THE ROLES OF CYCLOOXYGENASE-2 AND PROTEIN KINASE C DELTA IN MUTANT EPIDERMAL GROWTH FACTOR RECEPTOR NON-SMALL CELL LUNG CANCER

by

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ABSTRACT

Lung cancer causes more than 1 million deaths in every year worldwide, and is the leading cause of cancer-related death in the United States. Targeting epidermal growth factor receptor (EGFR) in non-small cell lung cancer (NSCLC) harboring activating mutations in EGFR is an effective treatment but is eventually limited by drug-resistance. This has led to a pressing need to identify alternative treatments or additional targets for resistant tumors. I found abundant activation of cyclooxygenase-2 (COX-2) signal in mutant EGFR NSCLC, suggesting that it might play tumorigenic roles in these tumors. Targeting COX-2 in mutant EGFR lung cancer cells and transgenic mouse models of mutant EGFR lung cancer reduced tumor cell growth, and was more effective in combination with EGFR inhibition. I found that COX-2 signaling regulates interleukin-6 (IL-6) transcription leading to signal transducer and activator of transcription 3 (STAT3) activation. These findings demonstrate that COX-2 modulates the IL-6/STAT3 signaling axis in mutant EGFR NSCLC and targeting COX-2 in combination with EGFR inhibition could be an effective strategy to treat mutant EGFR NSCLC.

Since COX-2 inhibition is a promising chemopreventive agent in other cancers; therefore, I next hypothesized that targeting COX-2 might be a viable strategy to prevent tumor formation. Using transgenic mouse models, I found that targeting COX-2 prior to tumor development delayed tumor formation, which suggests that COX-2 inhibition could be a potential strategy to delay or prevent development of mutant EGFR NSCLC.

In further efforts to identify other targets in mutant EGFR lung cancer, I found evidence of abundant activation of protein kinase C δ (PKCδ). This kinase normally functions as a tumor-suppressor, but it also is considered as a tumor-promoter in some contexts. I found reduced growth of mutant EGFR human lung cancer cells under PKCδ depletion. I also discovered that PKCδ promotes IL-6/STAT3 signal in mutant EGFR NSCLC, suggesting that PKCδ is a tumor-promoter in mutant EGFR NSCLC. Collectively, my data indicate that PKCδ is a potential target to treat mutant EGFR NSCLC.

In this dissertation, I identified COX-2 and PKCδ as novel targets for treating mutant EGFR NSCLC and defined their oncogenic roles in mutant EGFR NSCLC by regulating the critical oncogenic signal, IL-6/STAT3.

Dedicated to my family.

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CHAPTER 1

INTRODUCTION

Non-small cell lung cancer and mutant epidermal growth factor receptor

Lung cancer has been the leading cause of cancer-related death in the United States for decades (1, 2). Each year, this disease typically causes more than 1 million deaths worldwide (3). Lung cancer is histologically divided into two subtypes, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC is thought to originate in neuroendocrine cells and accounts for approximately 10-15% of all lung cancers (4). NSCLC originates in lung epithelial cells, accounts for 80% of primary lung cancers, and includes various histological subtypes such as adenocarcinoma, bronchioalveolar cell carcinoma, squamous cell carcinoma, anaplastic and large cell carcinoma (5). Smoking is considered a major risk factor for NSCLC. When compared with never-smokers, the risk of developing NSCLC is increased about 10- to 20-fold in smokers (6); however, globally, about 25% of NSCLC is found in never-smokers: 15% in men and 53% in women (3, 7). The major causes of NSCLC in never-smokers are likely related to environmental exposures such as second-hand tobacco smoke, radon and indoor coal burning (8, 9). These agents are thought to cause oncogenic genetic alterations in key regulatory genes such as epidermal growth factor growth factor receptor (*EGFR*) and *KRAS*. At least two lines of

evidence suggest that EGFR plays important roles in lung tumorigenesis. First, a number of somatic mutations have been identified in the coding region of *EGFR* in tumors from patients diagnosed with lung cancer. Second, retrospective analyses revealed that EGFR-overexpression is a feature of more than 60% of NSCLC cases (10, 11).

EGFR is a member of the ErbB family of receptor tyrosine kinases (RTKs) which are expressed on the cell surface and mediate signaling events elicited by extracellular growth factors (12, 13). Following binding of a ligand such as EGF or transforming growth factor-α (TGF-α), EGFR undergoes conformational changes that facilitate the formation of either homo- or hetero-dimers with other members of the ErbB family. These interactions result in activation of EGFR which occurs when tyrosine residues in the activation loop of the cytosolic domain of the receptor become phosphorylated. Activation of EGFR initiates a cascade of signaling events that affect key cellular functions such as proliferation and survival. The mechanisms involved in these responses involve participation of three major signaling pathways, including the phosphatidylinositol 3-kinase (PI3K)/Akt, Ras/mitogen-activated protein kinase (MAPK), and signal transducer and activator of transcription (STAT) axes (7, 14, 15). More than 90% of the *EGFR* mutations identified in NSCLC are accounted for by deletions in exon 19 and by a mutation in the kinase domain of the receptor that results in substitution of leucine 858 with arginine [L858R, (16, 17)]. These alterations in EGFR increase its basal and ligand-induced activity. These conclusions were derived from crystallographic and biochemical studies showing that the normal auto-inhibitory function of EGFR is disrupted in mutant forms of the receptor, leading to a 50-fold increase in kinase activity in mutant versus wild-type EGFR (18, 19). Hyper-activated, mutant forms of EGFR constitutively engage downstream signaling axes such as

PI3K/Akt, Ras/MAPK and STAT, contributing to NSCLC. As such, disrupting EGFR signaling appears to be a promising therapeutic strategy for the treatment of NSCLC harboring mutant forms of EGFR.

Targeting EGFR and drug resistance

NSCLC patients subjected to cytotoxic chemotherapies have moderate increases in survival, but this benefit is associated with significant toxicity (20). Molecularly targeted therapeutics are currently viewed as more promising and safer strategies to treat NSCLC. Distinct subsets of cancers are characterized by specific oncogenic mutations that are essential for tumorigenesis and cancer cell survival, and targeting the mutant proteins resulting from these mutations can result in cancer cell death with limited toxicity. Thus, targeting EGFR has emerged as a promising strategy to treat mutant EGFR NSCLC. Diverse approaches have been used to accomplish this, and various EGFR inhibitors have been developed and tested in human clinical trials. These include monoclonal antibodies that bind to the extracellular domain of EGFR (*e.g.*, cetuximab and matuzumab), and small molecules that block the tyrosine kinase activity of the receptor [*e.g.*, gefitinib, erlotinib, and other tyrosine kinase inhibitors or TKIs, (21)]. Specifically, gefitinib and erlotinib have been reported to be highly effective in NSCLC patients with activating mutations in EGFR (22, 23). Gefitinib and erlotinib act as reversible inhibitors of EGFR by competitively inhibiting binding of ATP to the kinase domain of the receptor (24, 25). While 50-80% of NSCLC patients harboring mutant EGFR initially respond to gefitinib or erlotinib, most patients become resistant to the drug in approximately 6-12 months (26, 27). Since treatment with gefitinib or erlotinib is initially effective, it is thought that tumor

relapse is the result of acquired resistance. Although this response can result from diverse mechanisms (*e.g.*, MET amplification), more than 50% of NSCLC patients who develop resistance to gefitinib or erlotinib develop a second EGFR mutation whereby threonine-790 is replaced by methionine [T790M, (13, 28-31)]. Development of T790M is thought to interfere with drug binding to the EGFR ATP pocket, which leads to resistance to gefitinib or erlotinib.

Diverse approaches have been tested in preclinical and clinical studies in order to inhibit EGFR and overcome resistance to TKI treatment in mutant EGFR NSCLC. Irreversible EGFR inhibitors such as neratinib and afatinib that bind covalently to EGFR have been developed as second generation TKIs; however, preclinical and clinical studies have shown that their effectiveness as single interventions is limited (32-34). Another strategy to overcome drug-resistance to TKIs in mutant EGFR NSCLC is targeting heat shock protein 90 (HSP90). Since it has been observed that association of mutant EGFR with HSP90 stabilizes the receptor, drugs such as geldanamycin have been used to disrupt the interaction between mutant EGFR and HSP90, which results in degradation of the receptor and stimulation of apoptosis of mutant EGFR NSCLC cell lines (35). Despite its preclinical effects, it is not known whether HSP90 inhibition is effective in humans with mutant EGFR NSCLC. In addition, studies using L858R+T790M mutant *EGFR* transgenic mice have tested whether combinations of two different types of EGFR inhibitors can effectively overcome drug-resistance. The combination of cetuximab and afatinib resulted in decreased tumor growth in this mouse model and in xenograft studies (33). These results indicate that combinations of TKIs with other inhibitors that disrupt additional signaling pathways involved in tumorigenesis can be a potentially effective strategy to overcome

drug resistance. In this dissertation, I will discuss my efforts to identify critical oncogenic signaling pathways that can be targeted to treat NSCLC patients whose tumors are characterized by deregulated signaling *via* EGFR.

Cyclooxygenase-2

A signaling pathway that may play important roles in mutant EGFR NSCLC is the cyclooxygenase-2 (COX-2) pathway which has been shown to participate in key events in tumorigenesis. COX-2 is an enzyme that contributes to inflammation owing to its role in the biosynthesis of prostaglandin precursors. Specifically, arachidonic acid generated from plasma membrane phospholipids is metabolized by COX-2 to prostaglandin endoperoxide H_2 (PGH_2). PGH_2 is then converted to diverse prostaglandin products by downstream enzymes such as prostaglandin E synthase (PGES), prostaglandin D synthase (PGDS) and prostaglandin F synthase (PGFS). COX-1 is constitutively expressed in a wide range of tissues in mammals. In contrast, COX-2 expression is induced in settings of inflammation, leading to prostaglandin biosynthesis and secretion. This series of events are required for responses such as fever and pain that are typical of physiological inflammation (36, 37).

Aside from its function in physiological inflammation, COX-2 appears to play a critical role in tumorigenesis. COX-2 overexpression has been reported in various types of solid tumors such as colon cancer, esophageal carcinoma, melanoma, pancreatic cancer and lung cancer (38-42). The contribution of COX-2 to tumorigenesis is further supported by the observation that COX-2 is a target of oncogenes, tumor suppressors and growth factors. COX-2 expression is regulated by the Wnt and Ras pathways (48). In addition, basic fibroblast growth factor (bFGF) stimulates COX-2 production (49), and COX-2

expression is frequently associated with p53 accumulation in ovarian adenocarcinomas (49). Recently, a large number of studies showed that COX-2 contributes to tumor initiation, promotion and metastasis of a diverse set of solid tumors (37, 43-47). Mouse models of colon cancer and breast cancer have shown that COX-2 overexpression is sufficient to initiate tumor formation (38, 51). In a transgenic skin tumor mouse model, overexpression of COX-2 was not sufficient to develop tumors, but led to increased susceptibility to carcinogenesis, suggesting a role for this enzyme in cellular sensitization to carcinogens (52). Additionally, in lung and breast cancers, COX-2 is highly upregulated in metastatic cells, and inhibiting COX-2 decreases metastatic phenotypes (53, 54). Mechanistically, studies by our group reported that activation of EGFR induces COX-2 expression, prostaglandin E_2 (PGE_2) biosynthesis, and release of the EGFR ligand transforming growth factor alpha (TGF-α). These events further activate EGFR, and represent a feed-forward autocrine loop that contributes to colon tumorigenesis (55). These observations formed the basis for studies in which I investigated whether deregulated COX-2 signaling is also a feature of mutant EGFR lung cancers. My studies showed that these malignancies are characterized by up-regulation of COX-2 expression and signaling, and led me to test the hypothesis that targeting COX-2 can have therapeutic utility for the treatment of mutant EGFR lung cancer.

Strategies that target EGFR and COX-2 in tumorigenesis

To inhibit COX-2, I have used celecoxib, the first COX-2 specific inhibitor originally used to reduce pain in osteoarthritis, rheumatoid arthritis, painful menstruation and ankylosing spondylitis. Celecoxib effectively relieves pain without the severe side

effects associated with the use of conventional nonsteroidal antiinflammatory drugs [NSAIDs, (56)]. Despite this advantage, there are potential risks associated with long-term celecoxib use, including increased cardiovascular risk and gastrointenstinal bleeding. Nonetheless, celecoxib is an attractive therapeutic approach to treat or prevent lung tumorigenesis due to its oral bioavailability, widespread use, relatively few side effects, and affordability compared to other cancer chemotherapy drugs. In fact, a large number of studies currently are evaluating whether celecoxib prevents tumor development in the lung, colon, breast, esophagus, pancreas, liver and brain (57).

A second approach commonly used to treat tumors is to target distinct oncogenic pathways through combination therapies. For example, combined administration of celecoxib and chemotherapeutic drugs such as paclitaxel and carboplatin enhances drug responses in patients with early stage NSCLC (58). Since combined drug strategies appear to be more effective than monotherapies in mutant EGFR lung cancer (33, 35), it seems logical to target additional components in the central oncogenic pathway in mutant EGFR NSCLC. As noted, COX-2 is likely to participate in tumorigenesis promoted by mutant EGFR. Therefore, in addition to testing the effect of single COX-2 inhibition, I assessed the impact of combined COX-2 and EGFR inhibition in the treatment of mutant EGFR NSCLC. Specifically, I tested whether administration of celecoxib in combination with a TKI is a reasonable approach to treat mutant EGFR NSCLC.

Protein kinase Cδ: another potential target

To evaluate additional contributors to the tumorigenic process that is typical of mutant EGFR NSCLC, I studied the role of protein kinase Cδ (PKCδ). The PKC family is

a group of serine/threonine kinases involved in the regulation of cell proliferation, survival, migration, and apoptosis. PKC isozymes are subcategorized in three groups based on the mechanism of activation: classical PKCs (PKCα, β, γ), novel PKCs (PKCδ, ε, η, θ), and atypical PKCs (PKCζ, ι). PKCs mature through a series of phosphorylation events, and mature, "primed," PKCs are translocated to the plasma membrane and activated by increased intracellular calcium and diacylglycerol (59-61). Importantly, I found that PKCδ is primed in mutant EGFR NSCLC, and this observation led me to investigate whether PKCδ contributes to the pathogenesis of mutant EGFR NSCLC.

PKCδ is generally recognized as a tumor suppressor. This is based on the observation that a constitutively-active catalytic fragment of PKCδ cleaved by caspase-3 migrates to the nucleus and contributes to apoptosis by phosphorylating DNA-dependent protein kinase (DNAPK), phospholipid scramblase 1 (PLS1) and lamin B (61-65). However, studies in K-Ras lung adenocarcinomas, pancreatic tumors, and ErbB2-driven breast cancers, reported that PKCδ can *promote* tumorigenesis in certain molecular contexts (66-68). These observations combined with our finding that primed PKCδ is a feature of mutant EGFR NSCLC led me to conduct studies aimed at characterizing potential tumor-promoting roles for PKCδ in mutant EGFR NSCLC, and evaluating whether targeting PKCδ could be an effective strategy to treat mutant EGFR NSCLC.

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CHAPTER 2

CYCLOOXYGENASE-2 INHIBITION REDUCES GROWTH OF MUTANT EGFR LUNG TUMORS

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Abstract

Targeted therapies reduce the growth of mutant epidermal growth factor receptor (EGFR) non-small cell lung cancers (NSCLC), but unfortunately most patients develop drug-resistance. This has led to efforts aimed at developing therapies that target resistant tumors. We investigated the role of cyclooxygenase-2 (COX-2) in mutant EGFR NSCLC using transgenic mice and human lung cancer cells harboring mutant EGFR and found abundant activation of the COX-2 signaling axis. Targeting COX-2 in human lung cancer cells reduced cell proliferation and anchorage-independent growth, and inhibiting COX-2 in mice harboring mutant EGFR significantly reduced lung tumor growth. Dual-targeting of COX-2 and EGFR had more pronounced effects. We found evidence that by generating prostaglandin E_2 (PGE_2), COX-2 promotes IL-6 transcription leading to activation of STAT3 in an EGFR-independent manner. Collectively, our studies demonstrate that COX-2 modulates oncogenic signaling in mutant EGFR lung tumors through the IL-6/STAT3 signaling axis, and that inhibiting COX-2 could be a viable strategy to treat mutant EGFR lung cancers when combined with EGFR inhibition.

Introduction

Lung cancer causes over 1 million deaths every year worldwide (1) and has been the leading cause of cancer-related deaths in the United States for decades (2, 3). NSCLC is thought to originate from lung epithelial cells and comprises about 80% of primary lung cancers. Patients with NSCLC treated with cytotoxic chemotherapy have moderate increases in survival, but that comes with significant toxicity (4). More promising strategies that target specific oncogenic mutations essential for tumorigenesis are being

developed. These therapies can result in cancer cell death with limited side effects. An example of this strategy is the use of specific kinase inhibitors to target tumors harboring activated mutant EGFR as a first line therapy. However, initial positive responses typically are followed by development of resistance to EGFR inhibitors (5). The most common cause of resistance is an acquired mutation involving a substitution of threonine to methionine at amino acid 790 (T790M) in EGFR (6-9).

EGFR resistance has been studied and tested in preclinical and clinical studies. First, second generation irreversible EGFR inhibitors have been developed (10-12), but despite promising preclinical data, inhibitors such as afatinib are only minimally effective in the context of T790M (13, 14). A second approach to overcome T790M-mediated and other forms of resistance is the use of EGFR inhibitors in combination with other inhibitors such as cetuximab (11), rapamycin (15), or 17-DMAG (16). In preclinical studies, these strategies led to significantly improved responses, demonstrating that T790M-mediated resistance can be overcome by a dual inhibitor approach. In addition, third generation EGFR inhibitors, such as AZD9291 and rociletinib, that specifically target T790M EGFR have been developed. These inhibitors had impressive results in clinical trials, but approximately 40-50% of patients failed to respond to them (14, 17). To identify additional options to treat mutant EGFR lung cancer, we sought to find other targetable signaling pathways that are important for mutant EGFR tumorigenesis and discovered that COX-2 acts in concert with mutant EGFR.

COX-2 is an inflammatory enzyme and its over expression has been reported in various types of solid tumors (18-20). COX-2 is not simply a marker of tumorigenesis as numerous studies have shown that it actively contributes to tumor initiation, promotion and

metastasis (21). Studies in cellular and mouse models have shown that COX-2 over expression contributes to malignant phenotypes as shown by deregulated cell growth, resistance to apoptosis, increased angiogenesis, metastasis, inflammation, and evasion of host immune surveillance (18, 22-26). In this study, we evaluated the role of COX-2 in the context of mutant EGFR NSCLC and found evidence that combined inhibition of EGFR and COX-2 is a promising strategy to treat mutant EGFR NSCLC.

Results

The COX-2 / PGE_2 axis is activated in mutant EGFR lung tumors

Because COX-2 is abundantly expressed in many types of solid tumors and participates in tumorigenesis (18-26), we evaluated the COX-2/PGE_2 axis in a mouse model of mutant EGFR lung cancer using *CCSP-rtTA; TetO-EGFR*$^{L858R+T790M}$ (*EGFR* $^{L/T}$) mice. These mice harbor a doxycycline-inducible *EGFR* $^{L/T}$ transgene that promotes lung tumorigenesis when the mice receive doxycycline (11). Control mice had the *EGFR* $^{L/T}$ transgene, but lacked the *rtTA* transgene necessary to induce *EGFR* $^{L/T}$ transcription. As expected, we found high levels of EGFR, abundant tyrosine phosphorylated EGFR (pEGFR), and strong induction of proliferating cell nuclear antigen (PCNA) in lung tissues of *EGFR* $^{L/T}$ mice, but not control mice (Fig. 2.1A-B). Lung lysates from *EGFR*$^{L/T}$ mice also demonstrated significantly increased levels of COX-2 and microsomal prostaglandin E synthase-1 (mPGES-1), an enzyme that couples with COX-2 to generate PGE_2 (Fig. 2.1B). Next, we evaluated activity of the COX-2/mPGES-1 synthesis pathway by measuring PGE_2 metabolites in lung lavage from the mice and found significantly increased levels of PGE_2 metabolites in *EGFR* $^{L/T}$ mice compared to control mice (Fig.

2.1C). This striking increase in PGE_2 is likely due not only to the high levels of COX-2 and mPGES-1, but also to reduced levels of 15-hydroxy prostaglandin dehydrogenase (15-PGDH), an enzyme that degrades PGE_2, (Supplementary Fig. 2.S1). Linkage between reduced expression of 15-PGDH and tumor progression has been reported in several cancers (28-30). In addition to the mouse model, we evaluated human lung cancer tissues harboring mutant EGFR and found high expression levels of COX-2 (Fig. 2.1D). Collectively, these data indicate that the COX-2 / PGE_2 axis is abundantly active in mutant EGFR lung tumors.

COX-2 inhibitors reduce proliferation and anchorage-independent growth of human mutant EGFR lung cancer cells

To evaluate the contribution of COX-2 to mutant EGFR lung tumorigenesis, we tested the effects of inhibiting COX-2 on *in vitro* tumor cell growth. We used the human mutant EGFR lung cancer cell lines H1975 or H1650 and treated them with celecoxib, a specific inhibitor of COX-2, either alone or in combination with afatinib, a second generation EGFR inhibitor. We found that celecoxib reduced cell proliferation by 50% when used alone, while combined inhibition with celecoxib and afatinib reduced proliferation by more than 80% (Fig. 2.2A). In anchorage-independent growth assays, we found that celecoxib reduced colony growth and that dual-treatment with celecoxib and afatinib generally had an even more pronounced effect on colony growth (Fig. 2.2B). These data support our hypothesis that COX-2 plays an important role in mutant EGFR lung cancer and suggest that targeting COX-2 either alone or in combination with EGFR inhibition is a potentially useful strategy to treat mutant EGFR lung cancer.

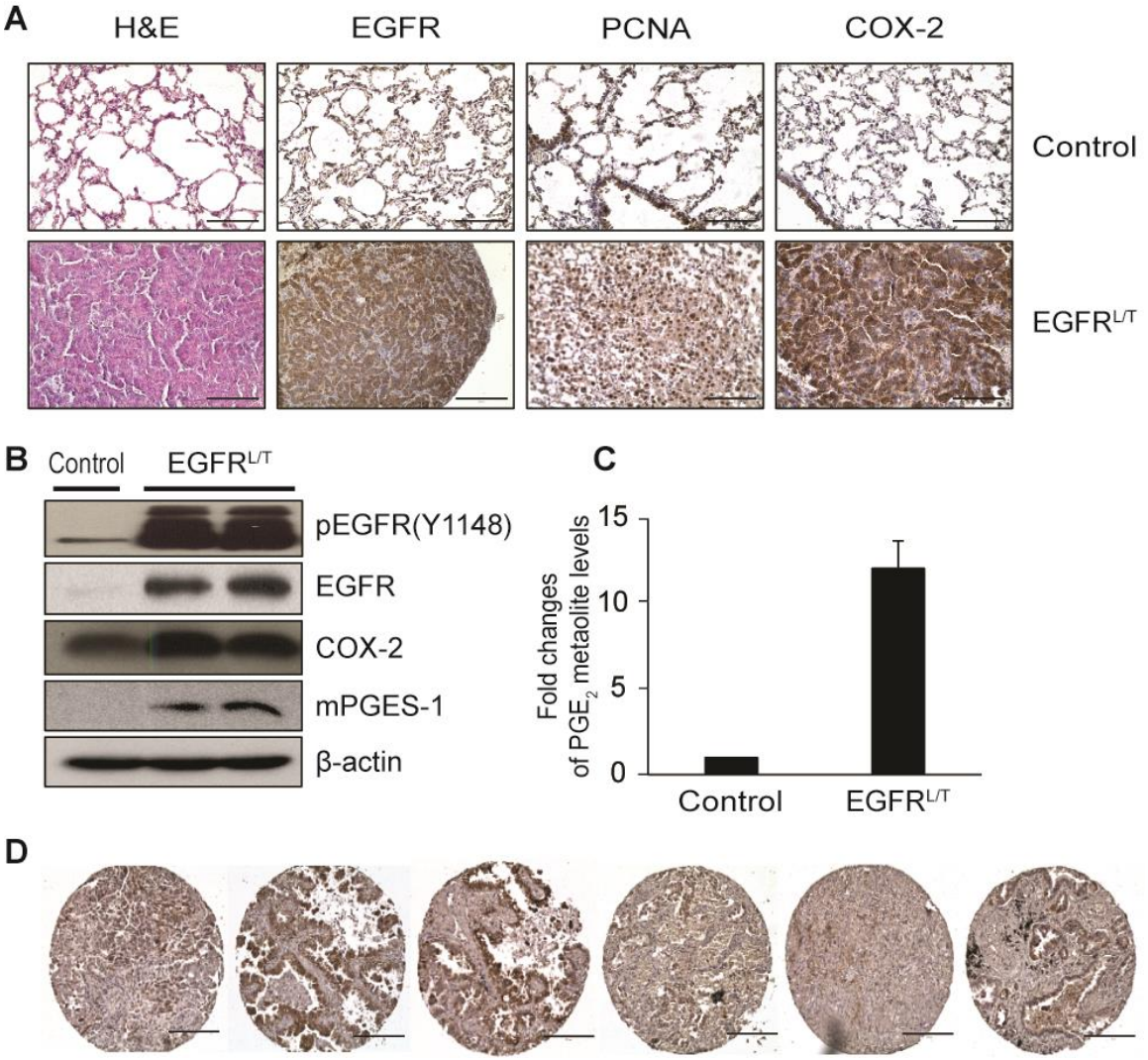

Figure 2.1. The COX-2/PGE$_2$ axis is activated in mutant EGFR lung tumors. A. H&E and IHC staining for EGFR, PCNA, and COX-2 of control or *EGFR$^{L/T}$* mouse lung tissues. Scale bar = 100μm. B. Western blots of pEGFR(Y1148), EGFR, COX-2, and mPGES-1 of control or *EGFR$^{L/T}$* mouse lung lysates. C. Relative levels of PGE$_2$ metabolites in mouse lung lavage fluid. *EGFR$^{L/T}$* mice had 12.1-fold higher levels of PGE$_2$ metabolites than control mice. Data are presented as mean ± SEM. n = 11 and 16 for control and *EGFR$^{L/T}$*, respectively. p = 0.000003. D. IHC staining for COX-2 in mutant EGFR lung tissues from 6 patients. Scale bar = 200μm.

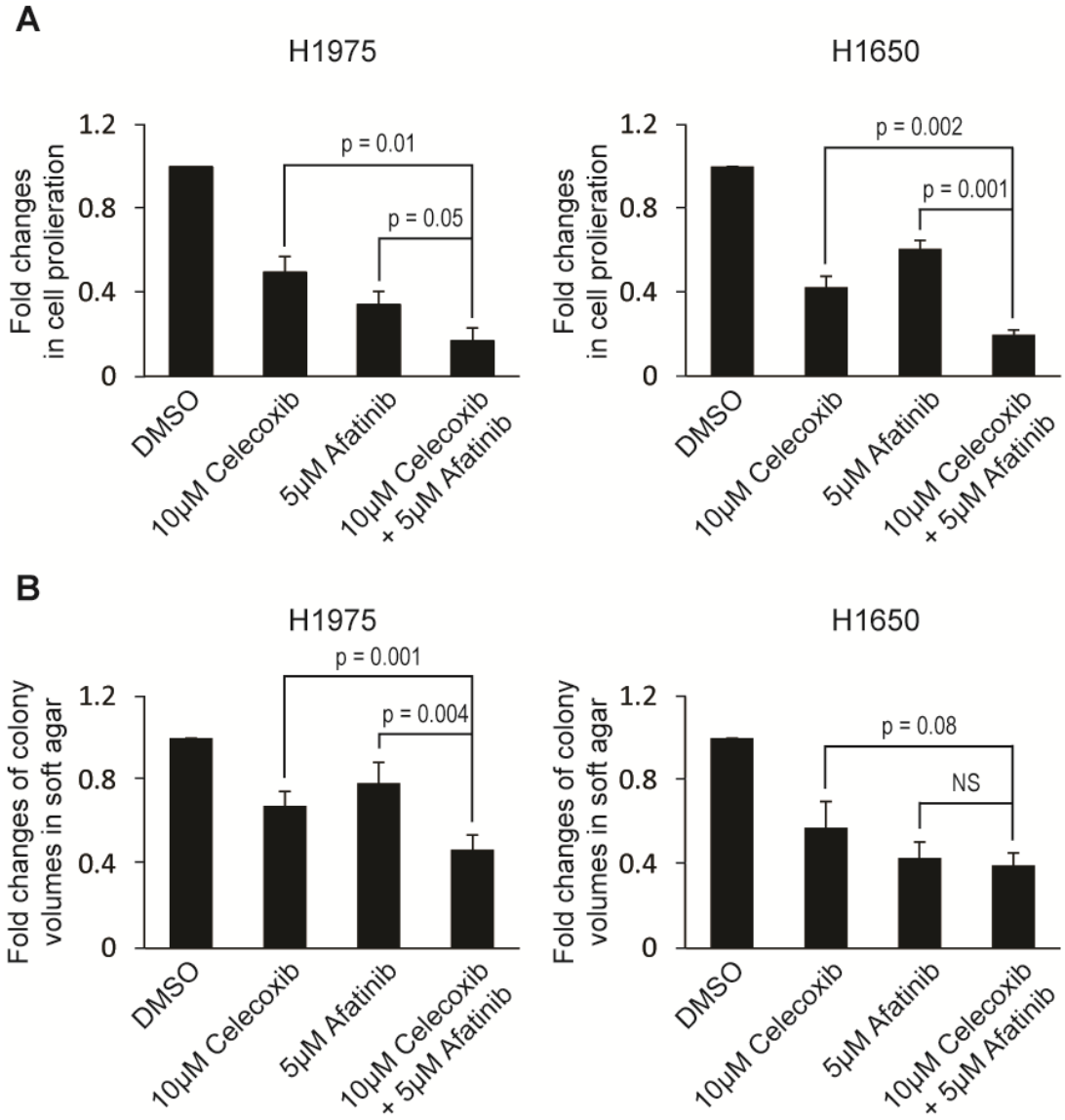

Figure 2.2. COX-2 inhibition reduces proliferation and anchorage-independent growth of human mutant EGFR lung cancer cells. A. Cell proliferation under celecoxib, afatinib, or both inhibitors. n = 3 individual experiments. p-values compared to control (H1975 - ≤ 0.004; H1650 - ≤ 0.0005). B. Colony volumes in soft agar under celecoxib, afatinib, or both inhibitors. n = 30 colonies (10 from 3 individual experiments). p-values compared to control (H1975 - ≤ 0.0005; H1650 - ≤ 0.01). NS represents not significant. All data are presented as mean ± SEM.

Dual-targeting of COX-2 and EGFR results in reduced tumor growth in mutant EGFR mice

To evaluate the effectiveness of combined COX-2 and EGFR inhibition *in vivo*, we used *EGFR* $^{L/T}$ mice. Control mice lacking the *rtTA* transgene necessary to induce *EGFR* $^{L/T}$ transcription or experimental *EGFR* $^{L/T}$ mice that harbored both transgenes were treated with doxycycline for 5 to 8 weeks followed by CT scanning to quantify tumor loads. Once tumor loads reached about 20% of lung tissue volume (average tumor loads were about 23%), mice were treated with celecoxib, afatinib, or both medications. After two and four weeks of treatment, all mice were subjected to CT scans to evaluate tumor load (Fig. 2.3A), and then after the fourth week scan, mice were sacrificied to collect tissue and lung lavage samples. Using the CT scans, we reconstructed three-dimensional images of the lungs and found that tumors in control mice grew rapidly during the four-week period. Single drug treatment with celecoxib or afatinib moderately reduced tumor growth compared to control mice, while combined treatment with celecoxib and afatinib more strikingly reduced tumor growth (Fig. 2.3B). Consistent with these changes, we found reduced PCNA levels in mice treated with both celecoxib and afatinib (Fig. 2.3C).

After quantifying the images at the beginning of treatment and after two and four weeks of treatment we found that untreated mice had an average 3.9-fold increase in tumor load during the four-week study (Fig. 2.4A and E). Single drug treatment with either celecoxib or afatinib slowed tumor growth, but tumor loads still increased in these mice by about 2.6-fold and 1.9-fold, respectively (Fig. 2.4B, C and E). In contrast to the tumor growth that occurred while the mice received single drug treatment, dual drug treatment with celecoxib and afatinib led to minimal changes in tumor load during the four-week

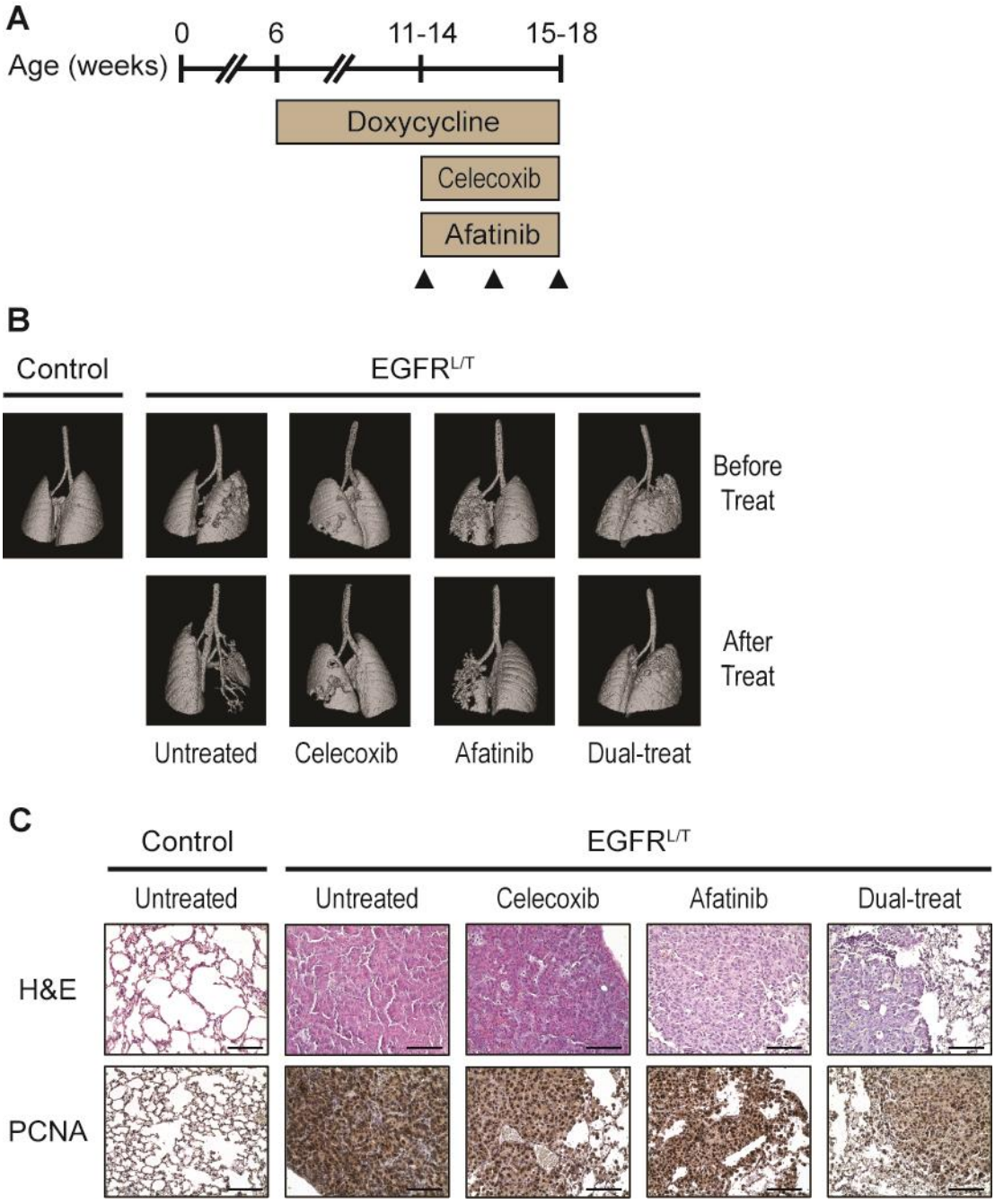

Figure 2.3. Dual-targeting COX-2 and EGFR reduces tumor growth in mutant EGFR mice. A. Timeline for tumor-induction in transgenic mice followed by drug treatment. Arrowheads indicate the time of CT scans. B. Reconstructed 3-D images of mouse lung tissues from different drug-treatment conditions. Reconstructed normal lungs are shown, and tumor tissue is shown as missing tissue. C. H&E and IHC staining for PCNA of mouse lung tissues. Scale bar = 100μm.

study (Fig. 2.4D and E). This result indicates that dual-targeting of COX-2 and EGFR could be an effective strategy to overcome EGFR inhibitor resistance in mutant EGFR lung cancer. Given the abundant activity of the COX-2 / PGE_2 axis and the reduced tumor growth under COX-2 inhibition, we next sought to identify mechanisms by which COX-2 contributes to mutant EGFR tumorigenesis.

The COX-2 / PGE_2 axis regulates IL-6/STAT3 signaling

To identify tumorigenic roles of COX-2 in mutant EGFR NSCLC, we first examined canonical EGFR downstream signaling pathways and found that the STAT3 signaling pathway was consistently activated in lungs from *EGFR* $^{L/T}$ mice (Fig. 2.5A). This is consistent with published studies showing elevated levels of activated STAT3 in human tumors harboring mutant EGFR (31). In that context, STAT3 is activated through the IL-6/JAK signaling axis (30), so we next evaluated IL-6 levels in lavage from *EGFR*$^{L/T}$ mice and found significantly increased levels of IL-6 in *EGFR* $^{L/T}$ mice compared to control mice (Fig. 2.5B). We also found abundant secretion of IL-6 from mutant EGFR lung cancer cell lines (not shown), which was consistent with published data (31), and loss of STAT3 phosphorylation in EGFR mutant cells exposed to a JAK inhibitor (Supplementary Fig. 2.S2). Collectively these data indicate that the IL-6/STAT3 axis is highly active in *EGFR* $^{L/T}$ mice.

Next, we sought to determine if there is a mechanistic connection between COX-2 and IL-6/STAT3 signaling by treating H1975 human lung cancer cells with exogenous dimethyl-PGE_2 to stably mimic COX-2 signaling. We found that dimethyl-PGE_2 increased phosphorylation of STAT3 and promoted IL-6 secretion (Fig. 2.5C and D). In the converse

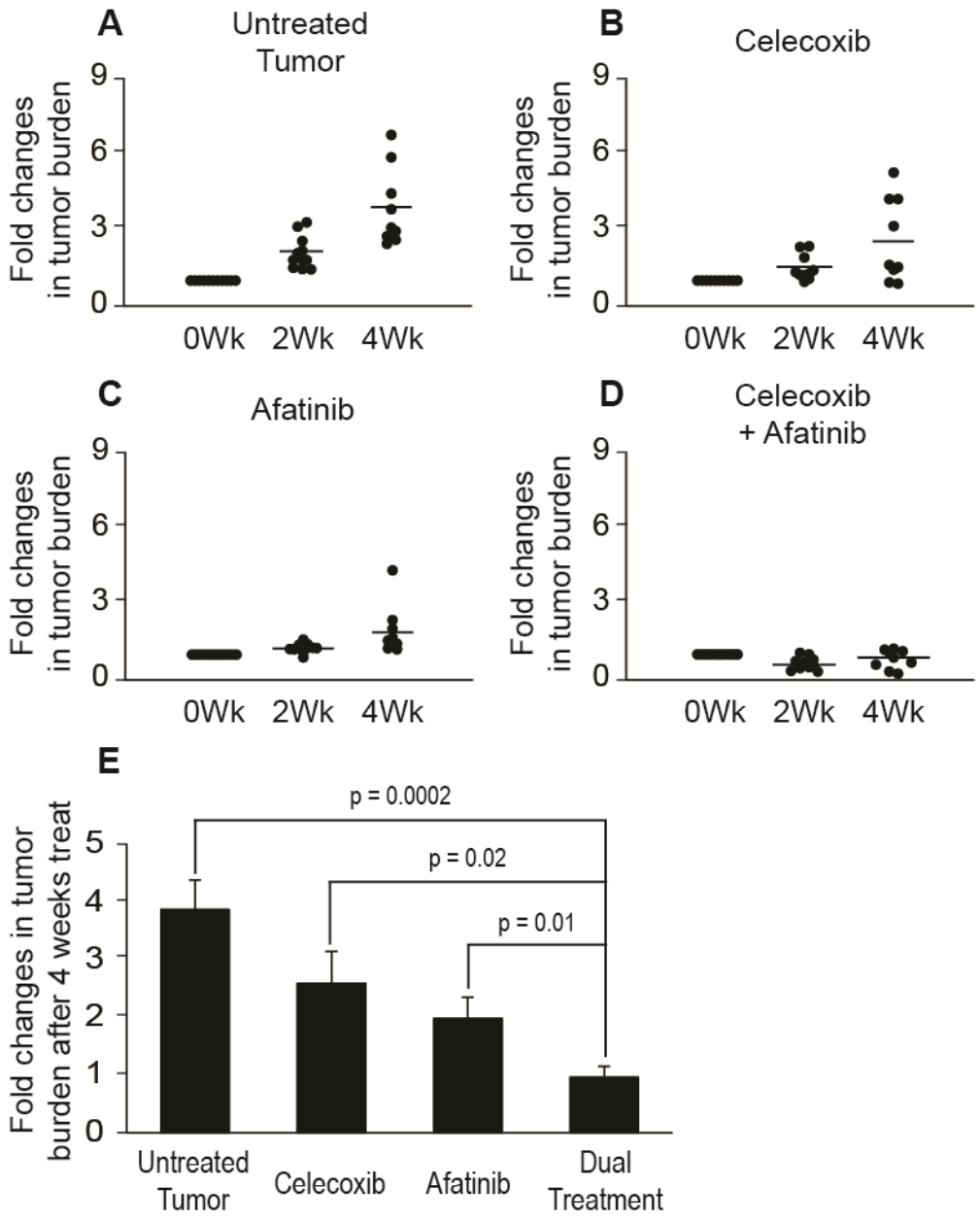

Figure 2.4. Reduced tumor burden in *EGFR*$^{L/T}$ mouse lungs after 4-week treatment with celecoxib and/or afatinib. Changes in tumor growth in: A. Untreated mice. p-values compared to 0-week treatment are ≤ 0.00001. n = 11 and 9 at 2 weeks and 4 weeks, respectively; B. Celecoxib-treated mice. p-values compared to 0 week treatment are ≤ 0.004. n= 10 and 9 at 2 weeks and 4 weeks, respectively. C. Afatinib-treated mice. p-values compared to 0 week treatment are ≤ 0.003. n = 11 and 8 at 2 weeks and 4 weeks, respectively; D. Combined treatment with celecoxib and afatinib. p = 0.0002 and 0.066 for 2 weeks and 4 weeks, respectively. n = 10. A-D. Data are presented as dots indicating fold changes in tumor burden of individual mice, and bars indicating mean of each set of mice. E. Tumor burdens in EGFR$^{L/T}$ mice are compared at the 4 weeks drug-treatment point. Data are presented as mean ± SEM.

experiment, we tested the effects of inhibiting COX-2 and found that celecoxib reduced both STAT3 phosphorylation and IL-6 secretion (Fig. 2.5E and F). These data suggest that the COX-2/PGE_2 axis activates STAT3 and IL-6 signaling.

Simultaneous inhibition of COX-2 and EGFR reduces IL-6/STAT3 signaling in mutant EGFR lung cancer

Given our evidence that inhibiting COX-2 reduces signaling through the IL-6 / STAT3 axis, we investigated the effects of dual inhibition of COX-2 and EGFR on IL-6 and STAT3 regulation in tissues from mutant EGFR mice and in human lung cancer cells. In mouse lung tissues, we found that both celecoxib and afatinib when used alone moderately reduced levels of phosphorylated STAT3 compared to control mice, while combined treatment with both inhibitors markedly reduced STAT3 phosphorylation (Fig. 2.6A). The increased levels of total EGFR, COX-2 and mPGES-1 in dual-treated mouse lung tissues indicate that reduced STAT3 activation occurred in tumor tissue. Similarly, single drug treatment with either celecoxib or afatinib reduced levels of IL-6 in mouse lung lavage fluid while combined treatment with both inhibitors significantly reduced IL-6 levels (Fig. 2.6B). And consistent with transgenic mice, we found in cultured cells that combined inhibition of COX-2 and EGFR reduced phosphorylation of STAT3 and secretion of IL-6 compared to single drug treatment (Fig. 2.6C-D). These results indicate that dual-targeting of COX-2 and EGFR can markedly downregulate IL-6/STAT3 signaling, suggesting that blocking this signaling axis contributed to the reduced lung tumor growth that we observed in *EGFR* $^{L/T}$ mice (Fig. 2.4) since this signaling axis has been shown to have a critical oncogenic role in mutant EGFR lung tumors (31).

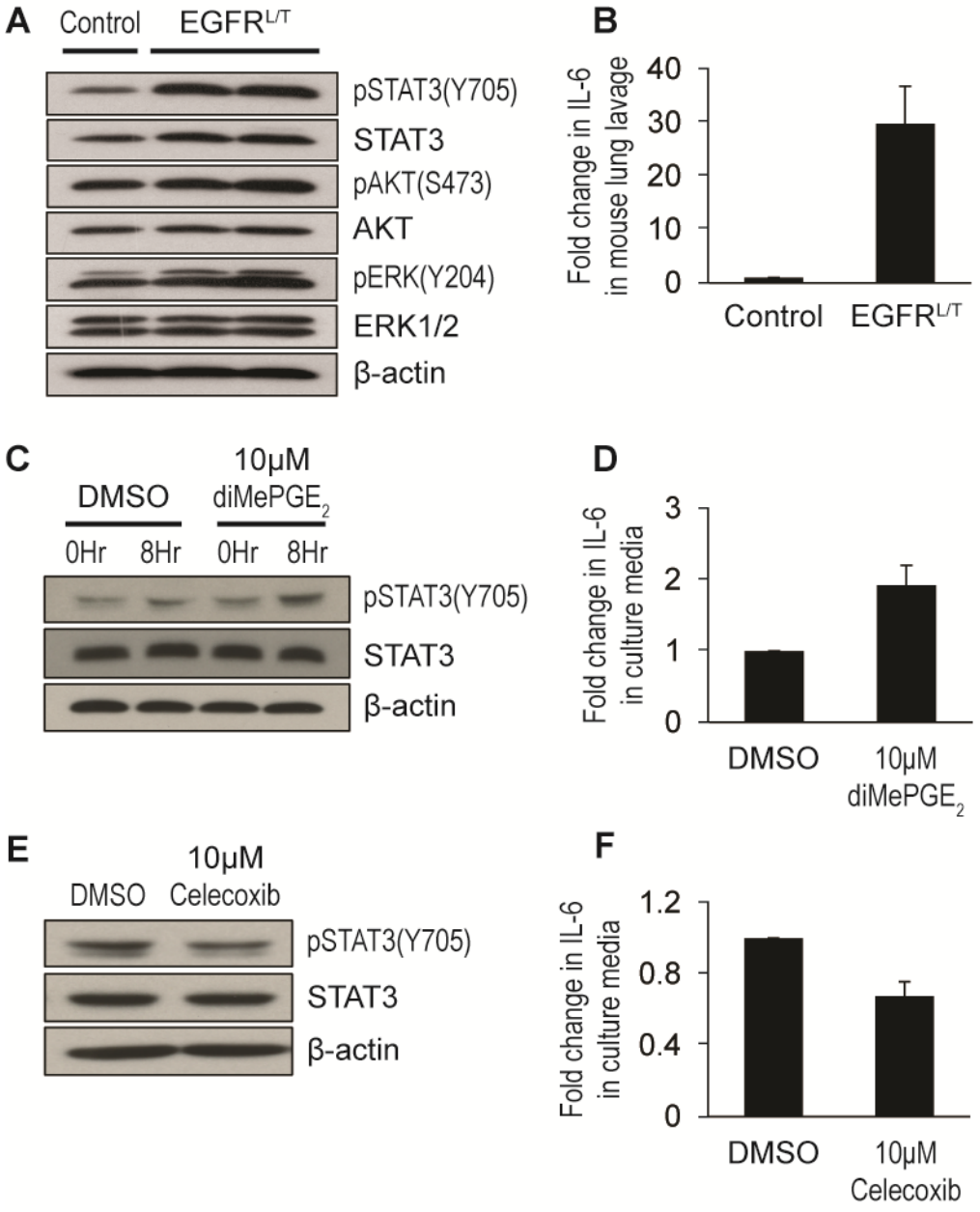

Figure 2.5. The COX-2/PGE_2 axis regulates IL-6/STAT3 signaling. A. Western blots of STAT3, AKT, and ERK in mouse lung lysates. B. Relative IL-6 levels in mouse lung lavage fluids. n = 10. p = 0.0005. C. Western blots of H1975 cells under 8-Hr di$MePGE_2$ treatment. D. Relative IL-6 levels in H1975 culture media under 8 hours treatment with di$MePGE_2$. n = 3 individual experiments. p = 0.015. E. Western blots of H1975 cells under 8 hours celecoxib treatment. F. Relative IL-6 levels in H1975 culture media under 8 hours treatment with celecoxib. n = 3 individual experiments. p = 0.001. Data in panels B, D, and F are presented as mean ± SEM.

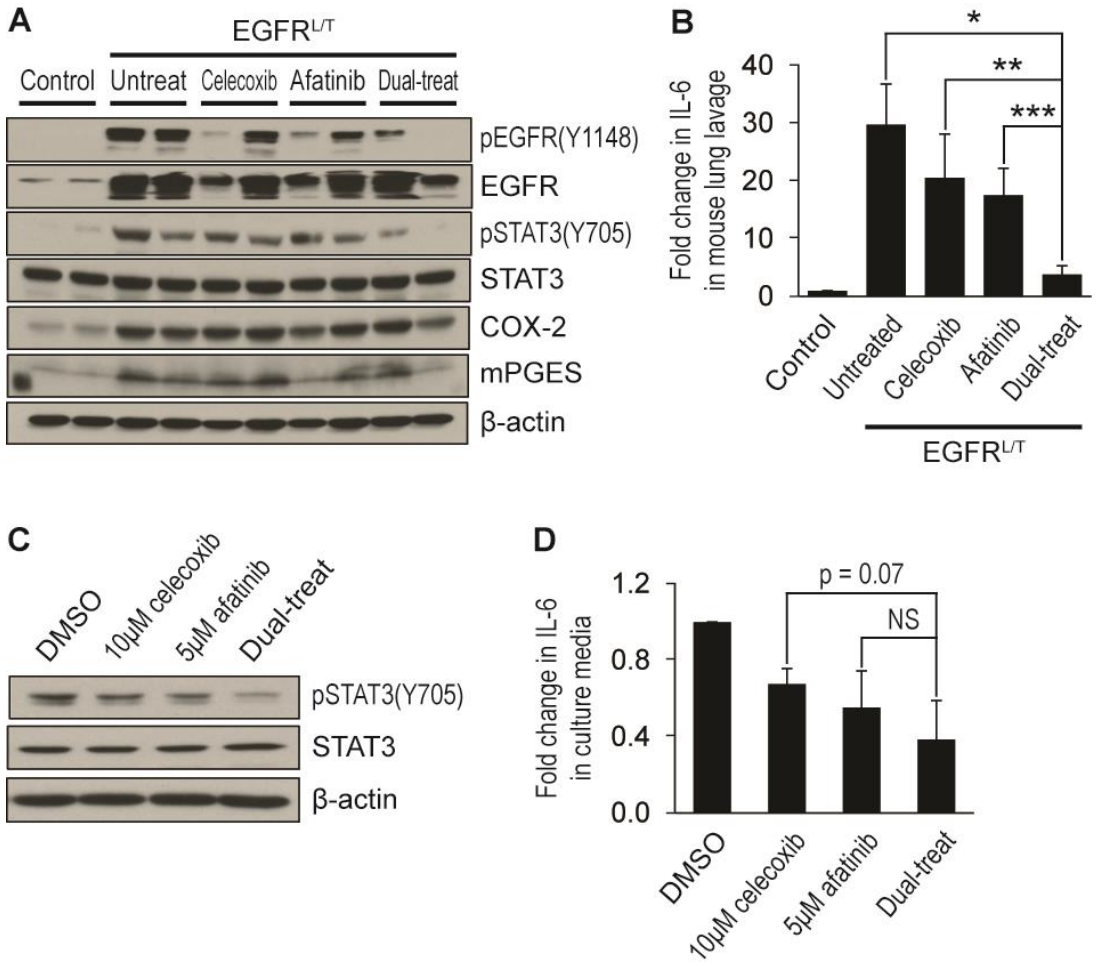

Figure 2.6. Dual-targeting COX-2 and EGFR reduces IL-6/STAT3 signaling in mutant EGFR lung tumors. A. Western blots of EGFR, STAT, and COX-2 in mouse lung lysates. B. Relative IL-6 levels in mouse lung lavage fluid. n = 7, 10, 9, 9, and 10 for control, untreated, celecoxib, afatinib, and dually-treated mice, respectively. p-values compared to control mice are ≤ 0.05. * = 0.0002, ** = 0.006, *** = 0.004. C. Western blots of H1975 cells under different drug-treatment conditions. D. Relative IL-6 levels in culture media. n = 3 individual experiments. p-values compared to control cells are ≤ 0.005. NS represents not significant. Data in panels B and D are presented as mean ± SEM.

COX-2 regulates IL-6 transcription in an EGFR-independent manner

Both COX-2 and EGFR have been shown to promote IL-6 transcription (31-33). To evaluate their effects in mutant EGFR lung cancer, we examined IL-6 promoter activity in H1975 cells treated with celecoxib, afatinib, or both inhibitors. Consistent with the effects on IL-6 secretion (Fig. 2.6D), we found that combined celecoxib and afatinib treatment reduced IL-6 promoter activity by about 55% compared to control cells (Fig. 2.7A). We also examined IL-6 mRNA levels under these conditions using real-time RT-PCR and found similar results (Supplementary Fig. 2.S3A).

By promoting PGE_2 synthesis, COX-2 has been shown to transactivate EGFR (26), suggesting that COX-2 might have been an upstream activator of EGFR in our experiments. To determine if EGFR is necessary for COX-2 to promote IL-6 transcription, we measured IL-6 promoter activity in H1975 cells treated with dimethyl-PGE_2 with or without afatinib. We found that while afatinib was responsible for a portion of IL-6 promoter activity, exogenous dimethyl-PGE_2 still increased levels of IL-6 promoter activity in the presence of afatinib (Fig. 2.7B). We also found similar changes in IL-6 mRNA levels in these conditions (Supplementary Fig. 2.S3B). Similarly, dimethyl-PGE_2 enhanced STAT3 phosphorylation in the presence of afatinib (Supplementary Fig. 2.S4). In contrast, dimethyl-PGE_2 did not promote STAT3 phosphorylation in the presence of baricitinib, a JAK inhibitor (Supplementary Fig. 2.S4). Collectively, these data suggest that the COX-2/PGE_2 signaling axis regulates IL-6/STAT3 signaling independently of EGFR (Fig. 2.7C).

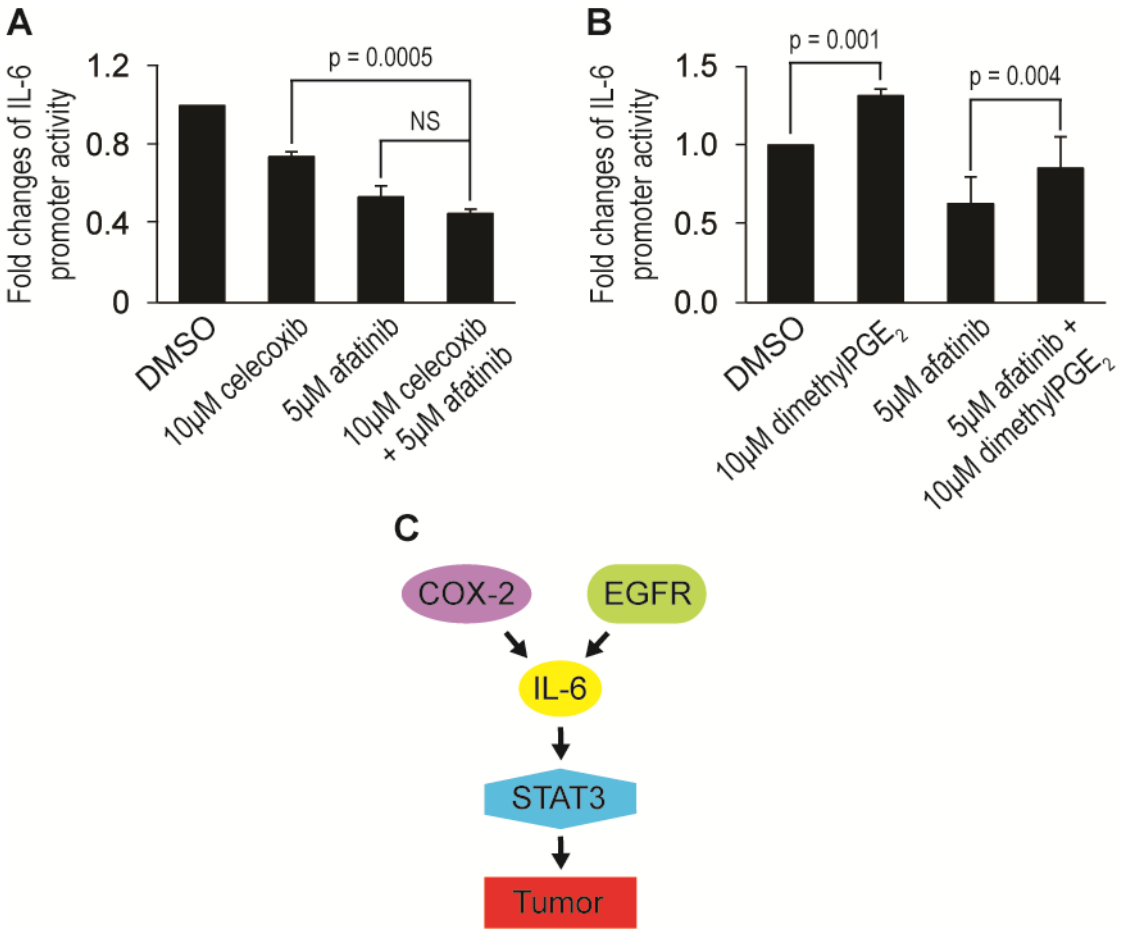

Figure 2.7. The COX-2/PGE_2 axis regulates IL-6 promoter activity in EGFR-independent manner. A. Changes in IL-6 promoter activity compared to DMSO-treated H1975 cells. n = 3 individual experiments. p-values compared to control cells are ≤ 0.0002. B. Changes in IL-6 promoter activity compared to DMSO-treated H1975 cells. n = 3 individual experiments. Data in panels A and B are presented as mean ± SEM. C. Model of COX-2-regulated IL-6 transcription leading to STAT3 activation in an EGFR-independent manner.

Discussion

Driver mutations in tumors such as those that activate EGFR are considered ideal targets for cancer therapy, and targeting EGFR has been shown to be superior to chemotherapies as first line therapy in lung tumors harboring mutant EGFR (34). Unfortunately, most patients who initially respond to EGFR inhibitors eventually develop resistance to them by a variety of mechanisms (5). To overcome this resistance it is has become imperative to find additional oncogenic signaling pathways that can be targeted to block the resurgent growth. In this study, we found that combined inhibition of COX-2 and EGFR reduced tumor loads in *EGFR* $^{L/T}$ mice and we discovered a novel tumorigenic role of COX-2 in the regulation of IL-6/STAT3 signaling in an EGFR-independent manner. Our findings suggest that combined inhibition of COX-2 and EGFR could be an effective strategy to treat inhibitor resistant, mutant EGFR lung cancer.

IL-6 has been implicated as a promoter of lung cancer growth and metastasis (35, 36), and the IL-6/JAK/STAT3 axis has been shown to be an essential oncogenic signaling pathway in human lung adenocarcinomas harboring mutant EGFR (31). But mechanisms of IL-6 expression have not been completely clarified in this context. Here, we found that COX-2/PGE_2 signaling promotes IL-6 expression, which leads to enhanced activation of the IL-6/STAT3 axis. Consistent with our findings, PGE_2 has been shown to stimulate IL-6 production and secretion in airway epithelial cells and cancer-associated fibroblasts (32, 33). Our observations suggest that IL-6 production is governed by signals emanating from both EGFR and the COX-2/PGE_2 signaling axis, such that combined inhibition of both pathways significantly reduced IL-6 production and secretion, which likely reduced tumor growth, cell proliferation, and anchorage-independent growth (Figs. 2.2 and 2.4). We are

currently trying to clarify how COX-2 modulates IL-6 transcription by assessing its role in activating known IL-6 transcription factors.

Many approaches have been tested in preclinical and clinical studies to overcome EGFR inhibitor resistance. Most of them have focused on intensively targeting EGFR such as developing next generation EGFR inhibitors (10, 37), dual-targeting of EGFR (11, 38), or inhibiting conformational maturation of EGFR (16). Although some of these approaches have been effective in preclinical studies, their performance in humans has been limited (39, 40). We considered the possibility that focusing solely on EGFR might not be an ideal approach to overcome resistance because this strategy would not block parallel oncogenic signaling pathways that bypass EGFR. Our observations suggest that one avenue would be to target COX-2, which contributes to mutant EGFR tumorigenesis by promoting production of IL-6, leading to STAT3 activation in an EGFR-independent manner.

The STAT3 pathway has been studied in the context of mutant EGFR and a previous study revealed high levels of phosphorylated STAT3 in mutant EGFR cell lines, human lung tumors, and xenografts (31). In that report, STAT3 was activated by abundant IL-6 secreted by lung cancer cell lines in an autocrine/juxtacrine manner, and inhibiting IL-6 significantly reduced cell and xenograft growth (31). Consistent with these findings, we found high levels of IL-6 in lung lavage fluid from *EGFR* $^{L/T}$ mice (Fig. 5B). The oncogenic role of IL-6 in mutant EGFR tumors has been attributed to activation of STAT3 in the tumor cells, but we also observed in *EGFR* $^{L/T}$ mice that celecoxib used alone or when combined with afatinib significantly reduced the number of CD11b-positive cells (presumably macrophages and neutrophils) in mouse lung tissues. In contrast, afatinib did not significantly change the abundance of CD11b-positive cells (not shown). This

observation suggests that COX-2 might promote tumorigenesis not only by activating the IL-6/STAT3 axis in the tumor cells, but also by modulating the inflammatory microenvironment through IL-6 and/or PGE_2.

Although we demonstrated that simultaneously targeting COX-2 and EGFR significantly reduced lung tumor growth, we did not monitor animal survival and, thus, cannot draw conclusions regarding this aspect of our treatment protocol. Nonetheless, we found marked reductions in tumor growth and we did not observe mortality in animals treated with both celecoxib and afatinib, while tumors continued to develop and several mice had to be sacrificed early in the control and single drug groups. Collectively, these observations lead us anticipate that dual-targeting of COX-2 and EGFR is likely to prolong survival in addition to reducing tumor growth. This outcome might be even more likely if celecoxib were combined with newer third generation EGFR inhibitors such as AZD9291 and rociletinib (14, 17). We were unable to test this possibility given the pronounced responses of $EGFR^{L/T}$ mice to these new inhibitors, which would make it difficult to measure additional effects caused by celecoxib.

Finally, our findings could carry immediate clinical implications because celecoxib is FDA approved, commonly used, and generic. As such, it could be quickly introduced into clinical studies and would be an inexpensive treatment. We found published clinical trials that tested combined COX-2 and EGFR inhibitors in lung cancer (41-45), but none of them were selected for EGFR mutation status. However, within each study, there was evidence that subsets of patients who either had EGFR mutations or had clinical profiles consistent with EGFR mutations (female nonsmokers) were more likely to respond. To highlight one study testing celecoxib and erlotinib: of seventeen patients in which EGFR

mutations were evaluated, five had EGFR mutations and all of them had a partial response. In contrast, two of twelve patients with wild-type EGFR had a partial response while the others had stable or progressive disease (45). These findings and our observations suggest that combined inhibition of COX-2 and EGFR could be a viable strategy to treat mutant EGFR lung cancer.

Materials and methods

Cell lines and culture

Human lung cancer cells expressing mutant EGFR, H1650 and H1975, were from American Type Culture Collection and grown in RPMI1640 (Invitrogen) with 10% FBS and antibiotics.

Cell growth and soft agar assay

For routine cell growth assays, 20,000 H1650 or H1975 cells were seeded in RPMI1640 with 1% FBS and antibiotics in 6cm cell culture dishes (Corning) followed by treatment with DMSO, 10μM celecoxib (Cayman Chemicals), 5μM afatinib (LC Laboratories), or combination of celecoxib and afatinib. After 5-6 days of culture, cells were harvested, and cell numbers were counted with a hemocytometer. For soft agar growth assays, 1,000 H1650 or H1975 cells were seeded on 0.5% Bacto-Agar (DIFCO) RPMI1640 with 10% FBS and antibiotics in 6cm culture dishes followed by treatment with DMSO, 10μM celecoxib, 5μM afatinib or combination of celecoxib and afatinib for 2 weeks. Volumes of 10 representative colonies from each condition were calculated by following formula:

Volume = (π/6) X longest diameter X shortest diameter X shortest diameter.

Luciferase assay

H1975 cells were transfected with the human IL-6 promoter luciferase reporter containing a 651-bp sequence upstream of the transcription start site of the human *IL6* gene (27) followed by treatment for 8 hours with DMSO, 10μM celecoxib, 5μM afatinib, or the combination of celecoxib and afatinib. In some experiments, cells were treated for 30 minutes with DMSO or 5μM afatinib followed by supplementation with 10μM dimethyl-PGE_2 (Cayman Chemical) for 8 hours. The cells were harvested in reporter lysis buffer (Promega) and mixed with Luciferase assay substrate (Promega). Luciferase activities were measured by a 2104 EnVision® Multilabel Plate Reader (PerkinElmer).

Real-time polymerase chain reaction

Total RNA was extracted using Trizol reagent (Invitrogen). Quantitative PCR was performed using the 7900HT Real-Time PCR system (Applied Biosystems), Power SYBR Green PCR master mix (Applied Biosystems), and the following primers:

Mouse 15-PDGH: 5'- GCCAAGGTAGCATTGGTGGAT-3' (forward)
and 5'- CTTCCGAAATGGTCTACAACT-3' (reverse);

Human IL-6: 5'-GGTACATCCTCGACGGCATCT-3' (forward)
and 5'-GTGCCTCTTTGCTGCTTTCAC-3' (reverse);

Human actin: 5'-AGGCACCAGGGCGTGAT-3' (forward)
and 5'-TCGTCCCAGTTGGTGACGAT-3' (reverse).

Amplifications were performed under following conditions: 95℃ 10min → 95℃ 1min (40 cycles) → 55℃ (15-PDGH) / 66℃ (IL-6) / 55℃ (actin) 30sec → 72℃ 45sec → 72℃ 5min (final extension).

Study mice and drug treatment

All mouse experiments were reviewed and approved by the University of Utah Institutional Review Board. Transgenic mice expressing the reverse tetracycline-controlled transactivator (rtTA) protein under control of the rat clara cell secretory protein (CCSP) gene promoter were obtained from The Jackson Laboratory (stock#006232). Mutant EGFR transgenic mice expressing $EGFR^{L858R+T790M}$ with a tetracycline promoter were kindly provided by William Pao (Vanderbilt University). Bi-transgenic *CCSP-rtTA*; $TetO\text{-}EGFR^{L858R+T790M}$ or control $TetO\text{-}EGFR^{L858R+T790M}$ mice were given doxycycline (1mg/L) in the water for 5-8 weeks followed by treatment with celecoxib (University of Utah Hospital Pharmacy) mixed into their chow (1mg/g food), afatinib (20μg/g of weight/day) by gavage, or combination of celecoxib and afatinib for 4 weeks. To evaluate and quantify tumor development before starting drug treatment, all mice were scanned in a small animal Quantum FX microCT (Perkin Elmer) for 4 minutes at 45-μm resolution, 90kV, with 160-μA current. Tumor growth in all mice was monitored by CT scans at two weeks of treatment and prior to sacrificing them after 4 weeks of treatment.

Histological analysis

Harvested mouse lungs were fixed in 10% neutral buffered formalin for at least 48 hours followed by paraffin embedding. Tissues were sectioned (5μm) and then stained

with hematoxylin (Vector Laboratories) and eosin (Sigma Aldrich) according to a standard protocol. Immunostaining was performed using the ABC reagent (Vector Laboratories) and anti-EGFR (Epitomics), anti-COX-2 (Santa Cruz Biotechnology), anti-CD11b (Epitomics), and anti-PCNA (Epitomics) according to instructions provided by the suppliers.

CT analysis

All scanned mouse lung images were obtained using Perkin Elmer Quantum FX software and analyzed with Analyze 11.0 (AnalyzeDirect) under supervision of two pulmonary experts, Matthew Topham M.D. and Aidin Iravani M.D. To calculate tumor volumes from the lung images, the volume of the entire lung tissue was obtained by manually outlining lungs every 0.45mm followed by summing up the entire lung volume with object propagation for the volume calculation. Tumor volumes were calculated by subtracting the volume of normal lung tissue obtained by extracting normal lung tissue based on the contrast threshold from the volume of the entire lung tissue.

Human and mouse IL-6 and PGE_2 assays

Cell culture media were aspirated at the time of harvest and analyzed for IL-6 using human or mouse IL-6 ELISA kits (eBioscience). Mouse lung bronchoalveolar lavage fluid samples were collected by dissecting the mice to expose the trachea, injecting cold PBS into the trachea, and then withdrawing the fluid. We then assessed levels of IL-6 and PGE_2 using mouse IL-6 ELISA and PGE_2 EIA kits (Cayman Chemical).

Western blots

Extracts from H1975 cells and mouse lungs were obtained using a cell lysis buffer (Cell Signaling), and aliquots (100μg) then were subjected to immunoblot analyses using specific antibodies: pEGFR(Y1148) (Epitomics), EGFR (Epitomics), COX-2 (Santa Cruz Biotechnology), mPGES-1 (Agrisera), pSTAT3(Y705) (Cell Signaling), STAT3 (Santa Cruz Biotechnology), pAKT(S473) (Cell Signaling), AKT (Cell Signaling), pERK(Y204) (Santa Cruz Biotechnology), ERK1 (Santa Cruz Biotechnology), ERK2 (Santa Cruz Biotechnology), or β-actin (ICN Biomedicals).

Acknowledgements

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Supplementary figures

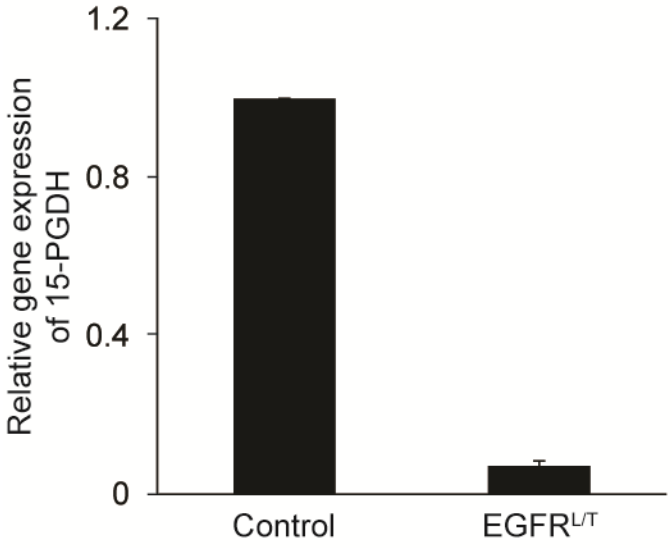

Figure 2.S1. Real time PCR relative quantification of 15-PGDH mRNA levels in control or *EGFR$^{L/T}$* mouse lung tissues. *EGFR$^{L/T}$* mice had lower levels of 15-PDGH mRNA compared to control mice. Data are presented as mean ± SEM. n for control = 3, n for *EGFR$^{L/T}$* = 7. p = 0.0000001.

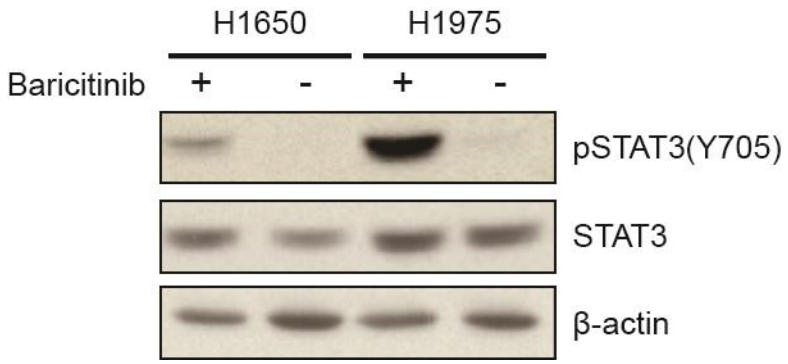

Figure 2.S2. JAK inhibition abolishes STAT3 activation in H1650 and H1975 cells. Western blots of STAT3 and pSTAT3(Y705) in H1650 and H1975 cells under 18-hour treatment of JAK inhibitor, 250nM Baricitinib.

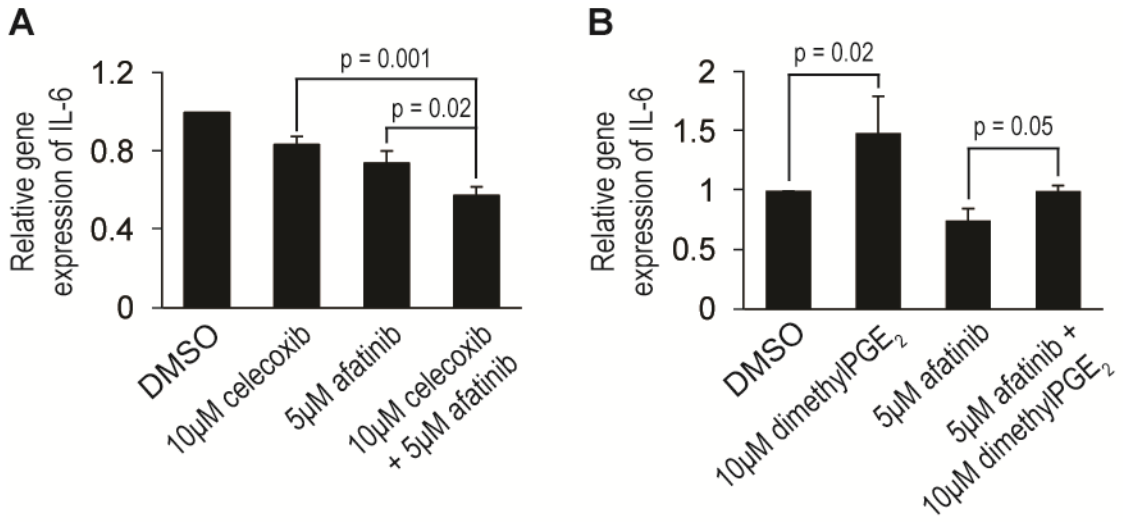

Figure 2.S3. Real time PCR relative quantification of IL-6 mRNA in H1975 cells. A. Levels of IL-6 mRNA were reduced in celecoxib, afatinib, and dual-treated H1975 cells. Data are presented as mean ± SEM. n = 6 individual experiments. p-values compared to control cells are ≤ 0.002. B. Levels of IL-6 mRNA in dimethyl-PGE_2, afatinib, and dual-treated H1975 cells. Data are presented as mean ± SEM. n = 3 individual experiments.

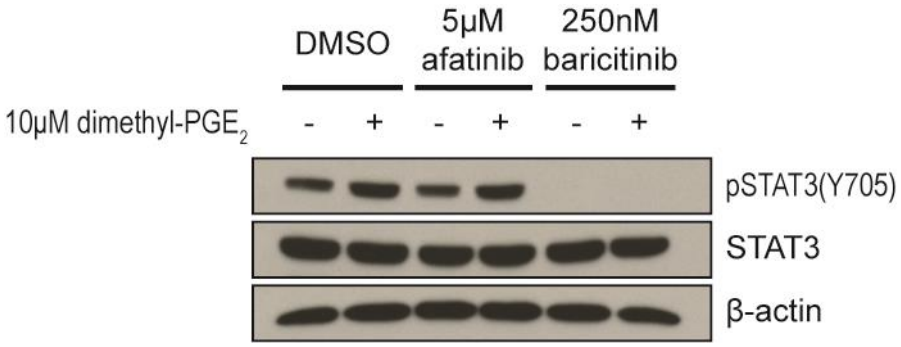

Figure 2.S4. Exogenous dimethyl-PGE_2 activates STAT3 in the presence of afatinib, but not in the presence of baricitinib. Western blots of STAT3 and pSTAT3(Y705) in H1975 cells under 8-hour treatment with dimethyl-PGE_2 treatment with or without afatinib or baricitinib.

CHAPTER 3

TUMOR PREVENTION BY TARGETING COX-2 IN *EGFR*$^{L858R+T790M}$ NSCLC MICE

Abstract

Up-regulation of COX-2 signaling in mutant EGFR NSCLC activates downstream oncogenic pathways such as the IL-6/STAT3 axis. Due to the importance of these events in mutant EGFR NSCLC, I hypothesized that targeting COX-2 with celecoxib is likely to be an effective strategy for prevention of this type of NSCLC. Using two different types of mutant EGFR transgenic mouse models in which mice express EGFR^{L858R} or EGFR$^{L858R+T790M}$ in the lung, I first investigated whether COX-2 inhibition had an effect on tumor formation. I observed no tumor formation following early treatment of *EGFR*^{L858R} mice with celecoxib. While this result was consistent with a preventive role for celecoxib in lung tumorigenesis, the study did not establish whether the animals in our cohorts would have developed tumors had they not been treated with the inhibitor. In the next series of experiments, I utilized computerized tomography (CT) analyses to monitor tumor development before celecoxib treatment. I found that targeting COX-2 prior to tumor development caused a slight delay on tumor formation in *EGFR*$^{L858R+T790M}$ mice compared to untreated *EGFR*$^{L858R+T790M}$ mice. These results point to the potential utility of targeting COX-2 as a strategy to delay or prevent development of mutant EGFR NSCLC.

Introduction

The inflammatory enzyme COX-2 is not detected in most tissues under basal conditions, but its expression is induced by inflammatory stimulation, leading to prostaglandin biosynthesis and the promotion of diverse physiological functions including fever and pain responses (1, 2). In addition to roles in inflammation, COX-2 has tumorigenic roles. In a diverse set of *in vivo* and *in vitro* studies, overexpression of COX-2 increased the malignant phenotype of cancer cells including stimulation of cell growth, increased resistance to apoptosis, increased angiogenesis and prometastatic effects, robust inflammation, and evasion of the host immune surveillance (3-7). Given its tumorigenic roles, COX-2 has been targeted in combination with other anticancer agents or with radiotherapeutic approaches to treat cancers of the breast, lung, colon, prostate, pancreas and skin with promising preclinical and clinical outcomes (8-13). In the previous chapter, I reported that dual-targeting of EGFR and COX-2 inhibits tumor growth, and that single-targeting of COX-2 moderately delays tumor progression in $EGFR^{L858R+T790M}$ mice, indicating that targeting COX-2 combined with EGFR inhibition is a promising strategy to treat mutant EGFR NSCLC.

In addition to its utility as a component of combination therapies for the treatment of advanced tumors, celecoxib has been studied as a chemopreventive agent. Results from several *in vivo* and *in vitro* studies suggest that celecoxib prevents tumor formation in diverse organs, including colon, skin, oral cavity and bladder (14-17). Given the potential of COX-2 inhibition as a chemo-preventive strategy, I conducted experiments aimed at determining if targeting COX-2 can prevent tumor formation in mutant EGFR NSCLC.

Results

Preventive effect of targeting COX-2 in *EGFR*^{L858R} mice

To investigate whether celecoxib has cancer-preventive properties in mutant EGFR NSCLC, I treated *EGFR*^{L858R} mice with celecoxib at very early stages of tumor development. I induced tumor development in 6-week-old transgenic mice expressing EGFR^{L858R} in the lung for 4 weeks, as this is normally considered as an initial point of tumor development. This was accomplished by supplementing doxycycline in the drinking water. The initial 4-week period was followed by a second phase during which mice were treated with celecoxib (1mg/g of chow) for 4 additional weeks; doxycycline was also administered during this phase of the experiment (Fig 3.1 A). This amount of celecoxib leads to clinically relevant serum levels (18). At the end of the study, I harvested lung tissues and assessed the levels of COX-2 and EGFR using immunoblot analysis. I found that tissues from control animals subjected to doxycycline treatment expressed high levels of EGFR and COX-2, as expected (Fig. 3.1.B, left panel). Expression of EGFR and COX-2 in lung tissues of mice treated with celecoxib was similar to that observed in lung tissues from non-transgenic mice (Fig 3.1 B). In addition, histological analyses revealed that the architecture of lung tissues from celecoxib-treated *EGFR*^{L858R} mice was similar to that of nontransgenic normal lung tissues (Fig 3.1 C). Collectively, these data indicate that targeting COX-2 during early stages of tumorigenesis may prevent tumor development.

Future studies should investigate the dynamics of tumor formation and progression before, during and after celecoxib treatment. Moreover, it will be important to assess whether prolonging treatment beyond 4 weeks continues to maintain EGFR and COX-2 expression at low levels, and to determine whether these features are associated with low

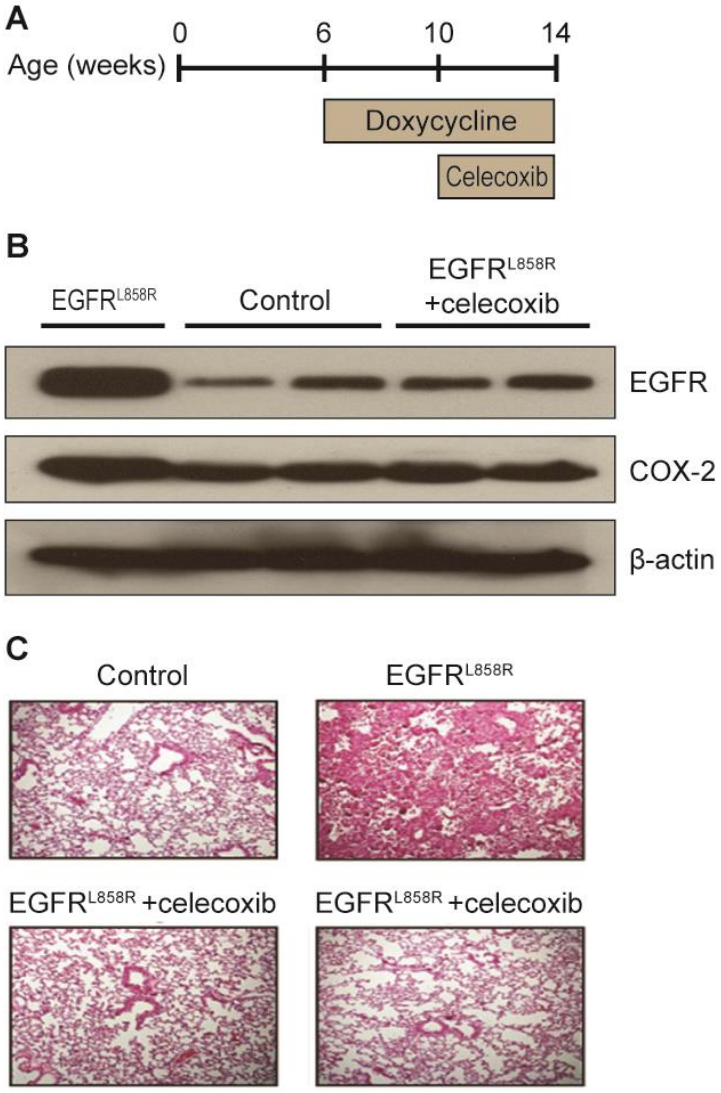

Figure 3.1. COX-2 inhibition is likely to prevent tumor development in *EGFR^{L858R}* mice. A. Timeline for tumor induction and celecoxib treatment in *EGFR^{L858R}* mice. Mice (6-week-old) were treated with doxycycline for 4 weeks followed by celecoxib treatment for additional 4 weeks. B. Western blots of EGFR, COX-2, and β-actin in mouse lung tissues present reduced levels of EGFR and COX-2 in celecoxib-treated *EGFR^{L858R}* mice. C. H/E staining of mouse lung tissues show the similar lung tissue architectures between control and celecoxib-treated *EGFR^{L858R}* mice.

or no tumor burden.

Verification of tumor development and regression in *EGFR*$^{L858R+T790M}$ mice

My next goal was to evaluate the reversibility of tumorigenesis in *EGFR*$^{L858R+T790M}$ mice. I utilized CT scanning methodologies and developed a new timeline of intervention. I investigated whether tumor induction and regression could be manipulated by altering the content of doxycycline on the drinking water. Specifically, I treated *EGFR*$^{L858R+T790M}$ mice with doxycycline for 5 weeks to induce tumor development, and confirmed this feature using CT scanning (Fig 3.2 A and left panel of B). To induce tumor regression, I withdrew doxycycline for the following 2 weeks and found that the lungs of these *EGFR*$^{L858R+T790M}$ mice appeared normal (Fig 3.2 A and right panel of B). These data confirmed the reversibility of lung tumorigenesis in *EGFR*$^{L858R+T790M}$ mice. This finding allowed me to next conduct chemopreventive studies in animals that had a demonstrated ability to develop lung tumors following induction of mutant EGFR expression.

Moderate preventive effect of targeting COX-2 in *EGFR*$^{L858R+T790M}$ mice

My next goal was to rigorously establish whether celecoxib has chemopreventive effects in *EGFR*$^{L858R+T790M}$ mice. Since withdrawal of doxycycline restored mouse lungs to an apparently normal state, I next treated *EGFR*$^{L858R+T790M}$ mice with both doxycycline and celecoxib for 10 weeks to test the tumor-preventive effects of targeting COX-2. Tumor formation and progression status were monitored by CT scans every 2 weeks (Fig 3.3 A).

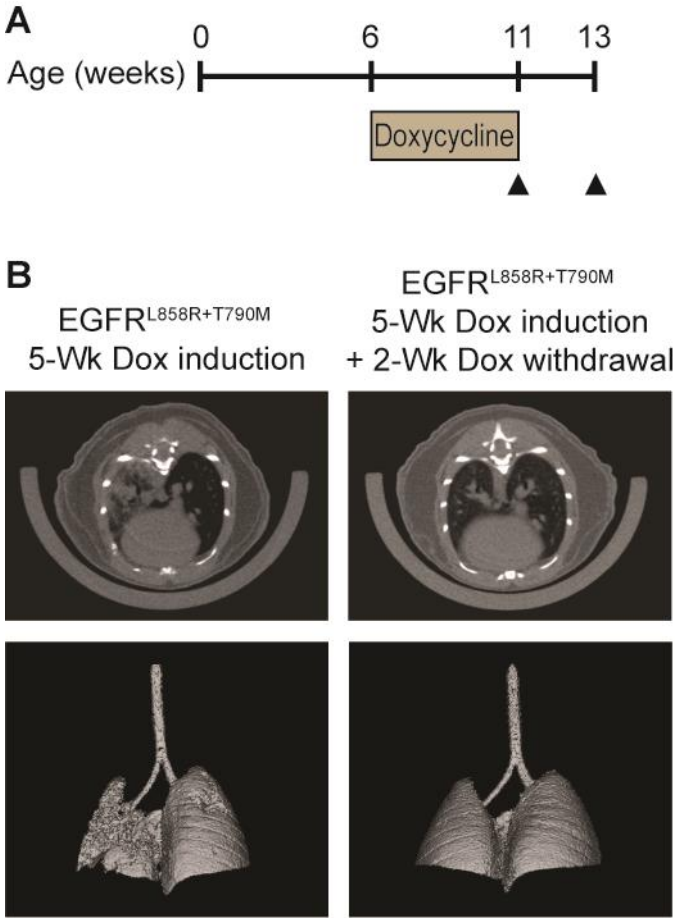

Figure 3.2. Verification of tumor development and regression in *EGFR*$^{L858R+T790M}$ mice. A. Timeline for tumor induction and regression in *EGFR*$^{L858R+T790M}$ mice. Mice (6-week-old) were treated with doxycycline for 5 weeks followed by withdrawal of doxycycline for the following 2 weeks. Arrowheads indicate the time of CT scan. B. CT images (upper panel) and reconstructed 3-D images (lower panel) of tumor-induced (left panel) and tumor-regressed (right panel).

In three-dimensional lung images reconstructed from CT scans, I found that an untreated mouse (F3 "19) developed tumors that, by the end of the 10-week treatment, accounted for 46% of the lung (Fig 3.3 B and C). Treatment with celecoxib delayed tumor formation and progression to various extents. Two animals (F3 "21 and F3 "32) displayed a slower rate of tumor progression compared with the untreated mouse. By the end of the 10-week treatment, tumor loads accounted for only 12% and 25%, respectively (Fig 3.3 C). The effects were less pronounced, but qualitatively similar, in a third animal (F3 "30, Fig 3.3 C). These findings point at chemopreventive properties of celecoxib and they suggest that COX-2 inhibition prior to tumor development is likely to delay tumor formation and progression in NSCLC cases that overexpress mutant forms of EGFR.

Discussion

Tumorigenic roles of COX-2 and inflammatory signals have been intensively studied in colorectal cancer, and the use of COX-2 inhibitors is likely to yield promising therapeutic outcomes for the prevention of colorectal cancer by reducing polyp multiplicity, tumor incidence, and tumor growth (14, 19-21). In addition to colorectal cancer, targeting COX-2 alone or in combination with other anticancer agents has been proposed to prevent tumor formation or recurrence in other types of cancers including those of the breast, head and neck, bladder, and lung (22-25). In the studies reported in this chapter, I found that targeting COX-2 with celecoxib is likely to impede tumor formation and growth when it is provided prior to tumor development in mutant EGFR mice. This observation has important clinical implications for patients diagnosed with lung tumors harboring mutant forms of EGFR. Since celecoxib is an inexpensive and moderately risk-

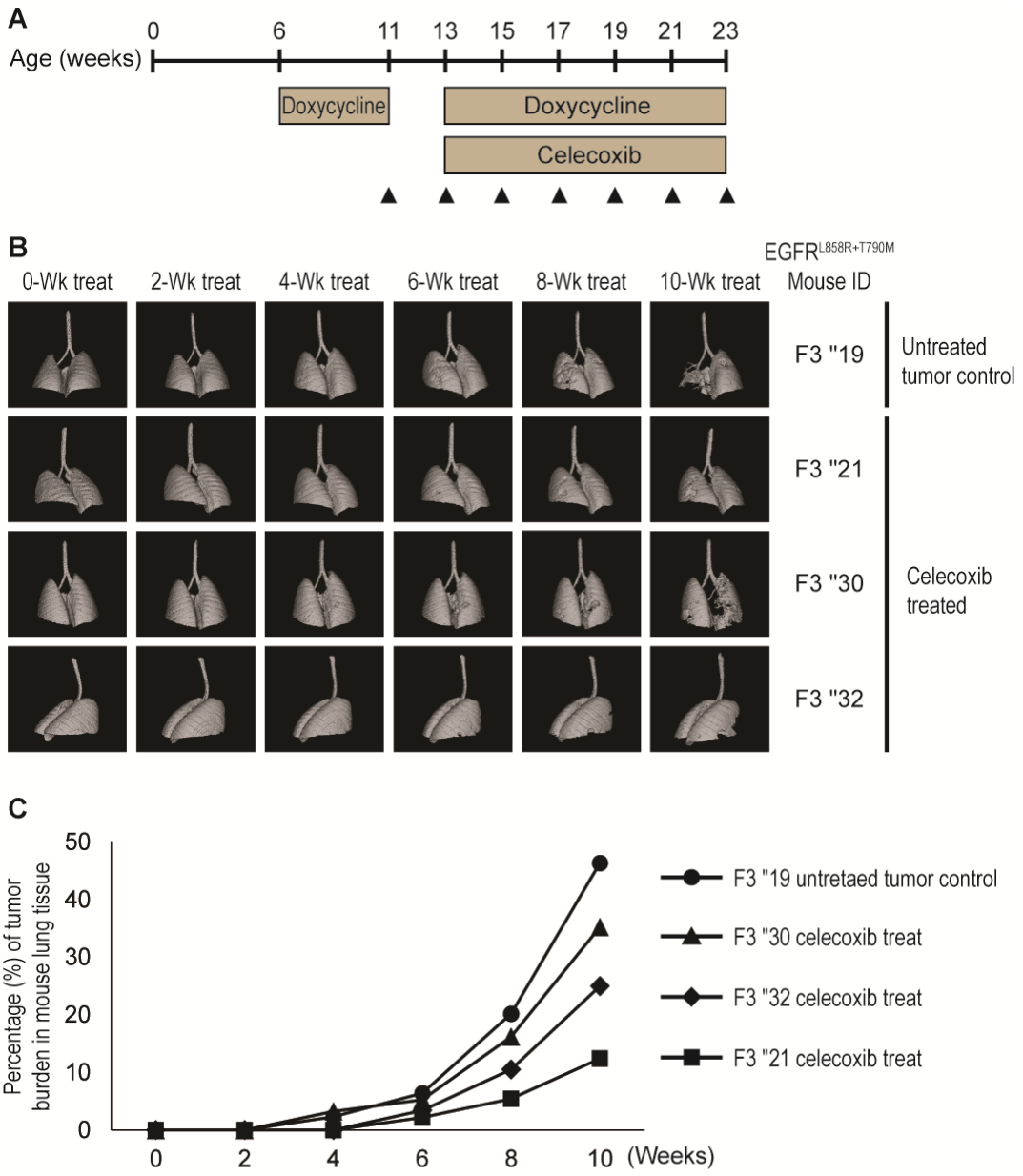

Figure 3.3. Tumor-preventive effect of COX-2 inhibition in *EGFR*$^{L858R+T790M}$ mice. A. Timeline for testing tumor-preventive effect of COX-2 inhibition. Mice (6-week-old) were treated with doxycycline for 5 weeks followed by withdrawal of doxycycline for the following 2 weeks. After confirming tumor-regression, mice were subjected to celecoxib treatment for 10 weeks. Arrowheads indicate the time of CT scan. B. Reconstructed 3-D images of mouse lungs. Celecoxib-treated mice (lower 3 panels) present delayed tumor development compared to untreated tumor control mouse (top panel). C. Percentages of tumor burden in mouse lung tissues. Untreated tumor control mouse developed tumor at 4-week point and show 46% tumor loads at 10-week point. Celecoxib-treated F3 "30 mouse developed tumor at 4-week point and show 35% tumor loads at 10-week point. Celecoxib-treated F3 "32 and F3 "21 mice developed tumor at 6-week point and show 25% and 12% tumor loads, respectively.

free drug, using it as a chemopreventive strategy could offer therapeutic benefit for mutant EGFR-positive lung cancer patients. I note that these findings were made in a limited number of mice (n for untreated $EGFR^{L858R+T790M}$: 1, n for celecoxib-treated $EGFR^{L858R+T790M}$: 3). Thus, it will be necessary to replicate these observations using larger mouse cohorts to firmly establish the potential of celecoxib or other COX-2 inhibitors as a chemotherapeutic strategy to treat mutant EGFR lung cancer patients.

Materials and methods

Mice and drug treatments

All mouse experiments were reviewed and approved by the University of Utah Institutional Review Board. The Jackson Laboratory provided transgenic mice expressing the reverse tetracycline-controlled transactivator (rtTA) protein under the control of the rat clara cell secretory protein (CCSP) gene promoter (stock#006232). Mutant EGFR transgenic mice expressing $EGFR^{L858R}$ and $EGFR^{L858R+T790M}$ with a tetracycline promoter were kindly provided by William Pao (Vanderbilt University). Bi-transgenic *CCSP-rtTA; TetO-EGFR^{L858R}* or control *TetO-EGFR^{L858R}* mice received doxycycline (1mg/L) in the drinking water for 4 weeks followed by celecoxib treatment (University of Utah hospital pharmacy, 200mg/200g) in the food supply for 4 additional weeks. Mice that had been treated for 4 weeks were sacrificed in a CO_2 chamber. Bi-transgenic *CCSP-rtTA; TetO-EGFR$^{L858R+T790M}$* or control *TetO-EGFR$^{L858R+T790M}$* mice received doxycycline (1mg/L) in the drinking water for 4-5 weeks to induce tumor development. This was confirmed by subjecting the animals to a small animal Quantum FX microCT, (Perkin Elmer) at 45-um resolution, 90kV, with 160-uA current. Induced mice were restored to an apparently

normal state by supplying normal water for 2 weeks. CT scans confirmed that tumors had regressed following doxycycline withdrawal. Inducible normal mice were treated with doxycycline and celecoxib for 10 weeks and tumor development and progression was monitored uisng by-weekly CT scans. Following completion of a 10-week treatment mice were sacrificed in a CO_2 chamber and the lungs were subjected to histological analyses.

Histological analysis

Harvested lungs were fixed in 10% neutral buffered formalin for more than 48 hours followed by paraffin embedding. Sectioned mouse tissues (5μm) were stained with hematoxylin (Vector Laboratories) for 10-20 seconds followed by washing with tap water. Eosin (Sigma Aldrich) staining was performed for 1 minute followed by washing with tap water. Stained sections were mounted with toluene-based liquid mount media (Triangle Biomedical Science). Stained section images were taken by EVOS FL (University of Utah Health Sciences Center core).

Western blots

Harvested mouse lungs were homogenized using a IKA® T10 basic ultra-turrax homogenizer in cell lysis buffer (Cell Signaling). Protein concentrations were quantified using BCA reagent (Thermo Scientifics), and tissue lysates (100μg of protein) were subjected to 10% SDS-PAGE at 35-50 mA for 4-5 hours followed by electroblotting to polyscreen PVDF membrane at 500 mA overnight. To block remaining protein binding sites, PVDF membranes were blocked in TBS with 5% milk-TBST for 2 hours. Separated proteins in PVDF membrane were blotted with primary antibodies, EGFR (Epitomics),

COX-2 (Santa Cruz Biotechnology), and β-actin (ICN Biomedicals), overnight at 4℃. Membranes were washed in TBST for 30 minutes by exchanging fresh TBST three times followed by secondary antibody interaction with mouse IgG HRP (Cell Signaling) or rabbit IgG HRP antibody (Cell Signaling) at room temperature for 2 hours. After washing membranes in TBST for 30 minutes by exchanging fresh TBST three times, Pierce® ECL western blotting substrates (Thermo Scientific) were applied to the membrane for 1 minute. Films (Carestream Biomax MR film) were exposed for various time periods and developed in Konica SRX-101A developer.

CT analysis

All scanned mouse lung images were obtained using Perkin Elmer Quantum FX software and analyzed with Analyze 11.0 (AnalyzeDirect) under supervision of two pulmonary experts, Matthew Topham M.D. and Aidin Iravani M.D. To calculate the tumor volumes (cm^3) from the mouse lung images, the volume of the entire lung tissue was obtained by manually outlining lungs every 0.45mm followed by summing up the entire lung volume with object propagation for the volume calculation. Tumor volumes were calculated by subtracting the volume of normal lung tissue obtained by extracting normal lung tissue based on the contrast threshold from the volume of the entire lung tissue.

Acknowledgements

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CHAPTER 4

ROLES OF PKCδ IN MUTANT EGFR LUNG CANCER

Abstract

PKCδ, a serine/threonine kinase, is known to regulate cell proliferation, survival, migration, and apoptosis. Previous studies established a proapoptotic role of PKCδ leading to the suggestion that PKCδ is a tumor suppressor. However, studies in pancreatic, breast, and lung cancers revealed positive correlations between PKCδ and cancer progression, and suggested tumor-promoting roles for PKCδ. Regulatory factors including localization and phosphorylation of PKCδ, and the presence of other proapoptotic or antiapoptotic signals are thought to define a role of PKCδ as a tumor suppressor or a tumor promoter. In this chapter, I analyzed lung tumors that developed in transgenic mice expressing $EGFR^{L858R+T790M}$ and found increased expression and activation of PKCδ, which led me to hypothesize that PKCδ acts as a tumor promoter in the context of mutant EGFR NSCLC. I conducted studies in human lung cancer cells in which I manipulated PKCδ levels using RNAi and overexpression approaches. I discovered that PKCδ regulates IL-6 expression leading to STAT3 activation. I also found that depletion of PKCδ decreased the growth of mutant EGFR human lung cancer cells. Collectively, these findings provide novel insights regarding tumor-promoting roles of PKCδ in mutant EGFR NSCLC and point at PKCδ as a novel and attractive target for the treatment of mutant EGFR NSCLC.

Introduction

The PKC family is composed of several serine/threonine kinases that regulate signaling events essential for cell proliferation, differentiation and apoptosis (1-4). PKCs were originally identified as kinases regulated by the phospholipid diacylglycerol (DAG) and calcium (5). Early observations showed that PKC isozymes are activated by tumor-promoting phorbol esters that are analogs of DAG. These findings suggested that PKCs might have tumor-promoting roles, which led to the proposition that at least some PKC isoforms could be suitable targets for cancer therapy (1, 6-7). Depending on the mechanism of activation, PKC isozymes are divided into three groups: classical PKCs (PKCα, β, γ), novel PKCs (PKCδ, ε, η, θ), and atypical PKCs (PKCζ, ι). Each PKC isozyme has a distinct tissue distribution, subcellular localization, and substrate specificity. PKCs mature through a series of phosphorylation events, and mature or "primed" PKCs are translocated to the plasma membrane and become activated by intracellular calcium and DAG (8-10). I found that PKCδ is the only member of the PKC family that is primed and becomes highly activated in $EGFR^{L858R+T790M}$ tumors. This finding led me to investigate whether PKCδ has tumor-promoting roles in mutant EGFR lung cancer.

Like other PKC isozymes, PKCδ is involved in the regulation of cell growth, differentiation and apoptosis. Notably, PKCδ is the first PKC isozyme identified as a caspase-3 substrate (11). A constitutively-active catalytic fragment of PKCδ cleaved by caspase-3 localizes to the nucleus and plays proapoptotic roles by targeting DNA-dependent protein kinase, phospholipid scramblase 1, and lamin B (10, 12-15). These initial findings suggested that PKCδ was a tumor suppressor, and a number of subsequent studies further supported this conclusion. For example, over-expression of PKCδ

decreased transformation of NIH 3T3 cells (16), and suppressed immunoresponsiveness (17). Further, PKCδ transgenic mice were resistant to 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced tumor promotion (18). Conversely, there was transcriptional repression of PKCδ in human squamous cell carcinoma (19) and loss of nuclear PKCδ in endometrial tumors (20). Collectively, these findings are consistent with a tumor-suppressing role of PKCδ.

In stark contrast to the tumor-suppressing roles described above, recent studies in K-Ras lung adenocarcinoma, pancreatic cancer, and ErbB2-driven breast cancer reported that PKCδ appears to have tumor-*promoting* roles. For example, over-expression of PKCδ increased the malignant phenotype of pancreatic cancer cells (21). Moreover, PKCδ is essential for mammary gland tumor development in ErbB2-overexpressing transgenic mice (22) and increased PKCδ expression has been reported in ErbB2-positive breast cancers. Knocking down PKCδ in K-Ras-driven NSCLC cells decreased cellular growth, migration, and transformation (23). Collectively, these findings indicate that PKCδ can act as a tumor-promoter in certain types of cancers. These observations, combined with our findings that PKCδ is primed and activated in mutant EGFR NSCLC, led me to investigate whether PKCδ has tumorigenic roles in this malignancy.

Results

PKCδ is "primed" and activated in *EGFR*$^{L858R+T790M}$ mouse and human lung cancer

My initial approach consisted of investigating the state of activation of canonical signaling pathways known to be involved in the EGFR axis, and that were previously

described in Chapter 2 of this dissertation. First, I found that STAT3 is highly active in mutant EGFR NSCLC (Chapter 2, Fig 2.5). Second, I discovered activation of PLCγ1 in *EGFR*$^{L858R+T790M}$ mice (Fig. 4.1A). Since PLCγ1 generates diacylglycerol (DAG), an activator of PKCs, I next investigated the activation state of PKC isozymes in *EGFR*$^{L858R+T790M}$ mice. In general, phosphorylation of PKCs in a hydrophobic motif, such as phosphorylation at serine 299 in PKCδ, primes PKCs for subsequent phosphorylation in catalytic domains in PKCs, leading to activation of PKCs. I found increased hydrophobic motif phosphorylation in PKCδ in lung tumors from *EGFR*$^{L858R+T790M}$ mice (Fig. 4.1B). In contrast, increased hydrophobic motif phosphorylation was not observed in other DAG-activated PKCs, including α, β, γ, ε and η (Fig. 4.1B). This observation indicates that PKCδ is selectively primed in EGFR$^{L858R+T790M}$ tumors, and that this may lead to further phosphorylation and full activation. To address this, I assessed the levels of active PKCδ by blotting S299-phosphorylated PKCδ and found high levels of activation in lung tumors from *EGFR*$^{L858R+T790M}$ mice (Fig. 4.1C). Interestingly, I found increased levels of *total* PKCδ protein in *EGFR*$^{L858R+T790M}$ compared to control mice that did not express mutant EGFR (Fig. 4.1B and C). These findings were confirmed in histological analyses that revealed increased PKCδ protein expression and activation in *EGFR*$^{L858R+T790M}$ compared with control mice (Fig. 4.1D and E). In addition, in human lung cancer tissue arrays used to evaluate mutation-specific monoclonal antibodies (24), I found that human mutant EGFR lung tumors present high expression levels of PKCδ (Fig. 4.1F). Collectively, these data indicate that PKCδ is upregulated in mutant EGFR lung cancer, which led us to hypothesize that PKCδ might have a prominent role in this malignancy.

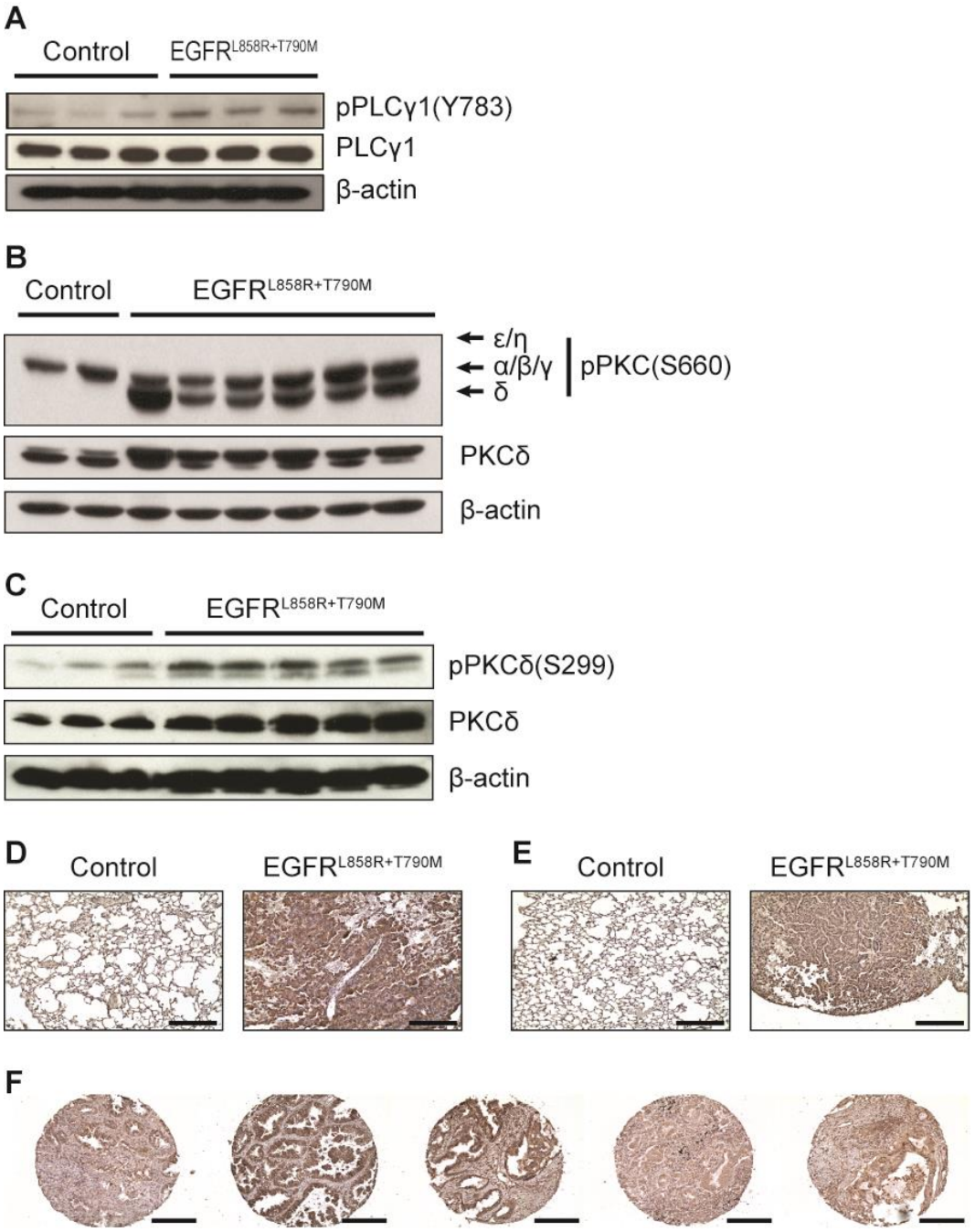

Figure 4.1. PKCδ is primed and activated in mutant EGFR lung cancer. A. Western blots of PLCγ1 in mouse lung tissues present increased activity of PLCγ1(Y783) in *EGFR*$^{L858R+T790M}$ mice. B. Western blots of pPKC(hydrophobic motif, Ser660) and PKCδ in mouse lung tissues present increased levels of pPKC(Ser660) in *EGFR*$^{L858R+T790M}$ mice. C. Active pPKCδ(Ser299) is highly expressed in *EGFR*$^{L858R+T790M}$ mice. D. IHC staining for PKCδ shows high expression of PKCδ in *EGFR*$^{L858R+T790M}$ mouse. E. IHC staining for pPKCδ(Ser299) shows high levels of active PKCδ in *EGFR*$^{L858R+T790M}$ mouse. F. IHC staining for PKCδ shows highly expressed PKCδ in mutant EGFR human lung tissues. Scale bars in C-E represent 200μm.

Depletion of PKCδ decreases mutant EGFR lung cancer cell growth

To test if PKCδ is an important oncogenic signal in mutant EGFR lung cancer, I examined the effects of PKCδ inhibition on cell growth. Human lung cancer cells harboring mutant EGFR, H1650 and H1975, were cultured under the condition of PKCδ depletion by using PKCδ RNAi oligonucleotides. Under depletion of PKCδ, cell growth of both cell lines was substantially reduced (Fig 4.2), indicating that PKCδ not only has an important oncogenic role in mutant EGFR lung cancer cell growth, but it might be a potential target to treat mutant EGFR lung cancer.

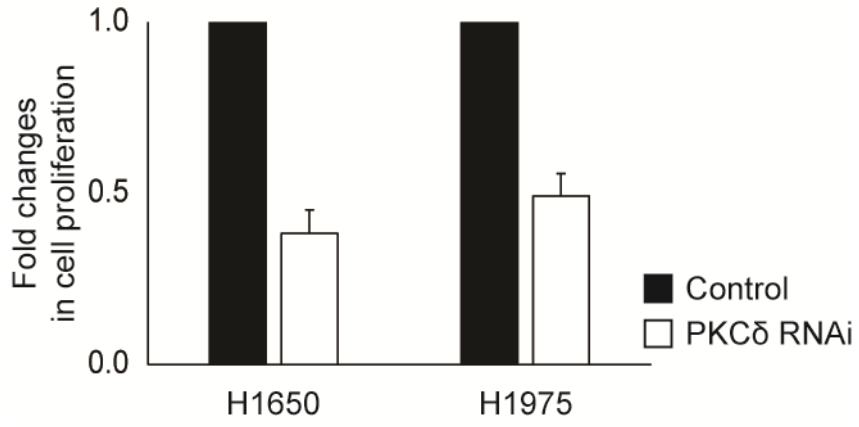

Figure 4.2. PKCδ RNAi decreases proliferation of H1650 and H1975 cells. Under PKCδ RNAi, H1650 and H1975 cells show 0.4- and 0.5-fold reduced cell proliferation, respectively. Data are presented as mean ± SEM. n = 3 individual experiments. p-values compared to control cells (H1650: 0.0003, H1975: 0.0001).

Depletion of PKCδ reduces STAT3 activation *via* janus kinases (JAKs)

Since PKCδ is an important oncogenic signal in mutant EGFR lung cancer, I investigated the mechanism of oncogenic PKCδ in the context of mutant EGFR lung cancer. First, I determined if there is a functional relationship between PKCδ and the critical oncogenic signal in mutant EGFR lung cancer, STAT3. To test this, I silenced PKCδ expression in H1650 and H1975 cells using RNAi, and then assessed the levels of STAT3 activation. I found that PKCδ RNAi virtually abolished STAT3 activation in both cell lines (Fig. 4.3A). I note that STAT3 inactivation following silencing of PKCδ is EGFR-independent because the levels of receptor activation remain high under these conditions (Fig 4.3A). It was previously demonstrated that STAT3 activation in mutant EGFR lung cancer is mediated by IL-6 *via* JAK (25). Thus, I utilized the JAK inhibitor baricitinib and confirmed that inhibition of JAK signaling also inhibited STAT3 activation, presumably through IL6 in H1650 and H1975 cells (Fig. 4.3B). Collectively, these data point at a potential role for PKCδ in the regulation of oncogenic events upstream of IL-6/JAK/STAT3 signaling.

PKCδ regulates IL-6 secretion in mutant EGFR lung cancer cells

My next goal was to directly test whether PKCδ regulates STAT3 signaling through IL-6. To address this issue, I determined whether silencing PKCδ expression affected the levels of IL-6 secreted by H1650 and H1975 into the culture media. I found that decreasing PKCδ reduced IL-6 secretion by 50% in both cell lines (Fig 4.4A), indicating that PKCδ regulates IL-6 secretion. Conversely, overexpression of PKCδ in H1650 and H1975 cells

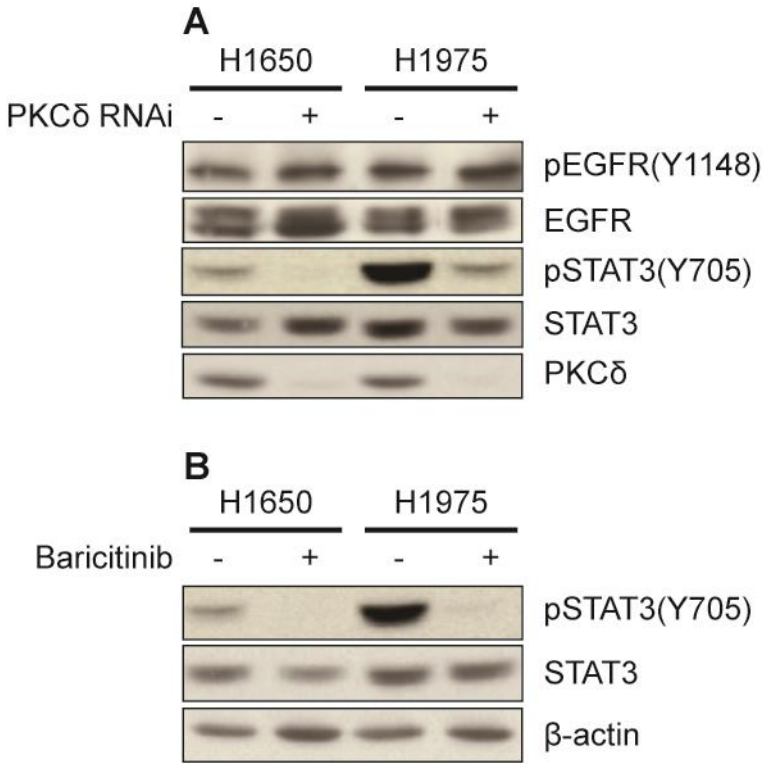

Figure 4.3. PKCδ RNAi reduces STAT3 activation *via* JAKs. A. Western blots of EGFR and STAT3 of H1650 and H1975 cells under PKCδ RNAi show that depletion of PKCδ reduces STAT3, but not EGFR activation. B. Western blots of STAT3 in H1650 and H1975 cells under JAK inhibition with 250nM baricitinib show that JAK inhibition abolishes STAT3 activation.

resulted in increased IL-6 secretion about 60% and 30% in H1650 and H1975 cells, respectively (Fig. 4.4B). I note that PKCδ overexpression increased pSTAT3 relative to total STAT3 levels (Fig. 4.4B), which is consistent with the findings of decreased STAT3 activation under PKCδ RNAi (Fig. 4.3A). Collectively, these findings demonstrate that PKCδ regulates IL-6 secretion in mutant EGFR lung cancer cells.

Transcriptional regulation of IL-6 by PKCδ

Next, to elucidate the mechanism of PKCδ-mediated regulation of IL6 in mutant EGFR lung cancer, I investigated whether transcriptional regulatory mechanisms were involved in this response, using silencing and overexpression approaches. I found that PKCδ RNAi decreased IL-6 mRNA levels by approximately 50% and 70% in H1650 and H1975 cells, respectively (Fig. 4.5A). Conversely, PKCδ overexpression increased IL-6 mRNA levels by approximately 4-fold and 2-fold in H1650 and H1975 cells, respectively (Fig. 4.5B). A caveat in the overexpression experiments is that sizeable variations in IL-6 mRNA levels resulted in differences that did not reach statistical significance ($p=0.23$ and 0.1 for H1650 and H1975 cells, respectively). However, PKCδ overexpression may not be an optimal experimental approach to assess cellular responses to enhanced PKCδ signaling. As previously noted, PKC isozymes must undergo a series of phosphorylation events for full activation, and variations in difficult-to-control culture conditions may alter the extent of PKCδ phosphorylation –and thus, activation– in separate experiments. To address this issue, we have generated a constitutively-active PKCδ construct (A147E) (26), which is expected to more consistently activate PKCδ signaling. Nonetheless, results from silencing and overexpression studies suggest that PKCδ regulates IL-6 transcription.

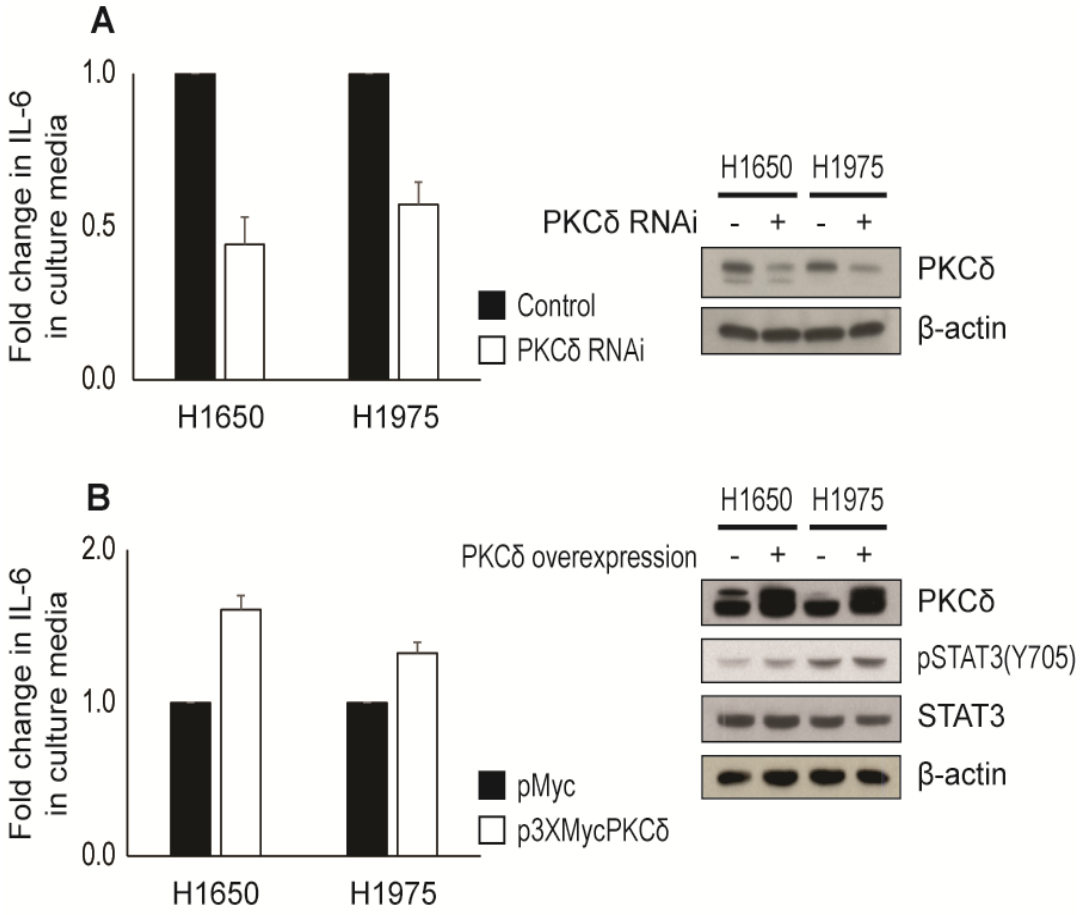

Figure 4.4. PKCδ regulates IL-6 secretion in mutant EGFR lung cancer cells. A. Relative IL-6 levels in culture media of H1650 and H1975 cells under PKCδ RNAi are 0.4- and 0.5-fold, respectively, lower than control cells. Data are presented as mean ± SEM. n = 3 individual experiments. p-values compared to control cells are ≤ 0.002. B. Relative IL-6 levels in culture media of H1650 and H1975 cells under PKCδ overexpression are 1.6- and 1.3-fold, respectively, higher than control cells. Data are presented as mean ± SEM. n = 3 individual experiments. p-values compared to control cells are ≤ 0.04.

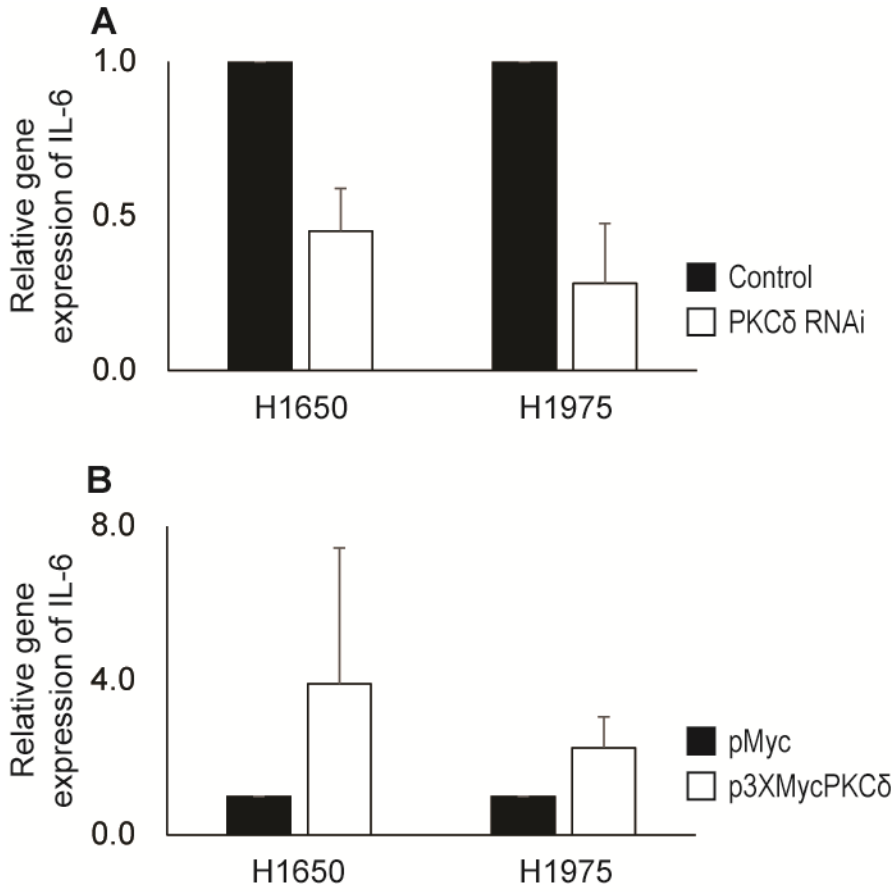

Figure 4.5. PKCδ regulates IL-6 transcription in mutant EGFR lung cancer cells. A. Levels of IL-6 mRNA in H1650 and H1975 cells under PKCδ RNAi are 0.45- and 0.28-fold reduced, respectively, compared to control cells. Data are presented as mean ± SEM. n = 4 individual experiments. p-values compared to control cells ≤ 0.05. B. Levels of IL-6 mRNA in H1650 and H1975 cells under PKCδ overexpression are 3.9- and 2.3-fold higher, respectively, than control cells. Data are presented as mean ± SEM. n = 3 individual experiments. p-values compared to control cells (H1650; 0.23, H1975: 0.1).

EGFR modulates the fate of PKCδ

My next goal was to contribute additional information regarding the role of PKCδ in mutant EGFR lung cancer, with a focus on factors that regulate proapoptotic versus prooncogenic roles for this PKC isoform. Since constitutively active mutant EGFR is the oncogenic driver in mutant EGFR lung cancer, I focused on the role of EGFR in the regulation of PKCδ functions. In this regard, it was recently reported that mutant EGFR limits the expression of BIM, a proapoptotic protein, through transcriptional and posttranslational regulation, leading to the suppression of apoptosis (27-30). In addition, BIM has been shown to induce apoptosis through a caspase-3-dependent mechanism in human mast cells (31). These findings led me to hypothesize that the oncogenic properties of PKCδ in mutant EGFR lung cancer could be explained on the basis of antiapoptotic effects resulting from mutant EGFR-mediated effects on BIM and caspase-3. To test this hypothesis, I assessed the effect of EGFR inhibition on PKCδ expression using the mutant EGFR lung cancer cell HCC827. I found that when EGFR signaling was inhibited with afatinib, the levels of constitutively-active catalytic fragments of PKCδ (δCF) were significantly increased (Fig 4.6A). This result is consistent with a model whereby inhibiting EGFR changes the function of PKCδ from prooncogenic to proapoptotic in mutant EGFR lung cancer cells. I note that EGFR is not involved in the regulation of PKCδ expression since there were no effects of EGFR inhibition on PKCδ protein levels. However, it is possible that EGFR may be involved in the generation of active PKCδ fragments by activating the proteolytic step, for example. Aside from the mechanism, the findings shown in Fig. 4.6A indirectly support the hypothesis that the antiapoptotic roles of EGFR participate in the definition of PKCδ as a prooncogenic contributor to mutant

EGFR lung cancer (Fig 4.6B). To rigorously establish whether EGFR controls prooncogenic versus antioncogenic roles for PKCδ, it will be required to assess detailed functional relationships among EGFR, BIM, caspase-3 and PKCδ.

Discussion

In this study, I discovered a novel prooncogenic function of PKCδ in the regulation of IL-6/STAT3 signal in mutant EGFR lung cancer. The studies suggest that PKCδ regulates IL-6 transcription at the transcriptional level. While over-expression studies were variable possibly owing to experimental limitations, this problem can potentially be solved using a plasmid construct expressing active pPKCδ (A147E) (26). This expression construct has been generated, and it will be utilized in future studies to further test the impact of pPKCδ on transcriptional regulation of IL6 expression in mutant EGFR lung cancer cells.

In this chapter, I also addressed the mechanism that leads to dual—prooncogenic and proapoptotic—roles of PKCδ in mutant EGFR lung cancer. I made the novel observation that EGFR inhibition leads to increased levels of δCF and, based on this result, I speculate that EGFR may determine whether PKCδ functions as a proapoptotic versus prooncogenic factor (Fig 4.6B). To further define the mechanism by which mutant EGFR modulates proapoptotic and prooncogenic functions of PKCδ in mutant EGFR lung cancer, our laboratory is aiming to address the following issues: (1) How does EGFR inhibition increase production of δCF? (2) How does EGFR signaling activate prooncogenic functions of PKCδ? (3) Does mutant EGFR alter the subcellular localization of PKCδ? (4) If this is the case, how does this response affect tumorigenesis?

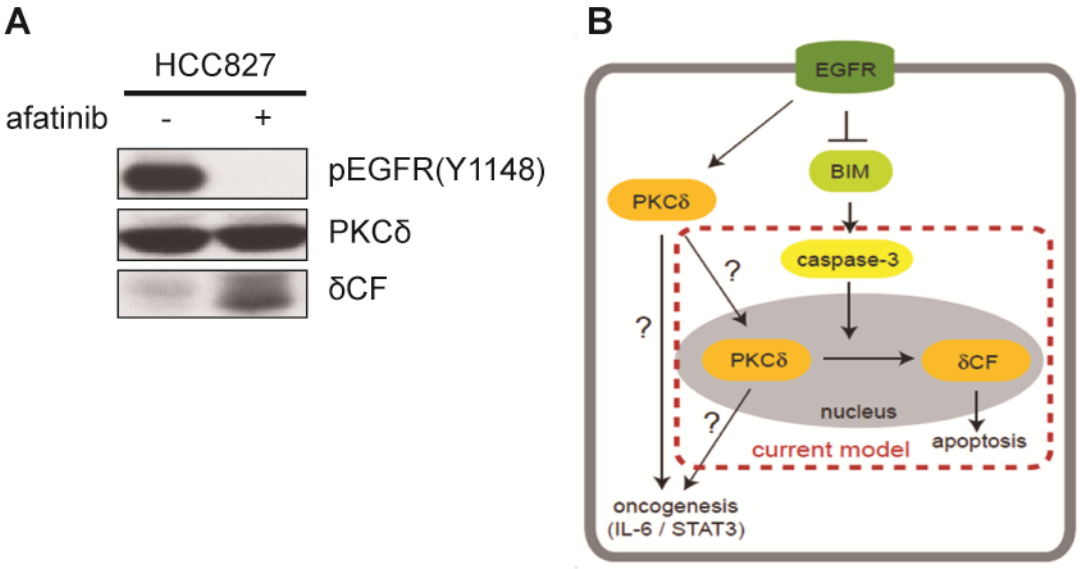

Figure 4.6. EGFR inhibition increases the generation of active PKCδ fragments in mutant EGFR lung cancer cells. A. Western blots of pEGFR(Y1148) and PKCδ show that active PKCδ fragments are increased under 1μM afatinib treatment for 18 hours in serum-free RPMI1640 culture media. B. The current model of an apoptotic role of PKCδ is shown in the red box. In the hypothetic model of an antiapoptotic role of EGFR, EGFR negatively regulates BIM/caspase-3 signaling axis, leading to downregulation of δCF production.

Finally, reduced cancer cell growth under PKCδ depletion implies that targeting PKCδ is a potential therapeutic strategy to treat mutant EGFR lung cancer, which is supported by recently published studies showing that inhibition or depletion of PKCδ leads to reduced proliferation and enhanced apoptosis in prostate, pancreatic, and cancer stem cells (32-34). In the previous chapter, I proposed, tested, and verified the efficacy of targeting both EGFR and COX-2, an additional oncogenic signaling axis, to treat mutant EGFR lung cancer. In addition, I discovered that COX-2 and PKCδ have similar oncogenic roles by regulating IL-6/STAT3 signal in the context of mutant EGFR lung cancer. These combined findings point at PKCδ as an additional target that, combined with EGFR inhibitors, could be used to treat mutant EGFR lung cancer. In order to test the impact of combined EGFR and PKCδ inhibition, future studies will assess the effect of afatinib or third generation EGFR inhibitors such as AZD9291 (35, 36) combined with specific peptides or small molecule PKCδ inhibitors (37, 38) on lung tumorigenesis, using mice expressing $EGFR^{L858R+T790M}$.

Materials and methods

Mice

All mouse experiments were reviewed and approved by the University of Utah Institutional Review Board. The Jackson Laboratory provided transgenic mice expressing the reverse tetracycline-controlled transactivator (rtTA) protein under the control of the rat clara cell secretory protein (CCSP) gene promoter (stock#006232). Mutant EGFR transgenic mice expressing $EGFR^{L858R}$ and $EGFR^{L858R+T790M}$ with a tetracycline promoter were kindly provided by William Pao (Vanderbilt University). Bi-transgenic *CCSP-rtTA;*

TetO-EGFR$^{L858R+T790M}$ or control *TetO-EGFR*$^{L858R+T790M}$ mice were given doxycycline (1mg/L) in the water for 10 weeks to induce tumor development. To confirm tumor development, doxycycline-induced mice were subjected to a small animal Quantum FX microCT, (Perkin Elmer) at 45-um resolution, 90kV, with 160-uA current. Tumor-harboring mice were sacrificed in CO_2 chamber for further analysis.

Histological analysis

Harvested mouse lungs were fixed in 10% neutral buffered formalin for more than 48 hours followed by paraffin embedding. An array of human lung cancer tissues was obtained from Memorial Sloan-Kettering Cancer Center. Sectioned mouse tissues (5μm) and human tissue arrays were immunostained with antibodies against PKCδ (Santa Cruz Biotechnology) and pPKCδ(S299 (Epitomics) using ABC reagent (Vector Laboratories) and ImmPACT™ DAB (Vector Laboratories) followed by counterstaining with hematoxylin (Vector Laboratories). Stained sections were mounted with toluene-based liquid mount media (Triangle Biomedical Science). Stained section images were taken by EVOS FL (University of Utah Health Sciences Center core).

Western blots

Harvested mouse lungs were homogenized using an IKA® T10 basic ultra-turrax homogenizer in cell lysis buffer (Cell Signaling). Harvested H1650, H1975 or HCC827 cells were lysed in cell lysis buffer. Protein concentrations were quantified using BCA reagent (Thermo Scientifics), tissue or cell lysates containing 100μg of total protein were subjected to 10% SDS-PAGE at 35-50 mA for 4-5 hours, followed by overnight transfer

to a polyscreen PVDF membrane at 500 mA. To block remaining protein binding sites, PVDF membranes were blocked in TBS with 5% milk-TBST for 2 hours. Separated proteins in PVDF membrane were blotted with primary antibodies, pEGFR(Y1148) (Cell Signaling), EGFR (Epitomics), PLCγ1 (Upstate biotechnology), pPLCγ1(Tyr783) (Cell Signaling), pPKC(S660) (Cell Signaling), pSTAT3(Y705) (Cell Signaling), STAT3 (Santa Cruz Biotechnology), PKC δ (Santa Cruz Biotechnology), pPKCδ(S299) (Epitomics), and β-actin (ICN Biomedicals) overnight at 4℃. Membranes were washed in TBST for 30 minutes by exchanging fresh TBST three times followed by secondary antibody interaction with mouse IgG HRP (Cell Signaling) or rabbit IgG HRP antibody (Cell Signaling) at room temperature for 2 hours. After washing membranes in TBST for 30 minutes by exchanging fresh TBST three times, Pierce® ECL western blotting substrates (Thermo Scientific) were applied to the membrane for 1 minute. Films (Carestream Biomax MR film) were exposed for various time periods and developed in Konica SRX-101A developer.

Cell growth assay

H1650 and H1975 cells (20,000/well) were seeded in RPMI1640 (Life Technologies) with 10% FBS (Life Technologies) and 1% Penicillin-Streptomycin (Life Technologies). The cells were subjected to PKCδ or control RNAi, as described below. The cells then were exposed to culture media supplemented with 1% FBS and 1% Penicillin-Streptomycin and allowed to proliferate for 5 days. We then harvested and counted the cells using a hemocytometer.

PKCδ RNAi

Cells were transfected with annealed RNAi oligonucleotides (20μM) using Oligofectamine® (Invitrogen) in Opti-MEM® (Lift Technologies) for 4-5 hours, and then grown in RPMI1640 with 10% FBS for 24-48 hours. Sequences of PKCδ RNAi oligonucleotides were as follows:

PKCδ_F: 5'-GGCUACAAAUGCAGGCAAUTT-3'

PKCδ_R: 5'-AUUGCCUGCAUUUGUAGCCTT-3'

PKCδ overexpression

pMyc-CMV empty vector (Clontech) or p3XMyc-PKCδ plasmids were transfected using Lipofectamine® (Invitrogen) in serum-free RPMI1640 for 5 hours. The cells were then grown in RPMI1640 with 10% FBS for 24-48 hours.

Human IL-6 ELISA

PKCδ-silenced and PKCδ-over-expressing H1650 and H1975 cells were serum-starved for 2 hours. The levels of IL-6 were determined in the culture media using the Human IL-6 ELISA Ready-SET-Go Kit (eBioscience), following the instructions provided by manufacturer.

Relative quantification of realtime RT-PCR

Total RNA from PKCδ-silenced and PKCδ-over-expressing H1650 and H1975 cells was extracted using Trizol reagent (Invitrogen). Extracted 2μg of RNA were employed to synthesize cDNA with OligodT primer (Invitrogen) and MMLV-reverse

transcriptase (Fermentas) at 37℃ for 1 hour. Synthesized cDNAs were mixed with Power SYBR Green PCR master mix (Applied Biosystems) and the following primers:

PKCδ (Forward): 5'-AAAGGCAGCTTCGGGAAGGT-3'

PKCδ (Reverse): 5'-TGGATGTGGTACATCAGGTC-3'

β-actin (Forward): 5'-AGGCACCAGGGCGTGAT-3'

β-actin (Reverse): 5'-TCGTCCCAGTTGGTGACGAT-3'

Realtime RT-PCR was performed with the 7900HT Real-Time PCR system (Applied Biosystems) at the University of Utah Health Sciences Center core.

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CHAPTER 5

DISCUSSION

Targeting EGFR is a promising therapeutic strategy to treat mutant EGFR NSCLC, and TKIs such as erlotinib and gefitinib are an effective approach to treat mutant EGFR NSCLC (1-4). However, most patients undergoing therapy with TKIs develop drug resistance within 6-12 months (5, 6). It has been proposed that combined inhibition of EGFR and additional oncogenic signaling pathways can potentially be a therapeutic strategy to overcome drug resistance in mutant EGFR NSCLC (7-9). In this dissertation, I identified COX-2 and PKCδ as novel additional targets for the treatment of mutant EGFR NSCLC and defined mechanisms whereby these enzymes contribute to oncogenesis in settings of mutant EGFR NSCLC.

Targetable COX-2 in mutant EGFR NSCLC

The goal of my studies was to identify key oncogenic pathways and to develop targeted strategies that can be utilized in combination with EGFR inhibitors to treat mutant EGFR NSCLC. In addition to its well-recognized functions in inflammation, COX-2 has been shown to have tumorigenic roles in various types of solid tumors (10-14). Previous work performed by members of our team identified a critical tumorigenic role for COX-2 in the transactivation of EGFR in the settings of colon cancer (15). The observation that

COX-2 is upregulated when EGFR is activated in colon cancer led me to conduct studies aimed at addressing whether COX-2 is upregulated in mutant EGFR NSCLC. First, I found that COX-2 expression is upregulated in models of mutant EGFR NSCLC. Second, I observed that signaling events regulated by COX-2 control IL-6 expression at the transcriptional level in mutant EGFR NSCLC. These observations suggested that COX-2 could have oncogenic roles in this disease and identified this enzyme as a novel therapeutic target for mutant EGFR NSCLC.

I directly tested the suitability of inhibiting COX-2, either alone or in combination with an EGFR inhibitor, in models of mutant EGFR NSCLC. I found that dual-targeting of COX-2 and EGFR inhibited tumor cell growth *in vivo* and *in vitro*. These findings are consistent with previous studies in models of colorectal, breast and lung cancers in which COX-2 inhibition was found to have promising anticarcinogenic effects when combined with chemotherapeutic or radiotherapeutic approaches (16). Importantly, my work also suggests that a regimen based on *single* COX-2 inhibition delays tumor formation in transgenic mice, pointing to potential chemopreventive uses for COX-2 inhibitors in NSCLC. This is in agreement with multiple studies that have consistently shown reduced polyp multiplicity and colorectal cancer incidence following COX-2 inhibition (17-19). The anticancer effects of targeting COX-2 on mutant EGFR NSCLC imply that COX-2 inhibition, when combined with EGFR inhibitor, is likely to be an attractive strategy to treat mutant EGFR lung cancer. Importantly, clinical studies aimed at assessing the efficacy of COX-2 inhibition in lung cancer could potentially be implemented in a straightforward manner. COX-2 inhibitors such as celecoxib are low cost, commonly used, orally active drugs that have been widely utilized and reported to have few side effects.

Role of COX-2 in the tumor-microenvironment

While conducting animal studies aimed at understanding the role of COX-2 in lung tumorigenesis, I made intriguing observations that led me to develop an additional set of studies related to the impact of COX-2 on immune responses. Specifically, I found that inhibition of COX-2 eliminated the characteristic infiltration of mouse lung tumors with CD11b+ cells (Fig. 5.1). Other groups also reported that COX-2 inhibition significantly decreased accumulation of CD11b+ cells and restored the activity of natural killer cells in other tissues including spleen, bone marrow, and skin (20, 21). CD11b+ cells are major producers of chemokines that function to recruit additional inflammatory cells (22). It was recently reported that infiltration of CD11b+ cells is markedly increased in tumor tissues, and that these cells establish a microenvironment that supports tumorigenesis by inhibiting T cell activation in colorectal and liver cancer (23, 24). My observations, combined with these reports, suggest that signaling events regulated by COX-2 may be involved in recruitment of CD11b+ cells to the tumor microenvironment, and that this is likely to favor tumor growth. The findings constitute an additional line of evidence supporting the utility of targeting COX-2 in mutant EGFR NSCLC.

PKCδ: Is it oncogenic and targetable in mutant EGFR NSCLC?

In related work, I investigated other potential strategies that could be used to prevent or overcome TKI-resistance in mutant EGFR NSCLC. I found that PKCδ is increased in protein and activity levels in mutant EGFR tumor mice. Although PKCδ is known to play proapoptotic roles in various types of cells, depletion of PKCδ resulted in reduced growth of human lung cancer cells harboring mutant forms of EGFR. This response

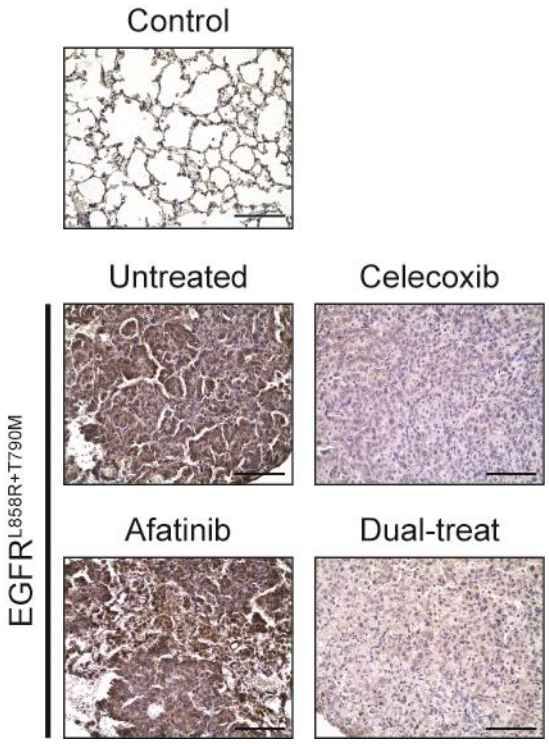

Figure 5.1. Eliminated recruitment of CD11b+ cells in mouse lung tissues under COX-2 inhibition. IHC staining for CD11b shows that COX-2 inhibition results in eliminated CD11b-staining in *EGFR*$^{L858R+T790M}$ mouse lung tissues. Sectioned mouse lung tissues (5μm) were immunostained with CD11b antibody (Epitomics) using ABC reagent (Vector Laboratories) and ImmPACT™ DAB (Vector Laboratories) followed by counterstaining with hematoxylin (Vector Laboratories). Scale bars represent 100μm.

likely involves downregulation of IL-6/STAT3 signaling. Considering its generally accepted roles as a proapoptotic protein, it was surprising that PKCδ had tumor-promoting effects in mutant EGFR NSCLC cells. I hypothesized that EGFR signals might participate in the definition of proapoptotic versus proliferative functions of PKCδ. Interestingly, I found that EGFR inhibition, which is known to induce apoptosis, also led to significantly increased levels of potentially proapoptotic PKCδ catalytic fragments. These correlative findings are consistent with a model whereby PKCδ promotes lung tumorigenesis by limiting apoptotic responses. This constitutes an additional line of evidence supporting inhibition of PKCδ as a strategy to treat mutant EGFR NSCLC.

Open question: Is there a connection between COX-2 and PKCδ?

In this dissertation, I found that COX-2 and PKCδ are potential targets to treat mutant EGFR NSCLC. Using mechanistic studies, I discovered that both COX-2 and PKCδ are involved in the regulation of IL-6 expression and STAT3 activation in mutant EGFR NSCLC. These observations suggest that there may be a functional link(s) between COX-2 and PKCδ, and that this may be important in the regulation of IL-6 levels. Interestingly, mechanistic studies in glioma and colon carcinoma cells revealed that expression of COX-2 is regulated by PKCδ in an EGFR-dependent manner (25, 26), suggesting that PKCδ may function upstream of COX-2. However, I found that COX-2 regulates IL-6 expression in an EGFR-independent manner. Thus, the EGFR-PKCδ-COX-2 axis proposed to function in some tumor models does not appear to operate in mutant EGFR NSCLC. In summary, it is unclear whether the regulation of IL-6 expression is regulated by COX-2 and PKCδ *via* shared or independent pathways, but I established that

COX-2-mediated control of IL-6 levels occurs through EGFR-independent mechanisms. Future studies establishing that inhibition and/or silencing of COX-2 affects PKCδ-mediated regulation of IL-6 expression would provide evidence for a COX-2→PKCδ functional link. Similar approaches can test whether PKCδ functions upstream of COX-2 to regulate IL-6 levels in mutant EGFR NSCLC.

Oncogenic role of the IL-6/STAT3 axis in mutant EGFR NSCLC

I found that both COX-2 and PKCδ contribute to regulation of IL-6 at the transcriptional level, which leads to further activation of STAT3 *via* JAK in mutant EGFR lung cancer cells. A large number of studies have demonstrated that IL-6 and STAT3 are involved in regulation of diverse cellular signaling pathways including differentiation, proliferation, apoptosis, angiogenesis and recruitment of immune cells (27-29). Consistent with my findings, activation of IL-6 and STAT3 has been linked to many types of cancers including myeloma, breast cancer, prostate cancer, head and neck cancer, and NSCLC (30-35). Even though STAT3 activation occurs transiently in normal cells, it has been demonstrated that STAT3 activation is persistent in most malignant cancer cells due to increased production of cytokines and cytokine receptors (34, 36). Persistent activation of STAT3 in cancer cells is involved in cancer development, progression and survival by regulating expression of genes governing cell cycle, survival and angiogenesis (37-39). Since I found persistent activation of STAT3 in mutant EGFR NSCLC, it is reasonable to speculate that the IL-6/STAT3 axis contributes to oncogenesis similar to its role in other cancers. As such, elucidating functional roles of IL-6/STAT3 axis in mutant EGFR NSCLC will be important to further understand mutant EGFR NSCLC oncogenesis to

establish strategies to treat this type of cancer. Indeed, due to its importance in tumorigenesis, targeting STAT3 and its upstream IL-6 and JAK has been proposed as a potential strategy to treat other types of cancer. Although most current STAT3 inhibitors are not potent enough to advance to clinical levels, monoclonal antibodies against IL-6 or its receptor and JAK inhibitors have shown promising outcomes in clinical trials (40). Hence, targeting IL-6, IL-6 receptor, or JAK in combination with an EGFR inhibitor could be another potential strategy to treat mutant EGFR NSCLC.

Future directions

My studies showing that COX-2 regulates IL-6 transcription in mutant EGFR NSCLC open avenues for future investigations to define the mechanism of this response. For example, it would be important to investigate participation of signaling events likely to function downstream of COX-2, such as PKA and β-catenin. The finding that recruitment of CD11b+ immune cells to lung tumors is robustly inhibited by inhibition of COX-2 suggests additional roles for this enzyme in the regulation of environmental events outside of tumor cells. In addition, studying the impact of CD11b+ cells recruitment to the tumor microenvironment is likely to provide additional insight into the role of COX-2 in mutant EGFR NSCLC. A second area for future development is related to studies showing that single inhibition of COX-2 delayed tumor formation in transgenic mice. While I recognize that these studies involved a small number of animals, this finding is potentially important for development of future chemotherapeutic prevention strategies. Finally, the discovery that PKCδ has tumorigenic effects in mutant EGFR lung cancer cells sets the stage for future studies aimed at dissecting the mechanism of this response. The potential

therapeutic utility of targeting PKCδ singly or in combination with TKIs to treat drug-resistant mutant EGFR NSCLC also should be evaluated. In this regard, it will be essential to elucidate the mechanism whereby EGFR signaling controls the switch between prooncogenic and proapoptotic effects of PKCδ.

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