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Protein Kinase Cι Expression and Oncogenic Signaling Mechanisms in Cancer

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Abstract

Accumulating evidence demonstrates that PKCι is an oncogene and prognostic marker that is frequently targeted for genetic alteration in many major forms of human cancer. Functional data demonstrate that PKCι is required for the transformed phenotype of NSCLC, pancreatic, ovarian, prostate, colon and brain cancer cells. Future studies will be required to determine whether PKCι is also an oncogene in the many other cancer types that also overexpress PKCι. Studies of PKCι using genetically defined models of tumorigenesis have revealed a critical role for PKCι in multiple stages of tumorigenesis, including tumor initiation, progression and metastasis. Recent studies in a genetic model of lung adenocarcinoma suggest a role for PKCι in transformation of lung cancer stem cells. These studies have important implications for the therapeutic use of aurothiomalate (ATM), a highly selective PKCι signaling inhibitor currently undergoing clinical evaluation. Significant progress has been made in determining the molecular mechanisms by which PKCι drives the transformed phenotype, particularly the central role played by the oncogenic PKCι-Par6 complex in transformed growth and invasion, and of several PKCι-dependent survival pathways in chemo-resistance. Future studies will be required to determine the composition and dynamics of the PKCι-Par6 complex, and the mechanisms by which oncogenic signaling through this complex is regulated. Likewise, a better understanding of the critical downstream effectors of PKCι in various human tumor types holds promise for identifying novel prognostic and surrogate markers of oncogenic PKCι activity that may be clinically useful in ongoing clinical trials of ATM.

Keywords

tumorigenesis; gene amplification; signal trandsuction; invasion; metastasis; aurothiomalate

Introduction

Protein kinase C (PKC) is a family of structurally related serine/threonine protein kinases whose catalytic activity is regulated by interaction with phospholipid co-factors, inter- and intra-molecular phosphorylation, and specific protein-protein interactions. The PKC enzyme family is divided into three subgroups: the conventional, calcium-dependent cPKCs [alpha (α), beta I (βI), beta II (βII), and gamma (γ)]; the novel, calcium-independent nPKCs [delta (δ), epsilon (ε), eta (η) and theta (θ)]; and the atypical aPKCs [zeta (ζ) and iota (ι) which is also known as lambda (λ) in mice]. This grouping is based on the presence or absence of functional domains that confer specific co-factor and activator requirements. Conventional

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PKCs are calcium-, diacylglycerol (DAG)- and phosphatidylserine-dependent due to the presence of conserved modular C1 and C2 domains within the regulatory region of the enzyme. Novel PKCs are DAG- and phosphatidylserine-dependent but do not require calcium. Due to the unique structure of their N-terminal regulatory region, atypical PKCs do not require calcium, DAG or phosphatidylserine for activation.

Biochemical and immunologic studies indicate that multiple PKC isozymes are expressed in virtually all cell and tissue types (reviewed in (Fields and Murray, 2008)). The expression of individual PKC isozymes is developmentally regulated and is responsive to the differentiation state of cells and tissues. For these reasons, PKC isozymes are thought to fulfill distinct, non-redundant functions within the cell (reviewed in (Dempsey et al., 2000; Reyland, 2009)). However, the similar activator requirements and substrate specificities of PKC isozymes *in vitro* have complicated the identification of physiologically relevant, isozyme-specific substrates and cellular functions. The ability to genetically manipulate expression of a specific PKC isozyme and to express mutant forms of individual PKC isozymes with altered kinase activity have proven successful in identifying PKC isozyme-specific functions. Using these genetic techniques, many laboratories, including our own, have demonstrated isozyme- and cell type-specific roles for PKC in cellular proliferation, differentiation, apoptosis and cell polarity (Chalmers et al., 2005; Gokmen-Polar and Fields, 1998; Jansen et al., 2001; Mischak et al., 1993; Murray et al., 1999; Oster and Leitges, 2006). The discovery that PKC is a cellular receptor for the tumor-promoting phorbol esters led to an intense interest in the role of individual PKC isozymes in cancer development (Castagna et al., 1982; Kikkawa et al., 1983). Indeed, the expression level and cellular localization of individual PKC isozymes has been shown to be altered during carcinogenesis in numerous tissue types (reviewed in (Fields and Gustafson, 2003)). However, despite exhaustive investigation, no somatic or germline mutations have been found in the coding regions of any PKCs that are associated with human cancers or other diseases, and to date, only one PKC isozyme, atypical PKCι, has been shown to satisfy the criteria of a human oncogene. This mini-review details the work from our laboratory and others characterizing the role of oncogenic PKCι in rodent carcinogenesis models and human cancer. We also discuss the status of our efforts to therapeutically target this critical oncogene for treatment of cancer patients.

I. PKCι Expression in Primary Human Tumors

PKCι is overexpressed and prognostic in multiple human cancer types—

Overexpression of PKCι has been demonstrated in many human cancers (Table 1). PKCι is frequently overexpressed in cancers of the lung (Regala et al., 2005b), pancreas (Scotti et al., 2010), stomach (Takagawa et al., 2010), colon (Murray et al., 2004), esophagus (Yang et al., 2008), liver (Du et al., 2009), bile duct (Li et al., 2008), breast (Kojima et al., 2008), ovary (Eder et al., 2005;Weichert et al., 2003;Zhang et al., 2006), prostate (Ishiguro et al., 2009) and brain (Patel et al., 2008). In addition to these published reports, meta-analysis of publicly available microarray data provides additional support for overexpression of PKCι in many of these tumor types, including lung (Landi et al., 2008) breast (Richardson et al., 2006), pancreas (Segara et al., 2005), prostate (Wallace et al., 2008), ovary (Cancer Genome Atlas) and liver (Wurmbach et al., 2007)(Table 2). Interestingly, increased PKCι in brain cancer was not supported by the available genomic data sets (Lee et al., 2006)(Table 2). Analysis of available microarray datasets revealed that, in addition to lung cancer (Landi et al., 2008), several other squamous-type cancers also express elevated PKCι, including head and neck (Ginos et al., 2004) and tongue cancer (Ye et al., 2008). Significant overexpression of PKCι was also detected in renal cancer (Gumz et al., 2007), bladder cancer (Dyrskjot et al., 2004;Sanchez-Carbayo et al., 2006), melanoma (Talantov et al., 2005) and leukemia

(Valk et al., 2004) (Table 2). These studies strongly indicate a role for PKCι in many major forms of human cancer.

In those tumor types where it has been examined, PKCι expression has also been shown to be of prognostic significance. High PKCι predicts poor patient survival in lung (Regala et al., 2005b) pancreatic (Scotti et al., 2010) bile duct (Li et al., 2008), ovarian (Eder et al., 2005; Weichert et al., 2003) and prostate (Ishiguro et al., 2009) cancer. Elevated PKCι expression predicts disease recurrence in gastric cancer (Takagawa et al., 2010) and metastasis in esophageal cancer (Yang et al., 2008). In our analysis of PKCι expression in lung cancer, PKCι emerged as a prognostic indicator that was comparable to tumor stage in its prognostic value. Interestingly, PKCι expression did not correlate with tumor stage in non-small cell lung cancer (NSCLC); rather PKCι levels were comparable in early and late stage disease indicating that elevated PKCι expression is a very early event in lung tumor development (Regala et al., 2005b). In contrast, PKCι expression correlates with tumor stage in ovarian, bile duct and liver cancer, suggesting that PKCι may regulate disease progression in these tumor types (Du et al., 2009; Li et al., 2008; Zhang et al., 2006).

Thus PKCι expression is elevated in a variety of tumor types, and in many cases, is predictive of poor clinical outcome. Therefore, PKCι expression profiling may identify patients at elevated risk of relapse or disease progression. Since many patients diagnosed with early stage cancer will eventually relapse, PKCι expression profiling may be useful in identifying high risk patients who would be candidates for more aggressive clinical management, perhaps, as will be discussed below, with PKCι-targeted therapy.

PKCι is a target for frequent tumor-specific gene amplification—DNA amplification is one mechanism by which oncogenes are activated in neoplastic tissue. The PKCι gene *PRKCI*, resides on chromosome 3q26, a chromosomal region frequently amplified in human cancers, particularly squamous cell carcinomas (Brass et al., 1996; Heselmeyer et al., 1997; Lin et al., 2006; Racz et al., 1999; Singh et al., 2002). Therefore, we examined NSCLC tumors for evidence of changes in *PRKCI* gene copy number (Regala et al., 2005b). *PRKCI* was found to be amplified in a tumor-specific fashion in 36% of the NSCLC tumors examined. Furthermore, *PRKCI* amplification correlates with PKCι mRNA and protein expression, and with poor outcome in NSCLC tumors (Regala et al., 2005b). Interestingly, *PRKCI* amplification was frequently found in lung squamous cell carcinoma (SCC) (~70%) but rarely in lung adenomcarcinoma (LAC) (Regala et al., 2005b), consistent with the distribution of chromosome 3q26 amplification in these tumor types which is confined to SCC (Balsara et al., 1997; Brass et al., 1997). Similar tumor-specific *PRKCI* amplification has also been observed in ovarian cancers of the serous sub-type (~70%) (Eder et al., 2005; Zhang et al., 2006) and esophageal squamous cell cancer (53%) (Yang et al., 2008). PKCι expression and *PRKCI* copy number also correlate with chromosome 3q26 gains in these tumors (Eder et al., 2005; Yang et al., 2008; Zhang et al., 2006), indicating that PKCι is a relevant target for tumor-specific chromosome 3q26 amplification. Since chromosome 3q26 amplification is one of the most common chromosomal changes in human cancers, including SCC of the head and neck (Snaddon et al., 2001) and cervix (Sugita et al., 2000), it is likely that PKCι expression and gene copy number are of prognostic significance in these tumors as well.

PRKCI amplification is not the only mechanism by which PKCι expression is elevated in human tumors. PKCι expression is elevated to the same degree and frequency in lung SCC and LAC tumors despite the fact that PKCι gene amplification is largely confined to SCC tumors (Regala et al., 2005b). Furthermore, PKCι is frequently over-expressed in other tumor types, including colon cancers (Murray et al., 2004), pancreatic cancers (Scotti et al., 2010) and leukemia (Gustafson et al., 2004) that do not harbor frequent chromosome 3q26

amplification. We recently demonstrated that Bcr-Abl transcriptionally activates PKCι through Ras/Mek-dependent activation of a specific Elk1 element within the proximal PKCι promoter in chronic myelogenous leukemia (CML) cells (Gustafson et al., 2004). A similar mechanism is likely at play in LAC and pancreatic ductal adenocarcinoma (PDAC) tumors that harbor oncogenic *KRAS* mutations. Indeed, we have observed a statistically significant positive correlation between the presence of *KRAS* mutation and PKCι expression in primary LAC tumors (unpublished observations). Likewise in PDAC, in which >90% of tumors harbor a *KRAS* mutation (Klimstra and Longnecker, 1994), we detected PKCι overexpressed in 96% of tumors (Scotti et al., 2010).

Another potential mechanism for oncogenic activation of PKCι is somatic mutation. However, sequence analysis of all 18 exons of the PKCι gene in 20 LAC cases and 20 SCC cases failed to detect any mutations, suggesting that somatic mutation of PKCι either does not occur or is extremely rare in NSCLC (unpublished observations). In summary, PKCι is the first, and to date only, PKC isozyme shown to be a bonafide human oncogene (Regala et al., 2005b). Current evidence strongly supports the oncogenic role of PKCι in NSCLC, PDAC, ovarian cancer and glioma. Given the widespread overexpression of PKCι in other major tumor types, it appears likely that PKCι will be shown to be an oncogene in many other tumor types as well.

II. PKCι in Cellular Transformation

PKCι as a survival gene in human cancer—The first demonstration that PKCι was important for the transformed phenotype of human cancer cells came from studies in CML cells (Murray and Fields, 1997). CML cells are highly resistant to the apoptotic effects of numerous chemotherapeutic agents as a result of expression of the chimeric tyrosine kinase oncogene Bcr-Abl, the transforming activity that causes CML (Bedi et al., 1995). We found that Bcr-Abl-positive CML cells express high levels of PKCι and that PKCι is activated in response to apoptotic stimuli such as treatment with the chemotherapeutic agent paclitaxel (Jamieson et al., 1999). Disruption of PKCι expression or activity sensitized CML cells to induction of paclitaxel-induced apoptosis (Murray and Fields, 1997). Subsequent studies have established a similar role for PKCι in the survival and chemoresistance of other tumor cell types including prostate (Win and Acevedo-Duncan, 2008), NSCLC (Jin et al., 2005) and glioblastoma (Baldwin et al., 2006).

PKCι and oncogenic ras mediated transformation of intestinal epithelial cells —Studies in mouse fibroblasts first established a functional link between aPKCs and cellular Ras. Ras can activate aPKCs (Diaz-Meco et al., 1994), and aPKC activity is necessary for Ras-mediated effects on the actin-based cytoskeleton in fibroblasts (Bjorkoy et al., 1997; Coghlan et al., 2000; Kampfer et al., 2001; Uberall et al., 1999). Since oncogenic *Ras* signaling can drive colon carcinogenesis, and oncogenic *Ras* mutations are detected in ~30% of colon cancers (Slattery et al., 2001; Takayama et al., 2001), we investigated the role of PKCι in *Ras*-mediated transformation of rat intestinal epithelial (RIE) cells (Murray et al., 2004). We found that oncogenic *Ras* activates PKCι when introduced into non-transformed RIE cells and that expression of a kinase-deficient, dominant negative PKCι mutant (kdPKCι) in *Ras* transformed RIE cells inhibits *Ras*-mediated invasion and anchorage-independent growth (Murray et al., 2004). These results provided direct evidence that PKCι is involved in the establishment of the transformed phenotype by *Ras* in epithelial cells.

PKCι is required for maintenance of the transformed phenotype of cancer cells—PKCι not only plays a key role in transformation induced by introduction of oncogenic *Ras* into non-transformed epithelial cells, but also in maintenance of the

transformed phenotype of established human cancer cells harboring oncogenic *KRAS* mutations (Frederick et al., 2008; Regala et al., 2005a; Scotti et al., 2010). Expression of kdPKCι or knock down of PKCι expression using lentiviral-mediated shRNA blocked transformed (anchorage-independent) growth and invasion of human NSCLC cells (Frederick et al., 2008; Regala et al., 2005a) and human PDAC cells (Scotti et al., 2010). Genetic disruption of PKCι also blocks the proliferative and invasive properties of prostate and glioma cell lines *in vitro* (Baldwin et al., 2008; Ishiguro et al., 2009; Patel et al., 2008). Disruption of PKCι expression also blocks tumorigenicity of NSCLC and PDAC cell tumors injected either subcutaneously, or orthotopically into the lung and pancreas, respectively (Regala et al., 2005a; Scotti et al., 2010). Analysis of human PDAC cells after orthotopic injection into the mouse pancreas revealed that PKCι-deficient tumor cells yielded significantly smaller tumors and significantly fewer metastases to the kidney, liver, diaphragm and mesentery, providing the first evidence that PKCι is important for tumor metastasis *in vivo* (Scotti et al., 2010). The role of PKCι in transformed growth is not restricted to cell harboring oncogenic *KRAS* mutations since NSCLC cell lines expressing wild-type *KRAS* but harboring *PRKCI* amplification also require PKCι for their transformed phenotype (Regala et al., 2005a; Regala et al., 2005b).

III. PKCι in Tumorigenesis *in vivo*

PKCι is necessary for oncogenic *Kras*- and mutant *APC*-mediated intestinal tumorigenesis—Several transgenic models of tumorigenesis have revealed that PKCι plays a critical promotive role in tumorigenesis *in vivo*. We established mice in which either kdPKCι or constitutively active (caPKCι) PKCι is expressed specifically in the intestinal epithelium (Murray et al., 2004). Expression of either PKCι mutant had no demonstrable effect on the basal proliferation or differentiation of the colonic epithelium (Murray and Fields, 1997; Murray et al., 2004). However, expression of caPKCι in the colon increased susceptibility to carcinogen (azoxymethane; AOM)-induced formation of colonic preneoplastic lesions, aberrant crypt foci (ACF), whereas expression of kdPKCι significantly inhibited AOM-induced ACF formation (Murray et al., 2004). In addition, caPKCι mice exhibited an increase in the number of AOM-induced colon tumors, and the majority of the tumors in these mice had progressed from benign adenoma to malignant intramucosal carcinoma (Murray et al., 2004). Similar results were obtained in an *in vivo* model of oncogenic *Kras*-mediated colon carcinogenesis, the $Kras^{LA2}$ mouse (Johnson et al., 2001). Expression of kdPKCι in the colonic epithelium of these mice inhibited oncogenic *Kras*-mediated ACF formation (Murray et al., 2004). These data demonstrated that PKCι is required for oncogenic *Kras*-mediated transformation of the intestinal epithelium *in vivo* and constitute the first evidence for a function role of PKCι in tumorigenesis *in vivo*.

Interestingly, PKCι is also elevated in intestinal tumors formed in $Apc^{Min/+}$ mice (Murray et al., 2009; Oster and Leitges, 2006). To determine if PKCι plays a role in tumor development in $Apc^{Min/+}$ mice, the mouse PKCι gene, *Prkci*, was inactivated in the intestinal epithelium of triple transgenic $Apc^{Min/+}$/$Prkci^{f/f}$/*villin-Cre* mice by Cre-mediated recombination. $Apc^{Min/+}$/$Prkci^{f/f}$/*villin-Cre* mice exhibited loss of intestinal epithelial PKCι expression and a significant decrease in the number of intestinal tumors compared to $Apc^{Min/+}$/$Prkci^{f/f}$ mice that harbor intact alleles of the *Prkci* gene (Murray et al., 2009). Thus, PKCι is important for $Apc^{Min/+}$-induced intestinal epithelial tumorigenesis, indicating that the role of PKCι in colon tumorigenesis *in vivo* is not limited to *Kras*-mediated tumors.

Role of PKCι in initiation of lung tumorigenesis: Is PKCι a cancer stem cell gene?—PKCι is an oncogene required for maintenance of the transformed phenotype of non-small cell lung cancer (NSCLC) cells (Frederick et al., 2008; Regala et al., 2005a; Regala et al., 2005b). To address whether PKCι is involved in lung tumor development, we

established a mouse model in which oncogenic *Kras*^{G12D} is activated by Cre-mediated recombination in the lung with or without simultaneous genetic loss of the mouse PKCι gene, *Prkci* (Regala et al., 2009). Genetic loss of *Prkci* dramatically inhibits *Kras*-initiated hyperplasia and subsequent lung tumor formation *in vivo*. This effect correlates with a defect in the ability of *Prkci*-deficient bronchioalveolar stem cells (BASCs) to undergo *Kras*-mediated expansion and morphological transformation *in vitro* and *in vivo* (Regala et al., 2009). BASC exhibit stem-like properties and are thought to be the tumor-initiating cells in this model of *Kras*-mediated lung tumorigenesis (Jackson et al., 2001). Thus, *Prkci* is required for oncogene-induced expansion and transformation of tumor-initiating, lung stem-like cells. These studies suggest that PKCι may serve a critical role in cancer stem cell biology. Whether PKCι plays a similar role in the maintenance of the cancer stem cell niche in human cancers remains an important topic for future study.

IV. Oncogenic PKCι Signaling Mechanisms

PKCι-mediated survival signaling—PKCι activates multiple survival pathways that confer resistance to apoptosis induced by many stimuli, including TNF-α, carcinogens and chemotherapeutic agents (Figure 1). PKCι is sufficient to mediate the anti-apoptotic effects of Bcr-Abl via transactivation of NF-κB (Jamieson et al., 1999;Lu et al., 2001). Interestingly, in CML cells Bcr-Abl induces PKCι expression through a Ras/Mek-dependent pathway involving a functional Elk1 transcription factor binding site within the proximal promoter of PKCι (Gustafson et al., 2004). Thus Bcr-Abl appears to regulate PKCι at multiple levels to induce a chemoresistant phenotype; not only does Bcr-Abl induce PKCι expression but PKCι is a key effector of Bcr-Abl-mediated survival signaling. PKCι-mediated survival of TNFα-treated prostate cancer cells is also mediated through a NF-κB-dependent mechanism. In prostate cancer cells, PKCι-mediated phosphorylation of IκK leads to activation of the canonical NF-κB pathway and cell survival (Win and Acevedo-Duncan, 2008). In glioblastoma cells, PKCι-mediated survival appears to result from PKCι-induced attenuation of p38 mitogen-activated protein kinase signaling, which protects these cells from cytotoxicity caused by chemotherapeutic agents (Baldwin et al., 2006). In NSCLC cells, the ability of PKCι to enhance resistance to NNK-induced apoptosis appears to be mediated through Src-dependent activation of PKCι, which phosphorylates the proapoptotic protein BAD (Jin et al., 2005). Therefore, PKCι can activate multiple signaling pathways that promote cell survival in different tumor cell types.

Rac1 is a critical downstream effector of oncogenic PKCι—The Rho family GTPase Rac1 is activated by oncogenic *Ras* and is essential for *Ras*-mediated transformed growth and cellular invasion in fibroblasts (Khosravi-Far et al., 1995; Qiu et al., 1995). PKCι is necessary for *Ras*-mediated Rac1 activation in RIE cells (Murray et al., 2004); thus, kdPKCι blocks oncogenic *Ras*-mediated Rac1 activation, and expression of a constitutively active Rac1 allele, RacV12, overcomes dnPKCι-mediated inhibition of cellular invasion (Murray et al., 2004). These studies placed PKCι downstream of oncogenic *Kras* and upstream of the critical *Kras* effector Rac1, which activates the Mek-Erk signaling axis to drive transformed growth and also mediates cytoskeletal rearrangement involved in cellular invasion (Murray et al., 2004) (Figure 1). Rac1 is also a critical downstream target of *Kras*-mediated, PKCι-dependent transformation in NSCLC (Regala et al., 2005a) and PDAC (Scotti et al., 2010). In NSCLC and PDAC cells, *KRAS* activates a PKCι-Rac1-Pak-Mek-Erk signaling axis that drives transformed growth *in vitro* and tumorigenicity *in vivo* (Regala et al., 2005a; Scotti et al., 2010). Interestingly, Rac1 also plays a critical role downstream of oncogenic PKCι in NSCLC cells that do not harbor mutant *KRAS* indicating that PKCι-Rac1 signaling is not specific to mutant *KRAS*-mediated transformation (Regala et al., 2005a; Regala et al., 2005b). Expression of RacV12 reconstituted cellular invasion and anchorage-independent growth in PKCι-deficient NSCLC and PDAC cells in a Mek-dependent manner

(Regala et al., 2005a; Scotti et al., 2010). Thus, Rac1 is a critical downstream effector of oncogenic PKCι in multiple cancer cell types and is not restricted to tumor cells harboring *KRAS* mutations.

The role of the PB1 domain of PKCι in oncogenic signaling—The N-terminal regulatory domain of aPKCs is unique in that it contains a Phox/Bem1 (PB1) domain that mediates homo- and heterotypic protein-protein interactions critical for activation and intracellular localization (Lamark et al., 2003). Par6 is a PB1 domain-containing protein that binds atypical PKCs via PB1:PB1 domain interactions (Etienne-Manneville and Hall, 2003; Wilson et al., 2003). Par6 links atypical PKC to cell polarity by forming a complex with atypical PKC and a Rho family GTPase, Rac1 or cdc42 (Etienne-Manneville and Hall, 2003; Joberty et al., 2000; Lin et al., 2000; Noda et al., 2001; Qiu et al., 2000; Suzuki et al., 2003; Suzuki et al., 2001). Since Rac1 is a critical downstream effector of oncogenic PKCι in multiple cell types including the colon, lung and pancreas (Murray et al., 2004; Regala et al., 2005a; Scotti et al., 2010), we assessed the role of the PB1 domain of PKCι in Rac1 activation and NSCLC cell transformation (Regala et al., 2005a). Expression of the PB1 domain of PKCι in NSCLC cells uncouples PKCι and Par6 from Rac1 activation and inhibits transformed growth. Likewise, RNAi-mediated knock down of PKCι, Par6 or Rac1 inhibits transformed growth and cellular invasion in NSCLC cancer cells (Frederick et al., 2008). Expression of wild-type PKCι in PKCι knock down cells restores transformation, whereas expression of a PB1 domain mutant of PKCι, PKCι-D63A, that cannot bind Par6, does not (Frederick et al., 2008). Similarly, expression of wild type Par6 in Par6 knock down cells restores transformation whereas expression of Par6 mutants that either cannot bind PKCι (Par6-K19A) or couple to Rac1 (Par6-ΔCRIB) does not (Frederick et al., 2008). Expression of RacV12 in PKCι- or Par6-depleted NSCLC cells restores transformed growth and cellular invasion (Frederick et al., 2008). The PKCι-Par6 complex functions to activate a Rac1-Mek-Erk signaling axis that drives the transformed growth of NSCLC cells (Frederick et al., 2008). These studies defined a novel PKCι-Par6 complex that is required for NSCLC transformation (Figure 1).

Ect2 binds and activates the PKCι-Par6 complex—Having identified Rac1 as a critical downstream effector of the oncogenic PKCι-Par6 complex, a key question became: how does this complex activate Rac1? To address this question, we utilized a proteomics approach to identify proteins that associate with the PKCι-Par6 complex in NSCLC cells (Justilien and Fields, 2009). The Rho family GTPase guanine nucleotide exchange factor (GEF) Ect2 was identified as a prominent component of the PKCι-Par6 complex (Fields and Justilien, 2010; Justilien and Fields, 2009). RNAi-mediated knock down of Ect2 inhibits Rac1 activity and blocks transformed growth, invasion and tumorigenicity of NSCLC cells, whereas expression of RacV12 restores transformation to Ect2-deficient cells (Justilien and Fields, 2009). Interestingly, the role of Ect2 in NSCLC transformation is distinct from its well-established role in cytokinesis. In fact, NSCLC cells appear to have acquired an Ect2-independent cytokinesis mechanism, which resembles that described in fibrosarcoma H1080 cells (Kanada et al., 2008). Rather, in NSCLC cells Ect2 is mislocalized to the cytoplasm where it binds the PKCι-Par6 complex. Knock down of either PKCι or Par6 causes redistribution of Ect2 to the nucleus and loss of transformed growth and invasion. Therefore, Ect2 and PKCι drive tumor cell proliferation through formation of an oncogenic PKCι-Par6-Ect2 complex. Interestingly, the Ect2 gene *ECT2* resides on chromosome 3q26 in close proximity to *PRKCI*. Studies in primary NSCLC tumors demonstrated that *PRKCI* and *ECT2* are co-amplified and overexpressed in NSCLC (Justilien and Fields, 2009). Thus, Ect2 and PKCι are genetically linked through coordinate gene amplification in NSCLC tumors, and biochemically and functionally linked in NSCLC transformation through

formation of an oncogenic PKCι-Par6-Ect2 complex that drives NSCLC cell transformation by activating Rac1 (Figure 1) (Justilien and Fields, 2009).

MMP10 is a critical downstream effector of the oncogenic PKCι-Par6-Rac1 signaling axis—We recently carried out a genomic analysis to identify genes whose expression is modulated by RNAi-mediated knock down of PKCι in NSCLC cells. The matrix metalloproteinase 10 (MMP10; stromolysin 2) emerged from this analysis as a genomic target of PKCι (Frederick et al., 2008). Depletion of PKCι, Par6 or Rac1 by RNAi inhibits MMP10 expression in NSCLC cells, and expression of exogenous wild-type Par6 in Par6 knock down cells restored MMP10 expression, whereas expression of Par6 mutants that either cannot bind PKCι or Rac1 did not, indicating the role of the PKCι-Par6-Rac1 complex in MMP10 expression. RNAi-mediated knock down of MMP10 blocks anchorage-independent growth and cell invasion in NSCLC cells, and the loss of transformed growth and invasion in PKCι knock down or Par6 knock down NSCLC cells can be rescued by the addition of catalytically active MMP10 (Frederick et al., 2008). Taken together, these data defined a PKCι-Par6-Rac1-Pak-Mek-Erk signaling axis that drives anchorage-independent growth and invasion of NSCLC cells, at least in part, through induction of MMP10 expression (Frederick et al., 2008). Interestingly, analysis of primary human lung tumor specimens demonstrated a strong correlation between PKCι and MMP10 expression in primary NSCLC tumors, suggesting a role for the PKCι-Par6-Rac1-Pak-Mek-Erk-MMP10 signaling axis in primary human lung cancers (Frederick et al., 2008). The molecular mechanism by which PKCι-mediated overexpression of MMP10 promotes transformation is currently unexplored and merits further investigation.

In a second genomic study, we conducted a meta-analysis of gene expression in primary lung adenocarcinomas (LAC) from three independent public domain datasets (Erdogan et al., 2009). Our analysis identified four genes, COPB2, ELF3, RFC4 and PLS1, whose expression correlates positively with PKCι in primary LAC tumors in all three databases. QPCR analysis of 60 primary LAC samples showed these four genes are highly overexpressed in tumors, and exhibit a strong positive correlation with PKCι expression (Erdogan et al., 2009). RNAi-mediated knock down of PKCι in LAC cell lines demonstrated that PKCι regulates expression of each of these genes. Furthermore, RNAi-mediated knock down of each of these genes led to significant inhibition of anchorage-independent growth and cellular invasion demonstrating that each of them is important for transformation in LAC cells (Erdogan et al., 2009). Finally, meta-analysis revealed that subsets of these PKCι-regulated genes are coordinately overexpressed with PKCι in other major tumor types including lung squamous cell carcinoma, breast, colon, prostate, pancreatic and glioblastoma cancers (Erdogan et al., 2009). This analysis revealed novel signaling mechanisms that participate in PKCι-mediated transformation (Figure 1) and provide potentially useful biomarkers of PKCι-mediated signaling which may serve as targets for the development of novel prognostic markers and/or therapeutic agents.

V. PKCι as a Therapeutic Target for Treatment of Cancer

The PB1-PB1 domain interaction between PKCι and Par6 is highly specific, and is required for the oncogenic PKCι-Par6-Rac1-MMP10 signaling axis that mediates anchorage-independent growth and invasion of human NSCLC cells *in vitro* and tumorigenicity *in vivo* (Frederick et al., 2008). Therefore, we reasoned that this interaction is an attractive target for development of novel mechanism-based therapeutics for treatment of NSCLC.

Using a novel fluorescence resonance energy transfer (FRET)-based assay we identified small molecular weight compounds that can disrupt the PB1-PB1 domain interaction between PKCι and Par6 (Stallings-Mann et al., 2006). Among the most potent inhibitors

identified were the gold-containing compounds aurothioglucose (ATG) and aurothiomalate (ATM), which are FDA-approved treatments for rheumatoid arthritis (Messori and Marcon, 2004). ATG and ATM exhibit dose-dependent inhibition of PKCι-Par6 binding with IC_{50}s of ~1μM (Stallings-Mann et al., 2006). Treatment of NSCLC cells with these compounds inhibits PKCι-mediated Rac1 activation and blocks anchorage-independent growth of NSCLC cells *in vitro* and tumorigenicity *in vivo* (Stallings-Mann et al., 2006). This inhibition can be rescued by expression of Rac1V12, indicating that ATM targets the interaction between PKCι and Par6 that couples PKCι to Rac1 (Stallings-Mann et al., 2006).

The precise mechanism of action of ATG and ATM in RA is still unknown, however a proposed mechanism of action is the formation of gold-cysteine adducts with target cellular proteins (Bratt et al., 2000; Jeon et al., 2000; Pia Rigobello et al., 2004; Yamashita et al., 2003). The PB1 domain of the atypical PKCs contains a unique cysteine residue, (Cys69) within the conserved OPR, PC and AID (OPCA) motif, which in the crystal structure of the PKCι-Par6 complex resides at the binding interface between PKCι and Par6 (Hirano et al., 2004; Lamark et al., 2003). Mutation of Cys69 to isoleucine (C69I) or valine (C69V), amino acids that frequently reside at this position in other PB1 domains, preserves Par6 binding but makes PKCι resistant to the inhibitory effects of ATM on Par6 binding *in vitro* (Erdogan et al., 2006). Expression of a C69I PKCι mutant in NSCLC cells supports transformed growth, but renders these cells resistant to the inhibitory effects of ATM on transformed growth (Erdogan et al., 2006). Thus, ATM inhibits PKCι-Par6 interactions *in vitro* and *in vivo*, and blocks NSCLC cell transformation by targeting Cys69 within the PB1 domain of PKCι.

Given the clinical potential of ATM as a therapeutic agent we assessed the inhibitory efficacy of ATM on the transformed growth of cell lines representing the major subtypes of lung cancer including lung adenocarcinoma (LAC), lung squamous cell carcinoma (LSCC), large cell carcinoma (LCC), and small cell lung carcinoma (SCLC) (Regala et al., 2008). ATM potently inhibited anchorage-independent growth in all lines tested with IC_{50}s ranging from ~300 nM to 100 μM. The lung cancer cell lines clustered into those that are highly sensitive to ATM (IC_{50}<5 μM) and those that are relatively insensitive to ATM (IC_{50}>40μM). Interestingly, ATM sensitivity did not correlate with tumor sub-type, *KRAS* mutation status or sensitivity to a panel of standard chemotherapeutic agents frequently used to treat lung cancer patients, including cisplatin, placitaxel and gemcitabine (Regala et al., 2008). Rather, elevated PKCι expression was the major molecular characteristic exhibited by lung cancer cells that were responsive to ATM (Regala et al., 2008). Consistent with our *in vitro* observations, ATM inhibits tumorigenicity of both sensitive and insensitive lung cell tumors *in vivo* at plasma drug concentrations consistent with the ATM IC_{50} of the cell lines *in vitro*. Furthermore, measurements of plasma drug concentrations demonstrated that both sensitive as well as insensitive cell lines exhibit an anti-tumor response to ATM at plasma levels routinely achieved in RA patients undergoing ATM therapy (Regala et al., 2008). Thus, ATM exhibits anti-tumor activity against major lung cancer subtypes, particularly tumor cells that express high levels of PKCι. PKCι expression profiling revealed that a significant subset of primary NSCLC tumors express PKCι at or above the level associated with ATM sensitivity *in vitro* (Regala et al., 2008). Therefore, PKCι expression profiling in lung tumor samples may be useful in identifying lung cancer patients most likely to respond to ATM therapy. In addition, the fact that PKCι is overexpressed in many other tumor types (see Table 1 and 2) suggest that ATM may be an effective treatment option for these tumor types as well. Several phase I and phase II clinical trials are currently accruing at Mayo Clinic to determine an appropriate dosing regimen for ATM, and to assess anti-tumor activity of ATM alone and in combination with other targeted therapeutic agents in NSCLC, ovarian cancer and pancreatic cancer.

Summary/Future Directions

Accumulating evidence demonstrates that PKCι is an oncogene that is frequently targeted for genetic alteration in many major forms of human cancer (Tables 1 and 2). Functional data indicate that PKCι is required for the transformed phenotype of NSCLC, pancreatic, ovarian, prostate, colon and brain cancer cells. Future studies will be required to determine whether PKCι is also an oncogene in other cancers. Studies of PKCι using genetically defined models of tumorigenesis have revealed a critical role for PKCι in multiple stages of tumorigenesis, including tumor initiation, progression and metastasis. Recent studies in a genetic model of lung adenocarcinoma suggest a role for PKCι in transformation of lung cancer stem cells. These studies have important implications for the therapeutic use of ATM, particularly if future studies validate PKCι as an important gene in the critical cancer stem cell niche. Significant progress has been made in determining the molecular mechanisms by which PKCι drives the transformed phenotype, particularly the central role played by the oncogenic PKCι-Par6 complex in transformed growth and invasion, and of several PKCι-dependent survival pathways in chemo-resistance. Future studies will be required to determine the composition and dynamics of the PKCι-Par6 complex, and the mechanisms by which oncogenic signaling through this complex is regulated. Likewise, a better understanding of the critical downstream effectors of PKCι in various human tumor types holds promise for identification of novel prognostic and surrogate markers of oncogenic PKCι activity that may be clinically useful in ongoing clinical trials of ATM. Such studies also hold promise of revealing novel therapeutic targets. Similarly, a more complete understanding of the signaling mechanisms by which *Kras* (and possibly other oncogenes) regulate PKCι expression in human tumors may reveal new therapeutic intervention strategies. Ultimately, ongoing and future clinical trials will be required to establish the usefulness of ATM as an anti-tumor agent, and allow validation of potential surrogate markers and predictors of therapeutic response to PKCι-directed therapy identified in pre-clinical models.

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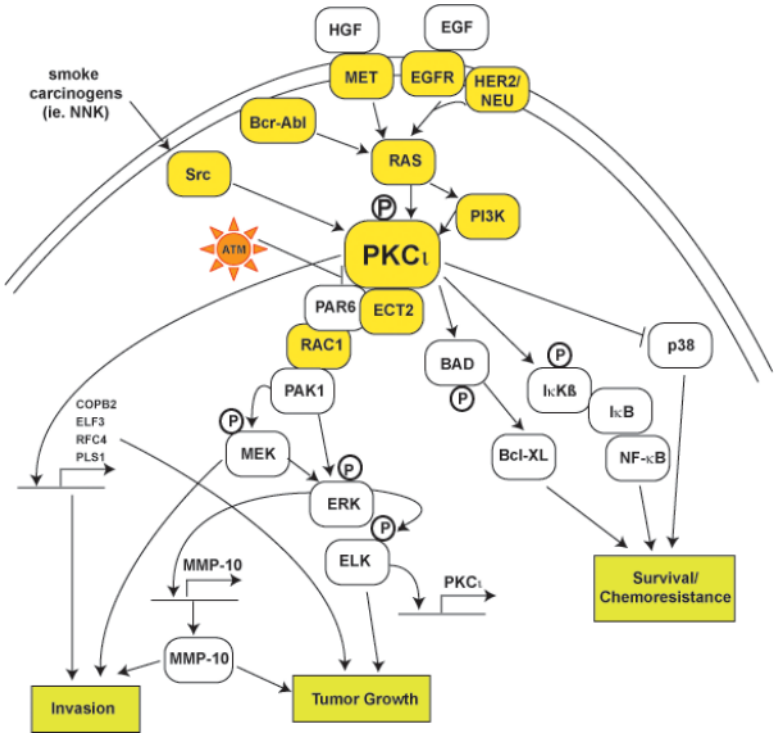

Figure 1. Schematic representation of key oncogenic PKCι signaling pathways

PKCι resides within several major signaling pathways implicated in human cancer. PKCι can be activated by known oncogenes such as Ras, Bcr-Abl, Src and PI3K, cytokines such as TNFα and IL-1, and growth factors such NGF and EGF. PKCι signals to downstream effectors such as Rac1 and NFκB which are important for different aspects of the transformed phenotype. Many components in PKCι-dependent signal pathways are mutated, often by multiple mechanisms (ie. gene amplification and somatic mutation), in human tumors (indicated by yellow boxes). Arrows indicate flow through signaling pathways; touching boxes indicate direct binding of signaling components. Phosphorylation events are indicated by circled Ps.

TABLE 1

PKCι Expression in Human Tumor Types

Tumor type	#Tumor / #Control (p=paired)	Type of Analysis	Result	Reference
Lung	74 / 74 p	blot; qPCR; IHC	Elevated; Amplified; High PKCι predicts poor survival	Regala et al., 2005b
PDAC	28 / 28 p	qPCR; IHC	Elevated; High PKCι predicts poor survival	Scotti et al., 2010
Gastric	177	IHC	Elevated; High PKCι predicts disease recurrence	Takagawa et al., 2010
Colon	5 / 5 p	blot	Elevated	Murray et al., 2004
Esophageal	108	IHC; FISH	Elevated; Amplified; gene amplification correlates with tumor size, stage, LN metastasis; High PKCι predicts metastasis	Yang et al., 2008
Hepatocellular carcinoma	43 / 43 p	RT-PCR; IHC	Elevated; High PKCι correlates with size, metastasis, invasion and stage	Du et al., 2009
Cholangiocarcinoma	41 / 9	IHC	Elevated; High PKCι correlates with differentiation, invasion, LN metastasis, stage; Prognostic for poor survival	Li et al., 2008
Breast	109	IHC	Elevated	Kojima et al., 2008
Ovarian	89	IHC; mRNA; array CGH	Elevated; Amplified; High PKCι correlates with stage	Zhang et al., 2006
Ovarian	235	Array CGH; qPCR; IHC	Elevated; Amplified in serous tumors and high copy # predicts poor survival; nonserous tumors: high PKCι predicts poor survival	Eder et al., 2005
Ovarian	67 / 15	IHC	Elevated; High PKCι predicts poor survival	Weichert et al., 2003
Prostate	29 / 29 p	qPCR, blot	Elevated; High PKCι predicts poor recurrence free survival	Ishiguro et al., 2009
Brain	21 / 12	blot	Elevated	Patel et al., 2008

TABLE 2

PKCι Expression in Publicly Available Human Tumor Microarray Datasets

Cancer Type	#Tumor / #Control	Significant in Tumor (P-value)	Reference
Head and Neck	41 / 13	Yes (0.04)	Ginos et al., 2004
Tongue	26 / 12	Yes (0.009)	Ye et al., 2008
Lung	58 / 49	Yes (0.006)	Landi et al., 2008
Superficial Bladder Cancer	28 / 48	No (0.248)	SanChez-Carbayo et al., 2006
Infiltrating Bladder Urothelial Carcinoma	81 / 48	Yes (5.99E-04)	SanChez-Carbayo et al., 2006
Superficial Bladder Cancer	28 / 9	Yes (3.94E-04)	Dyrskjot et al., 2004
Infiltrating Bladder Urothelial Carcinoma	13 / 9	Yes (2.57E-05)	Dyrskjot et al., 2004
Brain	22 / 76	No (0.068)	Lee et al., 2006
Pancreas	11 / 6	Yes (5.37E-04)	Segara et al., 2005
Melanoma	18 / 7	Yes (1.74E-04)	Talantov et al., 2005
Ovarian	38 / 10	Yes (2.75E-07)	TCGA Ovarian Not Published
Liver (HCC)	35 / 10	Yes (6.67E-04)	Wurmbach et al., 2007
Breast	40 / 7	Yes (0.02)	Richardson et al., 2006
Prostate	69 / 20	Yes (0.003)	Wallace et al., 2008
Acute Myeloid Leukemia	285 / 8	Yes (0.03)	Valk et al., 2004
Renal	10 / 10	Yes (0.003)	Gumz et al., 2007