Functional Role of Protein Kinase C Alpha in Endometrial Carcinogenesis

Alice Hsu
University of Nebraska Medical Center

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FUNCTIONAL ROLE OF PROTEIN KINASE ALPHA IN ENDOMETRIAL CARCINOGENESIS

By

Alice H. Hsu

A DISSERTATION

Presented to the Faculty of
the University of Nebraska Graduate College
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

Cancer Research Graduate Program

Under the Supervision of Professor Jennifer D. Black

University of Nebraska Medical Center
Omaha, Nebraska

April 2018

Supervisory Committee:
Adrian R. Black, Ph.D. Robert E. Lewis, Ph.D.
Adam R. Karpf, Ph.D. Jing Wang, Ph.D.
Kay-Uwe Wagner, Ph.D.
This work is dedicated

To Nik,
Who led me in to the door

To Scott,
Who showed me how to have fun

To Jenny and Adrian,
Who gave me guidance and freedom to grow
Acknowledgements

This work would not have been possible without the support and guidance from Jenny and Adrian, who have been and always will be my inspiration and role models. Under their, I have learned how to be critical with respect, be assertive but considerate, hold oneself to the highest standard but treat others with absolute grace. They have shown me the value of hard work and persistence, and tirelessly invested their time and energy to ensure my success in every experiment, every seminar, all the bits and pieces of writing and posters I have presented over the years, and most of all, as a scientist and a person. I truly could not have hoped for a better environment for my graduate training and am honored to be a member of the Black lab.

I have had the pleasure of working with wonderful colleagues from the Black lab, both past and present – Katie, Fang, Michelle, Misty, Xinyue, Navneet, Lisa, and Shane. Despite our different personalities, we have managed to create a cohesive, calming, productive, and accepting working environment that made it easy to hop and skip back to work the next morning following a long, frustrating day.

I would also like to express my gratitude to my committee members, Adrian Black, Adam Karpf, Rob Lewis, Jenny Wang, and Kay Wagner for the insightful comments regarding our project and monitoring the development of my graduate career. They have generously provided their perspective to better our research, as well as imparted wisdom and encouragement to make me a better scientist.

Additionally, I want to thank all the support I have received from UNMC especially Lijun Sun and Jiang Jiang from the Tissue Science Facility for their technical support for the extensive tissue work this project encompassed, our neighboring labs – the Dong lab, Lewis lab, and Rizzino lab for welcoming us into DRC and creating a harmonious research environment. I would also like to acknowledge those who have been my companions during my graduate studies, especially Erin, Beth, and Carter for challenging me both intellectually and personally.
In this long, testing journal of my graduate career, I have been blessed to be accompanied by an army of incredible friends. We have shared the important milestones of our lives with each other, celebrated many happy memories, but most important, when the going got hard, they have pulled me through the tough moments. I once read “Friends were family one chose”, and I am proud to say they are one of my best choices. Thank you Kelly L., Joanne, Simon, Daniel, Patrick, Tzu-an, Jay, Danny, Jackal, Moashih, Enkang, Wa, Fang, Dianmu, Varun, Wanchi, and many more.

Finally, I would like to thank my parents, brother, and husband for their unconditional trust and support, who kept their distance to allow me the space and freedom to be unapologetically me but always know when to step in to assure me they are still behind me and will always be. Hope I have done them proud.
Functional Role of PKCα in Endometrial Carcinogenesis

Alice H. Hsu, Ph.D.

University of Nebraska Medical Center, 2018

Supervisor: Jennifer D. Black, Ph.D.

Protein kinase Cα (PKCα) is a member of the PKC family of serine/threonine kinases that regulate many fundamental cellular processes, including cell proliferation, differentiation, survival and transformation. The impact of PKCα on tumorigenesis, and whether it acts as an oncogene or tumor suppressor, has been debated over the years. The overall goal of this study was to establish the functional role of PKCα in endometrial carcinogenesis. Results from this study broaden our knowledge of PKCα signaling and add to our understanding of its role in tumor development.

As understanding of the role of PKCα signaling in the uterus is limited, we first examined the expression and activation of PKCα in the normal endometrium. Our analysis revealed cyclical activation of PKCα, linked to hormonal fluctuations and negatively correlated with epithelial proliferation, suggesting that PKCα is a growth inhibitory kinase in the endometrium.

Historically, PKC isoforms have been recognized as oncoproteins as they are activated by the tumor promoting phorbol esters. However, accumulating evidence indicates that PKCs can be inhibitory in some cancers, with recent findings propelling a shift in focus to understanding tumor suppressive functions of these enzymes. Here, we report that PKCα acts as a tumor suppressor in PI3K/AKT-driven endometrial cancer. PKCα deficiency, due to transcriptional repression, is associated with aggressive disease and poor prognosis in endometrial cancer patients. Activation of PKCα induces PP2A activity, leading to hypophosphorylation of AKT and potently inhibiting the growth of endometrial cancer cells under anchorage-independent conditions. In murine models, PKCα loss is a rate-limiting event for initiation of endometrial tumorigenesis, as demonstrated by
increased tumor burden in mice lacking PKCα.

More than 90% of endometrial cancer cases are characterized by AKT hyperactivation as the result of mutations in multiple PI3K/AKT pathway components, including *PTEN*, *PIK3CA*, *PIK3R1*, and *AKT*. Mutations in these molecules often coexist in EC, indicating that EC development requires full, unopposed activation of PI3K/AKT signaling. Results from our study show that PKCα is a negative regulator of AKT activation in the endometrium, which can override hyperactivation of the PI3K/AKT pathway in endometrial cancer cells. Loss of this kinase in the context of other molecular aberrations, such as mutations in PTEN or PI3K, leads to further hyperactivation of AKT, enhancing tumor cell growth. Together, our data point to PKCα as a crucial tumor suppressor in the endometrium, with deregulation of a PKCα→PP2A signaling axis leading to robust AKT activation and enhanced endometrial tumorigenesis.
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<tr>
<td>17-AAG</td>
<td>17-N-allylamino-17-demethoxygeldanamycin</td>
</tr>
<tr>
<td>4E-BP1</td>
<td>eIF4E binding protein</td>
</tr>
<tr>
<td>ACF</td>
<td>Aberrant crypt foci</td>
</tr>
<tr>
<td>ANX8</td>
<td>Annexin VIII locus</td>
</tr>
<tr>
<td>AOM</td>
<td>Azoxymethane</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>APC(^{Min/+})</td>
<td>Adenomatous polyposis coli gene with ( min ) mutation</td>
</tr>
<tr>
<td>aPKC</td>
<td>Atypical protein kinase C</td>
</tr>
<tr>
<td>ATF4</td>
<td>Cyclic AMP-dependent transcription factor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BASCs</td>
<td>Bronchoalveolar stem cells</td>
</tr>
<tr>
<td>BCL-X(_L)</td>
<td>B-cell lymphoma-extra large</td>
</tr>
<tr>
<td>BH3</td>
<td>Bcl-2 homology 3</td>
</tr>
<tr>
<td>BIM-1/BisI</td>
<td>Bisindolylmaleimides</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>Calcium</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukemia</td>
</tr>
<tr>
<td>COX2</td>
<td>Cyclooxygenase 2</td>
</tr>
<tr>
<td>cPKC</td>
<td>Classical or conventional protein kinase C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DAG</td>
<td>sn-1,2-diacylglycerol</td>
</tr>
<tr>
<td>DGK</td>
<td>Diacylglycerol kinase</td>
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<tr>
<td>DiC$_8$</td>
<td>1,2-dioctanoyl-sn-glycerol</td>
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<tr>
<td>DMBA</td>
<td>7,12-Dimethylbenz[a]anthracene</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E$_2$</td>
<td>Estrogen</td>
</tr>
<tr>
<td>E2F</td>
<td>E2 factor</td>
</tr>
<tr>
<td>EC</td>
<td>Endometrial cancer</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>Estrogen responsive elements</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>ERαKO</td>
<td>ERα knockout</td>
</tr>
<tr>
<td>ERβKO</td>
<td>ERβ knockout</td>
</tr>
<tr>
<td>Ets2</td>
<td>Protein C-ets-2</td>
</tr>
<tr>
<td>EU</td>
<td>Ethylene uridine</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FKBP12</td>
<td>12-kDa FK506-binding protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead box O</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
</tr>
<tr>
<td>GADD45</td>
<td>Growth arrest and DNA damage 45</td>
</tr>
<tr>
<td>GLI1</td>
<td>Glioma-associated oncogene homolog 1</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>Hh</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hypoxia inducible factor-1 α</td>
</tr>
<tr>
<td>HSP90</td>
<td>Heat shock protein 90</td>
</tr>
<tr>
<td>Id</td>
<td>Inhibitor of DNA binding</td>
</tr>
<tr>
<td>IEC-18</td>
<td>Intestinal epithelial cell-18</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>INPP4B</td>
<td>Inositol polyphosphate-4-phosphatase</td>
</tr>
<tr>
<td>IP₃</td>
<td>Triphosphoate</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KD-PKCα</td>
<td>Kinase dead PKCα</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LCM</td>
<td>Lasercapture microdissection</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>LKB1</td>
<td>Liver kinase B1</td>
</tr>
<tr>
<td>LOF</td>
<td>Loss of function</td>
</tr>
<tr>
<td>LSCC</td>
<td>Lung squamous cell carcinoma</td>
</tr>
<tr>
<td>MARCK</td>
<td>Myristoylated alanine-rich protein kinase C substrate</td>
</tr>
<tr>
<td>MCM</td>
<td>Minichromosome maintenance protein</td>
</tr>
<tr>
<td>miRNAs</td>
<td>Micro RNAs</td>
</tr>
<tr>
<td>MMMT</td>
<td>Malignant mixed Mullerian tumors</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSI</td>
<td>Microsatellite instability</td>
</tr>
<tr>
<td>MSS</td>
<td>Microsatellite stability</td>
</tr>
<tr>
<td>Mst1</td>
<td>Macrophage-stimulating 1</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>mTORC</td>
<td>Mammalian target of rapamycin complex</td>
</tr>
<tr>
<td>Muc2</td>
<td>Mucin 2</td>
</tr>
<tr>
<td>myr-AKT</td>
<td>Myristoylated AKT</td>
</tr>
<tr>
<td>NaF</td>
<td>Sodium fluoride</td>
</tr>
<tr>
<td>NEAA</td>
<td>Non-essential amino acids</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>nPKC</td>
<td>Novel protein kinase C</td>
</tr>
<tr>
<td>NRF2</td>
<td>Nuclear factor erythroid 2-related factor 2</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
</tr>
<tr>
<td>OAG</td>
<td>1-Oleoyl-2-acetyl-glycerol</td>
</tr>
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<td>Abbreviation</td>
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<td>-----------</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimum cutting temperature</td>
</tr>
<tr>
<td>P₄</td>
<td>Progesterone</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>Pax</td>
<td>Paired box</td>
</tr>
<tr>
<td>PB1</td>
<td>Phox Bem 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PDAC</td>
<td>Pancreatic ductal adenocarcinoma</td>
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<td>PDBu</td>
<td>Phorbol 12,13-dibutyrate</td>
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<td>PDK1</td>
<td>Phosphoinositide-dependent kinase-1</td>
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<tr>
<td>PHLPP</td>
<td>Pleckstrin homology domain and Leucine rich repeat Protein Phosphatases</td>
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<tr>
<td>PIK3CA</td>
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<td>PKB</td>
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<td>Protein kinase C</td>
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<td>Protein kinase D</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>POLE</td>
<td>DNA polymerase ε</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
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<td>Progesterone receptor</td>
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<td>PRAKO</td>
<td>PR-A knockout</td>
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<td>Proline-rich Akt substrate of 40 kDa</td>
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<td>PRBKO</td>
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<td>PRPS6</td>
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<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
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<tr>
<td>RAC1</td>
<td>Ras-related C3 botulinum toxin substrate 1</td>
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<tr>
<td>RasGRP</td>
<td>Ras guanyl-releasing protein 1</td>
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<td>RB</td>
<td>Retinoblastoma protein</td>
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<tr>
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<td>Ribonucleic Acid</td>
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<td>Reverse transcription polymerase chain reaction</td>
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<tr>
<td>RUNX2</td>
<td>Runt-related transcription factor 2</td>
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<td>Ribosomal protein S6 kinase beta-1</td>
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<td>SEM</td>
<td>Standard error of the mean</td>
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<td>SHIP</td>
<td>Src homology 2 domain-containing Inositol 5'-Phosphatase</td>
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<td>siRNA</td>
<td>Small interfering RNA</td>
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<td>SIRT1</td>
<td>Sirtuin 1</td>
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<td>SOX2</td>
<td>Sex determining region Y-box 2</td>
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<tr>
<td>SREBP1c</td>
<td>Sterol regulatory element binding protein 1c</td>
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<tr>
<td>T-ALL</td>
<td>T cell-acute lymphoblastic leukemia</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
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<tr>
<td>TCGA</td>
<td>The Cancer Genome Atlas</td>
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<td>TCPA</td>
<td>The Cancer Protein Atlas</td>
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<td>TGFβ</td>
<td>Transforming growth factor β</td>
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<tr>
<td>TMA</td>
<td>Tissue microarray</td>
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<td>TPA</td>
<td>12-\textit{O}-tetra-decanoylphorbol-13-acetate</td>
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<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
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<tr>
<td>TRAMP</td>
<td>Transgenic adenocarcinoma of the mouse prostate</td>
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<tr>
<td>TSC2</td>
<td>Tuberous Sclerosis Complex 2</td>
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<td>USDA</td>
<td>United States department of agriculture</td>
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<td>WT</td>
<td>Wild type</td>
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Chapter 1: Introduction
1.1 The protein kinase C (PKC) family

Protein kinase C (PKC) is a member of the family of AGC kinases (including protein kinase A, G, and C) that phosphorylates serine and threonine residues. It was discovered in the brain by Nishizuka and colleagues in 1977 as a kinase whose activation is dependent on the presence of phosphatidylserine (PS) and calcium (Ca^{2+}) (Inoue et al., 1977; Kishimoto et al., 1977). In 1979, the same group showed that PKC can be activated by sn-1,2-diacylglycerol (DAG), a membrane-associated lipid generated by phosphoinositide hydrolysis, in the presence of low Ca^{2+} (Kishimoto et al., 1980; Takai et al., 1979). Later, PKC was identified as a cellular target for tumor promoting phorbol esters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA, also referred to as phorbol 12-myristate 13-acetate (PMA)) (Castagna et al., 1982; Kikkawa et al., 1983), that bind to PKC at the same site as DAG. This study demonstrated the first link of PKC to cell proliferation and pointed to its potential contribution to carcinogenesis. After years of intensive study of these isozymes, PKC has been shown to be involved in the regulation of many fundamental cellular processes such as cell cycle progression, differentiation, cell survival and apoptosis. Subsequent studies have established the pathogenic association of PKC to important health concerns such as cardiovascular disease and diabetes (Ferreira et al., 2011; Geraldes and King, 2010; Inagaki et al., 2006). In the late 1980s, multiple isoforms of PKCs were identified by molecular cloning (Coussens et al., 1986; Parker et al., 1986). More than 30 years since its debut, current understanding defines PKC as a family of serine/threonine kinase consisting of ten isozymes, categorized into three classes based on their structure and coactivator requirements and has isozyme-specific, context-dependent functions.

1.1.1 Structure, maturation, and activation of PKC isozymes

There are three classes of PKC isozymes: the classical or conventional PKCs (cPKCs), the novel PKCs (nPKCs), and the atypical PKCs (aPKCs) (Figure 1-1). All PKC isoforms share
structural similarities, with an N-terminal regulatory domain linked by a flexible hinge region to the C-terminal carboxyl kinase domain. The catalytic C3 and C4 domains are conserved in all the PKCs, which is similar to the catalytic domain of protein kinase A. However, each class of PKCs has a unique regulatory domain that determines cofactor requirements for activation. The pseudosubstrate domain is common to the regulatory domains of all PKCs, and plays a central role in regulating PKC activity (House and Kemp, 1987; House et al., 1987). It contains a sequence that binds to the substrate binding pocket of the catalytic domain and blocks the substrate binding, keeping the kinase in its inactive state (House et al., 1987; Orr and Newton, 1994). The regulatory domain of cPKCs consists of two cysteine-rich motifs (C1A and C1B) in the C1 domain and a Ca$^{2+}$-sensitive C2 domain. The C1 domain binds to DAG and phosphatidylserine, while the C2 domain binds to Ca$^{2+}$. The presence of Ca$^{2+}$ in the C2 domain increases the affinity of cPKCs for DAG.
Thus, activation of cPKCs (including PKCα, the splice variants PKCβI and βII, and PKCγ) requires DAG, Ca\(^{2+}\), and phosphatidylserine. While the nPKCs also have two cysteine-rich motifs in their C1 domain, they have an “atypical” C2 domain that lacks Ca\(^{2+}\) binding residues. Differing from cPKCs, activation of the nPKCs (PKCδ, ε, η, θ) requires DAG and phosphatidylserine but not Ca\(^{2+}\), as DAG exhibits higher binding affinity for the C1B component of nPKCs than for the C1A domain of cPKCs before Ca\(^{2+}\) binding. The activating phorbol ester, PMA, binds to the C1B motif of the regulatory domain of both cPKCs and nPKCs to enhance their membrane association and activation. Unlike other members of the family, activation of the aPKCs (PKCζ, λ/ι) is independent of both DAG and Ca\(^{2+}\). Atypical PKCs have a variant of the C1 domain that has only one cysteine-rich motif and does not bind to DAG or phorbol esters. They also lack the C2 domain in their regulatory region. Instead, aPKCs have a Phox Bem 1 (PB1) domain that is involved in protein-protein interactions, providing the regulatory forces in their activation.
Maturation of PKC requires a series of highly regulated, ordered phosphorylation events at three distinct sites of the kinase domain (Figure 1-2). While these events do not directly activate PKC, each phosphorylation induces a conformational change that stabilizes the kinase in its inactive form and protects it from phosphatase activity. The first of the three priming phosphorylations occurs at the activation loop mediated by phosphoinositide-dependent kinase-1 (PDK-1) facilitated by mTORC2 and HSP90. The mature, phosphorylated PKC is then released into the cytosol. Subsequently, PLC-mediated lipid hydrolysis generates membrane associated DAG and increased intracellular Ca\(^{2+}\) levels. Interaction with DAG creates a strong bond between the phospholipid in the membrane and the open, activated PKC, allowing it to act on appropriate downstream substrates. Adapted from (Wu-Zhang and Newton, 2013)

**Fig. 1-2: Sequential maturation and activation of PKCs**

The newly synthesized, immature PKC associates with the membrane where it is sequentially phosphorylated at the activation loop, turn motif, and hydrophobic motif mediated by PDK-1, and facilitated by mTORC2 and HSP90. The mature, phosphorylated PKC is then released into the cytosol. Subsequently, PLC-mediated lipid hydrolysis generates membrane associated DAG and increased intracellular Ca\(^{2+}\) levels. Interaction with DAG creates a strong bond between the phospholipid in the membrane and the open, activated PKC, allowing it to act on appropriate downstream substrates. Adapted from (Wu-Zhang and Newton, 2013)
The newly synthesized PKC associates with the membrane in its open conformation, in which the substrate binding cavity is free of the pseudosubstrate domain, allowing PDK-1 to access the threonine residue at the activation loop (Dutil and Newton, 2000). It has been shown that cells lacking PDK-1 have markedly reduced PKC levels (Balendran et al., 2000). This phosphorylation initiates the maturation process of PKC and is required for subsequent phosphorylation (Dutil and Newton, 2000; Dutil et al., 1998). The second phosphorylation at the turn motif requires the mammalian target of rapamycin complex 2 (mTORC2), as cells lacking mTORC2 have diminishing levels of PKC (Facchinetti et al., 2008; Ikenoue et al., 2008; Jacinto and Lorberg, 2008). Despite its essential role in the phosphorylation of the turn motif, mTORC2 was unable to phosphorylate PKC in vitro (Ikenoue et al., 2008), raising the question whether mTORC2 mediates direct phosphorylation of PKC. It has been proposed that mTORC2 might act as a chaperone to position the newly synthesized PKC for priming phosphorylation or activates another upstream kinase that phosphorylates the turn motif. The last step of this priming process is the autophosphorylation at the hydrophobic motif. Both heat shock protein 90 (HSP90) and mTORC2 have been shown to modulate the phosphorylation at this motif (Gould et al., 2009; Ikenoue et al., 2008; Ziegler et al., 1999). However, this might be because mTORC2 is required for mediating the prerequisite phosphorylating event that is necessary for hydrophobic motif autophosphorylation. Completion of these sequential phosphorylation results in a mature, catalytically competent form of PKC. The fully phosphorylated PKC is then released from the membrane into the cytosol with its pseudosubstrate domain occupying the active site, preventing full activation of the kinase (Newton, 2010).

Extracellular signals trigger signaling events leading to the generation of cofactors that are required for PKC activation. Binding of ligands to their cognate tyrosine kinase receptors or G-coupled protein receptors stimulates signal transduction that elicits intracellular molecular changes such as activation of phospholipase C (PLC). PLC then catalyzes the hydrolysis of the lipid
phosphatidylinositol 4,5-bisphosphate (PIP2) to generate inositol 1, 4, 5-trisphosphate (IP3) and DAG, the physiological agonist of both cPKCs and nPKCs. DAG binds to the C1 domain of PKC and anchors it to the plasma membrane. Thus, membrane translocation serves as a readout for PKC activation (Kraft et al., 1982). IP3 binds to receptors in the smooth endoplasmic reticulum (ER) and promotes the release of Ca\(^{2+}\) into the cytoplasm, resulting in elevated intracellular Ca\(^{2+}\) concentrations. To compensate for the weaker DAG binding affinity to cPKCs, Ca\(^{2+}\) enhances the binding between DAG and the C1 domain and is required for cPKC but not nPKC activation. Because of their rapid metabolism at the cell membrane, DAG and its analogs are only transient PKC-inducing signals. Works from our laboratory and others has shown that the activation of DAG-stimulated PKC is acutely terminated by ‘reverse translocation’ - i.e., redistribution of the kinase to the cytoplasm (Akita, 2002; Di Mari et al., 2005; Leitges et al., 1996; Lum et al., 2016). Additionally, prolonged activation of PKC such as that mediated by phorbol esters or chronic DAG stimulation, is terminated by enzyme degradation.

1.1.2 PKC modulating agents

As PKC signaling is associated with diseases such as cancer (Griner and Kazanietz, 2007), hypertension, and diabetes (Ferreira et al., 2011; Geraldes and King, 2010; Inagaki et al., 2006), deciphering the roles of individual PKCs in signal transduction and the dysregulation of these processes is pertinent to further our understanding of human disorders. Pharmacological agents that modulate PKC activity are valuable tools for the study of effects of this kinase family. Many PKC modulating agents have been widely used experimentally, allowing for easy manipulation of PKC activity. Yet, because of their promiscuous nature, these agents are also the culprits of exacerbating the inherent complexity of PKC signaling, adding confusion to the interpretation of results from their use. This following section provides an overview of the commonly used PKC modulating agents, their mechanism of action and concerns that should be taken into account when
utilizing these compounds in experimental settings. Other pharmacological agents that also affect PKC activity are reviewed in detail in (Wu-Zhang and Newton, 2013).

There are two major types of PKC agonists: DAG and its analogs and phorbol esters. Both are capable of activating cPKC and nPKC through their interaction with the C1 domain, but do not activate aPKC. As activation of PKC occurs at the plasma membrane (or possibly in other membrane compartments), all of these compounds promote membrane translocation of the enzyme, where it can interact with additional activating cofactors such as phosphatidylserine. In addition to binding to PKC, these compounds can also interact with other families of signaling molecules that also have the C1 domain (e.g. PKDs, RasGRPs, and DGKs) (Blumberg et al., 2010). DAG, a product of PLC-mediated lipid hydrolysis, is the physiological agonist for typical C1-containing PKC isozymes. Of note, elevated intracellular Ca\(^{2+}\) concentrations can also increase DAG levels (Kunkel and Newton, 2010). The two short-chain synthetic analogs of DAG, DiC\(_8\) (1,2-dioctanoyl-sn-glycerol) and OAG (oleoacetyle glycerol), have improved aqueous solubility compared with DAG and are more commonly used in laboratories. DAG and its analogs are rapidly metabolized in the cell, therefore, only inducing transient PKC activation. Although these compounds can bind and activate both classical and novel isozymes, they exhibit preferential binding to the C1B motif in nPKCs and interact with the C1A motif of cPKCs at reduced affinity. Interestingly, rather than serving as an activating signal, interaction with the lipid kinase, DGK, converts DAG to phosphatidic acid (PA) to abrogate its signaling ability (Baldanzi et al., 2016; Cai et al., 2009).

Tumor promoting phorbol esters are the active component derived from croton oil (Van Duuren and Sivak, 1968). The most commonly used phorbol ester is PMA (phorbol 12-myristate 13-acetate), also known as TPA (12-O-tetradecanoylphorbol 13-acetate). The identification of PKC as a receptor for PMA establishes its first association to carcinogenesis (Castagna et al., 1982; Kikkawa et al., 1983). The phorbol ester derivative, PDBu (phorbol 12,13-dibutyrate) has optimized potency and reduced lipophilicity comparing to PMA, making it more water soluble for therapeutic use. These phorbol esters activate PKC by binding to the C1B motif. They are much
more potent PKC activators than DAG, effective at the nanomolar range. Unlike DAG and its analogs, phorbol esters are not readily metabolized and are capable of generating prolonged activation of PKC. Despite their shared high potency as PKC activators, the phorbol ester derivatives can induce different cellular responses (Blumberg, 1980, 1981).

The C1 ligand, bryostatin-1 (Kunkel and Newton, 2010), provides an example of a PKC agonist that can produce different effects when compared with PMA. Bryostatins are macrocyclic lactones that also bind to the C1 domain. Although these large complex molecules elicit the same effects as phorbol esters, albeit transiently, they actually antagonizes tumor formation (Blumberg et al., 2010). It is unclear how bryostatin-1 acts like a phorbol ester (binding to the C1 domain) to activate PKC on the one hand, while failing to contribute to carcinogenesis on the other. However, this unique property has brought bryostatin into clinical trials for cancer and Alzheimer’s disease (Kortmansky and Schwartz, 2003; Mayer et al., 2010; Roffey et al., 2009).

The vast majority of effective PKC inhibitors are small molecules that target the ATP-binding site of PKC. These ATP-competitive PKC inhibitors have been the focus of the development of PKC-targeting drugs. There are two distinct chemical classes of these prototypical ATP-binding inhibitors, bisindolylmaleimides and indolocarbazoles. All of the currently known PKC inhibitors show some degree of promiscuity toward other kinases, despite some of them being highly selective for PKC. While these active site inhibitors target all three classical or novel PKCs, the inhibition effect is selective, rather than specific for certain isozymes (Wu-Zhang and Newton, 2013). For example, the non-isoenzyme specific inhibitors Gö6983 and bisindolylmaleimide 1 (BIM-1 or BisI), when used at 500 nM, have been shown to be more effective at inhibiting PKC activity than other kinases (Wu-Zhang and Newton, 2013). Other bisindolylmaleimides, including Ro-31-8220 and Ro-32-0432 developed by Roche, have been marketed as PKC inhibitors but also have the non-selective nature of other similar compounds. Among the ATP-competitive PKC antagonists, Gö6976 and staurosporine are the more widely used indolocarbazole-based inhibitors. The pharmacological properties of Gö6976 have been characterized in multiple kinase inhibitor
studies (Anastassiadis et al., 2011; Bain et al., 2007; Davies et al., 2000), demonstrating that it is highly selective for cPKCs at the nanomolar range while having limited effects on other isozymes or non-PKC kinases. Thus, Gö6976 is commonly regarded as a cPKC selective inhibitor. On the other hand, staurosporine is one of the most promiscuous commercially available kinase inhibitors. It binds to and potently inhibits all cPKC, nPKC, and even αPKC, as well as other non-PKC kinases (Anastassiadis et al., 2011; Davis et al., 2011; Karaman et al., 2008).

Structure-based PKC inhibitors targeting less conserved domains have been designed with the hope of inhibiting these kinases with improved specificity. One example of these allosteric inhibitors is BisIV. Despite its chemical similarity to BIM-1 and its specificity for PKC, it is a much less potent inhibitor. Other non-active-site inhibitors targeting DAG-binding sites (e.g. calphostin C), the PKC priming kinases PDK-1 and mTORC2, or the maturation process by inhibiting HSP90 (e.g. 17-AAG), have also been employed to inhibit PKC activity. In addition to weak PKC inhibition, these agents also affect other molecular processes and present their own sets of caveats as PKC inhibitors.

Incorporation of PKC modulating agents in understanding the signaling roles of these kinases is a double-edged sword for the PKC field. While these agents allow in vitro manipulation of PKC activities and alteration of its interaction with other signaling molecules, their selective but non-specific nature makes it difficult to link the induced effects to PKC function. Therefore, interpretation of results generated from experiments using PKC modulating agents must be critically reviewed and confirmed with genetic approaches to exclude the involvement of other molecules that might also be targeted by these compounds.

1.1.3 Functions of PKC isozymes

PKC signaling is involved in a wide variety of molecular pathways, among which impact on cell cycle control and apoptosis are the most well-studied cellular processes influenced by this kinase family. Progression through the cell cycle is driven by the enzymatic activity of cyclin-
dependent kinases (Cdks) regulated by their association with cyclins, whose levels are determined by the cellular environment (e.g., mitogenic signals or DNA damage) (Otto and Sicinski, 2017). PKC signaling can exert either positive or negative regulation of cell cycle progression primarily at the G1/S and G2/M transitions. At early G1, pocket proteins such as the retinoblastoma protein (RB), p107, and p130 bind to and inhibit the transcriptional activity of growth-related E2F transcription factors. Under appropriate mitogenic induction signals, Cdk4 and Cdk6 are activated by associating with one of the three D-type cyclins (D1, D2, and D3), leading to phosphorylation of pocket proteins, which relieves E2F repression and upregulates cyclin E (Cobrinik, 2005). The elevated cyclin E in late G1 binds and activates Cdk2 to initiate a program of transcriptional events necessary for S phase entry (Malumbres and Barbacid, 2005). Alternatively, when there are insufficient mitogenic signals for Cdk activation or pocket protein phosphorylation, the cell halts cell cycle progression and enters G0, the quiescent phase of the cell cycle. Continued inactivation of pocket proteins at the end of G1 phase allows accumulation of cyclin A. Once the cell enters S phase, cyclin E is degraded through a proteasome-mediated mechanism to avoid re-initiation of DNA replication. Instead, Cdk2 binds to cyclin A to facilitate completion and exit from S phase. Cyclin A also binds to Cdk1 (Cdc2) at the end of S phase to sequester its activation. As the cell progresses through G2, cyclin A is degraded while there is active synthesis and accumulation of cyclin B. This results in the association of Cdk1 and cyclin B, triggering the cell to enter mitosis.

The effect of PKC signaling on cell cycle progression is highly dependent on the timing and duration of PKC activation, the isoforms present in the cell, and the cellular environment. Of the crucial molecules involved in cell cycle progression, PKC has been shown to mediate its effect through regulation of cyclin D1 and p21 at the G1/S transition (Figure 1-3) and lamin B at G2/M (Figure 1-4). At early G1, PKC signaling can lead to increased transcription of cyclin D1 (Li et al., 2006; Soh and Weinstein, 2003) and the destabilization of p21 (Walker et al., 2006), resulting in hyperphosphorylation of pocket proteins and progression into S phase. However, activation of PKC signaling at mid-to-late G1 can decrease the transcription and
translation of cyclin D1 (Hizli et al., 2006; Pysz et al., 2009; Soh and Weinstein, 2003) and induce p21 expression (Black, 2000; Griner and Kazanietz, 2007), leading to inhibition of Cdk4/6 and Cdk2 and cell cycle arrest at G0/G1. Lamin B is one of the essential proteins for nuclear structure and function. During G2, PKCβII activation has been shown to result in lamin B phosphorylation and the disassembly of the nuclear envelope, allowing the cell to enter M phase and proceed to chromosome segregation (Thompson and Fields 1996). Alternatively, PKC can negatively regulate the G2/M transition by downregulation of Cdc25, preventing the dephosphorylation and activation of Cdk1 (Arita et al., 1998; Barth and Kinzel, 1994; Kosaka et al., 1993). PKC signaling can also induce p21 levels, likely through an ERK-dependent mechanism (Arita et al., 1998; Barboule et al., 1999; Oliva et al., 2008) causing a delay in G2/M transition
Fig. 1-3: PKC-mediated regulation of G₁/ S transition

PKC-mediated regulation of the G₁-S transition can have either positive (upper portion) or negative (lower portion) effects. In early G₁, PKC activation increases cyclin D1 levels but leads to destabilization of p21, resulting in hyperphosphorylation of pocket proteins (e.g., Rb and p107) and progression into S phase. However, activation of PKC in mid-late G₁ reduces cyclin D1 levels while elevating p21. This results in hypophosphorylation of pocket proteins and G₀ arrest.

Adapted from (Black, 2010).
Fig. 1-4: PKC-mediated regulation of G₂/M transition

Similar to its impact on the G₁-S transition, PKC signaling can have opposing effects on G₂-M progression. PKC-mediated phosphorylation of nuclear lamins promotes nuclear lamina disassembly and G₂-M transition as depicted in the upper portion. Alternatively, PKC activation can block Cdc25 phosphorylation and result in accumulation of p21, in order to inhibit progression into M phase (bottom portion). Adapted from (Black, 2010).

Apoptosis is another fundamental cellular processes that is regulated by PKC signaling. Appropriate induction of apoptosis is important for the maintenance of tissue homeostasis and prevents diseases relating to abnormal cellular growth such as cancer. Apoptosis
pathways are defined by their upstream initiation signals; the extrinsic pathway is related to the activation of death receptors (e.g., TRAILR or Fas) and the intrinsic pathway is initiated by stimuli that trigger cytochrome c release from the mitochondria (Figure 1-5) (Ichim and Tait, 2016). The opposing regulatory roles of PKCε and δ in apoptosis are a clear example of the differential response of PKC isozymes as described in (Griner and Kazanietz, 2007) and (Reyland and Bradford, 2010). The pro-survival effect of PKCε has been attributed to its ability to activate ERK and AKT, which are two well-known pro-mitogenic, pro-survival effector molecules (Lu et al., 2006). This signaling crosstalk leads to the upregulation of pro-survival factors BCL-XL and XIAP and protects the cell against TRAIL-induced apoptosis (Pardo et al., 2006). On the contrary, activation of PKCδ can promote both intrinsic and extrinsic apoptosis. Upon DNA-damaging insult, PKCδ undergoes caspase-3 mediated proteolysis, freeing the catalytic domain of the kinase to translocate to the nucleus where it induces the phosphorylation of apoptotic related substrates (Bharti et al., 1998; Cross et al., 2000; Frasch et al., 2000; Yuan et al., 1998). Additionally, PKCδ translocates to the mitochondria and leads to cytochrome c release, which further amplifies caspase-3 activation (Denning et al., 2002; Li et al., 1999a; Majumder et al., 2000). Therefore, PKCδ also serves a positive feedback signal to trigger intrinsic apoptosis.

Much of our understanding of PKC functions is based on studies using non-specific phorbol esters and pharmacological agents, which makes parsing the functional role of each of the ten PKC isozymes a constant challenge of the field. Other factors that have contributed to the discrepancies in the field include lack of antibody specificity, inclusion of validated controls in the analysis, discordant levels of mRNA and protein expression, and the activation status of the enzyme (Garg et al., 2014; Leitges, 2007). Studies using genetic approaches can provide important functional insights of PKC enzymes. Despite some phenotypic abnormalities, mice lacking PKC isozyme do not exhibit developmental defects with the exception of PKCι, in which deletion of this enzyme was embryonic lethal (Suzuki et al., 2003), making it possible to examine the effect of PKC deletion in combination with other genetic backgrounds (e.g. mutant Kras or p53 deletion). Therefore,
utilizing both pharmacological and molecular approaches with the incorporation of transgenic models is critical for the elucidation of the role of PKC isozymes, especially in disease such as cancer.

**Fig. 1-5: Mechanism of PKCδ-mediated apoptosis**

Upon DNA damage, either by exposure to UV or DNA-damaging agents, PKCδ is cleaved by caspase-3, resulting in the release of its catalytic domain, which is constitutively active without its regulatory domain. The catalytic fragment can then translocate to the mitochondria to stimulate cytochrome C release and initiate the intrinsic apoptotic pathway as shown in part a. Alternatively, the cleaved catalytic fragment of PKCδ can translocate into the nucleus to phosphorylate substrates involved in cell cycle or apoptosis. The full-length PKCδ can initiate extrinsic apoptosis by modulating the response of death receptors as shown in part b. Adapted from (Griner and Kazanietz, 2007).
1.2 PKC in cancer

The observation that exposure to PMA, which activates PKCs, promotes carcinogenesis in the presence of an additional initiating event (Barrett, 1993; Blumberg et al., 1983) sparked decades of research to develop PKC-targeted therapeutics for cancer. However, these earlier efforts have been futile as most of the inhibitors that were developed showed limited benefit in clinical trials (Mochly-Rosen et al., 2012). As shown in later studies, this strategic failure was inevitable, due to the complex, context-dependent signaling roles of PKCs, their effects on carcinogenesis, and the promiscuous nature of the pharmacological agents. The reported enzyme depletion under prolonged activation raises the question whether tumor promotion is the result of PKC activity or its loss under prolonged stimulation, further challenging the role of PKC as a tumor promoting kinase. Using gene targeting approaches, PKC isozyme-specific knock-out transgenic mouse models were developed to identify and analyze PKC functions in vivo (Leitges, 2007). From the observations made in genetically modified PKC models, it is evident that the effect of PKC isozymes on carcinogenesis is isozyme-specific and cancer type-dependent (Garg et al., 2014). In human tumors, alterations in PKC expression can result in either increased or decreased levels of the enzyme (Garg et al., 2014). The following section summarizes the evidence supporting oncogenic as well as tumor suppressive properties of PKC from in vivo models and discusses the recent shift in the paradigm to focus on its tumor suppressive effects.

1.2.1 PKC as a tumor promoter

The concept of PKC as tumor promoter stemmed from its activation by phorbol esters, a class of compounds well-known for their ability to promote skin papilloma formation. In the two-step mouse model of skin carcinogenesis, the mutagen 7,12-dimethylbenz[a]anthracene (DMBA) was first applied to the skin. Subsequently, repeated exposure to phorbol esters led to the development of papillomas at the sites previously treated with DMBA (Figure 1-6) (Griner and
Kazanietz, 2007). Of note, no tumor was observed in mice treated with either DMBA or phorbol esters alone, suggesting that both agents have unique contributions to skin carcinogenesis. Interestingly, reversing the sequence of exposure to these agents (repeated application of phorbol esters followed by DMBA), or lengthening the time between doses of phorbol ester, was insufficient to form papillomas. These observations indicated that, in this model, DMBA was an “initiator” that acted to induce cell transformation, but was insufficient for tumor development. Additional insults to the initiated cells, such as exposure to phorbol esters at the appropriate strength and timing, function as “promoters” and are necessary to complete the transformation and result in tumor formation. As signal transduction molecules, several PKC isozymes have been implicated in important pathways that promote tumor growth and proliferation, including crosstalk with RAS/RAF/ERK and AKT signaling axes.
Fig. 1-6: Phorbol ester as a tumor promoter

In a mouse model, skin papillomas were only observed when treatment with a mutagenic initiator (e.g. DMBA) was followed by repeated, frequent exposure to a tumor promoter (e.g., phorbol ester). No tumors were observed when only the initiator or promoter were applied, the order of initiator and promoter application was reversed, or the time between administrations of promoter was extended. This model defines the multi-stage nature of carcinogenesis and demonstrates the tumor promoting properties of phorbol ester. Adapted from (Griner and Kazanietz, 2007).
Elevated levels of PKCβII, one of the cPKCs, has been reported in colon cancer patients (Davidson 1998, Spindler 2009) (although a recent study reported the opposite trend (Dowling et al., 2016)). Transgenic mice overexpressing PKCβII in the intestinal epithelium led to hyperproliferation of the colonic epithelium and heightened sensitivity to carcinogens. These mice showed increased formation of aberrant crypt foci (ACF), preneoplastic lesions of the colon, after 5-week administration of azoxymethane (AOM) compared with the wild-type (Murray et al., 1999). In this context, PKCβII functions as an oncoprotein through 1) activation of the Wnt/adenomatous polyposis coli (APC)/β-catenin pathway leading to decreased glycogen synthase kinase 3β activity (GSK3β), a known inhibitor of AKT signaling (Murray et al., 1999); and 2) induction of ERK activation through a RAS-PKCι/RAC1-MEK signaling axis (Zhang et al., 2004). Interestingly, the colonic epithelial cells from PKCβII-overexpressing mice showed reduced levels of other cPKCs, PKCα and PKCβI, suggesting possible opposing functions of these isozymes (Gokmen-Polar et al., 2001).

PKCε, a member of nPKC subclass, is another oncogenic PKC isozyme that acts through activation of the RAF/MEK/ERK pathway (Cacace et al., 1996). Overexpression of PKCε has been reported in many human tumor types including breast cancer, non-small cell lung cancer (NSCLC), and prostate cancer, and is associated with more invasive disease (Bae et al., 2007; Cornford et al., 1999; Schleifenbaum et al., 2004). In the TRAMP prostate cancer model, deletion of PKCε inhibited cancer development and metastasis (Hafeez et al., 2013), whereas its deletion in the epidermis protected the skin from UV-induced transformation (Hafeez et al., 2016). Consistent with its tumor promoting role in the prostate, tissue-specific overexpression of PKCε regulated by the probasin promoter led to elevated phosphorylation of AKT and activation of its downstream effectors S6 kinase and mTOR, as well as an increase in preneoplastic lesions (Benavides et al., 2011). In the epidermis, PKCε overexpression resulted in induced inflammation, hyperkeratosis, and hyperplasia (Jansen et al., 2001; Wheeler et al., 2004).
In recent years, PKCι has gained tremendous momentum as an oncogene and a prognostic marker in cancer. Accumulating studies have consistently demonstrated PKCι as an oncogene in many human cancers (Murray et al., 2011; Regala et al., 2005b). Because of its distinct features as an aPKC, PKCι has attracted much attention as a therapeutic target as inhibitors of this kinase might not affect other PKC isozymes. Overexpression of PKCι has been reported in many cancer types as summarized in (Murray et al., 2011). In most of these cancers, high PKCι expression is predictive of poor patient survival, recurrence, and metastatic disease (Eder et al., 2005; Ishiguro et al., 2009; Li et al., 2008; Murray et al., 2011; Regala et al., 2005b; Scotti et al., 2010; Weichert et al., 2003). Elevated PKCι mRNA and protein can be attributed to gene amplification as reported in NSCLC (Regala et al., 2005b), serous ovarian tumors (Eder et al., 2005; Zhang et al., 2006), lung squamous cell carcinoma (LSCC) (Regala et al., 2005b), and esophageal squamous cell cancer (Yang et al., 2008), or the result of aberrant signaling leading to transcriptional activation of PKCι (Gustafson et al., 2004). While overexpression due to gene mutation is possible, it is an unlikely culprit in this case as the most frequent mutation of PKCι affects the substrate binding dibasic motif rather than increasing its expression (Linch et al., 2013; Murray et al., 2011). Functional analysis of PKCι in vivo has focused on its oncogenic role in NSCLC, pancreatic ductal adenocarcinoma (PDAC), and ovarian cancer. Disruption of PKCι expression in cells harboring KRAS mutation inhibited the tumorigenicity of NSCLC and PDAC cells in a subcutaneous or orthotopic model, respectively (Regala et al., 2005a; Scotti et al., 2010). In these tumors, PKCι acts as a critical effector downstream of RAS, activating the RAC1/MEK/ERK signaling axis to drive cellular transformation (Murray et al., 2004; Regala et al., 2005a; Scotti et al., 2010). In ovarian cancer, PKCι is required for the maintenance of the tumor-initiating phenotype (Wang et al., 2013). Additionally, PKCι has been found to co-amplify with SOX2 in LSCC, and is required for hedgehog (Hh) signaling and activation of the related transcription factor, GLI1 (Justilien et al., 2014). These studies provided strong evidence for the ability of PKCι to functions as an oncogene. Unlike other isozymes which could have opposing roles in carcinogenesis depending on the cellular
context, currently, no studies have reported PKCι as having tumor suppressive properties, further cementing PKCι as a bona fide oncogene.

1.2.2 PKC as a tumor suppressor

Although PKC is well-known for being activated by the tumor promoting phorbol esters, several of these isozymes have been shown to inhibit cellular transformation and suppress tumor growth. One example is PKCα; mice lacking this kinase exhibited enhanced intestinal and lung tumorigenesis (Hill et al., 2014; Oster and Leitges, 2006). Using the DMBA/PMA skin tumor model, PKCδ, one of the nPKCs, was similarly identified to function as a tumor suppressor. Mice with elevated PKCδ expression were resistant to chemically-induced transformation (Reddig et al., 1999), but PKCδ deficiency did not have the same protective effect against UV radiation-induced squamous cell carcinoma (Aziz et al., 2006). PKCζ has received little attention compared with the other aPKCs, PKCι. While both up- and down-regulation of PKCζ has been reported in human cancers (Garg et al., 2014), observation from the Par4-deleted model suggested a tumor suppressive role of PKCζ. As mentioned in the previous section (chapter 1.1.1.), aPKCs are activated by protein-protein interaction; interaction with Par4 leads to PKCζ activation. Without this activating protein-protein interaction, as seen in Par4 knockout transgenic mice, reduced PKCζ activation enhanced Ras-induced lung carcinoma (Joshi 2008), supporting the tumor suppressive function of this PKC isozyme.

A recent survey of cancer-associated PKC mutations has propelled the paradigm shift to focusing on the tumor suppressive function of PKCs. In Antal et al., despite the previously reported low mutation frequency in genes encoding PKCs, analysis of 50 of the now more than 1000 mutations identified in these isozymes revealed that the majority lead to loss of function (LOF) of the kinase (Antal et al., 2015). Two-thirds of identified mutations were found to be inactivating while the rest were inert. These mutations can render the kinase inactive by impeding the binding of second messengers or preventing priming phosphorylation. Most of these mutations are
heterozygous, yet mice completely devoid of individual PKC isozymes were not embryonic lethal (with the exception of PKCι knockout mice) and exhibited limited phenotypic aberrations. Taken together, these observations raise the possibility that mutant PKC might have dominant negative functions, or can act as a scaffold to modulate signal transduction (Newton and Brognard, 2017). It is also likely that maintenance of a threshold of PKC signaling is necessary for its oncogenic effect – hyperactivation or complete loss of a signaling pathway can be detrimental to tumorigenesis (Chen et al., 2015). In many of these tumors, the tumor suppression exerted by PKC is dominant even in context of potent oncogenes such as KRAS (Antal et al., 2015; Hill et al., 2014), suggesting a regulatory role of PKC in hindering the effect of oncogenes. Taken together, these paradigm-shifting, yet conflicting views on the tumor-related properties of PKC isozymes indicate an incomplete understanding of PKC functions in tumorigenesis, and warrant a thorough examination of each isozyme in a tumor-specific manner to better understand the signaling role of PKC and its impact on tumor biology.

1.3 Protein kinase C alpha (PKC\(\alpha\))

PKC\(\alpha\) is a member of the cPKC subclass of PKC isozymes that are activated by DAG (in the presence of \(\text{Ca}^{2+}\) and phosphotidylserine) and phorbol esters. Experimentally, PKC\(\alpha\) can be stimulated by pharmacological activators such as PMA, DiC\(\text{s}\), and bryostatin-1 and inhibited by bisindolylmaleimide I (Bim-1; a pan-PKC inhibitor) and Gö6976 (a classical PKC inhibitor). PKC\(\alpha\) activity is involved in the crosstalk of many signaling pathways that regulate a plethora of cellular processes including cell cycle progression, differentiation, and apoptosis (Nakashima, 2002). Because this kinase is ubiquitously expressed in many tissue, the role of PKC\(\alpha\) in normal tissue development as well as tumorigenesis has been the focus of many studies. However, these efforts have generated conflicting results and further mystified the signaling impact of PKC\(\alpha\). The current consensus in regard to the function of PKC\(\alpha\) (and most other PKC isozymes) is that whether it
positively or negatively impacts cell growth or acts to promote or suppress tumor formation is dependent on the cellular context (Garg et al., 2014). As summarized in Griner 2007, overexpression of PKCα has been reported in glioma, breast and bladder cancers, while its downregulation was observed in cancers such as in the colon and renal cell carcinoma (Griner and Kazanietz, 2007) (Figure 1-7). Specifically, 60% of colorectal tumors reported loss of or reduced PKCα expression (Suga et al., 1998). In T cell acute lymphoblastic leukemia (T-ALL), low PKCα levels were predictive of poor clinical outcome (Milani et al., 2014).

*Ex vivo* manipulation of PKCα expression has generated evidence for the pro-tumorigenic function of this kinase in some cancer types. In glioma cells, PKCα enhances proliferation and confers resistance to radiation and chemotherapy agents (Baltuch et al., 1995; Blackburn et al., 1998; Cameron et al., 2008; Mandil et al., 2001). Interestingly, depletion of PKCα but not the inhibition of its activity impairs growth of glioma cell lines, indicating PKCα functions independent of its activity to promote the growth of glioma cells (Cameron et al., 2008). Similarly, ectopic expression of PKCα in MCF-7 breast cancer cells led to phenotypic changes consistent with increased tumorigenic potential, including increased proliferation, enhanced anchorage-independent growth, and change in cell morphology that was indicative of EMT (Ways et al., 1995). In addition, PKCα has been implicated in facilitating the loss of estrogen receptor (ER) expression in breast cancer cells (Tonetti et al., 2000; Ways et al., 1995), leading to more aggressive disease that is insensitive to tamoxifen (Chisamore et al., 2001). Assessment of human breast tumors further corroborated the correlation of elevated PKCα levels with reduced progesterone receptor (PR) expression, poor response to endocrine treatment, and worse prognosis in patients (Assender et al., 2007; Lonne et al., 2010; Tonetti et al., 2003).
Despite the evidence supporting an oncogenic role of PKCα, transgenic models demonstrated its tumor suppressive effect in both intestinal and lung carcinogenesis (Figure 1-8). In an Apc<sup>Min/−</sup> background, mice lacking PKCα developed more aggressive intestinal tumors, larger in size, and had poor survival compared with their PKCα wild type littermate controls (Oster and Leitges, 2006). Additionally, deletion of PKCα alone increased the number of spontaneous intestinal lesions independently of the Apc<sup>Min/−</sup> mutation. Another key evidence of PKCα as a tumor suppressor was from Fields and colleagues (Hill et al., 2014). As reported in this study, PKCα is

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Fig. 1-7: PKCα expression in human cancers

PKCα expression levels in multiple human cancer types based on the RNA-seq data generated from The Cancer Genome Atlas. Graph obtained from The Human Protein Atlas.
frequently lost in the tumors of lung cancer patients, and is associated with more advanced disease. In mutant Kras-driven lung tumorigenesis, PKCα deletion enhanced tumor development and progression by facilitating the bypass of oncogene-induced senescence and expansion of the tumor-initiating bronchoalveolar stem cells (BASCs). Mechanistically, PKCα acts as a tumor suppressor in BASCs through activation of p38MAPK-TGFβ signaling axis, in part through inhibition of the expression of inhibitor of DNA binding 1-3 (Id1-3), which promote cell proliferation and inhibit differentiation (Ling et al., 2006). PKCα has also been shown to negatively regulate Id family protein expression in colon cancer (Hao et al., 2011; Pysz et al., 2009). In the skin, PKCα confers protection against skin tumor formation in a two-stage carcinogenesis system using DMBA as an initiator and PMA as a tumor promoter (as previously described in Chapter 1.2.1) (Hara et al., 2005). However, there was no difference in tumor growth or progression between PKCα−/− or wild type mice, suggesting PKCα is involved in the early events of carcinogenesis but not progression of chemically induced skin tumors (Hara et al., 2012)
1.3.1 PKCα in intestinal homeostasis and carcinogenesis

The mammalian intestinal epithelium comprises many crypt-villus units that undergo constant renewal every 4-5 days (for the small intestine). Within the crypt reside the proliferating cells whose self-renewal capability repopulates the mucosal compartment. Starting at the crypt bottom, the self-renewing stem cells give rise to proliferating progenitor cells, establishing the proliferation zone. As these cells travel upwards along the crypt onto the villus, they undergo cell cycle arrest and become committed differentiated cells. Near the tip of the villus, the cells undergo apoptosis and eventually bud off into the lumen of the intestine (Figure 1-11 Picture of the small intestine).

In the intestine, knockout of PKCα alone increased the number of spontaneous lesions formed. When paired with an initiating event such as in $APC^{Min/+}$ and mutant $Kras$ mouse models, loss of PKCα significantly enhances intestinal and lung tumorigenesis. Adapted from (Garg et al., 2014)

**Fig. 1-8: PKCα as a tumor suppressor**

In the intestine, knockout of PKCα alone increased the number of spontaneous lesions formed. When paired with an initiating event such as in $APC^{Min/+}$ and mutant $Kras$ mouse models, loss of PKCα significantly enhances intestinal and lung tumorigenesis. Adapted from (Garg et al., 2014)
Emerging from the extensive studies of the intestinal system are the crypt base columnar stem cell and the +4 stem cell models, whose namesake defined the cell population responsible for intestinal regeneration as described in (Clevers, 2013; van der Flier and Clevers, 2009). The uniquely, well-defined progression from proliferation to differentiation as the cells migrate along the crypt-villus axis makes the intestinal epithelium a valuable system for studying how the intestine maintains its homeostasis and regeneration, as well as the aberrant molecular changes associated with its malignant transformation.

One of the pivotal role of PKC isozymes in cellular growth is to regulate components of the cell cycle machinery, primarily at the G₁→S and the G₂-M transitions (Black, 2000). Research from our laboratory has focused on understanding the role of PKCs (and PKCα in particular) in the tightly regulated intestinal regenerative process and its link to intestinal tumor development (Figure 1-9). In the non-transformed, immortalized rat intestinal epithelial cell (IEC-18) model system, our biochemical analyses detected multiple PKC isozymes, including α, βII, δ, ε, η, ζ and ι (Saxon et al., 1994). Among these isozymes, several PKCs (α, βII, δ, and ζ) are activated at the crypt-villus junction where the cells cease to proliferate and start to differentiate, as marked by their translocation from the cytoplasm to membrane compartments (Frey et al., 2000). This observation suggested that several PKC isozymes may be involved in negative regulation of intestinal cell proliferation. Data from our laboratory have further shown that activation of PKCs, and PKCα in particular, triggers a cascade of growth regulatory events, partly through an ERK-dependent mechanism (Clark et al., 2004). Contrary to the more well-known growth promoting property of RAS/RAF/ERK signaling, sustained ERK activation as caused by prolonged PKCα activation resulted in growth inhibitory effects (Clark et al., 2004). Additionally, selective inhibition of PKCα or inhibition of ERK signaling abolished the ability of PKC agonists to induce cell cycle withdrawal (Clark et al., 2004). Treatment of unsynchronized IEC-18 cells with non-selective pharmacological PKC agonists led to cell cycle arrest in late G₁ phase, as well as delayed transition through G₂/M. PKC activation resulted in induction of the known growth inhibitory cyclin-dependent kinase
inhibitors p21 and p27, decrease expression of p107 but increase expression of p130, as well as alterations in the expression and alteration in the phosphorylation of pocket proteins (e.g. p107, pRb, and p130) (Frey et al., 2000; Frey et al., 1997). These effects could be recapitulated by selective activation of PKCα alone (Frey et al., 2000). Furthermore, PKCα-mediated ERK activation was shown to downregulate growth promoting effectors (e.g., cyclin D1 and Id-1) through translational and/or transcriptional repression. Cyclin D1 senses extracellular mitogenic signals and promotes G1 transition through association with cdk4 and cdk6. In intestinal epithelial cells, PKCα signaling induces the binding of the translational repressor 4E-BP1 with the cap-dependent translational machinery, sequestering cyclin D1 mRNA and preventing its translation (Guan et al., 2007; Hizli et al., 2006). In the case of the inhibitor of DNA binding (Id1-4) family proteins, their decreased expression is attributed to the negative impact of PKCα activation on their transcription (Hao et al., 2011). Members of Id family proteins are dominant negative antagonists of antiproliferative, differentiation-inducing transcription factors such as basic helix-loop-helix factors, Ets2, and Pax family members. Binding of Id1 negates the transcription activating function of these factors by blocking their interaction with DNA. Interestingly, Id1 expression is generally restricted to the proliferating crypt, opposite of the pattern observed for PKCα activity in the intestine. Our work thus far demonstrated that PKCα helps to maintain intestinal homeostasis by acting as a negative regulatory signal of epithelial proliferation.
Fig. 1-9: PKCα activation in the intestinal systems

PKCα activation, as indicated by its membrane association, correlates with intestinal epithelial cell growth arrest and differentiation. V, villus; C, crypt; P, progenitor cells; L, lumen. Adapted from (Saxon et al., 1994).
The importance of PKCα as a gatekeeper for intestinal epithelial growth is further supported by the loss of its expression in intestinal tumors (Figure 1-10). Reduced PKCα expression is observed in multiple genetic models that develop intestinal neoplasms due to mutation or loss of *Apc, β-catenin, KRas,* and *Muc2* (Pysz et al., 2009) and PKCα deficiency sensitizes the animals to azoxymethane-induced carcinogenesis (unpublished data). Loss or reduction of PKCα expression is also observed in human colon tumors (Chen et al., 2016; Gwak et al., 2009; Hao et al., 2011). These findings strongly suggest that loss of PKCα is a general characteristic of intestinal tumors and is a critical step in colon tumor development. While PKCα signaling reduces intestinal tumorigenicity by blocking anchorage-independent growth of colon cancer cells, the disruption of PKCα signaling results in a growth promoting cellular milieu, through modulation of the transcription and/or translation of cyclin D1 and Id1 (Hao et al., 2011; Pysz et al., 2009). While both PKCα and δ expression are reduced in intestinal tumors, restoration of PKCα confers stronger antitumor effects in colon cancer cells. Consistent with previous results in IEC-18 cells, the ability of PKCα to regulate cyclin D1 and Id1 in colon cancer cells requires ERK activation (likely via crosstalk at the level of Raf-1), and is independent of effects on PI3K/AKT signaling (Hao et al., 2011). Our findings, together with the enhanced tumor formation in PKCα KO animals (Oster and Leitges, 2006), provide strong *in vivo* and *in vitro* evidence for the growth and tumor suppressive role of PKCα in the intestine.
Fig. 1-10: PKCa expression in lost in intestinal tumors

Expression of PKCa is lost in A) multiple in vivo models of intestinal tumors; and B) human colon cancer cell lines; and C) human colorectal tumors. Adapted from (Pysz et al., 2009; Verstovsek et al., 1998)
1.3.2 Impact of PKCα signaling in cancer

As mentioned in previous sections, the active form of PKCα resides at the plasma membrane, allowing it to transduce signals from upstream receptors to many signaling pathways that are important in cancer, regulating major cellular processes like proliferation, apoptosis, differentiation, and motility. However, the biological outcome of PKCα signaling can be influenced by the duration of its activation, the interacting substrates available, and the molecular context of the cell. In breast cancer, PKCα may confer estrogen-independent, tamoxifen-resistant growth through Notch and ERK signaling (Pham and Tonetti, 2016; Tonetti et al., 2003; Yun et al., 2013), whereas PKCα-mediated ERK activation led to growth inhibition in hepatoma and intestinal epithelial cells (Clark et al., 2004; Wen-Sheng and Jun-Ming, 2005). Additionally, PKCα has also been shown to promote cell invasion and migration through NFκB activation (Cheng et al., 2009; Mahanivong et al., 2008; Mut et al., 2010), while crosstalk with Wnt (Gwak et al., 2009) and Hedgehog (Neill et al., 2003) signaling pathways inhibits tumor growth. PKCα activity has been shown to have opposing effects on AKT phosphorylation. Overexpression of PKCα has been shown to induce AKT activation in immune cells (Li et al., 1999b; Yang et al., 2010), keratinocytes (Li et al., 2006), and endothelial cells (Partovian and Simons, 2004). In other cellular systems, PKCα activity was found to suppress AKT phosphorylation (Motley et al., 2002) and dampen the activity of other components of the PI3K signaling pathway (Motley et al., 2003; Sipeki et al., 2006). Despite the overwhelming evidence showing that PKCα indeed intercepts a wide network of signaling axes, understanding the biological impact of PKCα involvement requires careful examination in the cell type of interest. Our study presented here focuses on the role of PKCα on PI3K/AKT signaling, specifically its impact on endometrial carcinogenesis driven by hyperactivation of AKT.
1.4 **AKT signaling network**

The discovery and cloning of AKT, which is also named protein kinase B (PKB) because of its similarity with PKA and PKC, identified it as a serine/threonine specific kinase activated downstream of PI3K (Bellacosa et al., 1991; Coffer and Woodgett, 1991; Jones et al., 1991; Staal, 1987). The mammalian genome encodes three isoforms of AKT - AKT1 (PKBα), AKT2 (PKBβ), and AKT3 (PKBγ). Many cell lines express all three isoforms of AKTs; however, AKT1 and AKT2 are more ubiquitously expressed. Defining the isoform-specific functions of AKTs has been an exciting new frontier for the AKT field. Thus far, data from isoform-specific knockout models and RNA interference experiments have shown that AKT1 has significant developmental roles, AKT2 is involved in glucose homeostasis, and AKT3 is important for brain development (Dummler and Hemmings, 2007). The importance of AKT as a signaling molecule is underscored by its numerous downstream targets that regulate key biological processes (e.g. metabolism, growth and survival) to establish a pro-proliferative cellular milieu. To promote cell survival, AKT blocks the function and expression of pro-apoptotic BH3-only proteins. AKT activity leads to activation of mTORC1 signaling, which in turn promotes protein translation and cell growth. Additionally, AKT is involved in responses to insulin and other metabolic events such as nutrient uptake and lipid and glucose metabolism. The following sections will discuss the regulation of AKT activation, the functions of its downstream targets GSK3β, PRAS40, and FOXO proteins, and hyperactivation of PI3K/AKT signaling in cancer.

1.4.1 **Regulation of AKT activation**

When growth factors interact with their cognate receptor tyrosine kinases (e.g. EGFR, HER2, and KIT) or G-protein coupled receptors (GPCRs) (Katso et al., 2001; Vanhaesebroeck and Waterfield, 1999), PI3K is recruited to the cytoplasmic portion of the receptors either by direct interaction with p85 (the regulatory subunit of PI3K) or through an adaptor protein. The catalytic
subunit of PI3K, p110, then phosphorylates the lipid second messenger phosphatidylinositol-3,4-P₂ (PIP₂) to generate phosphatidylinositol -3,4,5-P₃ (PIP₃) at the plasma membrane (Fruman et al., 1998). Due to the high affinity of PIP₃ for the N-terminal PH domain of AKT, the accumulation of PIP₃ relocates inactive AKT to the plasma membrane. While PIP₂ also binds to the PH domain, it only induces partial AKT activation (Franke et al., 1997; Frech et al., 1997; Klippel et al., 1997). Binding of PIP₃ induces a conformational change in AKT, exposing two key residues – Thr308 in AKT1 and 2/ Thr309 in AKT3 in the kinase domain and Ser473 in AKT1/ Ser474 in AKT2/ Ser472 in AKT3 in the C-terminal regulatory domain – giving kinases access to carry out phosphorylation at these two sites for maximal AKT activation (Alessi et al., 1996). PIP₃ also recruits the phosphoinositide-dependent protein kinase 1 (PDK-1) to the membrane and puts it in proximity with AKT, leading to the phosphorylation of Thr308 (Alessi et al., 1997; Andjelkovic et al., 1997). The full activation of AKT requires the mechanistic target of rapamycin complex (mTORC2) to phosphorylate AKT at Ser473 (Sarbassov et al., 2005). A phosphomimetic mutant of AKT (T308D/S473D) generates constitutively active kinase that is PI3K independent (Alessi et al., 1996). Both PDK-1 and mTORC2 also phosphorylate other AGC kinases such as PKC and S6K1. However, unlike other kinases that are constitutively phosphorylated by PDK-1 and mTORC2, phosphorylation of AKT is PIP₃-obligatory (Collins et al., 2003; Huang et al., 2008; McManus et al., 2005; Sarbassov et al., 2005). Recent evidence has shown that the pool of PIP₃ and PIP₂ found on the endosomal membrane can also lead to AKT redistribution and activation, suggesting there is an additional layer of spatial control of AKT phosphorylation and function yet to be explored (Jethwa et al., 2015).

Termination of AKT signaling is achieved by a number of phosphatases that either counteract upstream lipid synthesis or directly dephosphorylate AKT. In the late 1990s, the phosphatase and tensin homolog (PTEN) was cloned and identified as a key regulator of PI3K signaling (Frech et al., 1997; Li et al., 1997; Maehama and Dixon, 1998). PTEN possesses dual phosphatase function and can, therefore, act on both phosphoinositide and polypeptide substrates.
The lipid phosphatase function of PTEN dephosphorylates PIP$_3$ at the 3-position and converts it to P(4,5)P$_2$, thus decreasing membrane PIP$_3$ concentration and negating the effects of PI3K. Because of this intricate balance of PTEN and PI3K functions, the lipid synthesis in response to growth factor stimulation is often rapid and transient (Auger et al., 1989). Alternatively, the Src homology 2 domain-containing inositol 5'-phosphatase (SHIP) can dephosphorylate PIP$_3$ at the 5-position and convert it to P(3,4)P$_2$. The phospholipid is further hydrolyzed by inositol polyphosphate 4-phosphatase (INPP4B) to P(3)P. This step-wise dephosphorylation of P(3,4,5)P$_3 \rightarrow$ P(3,4)P$_2 \rightarrow$ P(3)P preferentially acts on phospholipids at endosomes and loss of INPP4B leads to AKT2 activation (Braccini et al., 2015; Fedele et al., 2010; Gewinner et al., 2009; Li Chew et al., 2015). These observations suggest there is spatial restriction on the species of PI3K lipids present on membranes that might be involved in isoform-specific regulation of AKT activation. Together, PTEN, SHIP, and INPP4B negatively regulate PI3K-mediated lipid synthesis and reduce the membrane concentration of P(3,4,5)P$_3$. This reduction in membrane P(3,4,5)P$_3$ not only affects the recruitment and activation of AKT but also other PH-domain containing proteins including PDK-1, thus further exerting negative pressure on PI3K/AKT signaling (Manning and Toker, 2017; Salmena et al., 2008).

Activation of AKT can also be terminated through direct modulation of its phosphorylation. Specific phosphatases act directly on AKT by removing the phosphate group from Ser473 and Thr308, whose phosphorylation is critical for AKT activation. Protein phosphatase 2A (PP2A) represents a family of holoenzymes that effectively dephosphorylate AKT (Andjelkovic et al., 1996)). This serine and threonine specific phosphatase is ubiquitously expressed in eukaryotic cells. Its involvement in major signaling pathways that regulate processes such as cell cycle, metabolism, migration, and survival demonstrates that reversible phosphorylation is a crucial means to ensure proper firing and fine tuning of signaling events in cells (Janssens and Goris, 2001; Perrotti and Neviani, 2013; Schonthal, 2001; Seshacharyulu et al., 2013). The PP2A holoenzyme consists of a core dimer of the structural subunit (A) and the catalytic subunit (C). Its substrate
specificity is dictated by the regulatory subunit (B), which binds to the subunit A/C dimer. In humans, 26 different B regulatory subunits have been identified and are categorized into 4 subfamilies: B/B55/PR55, B'/B56/PR61, B''/B72/PR72, and B'''/PR93 (SG2NA)/PR110 (Striatin). While the isoforms of the A and C subunits share high sequence homology, the B subunit subfamilies are distinct in their sequences and structures, which confers their substrate selectivity and contributes to the diverse functions and location of PP2A holoenzymes (Chen et al., 2013; Eichhorn et al., 2009; Sents et al., 2013). The PP2A-B55alpha holoenzyme has been shown to mediate AKT dephosphorylation at Thr308 (Kuo et al., 2008), while the PP2A-B56 holoenzyme acts to reverse phosphorylation of Ser473 (Rocher et al., 2007; Van Kanegan et al., 2005), making this phosphatase a prominent negative regulator of PI3K signaling. In 2005, the Pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) was identified as another family of serine/threonine specific phosphatases that act on Ser473 of AKT (Gao et al., 2005). There are three members in the PHLPP family: the splice variants PHLPP1alpha and PHLPP1beta and PHLPP2. The PHLPP isoforms display preferential dephosphorylation of AKT isoforms. PHLPP1 mainly acts on AKT2 and AKT3, while PHLPP2 mediates inactivation of AKT1 and AKT3 (Brognard et al., 2007).

In addition, other mechanisms triggered by active PI3K signaling can lead to negative feedback inhibition. AKT activates mTOR signaling to enhance protein translation and cell growth. As a result, mTORC1 promotes IRS1 and IRS2 degradation and phosphorylates GRB10 to attenuate insulin-induced signaling (Harrington et al., 2004; Hsu et al., 2011; Shah and Hunter, 2006; Tzatsos and Kandrör, 2006; Yu et al., 2011). Another kinase activated by mTOR signaling, S6K, has been shown to phosphorylate mTORC2 component Rictor and decrease phosphorylation of Ser473 of AKT by mTORC2 (Dibble et al., 2009; Julien et al., 2010; Liu et al., 2013a). Being one of the most potent growth promoting molecules, a multitude of regulatory pathways coordinated by phosphatases and intrinsic feedback responses ensure tight control of the timing and duration of AKT activation. As a result, activation of AKT often occurs rapidly after mitogen
stimulation and is usually short-lived. Defects in these regulatory mechanisms can result in detrimental developmental abnormalities and are associated with overgrowth syndromes and cancer in humans.

1.4.2 Downstream targets of AKT

As the key effector of the PI3K signaling axis, AKT acts on over 100 downstream substrates that contribute to the expanding repertoire of AKT functions. The enzymatic property of AKT kinases is fully activated by phosphorylation at Thr308 and Ser473, and the active enzymes then phosphorylate its downstream targets at the serine and threonine residues within the minimal consensus motif R-X-R-X-S/T-Φ (X is any amino acid, Φ is a large hydrophobic residue). This phosphorylation leads to activation or inhibition of downstream targets. As summarized in the review by Manning and Toker (Manning and Toker, 2017), the most recognized signaling role of AKT is mediated through three functional nodes: glycogen synthase kinase-3, TSC2/mTORC1, and Forkhead Box O transcription factors (Figure 1-11).
Fig. 1-11: AKT signaling network

When receptor tyrosine kinases are stimulated by extracellular signaling (e.g., insulin), PI3K is activated and converts PIP$_2$ to PIP$_3$, resulting in membrane recruitment of AKT to the proximity of its activating kinases (e.g., mTORC2 and PDK1). The phospholipid conversion can be reversed by PTEN to oppose AKT activation. The fully activated AKT phosphorylates its downstream targets such as GSK3β, PRAS40, and FOXO to regulate apoptosis, metabolism, translation, and protein and lipid synthesis.

Adapted from (Manning and Toker, 2017)
Glycogen synthase kinase-3 (GSK-3) was the first reported AKT substrate (Cross et al., 1995). This serine/threonine protein kinase is ubiquitously expressed in all tissues and highly conserved across species. In 1990, cloning of cDNA from rat brain identified two distinct genes that encoded two different GSK-3 isozymes – GSK-3α and GSK-3β. Overall, the two GSK-3 proteins share 85% sequence homology, with an almost identical kinase domain, making specific targeting of these two isoenzymes a challenge. GSK-3α and GSK-3β are mostly functionally redundant; however, some studies have identified isozyme-specific functions in certain tissues (Kaidanovich-Beilin and Woodgett, 2011). The multitude of GSK-3 functions is demonstrated by its extensive number of downstream targets, which are involved in biological processes ranging from development to circadian rhythm, affecting diseases such as diabetes and cancer (Kaidanovich-Beilin and Woodgett, 2011). Amongst the vast number of targets is glycogen synthase, the first known protein targeted by GSK-3. Initial discovery of GSK-3 identified it as the regulatory kinase for glycogen synthase, a rate limiting enzyme in glycogen formation whose activity is regulated by phosphorylation (Embi et al., 1980; Vandenheede et al., 1980). Unlike most kinases, GSK-3 is active under steady state and becomes inactivated by extracellular signaling such as insulin stimulation. Phosphorylation at tyrosine residue 270 for GSK3α and 216 for GSK3β, mediated by kinases such as FYN2 and PYK2 or by autophosphorylation (Cole et al., 2004; Lochhead et al., 2006), positively regulates GSK-3 activity. On the other hand, phosphorylation at a serine residue at the N-terminal (Ser 21 for GSK3α and Ser9 for GSK3β) mediated by AKT results in GSK-3 inhibition (Stambolic and Woodgett, 1994; Sutherland and Cohen, 1994; Sutherland et al., 1993). It has been shown that the phosphorylated serine generates an intramolecular pseudosubstrate that binds to the substrate binding pocket of the kinase and obstrucs actual substrate binding (Dajani et al., 2001; Frame and Cohen, 2001). Without stimulation, active GSK-3 has inhibitory effects on its targets either by blocking their activity or destabilizing the protein. Under the stimulation of extracellular factors, serine phosphorylation inhibits GSK-3 activity and releases the inhibitory regulation of GSK-3 targeted proteins. For example, GSK-3-
mediated phosphorylation hinders the activity of glycogen synthase and glycogen production. When AKT is activated upon insulin stimulation, AKT phosphorylates GSK-3 at its serine residue, causing it to dissociate from glycogen synthase. Following this relief, there is an induction in glycogen synthase activity and an increase in glycogen formation and deposition in multiple tissues (e.g., muscle and liver). This crucial involvement of GSK-3 in glycogen synthesis demonstrated its direct influence on cell metabolism. Later studies have identified additional GSK-3 targets such as sterol regulatory element binding protein 1c (SREBP1c), hypoxia inducible factor 1α (HIF-1α), and nuclear factor erythroid 2-related factor 2 (NRF2), showing that GSK-3 can also regulate metabolism in an indirect manner. For other targets, GSK-3-mediated phosphorylation results in their degradation. Proteins such as the pro-apoptotic protein Mcl-1 and pro-proliferative transcription factor c-Myc are first primed by kinases such as JNK and ERK respectively, which allows their phosphorylation by GSK-3 (Ding et al., 2007; Maurer et al., 2006; Morel et al., 2009; Sears et al., 2000; Welcker et al., 2003). GSK-3-mediated phosphorylation of Mcl-1 and c-Myc marks them for recognition by specific E3 ubiquitin ligases, directing them to proteosomal degradation. Therefore, activation of AKT and subsequent GSK-3 inhibition stabilize both Mcl-1 and c-Myc to promote cell survival and proliferation. Additionally, GSK-3 activity can be regulated by its subcellular localization. Although GSK-3 is mostly observed in the cytoplasm, there is evidence that its nuclear localization occurs dynamically depending on cell cycle phase (Bechard and Dalton, 2009; Liu et al., 2013b; Meares and Jope, 2007). It has also been reported that AKT can decrease nuclear levels of GSK-3 (Bijur and Jope, 2001, 2003). However, the significance of nuclear localization on regulation of GSK-3 activity and function remains unclear.

The second key signaling node regulated by AKT involves TSC2/mTORC1. Cellular growth requires increased production of proteins, lipids, and nucleotides. The mTOR signaling network, whose activation is controlled by AKT, is crucial in regulating the production of these biomolecules and the maintenance of metabolic balance in response to the cellular environment with the goal of promoting cell growth and division. Mechanistic (formerly known as mammalian)
target of rapamycin, or mTOR was first identified in 1994 as the direct binding target of the FKBP12-rapamycin complex (Brown et al., 1994; Sabatini et al., 1994; Sabers et al., 1995). This interaction inhibited mTOR kinase activity, thus hindering cell growth and proliferation. This inhibitory effect on growth attracted significant attention to the clinical potential of rapamycin as well as the pro-growth function of mTOR signaling. More than 20 years since its discovery, mTOR is now identified as the catalytic component of two distinct complexes, mTORC1 and mTORC2 differing in their interacting partners. The core complex of mTORC1 consists of mTOR, mLST8, and Raptor (Nojima et al., 2003; Schalm et al., 2003), which is substituted by Rictor in mTORC2 (Jacinto et al., 2006; Sarbassov et al., 2004). Functionally, the two mTORC complexes act at different points along the PI3K/AKT signaling axis. As previously mentioned, mTORC2 activates AKT by phosphorylating it at the Ser473 residue (Sarbassov et al., 2005). It is worth noting that PKC\(\alpha\) and other members of the PKC family are also phosphorylated by mTORC2 (Gan et al., 2012; Jacinto et al., 2004; Li and Gao, 2014; Sarbassov et al., 2004; Thomanetz et al., 2013). On the other hand, AKT acts upstream of mTORC1 to regulate its activation. Activated mTORC signaling leads to increased production of biomolecules essential for cellular growth (e.g. protein, nucleotides, and lipid) through phosphorylation of key effectors such as S6K1, 4EBP1, ATF4, Lipin1, and HIF-1\(\alpha\) and heightened glucose metabolism to meet the needs of a proliferating cell. Additionally, mTORC1 suppresses catabolic pathways such as autophagy, shifting the metabolic balance in favor of cellular growth. Under nutrient or serum deprivation, binding of the proline-rich AKT substrate of 40 kDa (PRAS40) keeps mTOR in an inactive state (Sancak et al., 2007; Vander Haar et al., 2007; Wang et al., 2007). As a direct substrate of AKT, PRAS40 is phosphorylated upon insulin stimulation. This modification significantly weakens the physical interaction between PRAS40 and mTORC1, thus relieving the inhibitory regulation on mTORC1, allowing it to activate the aforementioned downstream, pro-growth effectors (Sancak et al., 2007). To ensure unperturbed mTORC1 activation, phosphorylated PRAS40 is further sequestered by binding to 14-3-3.
The third important signaling node is through the Forkhead Box O (Fox) transcription factor family, specifically the “O” class proteins that are regulated by the insulin/PI3K/AKT signaling. The four members in this class – FOXO1, 3, 4, and 6 – share structural similarity and have four distinct functional motifs including forkhead, nuclear localization, nuclear export, and transactivation domains. Among the FOXO proteins, FOXO1, FOXO3, and FOXO4 are ubiquitously expressed, although at different levels (Anderson et al., 1998; Furuyama et al., 2000), whereas FOXO6 is primarily restricted to the central nervous system (Jacobs et al., 2003). The FOXO genes encoding FOXO 1, 3, and 4 were first identified in chromosomal translocation events in rhabdomyosarcoma and acute myeloid leukemia (Dumont et al., 2012; Linardic, 2008; Schmitt-Ney and Camussi, 2015; Wachtel and Schafer, 2015), suggesting their possible role in carcinogenesis. Soon after, studies of DAF-16, the homolog of FOXO proteins in *C. elegans* found the transcription factor to be crucial for the longevity of the organism and was negatively regulated by the insulin/PI3K/AKT signaling pathway (Hesp et al., 2015; Lin et al., 2001; Sun et al., 2017). In *C. elegans*, the phenotype caused by deletion of AKT1 and 2 can be rescued if DAF-16 is also lost (Paradis and Ruvkun, 1998). This negative regulation of DAF-16/FOXO proteins is evolutionarily conserved. In mice, FOXO deletion (mainly FOXO1 in the liver) can reverse the severe hepatic insulin resistance and hyperglycemia caused by liver-specific deletion of AKT 1 and 2 (Lu et al., 2012). *In vivo* genetic manipulation reveals that the different FOXO proteins have non-redundant functions. Mice lacking FOXO1 succumb to defects at embryonic day 10.5 due to incomplete vascular development (Hosaka et al., 2004). On the other hand, FOXO3 null mice are viable, despite lymphocyte proliferation and autoinflammation associated with abnormal regulation of NFκB signaling (Lin et al., 2004), age-dependent infertility due to abnormal ovarian follicle development (Hosaka et al., 2004), and a decline in the neural stem cell pool (Renault et al., 2009). Mice with FOXO4 deletion are also viable with exacerbated colitis in response to inflammatory stimuli (Zhou et al., 2009). As FOXO6 is mostly detected in the neural system, deletion of this isoform is reported to result in defects in memory consolidation (Salih et al., 2012). As they are
transcription factors, FOXO proteins are subjected to regulation by phosphorylation that alters their subcellular localization. Inactive FOXO proteins are found in the cytoplasm. Under oxidative or nutrient stress, kinases such as JNK, Mst1, or AMPK have been shown to phosphorylate FOXOs and induce their translocation from the cytoplasm to the nucleus (Coomans de Brachene and Demoulin, 2016; Wang et al., 2014). On the other hand, AKT-mediated phosphorylation at three conserved sites (T24, S256, S319 for FOXO1; T32, S253, S315 for FOXO3A; and T32, S197, S262 for FOXO4) generates a recognition motif for 14-3-3. This interaction facilitates the nuclear export of FOXO proteins and sequesters them in the cytosol, thus inhibits FOXO-regulated gene expression. Other post-translational modifications such as acetylation by SIRT1 and monoubiquitination have been shown to enhance FOXO-mediated transcription under oxidative stress (Boccitto and Kalb, 2011; Daitoku et al., 2011; Hariharan et al., 2010; Huang and Tindall, 2011). Similar to other transcription factors, the activity of FOXO proteins is also influenced by their binding to either coactivator or corepressors. FOXO-mediated transcription governs multiple cellular processes including proliferation, apoptosis, oxidative stress, etc. (Wang et al., 2014). In regard to cancinogenesis, FOXO proteins act as tumor suppressors. In colorectal cancer, there is decreased expression of FOXO4 (Xiang-qiang et al., 2011), while FOXO3 contributes to the repression of Myc function (Delpuech et al., 2007). Studies have shown that FOXO1 upregulates the expression of TNFα and FasL in prostate cancer and glioma, respectively (Ciechomska et al., 2003; Modur et al., 2002), to initiate apoptosis. In CML and breast cancer, FOXO3 increases Bim expression in response to the chemotherapeutic agents STI571 and paclitaxel and inhibits tumor growth (Essafi et al., 2005; Sunters et al., 2003). In HER2+ breast cancer, FOXO4 promotes G1 and G2 cell cycle arrest by induction of p27 and GADD45 (Yang et al., 2005). Both FOXO1 and FOXO4 have been shown to limit metastasis by inhibiting RUNX2 activity or increasing ANX8 expression, respectively (Lee et al., 2009; Zhang et al., 2011). On the other hand, FOXO1- and FOXO3-mediated transcription leads to expression of genes such as PIK3CA and confers chemoresistance in ovarian cancer and leukemic cells (Goto et al., 2008).
1.4.3 Hyperactivation of AKT in cancer

As described above, AKT alters a wide range of downstream effectors through GSK3β, mTOR, and FOXO signaling nodes to exert its pro-growth and pro-survival functions, making it a powerful oncogenic force in carcinogenesis. Indeed, hyperactivation of AKT is one of the most frequently observed molecular features in human solid and hematological malignancies (Samuels and Ericson, 2006; Shi et al., 2005). In cancer, AKT hyperactivation is often the result of defects in its upstream regulators, either through 1) amplification or oncogenic mutations in EGFR, HER2, or other RTKs, or kinases such as PDK1 and PI3K, and/or 2) inactivating mutations or loss of heterozygosity in genes that negatively regulate PI3K/AKT signaling such as PTEN, INPP4B, LKB1, and PHLPP as summarized in Table 1 (Cheung and Testa, 2013; Mayer and Arteaga, 2016). Interestingly, PI3KCA mutations alone do not always correlate with hyperactivation of AKT and mutations in multiple proteins involved in the PI3K/AKT signaling axis is often observed in tumors (Lien et al., 2017; Vasudevan et al., 2009). This suggests that components of the PI3K/AKT signaling network function co-operatively to regulate tumorigenesis. Although AKT mutation occurs at a lower frequency than its upstream regulators, an activating somatic E17K mutant has been observed in breast, colorectal, lung, ovarian, and endometrial cancers, as well as melanoma (Carpten et al., 2007; Davies et al., 2008; Rudolph et al., 2016). This mutation occurs in the PH domain of AKT and allows constitutive membrane localization of AKT (Carpten et al., 2007; Landgraf et al., 2008). Interestingly, the E17K mutant is insufficient for tumor transformation in the absence of other driver mutations (Lauring et al., 2010; Mancini et al., 2016), likely because the full activation of this AKT mutant still requires phosphorylation at the serine 473 and threonine 308 residues. However, tumors carrying mutant AKT have been reported to be more aggressive and have poorer prognosis (Bellacosa et al., 1995; Cheng et al., 1996; Parsons et al., 2005). In gastric cancer, AKT1 with E17K mutation correlated with resistance to cisplatin treatment (Liu et al., 2007) whereas a less common Q79K AKT1 mutant confers resistance to BRAF inhibitors in melanoma patients (Shi et al., 2014).
In accordance with hyperactive AKT being a prominent oncogenic driver, many pharmacological compounds have been developed to target AKT with the aim to suppress cancer growth. There are generally two classes of AKT inhibitors – the ATP-competitive inhibitors that target the ATP-binding pocket in its kinase domain and the allosteric AKT inhibitors that target the PH and catalytic domains of the kinase (Manning and Toker, 2017). Most of these agents are in either phase I or phase II clinical trials; however, results so far have been less than promising. Such outcomes could possibly be due to the associated toxicity of the agents or the lack of a patient stratification system as the molecular profile of the disease is likely to influence therapeutic response. Currently there are four ATP-competitive inhibitors of AKT - GSK690693, AZD5364, GDC0068 or Ipatasertib, and GSK2110183 or Afuresertib. All of these inhibitors target all three AKT isoforms except GSK2110183, which showed slight selective for AKT1 at subnanomolar concentrations and inhibition of AKT2 and 3 at the nanomolar range. Despite being efficacious in preclinical models, GSK690693 and GDC0068 did not fare well in phase I trials due to dose-limiting, on-target toxicity including hyperglycemia (Crouthamel et al., 2009; Rhodes et al., 2008) or lack of antitumor effects (Lin et al., 2013; Saura et al., 2017) respectively. GSK2110183 is currently being tested in phase I trials in combination with MEK inhibitors and appears to be well-tolerated (Dumble et al., 2014), while the efficacy of AZD5364 is being examined in phase II clinical trials for advanced breast cancer (Martini et al., 2014). Two allosteric inhibitors of AKT have been developed – MK2206 and ARQ092. Mechanistically, MK2206 targets the closed, inactive conformation of AKT, hindering its phosphorylation and activation (Barnett et al., 2005; Hirai et al., 2010). As a single agent, MK2206 failed to elicit clinical efficacy (Ma et al., 2016); however, it is being tested in phase I trials in combination with trastuzumab in HER2-positive solid tumors (Hudis et al., 2013). One caveat of targeting the inactive form of AKT is the reduced sensitivity of mutant AKTs such as the E17K mutant that are constitutively membrane-associated, bypassing the function of the PH domain (Parikh et al., 2012). The structural allosteric inhibitor ARQ092 has shown strong anti-tumor activity in multiple in vivo tumor models including ones
carrying E17K mutant AKT. Phase I trials are underway to evaluate ARQ092 in patients with AKT-E17K and PIK3CA mutations (Lapierre et al., 2016; Yu et al., 2015).

1.5 Dissertation objectives

Since the discovery of PKCs, the field has made tremendous advances in understanding the differential function of PKC isozymes in certain tissues. Previous work from our laboratory has established PKCα as a key regulator of intestinal cell growth whose loss contributes to the development of intestinal tumors (Frey et al., 2000; Frey et al., 1997; Pysz et al., 2009; Saxon et al., 1994). This dissertation describes our effort to profile PKCα in the endometrium as well as to establish its functional contribution to endometrial carcinogenesis.

The first study, reported in chapter three, is our examination of PKCα expression and activation in the cycling uterus. Within a cycle, the rise and fall of steroid hormones regulates a program of coordinated molecular and physiological changes in the endometrium, which are necessary for the constant turnover of the tissue. Although PKCα is detected in multiple tissues, little is known of its expression, activation, or function in the endometrium. Therefore, the first objective of this dissertation was to examine PKCα in the human menstrual cycle and the mouse estrous cycle, to provide a basis for our understanding of PKCα expression and activation in the endometrium.

In the second study, presented in chapter four, our goal was (a) to survey alterations in PKCα expression in human endometrial tumors and in lesions arising in mice harboring mutant Pten, which are commonly used as in vivo models for oncogenic PI3K/Akt driven endometrial cancer, and (b) to identify the signaling functions of PKCα and its impact on endometrial cancer development. In light of the recent renewed understanding of PKC function in cancer and the increasing health risk posed by rising endometrial cancer incidence, a thorough examination of the role of PKCα in endometrial cancer will add new insight into PKC signaling in hyperactive
PI3K/Akt driven cancer as well its potential as a molecular biomarker for stratifying endometrial tumors.

Taken together, findings in this dissertation expand current knowledge of PKCα in the uterus. Our profiling of PKCα in the endometrium identifies the kinase as a potential regulator of endometrial renewal and function, and its ability to modulate AKT activation points to tumor suppressive properties in this tissue. Such understanding warrants further studies to examine the relationship between steroid hormones and PKCα activation in reproductive biology, and to determine whether PKCα impacts the clinical outlook of endometrial cancer such as patient prognosis and response to AKT or other small molecule inhibitors.
Chapter 2: Materials and Methods
2.1 Cell lines and reagents

Human endometrial cancer cell lines with diverse genetic mutations were obtained from the following sources:

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Catalog #</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEC-1-A</td>
<td>HTB-112</td>
<td>ATCC</td>
</tr>
<tr>
<td>HEC-1-B</td>
<td>HTB-113</td>
<td>ATCC</td>
</tr>
<tr>
<td>HEC-50Co</td>
<td></td>
<td>Dr. Kimberly K. Leslie (University of Iowa)</td>
</tr>
<tr>
<td>KLE</td>
<td>CRL-1622</td>
<td>ATCC</td>
</tr>
<tr>
<td>AN3CA</td>
<td>HTB-111</td>
<td>ATCC</td>
</tr>
<tr>
<td>Ishikawa</td>
<td></td>
<td>Dr. Tim Hui-Ming (Ohio State University)</td>
</tr>
<tr>
<td>SK-UT-1B</td>
<td>HTB-115</td>
<td>ATCC</td>
</tr>
<tr>
<td>RL95-2</td>
<td>CRL-1671</td>
<td>ATCC</td>
</tr>
<tr>
<td>ECC-1</td>
<td>CRL-2923</td>
<td>ATCC (discontinued)</td>
</tr>
<tr>
<td>SNG-II</td>
<td>IFO50312</td>
<td>Japanese Collection of Research Bioresources (JCRB) Cell Bank</td>
</tr>
<tr>
<td>SNG-M</td>
<td>IFO50313</td>
<td>Japanese Collection of Research Bioresources (JCRB) Cell Bank</td>
</tr>
<tr>
<td>HEC-6</td>
<td>JCRB1118</td>
<td>Japanese Collection of Research Bioresources (JCRB) Cell Bank</td>
</tr>
<tr>
<td>HEC-59</td>
<td>JCRB1120</td>
<td>Japanese Collection of Research Bioresources (JCRB) Cell Bank</td>
</tr>
<tr>
<td>HEC-116</td>
<td>JCRB1124</td>
<td>Japanese Collection of Research Bioresources (JCRB) Cell Bank</td>
</tr>
</tbody>
</table>
All cells were maintained in culture conditions recommended by the providing source as described below:

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Culture Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEC-1-A</td>
<td>McCoy’s 5a + 10% FBS</td>
</tr>
<tr>
<td>HEC-1-B, SK-UT-1B</td>
<td>MEM + 10% FBS, 1% NEAA, 1 mM sodium pyruvate</td>
</tr>
<tr>
<td>HEC-50Co</td>
<td>DMEM + 10% FBS, 1 mM sodium pyruvate</td>
</tr>
<tr>
<td>KLE</td>
<td>DMEM F12 + 10% FBS</td>
</tr>
<tr>
<td>AN3CA, Ishikawa</td>
<td>DMEM + 10% FBS, 1% NEAA, 1 mM sodium pyruvate</td>
</tr>
<tr>
<td>RL95-2</td>
<td>DMEM F12 + 10% FBS, 10 mM HEPES, 0.005 mg/ml insulin</td>
</tr>
<tr>
<td>ECC-1</td>
<td>RPMI-1640 + 5% FBS, 10 mM HEPES, 10 mM glucose, 1 mM sodium pyruvate</td>
</tr>
<tr>
<td>SNG-II, SNG-M</td>
<td>Ham’s 12 + 10% FBS</td>
</tr>
<tr>
<td>HEC-6, 59, 116, 151, 251, 265</td>
<td>MEM + 15% FBS</td>
</tr>
<tr>
<td>HEC-108</td>
<td>MEM + 10% FBS</td>
</tr>
</tbody>
</table>
Mechanistic studies were performed in cultured cells with the following pharmacological agents dissolved in the solvent listed:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Solvent</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKC agonist</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMA</td>
<td>100 nM</td>
<td>EtOH</td>
<td>Biomol</td>
</tr>
<tr>
<td>DiC₈</td>
<td>20 µg/ml</td>
<td>acetonitrile</td>
<td>Caymen</td>
</tr>
<tr>
<td>cPKC inhibitor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gö6976</td>
<td>2.5 µM</td>
<td>DMSO</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>PI3K inhibitor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LY294002</td>
<td>40 µM</td>
<td>DMSO</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>AKT inhibitor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MK-2206</td>
<td>100 or 250 nM</td>
<td>DMSO</td>
<td>EMD Millipore</td>
</tr>
<tr>
<td>Phosphatase inhibitors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calyculin A</td>
<td>10 nM</td>
<td>DMSO</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>1 µM</td>
<td>DMSO</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>NSC 45586</td>
<td>50 µM</td>
<td>DMSO</td>
<td>NCI</td>
</tr>
<tr>
<td>NSC 117079</td>
<td>50 µM</td>
<td>DMSO</td>
<td>NCI</td>
</tr>
</tbody>
</table>

Because of its rapid metabolism in cells, DiC₈ was replaced every 30 minutes during the treatment period. All phosphatase inhibitors and cPKC inhibitor were added to cells 30 minutes prior to addition of PKC agonists, except for calyculin A, which was added 15 minutes prior to PKC activation.

2.2 Transgenic mice

The allele-specific Pten mutant mice (Pten⁺⁺/⁺⁺, PtenG129E/⁺⁺, and PtenC124R/⁺⁺) were generated and characterized by Dr. Gustavo Leone (Ohio State University) (Wang et al., 2010). The Pten conditional knockout animals were generated by mating Pten⁻⁻ and progesterone receptor promoter
driven- or lactoferrin promoter driven-Cre animals (*PR-Cre* or *Lf-Cre* respectively) by Dr. Takiko Daikoku and Dr. Sudhansu K. Dey (Cincinnati Children’s Hospital Medical Center) (Daikoku et al., 2008; Daikoku et al., 2014). Tissues from the aforementioned *Pten* transgenic models were provided by the corresponding laboratories for this study.

To generate *Pten* mutant mice lacking PKCα, SV129J/C57 BL6 *Prkca*+/− mice obtained from Dr. J. Molkentin (Braz et al., 2004) were crossed with mixed strains of *Pten*Δ4-5/+ mice provided by Dr. G. Leone (Wang et al., 2010). The *Prkca*+/−; *Pten*Δ4-5/+ mice were then intercrossed to generate animals with the following genotypes: *Prkca*+/+; *Pten*+/+, *Prkca*+/−; *Pten*+/+, *Prkca*+/−; *Pten*+/+, *Prkca*+/−; *Pten*Δ4-5/+, *Prkca*+/−; *Pten*Δ4-5/+, and *Prkca*−/−; *Pten*Δ4-5/+. The “Y” shaped uterine horn was halved at the cervix and cryopreserved in OCT or fixed in formalin for paraffin embedding. Uterine tissue was collected from female mice from all six genotypes at 3, 5, 7, and 9 months of age; however, 1- and 2-month uterine tissue was collected from *Prkca*+/+; *Pten*Δ4-5/+ and *Prkca*−/−; *Pten*Δ4-5/+ only. The functional effects of PKCα were only examined in *Prkca*+/+; *Pten*Δ4-5/+ and *Prkca*−/−; *Pten*Δ4-5/+ as we did not observe any uterine phenotypes in *Prkca* knockout only animals. The genotypes were confirmed by PCR using the following primers and conditions:

<table>
<thead>
<tr>
<th>Gene</th>
<th>5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Prkca</em></td>
<td></td>
</tr>
<tr>
<td>Primer 1</td>
<td>CCAAGTGTGAAGTGTGTGAG</td>
</tr>
<tr>
<td>Primer 2</td>
<td>AGCTAGGTCTGTGGAAGTCAA</td>
</tr>
<tr>
<td>Primer 3</td>
<td>GCGCATCGCCTTCTTTCGC</td>
</tr>
<tr>
<td>Cycling conditions</td>
<td>95°C 3 min</td>
</tr>
<tr>
<td></td>
<td>95°C 30 sec 35 cycles</td>
</tr>
<tr>
<td></td>
<td>57°C 30 sec</td>
</tr>
<tr>
<td></td>
<td>72°C 1 min</td>
</tr>
</tbody>
</table>
72°C  3 min
4°C   Hold

\textit{Pten}

CoA        GAATGCCATTACCTAGTAAAGCAAGG
CoB        GGGTTACACTAACTAAACGAGTCC
CoC        GAATGATAATAGTACCTACTTCAG

Cycling conditions

\begin{tabular}{ccc}
95°C & 3 min & \\
95°C & 30 sec & 35 cycles \\
58°C & 30 sec & \\
72°C & 1 min & \\
72°C & 3 min & \\
4°C & Hold & \\
\end{tabular}

2.3 Determination of estrous phases

The phases of the estrous cycle of the mouse can be determined from visual observation or cytology of vaginal discharge as described in (Byers et al., 2012). In this study, estrous cycle phases (diestrus, proestrus, estrus, metestrus) were determined based mainly on the cellular makeup of the vaginal smear, using visual observation as a supporting tool. To ensure accuracy of phase determination, vaginal smears were obtained from 5-6 week-old virgin, female mice (when they reach sexual maturity) twice a day (once in the light cycle, once in the dark cycle) for a week to track the progression of estrous cycling. The vaginal discharge was collected using Pasteur pipets whose tips had been rounded by flaming and cooled prior to use. The vaginal opening of the mice was rinsed with 20-50 µl of 1X PBS and the vaginal discharge was collected in an Eppendorf tube, stained with 100-200 µl of 10% toluidine blue, transferred onto microscope slides and overlaid
with a coverslip. The phase of the estrous cycle was then determined based on the presence or absence of leukocytes, nucleated or cornified epithelial cells.

2.4 Human tissue microarray

Human endometrial cancer TMAs were generated by Dr. Carl Morrison at Ohio State University (OSU) and Roswell Park Cancer Institute (RPCI). Two TMAs, displaying a total of 384 de-identified endometrial cancer tumors (304 endometrioid and 80 non-endometrioid), were generated at OSU with approval from OSU Institutional Review Board. All patients in the TMA were diagnosed with uterine malignancy between January 1, 1980 and July 31, 2003 at the Arthur James Cancer Hospital of the Ohio State University Medical School. Specimens for controls within the TMA consisted of 37 secretory endometrium, 30 proliferative endometrium, as well as multiple cores of normal tissue from 10 different organs including heart, colon, kidney, adrenal, ovary, myometrium, brain, thyroid, lung, and prostate. A third endometrial cancer TMA, consisting of 52 de-identified cases (26 endometrioid and 26 non-endometrioid) was generated at RPCI (RPCI_GYNCa09) with RPCI Institutional Review Board approval. Cases were diagnosed at RPCI between 1992 and 2011. For any case with variation in grade or cytological atypia, TMA cores of the donor block were always taken from the areas of the highest-grade tumor. Matching frozen tissue was also available for all of the RPCI cases and was used for determination of PKCα mRNA levels. Scoring of IHC staining was performed by three independent examiners who were blind to sample information. Patient survival analysis in correlation with PKCα expression was performed using the MDACC-endometrial-L3-S40 data set, consisting of 244 endometrial tumors of which 80% were of the endometrioid subtype or using TCGA data set. To perform parallel analysis of PKCα mRNA and protein levels in endometrial tumors, total RNA was isolated from frozen samples of 50 of the tumors included in the TMA. The correlation between PKCα mRNA and protein levels was determined by Spearman Rank Correlation analysis.
2.5 Immunohistochemistry

Murine uterine tissue was formalin-fixed, paraffin-embedded, and cross-sectioned or longitudinally-sectioned at 4 µm. Human and murine tissue sections were deparaffinized in xylene and rehydrated by incubation in serially diluted alcohol. Antigen retrieval used DAKO Targeting Retrieval Solution (DAKO S1699) for 30 minutes in a steamer. Sections were blocked with 0.03% casein for 30 minutes at room temperature before incubation with primary antibody conditions listed below:

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Concentration</th>
<th>Conditions</th>
<th>Source</th>
<th>Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKCα</td>
<td>1:3000 - 6000</td>
<td>4°C overnight</td>
<td>Abcam</td>
<td>32376</td>
</tr>
<tr>
<td>PTEN</td>
<td>1:100</td>
<td>4°C overnight</td>
<td>Cell signaling</td>
<td>9559</td>
</tr>
<tr>
<td>pAKT Ser473</td>
<td>1:300 - 600</td>
<td>Room temperature 1 hour</td>
<td>Cell signaling</td>
<td>4060</td>
</tr>
<tr>
<td>Id-1</td>
<td>1:1200</td>
<td>4°C overnight</td>
<td>Biocheck</td>
<td>BCH-1/195-14</td>
</tr>
<tr>
<td>Ki67</td>
<td>Stained by the Tissue Science Facility at UNMC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Following washes, incubation with secondary antibody (Vector BA-1000) was performed at room temperature for 30 minutes, followed by addition of Vectastain Elite ABC reagent (Vector Laboratories) and DAB chromogen solution (DAB Quanto; Thermo Scientific TA-060-QHDX). Sections were counterstained with hematoxylin. Specificity of these antibodies was validated in previous studies; PKCα and Id-1 were detected as described in (Hao et al., 2011), and pAKT Ser473 and PTEN IHC followed procedures detailed in (Wang et al., 2010). PKCα immunostaining specificity was further confirmed using PKCα knockout uterine tissue.
2.6 Immunofluorescence

Uterine tissue in various phases of the estrous cycle, as determined by vaginal smears, was collected and frozen in OCT. Frozen sections (4-6 µm) were fixed in 2% freshly depolymerized paraformaldehyde/PBS for 15 minutes at room temperature and 100% methanol for 10 minutes at -20°C, prior to blocking and antibody incubation. Antibodies were as follows: anti-PKCα (1:250; Abcam 32376), or Ki67 (1:1000; eBiosciences 11-5678) for 1 hour at room temperature, and anti-rabbit Alexa 594 (1:300; Invitrogen A-21207) for 30 minutes at room temperature. All images were obtained at the same exposure. Nuclei were detected by Hoechst dye.

A guide slide consisted of 2 serial sections (10 µm) was prepared and stained with immunofluorescence to use for identification normal vs. tumor regions for LCM. The tissue was stained with primary antibodies: anti-PKCα (1:250; Abcam 32376) or anti-pAKT (1:200; Cell Signaling 4060) for 1 hour and anti-rabbit Alexa 488 (1:300; Invitrogen A-32723) for 30 minutes at room temperature and stained with Hoechst dye for 5 minutes.

2.7 Western blot analysis

To prepare lysate for immunoblot analysis, cells were rinsed twice with cold 1X PBS before addition of pre-warmed lysis buffer (1% SDS, 10 mM Tris-HCl, pH 7.4). Cell lysates were collected, passed through a 27 g needle 5-10 times to shear DNA, and centrifuged at 16,000 x g for 20 minutes. Protein concentration was determined using the BCA Assay Kit (Pierce). Equal amounts of protein were subjected to SDS-PAGE and transferred to nitrocellulose membrane. Membranes were blocked with 5% milk and probed with primary antibodies at 4°C overnight as listed below. Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit or anti-
mouse IgG (1:2000) and detection used SuperSignal West (Pierce). Signal intensity was quantified using ImageJ.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Concentration</th>
<th>Source</th>
<th>Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKCα</td>
<td>1:10,000</td>
<td>Abcam</td>
<td>32376</td>
</tr>
<tr>
<td>PKCαα</td>
<td>1:20,000 - 30,000</td>
<td>Santa Cruz</td>
<td>SC-208</td>
</tr>
<tr>
<td>PKCβI</td>
<td>1:2000</td>
<td>Abcam</td>
<td>195039</td>
</tr>
<tr>
<td>PKCβII</td>
<td>1:1000</td>
<td>Santa Cruz</td>
<td>SC-210</td>
</tr>
<tr>
<td>PKCγ</td>
<td>1:1000</td>
<td>Santa Cruz</td>
<td>SC-211</td>
</tr>
<tr>
<td>PKCδ</td>
<td>1:1000</td>
<td>Santa Cruz</td>
<td>SC-213</td>
</tr>
<tr>
<td>PKCe</td>
<td>1:1000</td>
<td>Santa Cruz</td>
<td>Sc-214</td>
</tr>
<tr>
<td>PKCθ</td>
<td>1:1000</td>
<td>BD Transduction Laboratories</td>
<td>610084</td>
</tr>
<tr>
<td>PKCη</td>
<td>1:500</td>
<td>Santa Cruz</td>
<td>SC-215</td>
</tr>
<tr>
<td>PKCζ</td>
<td>1:8000</td>
<td>Santa Cruz</td>
<td>SC-216</td>
</tr>
<tr>
<td>PKCl</td>
<td>1:1000</td>
<td>BD trans</td>
<td>610175</td>
</tr>
<tr>
<td>PTEN</td>
<td>1:1000</td>
<td>Cell signaling</td>
<td>9559</td>
</tr>
<tr>
<td>pAKT Ser473</td>
<td>1:8000 - 10,000</td>
<td>Cell signaling</td>
<td>4060</td>
</tr>
<tr>
<td>pAKT Thr308</td>
<td>1:8000 - 20,000</td>
<td>Cell signaling</td>
<td>2965</td>
</tr>
<tr>
<td>AKT</td>
<td>1:1000</td>
<td>Cell signaling</td>
<td>9272</td>
</tr>
<tr>
<td>Antibody</td>
<td>Dilution</td>
<td>Source</td>
<td>Catalogue Number</td>
</tr>
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<td>--------------</td>
<td>-------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>pPRAS40</td>
<td>1:15,000 – 20,000</td>
<td>Cell Signaling</td>
<td>13175</td>
</tr>
<tr>
<td>PRAS40</td>
<td>1:10,000</td>
<td>Cell Signaling</td>
<td>2691</td>
</tr>
<tr>
<td>pFOXO1</td>
<td>1:1000</td>
<td>Cell Signaling</td>
<td>9401</td>
</tr>
<tr>
<td>FOXO1</td>
<td>1:1000</td>
<td>Cell Signaling</td>
<td>2880</td>
</tr>
<tr>
<td>pGSK3β</td>
<td>1:500</td>
<td>Cell Signaling</td>
<td>9323</td>
</tr>
<tr>
<td>GSK3β</td>
<td>1:10,000</td>
<td>BD Transduction Laboratories</td>
<td>610202</td>
</tr>
<tr>
<td>PP2AC</td>
<td>1:3000</td>
<td>Upstate</td>
<td>05-421</td>
</tr>
<tr>
<td>Id-1</td>
<td>1:1500-3000</td>
<td>Biocheck</td>
<td>BCH-1/195-14</td>
</tr>
<tr>
<td>β-actin</td>
<td>1:15000</td>
<td>Sigma</td>
<td>A2066</td>
</tr>
</tbody>
</table>

*a To assess PKCα levels in adenoviral vector mediated PKCα overexpression experiments.

2.8 Laser-capture microdissection (LCM)

Murine uterine tissue was placed in OCT and frozen immediately after dissection. For LCM, the tissue was longitudinally sectioned at 10 μm and mounted on PET-membrane FrameSlides (Leica). Slides were air-dried briefly, fixed in 70% EtOH for 30 seconds, washed in dH2O to remove excess OCT, stained with hematoxylin until tissue was visible, and washed in dH2O to remove excess dye, followed by incubation in 95% and 100% EtOH for 30 seconds. Stained PET-slides were air-dried and placed in a cold dessicator until LCM. Additional serial sections were prepared on charged glass slides for IF analysis for PKCα and pAKT to serve as a guide to distinguish normal vs. tumor regions. Laser capture was performed at optimal laser strength that ensures precise dissection while minimizing tissue damage. Dissected tissue was directly collected into 30 μl lysis buffer place on
the cap of the collection tube. RNA was isolated from dissected sections using the RNAqueous-MicroKit (Ambion) as directed by manufacturer’s instructions. All reagents used were RNAse free. Slides and working surface were cleaned with RNaseZap.

2.9 Quantitative RT-PCR analysis

RNA was isolated using the RNAspin mini RNA isolation kit (GE Health) or Trizol reagent (Thermo Fisher) and 10 ng total cellular RNA was analyzed using Brilliant II SYBR® Green QRT-PCR 1-Step Master Mix (Agilent). In the nascent RNA capture experiments, cDNA was generated using the iScript™ cDNA Synthesis Kit and quantified using iTaq Universal SYBR Green Supermix (BioRad). The primers used for each target are listed below:

<table>
<thead>
<tr>
<th>Target</th>
<th>5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPKCα</td>
<td></td>
</tr>
<tr>
<td>F’</td>
<td>GGAAGGGGACGAGGAAGGA</td>
</tr>
<tr>
<td>R’</td>
<td>TGATGACTTTTGTGCCAGCAG</td>
</tr>
<tr>
<td>hPHLPP1</td>
<td></td>
</tr>
<tr>
<td>F’</td>
<td>TGATGACTTTTGTGCCAGCAG</td>
</tr>
<tr>
<td>R’</td>
<td>AGTTCAATTAAGCCCCCTGGC</td>
</tr>
<tr>
<td>hPHLPP2</td>
<td></td>
</tr>
<tr>
<td>F’</td>
<td>TGTACGCAAGGGAAAGACCC</td>
</tr>
<tr>
<td>R’</td>
<td>AGCAAGGGAGTATTGCGT</td>
</tr>
<tr>
<td>18s rRNA</td>
<td></td>
</tr>
<tr>
<td>F’</td>
<td>CATTGGAGGGCAAGTCTGGT</td>
</tr>
<tr>
<td>R’</td>
<td>CTCCAAGCTCCAACTACGAG</td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
</tr>
<tr>
<td>F’</td>
<td>TGAAGGGTCGGAGTCAACGGA</td>
</tr>
</tbody>
</table>
2.10 RNA interference

For siRNA-mediated knockdown of PKCα or PP2AC, cells were transfected with 10 nM siRNA using RNAiMAX transfection reagent (Invitrogen) as per manufacturer’s protocol. Cell lysates were collected for Western blot analysis 72 hours after transfection. Knockdown of the catalytic subunits of PP2A was achieved by pooling PPP2CA and PPP2CB siRNAs. Information on the siRNAs used is provided below:

<table>
<thead>
<tr>
<th>siRNAs</th>
<th>Part/ Catalog number</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>siPKCα #1</td>
<td>4390827</td>
<td>Ambion Life Technologies</td>
</tr>
<tr>
<td></td>
<td>sense - GGAUUGUUCUUCUUUCUAATT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>antisense - UAUGAAGAAAGAACAAUCCGA</td>
<td></td>
</tr>
<tr>
<td>siPKCα #2</td>
<td>4390771</td>
<td>Ambion Life Technologies</td>
</tr>
<tr>
<td></td>
<td>sense - GAAGGGUUCUCGUAGUCATT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>antisense - UGACAUACGAGAACCCUCAAA</td>
<td></td>
</tr>
<tr>
<td>siPP2CA</td>
<td>ON-TARGET plus Human siRNA SMARTpool L-003598-01</td>
<td>GE Dharmacon</td>
</tr>
<tr>
<td>siPP2CB</td>
<td>ON-TARGET plus Human siRNA SMARTpool L-003599-00</td>
<td>GE Dharmacon</td>
</tr>
<tr>
<td>Non-targeting</td>
<td>D-001810-01-05</td>
<td>GE Dharmacon</td>
</tr>
</tbody>
</table>

Lentiviral vectors expressing PTEN targeting shRNAs were used to generate stable knockdown endometrial cancer cell lines. Cells were transduced with either or both clones of shPTEN vectors or a non-targeting vector at 20 moi (at $1 \times 10^6$ CFU/ml) in serum-free medium containing 8 µg/ml of polybrene and incubated at 37°C for 8 hours before adding 1 ml of culture medium. Cells were
passaged 4 days after transduction, selected with puromycin (10 µg/ml) on day 6, and cultured for at least 10 passages before collecting cell lysate for analysis or subsequent experiments.

Information on shRNAs used are listed as below:

<table>
<thead>
<tr>
<th>shRNA</th>
<th>Clone/ Catalog number</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>shPTEN #1</td>
<td>V2LHS_92317</td>
<td>Open Biosystems</td>
</tr>
<tr>
<td>shPTEN #2</td>
<td>V2LHS_231477</td>
<td>Open Biosystems</td>
</tr>
<tr>
<td>Non-targeting shRNA</td>
<td>RHS 4348</td>
<td>GE Dharmacon</td>
</tr>
</tbody>
</table>

2.11 Nascent RNA capture

Labeling and capture of nascent RNA was performed using the Click-iT Nascent RNA Capture Kit (Life Technologies) according to the manufacturer’s protocol. In brief, EU was added to cells for incorporation into nascent RNA to allow biotinylation of the synthesized RNA. The biotinylated RNA was then captured using Streptavidin magnetic beads and cDNA was generated using the iScript cDNA Synthesis Kit (Bio-Rad). cDNA for PKCα and GAPDH was quantified by qPCR using iTaq Universal SYBR Green Supermix.

2.12 Subcellular fractionation

To analyze PKCα subcellular partitioning after PMA-induced activation, cytosolic and particulate fractions were prepared from endometrial cancer cells after 6 hours of treatment with 100 nM phorbol 12-myristate 13-acetate (PMA; Biomol) dissolved in ethanol as previously described (Saxon et al., 1994). Cells were rinsed twice with PBS, lysed with digitonin buffer (0.5 mg/ml digitonin, 20 mM Tris-HCL pH7.4, 2 mM EGTA, 2 mM EDTA, 10 mM NaF, and 1:100 protease inhibitor cocktail) and centrifuge at 100,000 x g at 4°C for 30 minutes to separate the cytosolic (in the supernatant) and the membrane/cytoskeleton (in the pellet) fractions. Cytosolic proteins were
precipitated by adding 100% trichloroacetic acid (TCA) to a final concentration of 10% TCA and incubate briefly on ice. The pellet was washed with acetone four times and dissolved by incubation with 100 mM NaOH at 4°C overnight. The cytosolic fraction was then neutralized with an equal volume of 100 mM HCl. The pellet containing the particulate fraction was solubilized by addition of 200-300 µl of 1% SDS-digitonin buffer with 1:100 protease inhibitor cocktail. The pellet was fragmented by syringing and incubated on ice for 30 minutes at 4°C to further solubilize the particulate fraction. An equal ratio of cytosolic and particulate fractions (relative to total protein) was analyzed for the presence of PKCα.

2.13 PP2A activity assay

PP2A phosphatase activity was measured using a PP2A immunoprecipitation phosphatase assay kit (Millipore) and as previously described in (Guan et al., 2007). Specifically, cells were transfected with 30 pmole of non-targeting or a combination of two siRNAs targeting PKCα for three consecutive days to ensure sufficient knockdown. 24 hours after the last transfection, cells were treated with PMA or vehicle control for 10 minutes, washed twice in cold TBS, lysed in lysis buffer (20 mM imidazole-HCl, 2 mM EGTA, 2 mM EGTA, pH 7.0 with 1:100 protease inhibitor cocktail) and subjected to immunoprecipitation and measurement of free phosphate with malachite green following the manufacturer’s protocol.

2.14 Establishing EC cell lines stably expressing mutant AKT

To determine if the tumor suppressive effects of PKCα in the endometrium involves inhibition of AKT hyperactivation, EC cell lines stably expressing constitutively active mutants, either the AKT E17K mutant found in breast and endometrial cancers (Mancini et al., 2016; Rudolph et al., 2016; Shoji et al., 2009) or the phosphomimetic AKT DD (T308D, S473D) mutant, were generated via
retroviral vector-mediated integration. Retroviral vector pBabe-puro-AKT1 was a gift from Xinjiang Wang (RPCI, NY) and the E17K mutation was generated through site-directed mutagenesis. The plasmid encoding pEGFP-AKT-DD was a gift from Julian Downward (Addgene plasmid # 39536) (Watton and Downward, 1999) and was subcloned into retroviral vector pBMN-I-GFP, which was gifted by Garry Nolan (Addgene plasmid # 1736). First, pEGFP-AKT-DD was digested with BamH1 and pBMN-I-GFP was digested with NotI. The ends of the digested fragments were filled in by Klenow-mediated DNA polymerization. The cloning vector and insert were digested by Xhol and SalI, respectively, and ligated to generate pBMN-AKT-DD for subsequent viral vector production. Retroviral vectors expressing AKT mutants were generated by transfecting the packaging cell line 293GP (stably expressing gag and pol viral components) with pVSVG and either control vector (pBabe-puro or pBMN-I-GFP) or the mutant AKT encoding plasmids (pBabe-AKT-E17K or pBMN-AKT-DD) in DMEM medium containing 10% heat-inactivated serum. The viral supernatant was collected 48 hours after transfection and centrifuged to pellet cell components. The target EC cells were incubated with the cleared viral supernatant for 6-8 hours before changing to culture media. The infection was repeated 3 times to achieve sufficient viral transduction and robust mutant AKT expression (approximately 4-fold increase of endogenous level). EC cells transduced with pBabe-puro or pBabe-AKT-E17K were subjected to puromycin selection (10 µg/ml) and cells expressing pBMN-I-GFP or pBMN-AKT-DD were selected with 600 µg/ml of G418 for at least four passage before subsequent analysis by soft agarose colony formation assays.

2.15 Adenoviral-mediated overexpression and soft agarose colony formation assay

Adenoviral vectors expressing either LacZ, PKCa, or kinase-dead PKCa were amplified in HEK293 packaging cells and the titer of viral supernatants was determined using Adeno-X Rapid Titer Kit (Clonetech). Adenovirus expressing myr-AKT1 was purchased from Vector Biolabs.
Cells (5×10^5) were transduced with adenovirus at indicated moi in reduced (2.5%) serum medium for 24 hours and then incubated in complete culture medium. 48 hours post transduction, cells (1-5×10^3 cells per well) were plated in 0.6% low-melting agarose as described in (Pysz et al., 2009) in duplicate, and colonies were allowed to grow for 1-4 weeks prior to staining with crystal violet. Whole plates were then scanned and visible colonies were counted.

For AKT mutant-expressing cells lines, Cells (3×10^5) were transduced with the lowest moi (at which Ad-PKCα inhibits colony formation) in 2.5% serum medium for 24 hours. 2 days after infection, 2×10^4 cells were plated in 0.6% low-melting agarose as previously described, and allow to grow for 7 days before analysis. For analysis of the effect of co-expression of AKT mutants and PKCα, images from 4 independent fields per well were taken at 5X magnification at 4 different focal planes. Images of colonies in different focal planes were superimposed in Adobe Photoshop CC software and the number of colonies per field was determined using the particle analysis tool in ImageJ software.

2.16 Correlation between PKCα mRNA and protein levels

To perform parallel analysis of PKCα mRNA and protein levels in endometrial tumors, total RNA was isolated from frozen samples of 50 of the tumors included in the TMA. PKCα mRNA levels were measured by qRT-PCR. A score was given to the tumors based on PKCα signal intensity from the IHC analysis. Results from the mRNA analysis were paired with the corresponding IHC score for the 50 tumors. The correlation between PKCα mRNA and protein levels was determined by Spearman Rank Correlation analysis.

2.17 Quantification of tumor burden and pAKT intensity relative to PKCα levels
For quantification of uterine tumor burden in mice, two to four longitudinal sections per animal were stained for pAKT Ser473 to identify endometrial lesions (Wang et al., 2002) and positive signal was quantified using DEFINIENS software and expressed relative to total endometrial epithelium area (as %). For determination of pAKT intensity relative to PKCα expression, staining corresponding to negative-low, medium and high pAKT staining was determined for each slide without knowledge of corresponding PKCα staining. Analysis was performed using Adobe Photoshop CC. pAKT staining was abstracted from the image using the Color Range tool and copied to a separate layer. Thresholds corresponding to the boundaries between staining levels were set and used to trace areas corresponding to each level. Areas of positivity on slides probed for PKCα were similarly identified and abstracted to a separate layer without reference to pAKT staining. Staining layers from consecutive slides stained for PKCα and pAKT were overlaid and the area (in pixels) corresponding to each level of pAKT staining in PKCα positive and PKCα negative areas was determined.

2.18 Statistical analysis

Data are presented as the mean ± SEM of three or more independent experiments unless otherwise stated. Statistical analysis was performed using Microsoft Excel, GraphPad Prism or SAS software version 9.3 (SAS Institute Inc., Cary, NC). p-values of less than 0.05 were considered statistically significant. Significance was assessed using Student's t-test for comparison of means, Chi-square or Fisher’s exact test for categorical analysis of two groups, Wilcoxon rank-sum test for nonparametric comparison of two samples, or two-way ANOVA test for multiple comparisons. Pairwise comparisons were adjusted for multiple comparisons with Tukey’s method. Model assumptions were examined with residual plots.
Chapter 3: The Role of PKCα in the Endometrium

Portions of the content covered in this chapter are the subject of an article under consideration at *Cell Reports* by Hsu A.H. *et al.*
3.1 Introduction

3.1.1 Biology of the endometrium

The main function of the uterus, which develops from the Müllerian tubes (also referred to as the female ducts or paramesonephric ducts), is to house the developing embryo during its early life. The uterus consists of two compartments - the myometrium, the smooth muscle that controls uterine contraction, and the endometrium that lines the inner cavity of the uterus. Functionally, the endometrium facilitates the transport of the sperm and provides the nurturing environment for implantation and embryo development. The endometrium is one of the most dynamic tissues in the body, undergoing periodic remodeling throughout the reproductive period. In each cycle, both the stroma and epithelium of the endometrium are restructured in response to the rise and fall of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) produced from the pituitary gland, and estrogen (E₂) and progesterone (P₄) secreted by the ovaries. This intricate hormonal balance governs the proliferation, differentiation, and regression of the endometrium. Disruption of this tightly regulated process triggers the onset of menopause or, in the event of molecular dysregulation, results in uncontrolled, hyperplastic growth of the endometrium and the eventual development of endometrial carcinoma. This next section describes the regeneration of the endometrium during the menstrual cycle (human) and estrous cycle (mouse), and actions of E₂ and P₄ during these events.

3.1.2 Human estrous cycle

In humans, the endometrium regenerates every 28-30 days through a process that is defined as the menstrual cycle. This breakdown and rebuilding of the endometrium was first observed in other primates (Bartelmez, 1951; Bartelmez et al., 1946). Subsequent studies identified the basalis layer of the endometrium situated immediately atop the myometrium to be quiescent at the beginning of the cycle (the first day of spotting). The progenitor cells of the endometrium were
thought to reside in this layer as depletion of the basalis abolished the regenerative capability of the endometrium (Wyss et al., 1996). With the increase in E\textsubscript{2} levels in the follicular phase, a follicle starts to develop and mature in the ovary. This process generally spans the first 14 days of a typical 28-day cycle. During this time, the stromal and epithelial cells, as well as endothelial vasculature in the basalis of the endometrium, proliferate in response to estrogenic stimulation, giving rise to the functionalis layer of the thickening endometrium. This phase is referred to as the “proliferative” phase of the endometrium. At the end of this phase, E\textsubscript{2}, LH, and FSH reach peak levels and rapidly decline following ovulation or the release of the oocyte. This marks the entry to the ovarian luteal phase (or the secretory phase of the endometrium). At this time, although there is moderate amount of E\textsubscript{2}, P\textsubscript{4} is the predominant steroid, stimulating cell differentiation and secretion of growth factors in the endometrium and leading to decidualization. In the case of successful fertilization, P\textsubscript{4} levels are maintained throughout pregnancy. Otherwise, the fall of P\textsubscript{4} levels leads to shedding of the functionalis, leaving only the basalis to regenerate the endometrium for the next cycle. Because of advances in cell-based therapy, there has been effort to identify and isolate progenitor cells from the endometrium as a source of stem/progenitor cells (Emmerson and Gargett, 2016).

3.1.3 Rodent estrous cycle

The rodent uterus is a widely used model for reproductive studies because of the ease with which it can be physically and genetically manipulated. While the rodent endometrium shares many similarities with its primate counterpart, there are distinct features that maximize rodent fertility. The rodent uterus is a ‘Y’ shaped organ with horns that are joined at the cervix. This unique shape allows the implantation and development of multiple embryos in a single pregnancy. Unlike the human menstrual cycle, the rodent estrous cycle typically lasts 4-6 days and has four phases: proestrus, estrus, metestrus, and diestrus. Each phase can be differentiated by the physical appearance of the genital area and cellular makeup of vaginal smears (Byers et al., 2012). At the
beginning of proestrus, a new batch of eggs enters the maturation process. The accompanying surge of E2 at proestrus induces cell proliferation in the endometrium leading to the thickening and enlargement of the mouse uterus (Wood et al., 2007). In rodents, E2 peaks prior to ovulation and the surge of P4, LH, and FSH soon follows (Yoshinaga, 1973). Levels of these hormones sharply fall after ovulation. The rise of P4 converts the endometrium from a growth to a receptive state. Without implantation, the cycle proceeds to metestrus and diestrus at which time the endometrium is resorbed and primed to start the next cycle. These last two phases are equivalent to the secretory phase of the human menstrual cycle. Many studies have profiled the proliferation activities of the endometrium during the estrous cycle. Maximal proliferation is observed in proestrus followed by metestrus and diestrus, while estrus is repeatedly associated with minimal proliferation (Marusak et al., 2007; Mendoza-Rodriguez et al., 2003; Wood et al., 2007). In addition, increased apoptosis is also reported during estrus (Dery et al., 2003; Marusak et al., 2007). As part of our study to understand the role of PKCα in endometrial cancer, we examined the expression and activation of PKCα in the normal mouse endometrium and its correlation with proliferation as indicated by Ki67 immunohistochemistry.

3.1.4 Effects of steroid hormone signaling: estrogenic response

The cyclic endometrium remodeling is orchestrated by the fluctuation of follicular E2 and P4 and the activation of their cognate nuclear receptors. Rising E2, observed in proestrus of the estrous cycle or the proliferative phase of the menstrual cycle, initiates early events associated with the induction of uterine growth. Sustained estrogen receptor (ER) activation and E2 bioavailability then leads to late E2 responses that result in uterine epithelial proliferation. Thus, E2 functions as a pro-proliferative signal in the uterus. Increased P4 levels, following E2 induction, opposes ER activation to mediate cell differentiation, switching the uterus from a proliferative to a receptive state for possible implantation.
The surging E2 stimulates both physiological and molecular changes in the uterus (Barton et al., 1998). The early events, which occur between one to six hours after E2 induction, include increases in RNA and protein synthesis and nuclear ER occupancy (Hewitt et al., 2016). These changes prime the endometrial epithelial cells for subsequent proliferation. Physiologically, there is also an increase in water intake of the uterus (or water imbibition) and influx of blood and immune infiltration. Sustained E2 elicits late uterine responses that peak 24 to 72 hours after the initial E2 surge. At this time, there is continuing increase in DNA synthesis and significant mitosis in the uterus, which leads to an increase in uterine weight. There is also further accumulation of immune cells. Together, these events ensure that the physical fitness of the uterus is permissive for a subsequent P4-mediated switch from proliferating to differentiated endometrium.

Studies using transgenic models have provided strong evidence that these uterine changes in response to E2 are mainly mediated by ERα. There are two isoforms of ER expressed in the uterus, - ERα and ERβ - encoded by two distinct genes. Although the ligand-binding domain is conserved and E2 indiscriminately binds to both receptor subtypes, ERα is the predominant isoform expressed in the endometrium, while ERβ is expressed at a lower level (Lecce et al., 2001). Furthermore, the uteri of ERα knockout mice (ERαKO) were shown to be hypoplastic and unresponsive to E2 stimulation. In contrast, the uteri of ERβ knockout (ERβKO) mice were not different from those of wild type (WT) mice and were able to undergo cyclic remodeling when stimulated with E2 (Hewitt et al., 2016; Krege et al., 1998; Lubahn et al., 1993). Gene expression analysis has also shown that ERα is responsible for the E2-induced genomic responses. The ERβKO and WT mice had indistinguishable expression profiles in response to E2 stimulation, whereas the ERαKO uterus showed no changes in gene expression. The genes induced by E2 also clustered into two profiles, mirroring the previously described biphasic physiological response to E2 stimulation in the uterus (Hewitt et al., 2003). These observations indicate that E2 induces its major effects in the endometrium through ERα activation and that ERα but not ERβ is essential for normal endometrial development.
Ovariectomized mouse models are commonly used in studies of hormonal response *in vivo*, taking advantage of the ease of manipulation and the similarities between rodent and human reproductive biology. Studies have shown that E2 stimulates an ERα-dependent biphasic response in the uterus (Hewitt et al., 2003). In early response to E2 stimulation, events such as ER nuclear occupancy, transcription of early response genes (including *c-fos* and *c-jun*), increased protein and RNA synthesis, and water imbibition occur within hours. Nuclear ER occupancy peaks again approximately at 8 hours after E2 stimulation, followed by late responses such as transcription of late phase genes (e.g. *lactoferrin*), DNA synthesis, mitosis, accumulation of immune cells, and increase in uterine weight (Groothuis et al., 2007; Hewitt et al., 2003).

The binding of E2 triggers a conformational change in ER through which ER assumes its transcriptionally active state capable of promoting gene expression via interaction with estrogen responsive elements (ERE) and other transcriptional mediators (Hewitt et al., 2016). The activated ER leads to the expression of genes involved in crucial biological processes such as cell cycle progression, growth factor-mediated signaling, and apoptosis (Basu and Rowan, 2005; Murphy, 1991). In the case of ERα, one of the genes upregulated through direct interaction with ERE is *insulin-like growth factor 1 (Igf1)*, an important signaling molecule in stimulating uterine proliferation (Adesanya et al., 1999; Hewitt et al., 2009; Zhu and Pollard, 2007). Although elevation of IGF-1 production occurs in all compartments of the endometrium, it was most apparent in the uterine stroma (Cunha et al., 2004; Zhu and Pollard, 2007). IGF-1 is essential for E2-stimulated uterine proliferation, as administration of E2 fails to induce a proliferative response in mice lacking *Igf1* genes (Adesanya et al., 1999). IGF-1 binds to IGF-1 receptor (IGF-1R) to activate downstream signaling mediators and inhibit negative regulators including IRS1 (Richards et al., 1996), AKT, and GSK3β (Zhu and Pollard, 2007), leading to growth promoting effects such as nuclear accumulation of cyclin D1 (Tong and Pollard, 1999).

In addition to the IGF-1-mediated activation of PI3K/AKT signaling, E2 also exerts mitogenic effects through another arm of signal transduction that involves the MAPK/ERK
pathway. *In vitro* studies have shown that estradiol activates MAPK in breast, bone, and neuronal cells (Endoh et al., 1997; Migliaccio et al., 1996; Watters et al., 1997). In the endometrium, administration of E2 in ovariectomized mice leads to phosphorylation of ERK in uterine epithelium (Wang et al., 2015). The activation of ERK prominently upregulates the activity of AP-1 transcription factors such as c-Fos and c-Jun in response to E2 or tamoxifen stimulation (Babu et al., 2013; Karin et al., 1997; Treisman, 1996; Whitmarsh and Davis, 1996). AP-1 proteins then form active dimers (homodimers or heterodimers) to regulate genes that play important roles in cell proliferation and survival – for example, upregulation of cyclin D1 and cyclin D3 to promote growth or repression of growth inhibitory effectors such as p21, p27, and p53 (Shaulian, 2010; Shaulian and Karin, 2001; Zhuang et al., 2001).

Continuing research of E2 actions in the endometrium has revealed other mechanisms by which E2 promotes the proliferation of endometrial cells (e.g., induction of non-genomic effects through the G-protein coupled receptor 30 (GPR30)) (Bamberger et al., 2007; Gielen et al., 2007). Elevated exposure to E2 or tamoxifen is one of the main risk factors for endometrial cancer, increasing the risk of developing this malignancy (Morice et al., 2016) by supporting the pro-proliferative and -survival properties of E2 and ER signaling in the endometrium.

### 3.1.5 Effects of steroid hormone signaling: progesterone

The main role of progesterone (P4) in the uterus is to oppose the actions of E2 to maintain uterine homeostasis by inhibiting cell growth and inducing differentiation, through binding to the progesterone receptor (PR). Like ER, there are two isoforms of PR – PR-A and PR-B. Rather than being encoded by two separate genes (like ERα and ERβ), the two PRs share a single PR gene located on chromosome 11, regulated by different transcriptional start sites, thus generating two mRNA transcripts encoding either PR-A or PR-B (Conneely et al., 1989). While both PR-A and PR-B are detectable in endometrium, IHC analysis has found that PR-A is mainly in the stroma.
and PR-B is the predominant PR in the epithelial compartment (Mote et al., 2006; Mote et al., 1999). In the uterus, E₂ stimulation decreases PR levels in the epithelium while stromal PR is elevated in response to E₂ (Patel et al., 2015; Tibbetts et al., 1998). Yet, both epithelial and stromal PR act to antagonize E₂-responsive proliferation, while epithelial PR mediates expression of P₄-targeted genes and inhibition of proliferation (Franco et al., 2012; Liu et al., 2009b; Smid-Koopman et al., 2003; Zhang et al., 2013). With the understanding of differential PR isoform levels in the uterine compartments, this suggests that PR-A and PR-B have distinct functions in regulating uterine growth and homeostasis.

The predominance of each PR isoform in different endometrial compartments suggests there is differential response to estrogenic stimulation mediated by these molecules. Interestingly, elevated PR levels and activation, in turn, suppress ERα expression (Haluska et al., 1990; Mote et al., 2006), generating a feedback loop to maintain the intricate balance of ER and PR signaling and uterine homeostasis. The essential role of PR in the reproductive tract was further demonstrated by transgenic models. When lacking both PRs, the mice were infertile due to defects in ovulation and implantation. The animals also showed evidence of uterine hypertrophy and inflammation of the glandular epithelium of the endometrium (Mulac-Jericevic et al., 2000). Using isoform specific knockout models (PRAKO for PR-A knockout and PRBKO for PR-B knockout), it was shown that functional PR-A alone is sufficient and necessary for normal ovarian and uterine functions as PRAKO mice were infertile (similar to PR-null animals), while normal implantation and decidualization were observed in PRBKO mice (Mulac-Jericevic et al., 2003; Mulac-Jericevic et al., 2000). PRAKO mice also showed increase endometrial hyperplasia and inflammation (Mulac-Jericevic et al., 2000). Unexpectedly, P₄ stimulated abnormal epithelial proliferation in the uterus in PRAKO mice that was not observed in PR null mice, suggesting P₄ promotes uterine epithelial proliferation through PR-B activation, and that PR-A acts to limit PR-B-mediated proliferation (Mulac-Jericevic et al., 2003; Mulac-Jericevic et al., 2000). Together these observations
demonstrated 1) PR-A is required in the anti-proliferative response of PR signaling and; 2) the P₄-mediated uterine response is essential for maintenance of uterine homeostasis, blastocyst implantation and pregnancy.

P₄ can exert its functions in the uterus through a genomic pathway by binding and activating the transcriptional activity of PR or by non-genomic activation of signal transduction. The ligand bound PR (both PR-A and PR-B) can form either homodimers or heterodimers, leading to receptor phosphorylation, and subsequent interaction with hormone responsive promoters to regulate targeted gene expression (Conneely et al., 2003). In addition, P₄ also induces non-genomic responses through PR-dependent activation of Src/RAS/MAPK signal transduction and PR-independent activation of membrane receptors, whose physiological contribution is less clear (Ballaré et al., 2006; Mulac-Jericevic and Conneely, 2004). In general, P₄ antagonizes the action of E₂ by negatively impacting the regulation of genes activated by ER signaling (Yang et al., 2011). In contrast to the induction of AP-1 expression by E₂, P₄ downregulates growth promoting proteins such as the AP-1 family of transcription factors, particularly c-Jun, cyclin D1, and minichromosome maintenance proteins (MCMs) (Chen et al., 2005; Dai et al., 2003; Pan et al., 2017; Yang et al., 2011). P₄ also exerts its growth inhibitory effect through reprogramming of the AP-1 mediated transcriptional network, leading to opposing effects on many of the E₂-responsive genes. In addition to shunting AP-1 from binding to growth promoting effectors such as cyclin D1, P₄ also upregulates the expression of p53 and CDKIs such as p21, p27 by enhancing the binding of AP-1 to their respective promoters (Dai et al., 2003; Hagan et al., 2011; Yang et al., 2011). Other PR-responsive genes, such as FOXO-1 that mediates cell senescence, have been shown to be anti-proliferative (Kyo et al., 2011; Ward et al., 2008). Although P₄ binds to both PR-A and PR-B, the receptors have distinct, isoform-specific genomic functions, providing a likely explanation for the aforementioned differential physiological roles of PR isoforms. Studies have found that activated PR-A mainly acts as a dominant negative inhibitor of both ER and PR-B (Kraus et al.,
1997; McDonnell et al., 1994; Vegeto et al., 1993), while PR-B exhibits stronger transcriptional activating capability than PR-A (Jacobsen et al., 2002; Kumar et al., 1998; Leslie et al., 1997; Vegeto et al., 1993). However, only a subset of PR-responsive genes are regulated by PR-B. Among the genes related to implantation (e.g., calcitonin, histidine decarboxylase, lactoferrin), RNA analysis showed that PR-B alone was sufficient for P4-mediated transcriptional changes in histidine decarboxylase and lactoferrin but not calcitonin (Mulac-Jericevic et al., 2000). Interestingly, despite the hyper-proliferative effect of P4 in PRAKO mice, PR-B activation upregulated IGFBP-1, which exerts P4-induced anti-proliferative effects in cooperation with FOXO-1(Nakamura et al., 2013).

There is mounting evidence that steroid hormones can modulate gene expression profiles through regulation of microRNAs (miRNAs) (Nothnick et al., 2010). Alteration in miRNAs is associated with uterine abnormalities including endometriosis, uterine leiomyoma and endometrial carcinoma (Burney et al., 2009; Kuokkanen et al., 2010), strengthening the important regulatory role of miRNAs in the endometrium. In a high-throughput miRNA analysis in the endometrial epithelium of ovariectomized mice under hormone stimulation, P4 was found to upregulate many miRNAs with known biological importance, including inhibition of proliferation (Lim et al., 2005; Yuan et al., 2014). For example, miR-133a has been shown to target cyclin D2 to exert its P4-induced anti-proliferative response (Liu et al., 2008; Pan et al., 2017). Other P4-induced miRNAs, such as miR-145/143 and miR-152, are involved in P4 related growth inhibition by suppressing levels of cyclin D2 and Wnt-1, respectively (Yuan et al., 2015). It remains elusive whether there are PR isoform-selective effects on the regulation of miRNA and its downstream targets.

3.1.6 PKC signaling in the endometrium

Several PKC isozymes (e.g., PKCα, βII, δ, η, and ζ) have been detected in the rat uterus, particularly in the myometrium, where they have been implicated in mediating uterine contraction
PKC activity has been shown to upregulate urocortin production through activation of the urocortin promoter (Bamberger et al., 2007). The fine-tuning of PKC activation has been implicated in the control of myometrial contractility and determining the duration of gestation (Karteris et al., 2004; Vitale et al., 2016). Activated PKC has also been found to induce COX2 levels, allowing the production of prostaglandin to mediate uterine contractility (Wouters et al., 2014). In contrast to the inductive effect of PKC activation in uterine myometrium contractility, PKCα activity, not its abundance, was reported to significantly increase in preterm women (not in labor) when the myometrium is quiescent (Jofre et al., 2013), suggesting that PKCα is important in regulating the duration of pregnancy. This is in accordance with the role of PKCα as an inhibitor of smooth muscle contractility in the heart (Braz et al., 2004; Hambleton et al., 2006; Liu et al., 2009a). Clearly, further investigation is needed to elucidate the isozyme specific effects of PKCs on uterine contractility.

There is limited knowledge of the role of PKCs in the endometrium. PKC signaling has been shown to be involved in generating a suitable environment for embryo implantation. Many reproduction-related signaling molecules, such as Lewis Y antigens and calcitonin, which are actively secreted by the endometrial epithelial cells during implantation, were found to activate Ca2+-dependent PKCs to facilitate trophoblast-endometrial interaction (Kalbag et al., 1991; Li et al., 2008; Li et al., 2009). The work of Pollard and coworkers on hormone-regulated changes in the uterine epithelium suggested the involvement of PKC in the normal physiology of endometrial epithelium. They reported that E2 exerts mitogenic effects through 1) IGF-1 signaling that activates PI3K/AKT pathways, leading to cyclin D1 nuclear accumulation, cell cycle progression, and DNA synthesis (Zhu and Pollard, 2007); 2) retention and chromatin loading of minichromosome maintenance proteins (MCMs) to permit DNA replication licensing (Pan et al., 2006); and 3) regulation of protein synthesis by a PKC-ERK-mTOR signaling pathway (Wang et al., 2015). In addition, this group found that, while P4 inhibits DNA synthesis mediated by an IGF-
1/PI3K/AKT signaling axis, ERK-mTOR dependent protein synthesis was undisturbed (Wang et al., 2015). Interestingly, the induction of PKC activity, as indicated by the phosphorylation of MARCKS (myristoylated alanine-rich protein kinase C substrate), coincided with the switch from non-receptive to receptive state in the uterus, which is regulated predominantly by P₄ in cooperation with nidatory E₂ (Chen et al., 2003). Activation of PKC was found to be involved in P₄-mediated gene expression such as induction of immune response gene-1 (Irg1) (Chen et al., 2003), suggesting that the PKC signaling pathway may modulate steroid hormone responsiveness of the uterine epithelium. Together, these studies suggest that PKC signaling may have important roles in regulating the physiological changes of the uterus and female fertility, although little is known about isoform-specific functions in these processes.

While the main focus of our study is to decipher the functional role of PKCα in endometrial cancer, it is important to survey its expression in the normal endometrium. The following section reports our findings on PKCα levels and activation in the cycling human and mouse endometrium.

3.2 Results

3.2.1 Survey of PKCα expression in the endometrium

As a first step in characterizing PKCα expression in the endometrium, we analyzed PKCα levels in human uterine tissue using immunohistochemistry. While PKCα was detected in all samples, there was disparity in its levels among the different endometrial compartments (Figure 3-1A). In both the secretory and proliferative uterine tissue, there were high levels of PKCα in the stroma, as indicated by strong IHC signal. In contrast, PKCα was expressed at a much lower level in the epithelium when compared to adjacent stroma. It has been shown previously that the activation of PKCs is associated with membrane translocation of the enzyme (Black and Black, 2012). Therefore, a membrane-associated pattern of PKC staining is indicative of its activation. Interestingly, multiple regions of membrane-associated PKCα were observed in the secretory, but
not in the proliferative epithelium (Figure. 3-1B), suggesting that PKCα is activated when the endometrium switches from a proliferative to a secretory state and that activity of this enzyme might have a signaling role relating to the secretory uterine phenotype.

Fig. 3-1: PKCα expression in normal human uterus

(A) IHC analysis of PKCα expression in secretory and proliferative endometrium, S indicates the stromal compartment of the tissue. Arrow points to the endometrial epithelium. (B) Membrane associated PKCα in secretory endometrium. Arrow indicates epithelial regions with membrane-associated PKCα. Scale bar = 50 µm.
3.2.2 PKCα expression in mouse estrous cycle phases

The uterus undergoes significant physiological changes and remodeling throughout the estrous cycle. These changes are governed by an intricate network of signal transduction. As part of the effort to understand if PKCα plays a role in regulating uterine physiology, we performed IHC analysis on uterine tissues from all four estrous phases: proestrus, estrus, metestrus, and diestrus, aiming to assess PKCα expression in relation to the murine estrous cycle. The estrous phases of the animals were determined from the cytological makeup of the vaginal smear. Vaginal secretion was collected from sexually mature (5-6 weeks), virgin female mice and examined using microscopy. In our previous studies of the intestinal epithelium, we have shown that PKCα is activated at the crypt-villus junction where there is a program switch from proliferation to differentiation (Frey et al., 2000). As the cycling of the endometrium also involves a physiological switch between these states as it progresses through the estrous cycle, we hypothesized that PKCα may also be growth inhibitory in the endometrium and play a role in mediating this proliferation-to-differentiation switch. To test this hypothesis, we investigated the correlation between PKCα expression and proliferation, marked by positive Ki67 staining, in formalin-fixed, paraffin-embedded uterine tissue collected from mice at each phase. PKCα was detectable in all four phases of the estrous cycle (Figure 3-2). Levels of PKCα were relatively lower in metestrus, diestrus, and proestrus. The enzyme was predominantly cytoplasmic during these phases. However, in estrus, PKCα showed robust expression. Interestingly, there was reduced staining for the proliferation marker Ki67 in the same phase. These observations suggest a negative correlation between PKCα expression and proliferative activity in the epithelium of the uterus.
Fig. 3-2: Correlation between PKCα and Ki67 expression during the estrous cycle

IHC analysis of PKCα expression and proliferative activity as indicated by Ki67 staining in uterine tissues from mice at different phases of the estrous cycle: proestrus, estrus, metestrus, and diestrus, as determined by the cytology of vaginal smears.
3.2.3 Membrane-associated PKCα at estrous phase

Upon closer examination, we found that PKCα exhibited a distinct membrane-associated staining pattern in both late proestrus and estrous phases (Figure 3-3A). In late proestrus, PKCα was predominantly inactive, as most of the detected PKCα resided in the cytoplasm. However, the glandular epithelium not only had higher PKCα levels, but staining for PKCα displayed a membrane-associated pattern, indicating that the enzyme was in its active form. This membrane-associated PKCα staining intensified in estrous tissue, extending to both the glandular and luminal epithelium and providing evidence for continuous strengthening of PKCα activating signals as the cycle progresses from proestrus to estrus. However, PKCα maintained a cytoplasmic localization in both metestrus and diestrus indicating a cessation of activating signals in the later phases of the estrous cycle as shown in Figure 3-2. The membrane-associated staining in proestrous and estrous endometrium was further confirmed in frozen sections using immunofluorescence (Figure 3-3B).
Fig. 3-3: Membrane-associated PKCα at estrous

(A) IHC analysis of PKCα in the luminal (LE) and glandular (G) compartments of uterine tissue during late proestrus and estrus. Membrane-associated localization of PKCα indicates activation of the kinase. In proestrus, PKCα expression is cytoplasmic in the luminal compartment but membrane-associated in the glands (arrows). In estrus, membrane association of PKCα is detected in both the luminal and glandular epithelium (arrows). (B) Immunofluorescence staining for PKCα in frozen sections of luminal and glandular epithelium in estrus phase, confirming the localization seen in paraffin sections.
3.2.4 Inverse correlation between PKCα activation and cell proliferation

Thus far, our data have shown that membrane association of PKCα was most evident in the estrous phase, where the least proliferation was detected. In order to further confirm if there is a correlation between PKCα activation and cell proliferation, we examined PKCα expression/subcellular distribution and Ki67-marked proliferation by performing IHC analysis on serially sectioned uterine tissue at proestrus and estrus phases. As shown by images in Figure 3-4, proliferative activity was restricted to the luminal epithelium (indicated by arrowhead) and the glandular epithelium was completely devoid of Ki67 signal (shown by arrow). Interestingly, this compartmental delineation of staining correlated with PKCα levels and activation. Furthermore, in the lower panel, there was a striking correspondence between a Ki-67 negative region and strong membrane association of PKCα as indicated with arrows. Taken together, these observations demonstrated a negative correlation between PKCα activation and epithelial proliferation in the endometrium, suggesting that PKCα might have an inhibitory effect in the cycling uterus.
Fig. 3-4: PKCα expression during the estrous cycle

Serial sections from late proestrus and estrus phase uterine epithelium were analyzed for PKCα and the proliferation marker, Ki67. Top Panels: Survey sections show markedly reduced proliferation in the luminal epithelium (LE) during estrus phase; glands (G) are negative for Ki67 staining in both late proestrus and estrus phases. Block arrows in the lower panels indicate areas of PKCα membrane association/activation; arrowheads indicate areas that lack membrane-associated PKCα. Note the strong nuclear Ki67 staining in areas which lack membrane-associated PKCα. In contrast, areas with low levels of Ki67 exhibit strong membrane association/activation of the enzyme.
3.3 Discussion

The constant remodeling of the endometrium makes it one of the most dynamic tissues in the body. The cyclic breakdown and rebuilding of this tissue requires a complex signaling network that is intricately regulated to ensure appropriate proliferation, differentiation, and apoptosis of the epithelium. Molecular aberrations that tip this balance lead to outgrowth of the endometrium, resulting in tumor development in this tissue. As part of our effort to decipher the role of PKCα in endometrial cancer, we first profiled PKCα expression in the normal endometrium. Through IHC analysis, we found that PKCα is ubiquitously expressed in both human and mouse endometrium, regardless of the stage of the menstrual cycle or estrous cycle, respectively. However, PKCα levels are significantly lower in the endometrium when compared to the adjacent stroma in the human uterus and in the mouse uterus in estrous phase, while there was little difference between these two compartments in the mouse uterus in other phases. In the human tissue, membrane-associated PKCα was only observed in the secretory phase, while this staining pattern was detected in both late proestrus and estrus phases in the murine endometrium. This observation, which was consistent in our analysis of paraffin-embedded and frozen murine uterine tissues, suggests that activation of PKCα is initiated in proestrus and continues in estrus of the mouse estrous cycle. Interestingly, there was minimal epithelial proliferation, as marked by reduced Ki67-positive staining, when active PKCα was detected. This led us to hypothesize that PKCα signaling may be anti-proliferative and tumor suppressive in the endometrium. The data presented in this section provide the basis for future studies to further investigate the hormonal regulation of PKCα activation and the signaling effects of PKCα in the endometrium.

A limited number of studies have demonstrated that E2- and P2-mediated responses in the uterus require PKC activation (Chen et al., 2003; Wang et al., 2015). Chen et al. showed that PKC activity is involved in implantation-related uterine responses induced by the synergistic actions of P4 and E2 and the upregulation of P4-regulated genes. However, a subsequent study from the same group identified a PKC-ERK-mTOR signaling axis that activates E2-induced protein synthesis...
Because of the opposing actions of E$_2$ and P$_4$ in the endometrium, these findings raise questions on how these hormones regulate PKC activation in this tissue. Both of these studies assessed PKC activation using whole cell lysates prepared from uterine epithelial cells isolated from ovariectomized mice after hormone treatment \textit{in vivo}. PKC activity was then determined by the phosphorylation of MARCKS, shown by Western blotting. Although the hormone administration was performed \textit{in vivo}, assessment of PKC activity was indirect and used a non-selective PKC substrate. Additionally, previous studies have also shown uneven distribution of ER$\alpha/\beta$ and PR-A/B expression in the different compartments of the endometrium; thus, the purity of the epithelial isolate might be a confounding factor. Because of the high PKC levels in the endometrial stroma (in the human), it is not clear whether epithelial or stromal PKC is responsible for the hormone-induced responses in the uterus, or if there is isoform selectivity in PKC-mediated signaling programs in the endometrium. In our study, active PKC$\alpha$, demonstrated by its membrane-associated staining, was detected at late proestrus and estrus, suggesting that PKC$\alpha$ activation might be a late response to E$_2$ stimulation or the result of rising P$_4$. Staining of the uterine tissue directly assesses the presence of active enzyme in the uterine tissue and allows us to visualize its compartmental localization \textit{in vivo}. However, the current data are observational and can only demonstrate a correlative relationship between PKC$\alpha$ activation and the cycling endometrium. To establish a mechanistic link between hormonal stimulation and PKC$\alpha$ activation, we propose to examine PKC$\alpha$ localization in uterine tissue from ovariectomized mice after treatment with E$_2$, P$_4$ or E$_2$P$_4$.

In a previous study, estradiol was shown to stimulate endometrial proliferation and induce sustained PKC activation for at least 8 hours (Wang et al., 2015). Another study has demonstrated elevated PKC$\alpha$ mRNA in response to tamoxifen and E$_2$ in isolated human endometrial cells (Wu et al., 2005). However, it was unclear whether the enzyme was in its active state. Based on our examination of uterine sections from proestrus and estrus, PKC$\alpha$ activation was associated with reduced proliferation, which is thought to be a P$_4$-induced phenotype. Furthermore, PKC$\alpha$ activity
is increased in pregnant women when sustained P₄ is necessary to maintain pregnancy, which supports the notion that PKCα activation is more closely associated with PR signaling. Thus, although E₂ might induce activation of other PKC isozymes, PKCα activation is likely to be regulated by P₄. We expect to detect strongest membrane-associated PKCα staining in the uterus when the animals are given E₂P₄ synergistically as the presence of low E₂ levels is necessary for the proper activation of PR signaling (Chen et al., 2003). It is also possible that P₄ alone can induce PKCα activation; however, we expect it would be at a much lower level than in the E₂P₄ treated group. Meanwhile, the tissue will also be assessed for proliferation by Ki67 staining and for downstream signaling effects on cyclin D1, p-mTOR, and pRPS6, whose expression or phosphorylation have been shown to be hormonally regulated in the endometrium (Wang et al., 2015). If our data demonstrate PR-mediated activation of PKCα, this will support a role for this kinase in exerting anti-proliferative signaling in the endometrium and highlight its involvement in the transition from a proliferative to differentiated state. Alternatively, we might observe membrane-associated PKCα when mice are given E₂ alone if PKCα is activated by ER.

Together with the current data, results from the proposed studies will provide a more comprehensive view of the role of PKCα signaling in the biology of normal, cycling endometrium, as well as a basis for its effects during endometrial tumorigenesis. Further investigation in ER or PR isoform specific knockout models will be necessary to decipher isoform selective responses of PKCα.
Chapter 4: Crosstalk Between PKCα and PI3K/AKT Signaling is Tumor Suppressivve in the Endometrium

Portions of the content covered in this chapter are the subject of an article under consideration at Cell Reports by Hsu A.H. et. al
4.1 Endometrial cancer

Endometrial cancer (EC) is the most common gynecological malignancy in the United States, with an estimated 60,380 new cases and 10,920 deaths in 2017 (Siegel et al., 2017). With an overall 5-year survival rate of >80%, EC has attracted less public attention than other cancers. Most cases are diagnosed at an early stage when the patients experience unusual spotting, heavy bleeding or severe menstrual pain. When diagnosed early, patients are treated with surgery. For more advanced cases, chemotherapy or radiation may follow. However, advanced (which has spread beyond the uterine cavity) and recurrent disease is refractory to treatment and the prognosis for these patients is dismal, with survival estimates of less than one year (Engelsen et al., 2009). Because of its high incidence, EC is the sixth leading cause of cancer death in women, accounting for more deaths than melanoma, cervical cancer, glioblastoma, all lymphomas, or all leukemias (Siegel et al., 2017). Alarmingly, the incidence and mortality for EC are on the rise, with a >40% increase since 2005. Since obesity is a major risk factor for the disease (Fader et al., 2009), EC will become an even greater health concern as the effects of increased societal obesity become evident in coming years.

4.1.1 Clinical and molecular classification

EC has historically been classified into two histopathological subtypes. Type I tumors (85-90% of cases) are of endometrioid histology, while Type II non-endometrioid tumors can be further classified by histological appearance as serous, clear cell, mixed cell, or malignant mixed Mullerian tumors (MMMT), with serous being the predominant subtype in this category (Suarez et al., 2017). The original landmark paper published in 1983 described the clinical features observed in each of the subtypes (Bokhman, 1983). A majority of EC patients are diagnosed with Type I or endometrioid subtype. Many of these patients are post-menopausal women who are also likely to have elevated estrogen exposure (related to hormone replacement therapy, obesity, late onset of menopause, nulliparity), diabetes, hypertension, obesity, and hyperlipidemia. Formation of Type I
ECs follows a sequential course of progression initiated by hyperplasia, atypical hyperplasia, and benign hyperplasia, followed by premalignant neoplasia (Dedes et al., 2011). Disease in these patients often presents as low grade hyperplasia, with histology resembling the proliferative epithelium, anovulatory bleeding, and sometimes infertility (in women at reproductive age). Type I tumors have lower invasive potential, often only penetrating the superficial surface of the myometrium. These tumors are more sensitive to progesterone-mediated growth inhibition. Most of type I patients are successfully treated with surgery, progestin therapy, and radiation for more advanced cases. Patients with Type I disease have more favorable prognosis and have an overall 5-year survival rate of 85% (Morice et al., 2016).

As discussed above, Type II non-endometrioid tumors encompass several histological types including serous, clear cell, malignant mixed Mullerian tumors, and mixed epithelial types. These cancers generally have an accelerated course of disease progression, and tumors are more invasive with a higher likelihood of recurrence. Although Type II EC only accounts for ~20% of all endometrial cancer cases, it is responsible for 50% of EC-related mortality (Lobo and Thomas, 2016). At the time of diagnosis, these tumors have usually already invaded the myometrium and lymphovascular and are often metastatic. Despite the difference in disease type, patients with Type II EC also receive surgery as first line therapy. Due to the more aggressive nature of the disease, patients with Type II EC also receive adjuvant radiation and combinational chemotherapy to limit recurrence. Progestin therapy is rarely administered to these patients as Type II tumors are often insensitive to P₄. However, if the disease recurs, the tumors are often resistant to existing therapies and the prognosis for these patients is dismal (median survival of 8-16 months) (Dellinger and Monk, 2009). The simplistic classification of EC established in 1983 has guided clinical prognosis and treatment for this disease in the following decades. As the EC field gains more understanding of the biology of these tumors, the classification of ECs continues to refine this dualist model.
The first evolution of this two-category classification of EC disease was fueled by advances in the molecular understanding of ECs (Table 4-1) (Dedes et al., 2011). Molecular analysis of these tumors has identified genetic alterations that are more prevalent in each EC type. Because of the high prevalence of mutations in \textit{PTEN} (83%), loss of \textit{PTEN} function is considered a primary feature of Type I tumors (Mutter et al., 2000; Risinger et al., 1998). Additional oncogenic mutations are found in other molecules in the PI3K/AKT pathway, including activating mutations in PIK3CA (~30%) and AKT (3%) (Catasus et al., 2008; Miyake et al., 2008; Shoji et al., 2009). The frequent co-existence of mutations in PI3K subunits and \textit{PTEN} highlights the tumorigenic advantage of hyperactive AKT signaling in endometrial carcinogenesis (Oda et al., 2005). The microsatellite instability phenotype of Type I ECs has been attributed to inactivation of DNA repair genes (e.g. \textit{MLH1}, \textit{MSH2}, \textit{MSH6}, and \textit{PMS2}) (MacDonald et al., 2000; Mackay et al., 2010; Miturski et al., 2002) Other molecular aberrations commonly found in Type I ECs include \textit{KRAS} mutations, \textit{FGFR2} mutations, and nuclear accumulation of \(\beta\)-catenin (Koul et al., 2002; Lax et al., 2000; MacDonald et al., 2000; Pollock et al., 2007; Schlosshauer et al., 2000; Stefansson et al., 2004). In contrast, \textit{KRAS} mutations and \textit{FGFR2} mutations are mutually exclusive (O'Hara and Bell, 2012).
<table>
<thead>
<tr>
<th>Alteration</th>
<th>Prevalence in Type I (%)</th>
<th>Prevalence in Type II (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIK3CA mutation</td>
<td>~30</td>
<td>~20</td>
</tr>
<tr>
<td>PIK3CA amplification</td>
<td>2-14</td>
<td>46</td>
</tr>
<tr>
<td>KRAS mutation</td>
<td>11-26</td>
<td>2</td>
</tr>
<tr>
<td>AKT mutation</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>PTEN loss of function</td>
<td>83</td>
<td>5</td>
</tr>
<tr>
<td>Microsatellite instability</td>
<td>20-45</td>
<td>0-5</td>
</tr>
<tr>
<td>Nuclear accumulation of β-catenin</td>
<td>18-47</td>
<td>0</td>
</tr>
<tr>
<td>E-Cadherin loss</td>
<td>5-50</td>
<td>62-87</td>
</tr>
<tr>
<td>TP53 mutation</td>
<td>~20</td>
<td>~90</td>
</tr>
<tr>
<td>p16 loss of function</td>
<td>8</td>
<td>45</td>
</tr>
<tr>
<td>HER2 overexpression</td>
<td>3-10</td>
<td>32</td>
</tr>
<tr>
<td>HER2 amplification</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>FGFR2 mutations</td>
<td>12-16</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 4-1: Molecular alterations in endometrial cancer. Adapted from (Dedes et al., 2011)

The most commonly observed aberration in Type II EC disease is mutation in p53, which is found in 90% of Type II tumors, particularly in the serous subtype (Jia et al., 2008; Khalifa et al., 1994; Lax et al., 2000). In serous EC, somatic mutations of p53 are believed to be an initiating event (O'Hara and Bell, 2012). Interestingly, serous ECs share clinical and pathological similarities with serous ovarian tumors in which p53 mutations have significant pathogenic and therapeutic impact (Di Cristofano and Ellenson, 2007). Alterations in p16 levels are more commonly detected in Type II than in Type I ECs. p16\textsuperscript{ink4} negatively regulates progression into the G1/S phase by
binding to cyclin dependent kinases Cdk4 and Cdk6 and is, thus, considered a tumor suppressor (Liggett and Sidransky, 1998). Loss of p16 in EC is associated with aggressive disease with poor prognosis (Ignatov et al., 2008; Salvesen et al., 2000). However, other studies have reported overexpression of p16 in ECs (O'Hara and Bell, 2012). Elevated p16 is thought to be indicative of oncogene-induced senescence in benign or pre-malignant hyperplasia, or the result of the cell’s effort to arrest proliferation when the Rb pathway is defective (Romagosa et al., 2011). Other molecular alterations that are more commonly associated with Type II EC include loss of E-Cadherin and elevated levels of HER2 due to overexpression or gene amplification. Although molecular profiling provided a more detailed distinction of EC types, some aberrations are found in both types. For example, mutations in PIK3CA are found at similar frequencies in both Type I and II ECs (although there appears to be a preference for mutations in exon 20 in Type II disease, whereas Type I ECs have mutations in both exon 9 and 20 of PIK3CA) (Catasus et al., 2008). Interestingly, PIK3CA amplification is more prevalent in Type II than Type I ECs. The classical subtyping of EC disease based on tumor histology was particularly problematic for high-grade endometrioid tumors (Clarke and Gilks, 2010). Newly identified molecular alterations in ECs can serve as biomarkers to facilitate more accurate subtyping and better guidance in treatment selection (Alkushi et al., 2010; Darvishian et al., 2004; Yemelyanova et al., 2009). Using phenotypic markers such as MLH1, MSH2, p16, cyclin D1, ERBB2, and p53, 37% of grade 3 tumors exhibited molecular profiles of endometrioid tumors while 63% of the cases shared molecular similarities with serous carcinomas (Alvarez et al., 2012). This suggests that a comprehensive molecular profiling is needed to maximize the accuracy of the classification of ECs. Together, these findings provided new insights into the molecular alterations in ECs and the prognostic potential of these molecules, and also revealed opportunities for targeted therapy for the disease.

In 2013, The Cancer Genome Atlas project (TCGA) completed their integrated genomic, transcriptomic, and proteomic analysis of 373 ECs including 307 low or high grade endometrioid, 53 serous, and 13 mixed histology cases (Kandoth et al., 2013). The top most significantly mutated
genes for each subgroup are listed in Table 4-2.

<table>
<thead>
<tr>
<th>Molecular subtype</th>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Mutation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>POLE ultramutated</strong></td>
<td><em>POLE</em></td>
<td>Polymerase epsilon, catalytic subunits</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td><em>PTEN</em></td>
<td>Phosphatase and tensin homolog</td>
<td>94%</td>
</tr>
<tr>
<td></td>
<td><em>CSMD3</em></td>
<td>CUB and Sushi multiple domains 3</td>
<td>94%</td>
</tr>
<tr>
<td></td>
<td><em>TAF1</em></td>
<td>TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 250KDa</td>
<td>83%</td>
</tr>
<tr>
<td></td>
<td><em>FBXW7</em></td>
<td>F-box and WD repeat domain containing, E3 ubiquitin protein ligase</td>
<td>83%</td>
</tr>
<tr>
<td><strong>Hypermutated/microsatellite-unstable</strong></td>
<td><em>PTEN</em></td>
<td>Phosphatase and tensin homolog</td>
<td>88%</td>
</tr>
<tr>
<td></td>
<td><em>PIK3CA</em></td>
<td>Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha</td>
<td>54%</td>
</tr>
<tr>
<td></td>
<td><em>PIK3R1</em></td>
<td>Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)</td>
<td>40%</td>
</tr>
<tr>
<td><strong>Copy number low/microsatellite-stable</strong></td>
<td><em>PTEN</em></td>
<td>Phosphatase and tensin homolog</td>
<td>77%</td>
</tr>
<tr>
<td></td>
<td><em>PIK3CA</em></td>
<td>Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha</td>
<td>53%</td>
</tr>
<tr>
<td></td>
<td><em>CTNNB1</em></td>
<td>Catenin, beta 1, 88 KDa</td>
<td>52%</td>
</tr>
<tr>
<td></td>
<td><em>ARID1A</em></td>
<td>AT rich interactive domain 1A (SWI-like)</td>
<td>42%</td>
</tr>
<tr>
<td><strong>Copy number high/serous like</strong></td>
<td><em>TP53</em></td>
<td>Tumor protein 53</td>
<td>92%</td>
</tr>
<tr>
<td></td>
<td><em>PIK3CA</em></td>
<td>Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha</td>
<td>47%</td>
</tr>
<tr>
<td></td>
<td><em>PPP2R1A</em></td>
<td>Protein phosphatase 1, regulatory subunit A, alpha</td>
<td>22%</td>
</tr>
<tr>
<td></td>
<td><em>FBXW7</em></td>
<td>F-box and WD repeat domain containing, E3 ubiquitin protein ligase</td>
<td>22%</td>
</tr>
</tbody>
</table>

Table 4-2: Top 3 most significantly mutated genes in 4 molecular subtypes of EC. *High mutation frequency of this gene is likely a statistical artifact. Adapted from (Le Gallo and Bell, 2014)
While mutations in known EC-associated genes were detected, this comprehensive analysis revealed many more significantly altered genes that were not identified to drive endometrial tumorigenesis (e.g. BCOR, MECOM, ARID5B, and NKAP etc.). Based on results from this analysis, four molecular subgroups of ECs have been recognized: POLE ultramutated, hypermutated/microsatellite-unstable (MSI), copy number low/microsatellite-stable (MSS), and copy number high/serous like, as reviewed in (Le Gallo and Bell, 2014). This marked the second evolution of EC classification. DNA polymerase δ (POLE) exonuclease, encoded by the POLE gene, catalyzes the synthesis of the leading strand during DNA replication (Pursell and Kunkel, 2008). Mutations in the POLE gene, such as those identified in ECs, disrupt the enzyme’s proofreading ability leading to an ultramutated phenotype (232 × 10⁶ mutations/Mb; 867-9714 mutations/tumor), as well as high incidence of C>A transversions. Other studies have also reported POLE mutations in EC (Church et al., 2013). Despite the extraordinarily high mutation numbers in POLE ultramutated tumors, patients in this group have the most favorable prognosis among all EC subgroups. All POLE ultramutated tumors are of endometrioid histology, accounting for 10% of all endometrioid tumors examined (6.4% of low grade and 17.4% of high grade endometrioid tumors). Another subgroup that exclusively consists of endometrioid tumors is the hypermutated/MSI category. As its name indicates, the hypermutated phenotype in these tumors is the result of reduced expression of the mismatch-repair protein MLH1 and frequent methylation of the MLH1 promoter. This is also consistent with previous reports of high MSI frequencies in ECs (An et al., 2007; Konopka et al., 2007; Zighelboim et al., 2007). MSI tumors comprise 28.6% of low grade and 54.3% high grade endometrioid cases in the TCGA cohort. The next subgroup includes the copy number low/MSS tumors. The MSS tumors account for 60% of all low grade endometrioid tumors, making it the most common subgroup of endometrioid tumors. Unlike the previous two subgroups, the MSS subtype includes serous and mixed-histology carcinomas. In contrast to the higher mutation frequencies in KRAS and FGFR2 in MSI endometrioid tumors,
CTNNB1 mutations are more common in MSS endometrioid tumors (Byron et al., 2012). Despite the frequent occurrence of high grade endometrioid histology in MSI tumors and the inclusion of the more aggressive serous or mixed-histology MSS carcinomas, these two subtypes displayed similar trends in progression free survival. The most distinct subgroup of ECs is known as copy number high/serous-like. Almost all serous cases are categorized in this subgroup (97.7% of serous and 75% of mixed-histology). Phenotypically, these tumors are more similar to high-grade serous ovarian tumors than to other ECs (Merritt and Cramer, 2010). Genetic profiling of serous EC has also identified prominent mutations in TP53 in these tumors, mirroring serous ovarian tumors, which suggests that mutant p53 is the main driver for formation of serous EC. Mutation in other genes such as PTEN, CTNNB1, and PIK3R1 that are highly prevalent in other subgroups are significantly less frequent in serous EC, although they are still present. As expected, these tumors generally also have decreased pAKT levels, differing from the AKT hyperactivation that commonly drives carcinogenesis in other EC subtypes. In addition, TCGA analysis also identified focal amplification of MYC, ERBB2, and CCNE1 in 23-25% in this subgroup. Because of distinct molecular features and the close resemblance between serous endometrial and ovarian tumors, targeted therapies developed against high grade serous ovarian tumors might also be effective in copy number high/serous-like EC (Iijima et al., 2017).

The rapid advances in sequencing technology and high-throughput omics analysis sparked the second evolution of EC classification. The concerted effort by the TCGA research network provided a comprehensive view of EC tumor biology, and strongly urged re-definition of EC classification, which will also impact clinical decisions on appropriate treatments based on the disease subgroups.
4.2 Results

4.2.1 Transcriptional suppression of PKCα is associated with high-grade EC

As a first step toward understanding the role of PKCα signaling in EC, we performed IHC analysis to profile its expression in a panel of 436 human endometrial tumors (330 endometrioid; 106 non-endometrioid) in tissue microarray (TMA) format. While PKCα was detected in normal secretory and proliferative endometrium (as shown in the Figure 3-1) and was retained in some lesions, the enzyme was absent or markedly reduced in >60% of endometrioid ECs and >50% of all non-endometrioid tumors, with the proportion of PKCα deficient tumors ranging from 31% of MMMT to 70% of clear cell ECs (Figure 4-1, middle panel).

The availability of frozen tissue for a subset of these ECs (24 endometrioid; 26 non-endometrioid) allowed parallel quantification of PKCα mRNA by qRT-PCR. A significant correlation was noted between PKCα mRNA and protein levels in both tumor subtypes (Figure 4-1, bottom table), indicating that downregulation of PKCα protein in human EC is controlled primarily at the level of mRNA expression. Analysis of TCGA data confirmed that PKCα mRNA is markedly downregulated in human ECs (p=2.77e⁻⁹), and pointed to increased or decreased expression of several additional PKC family members in these tumors (Figure 4-2). Like PKCα, PKCβ, ε, η, and θ, showed reduced mRNA expression in tumors relative to normal endometrial tissue, while levels of other PKC isozymes were elevated (e.g. PKCδ and ξ) or unchanged (e.g. PKCγ and ι) (Figure 4-2). Note that PKCα is one of the most profoundly downregulated PKCs in this tumor type.
Fig. 4-1: PKCα expression in EC

IHC analysis of a panel of ECs including endometrioid and non-endometrioid subtypes. Decreased PKCα expression was found in over 60% of endometrioid tumors and 50% of non-endometrioid subtypes. PKCα signal in the stroma (S) serves as a positive control for IHC staining in Grade 3 tumors. Scale bars, 50 µm. Spearman Rank correlation analysis of PKCα protein and mRNA in endometrial tumors Analysis done with assistance from Adrian Black.
Fig. 4-2: Alterations in PKC isozyme mRNA expression in ECs

PKC isozyme mRNA levels in normal vs. tumor tissue based on analysis of TCGA database. Expression of PKCα, β, ε, η, and θ mRNA is reduced in endometrial tumors, while ECs have increased levels of PKCδ and ξ mRNA. Levels of PKCγ and ι mRNA are comparable in the normal endometrium and endometrial tumors.
To further examine the regulation of PKCα in EC, we determined its expression in a panel of 16 well-characterized human EC cell lines harboring a variety of genetic mutations characteristic of human endometrial tumors (Table 4-3) (Weigelt et al., 2013). Six of these cell lines were found to express markedly reduced levels of PKCα (Figures 4-3, top panel) and, consistent with findings in EC tissues, a significant correlation was observed between PKCα protein and mRNA levels across the panel of EC cell lines analyzed (Figure 4-3). To test for changes in mRNA transcription, RNA was pulse-labeled with 5-ethynyl uridine (EU) in two PKCα<sup>high</sup> (HEC-1-A; HEC-50Co) and two PKCα<sup>low</sup> (RL-95; Ishikawa) EC cell lines. PKCα<sup>low</sup> cells incorporated markedly lower amounts of EU into PKCα mRNA than PKCα<sup>high</sup> cells, with the extent of labeling closely paralleling the differences in overall levels of PKCα mRNA in the cells (Figure 4-4). Together, these data confirm that PKCα is downregulated in a significant fraction of human ECs and EC cell lines, and that PKCα deficiency primarily reflects a reduction in the transcription rate of the PKCα gene.

Further analysis of the TMA data revealed a positive correlation between decreased levels of PKCα and disease aggressiveness in the endometrioid EC subtype, with 56% of grade 1, 61% of grade 2, and 76% of grade 3 lesions showing loss of the enzyme (Figure 4-5, top panel). A significant association was also observed between loss of PKCα and risk of high-grade endometrioid disease (OR = 3.4) (Figure 4-5, bottom panel). PKCα deficiency also correlated with disease severity when only PTEN negative endometrioid tumors were considered (OR = 2.8), indicating that PKCα loss is a risk factor for aggressiveness in human EC even in the presence of activating mutations in the PI3K/AKT pathway. Consistent with this conclusion, reverse phase protein array analysis of 244 EC cases (~80% endometrioid (Liang et al., 2012)) from The Cancer Proteome Atlas (TCPA) showed that PKCα expression trends with enhanced survival of EC patients (Figure 4-6). In contrast, lower expression of PKCε or ι was associated with higher survival probability, consistent with the oncogenic properties of these isozymes in other systems (Bae et al., 2007; Benavides et al., 2011; Cacace et al., 1996; Eder et al., 2005; Murray et al., 2004; Regala et al., 2005a). Collectively, the data show that loss of PKCα can be an early event in EC
and that PKCα deficiency is associated with more aggressive disease, pointing to a potential tumor suppressive function of PKCα signaling in the endometrium.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PTEN</th>
<th>PIK3CA</th>
<th>PIK3R1</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEC-1-A</td>
<td>WT</td>
<td>G1049R</td>
<td></td>
</tr>
<tr>
<td>HEC-1-B</td>
<td>WT</td>
<td>G1049R</td>
<td></td>
</tr>
<tr>
<td>HEC-50</td>
<td>WT</td>
<td>G1049R</td>
<td>E468InsGEYDRLYE</td>
</tr>
<tr>
<td>KLE</td>
<td>WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AN3CA</td>
<td>R130fs</td>
<td></td>
<td>R557_K561&gt;Q</td>
</tr>
<tr>
<td>Ishikawa</td>
<td>V317fs; V290fs</td>
<td></td>
<td>L570P</td>
</tr>
<tr>
<td>RL95-2</td>
<td>M134I; R173H; N323fs</td>
<td></td>
<td>R386G</td>
</tr>
<tr>
<td>SNG-II</td>
<td>K6fs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNG-M</td>
<td>K164fs; V290fs</td>
<td></td>
<td>R88Q</td>
</tr>
<tr>
<td>HEC-6</td>
<td>V85fs; V290fs</td>
<td></td>
<td>R108H; C420fs</td>
</tr>
<tr>
<td>HEC-59</td>
<td>Y46H; R233X; P246L; L265fs</td>
<td></td>
<td>R38C; K567E; S460fs</td>
</tr>
<tr>
<td>HEC-108</td>
<td>K6fs; E288fs</td>
<td></td>
<td>A331V</td>
</tr>
<tr>
<td>HEC-116</td>
<td>R55_L70&gt;S; R173C</td>
<td></td>
<td>R88Q</td>
</tr>
<tr>
<td>HEC-151</td>
<td>I33del; Y76fs</td>
<td></td>
<td>C420R</td>
</tr>
<tr>
<td>HEC-251</td>
<td>S10N; E299X</td>
<td></td>
<td>M1043I</td>
</tr>
<tr>
<td>HEC-265</td>
<td>L318fs</td>
<td></td>
<td>H180fs; Q586fs</td>
</tr>
</tbody>
</table>

Table 4-3: Mutations in EC cell lines. Adapted from (Weigelt et al., 2013)
Fig. 4-3: Correlation between PKCα mRNA and protein levels in EC cell lines

Survey of PKCα protein and mRNA levels in a panel of EC cell lines using WB analysis and qRT-PCR respectively. The mRNA quantification was normalized against 18s rRNA. Vertical lines in the upper panel indicate rearrangement of lanes from a single membrane for clarity. Black bars: PKCα<sup>high</sup>. Grey bars: PKCα<sup>low</sup>. Spearman Rank correlation analysis of PKCα protein and mRNA in EC cell lines. Together, these data demonstrated a correlation between PKCα mRNA and protein levels. Data represent results from 2-3 biological replicates ± SEM. Analysis done with assistance from Adrian Black.
Fig. 4-4: Alterations in PKCa transcription in EC cells

RNA was pulse-labeled with EU in PKCa$^{\text{high}}$ and PKCa$^{\text{low}}$ EC cell lines. Levels of labeled PKCa mRNA were measured by qRT-PCR. PKCa$^{\text{high}}$ EC cells incorporated significantly higher amounts of EU than PKCa$^{\text{low}}$ cells, pointing to differential transcription rates in these cells. Data represent results from 2-3 biological replicates. Experiments performed by Adrian Black.
**Fig. 4-5: Loss of PKCα is associated with higher-grade EC**

The bar graph depicts PKCα expression status in endometrioid tumors of different grades. Included in the table is the odds ratio of tumor grade based on PKCα expression. Grade 1 and 2 tumors are considered low grade and Grade 3 tumors are high grade. **p < 0.01, *** p < 0.005 (2-sided Fisher’s exact test).**
Fig. 4-6: Survival probability of EC patients with high and low PKC expression

Patients with higher levels of PKCα have higher survival probability. Additionally, patients that have lower levels of PKCε or ι also have better survival prognosis. Results were obtained from analysis of TCPA (for PKCα) or TCGA (for PKCε and ι) databases.
4.2.2 PKCα expression is lost in mouse models of endometrial hyperplasia

The expression of PKCα was also explored in endometrial lesions arising in mice with single allele knockin of Pten mutations seen in Cowden syndrome patients (frameshift/truncation Pten^{Δ4-5/+} mutation, and missense Pten^{G129E/+} or Pten^{C124R/+} mutations). These mice develop endometrial hyperplasia with high penetrance upon loss of expression of the wild-type (WT) Pten allele (Figure 4-7A) (Wang et al., 2010). Loss of the WT allele is reflected in absence of PTEN staining in Pten^{Δ4-5/+} lesions and a reduction in PTEN staining in Pten^{G129E/+} or Pten^{C124R/+} lesions (Figure 4-7B, C). As expected, loss of PTEN function was accompanied by a marked increase in AKT activity, as indicated by enhanced pAKT^{Ser473} signal. Remarkably, these lesions uniformly lacked PKCα protein expression, with a perfect correspondence between PTEN deficiency, AKT activation, and PKCα loss. The sharp delineation between PKCα-expressing normal endometrial tissue and PKCα-deficient, pAKT positive lesions is clearly evident in these images (arrows and boxed areas, and Figure 4-7D). qRT-PCR analysis of normal and tumor tissue collected by laser capture microdissection demonstrated that loss of PKCα protein is associated with a reduction in PKCα mRNA in these early lesions (Figure 4-8). PKCα loss was also observed in uterine tumors of mice with cre-mediated conditional endometrial deletion of Pten in the epithelium and stroma (Pten^{−/−}) or the epithelium alone (Pten^{−/−}), regulated by the progesterone receptor promoter or the lactoferrin promoter, respectively (Figure 4-9) (Daikoku et al., 2008; Daikoku et al., 2014). Interestingly, these lesions expressed increased levels of Id1, a gene known to be suppressed by PKCα signaling (Figure 4-10) (Hao et al., 2011). The loss of PKCα in multiple in vivo models of PI3K/AKT-driven endometrial neoplasia further supports a role for PKCα deficiency in uterine tumorigenesis.
Fig. 4-7: Concurrent loss of PKCa and PTEN correlates with pAKT

See next page for figure legend
Immunohistochemical analysis of PTEN, pAKT, and PKCα expression in consecutive uterine sections from three strains of Pten-mutant mice - *Pten*Δ4-5/+ (A), *Pten*C124R/+ (B), and *Pten*G129E/+ (C). Arrows indicate hyperplastic lesions lacking functional PTEN and PKCα expression, and strongly positive for pAKT. In (B), inserted are magnified images of the boxed regions. (D) Image depicting the loss in PKC in the endometrial hyperplasia and its retention in the adjacent normal endometrium (NE) and endometrial hyperplasia (EH). *Scale bar*, 50 µm. Images are representative of all hyperplastic lesions observed in these animals. Experiments performed with assistance from Kathryn Curry and Kang-Sup Shim.
Fig. 4-8: Laser Microdissection of pAKT- and PKCα-labeled uterine epithelium for qRT-PCR analysis of PKCα mRNA levels.

Immunoflourescence analysis of serial sections of frozen uterine tissue from 9 month *Pten^{4l-5/+};Prkca^{+/+}* mouse identifies normal epithelium (PKCα^{pos}, pAKT^{neg}, indicated by large arrows) and tumor lesions (PKCα^{neg}, pAKT^{pos}, indicated by small arrows). The tissues were isolated by laser microdissection and qRT-PCR analysis was performed to quantify PKCα mRNA levels in the corresponding regions.
Fig. 4-9: Loss of PKCα in uterine tumors of mice with cre-mediated conditional endometrial deletion of Pten.

Immunohistochemical staining of PKCα in uterine tumors of mice with cre-mediated conditional endometrial deletion of Pten in the epithelium and stroma (Pten<sup>pro-/−</sup>) or the epithelium alone (Pten<sup>epi-/−</sup>), regulated by the progesterone receptor promoter or the lactoferrin promoter, respectively. Note the PKCα positive stroma and myometrium and PKCα negative endometrial tumor tissue. Scale bar, 50 µm.
**Fig. 4-10: Enhanced Id1 expression in endometrial hyperplasia correlates with loss of PKCα**

IHC analysis of PKCα and Id1 expression in endometrium from *Pten^G129E/+* and *Pten^C124R/+* mice. Note the strong nuclear staining for Id-1 in hyperplastic regions that lack PKCα (block arrows). Arrowheads indicate normal endometrial glands with high PKCα and low Id1 expression. IHC staining performed by Kathryn Curry.
4.2.3 PKC\(\alpha\) deficiency in EC is not a direct result of PTEN loss/ AKT activation and does not appear to drive loss of PTEN

To determine if PKC\(\alpha\) deficiency in EC is a direct consequence of PTEN loss of function/AKT activation, PTEN was stably knocked down in three PTEN WT, PKC\(\alpha^{\text{high}}\) human EC cell lines (Figure 4-11A). While the functional consequences of PTEN knockdown were confirmed by increased AKT phosphorylation, loss of PTEN did not affect the levels of PKC\(\alpha\) (or of any other PKC isozyme, Figure 4-12). Similarly, inhibition of aberrant AKT activity in PTEN mutant/PKC\(\alpha^{\text{low}}\) EC cell lines by restoration of PTEN expression (Figure 4-11B) or treatment with AKT or PI3K inhibitors (MK-2206 or LY294002, respectively) (Figure 4-11C), failed to affect PKC\(\alpha\) levels. Taken together, these data indicate that PKC\(\alpha\) deficiency is not a mere passenger event of PI3K/AKT hyperactivation, and suggest that loss of the enzyme may contribute directly to endometrial tumor development. To test the possibility that PKC\(\alpha\) deficiency drives loss of PTEN in endometrial cells, we explored the effects of PKC\(\alpha\) knockdown in PTEN WT, PKC\(\alpha^{\text{high}}\) EC cell lines. As shown in Figure 4-11D, PKC\(\alpha\) knockdown failed to affect PTEN levels in these cells.
Fig. 4-11: PKCα deficiency in EC is not a direct result of PTEN loss or AKT activation and does not affect PTEN levels

Western blot analysis of PKCα levels in EC cells with A) stable expression of PTEN shRNA, B) adenoviral restoration of PTEN, and C) treatment with AKT (MK-2206) or PI3K (LY294002) inhibitors. siRNA-mediated knockdown of PKCα, in turn, failed to affect PTEN levels (D). Data represent results from at least 3 biological replicates.
**Fig. 4-12: PTEN knockdown did not alter the expression of other members of the PKC family**

Western blot analysis of the expression of indicated PKC isozymes in EC cells stably expressing shRNA targeting PTEN. Data represent results from at least 3 biological replicates.
4.2.4 PKCα inhibits endometrial tumorigenesis in vivo and in vitro

To directly examine the effects of PKCα deficiency on endometrial tumor development, we generated Prkca+/+;PtenΔ4-5/+ and Prkca-/-;PtenΔ4-5/+ mice. Uterine tissue was collected at 1, 3, 5, 7, and 9 months and hyperplastic lesions were identified by pAKT Ser473 staining and histopathological analysis (Wang et al., 2002) (Figure 4-13, top and middle panels). Quantification of the proportion of pAKT positive endometrium pointed to a statistically significant 3-fold increase in tumor burden in PKCα-/- animals at both 1 and 3 months compared with PKCα+/+ littermate controls (Figure 4-13, bottom graph). However, this difference diminished at later times. Blinded histological analysis of H&E stained sections by a pathologist (Dr. Yuri Sheinin) confirmed this conclusion (not shown). Since hyperplastic lesions arising in Prkca+/+;PtenΔ4-5/+ mice uniformly lacked PKCα, the increased tumor burden in Prkca-/-;PtenΔ4-5/+ mice indicates that PKCα loss is a rate limiting step in endometrial tumorigenesis in this model.

To test the effects of PKCα on the transformed phenotype of human EC cells, PKCα expression was restored in PKCαlow cell lines by adenoviral transduction or silenced in PKCαhigh cells using siRNA, and anchorage-independent growth was assessed by colony formation in soft agarose. Exogenous PKCα markedly inhibited colony formation of PKCαlow EC cells, and colonies that did form were substantially smaller than those of LacZ-transduced cells (Figure 4-14). One infection unit/cell (moi) of PKCα adenovirus was sufficient to suppress anchorage-independent growth of these cells (Figure 4-15), pointing to a strong tumor suppressive activity of PKCα in human EC cells. The requirement for PKCα kinase activity in these effects was tested using kinase dead enzyme (KD-PKCα). Since KD-PKCα is unstable in cells (Pysz et al., 2009), 25 or 50 moi of KD-PKCα adenovirus were used to ensure levels of mutant protein expression comparable with WT PKCα at 1 moi. KD-PKCα failed to affect colony formation, even at the highest level of expression (Figure 4-15). The effects of PKCα knockdown were examined in PKCαhigh HEC-6 cells, which grow less efficiently in soft agarose than other EC cells in our panel (Figure 4-16). Notably, PKCα depletion led to an approximately two-fold increase in colony formation of these
Thus, PKCα kinase activity is a potent inhibitor of the transformed phenotype of human EC cells.

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**Fig. 4-13: PKCα inhibits endometrial tumorigenesis in vivo**

Uterine tissue from Pten<sup>Δ4-5+</sup>;Prkca<sup>+/+</sup> and Pten<sup>Δ4-5+</sup>;Prkca<sup>−/−</sup> transgenic mice aged for 1, 3, 5, 7, or 9 months was analyzed for pAKT by immunohistochemistry, as shown in the representative images from 1- and 3-month cohorts. At least two sections were analyzed for each animal. Percent pAKT-positive uterine epithelium was measured as a proportion of the total epithelial compartment of the endometrium. * indicates \( p < 0.05 \) as determined by Wilcoxon Rank Sum test. Grey circles, Pten<sup>Δ4-5+</sup>;Prkca<sup>+/+</sup>. Black circles, Pten<sup>Δ4-5+</sup>;Prkca<sup>−/−</sup>. Scale bar, 200 µM.
**Fig. 4-14: PKCα inhibits endometrial tumorigenesis *in vitro***

PKCα<sup>low</sup> endometrial cancer cell lines (Ishikawa, RL95-2, HEC-59, HEC-116, HEC-251) were infected with adenoviral vectors expressing either PKCα (α) or LacZ (Z) at a moi of 20 and analyzed for PKCα expression by immunoblotting; β-actin serves as a loading control (*upper panel*). Infected cells were plated in soft agarose and quantification of colony formation is presented relative to the LacZ control (*bottom graph*). Data are representative of at least 3 independent experiments.
Fig. 4-15: PKCα kinase activity is required for its tumor suppressive function

Assessment of anchorage independent growth of EC cells transduced with WT-PKCα or KD-PKCα. The higher mobility of KD-PKCα reflects the absence of priming phosphorylation of the enzyme (Carpenter et al., 2004). * p < 0.05, ** < 0.01, or ns (not significant) as calculated by 2-sided student’s t test. Error bars indicate mean ± SEM from 3 biological replications. β-actin serves as a loading control.
Fig. 4-16: siRNA-mediated knockdown of PKCα promotes tumorigenesis

Assessment of anchorage independent growth of EC cells with siRNA-mediated knockdown of PKCα.

* p < 0.05, ** < 0.01, or ns (not significant) as calculated by 2-sided student’s t test. Error bars indicate mean ± SEM from 3 biological replications. β-actin serves as a loading control.
4.2.5 PKCα activity inhibits PI3K/AKT signaling in EC cells via a PHLPP 1/2-independent, okadaic acid/ calyculin A-sensitive mechanism

Since a majority of ECs are highly dependent on PI3K/AKT signaling (Weigelt et al., 2013), we next examined the effects of PKCα activity on this pathway using a panel of human EC cell lines (Table 4-3). Treatment of PKCα\textsuperscript{high} cells with the PKC agonist phorbol 12-myristate 13-acetate (PMA) markedly decreased the phosphorylation of AKT as well as its downstream substrate, PRAS40 (Figure 4-17A). Similarly, the short-chain diacylglycerol 1,2-dioctanoylglycerol (DiC\textsubscript{8}), a more physiological PKC activator, also led to decreased pAKT\textsuperscript{Ser473} levels (Figures 4-17B). Consistent with the involvement of PKCα, PMA treatment did not affect AKT phosphorylation in PKCα\textsuperscript{low} cells (Figure 4-17C), an effect that was rescued by restoration of PKCα by adenoviral transduction (Figure 4-17D). Furthermore, PKCα inhibition with Gö6976, which targets the classical PKCs, blocked the ability of PMA to reduce pAKT in PKCα\textsuperscript{high} cells (Figure 4-18). Finally, knockdown of PKCα in PKCα\textsuperscript{high} cells with two different siRNAs prevented PMA-induced AKT hypophosphorylation (Figure 4-19). Since PKCα siRNA had no notable effect on the expression of other PKC isozymes (Figure 4-20), these data confirm a requisite role for PKCα in suppression of AKT activity in EC cells. Interestingly, PKCα knockdown also led to increased basal AKT\textsuperscript{Ser473} phosphorylation, pointing to a tonic repressive effect of the enzyme on PI3K/AKT signaling in these cells (Figure 4-19, compare lane 1 with lanes 3-6 in both panels). Full activation of AKT requires phosphorylation at both S473 and T308 residues. Both of these residues are targeted by PKCα in EC cells, as indicated by (a) the parallel decrease in their phosphorylation following PMA treatment, a sustained effect that is supported by the continued activation of PKCα (Figure 4-21), and (b) the robust increase in phosphorylation at both sites induced by PKCα knockdown in PKCα\textsuperscript{high} cells (Figure 4-22).
Fig. 4-17: PMA-induced activation of PKCα reduces AKT phosphorylation

Western blot analysis of the phosphorylation status of AKT and its downstream target PRAS40 in PKCα high and PKCα low EC cell lines treated with vehicle (C) or 100 nM PMA (P) for various times as indicated (A and C) or with 20 µg DiC₈ for 6 hr (B). DiC₈ was added in fresh medium every 30 min to account for its rapid metabolism in cells. (D) Adenoviral restoration of PKCα rescued the effect of PKC agonists in PKCα low cells. Data are representative of at least 3 biological replications. β-actin or total AKT serve as a loading control. Western blot analysis performed with assistance from Michelle Lum.
Fig. 4-18: The cPKC inhibitor Gö6976 blocks PMA-induced AKT hypophosphorylation

Treatment with Gö6976 (2.5 µM for 30 min prior to addition of PMA), which inhibits cPKCs, blocked the ability of PMA to reduce AKT phosphorylation. Data are representative of at least 3 biological replicates. β-actin serves as a loading control.
**Fig. 4-19: siRNA-mediated knockdown of PKCα prevents PMA-induced hypophosphorylation of AKT**

Western blot analysis of AKT phosphorylation in EC cells transfected with non-targeting (nt) or PKCα siRNAs (siRNA 1 or 2). Note the increase in steady-state levels of pAKT on PKCα knockdown. Data are representative of 3 biological replicates. β-actin serves as a loading control.

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Data are representative of 3 biological replicates. β-actin serves as a loading control.
Fig. 4-20: Knockdown of PKCα does not affect the expression of other members of the PKC family in EC cells

Western blot analysis of PKC isoforms in EC cells transfected with non-targeting (nt) siRNA or siRNA against PKCα (#1 or 2). Data are representative of 3 biological replicates. β-actin serves as a loading control.
**Fig. 4-21: The effects of PMA on PKC\(\alpha\) and AKT activity are sustained in EC cells**

A) Western blot analysis of the effects of PMA on the phosphorylation of both Thr308 and Ser473 residues of AKT in a time course PMA treatment. Despite the progressive downregulation of PKC\(\alpha\), AKT phosphorylation remains suppressed over the 6 h period. Immunofluorescence staining (B) and subcellular fractionation (C) confirmed the presence of active, membrane-associated PKC\(\alpha\) after 6 h of PMA treatment. \(\beta\)-actin and AKT serve as loading controls. Arrows in (B) indicate membrane-associated PKC\(\alpha\). In (C), *Particulate* indicates the membrane fraction. Data are representative of 3 biological replicates.
**Fig. 4-22: Knockdown of PKCα enhances steady-state AKT activation**

PKCα was silenced in KLE, HEC-6 and SNG-M EC cells and effects on phosphorylation/activity of AKT were determined by Western blot analysis of pAKT$^{473}$ and pAKT$^{308}$ and of AKT substrates FOXO1 or PRAS40. Data are representative of 3 biological replicates. Western blot analysis performed with assistance from Michelle Lum.
Coincident mutations in *PTEN*, *PIK3CA* and *PIK3R1/2* are commonly seen in EC (Weigelt et al., 2013). To determine if PKCα signaling can inhibit AKT activity in EC cells with coexisting PI3K/AKT pathway alterations, PTEN was knocked down in PKCα<sup>high</sup>, PTEN WT HEC-1-A and HEC-50Co cells, which harbor activating mutations in the catalytic (PIK3CA) and regulatory (PIK3R1) subunits of PI3K, respectively (Weigelt et al., 2013). As expected, PTEN knockdown further increased PI3K/AKT signaling, as indicated by enhanced basal levels of phosphorylated AKT (at both Ser473 and Thr308) and its downstream effector PRAS40 (Figure 4-23). However, loss of PTEN did not prevent PMA-induced hypophosphorylation of these sites, indicating that PKCα signaling can suppress AKT even in the face of multiple activating events in the pathway. This finding is consistent with the ability of PKCα to inhibit AKT in SNG-M and HEC-6 cells, which naturally harbor coincident mutations in PTEN and PI3K subunits. These data, combined with evidence for downregulation of the PKCα target gene Id1 (Figure 4-24), a master regulator of tumor aggressiveness (Hao et al., 2011), further support a role for PKCα as a tumor suppressor in EC.

Since PP2A and PHLPPs have been identified as the main phosphatases that reverse AKT phosphorylation (Gao et al., 2005; Van Kanegan et al., 2005), we investigated their involvement in PKCα-mediated AKT hypophosphorylation in EC cells. These phosphatases are expressed and functional in EC cells (Figure 4-25). A role for PHLPP1/2 in regulation of AKT in EC cells was confirmed by the ability of the PHLPP1/2 inhibitors NCS45586 and NCS117079 (Sierecki et al., 2010) to increase basal AKT phosphorylation (Figure 4-26A). Enhanced AKT phosphorylation was similarly observed following inhibition of PP1 and PP2A with calyculin A, or PP2A alone with okadaic acid (Figure 4-26B). PMA was still able to suppress pAKT in the presence of the PHLPP1/2 inhibitors, indicating that the effects of PKCα are independent of PHLPP1/2 (Figure 4-26A). In contrast, both calyculin A and okadaic acid blocked PMA-induced AKT hypophosphorylation, pointing to a PP2A-dependent mechanism (Figure 4-26B). More importantly, PMA led to an induction of PP2A activity, as determined by PP2A
immunoprecipitation phosphatase assays that was abolished by siRNA-mediated PKCα knockdown (Figure 4-27). The involvement of PP2A was further explored by siRNA-mediated knockdown of the catalytic subunit of the phosphatase (PP2AC). PP2AC knockdown enhanced basal AKT phosphorylation and inhibited the ability of PMA/PKCα to reduce pAKT levels (Figure 4-28), although the effect was less pronounced than seen with pharmacological inhibition, likely reflecting the incomplete knockdown of PP2AC achieved with siRNA. Together, these results indicate that PKCα inhibits PI3K/AKT signaling through a PHLPP-independent, PP2A-dependent mechanism. It should be noted that, although PP2A may preferentially dephosphorylate AKT on the T308 site, it can also dephosphorylate AKT on the S473 site, as described in numerous reports using a variety of biological systems (Andrabi et al., 2007; Li et al., 2003).
Fig. 4-23: PKCα signaling can inhibit AKT activity in EC cells with hyperactivation of the PI3K/AKT pathway due to coexisting alterations in more than one pathway component

EC cells harboring mutations in the catalytic (PIK3CA, HEC-1-A) or regulatory (PIK3R1, HEC50Co) subunits of PI3K and stably expressing non-targeting (nt) or two different PTEN (PTEN1+2) shRNA were treated with EtOH (C) or PMA (P) and subjected to Western blot analysis for the indicated proteins. Data are representative of 3 biological replicates. β-actin serves as a loading control. Western blot analysis performed with assistance from Michelle Lum.
Fig. 4-24: PKCα downregulates Id1 in EC cells

Western blot analysis showing downregulation of Id1, a growth/tumor promoter, following treatment with ethanol (C) or PMA (P) in PKCα\textsuperscript{high} but not PKCα\textsuperscript{low} cells. Data are representative of 3 biological replicates. β-actin serves as a loading control.
**Fig. 4-25: Expression of PHLPPs and PP2A in EC cells**

Assessment of the expression of two families of phosphatases that are known to modulate AKT phosphorylation, PHLPPs and PP2AC (the catalytic subunit of PP2A), in EC cells by qTR-PCR or Western blot analysis. The mRNA quantification was normalized against 18s rRNA. β-actin serves as a loading control for Western blots. Data are representative of 3 biological replicates and error bars are ± SEM.
Fig. 4-26: PP2A but not PHLPP inhibitors blocked PMA-mediated hypophosphorylation of AKT

EC cells were pretreated with A) 50 mM PHLPP inhibitor NSC 45586 or 117079 (15 min), or B) 10 nM calyculin A (CA) (15 min) or 2.5 µM okadaic acid (OA) (30 min) prior to treatment with vehicle (C) or 100 nM PMA (P) for 6 hr. Western blot analysis demonstrated the effects of phosphatase inhibition on AKT phosphorylation. β-actin serves as a loading control and data are representative of 3 biological replicates.
Fig. 4-27: PMA induces PP2A activity in EC cells

Measurement of PP2A activity using a malachite green based assay in PKCα knockdown EC cells (achieved by a combination of 2 siRNAs targeting PKCα) treated with vehicle (C) or 100 nM PMA (P) for 10 min. Non-targeting siRNA was used as a control. Results are representative of multiple biological replicates and error bars indicated mean ± SEM. *** p < 0.005, n.s. not significant. Experiment performed with assistance from Adrian Black.
Fig. 4-28: PP2A knockdown reduces the effects of PKCα signaling on AKT phosphorylation/activity in EC cells

Immunoblot analysis of AKT phosphorylation in EC cells transfected with non-targeting (nt) siRNA or a combination of two siRNAs targeting the catalytic subunit of PP2A (siPP2AC). β-actin serves as a loading control and data are representative of 3 biological replicates.
4.2.6 PKCα loss is associated with increased AKT activity in endometrial lesions in vivo

While universal loss of PKCα was seen in uterine lesions from Pten^{AT5/+}, Pten^{G129E/+} and Pten^{C124R/+} mouse models, PKCα-retaining areas were detected in lesions arising in the Pten^{pr-/-} mouse. This allowed comparison of AKT activity in PKCα-retaining and PKCα-deficient cells in situ. pAKT staining was stratified as none-low, medium or high and association between pAKT staining intensity and the presence or absence of PKCα signal was quantified in serial sections. PKCα expression was associated with significant differences in AKT staining intensity (p < 0.0001), with PKCα negative areas predominantly exhibiting high intensity pAKT staining, and PKCα positive areas largely coinciding with low intensity pAKT staining (Figure 4-29, compare areas indicated with block and open arrows). This correspondence supports a role for PKCα in suppression of PI3K/AKT signaling in endometrial tumor cells in vivo as well as in vitro.

Fig. 4-29: PKCα loss is associated with increased pAKT in endometrial lesions in vivo

Serial sections of uterine tissue from Pten^{pr-/-} mice immunostained for PKCα or pAKT(Ser473). Quantification of different intensities of pAKT staining (none-low, medium, or high) in PKCα positive and negative areas. Data are representative of 3 independent experiments. Error bars indicate SEM. Analysis performed with assistance from Adrian Black.
4.2.7 Inhibition of AKT activity is an important component of the PKCα tumor-suppressive axis in EC cells

The role of inhibition of PI3K/AKT signaling in the tumor suppressive effects of PKCα in EC cells was tested using constitutively active AKT: myristoylated AKT1 (myr-AKT), AKT-DD (T308D, S473D), and AKT-E17K (an active mutant identified in EC (Mancini et al., 2016; Rudolph et al., 2016)). These constructs were introduced into PKCαlow HEC-59, RL95-2, HEC-116, Ishikawa, or HEC-251 EC cells by adenoviral or retroviral transduction and the ability of exogenous PKCα to inhibit anchorage-independent growth was assessed. The amount of constitutively active AKT introduced into EC cells was titrated to produce a 2- to 5-fold increase in basal pAKT levels, as determined by quantification of immunoblots. In the context of strong endogenous AKT activation in EC cells, this increase in AKT activity had only slight effects on anchorage-independent growth on its own, with myr-AKT, AKT-DD, and AKT-E17K causing a modest increase in overall colony size (Figures 4-30, 4-31) and a small, non-statistically significant increase in colony number in some cell lines. In contrast, constitutively active AKT had a marked effect in cells transduced with PKCα, restoring both colony number and overall colony size, albeit to different extents in different cell lines (Figures 4-30, 4-31). While the inability of constitutive to fully reverse the effects of PKCα suggests that other factors may be involved, these data point to inhibition of PI3K/AKT signaling as a key mechanism mediating the tumor suppressive actions of PKCα in the endometrium.
Fig. 4-30: Inhibition of AKT activity is a component of the PKCα tumor-suppressive axis in EC cells

Colony formation in soft agarose of EC cells transduced with adenovirus expressing LacZ (moi = 50), myr-AKT1 (moi = 50), and/or PKCα (moi = 1) as indicated. * p < 0.05; ** p < 0.01 (2-sided student’s t test). Error bars indicate SEM from 3 independent experiments. Quantification was performed with assistance from Adrian Black.
Fig. 4-31: Mutant AKT partially rescues PKCα-mediated growth suppression

Colon formation in soft agarose of EC cells stably expressing mutant AKT generated by transduction with AKT-DD or E17K or empty retroviral vectors. Mutant AKT-expressing or control cells were subsequently transduced with Ad-LacZ or Ad-PKCa as indicated. * p < 0.05; **p < 0.01; ***p < 0.005 (2-sided student’s t test). Error bars indicate SEM from 3 independent experiments. Quantification was performed with assistance from Adrian Black and Michelle Lum.
4.3 Discussion

The current study provides the first evidence for a tumor suppressive role of PKCα signaling in PI3K/AKT-driven EC, via a mechanism that involves PP2A-mediated inactivation of AKT. The role of PKCα in tumorigenesis appears to be multifaceted. While the enzyme has been linked to tumor promotion in some systems, our studies add to a growing list of tumor types (e.g., colon and lung) in which PKCα signaling has tumor suppressive effects.

Our IHC and qRT-PCR analysis of human ECs revealed loss of PKCα protein and mRNA in a majority of cases. Notably, more than half of grade 1 ECs showed deficiency of the enzyme, indicating that PKCα suppression can occur early during human endometrial tumorigenesis. This notion is supported by our analysis of mouse models of PI3K/AKT-driven endometrial neoplasia, which demonstrated loss of PKCα expression in pre-malignant hyperplasia regardless of the nature of PTEN dysregulation. Additional supportive evidence is provided by previous transcriptome analysis of early Type I ECs, which demonstrated a 3-fold downregulation of PKCα mRNA in tumors relative to normal endometrium (Risinger et al., 2013; Saghir et al., 2010), and by IHC analysis of a limited set of grade 1 ECs, which revealed regions of low or undetectable PKCα protein in a subset of lesions (Haughian and Bradford, 2009).

Although loss of PKCα mRNA and protein has been noted in other tumor types (e.g., colon cancer (Verstovsek et al., 1998), non-small cell lung cancer (Hill et al., 2014), basal cell carcinoma (Neill et al., 2003), T-cell Acute Lymphoblastic Leukemia (T-ALL) (Milani et al., 2014)), the mechanisms underlying PKCα deficiency have yet to be defined. Our qRT-PCR and IHC analysis argues that decreased mRNA expression is the major mechanism involved in disabling PKCα functions in the endometrium, although TCGA data indicate that PKCα may be inactivated by mutation in up to 5% of ECs (Antal et al., 2015). We also show, for the first time, that PKCα loss can be mediated by transcriptional repression of the PKCα gene. Initial mechanistic analysis excluded methylation of the PKCα promoter in EC (data not shown) or differential promoter activity in cells with high or low levels of PKCα mRNA/protein. Recent analysis of the regulatory
region of PKCα found differential methylation of the CpG shores, defined as 2kb up- or down-
stream of the CpG island, with hypermethylation of the right CpG shores in cells with reduced
PKCα mRNA (Figure 4-32A). In line with this new finding, we have observed increased PKCα
expression when EC cells are treated with decitabine, a DNA hypomethylating agent (Figure 4-
32B). Experiments are underway to confirm these results in additional cells lines.
Fig. 4-32: Methylation of PKCα CpG shores in cancer cells

A) Methylation analysis showed hypermethylation in the right CpG shore in PKCα\textsuperscript{low} cell lines, as indicated in red, whereas the same region is generally hypomethylated in cell lines with high PKCα expression. B) Increased PKCα levels in PKCα\textsuperscript{low} cells (Ishikawa) treated with decitabine. Methylation analysis was performed with assistance from Xinyue Li, Chun-wei Chen, and Dr. Tim Huang.
The precise correspondence between PKCα loss, PTEN deficiency, and AKT activation in murine endometrial lesions suggested that transcriptional suppression of PKCα could be a direct result of hyperactivation of the PI3K/AKT pathway, at least in some ECs. However, modulation of PTEN expression or AKT activity in human EC cells excluded this possibility, indicating that PKCα downregulation is not merely a passenger effect of aberrant PI3K/AKT signaling but, instead, is an alteration that is selected for because of specific contributions to endometrial tumor development. The possibility that PKCα deficiency drives the loss of PTEN was also tested and excluded in PKCα knockdown experiments. Anchorage-independent colony formation assays provided direct evidence for a tumor suppressive function of PKCα in human endometrial cells: restoration of PKCα expression/activity profoundly inhibited colony formation of human EC cells in soft agarose, while PKCα knockdown enhanced the transformed properties of EC cells that retained expression of the enzyme. The importance of early loss of PKCα was highlighted by the marked acceleration in uterine hyperplastic transformation observed in PKCα deficient Pten<sup>Δ4-5/+</sup> mice, a finding that pointed to abrogation of PKCα signaling as a rate limiting step for tumor initiation in the endometrium. In advanced cases of human EC, the proportion of PKCα negative tumors increased to >75%, suggesting that PKCα deficiency defines a subset of ECs with high risk of progression, a notion that is consistent with TCPA data pointing to an association between reduced PKCα expression and decreased survival of EC patients. The finding that PKCα deficiency is associated with a 3-fold increase in risk of high grade disease is consistent with previous reports in other tumor types. Genetic deletion of PKCα led to increased tumor burden, adenoma-to-carcinoma progression, and reduced survival in the Apc<sup>Min/+</sup> mouse model of intestinal neoplasia (Oster and Leitges, 2006) and in Kras-mediated models of lung tumorigenesis (Hill et al., 2014). Low PKCα expression identified a new subgroup of childhood T-ALL with an extremely poor outcome (Milani et al., 2014) and appears to enhance the tumorigenic potential of Gli1 in basal cell carcinoma (Neill et al., 2003). It will be interesting to determine if common mechanisms mediate
PKCα loss in these tumor types.

A majority of ECs harbor coexisting alterations in two or more PI3K/AKT pathway components, with additive effects on AKT activation that are crucial for EC development (Chen et al., 2006; Oda et al., 2005; Weigelt et al., 2013). Remarkably, knockdown of PKCα in cells with genetic hyperactivation of the PI3K/AKT pathway further enhanced the activity of AKT, highlighting the ability of PKCα deficiency to contribute to the strength of PI3K/AKT signaling in EC cells. The demonstration that PKCα negative uterine regions in Pten<sup>−/−</sup> mice are associated with significantly higher levels of pAKT than PKCα-retaining regions indicates that PKCα loss also contributes to PI3K/AKT hyperactivation during endometrial tumorigenesis in vivo. PKCα activity may also restrain AKT in the normal endometrium, since increased expression and membrane association of the enzyme coincide with the reported downregulation of AKT activity during the proestrus-to-estrus transition in rats (Dery et al., 2003). These findings, together with the demonstration that agonist-induced PKCα activation suppresses AKT in EC cells, identify PKCα as a potent inhibitor of PI3K/AKT signaling in the endometrium, capable of disabling the AKT oncoprotein even in the context of multiple activating mutations in upstream signaling intermediates (e.g., PTEN and PIK3CA in HEC-251 cells; PTEN, PIK3CA and KRAS in SNG-M cells).

Inactivation of AKT appears to be a key component of the tumor suppressive actions of PKCα in endometrial cells, since constitutively active AKT [myr-AKT, AKTDD (T308D, S473D), and AKT(E17K)] markedly diminished the inhibitory effects of PKCα on EC cell colony formation in soft agar. Together, our findings support the idea that disruption of PKCα signaling may be critical for achieving the robust PI3K/AKT pathway activation that is required to drive EC development and progression (Figure 4-8D). To demonstrate the potential clinical impact of PKCα loss in EC, future experiments will investigate whether PKCα deficiency will enhance the sensitivity of EC tumors to AKT inhibitors or other targeted agents. The efficacy of agents that are currently under development or in clinical trials is largely limited by their dose-dependent toxicity.
As cells with \textit{PIK3CA} mutations exhibited increased sensitivity to agents targeting the PI3K/AKT pathway (e.g. PI3K inhibitors, AKT inhibitors, and mTOR inhibitors), the loss of PKCα-mediated negative regulation of AKT activation may similarly enhance the sensitivity of these cells to PI3K/AKT pathway inhibition. Thus, PKCα deficiency may provide an additional layer of patient stratification, indicative of therapeutic responsiveness to PI3K/AKT targeting agents in clinical trials.

Crosstalk between PKCα and the PI3K/AKT pathway has been noted in other systems. PKCα can inactivate upstream modulators of PI3K such as growth factor receptors and scaffolding proteins (Koese et al., 2013; Oriente et al., 2005) and can dampen PI3K activity directly (Hoshino et al., 2012; Sipeki et al., 2006). Here, we demonstrate that PKCα activates the serine/threonine phosphatase PP2A in EC cells and inactivates AKT via a PP2A-dependent mechanism. PKCα-induced AKT inactivation may involve direct effects of PP2A on activating phosphorylation at T308 and S473 (Manning and Toker, 2017), although potential effects on upstream regulators of AKT activity remain to be formally excluded. A role of PP2A in the antitumor effects of PKCα in the endometrium is consistent with known functions of the phosphatase as a \textit{bona fide} tumor suppressor, with key activities in maintenance of cellular homeostasis (Perrotti and Neviani, 2013). Future studies will explore the specific PP2A trimeric holoenzyme complex(es) responsible for mediating PKCα-induced inactivation of AKT.

Together, our findings highlight disruption of a PKCα→PP2A→AKT signaling module as a key step in the development of EC, ensuring robust, unopposed AKT activation and promoting transformation of endometrial cells (Figure 4-8D). These studies provide a foundation for exploring the prognostic value of PKCα expression in EC and the potential of harnessing the PKCα→PP2A module for therapeutic benefit in a subset of ECs.
Chapter 5: Conclusion
PKCα is ubiquitously expressed in many tissues and regulates the physiology of a cell by relaying signals to a wide molecular network. Understanding the functional role of PKCα in maintenance of tissue homeostasis and its involvement in the carcinogenesis process in different systems has been a long-standing interest of our laboratory. Past work from our lab, focusing on the intestinal system, found PKCα signaling to be growth inhibitory and tumor suppressive. In this study, we examined the expression and activation of PKCα in the normal endometrium. Previous studies have shown that PKCα mediates growth, invasion and survival response to DNA damage in endometrial cancer cells (Haughian and Bradford, 2009; Haughian et al., 2006; Haughian et al., 2009). However, its impact on oncogenic PI3K signaling, the driving force of the majority of endometrial tumors, remained elusive. Therefore, as part of our effort to understand the functional role of PKCα in cancer, we investigated how signaling mediated by this kinase impacts endometrial carcinogenesis driven by hyperactive PI3K/AKT signaling.

5.1 PKCα as a potential anti-proliferative regulator in the endometrium

The endometrium undergoes cyclic remodeling, in which the stroma and epithelium proliferate and regress/shed in response to hormonal fluctuation. Aberrations in this tightly regulated process disrupt the homeostasis of the endometrium and, eventually, results in uncontrolled growth and tumor formation. PKCα is among the several isozymes that have been reported to be expressed in the uterus (Jofre et al., 2013; Kim et al., 1999). However, little is known of its specific functions in this tissue. Unlike other isozymes, which have been shown to induce uterine contraction, activation of PKCα was observed in preterm uterus when the myometrium is quiescent and PKCα has been shown to inhibit myometrium contractility by regulating the expression of prostaglandin and urocortin, in order to maintain pregnancy (Bamberger et al., 2007; Jofre et al., 2013; Wouters et al., 2014). In the epithelium, PKC has been implicated in promoting protein synthesis through ERK-mTOR in response to estrogenic stimulation (Wang et al., 2015).
PKC activation was also shown to coincide with the switch to the secretory phase that is predominantly regulated by P₄ (Chen et al., 2013). Based on these studies, it is not clear how PKCα is involved in regulating E₂/P₄ responses and epithelial remodeling in the endometrium or which PKC isozyme(s) is the major regulator of these events.

Current understanding indicates that PKC functions are dependent on cellular context and that individual isozymes can have overlapping or opposing actions (Garg et al., 2014). To decipher the role of PKCα in cancer, it is crucial to first understand its involvement in the normal physiology in the tissue of interest. In our study, we observed PKCα expression in the stromal and epithelial compartments of the uterus, though expression was higher in the stroma in both human and murine uterine tissues (Figure 3-1). In the human endometrium, PKCα activation, as indicated by its membrane association, was observed in the secretory phase. In mouse, there was regional membrane-associated PKCα in the epithelium in proestrus, which became more prevalent in the subsequent estrous phase (Figure 3-3). This increase in PKCα activation negatively correlated with proliferation, as shown by the reduced Ki67 signal in estrus compared with other phases (Figure 3-2, 3-4). Interestingly, this timing coincided with a transition from massive proliferation to a state primed for embryo implantation. Together, our data linked the expression and activation of PKCα to hormone-driven cycling of the tissue, suggesting that PKCα signaling may trigger an anti-proliferative program that is involved in endometrial remodeling, implantation, and maintenance of pregnancy. Our IHC analysis also revealed a regional staining pattern for PKCα, suggesting that the endometrial epithelium might be heterogeneous, exhibiting differential responses or temporal discrepancies in their response to hormone fluctuation (Figure 3-3, 3-4). To further explore the role of PKCα in the endometrium, mechanistic studies focusing on how E₂ and P₄ influence PKCα signaling (and other PKCs) may reveal the role of this kinase in regulating endometrium regeneration, thus painting a more complete picture of how these enzymes function cooperatively to regulate uterine biology.
5.2 PKCα suppresses endometrial carcinogenesis

The question of whether PKCs promote or suppress cancer has been a subject of long-lasting debate in the field. Historically, PKCs are mostly recognized for their association with the carcinogenic phorbol esters, and their ability to promote tumor formation that encouraged the development of PKC inhibitors as cancer therapeutics of which had disappointing results in clinical trials (Garg et al., 2014; Mochly-Rosen et al., 2012). Subsequent studies revealed that these enzymes possess tumor suppressive ability, an observation that is bolstered by the recent survey of cancer-associated PKC mutations, which found these mutations to cause loss of kinase function (Antal et al., 2015; Hill et al., 2014; Newton and Brognard, 2017; Oster and Leitges, 2006).

Previous work from our laboratory has shown that PKCα inhibits intestinal tumor formation through downregulation of cyclin D1 and Id1 and increased expression of p21 (Frey et al., 2000; Hao et al., 2011; Pysz et al., 2009). In this study, we demonstrated that PKCα acts as a tumor suppressor in the endometrium by negatively regulating AKT activation. Together, our work further supports the current paradigm shift in the function of PKCs in cancer.

Our analysis and TCGA data both point to reduced PKCα expression in ECs due to alterations in its gene transcription (Figure 4-1 – 4-3). Although loss of PKCα protein and mRNA has been reported in other cancer types, we provided the first evidence that transcriptional repression is responsible for its absence in cancer (Figure 4-4). Furthermore, loss of PKCα correlated with poorer prognosis as it was associated with more aggressive disease and reduced patient survival probability (Figure 4-5, 4-6). In multiple mouse EC models carrying mutant PTEN or generated by allelic PTEN deletion, PKCα loss was uniformly observed in lesions arising in the endometrium (Figure 4-7, 4-9). However, it is unlikely that loss of PTEN led directly to reduced PKCα transcription as we did not observe changes in PKCα levels in EC cells with PTEN knockdown or overexpression, or with inhibition of PI3K/AKT signaling (Figure 4-11). Conversely, manipulation of PKCα expression did not affect PTEN levels, indicating that PKCα deficiency itself is unlikely to drive PTEN LOH to give rise to endometrial lesions. However, the
specific mechanisms that cause the reduction in PKC\(\alpha\) transcription remain elusive. Although our initial analysis has excluded methylation at the PKC\(\alpha\) promoter as a means of regulating its expression (data not shown), future experiments will explore other possible mechanisms such as differential binding of regulatory elements (e.g., transcription factors, co-activators, or co-repressors) or methylation of other regulatory regions (e.g., CpG shores).

Our study finds that PKC\(\alpha\) acts as a tumor suppressor and the reduced expression of this kinase diminishes protection against endometrial transformation. In mice carrying loss-of-function Pten mutations, concurrent PKC\(\alpha\) deficiency increased tumor burden by 3-fold at 1- and 3-months, although the differences diminished at later times of disease progression (Figure 4-13). This suggests that PKC\(\alpha\) acts as a “gatekeeper” and loss of this kinase is likely a rate-limiting event in endometrial tumorigenesis. Indeed, re-introduction of PKC\(\alpha\) markedly inhibits EC cell growth under anchorage-independent conditions (Figure 4-14), while PKC\(\alpha\) knockdown has the opposite effect. Interestingly, kinase-dead PKC\(\alpha\) had no effects, demonstrating that the tumor suppressive effects of PKC\(\alpha\) in EC are indeed due to its kinase function (Figure 4-15). Data from this study further demonstrated that PKC\(\alpha\) activation stimulates PP2A activity and leads to hypophosphorylation of AKT and its downstream targets PRAS40 and FOXO1 (Figure 4-17, 4-22). This negative regulation of AKT can be abolished by inhibitors of PKC\(\alpha\) or PP2A or by PKC\(\alpha\)/PP2A RNA interference (Figure 4-18, 4-22, 4-26, 4-28). However, the co-expression of constitutively active AKT (myr-AKT or AKT-DD) or hyperactive AKT found in breast and endometrial tumors (AKT-E17K) only partially rescues the growth inhibitory/tumor suppressive effects of PKC\(\alpha\) in EC cells (Figure 4-30, 4-31), suggesting that PKC can suppress EC tumorigenesis through other mechanisms in addition to negative regulation of AKT activity. PKC\(\alpha\) has been shown to downregulate the expression of Id1 family proteins (Figure 4-10, 4-24), which promote cell cycle progression, tumor cell growth and migration, through ERK-dependent signaling (Hao et al., 2011). Our analysis also demonstrated an inverse correlation between PKC\(\alpha\) and Id1 in EC tumors, presenting a possible additional mechanism for PKC\(\alpha\)-mediated suppression.
of EC. However, in-depth mechanistic studies are needed to confirm this hypothesis and also to explore other pathways by which PKCα functions as a tumor suppressor in EC.

The present study also showed that PP2A but not PHLPP is involved in PKCα-mediated hypophosphorylation of AKT (Figure 4-26). In contrast to previous studies showing a residue-specific dephosphorylation of AKT by PP2A and PHLPP on threonine 308 and serine 473, respectively, PP2A was responsible for PKCα-mediated dephosphorylation of AKT at both of these sites (Figure 4-26, 4-28). The specificity of the PP2A holoenzyme is determined by the B, or regulatory, subunits in the complex (Chen et al., 2013; Eichhorn et al., 2009; Sents et al., 2013). Although our data demonstrated that PKCα activation induces PP2A activity (Figure 4-27), we do not know the specific makeup of the holoenzyme involved in the effect. To determine how PKCα mediates PP2A activation, future studies will examine the formation of PP2A holoenzymes following PKCα activation, and how the individual holoenzyme(s) accounts for the dephosphorylation of AKT.

In comparison with other more widely studied cancer types, EC patients have a relatively optimistic prognosis as 1) many EC cases are symptomatic and detected at an early stage, and 2) the disease is usually contained in the uterus at the time of diagnosis, thus curative through hysterectomy (Engelsen et al., 2009). However, approximately 20% of EC patients experience recurrent disease that is resistant to radiation or currently available chemotherapeutics. Recurrent disease is usually aggressive and accounts for a majority of EC-related mortality (Engelsen et al., 2009). Molecular analysis of EC tumors has found most ECs are driven by hyperactivation of PI3K/AKT signaling, most commonly due to loss of PTEN, activating mutations in PI3K or AKT, or the combination of multiple genetic aberrations in this pathway (Dedes et al., 2011). As PTEN loss and AKT hyperactivation are prevalent in many cancers, small molecule inhibitors of AKT have received much attention as an emerging class of targeted therapeutic against ECs (Manning and Toker, 2017). However, results from clinical trials thus far have been disappointing as the efficacy of these agents is limited by their accompanying dose-limiting toxicity (Ma et al., 2016;
Saura et al., 2017). Apart from developing new agents that are less toxic, a system that can stratify patients who are more sensitive to AKT inhibition, and thus more likely to respond at a lower dosage, would also be beneficial to the clinical outcome of these trials (Manning and Toker, 2017). From our study of the role of PKCα in endometrial tumorigenesis, we have shown that PKCα acts as a negative regulator of AKT activation (Figure 4-17). While PTEN loss and oncogenic PI3K mutation indeed result in elevated phosphorylated AKT, PKCα signaling can still exert negative pressure on the kinase (Figure 4-23). Therefore, concurrent loss of PKCα leads to robust, unopposed AKT activation and more aggressive EC phenotypes (as depicted in our model in Figure 5-1). Our data strongly supports a PKCα-PP2A-mediated mechanism that acts directly on AKT; however, the possibility that crosstalk exists between PKCα-PP2A and other components of PI3K signaling (e.g. upstream receptor tyrosine kinases or PTEN) should also be considered. Additionally, future experiments examining whether PKCα status affects the sensitivity of EC cells to currently available AKT inhibitors could improve the clinical benefits of these inhibitors. Based on our findings, PKCα is able to suppress AKT phosphorylation regardless of the status of PTEN or PI3K, suggesting it is a potent inhibitor of AKT activation. It is likely that EC cells that have PKCα and, by extension, retain part of the regulation of AKT activation, could be more sensitive to small molecule AKT inhibitors, allowing them to inhibit tumor growth with limited, better tolerated side effects.
Fig. 5-1: Schematic representation of PI3K/PTEN and PKCα as regulators of AKT activation in endometrial cancer.

The model depicts that in addition to the well-known PI3K/PTEN signaling pathway, PKCα-PP2A mediates another regulatory axis that maintains balanced activation of AKT in EC cells. Mutations of PI3K and PTEN have been shown to result in hyperactivation of AKT. However, our study demonstrated that disruption of the PKCα-PP2A regulatory axis ensures robust, unopposed AKT activation and promotes the transformation of endometrial cells.
Appendix A: Estrogenic stimulation of PKCα expression

**Rationale:**

Based on our examination of normal mouse endometrium under estrous cycle, PKCα expression increased during later proestrus and was robustly expressed during estrous, coinciding with the peak of estrogen level.

**Results/Discussion:**

To yesy whether estrogenic stimulation induces PKCα expression, Ishikawa, an EC cell line that retained ER expression, was treated with estradiol (E2). Both PKCα mRNA and protein were examined at 3 and 24 hours following E2 stimulation. Cells were maintained in charcoal-cleared culture medium and experiments were performed with assistance from the laboratory of Dr. Vimla Band.

Results from our analysis showed that E2 robustly increased PKCα mRNA expression after 24 hours but no change in protein level was observed. The induction of cyclin D1, a well-known E2-responsive target, following E2 treatment demonstrated that the cells were indeed responsive to the stimulation. Our finding is consistent with previous report showing that estrogenic stimulation induced PKCα expression in primary endometrial cancer cells (Wu et al., 2005). However, failure to translate PKCα mRNA to protein indicated possible translational defect in Ishikawa, a human EC cell line, which might account for the decreased PKCα expression in these cells.
Fig. A.1: PKCα expression following E2 stimulation of EC cells.

The graph showed PKCα mRNA levels at 3 and 24 hours after stimulation with E2 or vehicle control (Ethanol) measure by qRT-PCR analysis relative to 18sRNA. Shown in bottom panel is Western Blot analysis of PKCα protein levels following E2 stimulation. Cyclin D1, a well-known E2 target gene, was assessed as demonstration of E2 effect.
Appendix B: Regulation of PKCα Expression

Rationale:

Parallel analysis of PKCα mRNA and protein from human endometrial tumors, mouse endometrial lesions, and endometrial cancer cell lines revealed that reduced PKCα mRNA correlated with lower protein levels. We explored possible mechanisms that might be involved in regulating PKCα expression in EC cells including differential promoter activity, mRNA stability, and protein stability.

Results/Discussion:

To explore mechanism that might be involved in regulating PKCα expression in EC cells, we assessed promoter activity, mRNA stability, and protein stability of PKCα using methods described below:

1) PKCα promoter activity assay

Plasmids encoding firefly luciferase regulated by PKCα promoters (-1571/+277, -1571/+77, -331/+77, -279/+77, -260/+77) were transfected into EC cells using Fugene 6 or XtremeGENE 9 as per manufacturer’s protocol. Plasmids encoding renilla luciferase regulated by thymidine kinase promoter was transfected in all samples and served as normalization for transfection efficiency PKCα promoters within a cell line. Promoter activity was expressed at relative to the minimal PKCα promoter (-260/+77)

2) mRNA stability

To examine PKCα mRNA stability, EC cells were treated with α-amanitin, a selective RNA polymerase II and III inhibitor, or vehicle control (ethanol) for 12 hours. PKCα mRNA levels were assessed by qRT-PCR and normalized to the vehicle control.

3) Protein stability

Protein stability was examined by a time course (6 hour interval in 24 hours) treatment
with cycloheximide, which inhibits protein synthesis or the vehicle control (DMSO). PKCα protein levels were assessed by Western Blotting analysis.

Results from these experiments did not present a general mechanism that regulates the broad alteration of PKCα expression in these cell lines. The promoter analysis showed possible repressive element binding to the PKCα promoters in 4 out of the 7 EC cell lines (SK-UT-1B, Ishikawa, AN3CA, KLE) examined, as demonstrated by the decreasing luciferase activity with increased PKCα promoter length (Figure B.1.). In three EC cell lines (SN3CA, Ishikawa, SK-UT-1B), there was reduced PKCα mRNA when mRNA synthesis was inhibited by α-amanitin (Figure B.2.). Interestingly, despite the long half-life of PKCα protein, it was more readily degraded in AN3CA than other PKCα low cells (e.g. SK-UT-1B) (Figure B.3.). Together, these results demonstrated that multiple mechanisms may be involved in the regulating PKCα expression in EC cells. However, the mechanisms explored here did not explain the broad PKCα loss in EC cells or tumors.
**Fig. B.1: Figure PKCa promoter activity in human EC cells.**

Plasmids of PKCa promoter-regulated firefly luciferase plasmids were used to measure promoter activity. All expressions were represented as relative to the minimal promoter (-260/+77) of each cell lines and normalized to renilla luciferase expression to account for transfection efficiency. Error bars indicate mean ± SEM from 3 biological replications.
Fig. B.2: PKCα mRNA levels under RNA polymerase inhibition.

PKCα mRNA was measured using qRT-PCR after 12 hours of treatment with the a-amanitin, a RNA polymerase inhibitor or vehicle control (ethanol), and quantified relative to 18rRNA. Error bars indicate mean ± SEM from 3 biological replications.
Fig. B.3: PKCα levels under inhibition of protein synthesis.

EC cells treated with cycloheximide to inhibit protein synthesis or vehicle control (DMSO) in a time course. PKCα protein levels were analyzed by Western Blotting.
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