Repression of Protein Kinase C Delta in Human Squamous Cell Carcinomas by Ras, Fyn and NF-Kappa B Signaling

Vipin Yadav
Loyola University Chicago

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LOYOLA UNIVERSITY CHICAGO

REPRESSION OF PROTEIN KINASE C DELTA
IN HUMAN SQUAMOUS CELL CARCINOMAS
BY RAS, FYN AND NF-KAPPA B SIGNALING

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN MOLECULAR BIOLOGY

BY

VIPIN YADAV
CHICAGO, ILLINOIS
MAY 2011
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<td></td>
</tr>
<tr>
<td>AK</td>
<td>Actinic Keratosis</td>
<td></td>
</tr>
<tr>
<td>BCC</td>
<td>Basal Cell carcinoma</td>
<td></td>
</tr>
<tr>
<td>BD</td>
<td>Bowen’s Disease</td>
<td></td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
<td></td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunopreipitation</td>
<td></td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
<td></td>
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<tr>
<td>DMBA</td>
<td>7,12-dimethylbenz(alpha)anthracene</td>
<td></td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
<td></td>
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<tr>
<td>ECM</td>
<td>Extra cellular matrix</td>
<td></td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
<td></td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
<td></td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal regulated kinase</td>
<td></td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
<td></td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
<td></td>
</tr>
<tr>
<td>GEFs</td>
<td>Guanine nucleotide exchange factors</td>
<td></td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
<td></td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphatases</td>
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</tr>
<tr>
<td>HK</td>
<td>Human keratin</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
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<tr>
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<tr>
<td>IKK</td>
<td>IκB Kinase</td>
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<td>IL-1</td>
<td>Interleukin-1</td>
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<td>K1</td>
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<td>KC</td>
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<td>LCM</td>
<td>Laser capture microdissection</td>
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<tr>
<td>MARCKS</td>
<td>Myristoylated alanine rich C kinase substrates</td>
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<tr>
<td>MMP</td>
<td>Matrix metallo-proteases</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappaB</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcriptase polymerase chain reaction</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
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<tr>
<td>PDK-1</td>
<td>Pyruvate dehydrogenase kinase isozyme 1</td>
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<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
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<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
<td></td>
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<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
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<tr>
<td>RACKS</td>
<td>Receptors for Activated C Kinase</td>
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<tr>
<td>RalGDS</td>
<td>Ral guanine nucleotide-dissociation stimulator</td>
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<tr>
<td>RBD</td>
<td>Ras binding domain</td>
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<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
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<td>SDS-PAGE:</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
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<tr>
<td>SFK</td>
<td>Src family of tyrosine kinases</td>
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<tr>
<td>SH</td>
<td>Src-homology</td>
<td></td>
</tr>
<tr>
<td>SiRNA</td>
<td>Small interfering RNA</td>
<td></td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming growth factor-alpha</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
<td></td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
<td></td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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ABSTRACT

The delta isoform of Protein Kinase C (PKC-δ) is widely expressed in many normal tissues, including epidermal keratinocytes, and has a critical role in UV-induced apoptosis. However, PKC-δ is frequently lost in chemically or UV-induced mouse skin tumors, as well as in human cutaneous squamous cell carcinomas (SCC). Furthermore, re-expression of PKC-δ in human SCC lines is sufficient to induce apoptosis and suppress tumorigenicity, making PKC-δ a potential tumor suppressor gene for SCCs. The objective of this dissertation is to investigate the mechanism of PKC-δ loss in human SCCs.

To determine the mechanism of PKC-δ loss in human SCCs, we used Laser Capture Microdissection to isolate cells for RNA and DNA analysis from 3 normal epidermis and 14 human SCCs with low PKC-δ protein. Using this more selective approach, we found the tumor suppressor PKC-δ is lost at the mRNA level in human SCCs, and that the PKC-δ gene is rarely deleted suggesting that the mechanism of down-regulation of PKC-δ in SCCs is likely to be primarily at the level of gene transcription. To further explore the mechanism of PKC-δ down-regulation, we studied Ras-transformed immortalized human keratinocytes (HaCaT-Ras), which have selective down-regulation of the PKC-δ isoform at both protein and mRNA levels. Ras significantly repressed human PKC-δ promoter activity in HaCaT cells (85% reduction, p<0.05). Mutagenesis and ChIP studies of the PKC-δ promoter revealed that Ras
activation represses PKC-δ promoter activity by activation of nuclear factor kappa B (NF-κB) and recruitment of repressive NF-κB subunits (p50 and c-Rel).

We also found that Fyn tyrosine kinase activation was necessary and sufficient for NF-κB activation and PKC-δ repression. In addition, Fyn was over-expressed in human SCCs and HaCaT-Ras cells. Furthermore, activation of PI3K/AKT pathway was necessary and sufficient for Ras-induced up-regulation of Fyn expression in HaCaT cells. Thus, a Ras→PI3K→Fyn→NF-κB pathway leads to PKC-δ repression in human keratinocytes. Our results have implications for the development of therapeutic strategies abrogating this signaling pathway to trigger the re-expression of pro-apoptotic PKC-δ to induce apoptosis in SCCs.
CHAPTER I
INTRODUCTION

1.1 The Human Skin

1.1.1 The Anatomy of the Skin

Skin is the largest and one of the most multifaceted organs of the human body. It senses our surroundings and acts as a protective barrier against many different insults, such as ultraviolet radiation, trauma, temperature extremes, toxins and pathogens. Other important functions include sensory perception, immunologic surveillance, thermoregulation, control of fluid loss and pigmentation. Skin is composed of several cell types of different embryonic origin. The ectoderm gives rise to the epidermis, hair follicles, sebaceous glands and the nails. The neural crest gives rise to melanocytes, nerves, and sensory receptors, while fibroblasts, adipocytes, blood vessels and immune cells are derived from mesoderm.

Human skin has two primary layers, the dermis and the epidermis, that encompass a fatty subcutaneous layer called the hypodermis (Dale and Holbrook, 1987; Presland and Dale, 2000). The epidermis represents the outermost layer of the skin, and is largely made up of
epithelial cells known as keratinocytes (KCs). The epidermis consists of several distinct layers of keratinocytes at various stages of differentiation. These layers include the innermost basal layer, which contains self-renewing undifferentiated KCs, the intermediate spinous and granular layers, and the outermost layer, known as stratum corneum, which contains dead KCs (Dale and Holbrook, 1987; Presland and Dale, 2000). The stratum corneum provides a protective barrier against infections and the environment, and maintains a waterproof barrier that prevents loss of water from the human body. The cells in the basal layer are less differentiated and are distinguished by expression of keratin 5 and 14. The basal layer is the main site of proliferation in the epidermis. KCs in the basal layer are constantly renewed as they migrate towards the outer layers and undergo differentiation in a Ca\(^{2+}\) dependent manner (Fuchs and Raghavan, 2002). The differentiated KCs in the spinous layers of the epidermis are characterized by the expression of keratin 1 and 10, and granular layer KCs express terminal differentiation markers such as profilaggrin, involucrin and transglutamase. This continuous self-renewal process of the epidermis, though tightly regulated, can sometimes malfunction leading to disorders such as atopic dermatitis and cancer.

In addition to KCs, the epidermis also contains pigment producing melanocytes that are scattered throughout the basal layer, Langerhans' cells that are part of the skin's immune system and the pressure-sensing Merkel cells. The dermis, in contrast to epidermis, is a thick, relatively acellular layer that comprises extra-cellular matrix, nerve endings, fibroblasts, hair follicles, sebaceous glands (sweat and oil glands) and blood vessels.
Immediately above the dermis lies the basement membrane that separates the epidermis from the dermis. It plays an important role in adhesion between basal layer of the epidermis and dermis and controls epidermal differentiation (Inoue, 1989). The basement membrane is composed mainly of laminins, type IV collagen, type VII collagen, nidogen, and perlecan, etc (Burgeson and Christiano, 1997). In cancer, neoplastic KCs or melanocytes secrete matrix-metalloproteases (MMPs) that degrade the basement membrane, aiding the invasion of the transformed cells into the dermis and eventually into the blood vessels.

1.1.2 Skin Cancer

Cancer of the skin is the most common type of human malignancy, with more than a million new cases diagnosed every year in United States (American Cancer Society 2008). The incidence and mortality of skin cancer have increased progressively during the past decades, and every year the figure mounts. Ultraviolet (UV) radiation from the sun or tanning beds is the main etiological agent of skin cancers (Epstein and EPSTEIN, 1963; Fisher and Kripke, 2002; Cleaver and Crowley, 2002). The alarming skin cancer statistics are further exacerbated by the increased UV irradiation reaching the earth’s surface due to increased ozone depletion in the earth’s atmosphere regions (de Gruijl, 1999).

Skin cancers are broadly classified into melanoma and non-melanoma, with non-
melanoma skin cancers further divided into squamous cell carcinoma (SCC) and basal cell carcinoma (BCC). BCC is the most common subtype of skin cancer affecting ~800,000 Americans each year. BCC is locally invading and originates in the basal layer or the hair-follicle derived KCs and almost never metastasizes (Fan et al., 1997). BCCs occur mostly on the sun exposed areas of the body such as the face, ears, neck, scalp, shoulders and back, and causes considerable morbidity. Non-melanoma skin cancers are slow-growing, easy to recognize, and relatively easy to treat. SCCs are the second most common subtype of skin cancer affecting more than ~250,000 Americans each year. SCCs arise from the transformed KCs of the epidermis that produce significant number of squamous differentiating KCs. SCC can occur on all areas of the body including mucous membranes, but in most cases it occurs in the sun-exposed areas of the body. SCC is easily curable with surgery and localized treatments, but it can metastasize and in some cases can also be fatal if left untreated. Although the mortality rate is only 5-10% per year, the morbidity from treatment is tremendous in terms of cosmetic deformity, loss of function and adverse psychological effects. In contrast, melanoma is a cancer of melanocytes and is highly malignant, but has a much lower incidence. Nevertheless, melanoma is responsible for majority of the skin cancer deaths, estimated to be around ~8400 in 2008 in United States alone.

1.1.3 Histopathology of Human SCCs

Cutaneous SCC arises from the malignant transformation of the KCs in the epidermis. The neoplasm develops predominantly on the sun-exposed areas of the skin.
Pathologically SCCs are classified into the following stages of progression: Actinic Keratosis (AK) and SCC *in situ* where the neoplasm is confined to the epidermis and invasive SCC where the neoplasm extends beyond the epidermis (Croxton et al., 2002). These two forms differ in the histology and carry different clinical features (Bhawan, 2007).

1.1.3.1 Actinic Keratosis and SCC *in situ*

Actinic Keratosis (AK) are a common type of pre-malignant lesion in the skin (Arora and Attwood, 2009). AKs are confined to the epidermis and develop largely on sun-exposed areas of the skin. Pathological features of AK include thickening of the epidermis and sometimes epidermal dysplasia (Fenske et al., 2010). In some cases AK (5%), if left untreated, eventually progresses into an invasive SCC. SCC *in situ* is also known as Bowen’s Disease (BD). BD is a type of SCC *in situ* with largely epidermal dysplasia in all cases (Arora and Attwood, 2009). BD usually occurs on the mucosal areas. Carcinogens, arsenic and HPV infections are the main cause of this malignancy.

1.1.3.2 Invasive SCC

Pathologically, invasive SCCs are characterized by the malignant proliferation of KCs from the epidermis into the dermis and deeper tissue. Based on the degree of differentiation, SCCs are classified as well-differentiated, moderately-differentiated and
poorly-differentiated (Takata and Saida, 2005). Well-differentiated SCCs are characterized by highly keratinized atypical KCs forming eostonophilic keratin pearls in the tissue. On the other hand, lack of keratinization, increased spindle shaped cells, increased cellular polymorphism, frequent atypical mitosis and occasional necrotic cells are the features of moderately to poorly-differentiated SCCs. In addition, depending on the depth and the age of the tumor, the inflammatory immune infiltrate mainly comprising of lymphocytes and eosinophils is usually found in the dermis surrounding the neoplasm.

Most invasive SCCs are associated with or preceded by an AK or BD. However some SCCs arise de novo and are not associated with an in situ component. De novo SCCs are usually associated with poor prognosis and are considered high-risk lesions. Another form of invasive SCC is keratoacanthomas (KA). These are a neoplastic proliferation of KCs extending from the acanthotic epidermis into the dermis. Histologically the KA resembles well-differentiated SCCs. KAs usually self-regress in couple of months as a result of host immune response (Kossard et al., 2008).

SCCs can metastazie to lymph nodes and other organs, and can cause significant morbidity and in some cases even death (Weinberg et al., 2007). Prognostically, SCCs are classified into different risk categories based on the metastatic rate. Low risk SCCs include in situ SCCs, invasive SCCs derived from AK and keratoacanthomas. Intermediate risk SCC include acantholytic invasive SCC. High-risk subtypes include de
novel invasive SCCs and SCCs associated with radiation and burns. SCCs are also associated with immunosuppression and are common in organ transplant recipients on long-term immunosuppressive drugs (Boukamp, 2005). Well-differentiated SCCs have a cure rate of 99%, whereas the poorly differentiated invasive SCC have a success rate of 42% (Takata and Saida, 2005).

1.2 Skin Carcinogenesis

1.2.1 Apoptosis and Skin Carcinogenesis

Apoptosis, also referred to as programmed cell death, plays a central role in several processes such embryonic development and homeostasis. Apoptosis can be initiated through activation of death receptors (receptor pathway) or release of cytochrome-c and other pro-apoptotic molecules from mitochondrial membrane (mitochondrial pathway), resulting in activation of caspases (family of cysteine proteases) and consequently cell death (Ferri and Kroemer, 2001). Apoptosis is the major form of defense mechanism against uncontrolled proliferation and un-repairable DNA damage by agents such as UV radiation. If the damage is unreparable, UV-exposed cells undergo apoptosis thus inducing the formation of sun-burned or apoptotic KCs in the skin (Ziegler et al., 1994). Sometimes, cells can acquire UV-induced mutation in tumor suppressor genes (e.g. p53), thus decreasing the rate of apoptosis in these cells and providing them with a growth advantage over normal KCs (Ren et al., 1996; Berg et al., 1996). Over a period of time these mutant KCs expand and in the case of excessive UV exposure, acquire oncogenic mutations (e.g. Ras), resulting in transformation and squamous cell carcinoma (Zhang et
al., 2001). P53 mutations are frequently present in AKs, BCCs and SCCs. Other tumor suppressor genes such as PKC-δ, a novel member of the protein kinase C family, function as a key surveillance molecule and suppress transformation of KCs. Previous studies have shown that re-expression of tumor suppressor PKC-δ is sufficient to suppress the tumorigenicity and inducing apoptosis in human SCC cells (D’Costa et al., 2006). BH domain containing Bcl-2 family members play a key regulatory role in apoptosis. BH3 domain containing proteins such as NOXA, Bax, Bad and Bid promote apoptosis by inducing release of cytochrome c from mitochondria and subsequently resulting in irreversible activation of caspases. Whereas, pro-survival Bcl-2 family members such as Bcl-2, Bcl-XL, Mcl-1, that are frequently elevated in cancers including human SCCs, bind and sequester proapoptotic BH3-only proteins and inhibit apoptosis (Adams and Cory, 1998). Furthermore, suppression of apoptosis by targeted disruption of Bcl-xL in mouse keratinocytes inhibits both UVB- and chemically induced skin carcinogenesis (Kim et al., 2009a). In addition, disruption of pro-apoptotic gene Bax alters the epidermal response to ultraviolet irradiation and in vivo induced skin carcinogenesis thus further supporting a key role of apoptosis in tumor suppression (Cho et al., 2001).

1.2.2 The Pathogenesis of SCCs

Skin carcinogenesis is divided into three stages: initiation, promotion and progression. Carcinogenesis in mice can be induced by tumor initiators (e.g. DMBA) and chronic application of tumor promoters (e.g. TPA) as per the 2-stage chemical carcinogenesis
protocol (Zhan et al., 1997) or by chronic UV-exposure, also referred to as photocarcinogenesis.

1.2.2.1 Chemical Carcinogenesis

In the 2-stage chemical carcinogenesis model, initiation is caused by a single application of DMBA (7,12-dimethylbenz-α-anthracene) on mouse skin. In the second stage, tumor promoters such as the phorbol ester TPA (12-O-tetradecanoylphorbol-13-acetate) are used. Repetitive treatment with tumor promoter results in the formation of benign tumors, also called as papillomas (Karen et al., 1999). These squamous papillomas display hyperplasia, delayed terminal differentiation and altered expression of KC differentiation markers. Papillomas usually develop within 10 weeks and almost all (>90%) of these tumors have activating mutation in the Ha-Ras oncogene (Green and Khavari, 2004). Tumor initiation is an irreversible process, whereas tumor promotion is epigenetic in nature and can be reversed. Withdrawal of the tumor promoter results in regression of the papillomas in the early stages. Lastly, within 20-50 weeks a small percentage of these benign papillomas acquire further mutations and spontaneously progress into malignant carcinomas. The chemical carcinogenesis and progression of malignant SCCs is accelerated in mice that harbor inactivating mutations or deletions in tumor suppressor genes such as p53 (Wang et al., 1998). The progression of papillomas to SCCs is also associated with aberrant expression and activity of a variety of proteins such as integrins, keratins, EGFR, PKCs, AP-1 and Cyclins that promote proliferation, survival, migration
and invasion of the malignant KCs (Yuspa, 1986).

1.2.2.2 Photocarcinogenesis

UV exposure induces inflammation, oxidative stress and formation of apoptotic cells in the skin also referred to as a sunburn cells. The UV radiation from the sun is classified into three subtypes: UVC (short wave, 200-290 nm), UVB (mid-wave, 290-320 nm) and UVA (long wave, 320-400 nm). UVC is the most potent of them all; however, it is absorbed efficiently by the ozone layer. In contrast, UVB and UVA both reach the surface of the earth in amounts sufficient to have biological affects on the skin. UVB, and to a lesser extent UVA, is carcinogenic in humans (de Gruijl et al., 1993). Exposure of DNA to UVB radiation generates DNA adducts, such as thymine dimers and (6-4) photoproducts. In addition, UVB induces oxidative damage to the DNA by forming 8-oxo-2'-deoxyguanosine residues (Hemminki, 1993).

UV radiation acts as both tumor initiator and tumor promoter (Munger et al., 1992). UV can initiate carcinogenesis by inducing mutational inactivation of tumor suppressor genes (such as p53). These mutant cells have a selective growth advantage over normal KCs as they are resistant to UV-induced apoptosis, thus increasing target cell population for subsequent rate-limiting genetic changes (Zhang et al., 2005). Several tumor suppressor genes involved in UV-induced skin cancers are p16 (melanoma), p53 (SCC) and PTCH (BCC) (Situm et al., 2008; Popp et al., 2002). Over a period of time either spontaneously
or upon further UV exposure, these UV-transformed KCs accumulate more genetic alterations in oncogene, tumor suppressor and cell-cycle regulators and progress into invasive SCCs. Several tumor promoting oncogenes involved in photocarcinogenesis include Ras, Bcl-2 and MMPs (Knezevic et al., 2007; Soehnge et al., 1997; Rittie and Fisher, 2002). In addition, UV radiation induces immunosuppression in skin and these effects have been strongly linked to the progression of SCCs.

1.3 Ras Oncogene

1.3.1 The Ras Isoforms

Ras proteins are membrane bound GTPases that receive signals from cell surface receptors and extracellular matrix proteins, and transmit them to the nucleus (Shields et al., 2000). Ras proteins, first identified from Harvey and Kirsten strains of rat sarcoma viruses, were found to have cellular proto-oncogene homologs, H-Ras and K-Ras respectively. In mammals, K-Ras exists as two alternative splice variants, K-Ras4a and K-Ras4b. The third proto-oncogene homolog of Ras, known as N-Ras, was originally isolated from human neuroblastoma and leukemia cells (Taparowsky et al., 1983). The Ras family of GTPases encompasses 36 genes that encode 39 small GTPase proteins (20-29 kDa) in the human genome. Ras proteins regulate many important cellular processes, such as growth, motility, differentiation, cytoskeletal rearrangements and adhesion. Ras isoforms have different functions in different tissues, despite highly similar structural, biophysical and biochemical properties. For example, K-Ras4B, and not K-Ras4a is
essential for embryogenesis in mice (Johnson et al., 1997; Koera et al., 1997). H-Ras and/or N-Ras deficient mice are viable and develop normally, indicating a functional redundancy among Ras proteins (Esteban et al., 2001; Umanoff et al., 1995).

1.3.2 Regulation of Ras Signaling

Ras activity is regulated by several cell surface receptors such as EGFR and integrins. These receptors transmit signal to Ras via adaptor proteins such as Grb2 and Gap1, and nucleotide exchange factors such as SOS (Mitin et al., 2005). The classical Ras genes (H, K, N-Ras) are the most frequently mutated genes in cancer, with a frequency of 30% of all human tumors analyzed. Ras is commonly mutated in the codons 12, 13 and 61, leading to a constitutive GTP-binding and activation. Ras is also activated in cancers by overexpression or amplification of the Ras genes, or by aberrant upstream signaling such as EGFR.

Ras proteins are 100% identical in the first 85 amino acids in the N-terminus, whereas the C terminus is variable and is responsible for different subcellular localization and consequently different downstream effectors. The Ras proteins undergo lipid modifications, such as palmitoylation and farnesylation, at the conserved C-terminus CAAX domain. This post-translational lipid modification is another key determinant of the subcellular localization and function of Ras isoforms. For example plasma membrane localized K-Ras induces transformation, whereas mitochondrial localized K-Ras induces
apoptosis (Bivona et al., 2006). Furthermore, only ER-associated H-Ras, and not Golgi-tethered Ras proteins, has the ability to activate MAPK signaling and induce transformation in cells (Chiu et al., 2002; Matallanas et al., 2006). This indicates that subcellular distribution of Ras and its effectors is a key determinant the Ras signaling and its downstream effectors.

1.3.3 Ras Downstream Effector Signaling

Ras proteins are functionally redundant across species, and signal via variety of downstream effector pathways such as Raf kinases, Phosphotidylinositol-3 kinases (PI3Ks), and Ral guanine nucleotide exchange factors (RalGEFs) (Rajalingam et al., 2007; Shields et al., 2000). The contribution of these Ras effector pathways and their relative roles vary with cell and tissue type (Gille and Downward, 1999). The Raf-MEK-ERK, also known as the mitogen-activated protein kinase (MAPK) pathway, and PI3K-Akt signaling transduce a variety of external signals, leading to a range of cellular responses, including growth, proliferation, survival, inflammation and differentiation (Figure 1) (Rajalingam et al., 2007). These Ras effector pathways are well known to be sufficient and necessary for Ras-induced transformation of many human and murine cells.

1.3.3.1 MAPK Pathway
The MAPK signaling involves recruitment of Raf kinase to the plasma membrane and subsequent activation by binding to active Ras proteins via a conserved Ras binding domain (RBD). Raf kinase (MEKKK) phosphorylates and activates MEK. MEK (MEKK) phosphorylates and activates ERK (MAPK). Activated ERK translocates to the nucleus and, in association with many transcription factors, regulates the expression of many important cell-cycle and pro-survival genes such as AP1/c-fos (Khavari and Rinn, 2007). In addition to the activating Ras mutations, MAPK activating B-Raf mutations were also discovered in many cancers (such as melanoma and colon cancer) thus emphasizing an important role of aberrant MAPK signaling in oncogenesis (Wellbrock et al., 2004).

1.3.3.2 PI3K-Akt Signaling Pathway

The PI3Ks are lipid kinases and catalyze the conversion of phosphatidylinositol-3,4-bisphosphate (PtdIns-4,5-P2) to phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P3). A number of plasma membrane receptors, especially the ones with tyrosine kinase activity, can activate PI3Ks. PI3K pathway initiates normal cellular processes that are frequently dysregulated in cancer: growth, proliferation, migration, metabolism and metastasis (Carnero, 2010; Manning and Cantley, 2007). In addition to carcinogenesis, the PI3K signaling has been implicated in angiogenesis. The PI3K family is classified into Class I, II and III that can be activated by different stimuli such as tyrosine kinases, cytokine receptors, GPCRs, integrins, chemokines and Ras proteins.
FIGURE 1: Ras Effector Pathways

Major Ras effector pathways are shown. GTP-bound Ras binds and activates Raf, which in turn, activates MEK and subsequently ERK/MAPK, which then translocates to the nucleus and activates a variety of transcription factors. In addition, Ras binds to the catalytic subunits of PI3K, leading to their activation. Active PI3K induces PDK-1 activation, and ultimately leads to activation of Akt/PKB. Furthermore, Ras can activate EGFR via an autocrine mechanism involving increased expression and secretion of EGFR ligands. Ras/EGFR autocrine signaling inactivates pro-apoptotic PKC-δ protein in a SFK-dependent manner. Loss of PKC-δ activity and activation of PI3K signaling result in suppression of apoptosis and increased cell survival. Also shown is the mechanism of PKC-α activation via EGFR mediated PLC-γ activation and upregulation of DAG & phosphoinositol levels.
The class I PI3K constitutes a regulatory (p85) and catalytic subunits (p110). The Ras-PI3K signaling involves recruitment of the p110 catalytic subunit of the class I PI3Ks to the plasma membrane and binding to activated Ras via a Ras binding domain (RBD) (Ramjaun and Downward, 2007). PI3K, once on the membrane, subsequently phosphorylates phosphoinositol lipids. These specialized lipids when phosphorylated, recruit pleckstrin homology (PH) domain containing proteins such PDK1 and Akt to the plasma membrane. At the plasma membrane, Akt is phosphorylated and activated by PDK1. Thus Akt, also a serine threonine kinase, functions as a key downstream target of PI3K. Akt phosphorylates and regulates the activity of variety of targets including transcription factors, kinases and other cellular pathways such as m-TOR.

The PI3K signaling is dysregulated in cancers by several mechanisms. Activating mutation, amplification or overexpression of the PI3K gene can result in increased PI3K signaling (Bunney and Katan, 2010; Gabelli et al., 2010). Furthermore, activation of upstream regulators such as EGFR or Ras, which are commonly overexpressed and mutated in cancers, have been shown to increase PI3K activity (Bunney and Katan, 2010). PI3K signaling is also activated by the loss of phosphatase and tumor suppressor known as PTEN. PTEN is commonly down-regulated by gene deletion or inactivation in cancers (Zhang and Yu, 2010).

1.3.3.3 RalGDS-Ral Pathway
The RalGDS-Ral pathway involves Ras-induced activation of guanine nucleotide exchange factors for the Ras-like (Ral A and Ral B) small GTPases (Ral guanine nucleotide-dissociation stimulator (RalGDS) and RalGDS-like protein (RGL)) (White et al., 1996; Urano et al., 1996). The RalGDS pathway has a limited role in Ras-induced transformation. Recent studies have suggested that activation of RalGDS alone and not PI3K or MAPK pathways, is sufficient to promote transformation of human kidney cells (Hamad et al., 2002). Furthermore, recent evidence indicates prominent but distinct roles of the RalA and RalB GTPases in regulating cell proliferation and inhibiting apoptosis, respectively (Chien and White, 2003). However, the downstream signaling and the mechanistic details of RalGDS-Ral pathway is not clearly understood.

### 1.3.4 Ras Function in Epidermis

The role of Ras and its effectors in the epidermal cell is not clear due to difficulty in obtaining a genetic deletion of epidermal Ras function. The H-Ras\(^{-/-}\), N-Ras\(^{-/-}\), H-Ras\(^{-/-}\)/N-Ras\(^{-/-}\) and K-Ras\(^{-/-}\)/N-Ras\(^{-/-}\) chimeras do not exhibit any detectable phenotype in the skin (Umanoff et al., 1995; Esteban et al., 2001; Johnson et al., 1997). H-Ras deficient skin shows compensatory increase in other Ras isoforms, thus indicating that a lack of phenotype due to Ras deletion could most likely be due to functional redundancy among Ras proteins (Ise et al., 2000). However, inhibition of Ras signaling in mouse epidermis by expression of dominant negative Ras significantly decreases basal cell proliferation, induces terminal differentiation and leads to complete loss of epidermal self-renewal
leading to death, thus suggesting that Ras is required for normal epidermal function including proliferation and control of differentiation (Dajee et al., 2002). Furthermore, Ras gain-of function studies have shown that conditional activation of Ras in the epidermis promotes growth and opposes differentiation (Dajee et al., 2002; Tarutani et al., 2003).

1.3.5 Ras and Epidermal Tumorigenesis

1.3.5.1 Ras and Pathogenesis of SCCs

Ras genes play a key role in human epidermal carcinogenesis. Activating mutations of Ras isoforms are found in ~58% of human SCCs (Quintanilla et al., 1986). In addition, the majority of the human SCCs that harbor wildtype Ras genes display increased levels of active Ras protein and activity (Dajee et al., 2003). cDNA gene expression profiling of human SCC patient tissue showed strong induction of known Ras target genes, indicating a key role of Ras signaling in the pathogenesis of human SCCs (Higashikawa et al., 2008; Tomioka et al., 2006). Studies in mice models of SCCs, using chemical carcinogenesis, demonstrated that ~100% of the DMBA induced mice skin tumors harbor activating mutations in the Ha-Ras gene(Pierceall et al., 1991). Expression of mutant H-Ras induces benign skin tumors in mice, thus supporting the role of Ras as an initiator of SCCs (Roop et al., 1986). Furthermore, targeted expression of mutant Ras in the epidermis substitute for the effects of DMBA on mouse skin, and tumors develop spontaneously after application of tumor promoting agents (Brown et al., 1986).
1.3.5.2 Ras signaling and Malignant Transformation of KCs

Loss of function studies has identified many regulators of Ras signaling that are critical for proliferation, differentiation and apoptosis in skin. Some of these are p63, Notch, EGFR, β1 integrins and PKCs (Brakebusch et al., 2000; Li et al., 2001; Blanpain et al., 2006; Li et al., 2000; Mills et al., 1999). Also, Cyclin D, Cdk 4, Cdk 6, p16, Rb, AP-1/Jun are some of the downstream targets of Ras that are critical for malignant progression of KCs (Rodriguez-Puebla et al., 1999; Hirai et al., 1994; Zhang et al., 2004; Efimova et al., 2004). Primary mouse KCs expressing v-rasHa develop papillomas when grafted orthotopically onto nude mice and are resistant TPA or Ca2+ induced terminal differentiation (Roop et al., 1986). In contrast, TPA induces proliferation in Ras-transformed KCs. Furthermore, transduction of v-rasHa is associated with aberrant PKC activity. PKCs are downstream targets of Ras signaling and play an important role in Ras mediated altered terminal differentiation in KCs (Dlugosz et al., 1994; Dlugosz and Yuspa, 1994).

Previous studies have found that Ras activation is associated with increased activity of PKC-α, the pro-survival and only Ca2+ dependent PKC isozyme expressed in KCs, and inhibition of PKC-δ, Ca2+ independent and pro-apoptotic novel PKC member (Dlugosz et al., 1994; Denning et al., 1993; Denning et al., 1996). These studies revealed that a Ras-TGFα-EGFR autocrine signaling was responsible for the inhibition of PKC-δ and
increased PKC-α activity (Figure 1). Ras-transformed KCs exhibit elevated DAG and IP3 levels and subsequently increased PKC-α activation (Punnonen et al., 1994). EGFR ligands are fundamental regulators of epithelial growth and neoplastic transformation. TGF-α, like EGF, is an EGFR ligand. Many tumor promoters including TPA, induce TGF-α production in mice (Krasagakis et al., 2004). Furthermore, chronic treatment or overexpression of TGF-α in the epidermis bypasses the need for a tumor initiation step in chemical carcinogenesis protocol in mice, and tumors develop upon subsequent application of TPA alone (Vassar et al., 1992). Furthermore, papillomas produced by grafting EGFR null KCs transduced with \( v^{-ras} \) were significantly smaller than those by \( v^{-ras} \) alone, indicating that TGFα/EGFR autocrine loop is involved in the growth of papillomas by Ras (Threadgill et al., 1995; Dlugosz et al., 1997; Dlugosz et al., 1995).

The proto-oncogene c-Fos/AP-1 is one of the main nuclear target of many signaling pathways such as Ras, and plays a critical role in growth and differentiation of KCs (Domann, Jr. et al., 1994; Greenhalgh et al., 1990). Activation of \( v^-{fos} \) is sufficient for development of malignant SCCs from papillomas (Greenhalgh et al., 1990). Genetic inhibition of \( c^-{fos} \) blocks the development of malignant SCCs in \( v^-{ras} \) mice, without affecting the formation of benign papillomas (Saez et al., 1995). This indicated that c-fos and AP1 transcription factors play a key role in Ras-mediated malignant transformation and progression of squamous papillomas and not necessarily in tumor initiation and papilloma formation. Thus, active Ras signaling is indispensable for pathogenesis of SCCs.
1.4 The Protein Kinase C Family

1.4.1 The Structure and Function of PKCs

Protein Kinase C (PKC) is a family of serine/threonine kinases and was first discovered in late 1970’s in bovine brain extracts as a proteolytically activatable kinase (Mellor and Parker, 1998; Inoue et al., 1977). PKCs play an important role in cell growth, differentiation, cell cycle, apoptosis and angiogenesis (Parker and Murray-Rust, 2004a). Structurally PKCs consists of a regulatory and a catalytic region. The catalytic half consists of C3 and C4 domains (Newton, 1997). C3 functions as an ATP binding region and the C4 domain transfers the γ-phosphate from the bound-ATP onto the serine/threonine amino acid in the PKC substrate protein. The regulatory half of the PKC consists of C1 and C2 that are essential for activation of PKCs, and a pseudosubstrate domain which binds to the catalytic domain and inhibits enzyme activation (Hunn and Quest, 1997). The pseudosubstrate region binds to the catalytic site of the PKCs and maintains an inactive/closed conformation of PKCs. Mutation of the pseudosubstrate region or using blocking antibodies can also induce PKC activation by a similar a mechanism (Pears et al., 1990; Makowske and Rosen, 1989). C1 domains bind to diacylglycerol (DAG) and other analogues of DAG such as phorbol esters (e.g. TPA). DAG is produced as a result phosphoinositol bisphosphate cleavage reaction by phospholipase C (PLC) (Berridge and Irvine, 1984). DAG is membrane localized and activates PKCs by binding and inducing an open conformation and consequently releases the pseudosubstrate domain from binding to the catalytic domain. In addition to DAG,
the PLC reaction generates inositol triphosphate which causes the release of $\text{Ca}^{2+}$ from the ER. The C2 domain binds $\text{Ca}^{2+}$ and activates classical PKCs ($\alpha, \beta, \gamma$). PKCs also have variable V3 and V5 domains. The V3 domain is the flexible hinge region connecting the regulatory domain and catalytic region. The hinge region includes a protease cleavage site that is important for proteolytic activation of some of the PKCs isoforms (Remenyik et al., 2003). The V5 domain is important for subcellular localization of PKCs and binds to scaffold protein such as RACKs (Chipuk et al., 2003).

FIGURE 2: PKC Classification and Regulatory Domain Structures

Shown is the basic domain structure of PKCs. PKCs are classified into classical, novel and atypical subtypes based on the mechanism of activation and domain makeup. The C1 domains bind DAG and activate classical and novel PKCs. The C2 domain binds $\text{Ca}^{2+}$ and activates only classical PKCs. The atypical PKCs are not activated by $\text{Ca}^{2+}$ or DAG. The PB1 domain on atypical PKCs is required for protein-protein interactions. The C2-like domain on novel PKCs also facilitates protein-protein interactions. The C2-like domain of novel PKC member, PKCδ, is a phosphor-tyrosine binding domain. C3 is the ATP binding domain, and C4 is the kinase domain. The hinge region of novel PKCs contains a caspase cleavage site (DMQD).
1.4.2 PKC Isoforms and Mode of Activation

The PKC family is comprised of 9 isoforms that differ in their substrate specificity, tissue distribution, subcellular localization, mode of activation and structure (Newton, 1995; Parker and Murray-Rust, 2004b). Based on the structure and mode of activation, the PKC family is classified into 3 subtypes (Stabel and Parker, 1991): Classical, Novel and Atypical PKCs (Figure 2). The classical PKCs comprise PKC-α (alpha), PKC-βI and βII (beta), and PKC-γ (gamma) and are activated by Ca\textsuperscript{2+} and DAG. The novel PKCs include PKC-δ (delta), PKC-ε (epsilon), PKC-η (eta) and PKC-θ (theta). The novel PKCs can be activated by DAG but not Ca\textsuperscript{2+} as they lack the Ca\textsuperscript{2+} responsive C2 domain. Instead, the novel PKCs contain a C2-like domain. However, the precise role of C2-like domain is not well understood and is likely to play an important role in substrate specificity (Lopez-Lluch et al., 2001). In addition, C2-like domain of PKC-δ has been reported to function as a phosphotyrosine binding domain and plays a key role in protein-protein interactions (Benes et al., 2005). The atypical isoforms include PKC-ζ (zeta) and PKC-ι/λ (iota/lambda) and lack both C2 and C1b domains, and thus are not activated by either Ca\textsuperscript{2+} or DAG. In addition to DAG and Ca\textsuperscript{2+}, PKCs can also be activated by preoteolytic cleavage in the hinge region, and consequently releasing the constitutively active catalytic domain from the inhibitory pseudosubstrate domain (Stabel and Parker, 1991; Denning et al., 1998). PKC-δ, PKC-θ and PKC-ε have been reported to be activated by this proteolytic mechanism (Denning et al., 1998; Datta et al., 1997; Ghayur et al., 1996; Remenyik et al., 2003).
The PKCs must be primed by phosphorylation at three sites: the activation loop, turn motif and the hydrophobic motif (Han et al., 2004). For maximum activity, PKCs require phosphorylation of conserved serine and threonine residues, such as TFCGT motif, within the activation loop (Craig, 2002). For example, phosphorylation of PKC-α and PKC-β at Thr 497 and Thr 500 residue by PDK1 is required for their activation (Cuconati et al., 2003). PDK-1 has been shown directly responsible for activation loop phosphorylation on PKCs (Balendran et al., 2000). Followed by PDK-1 phosphorylation, PKCs can be phosphorylated on the hydrophobic motif and turn motif by mTOR complexes or by autophosphorylation (Parker and Murray-Rust, 2004a; Li et al., 1997).

1.4.3 PKC Substrates and Role in Normal Epidermis

RXXS/TXRX is the consensus substrate phosphorylation site for PKC (Zhou et al., 1997). Here X indicates any amino acid. PKC substrate peptide usually comprises a hydrophobic amino acid at position +1 position and a basic residue at the -3 position (Nishikawa et al., 1997). MARCS (myristilated alanine rich C kinase substrate) was the first PKC substrate to be identified (Letai et al., 2002). Phosphorylation of MARCS by PKCs in the effector domain is required for its binding to calmodulin and membrane localization (Letai et al., 2002). PKCs can phosphorylate a variety of proteins such as ion channels, cell surface receptors, transcription factors etc (Eckert et al., 2004). Raf-1, Eif-1 and p53 are some of the well known PKC substrates.
PKCs play a key role in proliferation, differentiation and survival of many cell types including KCs. Five PKC isoforms (PKC-α, PKC-δ, PKC-ε, PKC-η and PKC-ζ) are expressed in human keratinocytes (Ma et al., 2003). PKC-α plays a key role in KC differentiation and growth arrest. PKC-α is required for terminal differentiation-associated growth arrest of KCs in the suprabasal layer (Kashiwagi et al., 2002; Jerome-Morais et al., 2009). This function of PKC-α is consistent with the role of extracellular Ca\(^{2+}\) in KC differentiation and expression of late differentiation markers (Denning et al., 1995; Lee et al., 1997). In addition, PKC-α is strongly linked to cutaneous inflammation and cytokine release. Overexpression of PKC-α in the basal layer strongly induces neutrophil infiltration and pro-inflammatory cytokine release (Wang and Smart, 1999; Cataisson et al., 2003).

PKC-δ is expressed throughout the epidermis (Denning et al., 1995). PKC-δ functions as a pro-apoptotic protein and is necessary and sufficient for UV-induced apoptosis. PKC-δ also plays an important role in G2/M growth arrest and DNA damage response (Lagory et al., 2009; Watanabe et al., 1992; Ishino et al., 1998). Multiple PKC-δ substrates implicated in apoptosis have been identified such as pro-survival Bcl-2 family member Mcl-1 and Scramblase-3 (Sitailo et al., 2006; Liu et al., 2003; He et al., 2007). In addition to the pro-apoptotic effects, PKC-δ also induces differentiation marker expression in TPA treated KCs, but the precise role PKC-δ is differentiation is not clear (Ohba et al., 1998; Efimova and Eckert, 2000).
PKC-ε is strongly associated with proliferation and survival of KCs (Jansen et al., 2001). Overexpression of PKC-ε in mice epidermis induces basal hyperplasia and promotes TPA-induced proliferation of KCs (Jansen et al., 2001; Papp et al., 2004). PKC-η is exclusively expressed in the granular layer and is associated with terminal differentiation and cell cycle withdrawal (Cabodi et al., 2000; Ohba et al., 1998; Ueda et al., 1996; Ishino et al., 1998). The atypical isoform PKC-ζ is frequently associated wound healing (Chida et al., 2003).

1.4.4 PKC and Epidermal Carcinogenesis

Tumor promoter phorbol esters such as TPA are potent activators of PKCs. Phorbol esters are analogs of DAG and activate PKCs by binding to the C1 domain and relieving inhibition from pseudosubstrate domain, and stabilizing its membrane localization. Phorbol esters cause an immediate activation of PKCs followed by a rapid and sustained proteolytic down-regulation, which is maintained for 3-4 days (Sakaguchi et al., 2003). Furthermore, direct application of TPA perturbs the PKC function, and promotes the formation of skin tumors in mice in 2-stage chemical carcinogenesis protocol (Hsieh et al., 1990). Previous work has shown that down-regulation of PKCs, especially PKC-δ, by TPA or Ras, is associated with tumor promotion in chemical carcinogenesis model. For example,, aberrant PKC activity is strongly linked to transformation of v-ras<sup>Ha</sup> transduced KCs (Griner and Kazanietz, 2007).
PKC isoforms exhibit overlapping, different, and sometimes opposite functions, especially in the context of transformation and epidermal tumorigenesis. PKC-α has been linked to differentiation-induced growth arrest, and TPA-induced and NF-κB dependent epidermal inflammation in KCs (Tibudan et al., 2002; Wang and Smart, 1999; Cataisson et al., 2003). PKC-α signaling is altered in Ras-transformed KCs, and is associated with aberrant differentiation marker expression in granular and spinous layers in SCCs (Dlugosz et al., 1994; Yang et al., 2003). The role of PKC-α in growth-arrest, differentiation and inflammation makes a strong case in favor of PKC-α as a potential tumor suppressor in SCCs. Furthermore, the PKC-α null mice are more susceptible to chemical carcinogenesis than the wild type, consistent with the role of PKC-α as a tumor suppressor in SCCs (Hara et al., 2005). In constrast, PKC-α has also been linked to tumor promotion. PKC-α transgenic mice developed SCCs in response to DMBA and low-dose TPA promotion, whereas the wildtype mice did not develop any tumors (Cataisson et al., 2009). Molecular genetic experiments have also implicated PKC-ε in skin carinogenesis (Verma et al., 2006; Aziz et al., 2006). PKC-ε transgenic mice are highly susceptible to chemically or UV-induced skin carcinogenesis (Reddig et al., 2000; Wheeler et al., 2003; Wheeler et al., 2004). PKC-ε binds and regulates the UV-induced activation of STAT-3 (Aziz et al., 2007). STAT-3 regulates the expression of TNF-α, which plays a key role in skin carcinogenesis (Kataoka et al., 2008). PKC-η is associated with growth arrest and activation of terminal differentiation genes such as involucrin and transglutaminase (Cabodi et al., 2000; Ohba et al., 1998; Ishino et al., 1998). PKC-η null mice display
increased TPA-induced hyperplasia and tumor formation indicating that PKC-η might function as a tumor suppressor in SCCs (Chida et al., 2003).

The role of PKC-δ as a tumor suppressor in SCCs is firmly established. PKC-δ functions as a tumor suppressor in the epidermis, and is necessary and sufficient for UV-induced apoptosis (Li et al., 1999; Denning et al., 2002; D'Costa and Denning, 2005; D'Costa et al., 2006; Lagory et al., 2009). PKC-δ is down-regulated in human SCCs, and loss of PKC-δ activity is required for transformation of KCs (D'Costa et al., 2006; Popp et al., 2002). The chemically induced mice skin tumors that harbor Ras mutations are also deficient in PKC-δ protein levels and activity (Reddig et al., 1999). Furthermore, transgenic mice overexpressing PKC-δ are resistant to chemically induced SCCs, and re-expression of PKC-δ in human SCC lines is sufficient to induce apoptosis and suppress tumorigenicity (Reddig et al., 1999; D'Costa et al., 2006). Although the role of PKC-δ as a tumor suppressor in chemical carcinogenesis model is well-established, the tumor suppressive role of PKC-δ in photocarcinogenesis model is not clear. Aziz and colleagues showed that PKC-δ transgenic mice failed to suppress tumorigenesis in response to repeated UV exposure thus the authors ruled out PKC-δ as a tumor suppressor in their model (Aziz et al., 2006). However, the UV-induced skin papillomas generated in PKC-δ overexpressing mice exhibited loss of PKC-δ protein levels in tumor cells (Aziz et al., 2006). This is consistent with the loss of PKC-δ in chemical carcinogenesis model and reaffirms that loss of PKC-δ is strongly associated with development of SCCs.
1.5 Src Family of Non-Receptor Tyrosine Kinases

1.5.1 The Structure and Function of SFKs

SFK is a multigene family of 52-62 kDa membrane-associated non-receptor tyrosine kinases. SFKs are required for a variety of biological processes, especially cell migration and invasion due to their role regulating cell-matrix adhesion, cell-cell adhesion and actin reorganization. SFKs are key signaling intermediates for receptor tyrosine kinases, cytokine receptors, and integrin extracellular matrix receptors. Activation of G-Protein Coupled Receptors (GPCR), integrins, CAMs (immunoglobulin super family cell adhesion molecules) result in the phosphorylation or activation of SFKs. Numerous targets for SFK substrates have been implicated in cell-matrix adhesion, migration, proliferation, survival and invasion (Kim et al., 2009b; Thomas and Brugge, 1997).

The SFK family consists of ten members (Src, Fyn, Yes, Yrk, Blk, Fgr, Hck, Lck, Lyn and Frk) that differ in the subcellular localization, substrates, and pattern of expression. The SFKs are divided into three subgroups based on the pattern of expression. The Src, Fyn and Yes are ubiquitously expressed and form the first and the most widely studied subgroup of SFKs. The second group of SFKs, Blk, Fgr, Hck, Lck and Lyn, are expressed primarily in hematopoietic cells (Bolen and Brugge, 1997). The third group, the Frk-related kinases Frk/Rak and Iyk/Bsk, are primarily expressed in epithelial derived cells (Cance et al., 1994; Lee et al., 1994; Thuveson et al., 1995). In addition to being differentially expressed, the SFKs also differ in their subcellular localization. For
example, Src is largely localized to focal adhesions and endosomes, whereas Fgr and Frk are localized in the nucleus (Robbins et al., 1995; Cance et al., 1994; Kaplan et al., 1992; Thuveson et al., 1995).

SFKs are composed of multiple src-homology domains: SH4, SH3 and SH2. In addition, SFKs have a catalytic domain, a ‘unique region’ and a short negative regulatory tail (Figure 3) (Brown and Cooper, 1996). SFKs undergo lipid modification such as palmitoylation or myristolation in the SH4 domain. These lipid modifications are responsible for differential subcellular localization within the cell (Oberg-Welsh and Welsh, 1995; Resh, 1993). SFKs are activated by receptors primarily via protein-protein interactions. The unique domain is distinct for each member and is important for protein-protein interactions and sometimes harbor phosphorylation sites (Engen et al., 2008). The precise role of unique domain is currently unclear. The SH3 and SH2 are protein binding domains and regulate SFK catalytic activity and interaction with substrates (Engen et al., 2008). The SH3 domain contains a P-X-X-P sequence, where X represents any amino acid, and binds to proline rich amino acid sequences in other proteins. For example, p85 PI3K and paxillin bind to SFKs via the SH3 domain (Liu et al., 1993; Taylor and Shalloway, 1994). SH2 domain binds to phosphotyrosine containing sequences. For example, Src interacts with Focal Adhesion Kinase (FAK), p130Cas, p85 PI3-K via its SH2 domain (Fukui and Hanafusa, 1991; Petch et al., 1995; Taylor and Shalloway, 1994).
FIGURE 3: Structure of Src Family of Tyrosine Kinases

The structural domains of Src are shown. U represents the unique domain. R is the regulatory domain containing the inhibitory Tyr 527 phosphorylation site. The kinase (SH4) domain contains the Tyr 416 autophosphorylation site associated with activation. Also shown are the interaction domains SH2 and SH3.
SH2 and SH3 domains also regulate the catalytic activity of SFKs as well. In the inactive state, the Y-527 (Src) residue in the negative regulatory tail region of SFKs is phosphorylated by Csk, and subsequently binds to SH2 domain thus maintaining a closed inactive conformation. In addition, the SH3 domain interacts with the linker region and the catalytic domain and negatively regulates the catalytic activity of SFKs. Deletion of Y-527 residue or knockdown of Csk results in activation SFKs in many cell types (Reynolds et al., 1987; Nada et al., 1993). In contrast to Y-527, the phosphorylation of Y-416 residue induces an open conformation in SFKs and induces activation (Roskoski, Jr., 2005; Roskoski, Jr., 2004). The Y-416 is the autophosphorylation site in SFKs, and is constitutively phosphorylated in oncogenic Src mutants (Cooper et al., 1986; Parsons and Weber, 1989). Furthermore, mutation of Y416 dramatically inhibits the activity of v-src and oncogenic Src mutants indicating this phosphorylation event is required for maintaining the activity of SFKs (Piwnica-Worms et al., 1987). Isoleucine 338 is highly conserved across species and lies in the hinge region between the N-terminal and C-terminal lobes. This residue regulates the recognition and binding of ATP analogues, and when mutated to threonine induces change in confirmation and resders the kinase constitutively active.

1.5.2 Regulation of SFKs in Cancer

Src was first identified as transforming agent (v-src) in the Ras sarcoma oncogenic retroviruses (Thomas and Brugge, 1997). v-src differs from c-Src in the C-terminal
sequence. \(v-src\) lacks the amino acids that normally bind to SH domains and maintain an inactive closed conformation. In addition, \(v-src\) also harbors mutations within the SH3 domain that result in increased catalytic activity (Frame, 2004). Many \(v\)-Src substrates are proteins that are usually phosphorylated in response to activation of cell surface receptors. For example, cytoskeletal proteins such as FAK, paxillin, p130cas and tensin are well known substrates of SFKs and are activated in response to integrins, PDGFR and EGFR activation. In addition, a variety of structural proteins (such as caveolin, connexins, catenins), kinases (such as PKC-\(\delta\)), phosphatases (shp-1, PP2A) and some tyrosine kinase receptors (such as EGFR) are regulated by SFKs (Thomas and Brugge, 1997).

Majority of the human cancers, including breast, pancreatic, colonic and skin, show increased SFK activity (Ayli et al., 2008; Aligayer et al., 2002; Cam et al., 2001; Lutz et al., 1998; Yezhelyev et al., 2004). However, the mechanisms responsible for elevated SFK activity in human carcinomas is not clearly understood. These mechanisms involve mutations in SFK genes, elevated expression, increased stability or loss of negative regulators (e.g. Src Activating and Signaling Molecule (Srcasm)). The activating mutations in SFKs are very rare in human cancers (Daigo et al., 1999; Irby et al., 1999; Talamonti et al., 1993). Activated Src is less stable than wild type form and is downregulated by poly-ubiquitination and proteosomal degradation (Hakak and Martin, 1999). Cbl, an E3 ubiquitin ligase, is responsible for ubiquitination and subsequent degradation of Src. Cbl is also negatively regulated by Src, and tyrosine phosphorylation
of Cbl by Src induces autoubiquitination and subsequently targets it for degradation (Fujita et al., 2002).

1.5.3 Downstream SFK signaling

Oncogenes such as Src and Ras, induce a switch from an epithelial-like to a mesenchymal-like phenotype in the cells that is associated with the loss of cell-cell adhesion proteins such as E-Cadherin, and increase of integrin-dependent extra-cellular matrix contacts (Thiery, 2002). This process is called EMT or epithelial to mesenchymal transition. Active Src blocks the translocation of E-Cadherin to the membrane by inducing ubiquitination and subsequent degradation of E-Cadherin via Hakai, an E3 ubiquitin ligase (Fujita et al., 2002). In addition, \( v-src \) substrates such as Arf6 GTPase and catenin p120CTN, negatively modulate E-Cadherin function and assembly at the cell-cell contacts when activated (Palacios et al., 2001; Ozawa and Ohkubo, 2001). PI3K signaling plays a crucial role in \( v-src \) induced EMT and invasion. Expression of PTEN, the negative regulator of PI3K signaling, stabilizes adherens junctions and blocks the invasive capacity of \( v-src \) transformed cells without affecting the \( v-src \)–induced down-regulation of E-Cadherin (Kotelevets et al., 2001). Src also affects integrin function by tyrosine phosphorylation. Src-mediated phosphorylation of integrin \( \beta 1 \) at the tyrosine residues 783 and 785 contributes to reduced adhesiveness during cell transformation (Sakai et al., 2001). Src can also phosphorylate integrin \( \beta 3 \) and inhibit \( \alpha v \beta 3 \) mediated adhesion to fibronectin (Datta et al., 2002). Furthermore, Src can also indirectly regulate
integrin function by directly phosphorylating R-Ras, a Ras superfamily GTPase member that controls integrin function (Zou et al., 2002). FAK is a key substrate of Src and regulates Src-mediated increased migration and invasion (Hauck et al., 2002a). Expression of FRNK, an endogenous inhibitor of FAK, in Src transformed cells inhibits src/FAK binding and consequently suppresses invasion of Src transformed cells without affecting anchorage-independence and tumor growth in nude mice (Hauck et al., 2002b). In addition, Src-mediated expression of matrix metalloproteases (MMP), the ECM remodeling enzymes, is necessary for the increased invasive phenotype of Src transformed cells (Hsia et al., 2003; Hauck et al., 2002b). SFKs alter cell morphology by activating RhoA-ROCK pathway that controls actin filament assembly (Pawlak and Helfman, 2002). In addition to regulating cell-cell adhesions, Src also disrupts Gap junctions by tyrosine phosphorylation of gap junction protein Connexin-43 (Warn-Cramer et al., 2003).

In addition to migration and invasion, elevated expression of SFKs is also linked to increased survival and proliferation of many cells (Frame et al., 2002; Thomas and Brugge, 1997). For example, in colon cancer cells, Src inhibits anoikis via activation of PI3K signaling (Windham et al., 2002). The association of Src with cell surface receptors such as PDGFR or EGFR is required for receptor-mediated increased proliferation and expression of many cell cycle regulators such as Myc (Broome and Hunter, 1996; Erpel et al., 1996; Barone and Courtneidge, 1995). Also, phosphorylation of Shc or EGFR by Src activates MAPK pathway which is important for cell proliferation and survival
(Sasaoka et al., 1994; Luttrell et al., 1997). In addition, Src can directly bind and phosphorylate p85 subunit of PI3K and consequently induce PI3K activation leading to increased cell survival in many cell types (Chang et al., 1997; Luttrell et al., 1996). SFKs can suppress apoptosis in many cells by mimicking the effects of integrins, cytokines and growth-factor receptors (Anderson et al., 1990; Frisch and Francis, 1994; Basu and Cline, 1995). SFKs are also required for activity of several transcription factor pathways. Transcription of several STAT3 targets such as Cyclin D, Myc, PDGF requires SFK activity (Barone and Courtneidge, 1995; Bowman et al., 2001; Twamley-Stein et al., 1993). Many regulators of NF-κB signaling have been implicated as substrates of SFK (Fan et al., 2003; Huang et al., 2003). In addition, SFKs are required for hypoxia-induced VEGF induction (Shweiki et al., 1992). Src is also required downstream of VEGF for the survival of endothelial cells and angiogenesis (Eliceiri et al., 1999).

1.5.4 The Fyn Oncogene

Fyn is a 59 kDa SFK located on chromosome position 6q21. Fyn was originally identified in 1986 through probes derived from v-lyn and v-fgr (Davidson et al., 1994; Popescu et al., 1987). Fyn undergoes post-translational lipid modifications such as palmitoylation and myristolation, and as a result is localized to lipid rafts and subcellular domains associated with integrins and EGFR (Li et al., 2005; Simons and Toomre, 2000). Active Fyn largely functions by interacting and phosphorylating a variety of substrates such as integrins, Paxillin and FAK. In contrast to other SFKs that are largely activated in
cancers by post-translational modifications or mutations, Fyn mRNA is selectively overexpressed in many cancers such as prostate, glioblastoma, melanoma and SCC (Posadas et al., 2009; Talantov et al., 2005; Lu et al., 2009; Saito et al., 2010; Ban et al., 2008). However, the mechanism and the role of Fyn induction in cancer are not clear. Recent studies in chronic myelogenous leukemia cells found that Fyn mRNA is up-regulated in response to Bcr-Abl1-induced oxidative stress, and this transcriptional mechanism involves the redox-sensitive Egr1 transcription factor (Gao et al., 2009).

Fyn is ubiquitously expressed and regulates many cellular functions such as cell cycle, proliferation, growth and integrin mediated migration and invasion (Saito et al., 2010). While the role and regulation of Src has been heavily characterized in cancer, other SFK members such as Fyn have received less attention. Like Src, Fyn is a proto-oncogene and induces transformation in many cell types such as NIH-3T3 cells (Kawakami et al., 1988). PP1, a SFK inhibitor, specifically inhibits Fyn over Src at lower concentrations and blocks malignant transformation of cells by v-rasH	extsuperscript{Ha} (He et al., 2000). Also, overexpression of Fyn in fibroblasts induces anchorage-independent growth indicating a critical role of Fyn is malignant transformation of normal cells (Wary et al., 1998). Expression of active Fyn is the epidermis (K14-FynY528F) spontaneously induces cutaneous SCCs in mice, suggesting Fyn is a potential oncogene for SCCs (Zhao et al., 2009). Inhibition of Fyn has been associated with decreased cell growth (Zhao et al., 2009). For example, expression of dominant-negative Fyn reduces skin tumors in mice (Li et al., 2003). Furthermore, Fyn is strongly linked with increased cell survival via
PI3K signaling (Noronha et al., 2007). In addition, knockdown of Fyn blocks EGF-induced Akt activation indicating Fyn is required for activation of PI3K pathway (Chen et al., 2001). Furthermore, Fyn mRNA is upregulated in imatinib-resistant chromic myeloid leukemia cells indicating that Fyn might also play a role in drug resistance of cancer cells (Ban et al., 2008).

Fyn plays an integral role in migration, invasion and metastasis of many cancer types. Fyn has been shown to mediate extracellular interactions by regulating IL-8, c-Met, EGFR and integrins (Chang et al., 2007). Like Src, Fyn has been shown to co-immunoprecipitate with FAK (Cary et al., 1996). At focal adhesions, FAK is recruited to the β subunits of integrins and upon association with SFKs undergoes activation and autophosphorylation at Y397 residue (Mitra et al., 2005). Activation of FAK by Fyn-mediated phosphorylation of Y861 and Y925 residues is required for PI3K activation within the lipid rafts (Baillat et al., 2008). The Fyn/FAK/Integrin assembly is the center of many cellular functions such as proliferation, migration and invasion. Fyn is also linked to Rac and Rho family GTPases. Interaction of Fyn and Rho family members such as Rac1, Rho A and Cdc42 and is shown to control morphology-induced differentiation of many cells such as oligodendrocytes (Liang et al., 2004). Fyn deficient mast cells exhibit decreased lamellipodia formation indicating that Fyn is required for regulation of cell spreading and motility (Samayawardhena et al., 2007). In addition, inactivation of Fyn by PTEN is required for PTEN-mediated loss of Rac-GTPase activity, consistent with the role of Fyn in regulating cell shape and motility (Dey et al., 2008). Fyn also regulates
MMP production. Activation of Fyn by β6 integrin in required for MMP-3 production leading to increased cell proliferation and tumor progression in vivo (Li et al., 2003). Overall, these findings describe a key role of Fyn in tumor growth and metastatic progression.

Fyn plays a critical role is epidermal tumorigenesis. Fyn is required for EGFR mediated inhibition of β4 integrin function and dissociation of hemidesmosomes in human KCs (Mariotti et al., 2001). Fyn is overexpressed in human SCCs and inhibition of Fyn suppresses migration and invasion of SCC cells (Ayli et al., 2008; Mariotti et al., 2001). However, the mechanism of Fyn induction and downstream signaling in epidermal tumors is not clear. Fyn induced epidermal tumors exhibit increased activation of MAPK and PI3K pathways, the key signaling responsible for increased cell proliferation and cell survival, respectively (Zhao et al., 2009). Furthermore, inhibition of Fyn with myricetin inhibits UV-induced skin tumors in mice (Jung et al., 2008). Together, these findings indicate that induction of Fyn and activation of Fyn downstream signaling is very critical for the development of cutaneous SCCs.

1.6 Nuclear Factor kappaB Transcription Factors

1.6.1 The Structure and Function of NF-κB

NF-κB was first identified in B cells as an enhancer element binding protein in the immunoglobulin kappa light chain gene (Sen and Baltimore, 1986). NF-κB transcription
factors play a critical role in inflammation, infection, apoptosis, and cancer progression (Baldwin, Jr., 1996; Gilmore et al., 1996). The NF-κB family consists of five structurally related proteins that are highly conserved across species: p50 (NF-κB1), p65 (Rel A), c-Rel, p52 (NF-κB2 or lyt-10) and RelB. These subunits form homodimers or heterodimers, and bind to the κB motif sequences in the target promoter sequence to regulate gene expression by recruiting co-repressors (e.g. HDAC) or co-activators (e.g. p300) (Gilmore et al., 1996; Matthews and Hay, 1995; Elsharkawy et al., 2010). All NF-κB proteins contain a conserved ~300 amino acid long DNA-binding and dimerization domain known as Rel homology domain (RHD). p65, c-Rel and Rel B contain a non-homologous transactivation domain in the c-terminus that can induce target gene expression. p50 and p52 subunits of NF-κB lack the c-terminal transactivation domain, and are generated as a result of proteolytic cleavage of p105 and p100 proteins, the products of NF-κB1 and NF-κB2 genes, respectively. NF-κB dimers consisting of the p65 subunit function as activators of gene expression (Matthews and Hay, 1995). However, NF-κB dimers containing the p50 subunit largely functions as transcriptional repressors (Matthews and Hay, 1995; Elsharkawy et al., 2010; Cao et al., 2006).

Activity of NF-κB is regulated by the family of IκB proteins (Karin, 2006). Under control conditions, NF-κB transcription factors are retained in the cytoplasm by binding to the inhibitory IκB-α proteins such as IκB-α, IκB-β and IκB-γ. IκB proteins contain ankyrin repeats in their C-terminus that bind and sterically block the nuclear localization signal sequences on NF-κB proteins, thus sequestering them in the cytoplasm. Unprocessed
p100 and p105 proteins also contain the ankyrin repeats, similar to IκB, and thus are capable of binding and retaining NF-κB proteins in the cytoplasm. In contrast, Bcl-3, an IκB-like protein, functions as a transcriptional co-activator of p50 and p52 (Palmer and Chen, 2008). Upon IκB degradation, NF-κB is relieved of its inhibition, allowing it to translocate to the nucleus where it can be further regulated by acetylation, phosphorylation and interactions with co-repressors and co-activators to transcribe or repress target gene expression (Nakshatri and Goulet, Jr., 2002).

IκB proteins are targets of many upstream kinases such as IκB kinase (IKK), PKCs and in some cases SFKs. IKK exist as a complex of three subunits, the catalytic IKKα and IKKβ, and the regulatory IKKγ (NEMO). In response to stimulation, such as tumor necrosis factor (TNFα) and interleukin-1 (IL-1), signaling pathways are activated that phosphorylate and activate the IKK. When activated IKK complex phosphorylates IκB and targets it for ubiquitination and subsequent proteosomal degradation. For example, IκBα is phosphorylated on serine 32/36 by IKKβ and is consequently poly-ubiquitinated on lysine 21/22 by E3 ubiquitin ligases resulting in degradation by 26S proteosome. The primary function of IKK-β is to phosphorylate and inhibit IκB proteins, whereas IKK-α has several other functions including p100 phosphorylation and regulation of histones (Israel, 2003).

1.6.2 Constitutive Activation of NF-κB
Activation of the NF-κB signaling pathway is classified as either inducible (e.g. TNF-α, TPA, UV, IL-1) or constitutive (e.g. mutant Ras) (Sethi et al., 2008; Liou et al., 1994). Normally NF-κB activation is dependent upon stimulation, for example TNF-α activates NF-κB in some cell types and IL-1 or hypoxia in others. The signaling and regulation of inducible NF-κB activation has been well characterized and largely involves transactivation of target genes by p65 NF-κB dimers. However, the signaling and regulation of constitutive NF-κB activation is not well understood.

Constitutive activation of NF-κB occurs upon constitutive down-regulation of inhibitory IκB proteins and sustained nuclear localization of NF-κB subunits. In contrast to inducible NF-κB activation where many types of stimuli have been identified, the precise stimulus and mechanism responsible for constitutive NF-κB activation is not clear. Aberrant IKK activity and loss of IκB inhibitory function via inactivating mutations or increased protein turnover are some of the possible causes for constitutive NF-κB activation (Wood et al., 1998; Gasparian et al., 2002; Miyamoto et al., 1998). Several signaling pathways such as EGFR, Ras, and PI3K have been strongly linked with constitutive NF-κB activation in many cell types and cancers (Finco et al., 1997; Cox and Der, 2003; Biswas et al., 2000; Pianetti et al., 2001). In addition, many regulators of constitutive NF-κB signaling have been implicated as direct targets of tyrosine kinases such as SFKs and EGFR, and integrin signaling (Nikolopoulos et al., 2004; Reuther et al., 1998; Sethi et al., 2007; Fan et al., 2003; Huang et al., 2003).
NF-κB is constitutively activated in many tumors including breast, pancreatic, ovarian, colon and SCC, and in some cases are accompanied by increased expression of NF-κB subunits (Wang et al., 1999; Bours et al., 1994; Sovak et al., 1997; Dejardin et al., 1995; Duffey et al., 1999; Nakshatri et al., 1997; Budunova et al., 1999). Studies in B cells have found that constitutive NF-κB activation comprises mostly of p50/c-Rel complexes (Liou et al., 1994; O'Connor et al., 2004; Grumont and Gerondakis, 1994). Constitutive activation of NF-κB have been strongly linked to resistance of apoptosis, whereas inducible NF-κB activation is associated with induction of pro-apoptotic proteins. NF-κB activation by cellular stresses such as UV, ionizing radiation and chemotherapeutic agents is required for apoptosis in many cell types (Sethi et al., 2008). In contrast, tumor cells exhibiting constitutive NF-κB activation are resistant to many chemotherapy and radiation treatments (Sethi et al., 2008).

1.6.3 NF-κB Signaling and Epidermal Tumorigenesis

NF-κB was first linked to cancer upon identification of v-rel, the oncogenic homolog of cellular NF-κB subunits c-Rel (Gilmore, 1999). NF-κB regulates the expression of many oncogenes, growth factors, and pro-apoptotic genes, and thus functions as a tumor promoter or a tumor suppressor based on the cell conditions and the target genes (Karin, 2006; Gilmore et al., 1996). Several genes that are implicated in cell proliferation are regulated by NF-κB, for example Cyclin D, c-Myc and p21 (Sethi et al., 2008). The expression of cyclooxygenase-2 (COX-2), which plays a key role in PGE2 production
and proliferation of tumor cells, is also regulated by NF-κB (Yamamoto et al., 1995). Also, NF-κB activity is required for EGF and PDGF induced growth and survival of cancer cells (Habib et al., 2001; Romashkova and Makarov, 1999). Expression of prosurvival genes such as IAPs, TRAFs and various members of the anti-apoptotic Bcl-2 family is also regulated by NF-κB (Ahn and Aggarwal, 2005). Furthermore, NF-κB signaling is strongly linked to expression of genes such as MMPs, uPA, IL-8 and VEGF, which are involved in angiogenesis and invasion of various transformed and tumor cells including transformed KCs (Bond et al., 1998; Novak et al., 1991; Aggarwal et al., 2006; Levine et al., 2003; Kim et al., 2008). NF-κB is a target of Ras signaling and cells expressing active Ras have been shown to exhibit sustained IKK activation and reduced IκB-α steady state levels. Inhibition of Ras function by expression of dominant negative Ras down-regulates NF-κB activity (Koong et al., 1994; Folgueira et al., 1996). In addition, NF-κB is constitutively activated in transformed cell lines including Ras-transformed KCs (Finco et al., 1997; Tobin et al., 1996; Folgueira et al., 1996), and required for Ras-induced transformation of many cell types such as NIH-3T3 (Finco et al., 1997; Norris and Baldwin, Jr., 1999). Furthermore, NF-κB activity is also required for maintaining the apoptotic resistance phenotype of the Ras-transformed cells, suggesting that NF-κB may be required for repression of pro-apoptotic genes or induction of anti-apoptotic genes (Millan et al., 2003).

NF-κB is a well known mediator of epidermal hyperplasia and inflammation, which plays a key role in skin tumor promotion (Karin, 1998; Slaga et al., 1996). NF-κB signaling is
strongly activated in response to skin tumor promoters such as phorbol esters and okadaic acid (Tobin et al., 1996; Folgueira et al., 1996; Koong et al., 1994). NF-κB is constitutively activated in SCCs and melanomas, and NF-κB signaling is required for apoptotic resistance and malignant transformation of KCs (Qin et al., 1999; Ren et al., 2006; Nair et al., 2003; Jackson-Bernitsas et al., 2007). In addition, the NF-κB pathway is constitutively activated in mouse skin tumors, and inhibition of NF-κB signaling prevents UV-induced skin tumors in mice, consistent with the role of NF-κB as an oncogene in the epidermis (Budunova et al., 1999; Gottipati et al., 2008).

NF-κB has also been implicated as a tumor suppressor in the epidermis. Inhibition of NF-κB activation by ectopic expression of dominant negative IκBα super-repressor promotes KCs transformation and development of SCCs (Dajee et al., 2002; Dajee et al., 2003). In addition, inducible activation of NF-κB signaling by UV or TNF-α promotes apoptosis and transactivates expression of pro-apoptotic genes such as PKC-δ (Kaufman and Fuchs, 2000; Liu et al., 2006). Furthermore, p65 and NF-κB activation were shown to be critical for p53 mediated apoptosis (Ryan et al., 2000). Recent studies suggest that NF-κB may function as pro-apoptotic signaling pathway during the early stages of tumorigenesis, however, due to further mutations and activation of the oncogenic pathways, NF-κB may get constitutively activated and subsequently become tumor promoting during the later stages of cancer (Rocha et al., 2003b; Rocha et al., 2003a). This shows that our knowledge of NF-κB signaling and its function is still not clear and requires a more careful analysis.
1.7 PKC-δ: Tumor Suppressor for Human SCCs

1.7.1 PKC-δ Structure and Function

PKC-δ was first cloned in 1987 from a rat brain cDNA library (Ono et al., 1987). PKC-δ is a 78 kDa serine/threonine kinase and phosphorylates substrates on the following consensus sequence: S/T.X.X.R/K, where X represents any amino acid. PKC-δ, a ubiquitously expressed PKC isoform, is activated by DAG/phorbol esters in a Ca^{2+} independent manner. PKC-δ is unique in comparison to other members of the PKC family. PKC-δ contains a unique and conserved nuclear localization signal in the C4 domain, which plays an important role in nuclear localization of PKC-δ during apoptosis (DeVries et al., 2002; Scheel-Toellner et al., 1999; Yoshida et al., 2003; Eitel et al., 2003). PKC-δ contains the C2-like domain, which has been reported to function as a phosphotyrosine binding domain and may play a key role in protein-protein interactions and substrate specificity (Benes et al., 2005). PKC-δ is capable of activation by proteolytic cleavage thus avoiding the need for membrane translocation (Steinberg, 2004). PKCs such as PKC-α, PKC-ε and PKC-ζ have been largely associated with suppression of apoptosis, while PKC-δ is a critical pro-apoptotic signal in many cells (Reyland, 2007). Furthermore, PKCs such as PKC-α and PKC-β required PDK-1 mediated phosphorylation in the activation loop for maximum activity, however, mutation of Thr505 in the activation loop of PKC-δ does not affect its function indicating PKC-δ does not require PDK-1 mediated phosphorylation for its activity (Zhang et al.,
In contrast, mutation of Glu500 to Val within the activation loop dramatically suppresses the activity of PKC-δ indicating that PKC-δ activation is regulated by a mechanism different than other PKCs (Zhang et al., 2002).

PKC-δ activity is frequently regulated by tyrosine phosphorylation within the activation loop, hinge region or the regulatory domain. Tyrosine phosphorylation of PKC-δ was first reported in v-rasHa transformed mouse KCs (Denning et al., 1993). Further studies revealed that PKC-δ can be phosphorylated at Tyr 52, 187, 311, 512 and 523 residues (Szallasi et al., 1995; Li et al., 1996; Konishi et al., 1997; Gschwendt et al., 1994). PKC-δ is a direct substrate of tyrosine kinases such as SFKs which regulate PKC-δ activity depending on the upstream stimuli and the site of phosphorylation (Denning et al., 1996; Zhu et al., 2008; Lu et al., 2007). Furthermore, inhibition of PKC-δ activity by SFK-mediated tyrosine phosphorylation is associated with induction of neoplastic phenotype of Ras-transformed mouse KCs, thus linking PKC-δ tyrosine phosphorylation to neoplastic transformation of KCs (Joseloff et al., 2002). Tyrosine phosphorylation of PKC-δ is usually associated with inhibition of its activity; however, in some cases (Tyr 311) it could also promote PKC-δ function (Lu et al., 2007). Furthermore, PKC-δ is also tyrosine phosphorylated in response to EGFR and Ca\(^{2+}\) during KC differentiation thus associating PKC-δ tyrosine phosphorylation to differentiation as well (Denning et al., 2000). In addition to Ras or SFK, PKC-δ is tyrosine phosphorylated in response to a variety of stimuli such as TGF-α, TPA, H\(_2\)O\(_2\), PDGF and IgE ligand (Li et al., 1994a; Li et al., 1994b; Denning et al., 1996; Haleem-Smith et al., 1995; Konishi et al., 1997;
PKC-δ activity and function in normal and transformed cells.

PKC-δ is also regulated by proteolysis. PKC-δ contains a unique caspase cleavage site, DMQD_{330}N, in the hinge region between the regulatory and catalytic domains. Caspase-3 is the primary caspase responsible for this cleavage; however, caspase-2 has also been recently described to cleave PKC-δ at the same site (Panaretakis et al., 2005). When cleaved at this position, PKC-δ generates a regulatory and a 40 kDa constitutively active catalytic domain (Emoto et al., 1995). The catalytic fragment of PKC-δ have been shown to be necessary and sufficient for variety of tumor suppressive functions of PKC-δ such as apoptosis and cell-cycle arrest, across many cell types including Kcs (Sitailo et al., 2006; Lagory et al., 2009). PKC-δ is also regulated at the mRNA level. Eight different alternative splice forms of PKC-δ exists across species. Some of which, such as PKC-δ VIII lack the caspase-3 cleavage site and are resistant to activation by proteolytic cleavage. PKC-δ VIII functions as a pro-survival signal in the cell. For example, PKC-δ VIII rescues NT2 cells from etoposide-induced apoptosis (Patel et al., 2006).
FIGURE 3: Caspase-3 Mediated Cleavage of PKC-δ

The hinge region of novel PKCs contain a caspase cleavage site (DMQD). PKC-δ, when cleaved at this site, releases a 40 kDa constitutively active catalytic fragment. Activation of PKC-δ via caspases-3-mediated cleavage is required for UV-induced apoptosis.
1.7.2 Regulation of PKC-δ Gene Expression

PKC-δ gene is evolutionary conserved and resides on the 3p21.31 region of the human chromosome (Huppi et al., 1994; Suh et al., 2003). PKC-δ gene is 20 kb long and consists of 18 exons in mice and humans. The translational start site is located within the exon 2, which is separated from exon 1 by a 17 kb long intron (Suh et al., 2003). Previous studies in rat cells have shown that PKC-δ undergoes inhibitory translational regulation due to formation of secondary structures in the unusually long 5'-UTR region (Morrish and Rumsby, 2002). PKC-δ gene contains the C2-like domain in the 3rd exon. The inhibitory psuedosubstrate domain resides in the 5th exon of PKC-δ gene. The C1 domain is encoded within exon 5-exon 9, and kinase domain spans 8 exons from 11th-18th exon.

PKC-δ promoter activity and gene expression is regulated by several cellular stimuli such as androgens, Vitamin D3, UV exposure, estrogens or mechanical stress (Gavrielides et al., 2006; Liu et al., 2006; Berry et al., 1996; Shanmugam et al., 1999; Geng et al., 2001). The promoter of mouse PKC-δ contains several putative transcription factor binding sites. These transcription factors are well known to regulate various cellular pathways such as spermatogenesis (SRY), development (MYOD, CDXA, CEBP), embryogenesis (XFD, GATA), immune response (LYF) and most importantly oncogenesis (NF-κB, AML, p53, MZF, SP1) (Suh et al., 2003). In mouse KCs, PKC-δ gene expression is induced upon TNF-α treatment suggesting a role of NF-κB in PKC-δ gene expression in KCs. Studies in mice fibroblasts have found that NF-κB  Rel-A signaling plays a critical
role in basal PKC-δ expression (Liu et al., 2006). Liu and colleagues showed that pre-existing Rel-A NF-κB complex on the PKC-δ promoter regulates UV-induced JNK activation via induction of PKC-δ. This induction of PKC-δ and JNK via NF-κB was found to be critical for UV-induced apoptosis (Liu et al., 2006). Recently, a study identified that p63 and p73, members of p53 family can bind and activate human PKC-δ promoter in KCs (Ponassi et al., 2006). While the promoter of mice PKC-δ gene has been well characterized, little is known about the regulation of the human PKC-δ promoter.

1.7.3 PKC-δ Downstream Signaling and Role in Apoptosis

PKC-δ plays a critical role in a variety of cellular functions such as apoptosis, cell cycle, migration and differentiation. PKC-δ is activated in response to a variety of apoptotic stimuli such as genotoxins, radiation and oxidative stress (Reyland et al., 1999; Majumder et al., 2001; Khwaja and Tatton, 1999; D’Costa and Denning, 2005). In response to apoptotic stress PKC-δ is proteolytically activated by Caspase-3 mediated cleavage, releasing the constitutively active catalytic fragment, and resulting in growth arrest or apoptosis in many cell types, including KCs (Lagory et al., 2009; Denning et al., 1998; Sitailo et al., 2004). Inhibition of PKC-δ by Rottlerin or by expression of dominant negative kinase dead form inhibits apoptosis (Reyland et al., 1999; Matassa et al., 2001). In addition, PKC-δ deficient mice are resistant to etoposide and radiation induced apoptosis indicating that PKC-δ is also critically important for apoptosis in vivo (Humphries et al., 2006). Several downstream substrates of PKC-δ have been implicated
in apoptosis. PKC-δ activates p53 by phosphorylating Ser 15 and Ser16 residues (Lee et al., 2006; Yoshida et al., 2006a). In addition, PKC-δ phosphorylates Btf (Bcl2 associated transcription factor) and transactivates p53 expression (Liu et al., 2007). PKC-δ also regulates transcription of pro-apoptotic STAT-1 gene (Ren et al., 2002). Anti-apoptotic Bcl-2 proteins such as Mcl-1 suppress apoptosis by binding and sequestering pro-apoptotic BH3-only proteins such as Bax. In response to apoptotic stimuli, PKC-δ phosphorylates Mcl-1 and targets it for degradation, and consequently induces Bax activation resulting in apoptosis (Sitailo et al., 2004; Sitailo et al., 2006). In addition, PKC-δ promotes apoptosis by activating the pro-apoptotic stress kinase p38 and by aiding the breakdown of nuclear envelope by phosphorylation of Lamin B (Tanaka et al., 2003; Cross et al., 2000). In KCs, caspase-3 mediated activation of PKC-δ is required for UV-induced apoptosis and subsequent elimination of pre-cancerous keratinocytes (D’Costa and Denning, 2005; Denning et al., 2002; Matassa et al., 2001; Matsumura et al., 2003). The pro-apoptotic function of PKC-δ is not limited to human skin. Recent studies have described the proapoptotic role of PKC-δ in neuronal, renal and breast cell lines as well (Lelongt, 2010; Kanthasamy et al., 2003; Chen et al., 2010). In addition to mitochondrial-induced apoptosis, PKC-δ has also been implicated in death receptor-mediated apoptosis (Gonzalez-Guerrico and Kazanietz, 2005). For example, in prostate cancer cells, PKC-δ is required for TNF-α and TRAIL induced apoptosis (Fujii et al., 2000). Thus, multiple effectors of PKC-δ-induced apoptosis have been described by multiple groups, yet no consensus have been reached regarding the critical targets
Recent studies have strongly implicated PKC-δ in cell cycle control and DNA damage checkpoint activation. PKC-δ can arrest cell cycle at various stages in response to different stimulus or DNA damage. Upon serum starvation, PKC-δ induces p27 and delays Cyclin D expression in endothelial cells indicating a role of PKC-δ in G1/S growth arrest (Ashton et al., 1999). Also, knockdown of PKC-δ inhibits TPA induced G1 arrest and p27 induction in thyroid cancer cells, indicating that PKC-δ is required for G1 arrest in these cells (Afrasiabi et al., 2008). PKC-δ is a key substrate of the DNA damage checkpoint kinase, ATM. In response to DNA damage, PKC-δ phosphorylates and activates Rad9 in an ATM-dependent manner thus promoting DNA repair signaling (Yoshida et al., 2003). PKC-δ also regulates other proteins in the DNA repair signaling such as DNA-PK and Topoisomerase IIα (Bharti et al., 1998; Yoshida et al., 2006b). In response to UV radiation, PKC-δ can also induce G2/M growth arrest, consistent with the DNA damage cell cycle checkpoint (Watanabe et al., 1992; Ishino et al., 1998; Lagory et al., 2009). Recent studies have shown that PKC-δ is required for UV induced DNA damage checkpoint activation resulting in G2/M growth arrest and apoptosis (Lagory et al., 2009). Furthermore, induction of G2/M arrest by PKC-δ-cat was shown to be independent of ATM/ATR checkpoint kinases indicating that PKC-δ might be directly involved in G2/M signaling (Lagory et al., 2009).

1.7.4 Loss of PKC-δ in Human SCCs

Loss of tumor suppressor function, either by gene deletion or inactivation, is necessary
for the progression of human cancers. PKC-δ functions as a tumor suppressor in mouse and human SCCs, and other cell types such as transformed colon and fibroblast cells (D'Costa et al., 2006; Reddig et al., 1999; Gschwendt, 1999; Dlugosz et al., 1994; Perletti et al., 1999). HaCaT cells are spontaneously immortalized human KCs but are non-tumorigenic when transplanted into nude mice (Lehman et al., 1993). In addition, HaCaT cells display normal KC differentiation and express differentiation markers such as K1, K10, involucrin and filaggrin (Boukamp et al., 1988). Thus, HaCaT cells serve as a good model system to study KC biology. The tumor suppressive function of PKC-δ has been described in primary as well as HaCaTs, which have mutant p53, indicating a p53-independent pro-apoptotic function of PKC-δ in the skin (Sitailo et al., 2004; Sitailo et al., 2006; Denning et al., 2002). Furthermore, transgenic mice overexpressing PKC-δ are resistant to chemically induced SCCs, and re-expression of PKC-δ in human SCC lines is sufficient to induce apoptosis and suppress tumorigenicity (Gschwendt et al., 1995; D'Costa et al., 2006).

Although the role of PKC-δ as a tumor suppressor in chemical carcinogenesis model is well-established, the tumor suppressive role of PKC-δ in photocarcinogenesis model is not clear. Aziz and colleagues showed that PKC-δ transgenic mice failed to suppress tumorigenesis in response to repeated UV exposure thus the authors ruled out PKC-δ as a tumor suppressor in their model (Aziz et al., 2006). However, the UV-induced skin papillomas generated in PKC-δ overexpressing mice exhibited loss of PKC-δ protein levels in tumor cells (Aziz et al., 2006). This is consistent with the loss of PKC-δ in
chemical carcinogenesis model and reaffirms that loss of PKC-δ is strongly associated with development of SCCs. Furthermore, inhibition of PKC-δ activity transforms rat 3Y1 fibroblasts indicating that PKC-δ is necessary for transformation (Popp et al., 2002). However, the study in fibroblasts was performed by using Rottlerin which is not a specific inhibitor of PKC-δ. Although, PKC-δ is down-regulated in chemically and UV-induced mouse SCCs and in ~30% of human SCCs (Gschwendt et al., 1995; D'Costa et al., 2006), the mechanism of PKC-δ down-regulation is not clear. PKC-δ down-regulation might involve increased protein turnover or reduced gene expression due to gene deletion, increased mRNA turnover or repression of promoter activity in the human SCCs. The 3p region of the human chromosome that harbors PKC-δ gene is frequently deleted or methylated in cancers (Sikkink et al., 1997; Zabarovsky et al., 2002), thus raising a strong possibility that PKC-δ gene expression is lost due to gene deletion or promoter methylation.

Activation of Ras oncogenes by mutation or amplification is one of the most common dominant oncogenic events in human cancers (Karnoub and Weinberg, 2008). Ras is active in ~60% of human SCCs and >90% of chemically induced skin tumors in mice (Quintanilla et al., 1986; Pierceall et al., 1991). These chemically induced mice skin tumors that harbor Ras mutations are also deficient in PKC-δ protein levels and activity indicating that Ras signaling is associated with the loss of PKC-δ (Reddig et al., 1999). Furthermore, autocrine TGF-α/EGFR signaling is strongly linked to the inhibition of PKC-δ activity (DiGiovanni et al., 1994; Denning et al., 1996; Li et al., 1994a; Lu et al.,
In addition, expression of mutant H-Ras or activation of EGFR significantly reduces PKC-δ protein and mRNA levels in HaCaT cells, without affecting other PKC isoform levels, strongly indicating a role for Ras/EGFR signaling in the loss of PKC-δ gene expression in human SCCs (Yadav et al., 2010; D'Costa et al., 2006; Geiges et al., 1995). This raises a strong possibility of Ras-mediated down-regulation of PKC-δ transcription. Ras regulates the expression of many pro-survival and tumor suppressor genes via activation of transcription factors such as Ets, AP-1 and NF-κB (Finco et al., 1997; Mavrothalassitis and Ghysdael, 2000). However, the precise transcription factor signaling responsible for the loss of PKC-δ promoter repression by Ras is not known. Like Ras, SFK activity is also strongly linked to the loss of PKC-δ activity, and the loss of PKC-δ activity by SFK members c-Src and c-Fyn, is required for maintaining the neoplastic phenotype of ras-transformed keratinocytes (Blake et al., 1999; Joseloff et al., 2002). Reexpression of PKC-δ in the SCC lines and Ras-transformed KCs is sufficient to induce apoptosis and reduce tumorigenecity in nude mice strongly indicating that therapeutic strategies targeting PKC-δ down-regulation signaling might be efficacious against cancers such as SCCs (D'Costa et al., 2006).

1.8 Hypothesis and Aims

1.8.1 Hypothesis

PKC-δ is down-regulated at the transcriptional level by oncogenes (such as Ras, Fyn) in human SCCs. Interfering with the PKc-δ down-regulation signaling is a potential
therapeutic target for treatment of human SCCs

1.8.2 Aims

1.8.2.1 Aim 1
To determine if PKC-δ gene expression is down-regulated in human SCCs

1.8.2.2 Aim 2
To unravel the mechanism of Ras-mediated loss of PKC-δ gene expression in HaCaT cells

1.8.2.3 Aim 3
Identify novel molecular targets for the treatment of human SCCs.
CHAPTER II
MATERIALS AND METHODS

2.1 Cell Culture and Reagents

All cell lines were maintained in DMEM (Invitrogen, 11965-092) with 10% FBS and 1% Pen-Strep (Invitrogen). HaCaT, HaCaT-RasII-4 and HaCaT Ras I-7 cells were a gift from Dr. Norbert Fusing, and were authenticated as keratinocytes by cytokeratin staining. MDA-MB-231 cells were a gift from Dr. Clodia Osipo. PP1 (Calbiochem, 529579), PP2 (Invitrogen, PHZ1223), AG1478 (Calbiochem, 658552), U0126 (Cell Signaling) and LY294002 (Axxora, ALX-270-038-M005) were purchased from the indicated companies.

2.2 Immunoblotting

Western blotting was performed as described previously and was visualized by the LI-COR Infrared Imaging System (Voris et al., 2010). Briefly, cell lysates were collected by scraping cells in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% Sodium Deoxycholate). Lysates were briefly sonicated and centrifuged at 14,000 rpm for 5 min to remove cellular debris. Protein concentrations were determined.
using standard Bradford Reagent methodology. Primary antibodies against c-Fyn (SC-16, SC-434), IκBα (SC-371), c-Src (SC-19), c-Rel (SC71X), p65 (SC-372X), p50 (SC-1190X), FAK (SC-558), and P-FAK (SC-11765) were obtained from Santa Cruz Biotechnology Inc. Antibodies against P~ERK1/2 (9106S), P~Akt1 (S473) (4051S), P~EGFR (Y1068) (2236S) were obtained from Cell Signaling Technology. Anti-β-Actin (ICN, 691001), Anti-α-Tubulin (Upstate, 05-829), Anti-Ras (Upstate, 05-516) and p52 (Abcam, AB7972) were obtained from the indicated companies. Secondary antibodies used were goat anti-rabbit IgG-Alexa Fluor 680 (Invitrogen, A21076), donkey anti-rabbit IgG-IRDye 800 (Rockland, 611-732-127) and goat anti-mouse IgG conjugated to AlexaFluor-680 (Molecular Probes, A21057) and donkey anti-mouse IgG IRDye 800 (Rockland, 610-732-124) for detection using the LI-COR infrared scanning system (LI-COR Biosciences).

2.3 Retroviral Infections

Active H-Ras(G12V) was expressed from the LZRS retroviral vector, and the active Fyn (I338T) cDNA was a gift from Dr. Tadashi Yamamoto, and cloned into the pMV7 retroviral vector. Constitutively active Akt (Myr-Akt) lentiviral vector was kindly provided by Dr. Maria Soengas. Upon infection with the LZRS retrovirus the LTR sequences, along with any intervening sequences, are incorporated randomly into the genome of the infected cell. The 5’ LTR then drives transcription of the target gene of interest. Retroviral supernatant was generated by calcium phosphate-mediated
transfection of Phoenix-Ampho packaging cells as described previously (Sitailo et al., 2002). Following transfection, Phoenix-Ampho cells were positively selected for plasmid incorporation by treatment with 1 µg/mL puromycin until the culture dishes reached approximately 75% confluency. Phoenix-Ampho cells were then refed with DMEM minus puromycin and incubated at 32 °C overnight to generate retrovirus. Retroviral infection of target cells using Phoenix-Ampho retroviral supernatant was done for 1 hr at 300xg and 32°C.

2.4 Luciferase Assays

Luciferase assays were performed as described previously (Qin et al., 2004). Briefly, cells were plated onto 12-well dishes and co-transfected with 0.5 µg of pGL3 construct containing the firefly luciferase gene and 0.05 µg of pRLTK plasmid DNA that contained the renilla luciferase gene to normalize the transfection. Transfections were carried out using Fugene 6 transfection reagent (Roche, 18-14443) as per manufacturer instructions. The empty vector pGL3-Basic was used as a negative control in the assay. Cell lysates were prepared 48 hours post-transfection using the Dual-Luciferase Assay Kit (Promega, E1910) as per manufacturer instructions. The luciferase assay was performed by using the Sirius Luminometer (Berthhold Detection Systems). The NF-κB transcriptional activity was determined by using a pNF-κB-LUC plasmid (Clontech Laboratories) containing a firefly luciferase gene driven by a NF-κB enhancer. The NF-κB reporter assay was performed as described previously (Qin et al., 1999).
2.5 Subcloning and Site-Directed Mutagenesis

A BAC clone (RPCI-11-82B23, BACPAC Resources, Children’s Hospital Oakland Research Institute) containing the human PKC-δ gene was digested with XhoI/EcoRV (New England Biolabs, Ipswitch, MA) to generate a 7.4 kb fragment containing the PKC-δ promoter and 3 kb of the PKC-δ gene. This was cloned into a pKS-Bluescript vector to generate pKS-PKCδ-Bac. Digestion of pKS-PKCδ-Bac with XhoI/SacII (New England Biolabs) generated a 4400 bp fragment that was subcloned into the firefly luciferase reporter pGL3-Basic vector (Promega) to generate pGL3-hPKCδ-4.4. The pGL3-hPKCδ-1.6 kb luciferase construct, containing 1648 bp of PKC-δ promoter, was generated by NheI/PstI digestion of the pGL3-PKCδ-4.4kb construct. The pGL3-hPKCδ-0.4kb luciferase construct, containing 415 bp of PKC-δ promoter, was generated by SacI digestion of the pGL3-PKCδ-4.4kb construct. To generate the pGL3-hPKCδ-4.4kb-NFκBMut vector, the NF-κB consensus binding site (-260) in the 4.4kb long PKC-δ promoter was mutated (CGGGGGAACC → CGAATTCACC) to generate an EcoRI site, using the Quickchange Site-Directed Mutagenesis kit (Stratagene, 200519) as per manufacturer instructions. The mutation was verified by EcoRI digestion and DNA sequencing.

2.6 Chromatin Immuno-Precipitation Assay
Chromatin Immuno-precipitation (ChIP) assay was performed on lysates from formaldehyde cross-linked cells using the EZ-ChIP kit (Millipore, 17-371) as per manufacturer instructions. Briefly, the cell were treated with 37% formaldehyde (final concentration 1%) for 10 mins at room temperature followed by 10X Glycine treatment to neutralize the unreacted formaldehyde for 5 mins at room temperature. Cells were then washed with ice-cold PBS and lysed in SDS Lysis Buffer (EZ-ChIP kit, Millipore, 17-371). To shear chromatin DNA the cross-linked cells were sonicated 8-10 times with 10 sec pulses in ice using an ultrasonic processor (GEX-130PB, 50% of maximum power). Crosslinked DNA:Protein were immunoprecipitated using Protein G agarose beads and respective antibodies (overnight incubation at 4°C) followed by a series of single washes with low-salt immune complex buffer, high-salt immune complex buffer, LiCl immune complex buffer and TE buffer as per manufacturer instructions. The DNA:Protein complexes were eluted by incubating the beads in elution buffer for 15 min at room temperature followed by reverse-crosslinking reaction by incubating the samples in 5M NaCl at 65°C for 6 hours. The DNA was then incubated with the binding reagent and purified using Spin filters provided in the kit. The PKC-δ promoter sequence was detected in precipitated DNA by performing qPCR using GeneAmp 5700 real time PCR system (Applied Biosystems). The sequences of the primers used for detection of the PKC-δ promoter region containing the -260bp NF-κB binding site are as follows:

Forward: AGCTCCAGCCAACAGGAA,
Reverse: GCATCCTCCGCACCCATTAG.
2.7 SiRNA Transfections

Cells were transfected with control siRNA (Qiagen, 1027281) or Fyn siRNA (Santa Cruz, SC-29321) using the Lipofectamine 2000 transfection reagent (Invitrogen, 11668-027) in antibiotics-free DMEM. Cells were plated at a density of 100,000 cells per well in a 6-well plate the day prior to transfection. The next day, 100 pmol of siRNA per well was combined with 250 µL of Opti-MEM serum free medium and mixed gently. Meanwhile, 5 µL per well of Lipofectamine 2000 was diluted in 250 µL Opti-MEM and incubated at room temperature for 5 min. After incubation, the diluted Lipofectamine 2000 solution was mixed with the diluted siRNA solution, and incubated at room temperature for 20 min. 500 µL of the Lipofectamine/siRNA solution was added to each well containing 1.5 mL of Opti-MEM. After overnight incubation, cells were washed and refed with normal media. 48 hrs after transfection, cells were analyzed by western blotting to verify successful knockdown.

2.8 RNA Isolation, mRNA Half-life Analysis and Reverse Transcriptase PCR

Total RNA was isolated using the PicoPure™ RNA Isolation Kit (Arcturus Biosciences, KIT0204) from LCM samples, and by Trizol (Gibco, 15596-018) from cultured cells. The amount of RNA isolated was on the order of 50-500 ng per sample. Complementary DNA was synthesized by reverse transcription of total isolated RNA (Superscript™ First Strand Synthesis, Invitrogen). The PKC-δ mRNA half-life analysis was performed by
treating the cells with Actinomycin-D (5 µg/ul) for 0-6 hours, followed by total RNA isolation and qRT-PCR analysis. Quantitative RT-PCR was performed using a GeneAmp 5700 sequence detection system (Applied Biosystems) with Platinum SYBR Green PCR reagents (Invitrogen, 11733-038). The GAPDH and Actin was used to normalize the expression levels. Relative mRNA expression was calculated using the ΔΔCt method. The primers used are as follows:

PKC-δ Forward: 5’-AAAGGCAGCTTCGGGAAGGT-3’,
PKC-δ Reverse: 5’-TGGATGTGGTACATCAGGTC-3’
Fyn Forward: 5’ CTCAGCACTACCCCAGCTTC-3’,
Fyn Reverse: 5’- ATCTCCTTCCGAGCTGTTCA-3’,
GAPDH Forward: 5’-GCACCGTCAAGGCTGAGAAC-3’,
GAPDH Reverse: 5’-GCCTTCTCCATGGTGTTGAA-3’.

2.9 Migration and Invasion Assays

Migration and invasion assays were performed using 24-well chambers with 8 µm FluoroBlok cell culture inserts (BD Biosciences, 351157). For invasion assays, inserts were pre-coated with Matrigel (1:3 dilution, BD Biosciences, 356234). Cells were pretreated with 10 µM CFDA-SE fluorescence tracer (Molecular Probes, V12883) for 30 minutes at 37° C and 2.5 x 10^5 cells were seeded in serum-free DMEM on the upper compartment of the FluoroBlok chambers. DMEM with 10% FBS was added to the lower compartment. Fluorescence of the cells in the lower side of the insert was
measured using a POLARstar Omega microplate reader (BMG Labtech) at 0, 24 and 48 hours using 485 nm excitation filter and 520 nm emission filter. Values shown are the mean ± SD.

2.10 Immunohistochemistry

Tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections (10 µm) were deparaffinized followed by antigen retrieval (10 mM citrate buffer, pH 6.0, microwave 500 W; 15 min). The samples were stained for PKC-δ (SC-937, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and HK-14 antibody (PRB-155P, Covance), by immunohistochemical methods using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). All human tissue samples were obtained with approval from the Loyola University Medical Center's Institutional Review Board. Immunohistochemistry was performed on tissue microarray (IMH-323, Imgenex Corp.) using purified mouse anti-Fyn (BD Transduction) as per the instructions in the Vectastatin ABC kit (Vector Laboratories).

2.11 Laser Capture Microdissection

LCM was performed using the PixCell II and Arcturus-XT apparatus (Arcturus Biosciences) as per manufacturer’s instructions. Formaldehyde-fixed, paraffin-embedded tissue sections (10 µm) were deparaffinized and stained with hematoxylin and eosin.
Stained sections were dehydrated in 100% xylene immediately before performing LCM. For each sample, LCM was performed on 8-10 tissue sections and isolated approximately 300-500 cells per section, the sections were pooled to yield ~3000-5000 cells per sample.

2.12 PCR-Based Gene Deletion Analysis

Total DNA was purified from LCM samples using the PicoPure™ DNA Isolation Kit (Arcturus Biosciences, KIT0103). The amount of DNA isolated was on the order of 10-50 ng per sample. Gene-specific primers were designed with identical annealing temperatures to amplify 100 bp regions of intron-1 in the PKC-δ gene or the GAPDH gene. qPCR was performed using a GeneAmp 5700 sequence detection system (Applied Biosystems) with Platinum® SYBR Green PCR reagents (Invitrogen). The initial denaturation was performed at 95°C for 10 min, followed by 50 cycles each consisting of denaturation at 95°C for 25 s, annealing at 56°C for 1 min and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. DNA was analyzed by qPCR from each LCM sample 3-4 times. A single 100 bp amplicon was confirmed by post-amplification dissociation curve analysis and by 3% agarose gel electrophoresis. DNA from each LCM sample was amplified in 3-4 independent qPCR reactions for quantitation. Relative PKC-δ gene levels were calculated by the ΔΔCt method.

The sequences of PKC-δ and GAPDH gene specific primers are as follows:

PKC-δ Intron 1 Forward: 5’-ACGAAGAAGGTCAGCAGCAGTTAG-3’,
PKC-δ Intron 1 Reverse: 5’-TGTCCCTACCTCCTCAACACT-3’,
GAPDH Forward: 5’-AGTGAGTGGAAGACAAGACATGGAA-3’,
GAPDH Reverse: 5’-CCATATTGAGGGACACAAGGT-3’.
PKCδ-Intron 16 Forward – 5’- TCAATCTTTAGCTGGGTATTTGC-3’
PKCδ-Intron 16 Reverse – 5’-TGAGTTCTAGGTCTCTGAAGCTG-3’
PKCδ-Exon 14 Forward – 5’- TACACATTCTCTGTGGACCTGGTG-3’
PKCδ- Exon 14 Reverse – 5’- CTCGAGAGTTCATCCTCATCAT -3’

2.13 Soft Agar Colony Formation Assay

The media/soft agar mixture was prepared by combining 40 mL of 2x DMEM with antibiotics, 10 mL FBS, and 40 mL of melted 44 °C, 1.25% Difco agar for a final concentration of 0.5% agar medium. 1 mL of prepared DMEM-agar medium was poured into each well of a 6-well plate and allowed to solidify at room temperature for 30 min. Meanwhile, cells were resuspended to a final concentration of 100,000 cells per mL of DMEM-agar medium, and 1 mL of this suspension was added on top of each well. Cells were then grown in soft agar over 14 days then stained using a 1 mg/mL solution of p-iodonitrotetrazolium violet (INT) staining to label metabolically active colonies. 500 µL of INT solution was added on top of each well, and plates were incubated at 37 °C overnight. Labeled colonies were counted using a bright field microscope.

2.14 SFK IP-Kinase Assay
500 μg of protein extract was immunoprecipitated with Fyn (sc-16) or Src (sc-19) specific antibodies using protein A/G Sepharose beads (Santa Cruz Biotech. Inc.). The immunoprecipitates were washed once with lysis buffer (D’Costa et al., 2006), twice with assay buffer (100 mM Tris HCl pH 7.2, 125 mM MgCl₂, 2 mM EGTA, 250 μg/μl BSA) and incubated with 300 μM of Src/Fyn substrate peptide (Millipore) in the presence of 1 μCi {γ-³²P}-ATP for 5 or 20 minutes at 30°C, as indicated. After the enzyme reaction, the beads were spun and 25 μl of supernatant spotted onto P81 phosphocellulose filters and washed 3 times with 0.45% phosphoric acid prior to scintillation counting.
CHAPTER III

LOSS OF PKC-δ GENE EXPRESSION IN HUMAN SCC

3.1 Abstract

Loss of tumor suppressor function, either by gene deletion, transcriptional repression, increased mRNA or protein turnover, or by inhibition of protein activity, is necessary for the progression of human cancers. PKC-δ has been implicated as a tumor suppressor in both human and mouse SCCs (D'Costa et al., 2006; Reddig et al., 1999). Previous studies have found that PKC-δ protein is reduced in ~30% of human SCCs, as well as the majority of chemically and UV-induced mouse skin tumors (D'Costa et al., 2006; Reddig et al., 1999; Aziz et al., 2006). The mechanism of human PKC-δ down-regulation/inactivation in human SCCs is unknown, and may involve regulation at multiple levels. In this study, we identified transcriptional repression, not gene deletion as is common for many tumor suppressor genes, as the mechanism of PKC-δ loss in human cutaneous SCCs (Yadav et al., 2010).
3.2 Laser Capture Microdissection and RNA analysis

Definitive understanding of the mechanism of tumor suppressor gene loss is complicated by tumor heterogeneity and the presence of other cell types within and adjacent to the tumor which may express the tumor suppressor gene at high levels. To determine if PKC-δ gene expression is reduced in human SCCs, we used Laser Capture Microdissection (LCM) to isolate epithelial cells from the epithelial compartment of 3 normal human skins and 14 human SCC samples. The selected SCCs all had reduced PKC-δ protein as determined by immunohistochemistry (D'Costa et al., 2006). LCM was used to exclude stromal cells, inflammatory cells and other normal cells, which can express high levels of PKC-δ, allowing us to isolate individual cancerous cells for expression studies (Espina et al., 2006). Histological analysis of the 14 human SCC samples was performed in order to identify the tumor regions and non-tumor regions (Figure 5). All tumor biopsies were confirmed as SCCs with a highly variable histology, ranging from invasive to well-differentiated. Parallel immunohistochemical staining was carried out for PKC-δ and an epithelial marker (Keratin-14) as shown in Figure 5. We identified regions of Keratin-14 positive SCC cells with reduced PKC-δ staining compared to normal epidermis (Figure 5).

We performed LCM to specifically isolate SCC cells and normal epidermal cells from tumor sections and control normal skin sections, respectively (Figure 6A). For every SCC and normal epidermis sample, ~10^4 cells were captured, total RNA was isolated, and
PKC-δ mRNA was quantitated by qRT-PCR. To ensure the integrity of the PKC-δ mRNA was preserved and the specificity of the PCR, we performed PKC-δ RT-PCR from total RNA isolated from a normal epidermis, collected by LCM, in presence and absence of reverse transcriptase. A single, specific RT-PCR product of the expected ~260 bp was obtained only in the presence reverse transcriptase, thus ruling out genomic DNA contamination (Figure 6B).
FIGURE 5: Loss of PKC-δ protein in human SCCs

Hematoxylin and eosin staining of normal human skin (NN363) and human SCC (case 234) is shown on top. PKC-δ staining of normal human epidermis and human skin SCCs by immunohistochemistry is shown in the middle row. Keratin-14 staining of sections of normal human epidermis and human skin SCCs is shown at the bottom. Keratin-14 staining was used as an epithelial marker to differentiate between cells of epithelial and non-epithelial origin. Scale bars denote 30 μm.
FIGURE 6: Laser Capture Microdissection and RNA Isolation

A. Isolation of pure SCC and normal epidermis cells by LCM. The tissue before and after LCM are shown. The isolated region containing normal epidermal cells and SCC cells on the cap are shown. B, Detection of PKC-δ RNA. RNA was purified from normal human epidermis isolated by LCM. The integrity and specificity of the PKC-δ RNA sample was verified by performing RT-PCR in presence and absence of reverse-transcriptase as shown. The predicted 260 bp PCR fragment was obtained upon performing PKC-δ RT-PCR in the presence of reverse transcriptase.
3.3 PKC-δ mRNA is Lost in Human SCCs

We analyzed a total of 14 human SCC clinical samples and 3 normal epidermises for PKC-δ RNA levels. The PKC-δ RNA levels were normalized to GAPDH RNA levels for each tumor and normal epidermis sample as determined by qRT-PCR (Figure 7). PKC-δ mRNA was undetectable or reduced in all the tumors analyzed compared to normal epidermises (Figure 7). On an average, PKC-δ RNA levels were reduced by 90% in human SCCs compared to normal epidermises. One SCC (sample 158) had PKC-δ RNA levels reduced only 20% below the lowest PKC-δ expressing normal epidermis (NN364) despite having almost undetectable PKC-δ protein by immunohistochemistry. Thus post-transcriptional mechanism may contribute to the reduced PKC-δ protein levels in some human SCCs. In order to rule out the possibility that GAPDH expression might itself be deregulated in SCCs, we confirmed our findings by using Actin normalization on 6 tumors and one normal human epidermis. A similar reduction in PKC-δ mRNA was observed upon normalization against Actin RNA levels in the LCM samples (Figure 8). Thus, human SCCs with low PKC-δ protein have reduced PKC-δ mRNA levels, suggesting either gene deletion/loss or transcriptional silencing.
FIGURE 7: Quantitation of PKC-δ mRNA in human SCCs

PKC-δ RNA is reduced in human SCC cells compared to normal human epidermis. Keratinocytes from 14 human SCC samples and 3 normal epidermises were isolated by LCM and analyzed for PKC-δ RNA by qRT-PCR. The PKC-δ RNA levels were normalized to the GAPDH RNA for each sample. Relative mRNA expression was calculated using the ΔΔCt method.
FIGURE 8: Normalization of PKC-δ RNA levels with Actin levels in human SCCs

In order to confirm that loss of PKC-δ is not due to a relative changes in GAPDH, we re-analyzed 6 tumors and one normal epidermis for relative PKC-δ and Actin mRNA levels. PKC-δ and Actin RNA levels were quantitated in human SCC and normal skin sections as described in materials and methods section. The PKC-δ RNA levels were normalized to the Actin RNA levels for selected tumor and normal epidermis samples. Relative mRNA expression was calculated using the ΔΔCt method.
3.4 PKC-δ Gene Deletion Analysis

PKC-δ resides at 3p21.31 and the 3p region has been reported to be deleted in many cancers including cutaneous SCC (Sikkink et al., 1997). Thus loss of PKC-δ by gene deletion in human SCCs is a possible mechanism for reduced PKC-δ protein and mRNA levels. To determine the status of PKC-δ gene, we extracted genomic DNA from LCM-isolated keratinocytes from the same 14 human SCC samples and 4 normal epidermises and performed qPCR based gene deletion analysis. Only 9 of the 14 tumors were informative for the genomic DNA analysis. We found that PKC-δ gene was present in 8 out of 9 tumors, while the GAPDH fragment was detected and reproducibly amplified in all samples (Figure 9). Partial gene deletion or deletion of promoter region is also a possibility that could account for reduced PKCδ RNA in the PKC-δ deficient SCCs. To address the partial gene deletion, we amplified fragments of Intron 16 and Exon 14 near the 3’-end of PKC-δ gene from isolated SCC cells and confirmed that that full length PKC-δ gene was intact in the five randomly selected tumors analyzed (Figure 10). These findings suggest that the mechanism of down-regulation of PKC-δ in SCCs is likely to be primarily at the level of gene transcription and not gene deletion.
FIGURE 9: Analysis of PKC-δ Gene in human SCCs

PCR-based gene deletion assay was performed on cells isolated by LCM from 9 human SCCs and 4 normal human epidermises. PKC-δ gene fragment was detected by specific primers for a 100 bp region inside intron 1 as described in “Material and Methods”. GAPDH gene fragment was amplified to normalize for DNA integrity and amount. The sample numbers are shown on the x-axis, and the relative PKC-δ gene levels are indicated above each bar. Error bars denote standard deviation for 3-4 independent qPCR reactions. Products from a representative qPCR reaction for PKC-δ and GAPDH were run on agarose gels and are shown below the bar graph. Note that only one SCC (205) had a deletion in the PKC-δ gene.
FIGURE 10: PKCδ Gene deletion analysis using alternative primers

PKC-δ gene fragments were detected by using specific primers designed to amplify 100 and 102 bp regions inside exon 14 and intron 16 respectively. GAPDH gene fragment was amplified to normalize for DNA integrity and amount. Intron 16 and exon 14 fragments were chosen in order to detect partial gene deletions towards the 3’-end of the gene. The tumors samples are shown on the x-axis, and the relative PKC-δ gene levels are indicated above each bar. Error bars denote standard deviation for 3 independent qPCR reactions.
3.5 PKC-δ mRNA is Reduced in Ras-Transformed Cells

Ras activation has been shown to down-regulate PKC-δ protein and RNA in human keratinocytes (Geiges et al., 1995; D'Costa et al., 2006) and activation of Ras oncogene was found in 58% of human SCCs (Pierceall et al., 1991). Furthermore, EGFR activation correlates with PKC-δ protein down-regulation in human SCCs, supporting an inverse relationship between the EGFR/Ras pathway and PKC-δ (D'Costa et al., 2006). To explore the potential transcription mechanism of PKC-δ down-regulation in human SCCs, we used the HaCaT cell line and Ras transformed HaCaT cells (HaCaT Ras II-4 & HaCaT-Ras I-7) (Henseleit et al., 1997; Breitkreutz et al., 1991). HaCaT cells are spontaneously immortalized human KCs but are non-tumorigenic when transplanted into nude mice (Lehman et al., 1993). In addition, HaCaT cells display normal KC differentiation and express differentiation markers such as K1, K10, involucrin and filaggrin (Boukamp et al., 1988). Thus, HaCaT cells serve as a good model system to study KC biology. The tumorigenic HaCaT Ras II-4 and HaCaT Ras I-7 cells have been shown to have reduced PKC-δ protein levels and their tumorigenecity is significantly inhibited by re-expression of PKC-δ (D'Costa et al., 2006). We analyzed PKC-δ RNA levels in HaCaT Ras II-4 and HaCaT Ras I-7 by qRT-PCR and confirmed that PKC-δ RNA is reduced relative to HaCaT cells (Figure 11).

3.6 PKC-δ mRNA Stability is Not Reduced in Ras-Transformed Cells
To investigate if PKC-δ mRNA stability is being regulated by Ras, we estimated the half-life of PKC-δ mRNA in HaCaT-Ras cells relative to the control HaCaT cells. We performed the PKC-δ mRNA half-life analysis by treating the cells with Actinomycin-D (5 µg/ul) for 0-6 hours, followed by total RNA isolation and qRT-PCR analysis. We found that the half-life of the PKC-δ mRNA was 2.6 hours in HaCaT cells and 4 hours in HaCaT-Ras cells, indicating that regulation is PKC-δ mRNA is not responsible for loss of PKC-δ gene expression as Ras does not increase PKC-δ mRNA turnover (Figure 12).
FIGURE 11: PKC-δ RNA is Reduced in Ras transformed HaCaT Cells

Total RNA was isolated from HaCaT, HaCaT Ras I-7 and HaCaT Ras II-4 cells and analyzed for PKC-δ RNA by qRT-PCR. Shown is the mean and standard deviation from experiments performed in triplicate. *p<0.001.
FIGURE 12: PKC-δ mRNA half-life in Ras transformed HaCaT cells

Cells were treated with 5 µg/ul of Act-D for the indicated times. Total RNA isolation, cDNA synthesis and qPCR amplification was performed as described in the text. The level of PKC-δ mRNA at each time point was calculated relative to untreated HaCaT cells and plotted on a semi-log scale. Exponential curve fitting was used to calculate the half-life from the slope of the curve using T1/2=\{-0.693/K\} formula. Shown is mean of two independent replicates at each time point.
3.7 PKC-δ Promoter Activity is Down-Regulated upon Ras and EGFR Activation

We then performed PKC-δ promoter reporter assays in HaCaT and Ras-transformed HaCaT cells using 4.4 kb of the human PKC-δ promoter. We found that PKC-δ promoter activity was significantly (p<0.001) reduced in HaCaT I-7 and HaCaT Ras II-4 compared to control HaCaT cells (Figure 13). We also found that PKC-δ reporter activity is decreased in HaCaT cells that were treated with EGF or actively transduced with a Ha-Ras (G12V) retrovirus (Figure 14). The EGFR inhibitor AG1478 (500 nM) was able to significantly attenuate the suppression of PKC-δ reporter activity by EGF (Figure 14). However, AG1478 (500 nM) failed to block PKC-δ repression in HaCaT-Ras cells (data not shown), thus suggesting that the Ras/MAPK/TGF-α/EGFR autocrine activation is not a possible mechanism of PKC-δ repression. These results suggest that transcriptional repression of PKC-δ might be responsible for down-regulation of PKC-δ in human SCCs as well (Yadav et al., 2010). However, reporter analysis does not rule out the potential epigenetic mechanisms such as promoter methylation or chromatin remodeling which could also explain loss of PKC-δ gene expression.
FIGURE 13: PKC-δ promoter activity is down-regulated in Ras transformed HaCaT cells

pGL3-Basic or pGL3-hPKCδ-4.4 was transiently co-transfected with pRL-TK into HaCaT and HaCaT-Ras cells. After 48 hours, luciferase activity in cell lysates was measured. Shown is the mean and standard deviation from experiments performed in triplicate. *p<0.001.
HaCaT cells were retrovirally transduced with c-Ras\textsuperscript{Ha} (G12V) to obtain an independent HaCaT-Ras cell line, and PKC-δ promoter activity was measured by dual-luciferase assay. HaCaT cells were treated with EGF (15 ng/ml) for 48 hours in presence and absence of AG1478 (EGFR inhibitor, 500 nM). After 48 hours, luciferase activity in cell lysates was measured. Shown is the mean and standard deviation from experiment performed in triplicate. Similar results were obtained in 2 additional experiments. Student t-test was performed on ‘#’ group, p<0.05. Bonferroni corrected t-test was performed on * group p<0.025, EGF, Ras vs control.

**FIGURE 14:** PKC-δ promoter activity is repressed in HaCaT cells upon EGF treatment or expression of constitutively active c-Ras\textsuperscript{Ha} (G12V)
CHAPTER IV

REPRESSION OF PKC-δ GENE EXPRESSION BY RAS AND NF-κB SIGNALING

4.1 Abstract

Ras signaling is strongly linked to the loss of PKC-δ gene expression (Yadav et al., 2010; Geiges et al., 1995). Furthermore, Ras regulates the expression of many pro-survival and tumor suppressor genes via activation of transcription factors such as Ets, AP-1 and NF-κB (Finco et al., 1997; Mavrothalassitis and Ghysdael, 2000). However, the transcription factor signaling responsible for the loss of PKC-δ promoter repression by Ras is not known. Here we show that constitutively active NF-κB signal is responsible for down-regulation of tumor suppressor PKC-δ gene expression by Ras.

4.2 PKC-δ Promoter Activity is Down-Regulated by Ras

To study the mechanism responsible for the loss of PKC-δ gene expression in transformed keratinocytes, we generated a stable HaCaT-Ras cell line through retroviral transduction of HaCaT cells with mutant H-Ras (G12V) (Figure 16A). We confirmed the transformed phenotype of HaCaT-Ras cells by morphology and increased soft agar colony formation (Figure 16B). To explore the potential mechanism responsible for the
loss of PKC-δ gene expression by Ras, we performed deletion of the 4.4 kb region of the PKC-δ promoter and generated 1.6 kb and 0.4 kb long PKC-δ promoter fragments for reporter analysis (Figure 15). Reporter analysis of deletion constructs revealed that the 400 bp region upstream of human PKC-δ exon1 was sufficient for the down-regulation of the PKC-δ promoter by Ras (Figure 17A). TFSEARCH analysis of the human PKC-δ promoter identified many potential transcription factor binding sites (such as NF-κB, c-Ets, AP-1) that could be regulated by Ras (Figure 15) (Heinemeyer et al., 1998). We investigated if the NF-κB binding motifs located in the minimal 400 bp region are involved in Ras-mediated repression of the PKC-δ promoter. Site-directed mutagenesis of the potential NF-κB binding motif, located 260 bp upstream of the PKC-δ gene, resulted in 20-fold up-regulation of PKC-δ promoter activity in HaCaT-Ras cells but not in HaCaT cells (Figure 17B).
Potential transcription factor binding sites are shown on the 4.4 kb region of human PKC-δ promoter, as predicted by the TFsearch program. Also, schematic representation of the 4.4 kb, 1.6 kb and 0.4 kb fragments of PKC-δ promoter is shown. The 1.6 kb and 0.4 kb promoter fragments were generated by restriction digestion of the parent 4.4 kb fragment as described in “Material and Methods”.
FIGURE 16: Generation of HaCaT-Ras Cells

A. HaCaT cells were retrovirally transduced with active H-Ras G12V to generate stable HaCaT-Ras cells. Morphology of HaCaT and HaCaT-Ras is shown at 10X magnification. B, Ras induces soft agar colony formation in HaCaT cells. Survival and growth of HaCaT and HaCaT-Ras cells was determined as described in Material and Methods. Soft agar colonies were scored at 2 weeks in triplicate samples. Shown is the mean and SD from experiments performed in triplicates. *P<0.001.
FIGURE 17: Ras Down-regulates PKC-δ Promoter Activity via NFκB

A. 400 bp promoter region upstream of PKC-δ gene is sufficient for Ras-mediated repression of PKC-δ promoter. pGL3-Basic, pGL3-hPKCδ-4.4kb, pGL3-hPKCδ-1.6kb or pGL3-hPKCδ-0.4kb were transiently co-transfected with pRL-TK into HaCaT and HaCaT-Ras cells, and luciferase activity was determined as described in Material and Methods. B, Mutation of the putative NF-κB binding site rescues PKC-δ repression in HaCaT-Ras cells. Potential NF-κB binding motif, located ~260 bp upstream of PKC-δ exon 1, was mutated in the pGL3-hPKCδ-4.4kb luciferase reporter construct. pGL3-hPKCδ-4.4kb with or without the mutant NF-κB binding site was transiently co-transfected with pRL-TK into HaCaT and HaCaT-Ras cells, and luciferase activity was determined as described in “Material and Methods”. Shown are the mean and SD from experiments performed in triplicates. *P<0.01.
4.3 NF-κB Signaling is Constitutively Activated in HaCaT-Ras Cells

NF-κB transcription factors are sequestered in the cytoplasm by the inhibitory IκB-α protein. However, in response to activating stimuli (such as active Ras, TNF-α, UV radiation), IκB-α is phosphorylated and subsequently degraded resulting in NF-κB activation (Gilmore et al., 1996). To confirm NF-κB activation by Ras, we analyzed the total IκB-α protein levels and detected reduced IκB-α in HaCaT-Ras cells compared to control HaCaT cells (Figure 18A). Furthermore, we investigated the transcriptional activity of NF-κB by using a NF-κB-driven luciferase reporter. HaCaT-Ras cells exhibited dramatic induction of NF-κB-driven promoter activity compared to HaCaT cells, indicating a constitutive activation of NF-κB signaling by Ras (Figure 18B).
**FIGURE 18: Constitutive Activation of NF-κB in HaCaT-Ras Cells**

* A, IκBα protein levels are reduced in HaCaT-Ras cells. Western blot displaying the IκB-α protein levels in HaCaT and HaCaT-Ras cells is shown. Tubulin is shown as a loading control. *B*, HaCaT and HaCaT-Ras cells were transiently co-transfected with NF-κB driven- luciferase vector pNF-κB-LUC and control plasmid pRL-TK, and luciferase activity determined as described in “Material and Methods”. Shown are the mean and SD from experiments performed in triplicates. *P*<0.01.
4.4 Ras Induces Recruitment of p50/c-Rel NF-κB Subunits to the PKC-δ Promoter

NF-κB transcription factors are comprised of multiple subunits (p50, p65, c-Rel, p52 and RelB) that dimerize and bind to the target promoter sequence, and regulate gene expression by recruiting co-repressors (e.g. HDAC) or co-activators (e.g. p300) (Gilmore et al., 1996; Matthews and Hay, 1995; Elsharkawy et al., 2010). To determine the specific NF-κB subunit(s) responsible for PKC-δ repression, we performed chromatin immunoprecipitation (ChIP) using PCR primers that flank the NF-κB site at -260 and found that p50 and c-Rel are specifically recruited to PKC-δ promoter by Ras (Figure 19A and 19B). This is consistent with the previous reports that c-Rel and p50 function as repressors of gene expression in many cells (Fu et al., 2009; Plaksin et al., 1993; Cao et al., 2006). NF-κB subunit p65, a classic transcriptional activator, was not recruited to the PKC-δ promoter in the HaCaT-Ras cells (Figure 19B). The binding of p52 to the PKC-δ promoter was not different between HaCaT and HaCaT-Ras cells. Acetyl-H3 binding to constitutively active GAPDH promoter was measured as a positive control, and did not change.
FIGURE 19: Ras Induces Recruitment of p50/c-Rel NF-κB Subunit to PKC-δ Promoter

ChIP assay was performed on lystaes from formaldehyde crosslinked HaCaT and HaCaT-Ras cells using specific antibodies against the indicated proteins. Precipitated PKC-δ promoter DNA was detected by regular PCR (A), and quantified by qPCR (B). Precipitation of transcriptionally active GAPDH promoter by anti-Acetyl-H3 antibody is shown as a positive control.
CHAPTER V

FYNS IS CRITICAL FOR RAS-MEDIATED NF-κB ACTIVATION AND PKC-δ REPRESSION

5.1 Abstract

SFKs have been linked to the loss of PKC-δ activity and are activated in Ras-transformed cells (Denning et al., 1996; Blake et al., 1999; Joseloff et al., 2002). However, the role of SFKs in regulation of PKC-δ gene expression has not been reported. Here we show that the SFK member Fyn is required and sufficient for NF-κB activation and PKC-δ repression by Ras.

5.2 SFK Activity is Required for NF-κB Activation and PKC-δ Repression by Ras

We explored whether SFK activity is required for repression of the PKC-δ promoter activity by Ras. The SFK inhibitor PP1 significantly blocked the repression of PKC-δ promoter in HaCaT-Ras cells, indicating that SFK activity is required for reduced PKC-δ promoter activity in HaCaT-Ras cells (Figure 20A). SFKs can activate NF-κB signaling via tyrosine phosphorylation of many regulators of NF-κB signaling, such as NIK, IKK and IκB-α, subsequently targeting IκB-α for proteosomal degradation (Fan et al., 2003; Huang et al., 2003). We further explored the mechanism of PKC-δ repression in HaCaT-
Ras cells by examining if SFK activity is required for constitutive NF-κB activation by Ras. Total IκB-α protein levels were increased by PP1 treatment of HaCaT-Ras cells (Figure 20B), indicating that SFK activity is required for Ras induced IκBα loss.

### 5.3 Expression of Fyn, and not Src, is induced by Ras

To identify if SFK activity is being regulated by Ras, we measured c-Src and c-Fyn kinase activity in HaCaT-Ras cells. We found that HaCaT-Ras cells had elevated Fyn enzymatic activity, as determined by immunoprecipitation kinase assays (Figure 21B). H-Ras did not increase the activity of Src (Figure 21C), indicating that the effects of H-Ras were relatively selective for Fyn. Figure 21A shows that both HaCaT-Ras cells and the independently generated HaCaT Ras II-4 cells also had elevated levels of Fyn protein relative to HaCaT cells.
A. SFK activity is required for repression of PKC-δ promoter activity. HaCaT and HaCaT-Ras cells were transiently co-transfected with pGL3-hPKCδ-4.4kb and pRL-TK vector, and left untreated or treated with PP1 (10 µM). After 48 hours, luciferase activity was determined as described in Material and Methods. Shown is the mean and SD from experiments performed in triplicates. *P<0.01. B, SFK activity is required for the proteosomal degradation of IkB-α by Ras. HaCaT and HaCaT-Ras cells were untreated or treated with PP1 (10 µM). After 48 hours, IkB-α protein levels were examined by western blotting as shown.
FIGURE 21: Active Ras up-regulates Fyn protein levels

A, Fyn protein levels were examined in HaCaT, HaCaT-Ras, and HaCaT Ras II-4 cells using western blotting. Tubulin levels are shown as a loading control. B and C, Activity of Fyn and Src was analysis by immunoprecipitation-kinase assay using SFK substrate peptide for either 5 or 20 mins as described in “Material and Methods”.
5.4 Fyn is Required for NF-κB Activation by Ras

We explored if Fyn is required for NF-κB activation in HaCaT-Ras cells, by specific knockdown of Fyn by siRNA (Figure 22). IκB-α protein levels were stabilized in HaCaT-Ras cells upon Fyn knockdown or SFK inhibition with PP2 (Figure 22). Knockdown of Fyn did not affect the protein levels of Src in HaCaT and HaCaT-Ras cells. This indicates that Fyn is required for the constitutive activation of NF-κB signaling by Ras.

5.5 Fyn is Sufficient for HaCaT Cell Transformation

To determine if Fyn activity is sufficient for PKC-δ repression, we generated a stable HaCaT-Fyn cell line by retroviral transduction of HaCaT cells with constitutively active Fyn (I338T). HaCaT-Fyn cells developed significantly higher number of colonies in soft agar as compared to HaCaT cells and appeared morphologically transformed (Figure 23). Treatment of the HaCaT-Ras and HaCaT-Fyn cells with PP2 or LY294002 (PI3K/Akt inhibitor) dramatically inhibited their colony forming capacity compared to the untreated cells (Figure 23). This indicates that activation of Fyn or expression of Fyn via PI3K signaling is required for the transformed phenotype of HaCaT cells in culture.
FIGURE 22: Fyn is necessary for the degradation of IκB-α by Ras

HaCaT and HaCaT-Ras cells were treated with control siRNA, Fyn specific siRNA or PP2 (10 μM) for 48 hours. Levels of the indicated proteins were analyzed by western blotting as shown.
Induction of Fyn is necessary and sufficient for the transformation of HaCaT cells.

Fyn induces soft agar colony formation in HaCaT cells. HaCaT, HaCaT-Fyn and HaCaT-Ras cells were untreated or treated with PP2 (10 μM), or LY294002 (20 μM) assessed for survival and growth on soft-agar as described in Material and Methods. Soft agar colonies were scored at 2 weeks in triplicate samples. Shown is the mean and SD from experiments performed in triplicates. T-Test was performed on the indicated groups (*, #), p<0.01.
5.6 Fyn is Sufficient for Repression of PKC-δ Promoter by Ras

Furthermore, we evaluated the PKC-δ promoter activity in the HaCaT-Fyn cells and found that PKC-δ promoter activity was significantly down-regulated in HaCaT-Fyn cells as compared to HaCaT cells (Figure 24). Thus Fyn activity is sufficient for PKC-δ promoter repression in HaCaT cells. In addition, mutation of the NF-κB binding site on the PKC-δ promoter significantly increased PKC-δ promoter activity in HaCaT-Fyn cells (Figure 24). Further analysis of the PKC-δ promoter revealed that Fyn is sufficient to induce specific recruitment of p50 and c-Rel to the PKC-δ promoter in the HaCaT cells (Figure 25A and 25B). Thus Fyn, like Ras, mediates PKC-δ promoter repression via NF-κB activation and recruitment of repressive p50/c-Rel subunits to the PKC-δ promoter.
FIGURE 24: Fyn is sufficient for the PKC-δ promoter repression

Fyn represses PKC-δ promoter via constitutive NF-κB signaling. pGL3-hPKCδ-4.4kb vector with or without the mutant NF-κB binding site was transiently co-transfected with pRL-TK into HaCaT, HaCaT-Ras and HaCaT-Fyn cells, and luciferase activity was determined as described in Material and Methods. Shown is the mean and SD from experiments performed in triplicates. T-Test was performed on the indicated groups (*, #), p<0.01.
FIGURE 25: Fyn is sufficient for recruitment of p50 and c-Rel to the PKC-δ promoter

A & B, ChIP assay was performed on lystaes from formaldehyde crosslinked HaCaT, HaCaT-Ras and HaCaT-Fyn cells using specific antibodies against c-Rel and p50. Precipitated PKC-δ promoter DNA was detected by qPCR as described in Material and Methods section.
CHAPTER VI

FYN IS INDUCED BY RAS/PI3K/AKT SIGNALING AND IS REQUIRED FOR ENHANCED INVASION/MIGRATION

6.1 Abstract

Src family kinases (SFKs) are frequently over-expressed and/or activated in human cancers, and play key roles in cancer cell invasion, metastasis, proliferation, survival and angiogenesis. Allosteric activation of SFKs occurs through well-defined post-translational mechanisms, however the SFK member Fyn is over-expressed in multiple human cancers (prostate, melanoma, pancreatic, glioma, chronic myelogenous leukemia) and the mechanism of increased Fyn expression is unclear. We have shown that Ras can induce Fyn expression, and Fyn is necessary and sufficient for NF-κB activation and PKC-δ repression (Chapter V). However, the mechanism responsible for Ras-mediated Fyn induction is not clear. Here we show that the Ras/PI3K/Akt pathway can account for Fyn over-expression in cancers, and Fyn is a critical mediator of the Ras-stimulated invasive cell phenotype.
6.2 Mechanism of Fyn induction

We evaluated if either of two major Ras effector pathways (Raf/MEK/ERK, PI3K/Akt) were involved in the induction of Fyn by H-Ras. Figure 26A shows that HaCaT-Ras cells had elevated P-ERK1/2 and P-Akt1 relative to HaCaT cells. The over-expression of Fyn in HaCaT-Ras cells was inhibited by the PI3K inhibitor LY294002, but not the MEK1/2 inhibitor U0126 (Figure 26B). Oncogenic H-Ras also mediates some of its effects by activating EGFR via autocrine EGFR ligand production (Dlugosz et al., 1997). H-Ras induced a slight increase in P-EGFR, however, the EGFR inhibitor AG1478 was not able to reduce Fyn levels in HaCaT-Ras cells despite inhibiting P-EGFR levels. Note the PI3K and MEK1/2 inhibitors also inhibited P-EGFR, suggesting a role for these Ras-effector pathways in autocrine EGFR ligand production. Thus, the induction of Fyn by H-Ras appears to require PI3K/Akt signaling.
FIGURE 26: PI3K Signaling is Required for Induction of Fyn Protein by Ras

A. HaCaT and HaCaT-Ras cells were evaluated for induction of indicated Ras effector pathways by western blotting. Increases in P~ERK1/2, P~Akt1 (S473) and P~EGFR (Y1068) are shown. B, HaCaT-Ras cells were treated with the PI3K inhibitor LY294002 (20 μM), MEK inhibitor (U0126, 10 μM) and EGFR inhibitor (AG1478, 5 μM) for 48 hours and Fyn protein levels were examined by western blotting. Protein levels of Fyn, P~Akt1 (S473), total Akt, P~EGFR (Y1068), and Actin are shown.
6.3 Activation of PI3K/Akt Signaling is Required for Induction of Fyn mRNA

We further explored the mechanism of Fyn induction by examining Fyn mRNA levels. Figure 27A shows that Fyn mRNA was undetectable in HaCaT cells, but was strongly up-regulated in HaCaT-Ras cells. The PI3K inhibitor LY294002 was able to inhibit the over-expression of Fyn in HaCaT-Ras cells at both the protein and mRNA level, and inhibited H-Ras-induced P–Akt1 (S473) levels (Figures 26B and 27A). Quantitation of Fyn mRNA by qRT-PCR in Figures 27B and 27C showed dramatic induction of Fyn mRNA levels by H-Ras and >97% inhibition by LY294002. Transduction of HaCaT cells with a virus expressing constitutively active Akt induced Fyn mRNA levels (Figure 27C). Taken together, these results demonstrate that PI3K/Akt signaling is necessary and sufficient for induction of Fyn expression by H-Ras.
FIGURE 27: PI3K/Akt signaling is required for the induction of Fyn mRNA by Ras

A, HaCaT-Ras cells were treated with the PI3K inhibitor LY294002 (20 µM) for 48 hours and Fyn mRNA levels were examined by RT-PCR. Total Akt1 and P~Akt1 (S473) levels were also examined by western blotting after 48 hours of LY294002 treatment. B, Akt activation is necessary and sufficient for Fyn mRNA induction. HaCaT and HaCaT-Ras cells were transduced with either constitutively active Akt or treated with LY294002 (20 µM). After 48 hours, Fyn mRNA levels were analyzed by qRT-PCR normalized to GAPDH. Data is represented as mean ± SD from a representative experiment performed in triplicate. The numbers over each bar represent relative Fyn mRNA levels relative to untreated HaCaT cells.
6.4 Role of Fyn in Ras-Mediated Increased Migration and Invasion

Many oncogenes, especially SFKs such as Fyn, are able to promote tumor cell migration and invasion. We therefore measured migration and invasion through Matrigel of HaCaT cells and HaCaT cells transduced with either H-Ras or active Fyn. Over-expression of Fyn was confirmed in both HaCaT-Ras and HaCaT-Fyn cells by western blot (Figure 28A). Figures 28B and 28C show that both H-Ras and Fyn induced significant migration and invasion of HaCaT cells over 24-48 hours. To test if Fyn was required for the increased migration and invasion of HaCaT-Ras cells, Fyn was knocked-down with siRNA (Figure 30). Figure 30 shows that both migration and invasion were significantly (p<0.01) inhibited by Fyn knock-down. The SFK inhibitor PP2 also significantly (p<0.01) inhibited migration of HaCaT-Ras and HaCaT-Fyn cells (Figure 29).
FIGURE 28: Fyn is sufficient for Ras-induced migration and invasion of HaCaT cells

A, Fyn protein levels in HaCaT, HaCaT-Ras and HaCaT-Fyn cells were determined by western blotting. Protein levels of α-Tubulin are shown as a loading control. B and C, The indicated cells were pretreated with CFDA fluorescence tracer and plated on FluoroBlok inserts without (B) or with (C) Matrigel coating. Migration and invasion were measured at 0, 24 and 48 hours. T-test was performed on the indicated groups (*, #), p<0.01.
FIGURE 29: SFK Activity is Required for Ras-induced migration of HaCaT cells

A. Src family kinases are essential for increased migration by Ras: Cells were treated with the SFK inhibitor (10 μM), and migration was measured after 24, respectively. After 24 hours, cells in the upper chamber were scraped off, and cells migrating to the lower chamber were fixed with 10% formalin and stained with hematoxylin. B, Cells in the lower side of the filter were counted and the numbers plotted graphically. Data is represented as mean ± SD from a representative experiment performed in triplicate. T-test was performed on the indicated groups (*, #), p<0.01.
FIGURE 30: Fyn is Necessary for Ras-induced migration and invasion of HaCaT cells

HaCaT-Ras cells were transfected with either control or Fyn specific siRNA, and migration and invasion were measured after 24 and 48 hours, respectively. Data is represented as mean ± SD from a representative experiment performed in triplicate. T-test was performed on the indicated groups (*, #), p<0.01.
6.5 Fyn is Necessary and Sufficient for FAK Activation by Ras

FAK is located at cell-matrix adhesions and plays a key role in cell migration and proliferation (Westhoff et al., 2004). FAK is over-expressed in many cancers including human SCCs and is activated by SFKs (Agochiya et al., 1999). Upon activation by SFK, FAK undergoes auto-phosphorylation at Tyrosine 397 (Westhoff et al., 2004). We explored if FAK is over-expressed and/or activated in HaCaT-Ras cells by analyzing total FAK and pY397-FAK protein levels. Interestingly, we found FAK is activated, but not overexpressed in HaCaT-Ras cells compared to HaCaT cells (Figure 31A). Furthermore, we evaluated if Fyn was responsible for the activation of FAK by H-Ras. FAK became auto-phosphorylated (Y397) in both HaCaT-Ras and HaCaT-Fyn cells (Figure 31A), indicating that Fyn is sufficient for FAK activation in HaCaT cells. Furthermore, inhibition of SFK activity with PP2 or knockdown of Fyn with siRNA inhibited FAK auto-phosphorylation by H-Ras (Figure 31B). These results indicate that Fyn is necessary and sufficient for activation of FAK by active-H-Ras.
FIGURE 31: Fyn is necessary and sufficient for FAK activation by Ras

A, Cells were treated with the Src family kinase inhibitor (PP2, 10 µM) for 48 hours and P-FAK (Y397) and total FAK levels examined by western blotting. Levels of Actin are shown as a loading control. B, HaCaT-Ras cells were transfected with control or Fyn-specific siRNA. After 48 hours, levels of Fyn, P-FAK (Y397), and total FAK were examined by western blotting. Actin is shown as a loading control.
6.6 Overexpression of Fyn in Human SCCs and Ras-Transformed Tumor Cells

We analyzed human SCC samples and normal epidermises for Fyn protein levels by immunohistochemistry (Figure 32). Fyn was over-expressed in 15% of the SCCs (7 of 44) examined and none of the normal epidermises (0 of 10). We also explored whether Ras/PI3K/Akt signaling was involved in Fyn expression in human tumor cell lines with activated Ras. We analyzed Fyn mRNA levels by qRT-PCR in MDA-MB-231, a well characterized human breast cancer line with activated K-Ras (Gilhooly and Rose, 1999), and found that inhibition of PI3K activity reduced expression of Fyn mRNA (Figure 33A). In addition, the invasive capacity of MDA-MB-231 cells was significantly inhibited by Fyn siRNA knockdown, indicating that Fyn is involved in invasion of these human tumor cells harboring active K-Ras (Figure 33B).
FIGURE 32: Overexpression of Fyn in human SCCs

Fyn is overexpressed in human SCCs. Fyn staining of normal human epidermis and human SCCs by immunohistochemistry is shown. The bottom left panel is hyperplastic skin.
FIGURE 33: Fyn regulation and role in invasion in MDA-MB-231 cells

A, Expression of Fyn in MDA-MB-231 cells in PI3K dependent. Cells were treated with LY294002 (20 µM). After 48 hours, Fyn mRNA levels were analyzed by qRT-PCR normalized to GAPDH. Data is represented as mean ± SD from a representative experiment performed in triplicate. B, MDA-MB-231 cells were transfected with either control or Fyn specific siRNA (dotted line) or treated with SFK inhibitor PP2 (5 µM), and invasion was measured after 48 hours. Data is represented as mean ± SD from a representative experiment performed in triplicate. T-test was performed on the indicated groups (*, #), p<0.01.
CHAPTER VII

DISCUSSION

7.1 PKC-δ Gene Expression is Reduced in Human SCCs

PKC-δ functions as a tumor suppressor, and while the loss of PKC-δ in human SCCs has been well documented, the mechanism is still unclear (Reddig et al., 1999; D'Costa et al., 2006). The 3p region of the human chromosome that harbors PKC-δ gene is frequently deleted or methylated in cancers (Sikkink et al., 1997; Zabarovsky et al., 2002), thus raising a strong possibility that PKC-δ gene expression is lost due to gene deletion or promoter methylation. Definitive understanding of the mechanism of PKC-δ loss is complicated by tumor heterogeneity and the presence of other cell types within and adjacent to the tumor which may express the PKC-δ at high levels. We used LCM to isolate relatively pure SCC and epidermal cells for RNA and DNA analysis. Using this more selective approach, we found the tumor suppressor PKC-δ is lost at the mRNA level in human SCCs, and that the PKC-δ gene is rarely deleted. Furthermore, we showed that the human PKC-δ promoter is negatively regulated in Ras-transformed keratinocytes. Together, these results strongly support a transcriptional repression mechanism for silencing PKC-δ expression. The PKC-δ gene resides at 3p21.31, and the 3p region has been reported to be deleted in many cancers, including cutaneous SCC.
(Sikkink et al., 1997; Dobler et al., 1999). Based on the qPCR data in Figure 9, we have ruled out PKC-δ gene deletion as a major mechanism of PKC-δ loss in human SCCs.

7.2 Role of Constitutive NF-κB Signaling in the Repression of PKC-δ Gene Expression

Ras signaling is strongly linked to the loss of PKC-δ gene expression (Playford and Schaller, 2004; Geiges et al., 1995). We showed that NF-κB signal is responsible for down-regulation of tumor suppressor PKC-δ gene expression by Ras (Chapter IV). This is consistent with the previous reports that Ras regulates the expression of many pro-survival and tumor suppressor genes via activation of transcription factors such as Ets, AP-1 and NF-κB (Finco et al., 1997; Mavrothalassitis and Ghysdael, 2000). However, mutation of NF-κB site did not completely rescue the PKC-δ promoter repression suggesting that other transcription factor binding sites could also be involved in PKC-δ repression. Ras is the most commonly mutated gene across human cancers, including cutaneous SCCs, and is activated by multiple growth factor receptor pathways (Rajalingam et al., 2007; Denning et al., 1996; Li et al., 1994a; Lu et al., 1997). Furthermore, NF-κB regulates the expression of many oncogenes, growth factors, and pro-apoptotic genes, and thus functions as a tumor promoter or a tumor suppressor based on the cell conditions and the target genes (Karin, 2006; Gilmore et al., 1996). NF-κB pathway is a known target of mutant Ras signaling (Tobin et al., 1996; Folgueira et al., 1996), and NF-κB activation is required for malignant transformation of many cells
including HaCaTs, indicating that NF-κB functions as an oncogene in the epidermis (Qin et al., 1999; Ren et al., 2006). Furthermore, the NF-κB pathway is constitutively activated in mouse skin tumors, and inhibition of NF-κB signaling prevents UV-induced skin tumors in mice (Budunova et al., 1999; Gottipati et al., 2008). Our findings showing that Ras induces NF-κB activation and subsequently loss of PKC-δ gene expression have strong therapeutic implication targeting Ras-NF-κB pathway in tumors and inducing re-expression of PKC-δ and reduce tumorigenecity of the Ras-transformed cells.

We showed that Ras induces constitutive NF-κB activation and recruitment of p50 and c-Rel subunits to the PKC-δ promoter (Figure 19). Among the different NF-κB subunits, p50 functions as the major repressive subunit, and p65 acts as the major transcriptional activator (Matthews and Hay, 1995; Elsharkawy et al., 2010; Cao et al., 2006). We found that p50 is specifically recruited to PKC-δ promoter and not p65 supporting the repressive function of p50 in the regulation of target gene expression. The signaling and regulation of constitutive NF-κB activation is less well understood. Our findings that NF-κB is constitutively activated by Ras in HaCaTs is consistent with previous reports linking mutant Ras with constitutive NF-κB activation (Tobin et al., 1996; Folgueira et al., 1996). Previous studies in mice have shown that NF-κB is constitutively activated in skin tumors and is accompanied by increased p50 expression (Budunova et al., 1999). In addition, studies in B cells have found that constitutive NF-κB activation comprises mostly of p50/c-Rel complexes (Liou et al., 1994; O'Connor et al., 2004; Grumont and
Gerondakis, 1994). This is also consistent with our current findings that p50/c-Rel is specifically recruited to PKC-δ promoter is Ras-transformed cells.

The NF-κB binding site (-260) on the PKC-δ promoter is well conserved across species, and has been previously characterized (Suh et al., 2003; Liu et al., 2006). Studies in mouse embryo fibroblasts have found that Rel-A NF-κB signaling plays a critical role in basal PKC-δ expression (Liu et al., 2006). Activation of PKC-δ gene expression is a critical step in UV-induced apoptosis, and is mediated by p65 homodimers (Liu et al., 2006). In contrast, here we show that in response to oncogenic stimuli (Ras or Fyn) NF-κB is constitutively activated and reduces PKC-δ gene expression using repressive p50/c-Rel subunits (Figure 32 & 33). Interestingly, no significant difference in the total protein levels of NF-κB subunits was detected in HaCaT-Ras cells as compared to control HaCaT cells, thus raising a critical question of how are different NF-κB subunits recruited to the same NF-κB binding site under different conditions? The answer lies in a detailed analysis and understanding of the NF-κB interacting partners that possibly mediate recruitment of different NF-κB dimers to the same promoter in response to different activating stimuli (i.e inducible or constitutive). For instance, CDK2 can bind to c-terminus of p300 and inhibit NF-κB dependent transcription (Perkins et al., 1997). In addition, ING4, a tumor suppressor in gliomas, interacts with NF-κB and specifically inhibits the expression of pro-angiogenesis genes such as Interleukin-8 (Nozell et al., 2008; Garkavtsev et al., 2004). Further analysis of constitutive NF-κB signaling and identification of proteins that specifically interact and regulate the translocation of NF-κB
subunits should provide insight into the mechanism of PKC-δ repression at the level of the gene.

7.3 Fyn is a critical node in the Ras/Akt effector pathway involved in tumor cell invasion

While the over-expression and oncogenic activity of Fyn in human and experimental tumors is well-documented, the mechanism of how Fyn is over-expressed in cancers is less clear (Saito et al., 2010; Zhao et al., 2009; Ban et al., 2008; Talantov et al., 2005; Chen et al., 2009; Posadas et al., 2009). Here we found that oncogenic H-Ras dramatically induced the expression of Fyn through the PI3K/Akt Ras effector pathway. Src was not activated or up-regulated (Figure 21 & 22) by H-Ras transduction, indicating some specificity of this effect among SFKs for Fyn. The induction of Fyn by Ras is highly significant since Ras genes are among the most commonly mutated oncogenes in human cancers, and multiple growth factor receptor pathways activate Ras and PI3K/Akt signaling in tumors, even tumors with wild-type Ras alleles (Karnoub and Weinberg, 2008).

Fyn is rather unique among SFKs since it is up-regulated at the mRNA level in multiple cancers, including glioblastoma, head and neck squamous cell carcinoma, melanoma, chronic myelogenous leukemia, and during prostate cancer progression (Saito et al., 2010; Ban et al., 2008). In addition, we performed immuno-histochemical staining of
human SCC tissue array and showed that Fyn was overexpressed in ~15% of human SCCs (Figure 32). We also found that Fyn was overexpressed particularly in metastatic SCCs (data not shown), thus suggesting Fyn involvement in increased invasion of SCC cells. However, the mechanism of Fyn overexpression in human SCCs is not clear and might involve a Ras effector signaling as Ras is frequently mutated in many tumors including SCCs. Fyn mRNA is up-regulated by Bcr-Abl1-induced oxidative stress in chronic myelogenous leukemia cells, and this transcriptional mechanism involves the redox-sensitive Egr1 transcription factor (Gao et al., 2009). Akt activation also induces oxidative stress, and thus a similar mechanism may be responsible for Fyn induction in other cancers with elevated Fyn expression (Nogueira et al., 2008; Govindarajan et al., 2007). We also found elevated Fyn kinase activity in HaCaT-Ras cells (Figure 20B), most likely due to the large increase in Fyn expression. Fyn protein levels can also be down-regulated by the Src-activating and signaling molecule Srcasm, but since Srcasm does not influence Fyn mRNA levels (Li et al., 2007), it cannot account for regulation of Fyn by active Ras we observed.

We found that PI3K/Akt signaling was involved in Fyn induction by H-Ras, and active Akt was sufficient to induce Fyn expression (Figures 21 and 22). The Ras effector pathway ERK was not required for Fyn induction. Ras binds directly to the p110α catalytic subunit of PI3K to activate Akt (Gupta et al., 2007). In addition, Akt is activated via phosphatidylinositol-3,4,5-trisphosphate generated by PI3K in response to growth factor/receptor tyrosine kinase activation or loss/repression of the PTEN dual-
specificity phosphatase (Manning and Cantley, 2007). Direct phosphorylation by PDK1 also activates Akt in response to growth factors (Manning and Cantley, 2007). Activation of these signaling pathways are common in human cancers, making Akt a major survival mechanism active in most tumor cells (Bhaskar and Hay, 2007).

None of the well characterized Akt effectors, including mammalian target of rapamycin, GSK3, and FOXO, have been described as being able to regulate Fyn (Manning and Cantley, 2007). Akt can repress Egr1, the transcription factor shown to induce Fyn in chronic myelogenous leukemia cells, but this cannot explain the induction of Fyn by Akt (Gao et al., 2009). We also demonstrated that Fyn expression in active K-Ras expressing MDA-MB-231 cells was dependent on PI3K (Figure 5). Thus the role of the Akt/Fyn pathway we described in the HaCaT-Ras model is functional in other human cancers, although the detailed molecular mechanism requires further investigation.

We showed that Fyn activity was essential and sufficient for Ras-induced anchorage independence in HaCaT cells, suggesting that Fyn plays a key role in inducing a transformed phenotype by Ras in culture (Figure 23). This is consistent with the previous findings that SFKs are required for the transformation of KCs by Ras (Joseloff et al., 2002). However, the role of Fyn in normal KCs might be different then that in transformed KCs. While Fyn activity is essential for maintaining the transformed phenotype of Ras-transduced KCs in culture, in normal mouse KCs Fyn has been strongly implicated in differentiation and growth arrest (Cabodi et al., 2000; Calautti et
al., 1995). Previous studies have shown that in Fyn is activated by PKC-η, and is sufficient for suppression of growth in mouse KCs and induction of differentiation marker transglutamase (Cabodi et al., 2000). Thus, PKC-η dependent Fyn activation would occur in the suprabasal granular layers, thus post-mitotic KCs may respond differently then basal proliferating KCs. This is in contrast to our findings, and indicates that the role of Fyn might be context dependent and varies with the type of upstream activating signals and downstream effectors. In addition, the above mentioned studies were performed in mouse KCs instead, and this could also explain the contrasting roles of Fyn in human KCs (i.e. HaCaTs).

We also demonstrated that the enhanced migration and invasion in H-Ras-transduced HaCaT cells was due to induction of Fyn (Figure 24 & 25), and that Fyn was sufficient to enhance HaCaT cell migration and invasion (Figure 23 & 24). We also found that Fyn was sufficient for loss of E-Cadherin and inhibition of GSK-3β, suggesting that Fyn is sufficient for induction of EMT in HaCaT cells (data not shown). Fyn was also important for invasion of MDA-MB-231 cells (Figure 28). These findings are consistent with the established role of SFKs in integrin and growth factor receptor signaling resulting in FAK activation, actin cytoskeleton reorganization, and enhanced cell migration. We also demonstrated that both Ras and Fyn can increase the level of phospho-FAK (active), and that Fyn was important for the elevated phospho-FAK in HaCaT-Ras cells (Figure 26). H-Ras activates multiple effectors capable of promoting cell migration and invasion, including p190 Rho-GAP and AF6 (Karnoub and Weinberg, 2008), and our findings that
Fyn is required for enhanced invasion in oncogenic Ras-expressing cells is noteworthy (Figure 34). In addition, SFKs have been implicated as a potent inducer of tumor angiogenesis, and thus play multiple roles in neoplastic progression (Kim et al., 2009b).

7.4 Role of Fyn is Activation of NF-κB and Loss of PKC-δ

Multiple effector pathways downstream of Ras such as Raf/MEK/ERK, PI3K/Akt and Ral-GEFs are responsible for Ras-mediated cell proliferation, survival, transformation and neoplastic growth (Rajalingam et al., 2007). Fyn and Src play an important role in keratinocyte growth and differentiation, and are required for maintaining the neoplastic phenotype of Ras-transformed cells (Joseloff et al., 2002). Fyn is overexpressed in many cancers, including human SCCs (Ayli et al., 2008; Grosso et al., 2009; Posadas et al., 2009; Talantov et al., 2005; Lu et al., 2009), and transgenic mice expressing constitutively active Fyn (K14-Fyn Y538F) spontaneously develop skin tumors, indicating that Fyn is a potential oncogene in human SCCs (Zhao et al., 2009). Furthermore, activation of SFKs such as Src and Fyn is linked to the loss of PKC-δ activity (Joseloff et al., 2002; Blake et al., 1999), but the role of SFKs in loss of PKC-δ gene expression has never been reported. Previous studies have shown that pharmacological inhibition of SFKs blocks Integrin-mediated constitutive NF-κB signaling indicating a role of SFK in activation of NF-κB signaling (Courter et al., 2005). In Figures 30-34, we describe a novel role of Fyn signaling in the regulation of PKC-δ gene expression. We show that Fyn is required for the constitutive activation of NF-κB
signaling in HaCaT-Ras cells (Figure 30). In addition, we show that Fyn is sufficient for the recruitment of p50/c-Rel subunits to the PKC-δ promoter and subsequent repression of PKC-δ promoter activity (Figure 32, 33 & 34). We believe this is the first report of SFK involvement in Ras induced constitutive NF-κB activation. Although many regulators of NF-κB signaling have been implicated as substrates of SFK (Fan et al., 2003; Huang et al., 2003), the precise mechanism of NF-κB activation by Fyn is unknown at this time.

7.5 Significance: Fyn as a novel therapeutic target in human SCCs

SFKs are over-expressed in many cancers, including human squamous cell carcinomas, and these studies provide mechanistic insights into Fyn induction (Zhao et al., 2009; Ayli et al., 2008). Over-expression and activation of Fyn has a dominant function in tumor cells, and Fyn selective inhibitors should have a good therapeutic window and be useful in a wide range of human cancers. Orally available small-molecule SFK inhibitor such as Dasatinib, FDA-approved for imatinib-resistant chronic myelogenous leukemia, are effective against a variety of cancers, and currently numerous clinical trials are underway to evaluate their efficacy in additional cancer types (Kim et al., 2009b).

Our identification of Fyn as a key mediator of Ras/Akt oncogenic signaling provides additional rationale for developing and characterizing SFK-targeted therapeutics. Directly targeting Ras has proven difficult, and Akt inhibitor development has had to deal
with metabolic side-effects due to the central role of Akt in energy metabolism (Nogueira et al., 2008). Thus, selective targeting of Fyn may prove to be especially effective given the role of Fyn in tumor progression (invasion, metastasis, anchorage independence) by activation of oncogenic NF-κB signaling and loss of tumor suppressor PKC-δ. We have previously shown that re-expression of PKC-δ increases spontaneous apoptosis in SCC cells and reduces the tumorigenecity of Ras-transformed HaCaT cells in nude mice (D'Costa et al., 2006). Furthermore, inhibition of Fyn activity suppresses UV-induced skin cancer in mice (Jung et al., 2008). Thus, our findings describing a novel crosstalk between Ras, Fyn and NF-κB, which is responsible for the loss of tumor suppressor PKC-δ gene expression has tremendous therapeutic implications in SCCs (Figure 34). Drugs blocking Fyn induction and/or activity, and subsequently inducing PKC-δ gene re-expression might be especially effective against skin cancers, due to the topical route of administration, thus minimizing toxicities and limiting the delivery to tumor cells.
FIGURE 34: Mechanism of PKC-δ promoter repression by Ras

Fyn expression is induced by Ras via activation of the PI3K/Akt signaling pathway. Induction and activation of Fyn is required for FAK activation and increased migration/invasion by active Ras. In addition, induction and activation of Fyn is required for IκB-α degradation and constitutive NF-κB activation. Furthermore, the constitutive activation of NF-κB leads to the recruitment of repressive NF-κB subunits p50 and c-Rel and possibly other co-repressors, to the PKC-δ promoter resulting in promoter repression. Thus, Ras down-regulates PKC-δ gene expression by induction of Fyn and subsequent activation of constitutive and repressive NF-κB signaling.


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Vipin received the Arthur J. Schmitt dissertation fellowship for the 2009-2010 academic year. Vipin was also awarded the Albert M. Kligman Travel Fellowship to attend the 2009 Annual Society for Investigative Dermatology Meeting in Montreal, Quebec, Canada. Dr. Vipin Yadav successfully defended his Ph.D. dissertation on October 22nd 2010.