R
DMSO
Wortmannin
LY294002
DMSO
Wortmannin
LY294002
DMSO
Wortmannin
LY294002
pAkt
Akt
pPKCt
PKCt
Actin

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

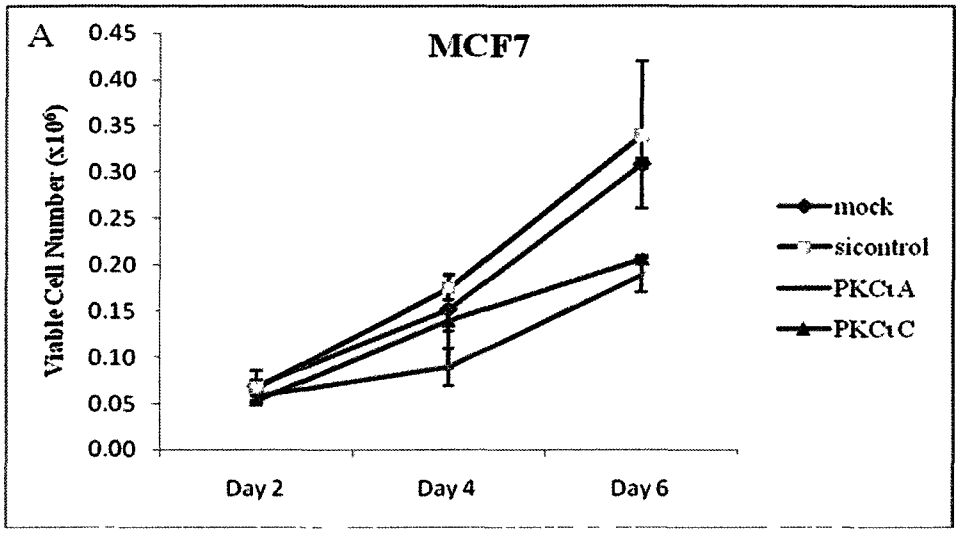
In order to determine if PKCι promotes proliferation in breast cancer lines, we used two different siRNA sequences to target PKCι and downregulate protein levels. We used non-targeting siRNA and mock transfected cells as controls in each experiment. Proliferation was measured by viable cell counts with 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.

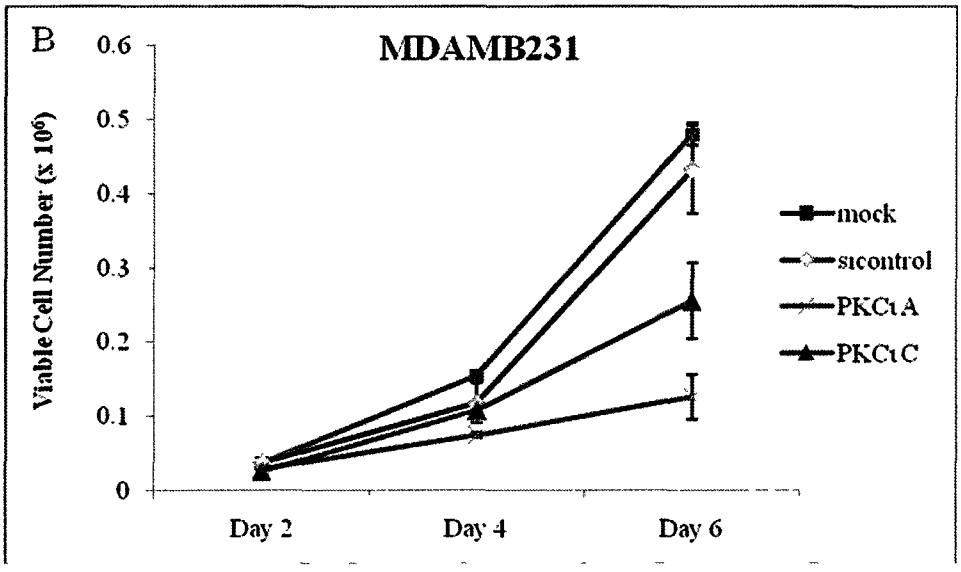
3.6 Down-regulation of PKCι does not cause apoptosis

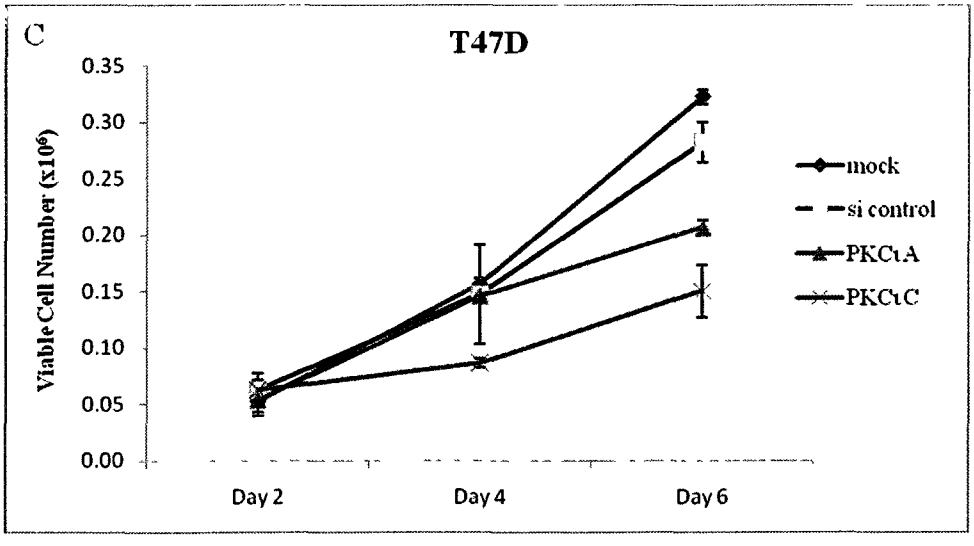
There was little change in the ratio of viable cells to total cells between any of the conditions in the proliferation assays, indicating no increase in apoptosis with siRNA treatment. To confirm that no apoptosis was occurring, flow cytometry was performed on cells treated with siRNA to PKCι. Propidium 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 PKCι inhibits proliferation in MCF7, T47D and MDAMB231 cell lines

Cells were plated in duplicate for each condition and treated with siRNA to PKCι. 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.


A
MCF7
Viable Cell Number (x10⁶)
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Day 2
Day 4
Day 6
mock
sicontrol
PKCtA
PKCtC


B
MDAMB231
Viable Cell Number (x 10⁶)
0.6
0.5
0.4
0.3
0.2
0.1
0
Day 2
Day 4
Day 6
mock
sicontrol
PKCtA
PKCtC


C
T47D
Viable Cell Number (x10⁶)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Day 2
Day 4
Day 6
mock
si control
PKCtA
PKCtC

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 PKCι does not induce apoptosis in these cell lines at the time point analyzed.

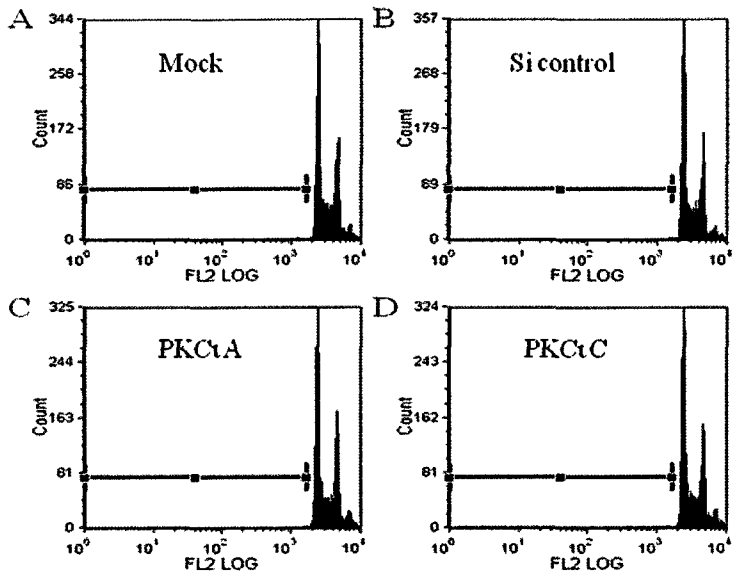
3.7 Knock down of PKCι in breast cancer cells causes induction of premature senescence

To determine if senescence was a factor in the decreased proliferation seen with down-regulation of PKCι, we assayed for SA-β-Gal activity. MCF7 and T47D cells were plated on gelatin-coated coverslips and treated with siRNA to PKCι. 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 PKCι, 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 PKCι. MCF7 and T47D cells are generally more round or cuboidal in shape and grow tightly packed in clusters. When PKCι 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 PKCι 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 PKCι A siRNA and D is PKCι C siRNA transduced cells respectively. The red line on each panel represents where subG1 cells would be located.

MDAMB231



MCF7

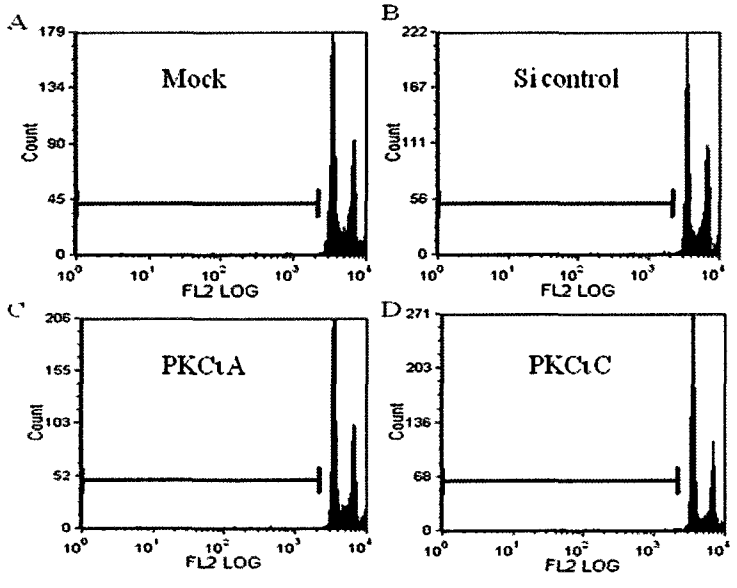
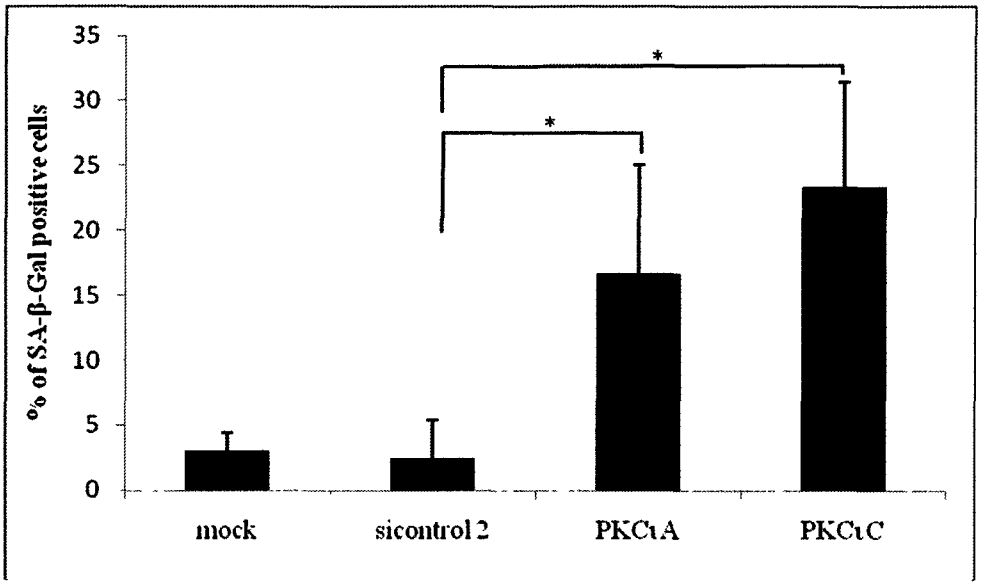


Figure 3.8 Knockdown of PKCι increases SA-β-Gal staining in MCF7 and T47D cell lines

MCF7 or T47D cells were plated on gelatin-coated coverslips and treated with siRNA to PKCι. 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 $p<0.01$)

A


% of SA-β-Gal positive cells
35
30
25
20
15
10
5
0
*
*
mock
sicontrol 2
PKCιA
PKCιC

B

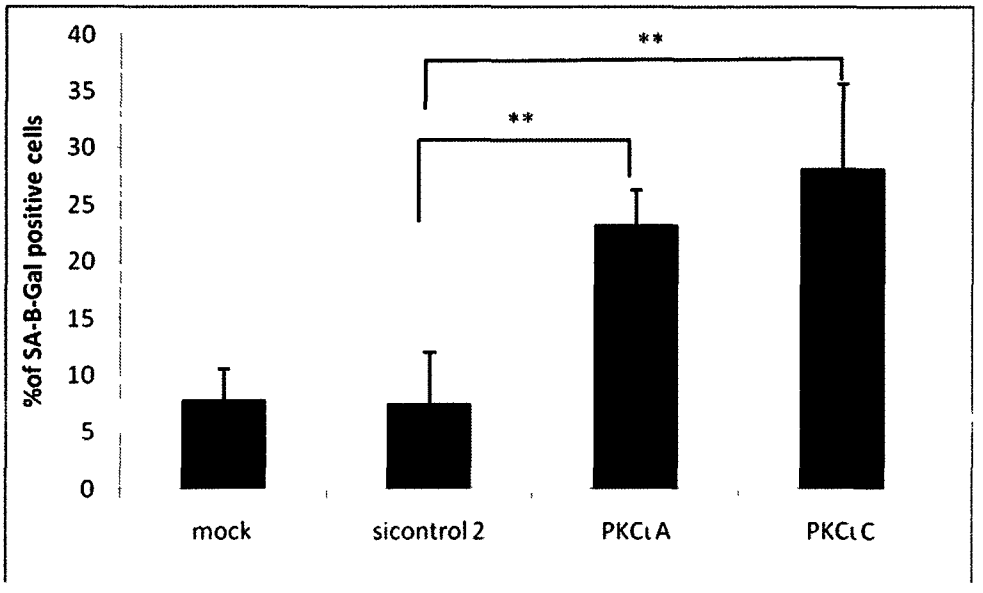
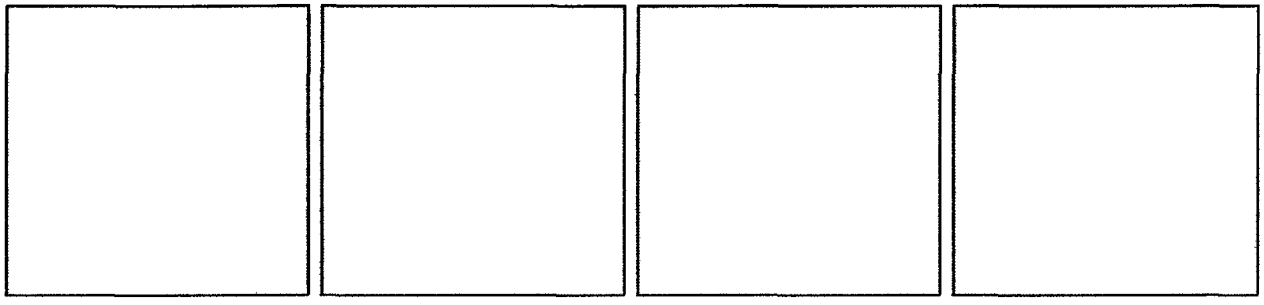

%of SA-B-Gal positive cells
40
35
30
25
20
15
10
5
0
**
**
mock
sicontrol 2
PKCιA
PKCιC

Figure 3.9 Knockdown of PKCι induces morphology changes characteristic of senescence

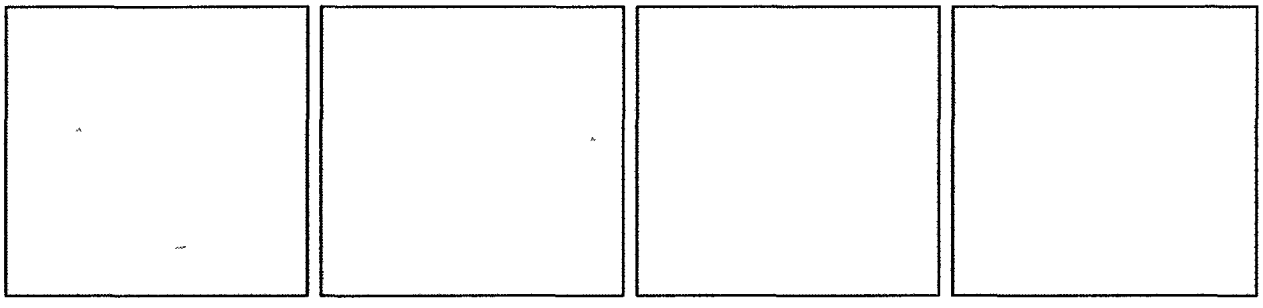
Cells were plated in gelatin-coated coverslips and treated with siRNA to PKCι. Five days later, cells were fixed with paraformaldehyde and stained with X-gal overnight at 37°C. Coverslips were then mounted on slides and visualized using bright-field microscopy.

T47D



Mock Sicontrol PKCι A PKC ι C

MCF7



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

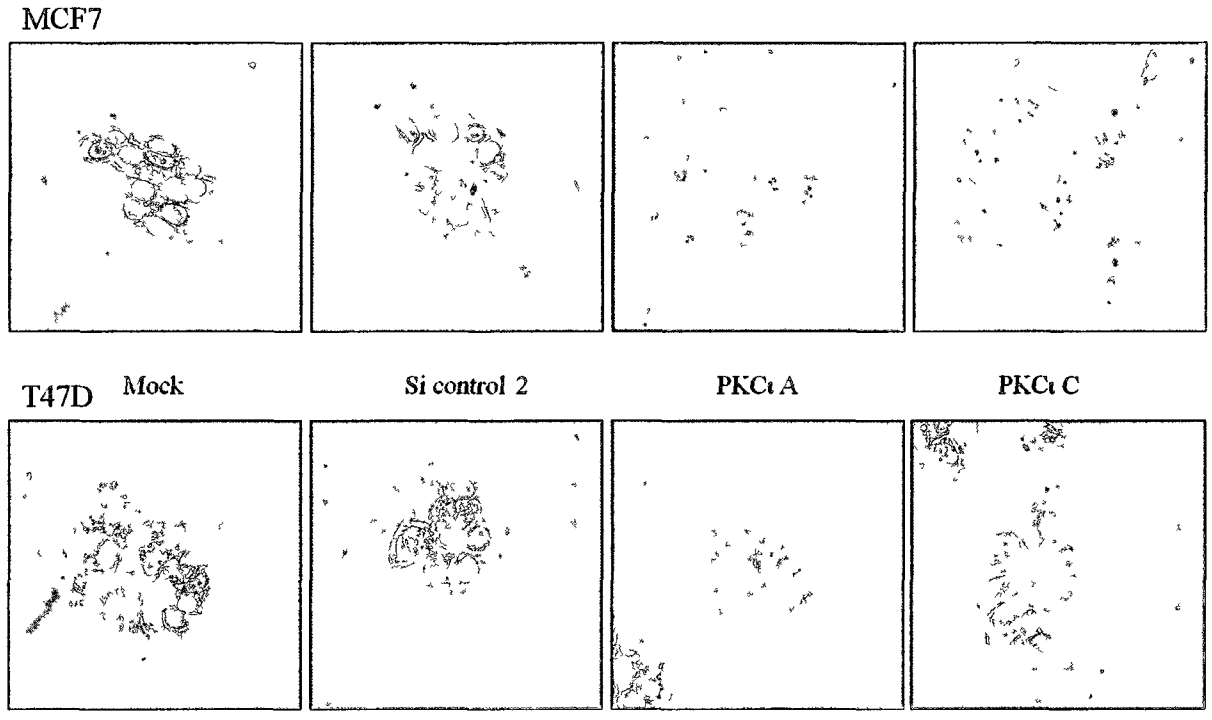
Figure 3.10 Depletion of PKCι decreases BrdU incorporation in MCF7 and T47D cells

A. Representative pictures of MCF7 and T47D cells mock transfected or transfected with control siRNA or PKCι 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 $p<0.05$)

A.


MCF7
T47D
Mock
Si control 2
PKCι A
PKCι C

B.

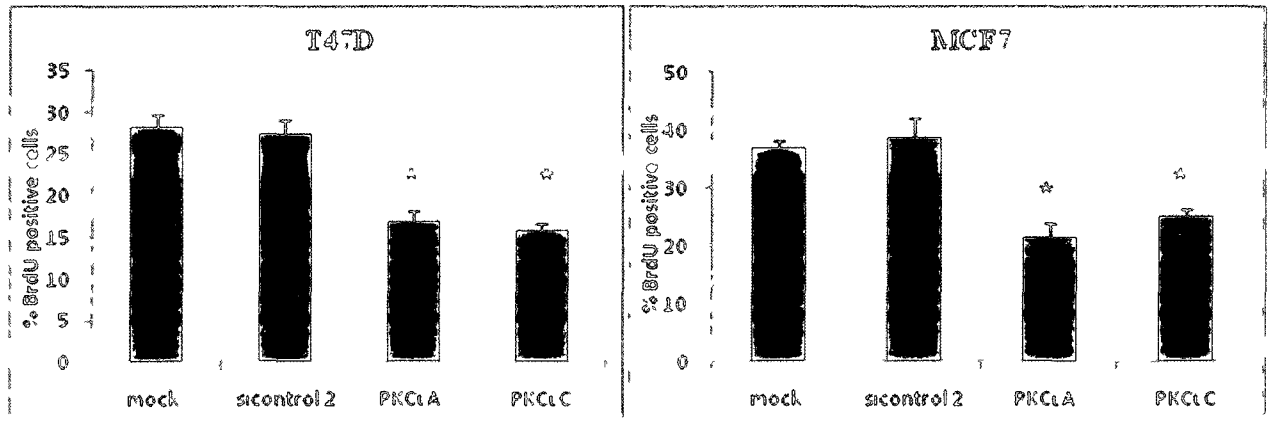
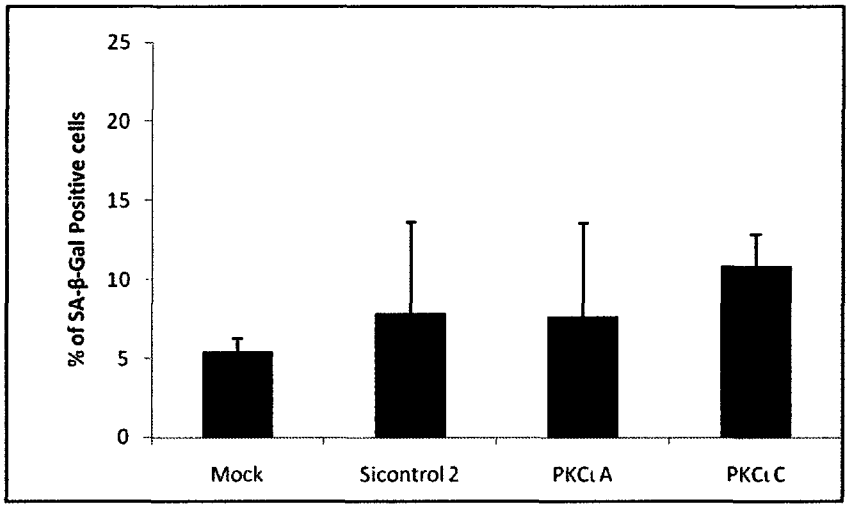

T47D
MCF7
% BrdU positive cells
mock
sicontrol 2
PKCι A
PKCι C

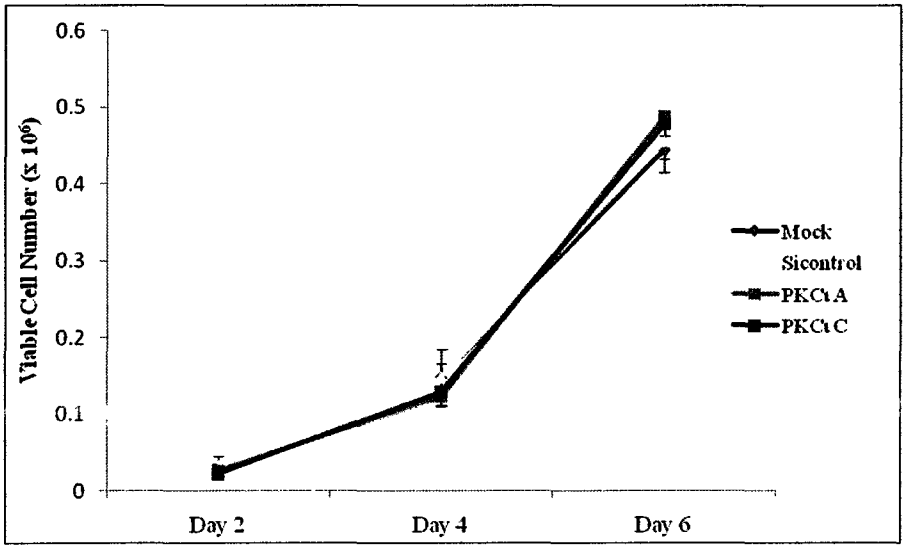
Figure 3.11 PKCι does not promote proliferation in normal mammary breast cells by inducing senescence

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 condtion. For the proliferation assays, hTERT-HMEC cells were plated in duplicate for each condition and treated with siRNA to PKCι. Cells were then counted 48 hours, 96 hours and 144 hours using trypan-blue exclusion to determine cell viability and cell number.

MCF10A



hTERT-HMEC



collected protein at Day 2, Day 4 and Day 6 post-siRNA transfection. Even six days after siRNA transfection, we see knockdown of PKCι (**Figure 3.12**). Similar knockdown in MCF7 cells is seen with siRNA treatment (**Figure 3.12**). This indicates that PKCι 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 PKCι does not increase p27 levels

It has previously been demonstrated in our lab that PKCι regulates cyclin dependent kinase inhibitor p27 levels in glioblastoma (M.Sc thesis, Jana Gilles). In glioblastoma cell lines depleted of PKCι 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 PKCι 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 PKCι, 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 PKCι 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 PKCι depletion and MEK inhibition. Therefore, PKCι does not appear to regulate p27 levels in breast cancer.

Figure 3.12 PKCι 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, PKCι A, and PKCι 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 PKCι expression. GAPDH was used as a loading control.

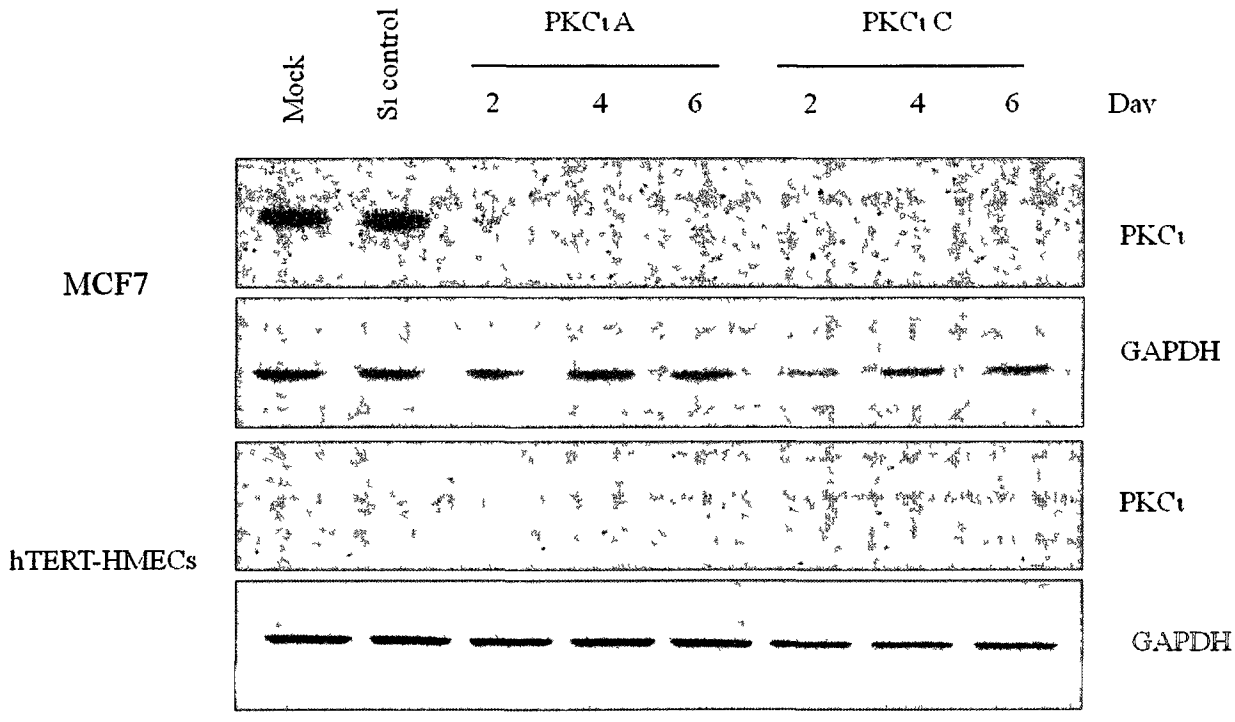
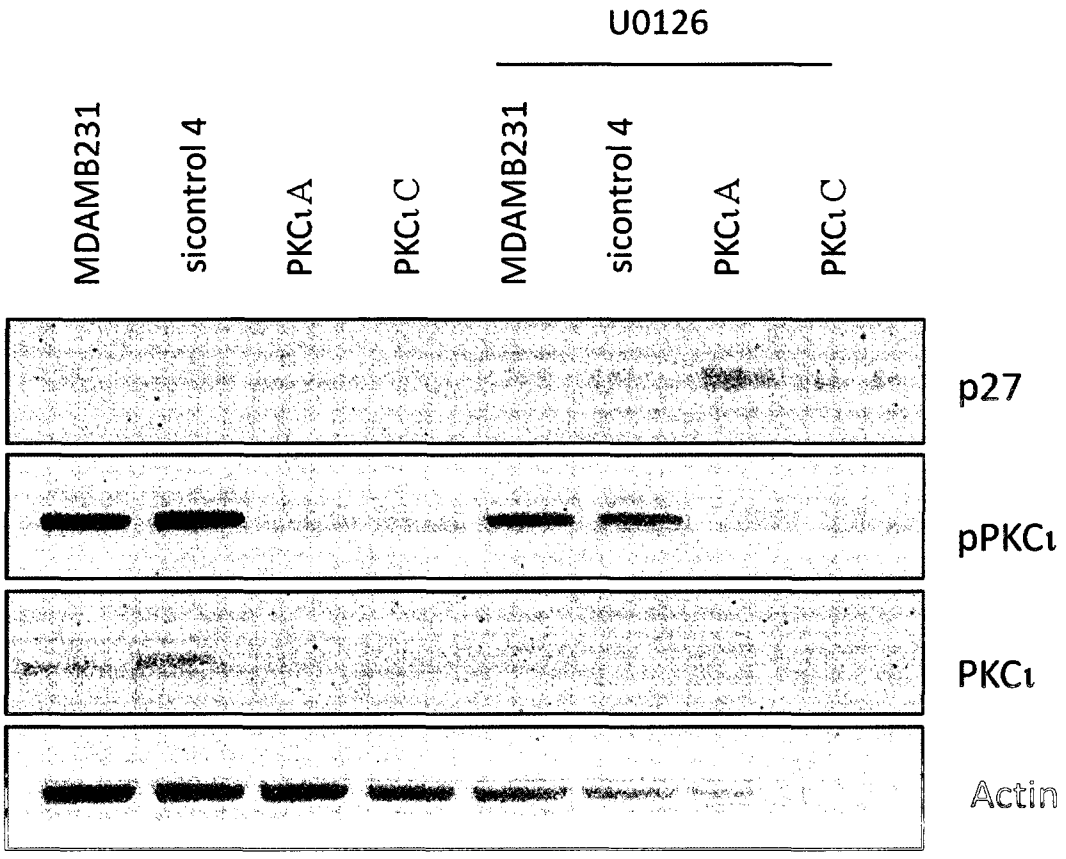

Mock
Si control
PKCι A
PKCι C
2
4
6
2
4
6
Dav
MCF7
PKCι
GAPDH
hTERT-HMECs
PKCι
GAPDH

Figure 3.13 Depletion of PKCι 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 μM U0126 or an equal volume of DMSO as a control. 24 hours later, cell lysates were collected and analyzed by Western Blotting.


U0126
MDAMB231
sicontrol 4
PKCιA
PKCιC
MDAMB231
sicontrol 4
PKCιA
PKCιC
p27
pPKCι
PKCι
Actin

3.10 Knockdown of PKCι does not increase levels of γH2AX

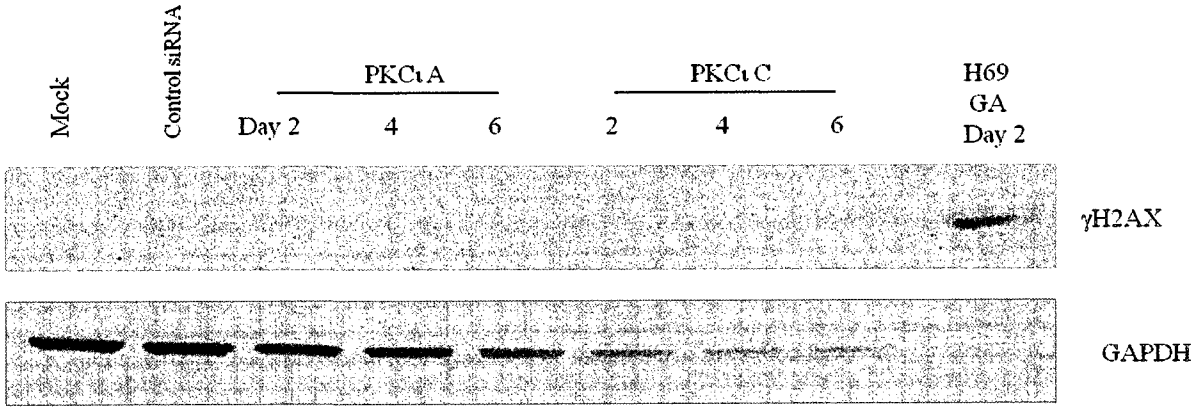
DNA damage, either telomeric or non-telomeric, is the currently best characterized inducer of senescence[84]. DNA damage checkpoints are activated by DNA double-strand breaks and can initiate senescence through activation of several important proteins such as p53, ATM, CHK2 and chromatin regulators such as phosphorylated histone H2AX (γ-H2AX) [82]. γ-H2AX is used as a marker of DNA-damage foci using Western blotting or by immunofluorescence. No induction in γ-H2AX was observed with PKCι 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 γ-H2AX[110]

3.11 Systems to analyze PKCι function in vivo

Our lab has used transient RNAi knockdown or stably-expressing RNAi cell lines to analyze the effects of PKCι 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 PKCι 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 PKCι depletion does not increase levels of γH2AX in MCF7 cells

We used MCF7 cell lysates, previously used to show PKCι knockdown, to determine if γH2AX levels increased. Lysates were analyzed by Western blotting and antibodies to γH2AX and GAPDH.

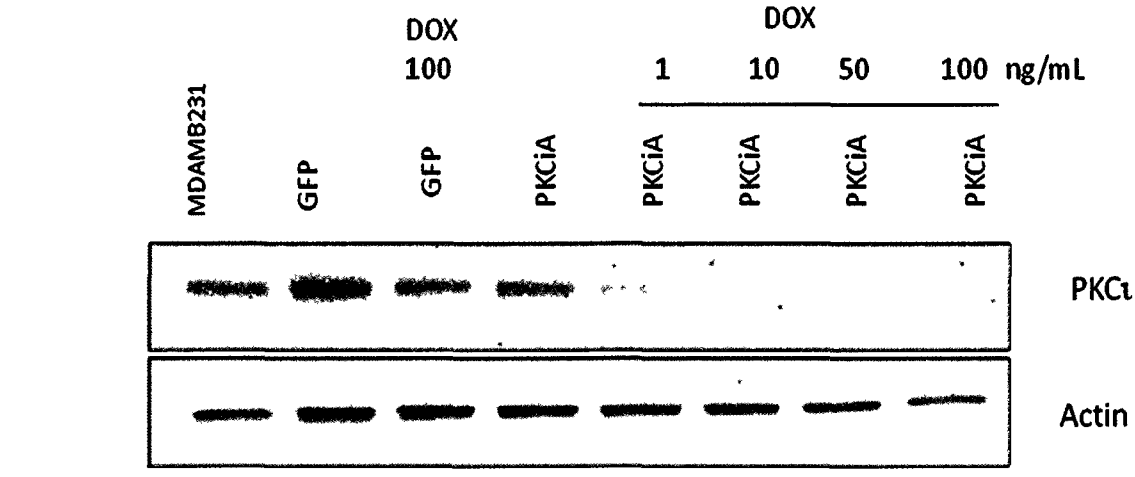

Mock
Control siRNA
PKCι A
Day 2
4
6
PKCι C
2
4
6
H69
GA
Day 2
γH2AX
GAPDH

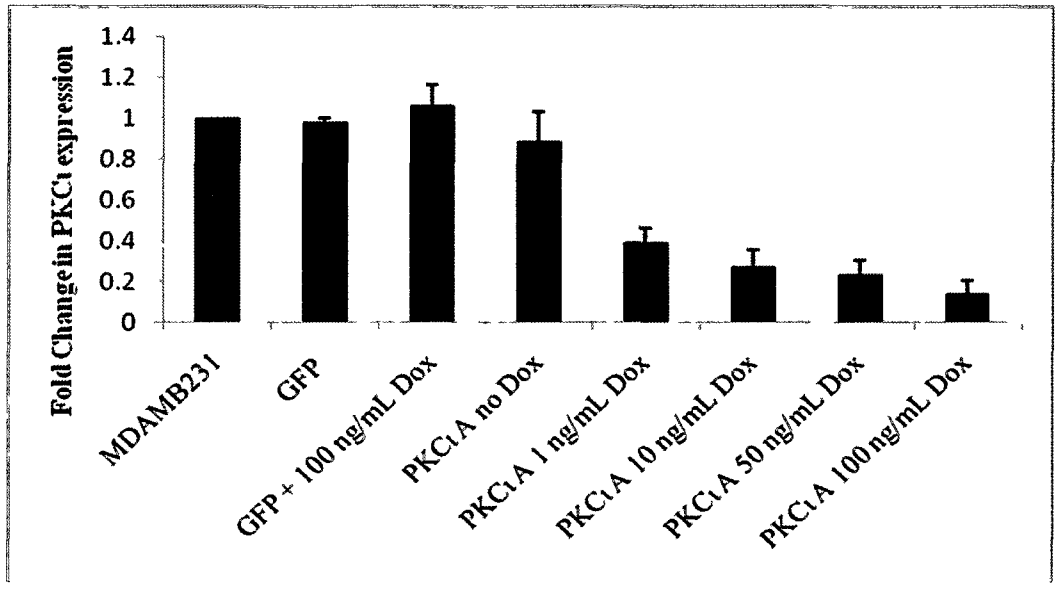
hairpin RNA both *in vitro* and *in vivo* and can mediate a longer duration of knockdown compared to RNAi[111, 112]. The lentiviral system we have used is an inducible system which has all the components in one vector: tetracycline repressor protein, shRNA sequence and puromycin resistance[113]. Lentivirus 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 PKCι by Western blotting. With the lentiviral system, after three days we see knockdown of PKCι 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 PKCι by approximately 60% with higher amounts of crude virus.

Figure 3.15 Lentiviral and Adenoviral Systems to express shRNA to PKCι

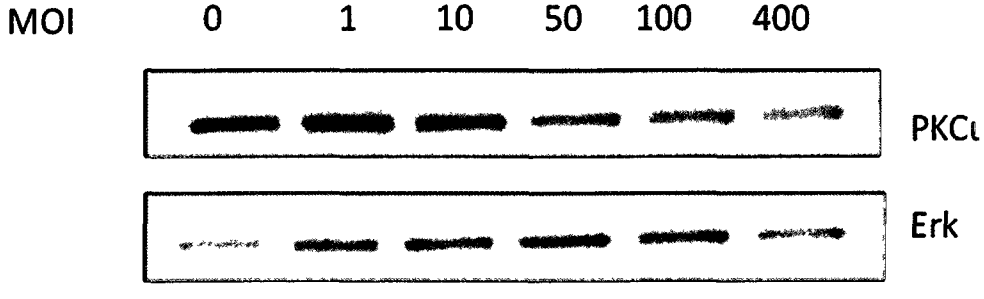
A. *Lentiviral System.* MDAMB231 cell lines were generated and tested with increasing amounts of Dox for 72 hours before cell lysates were collected. Three separate experiments were quantified to determine amount of knockdown.

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.


DOX
100
DOX
1
10
50
100 ng/mL
MDAMB231
GFP
GFP
PKCiA
PKCiA
PKCiA
PKCiA
PKCiA
PKCι
Actin


Fold Change in PKCι expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
MDAMB231
GFP
GFP + 100 ng/mL Dox
PKCι A no Dox
PKCι A 1 ng/mL Dox
PKCι A 10 ng/mL Dox
PKCι A 50 ng/mL Dox
PKCι A 100 ng/mL Dox

B.


MOI
0
1
10
50
100
400
PKCι
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.

PKCι is a known downstream effector of the PI3K pathway. PKCι 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 PKCι in breast cancer. Only one study has previously looked at PKCι expression in breast cancer tissue samples and they only investigated IDC samples[68]. It is important to further examine PKCι expression not only to validate this result but also to determine if PKCι 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 PKCι 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 PKCι is over-expressed in approximately 70% of breast cancers (n=157, **Table 3.1**). This is comparable to the previous study which found PKCι over-expressed in 80% of IDC cases (n=110) [68]. Over-expression of PKCι suggests that PKCι could be an important downstream effector in malignant breast cancers.

PKCι 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 PKCι expression[68]. Elevated PKCι 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 PKCι was present in colon tumours when compared to normal tissue[58]. In some tumour types, such as ovarian and lung, PKCι gene amplification was found[57, 64]. This has not been assessed in breast cancers and may also contribute to the upregulation of PKCι expression. However, over-expression of PKCι 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 PKCι 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 PKCι 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 PKCι expression compared to MCF10A and hTERT-HMEC normal mammary epithelial cells. MDAMB231 cells had a 2.5-fold increase of PKCι while the BT549 cells did not have increased expression of PKCι. The elevated expression of PKCι in breast cancer cell lines mirrors the PKCι expression pattern seen in the breast cancer tissue microarrays. When pPKCι levels were normalized to PKCι 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 PKCι activation. PKCι 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 *PIK3CA* activation mutations, E545K and H1047R respectively. We tested whether *PIK3CA* mutations alone are sufficient to increase in PKCι activation and expression *in vitro*. Using retroviral transduction, we found that *PIK3CA* mutations increased expression and activation of PKCι 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 PKCι. We did not see an increase in PKCι 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 PKCι 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 PKCι 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 PI3-kinase found in MCF10A, causing the large decrease in activation shown. Another possibility is that there is also a PI3K-independent mechanism of PKCι activation that could be responsible for the low level of activity in wild-type PI3K cells. While not extensively studied, PI3K-independent activation of PKCι 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 PKCι 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]. We

were able to detect PKCι over-expression in DCIS and in cases of hyperplasia and this could be attributed to mutations in *PIK3CA* at these early stages. In a cell model system for studying breast cancer progression, it was demonstrated that *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 PKCι activity also contributes to tumour progression.

We observe a 70% overexpression of PKCι, yet *PIK3CA* mutations are only present in approximately 30% of breast cancers[17, 26-29]. Therefore there must be alternate mechanisms to increase PKCι 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 PKCι and provide an explanation why we see higher frequency of expression of PKCι in the tissue microarrays than would be expected for *PIK3CA* mutations alone.

To investigate possible functions of PKCι in breast pathogenesis, we utilized siRNA to silence the expression of the kinase. In proliferation assays, we found that knocking down PKCι 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 PKCι and observed a decrease in proliferation in MCF7 cells after seven days[70]. The involvement of PKCι in promoting proliferation has also previously been demonstrated in

glioma by two separate groups[103, 124]. They both found that targeting PKCι 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 PKCι only affected anchorage-independent growth[63, 125]. This discrepancy could be due to cell-type specific functions of PKCι.

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 PKCι did not induce apoptosis using flow cytometry (**Figure 3.7**). There was no increase in hypodiploid cells with knock down of PKCι in MCF7 or MDAMB231 cells. These results are consistent with findings in intestinal epithelial cells, pancreatic carcinoma and NSCLC where depletion of PKCι did not induce apoptosis [57, 58, 125]. It was also previously shown *in vivo* that pharmaceutical PKCι 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 PKCι. SA-β-Gal is the most commonly used and consistent marker of senescence and has been used to detect senescence both *in vitro* as well as *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-β-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 PKCι (**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 PKCι knockdown causes premature senescence, BrdU incorporation was analyzed to show that there is a decrease in actively proliferating cells when PKCι is depleted (**Figure 3.10**). There was a decrease of approximately 10 to 15% in cells depleted of PKCι 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 PKCι 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 PKCι, 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 PKCι 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 PKCι (**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 PKCι (**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 PKCι 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 PKCι was being depleted over the time course analyzed (**Figure 3.12**). While there was a depletion of PKCι protein levels, there was no effect on proliferation or SA-β-Gal activity. This suggests that the induction of premature senescence upon PKCι 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 PKCι prevents the premature induction of senescence in breast cancer cells. While this is a completely novel function for PKCι, inhibition or depletion of PKCι 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 PKCι causes senescence is unknown. Determining the molecular mechanism by which premature senescence is being induced with PKCι knockdown will be difficult for a few reasons. Few downstream targets of PKCι 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 $HRAS^{G12V}$ and BRAF-driven senescence in human melanocytes does not require p53 or p16[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[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 PKCι 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 PKCι.

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[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[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 PKCι 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 PKCι 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]. PKCι is a downstream kinase in the Akt pathway and may mediate this nuclear translocation event. PKCι may function as an important kinase to phosphorylate either one of these sites to promote cell cycle progression. When PKCι 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 i*n vivo* significance of PKCι knockdown we needed to develop suitable systems to target PKCι that could be used in animal models. While there are small molecule PKCι inhibitors being developed, to date these are not effective or selective. Therefore we decided to develop systems which can express shRNA to PKCι. 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 PKCι 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 PKCι 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 1ng/mL of dox. There is a dose-dependent induction of the shRNA as we see the highest knockdown of PKCι with 100 ng/mL of dox. While not as effective as the lentiviral system, we also developed an adenoviral system to express shRNA to PKCι. With the assistance of the McKay lab, we were able to prepare crude adenovirus. We infected MDAMB231 cells and saw approximately 60% knockdown of PKCι 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 *in vivo* but also be able to have consistent long-term expression of shRNA during *in vitro* experiments. In addition, SA-β-Gal and BrdU are established markers of senescence *in vivo* [reviewed in[88, 156]] and would enable the *in vitro* results in this study to be further assessed in xenograft models of breast cancer.

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

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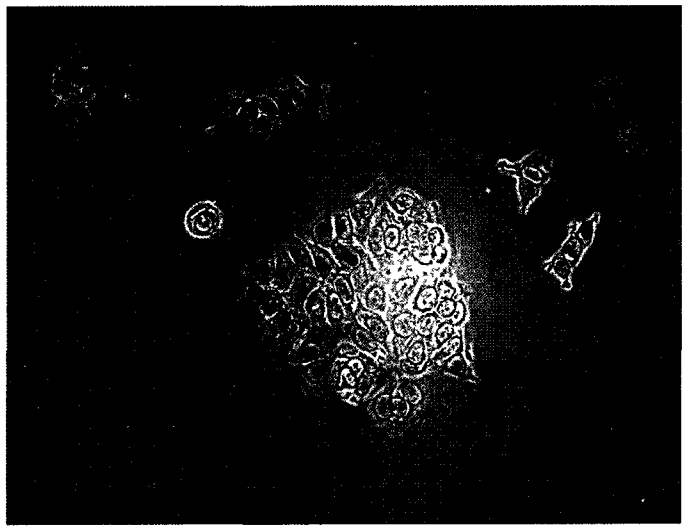
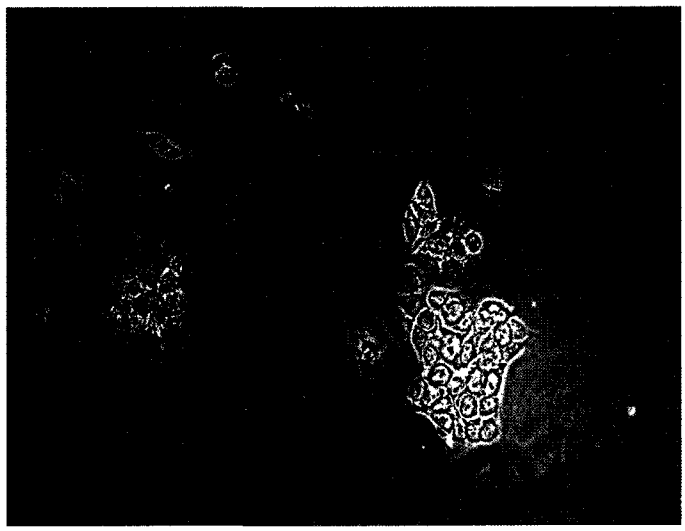
Appendix I



MCF7



T47D

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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Published Abstracts

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The Expression of PKCι in Breast Cancer
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