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Role of IKKalpha in the EGFR Signaling Regulation

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14. ABSTRACT Overexpression of EGFR is frequently linked to more aggressive tumor behavior, including increased proliferation, metastasis, and therapeutic resistance. Here, we identified a molecular linkage between IKK α and EGFR signaling in breast cancer cells. Inhibition of IKKs activity elevates EGFR tyrosine phosphorylation. In addition, IKK α forms a specific interaction with EGFR in Golgi apparatus and catalyzes EGFR S1026 phosphorylation. We found that EGFR S1026A possess a stronger tumorigenesis phenotype compare with wild type EGFR suggesting a negative regulation of IKK α in EGFR signaling. In agreement with an earlier finding where conditional ablation of IKK α in the mice Keratinocytes elevates the autocrine loop of EGFR, our results further provide a potent role of IKK α kinase activity in preservation of EGFR activity.					
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1. Introduction

Epidermal growth factor receptor (EGFR), an essential mediator for various growth factors, plays a pivotal role in regulating cell proliferation, migration, and cell cycle progression through activation of distinct signaling pathways. Posttranslational modifications of EGFR such as phosphorylation, ubiquitination, and neddylation confer EGFR a multipotent player and arbitrate the fate of EGFR in mediating signal transduction, subcellular localization, or protein degradation (1). Upon Ligand binding, EGFR dimerizes, autophosphorylates, and trigger a myriad of downstream signaling, such as the activation of phosphoinositide 3 kinase-protein kinase B (PI3K-PKB/AKT), mitogen-activated protein kinase (MAPK), Jak/the signal transducers and activators of transcription (STAT), Rho family GTPase (VAV2), and Phospholipase C (PLC γ) (2). These signaling activities regulate cell proliferation, mobility, and differentiation in many different cell types. To date, tyrosine phosphorylation of EGFR is relatively well characterized and most of them respond to ligand stimulation. For example, Y845 (Tyr845) phosphorylation by c-Src is implicated in stabilizing the activation loop, maintaining the enzyme activity and providing a binding surface for the STAT5b (3) (4). Y992 is a direct binding site for the PLC γ SH2 domain, resulting in activation of PLC γ -mediated downstream signaling (5). Y1045 phosphorylation creates a major docking site for c-Cbl, an ubiquitin E3 ligase, leads to receptor ubiquitination and degradation. Y1068 phosphorylation recruits Grb2 (6), whereas Y1148 and Y1173 provide a docking site for SHC binding (4). Except for extensive knowledge on tyrosine phosphorylation, serine and threonine phosphorylation of EGFR is less understood, and they usually related to negatively regulation of EGFR. For example, the S1046 and S1047 phosphorylation by CaM kinase II attenuates EGFR kinase activity, and mutation of either of them enhances EGFR kinase activity (7).

In this proposal, we found a major inflammation regulator, IKK α , potentially inhibits EGFR activity through a novel signaling pathway in breast cancer cells. IKK α directly interacted with and phosphorylated EGFR at S1026. Inhibition of IKK activity led to hyperphosphorylation of EGFR Y845 and STAT3. Therefore, we hypothesized that IKK α phosphorylates EGFR resulting in a functional inactivation of EGFR at Y845, thereby inactivating STAT3 activity.

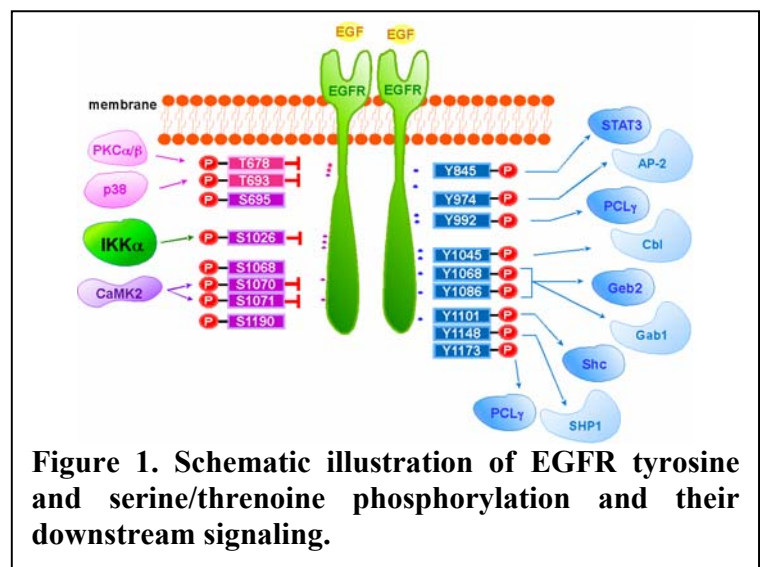


Figure 1. Schematic illustration of EGFR tyrosine and serine/threonine phosphorylation and their downstream signaling.

2. Body

2.1 IKK Inhibits EGFR Signaling

To understand the underlying mechanism of EGFR regulation, various inhibitors were selected and tested their effect on EGFR tyrosine phosphorylation using an anti phospho-tyrosine antibody (4G10) (Fig. 2A). Among all tested inhibitors, Bay 11-7082 (Calbiochem), an IKK kinase inhibitor, unexpectedly enhance EGFR tyrosine phosphorylation in the presence of 25 ng/ml EGF treatment (Fig. 2B). To determine the tyrosine residue might respond to the EGFR activation, six well known anti phospho-tyrosine antibodies of EGFR (pTyr845, pTyr992, pTyr1068, pTyr1086, pTyr1148, and pTyr1173) were also examined. We found that Bay 11-7082 drastically enhances Tyr845 activation, whereas others remain virtually no activation in MDA-MB-468 cells (Fig. 2C). We also examine the activation of EGFR downstream targets PLC γ 1 (pTyr783), VAV2 (pTyr172), STAT3 (pTyr705), AKT (pSer470), and ERK (pTyr204) in the presence of Bay 11-7082 treatment. Surprisingly, inhibition of IKK results in specific activation of EGFR Tyr845 and STAT3 Tyr705 (Fig. 2C). To exclude out the off-target effect using chemical inhibitors, we used siRNA to knockdown IKK α and IKK β expression. Consistent with our earlier finding, downregulation of IKK α elevates EGFR Tyr845 phosphorylation and p-STAT3 status, supporting the notion that EGFR activity is negatively regulated by IKK α .

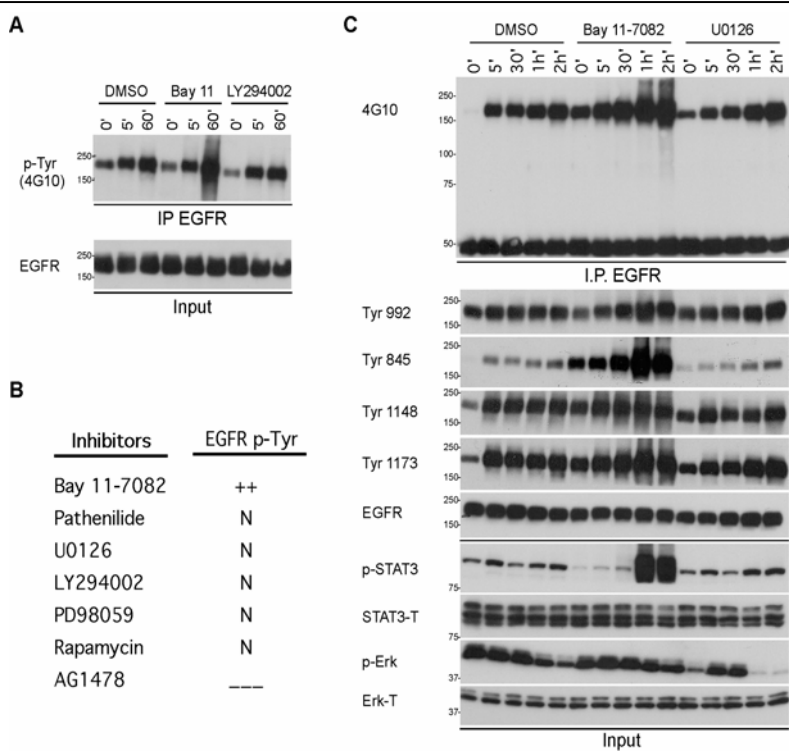


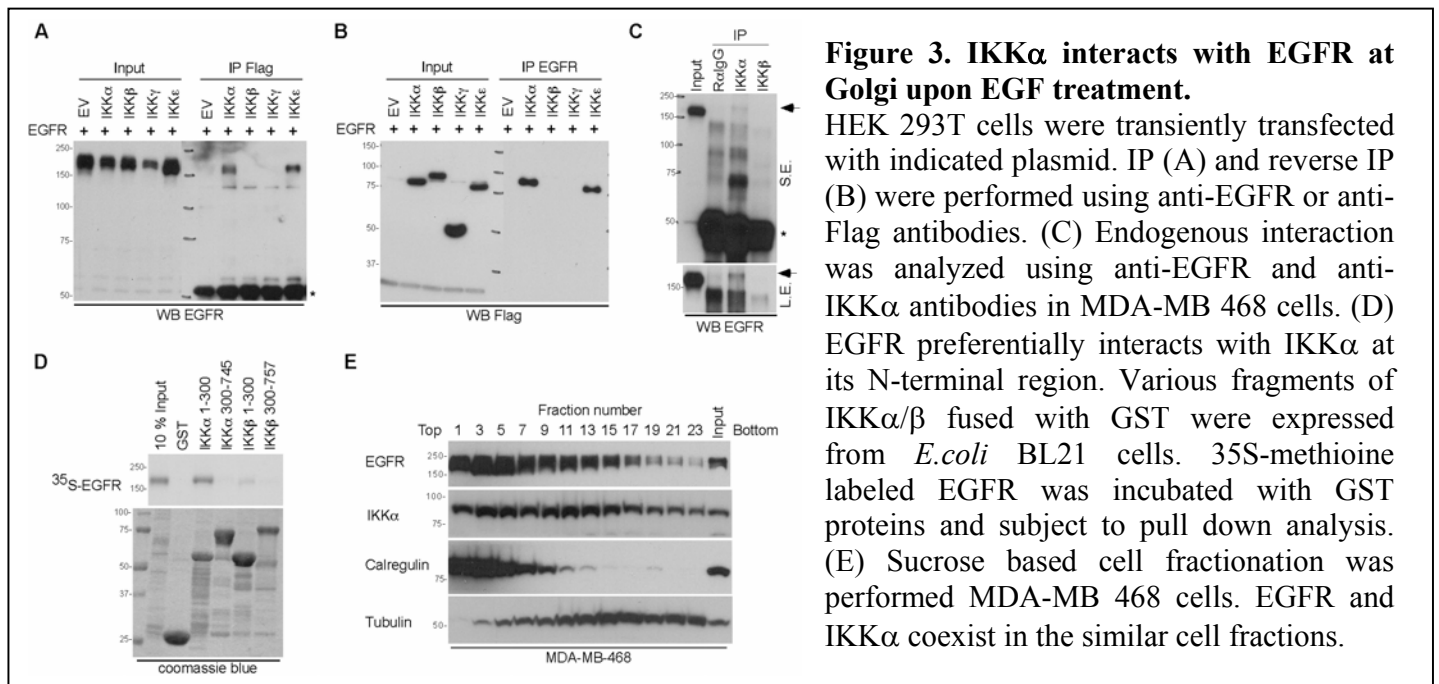
Figure 2. Inhibition of IKK activity enhances EGFR signaling.

(A) MDA-MB-468 cells were pretreated with Bay 11-7082 and LY294002 for 45 mins. Total tyrosine phosphorylation of EGFR IP and examined using anti-Tyrosine phosphorylation antibody (4G10). (B) Similar experiment procedure was performed in MDA-MB-468 cells with various protein kinase inhibitors. As indicated, inhibition of IKK activity enhances EGFR phosphorylation. (C) Inhibition of IKK activates EGFR Tyr845/p-STAT3 signaling axis. MDA-MB-468 cells were pretreated with BAY 11-7082 before 30 ng/ μ l EGF treatments for various time points. EGFR tyrosine phosphorylation and its downstream targets expression were examined.

2.2 IKK α Physically Interacts with EGFR

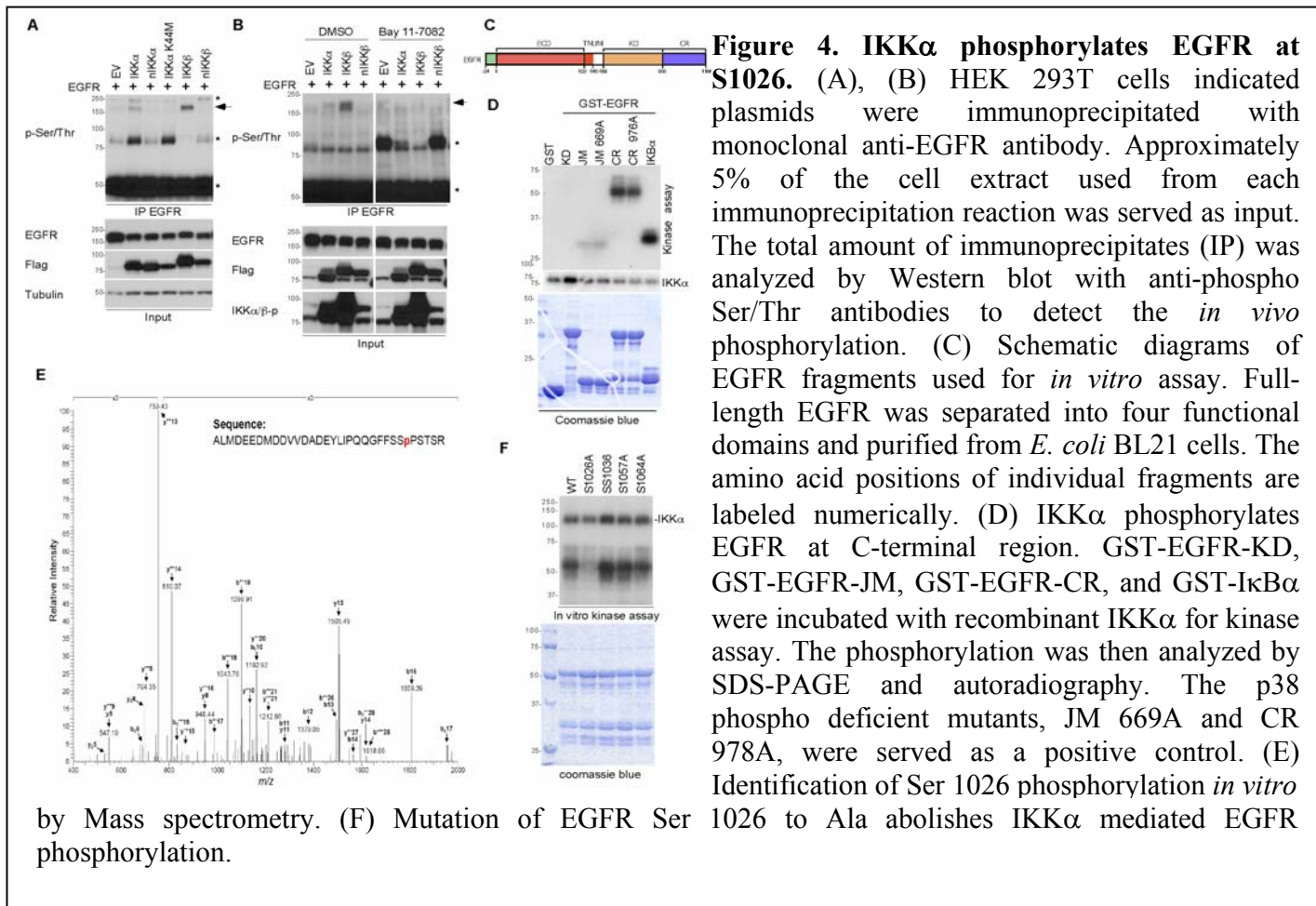
Our finding that inhibition of IKK activates EGFR signaling prompted us to investigate the mechanism by which IKK mediated downregulation of EGFR. Since the IKK complexes are the upstream molecules responsible for the regulation of NF κ B activity, we investigated whether the regulation of EGFR by NF κ B pathway via IKK complex (IKK α , IKK β , and IKK γ). To do so, HEK 293 cells were transiently transfected with

EGFR together with Flagged-IKK α , IKK β , and IKK γ . Coimmunoprecipitation (Co-IP) experiments showed that IKK α but not IKK β or IKK γ physically associates with EGFR (Fig. 3A). Similar results were confirmed using a reverse IP (Fig. 3B). The specific interaction between IKK α and EGFR was also observed at endogenous level in MDA-MB-468 cells using specific antibodies against IKK α and EGFR (Fig. 3C). To test the direct physical interaction, an *in vitro* GST pull down assay was conducted and identified EGFR preferentially interacts with IKK α N-terminal domain (Fig. 3D). In addition to their physical interaction, cellular fractionation assay reveals that both IKK α and EGFR colocalized in the Golgi apparatus under EGF stimulation (Fig. 3E). Since EGFR undergo ligand dependent internalization, we hypothesis the physical contact of EGFR and IKK α appears at Golgi apparatus.



Given the physical association between IKK α and EGFR, we examined whether EGFR is a physiological substrate of IKK α . To test this hypothesis, EGFR together with IKK α , nIKK α (dominant negative), K144M (kinase dead) and IKK β were overexpressed in HEK 293 cells and subjected to IP of EGFR. We found that IKK α catalyzed EGFR serine phosphorylation using an anti-phosphoserine antibody (Fig. 4A) and treatment with Bay 11-7082 abrogates this phosphorylation (Fig. 4B). Since IKK α / β activates one another in the transient transfection system, we utilized an *in vitro* kinase assay in the following experiment. We found that the EGFR C-terminal domain (CR, 978 aa-1211 aa) was strongly phosphorylated by IKK α , whereas other domains (JM, aa 650-718 and KD, aa 718-978) were not (Fig. 4C and 4D). Although S669A and S976A of EGFR complete abolishes P38^{MAPK} phosphorylation, they do not affect IKK α mediated EGFR phosphorylation. To identify the IKK α mediated EGFR phosphorylation, mass spectrometry analysis of *in vitro* kinase assay reveals that EGFR Ser1026 is phosphorylated by IKK α (Fig. 4E). Mutation of Ser residue into Ala (S1026A) abolished the

phosphorylation by IKK α but not other known phosphorylation sites (Fig. 4F), suggesting that IKK α directly phosphorylates EGFR at S1026. To assess whether this phosphorylation occurs *in vivo*, mass spectrometry analysis was performed and confirmed EGFR S1026 phosphorylation in MDA-MB-468 cells (data not shown).



2.4 Characterize the Biological Activity of EGFR S1026A

The biological activities of IKK α mediated EGFR S1026 phosphorylation was vigorously achieved. As proposed in the specific Aim 1, we established EGFR stable transfectants with EV (empty vector), EGFR wild type, EGFR DN (dominant negative, D837A), a well-known patient mutation (L858R) and S1026A in NIH3T3, CHO and MCF7 breast cancer cell lines, in which the basal level of EGFR is low. In fact, the functional analysis of EGFR utilizing these cells for various purposes has been well established in our lab. Therefore, these cell lines served as good recipients for establishing stable transfectants of EGFR and its variants. To create EGFR stable clones, replication incompetent retroviruses produced from pBABE-puro-EGFR vectors by cotransfection in the HEK 293 phoenix packaging cell line (Clontech). Cells were then selected using 2 μ g/ μ l puromycin for 10 days to remove low- or non-infected cells. To rule out the possibility that protein conformational misfolding may occur by amino acid substitution, we analyzed membrane associated EGFR (Fig. 5A) and their ligand binding ability (data not shown). As shown in the Figure 5A, the distribution of membrane

bound EGFR are similar in all stable clones compared to the cells infected with empty vector, suggesting that mutation of EGFR does not influence its cellular property.

To investigate the activation of EGFR signaling, EGFR stable transfectants were serum-starved overnight and treated with 25 ng/ml for various time points. Indeed, EGFR WT activated a good induction of p-STAT3, p-AKT, or p-Erk in response to EGF treatment, whereas EGFR DN failed to do so. Consistent with our hypothesis, EGFR S1026A showed a higher activation of Tyr845 and p-STAT3 with similar degree to that of EGFR L858R. In fact, EGFR S1026A mediated hyperactivation is specific, because p-AKT and p-Erk status did not change compared with EGFR WT (Fig. 5B).

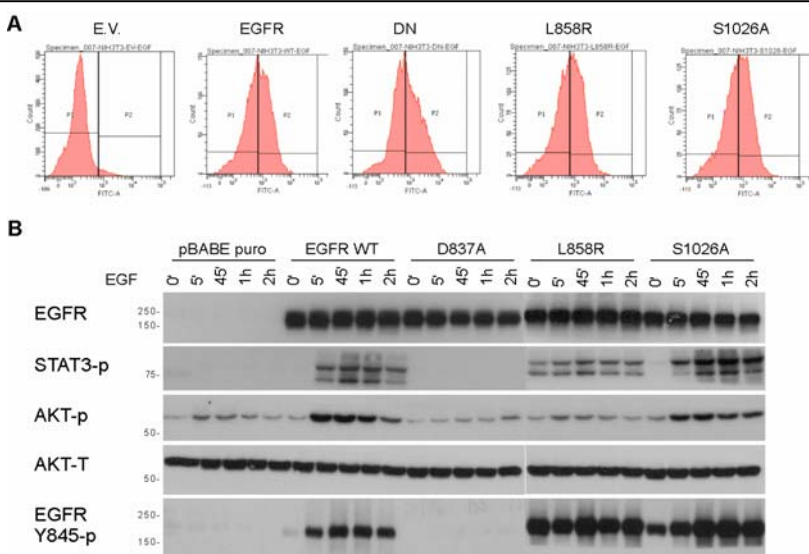


Figure 5. EGFR S0126A exhibits hyperactivation in p-EGFR Y845 and p-STAT3.

(A) NIH3T3 stable clones carry various EGFR variants were subjected to FACS analysis to identified their membrane EGFR localization. As wild type NIH3T3 cells harbor low EGFR expression, the EGFR variants stable clones showed similar expression and localization. (B) EGFR S1026A shows hyperactivation of Y845 and p-STAT3. EGFR Stable clones were serum starved for overnight and treated with 30ng/ul EGF at indicated time points. EGFR tyrosine phosphorylation and its downstream signaling were analvzed by Western blot.

2.5 The Functionality of EGFR S1026A

To test the biological behavior of EGFR S1026A, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assays was performed. We observed that MCF7-EGFR (S1026A) cells grew more quickly than MCF7-EGFR (WT) cells, whereas MCF7-EGFR DN showed a reduced growth (Fig. 6).

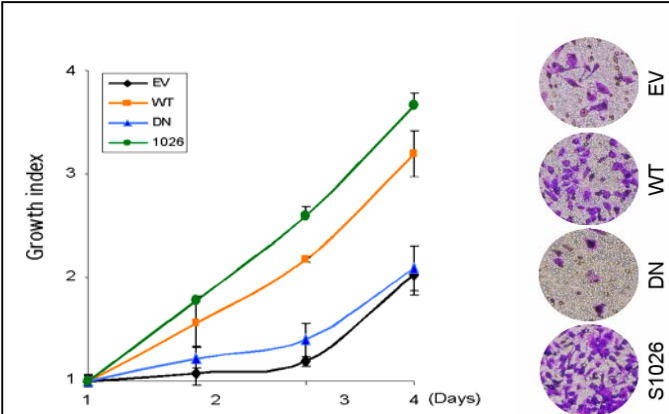
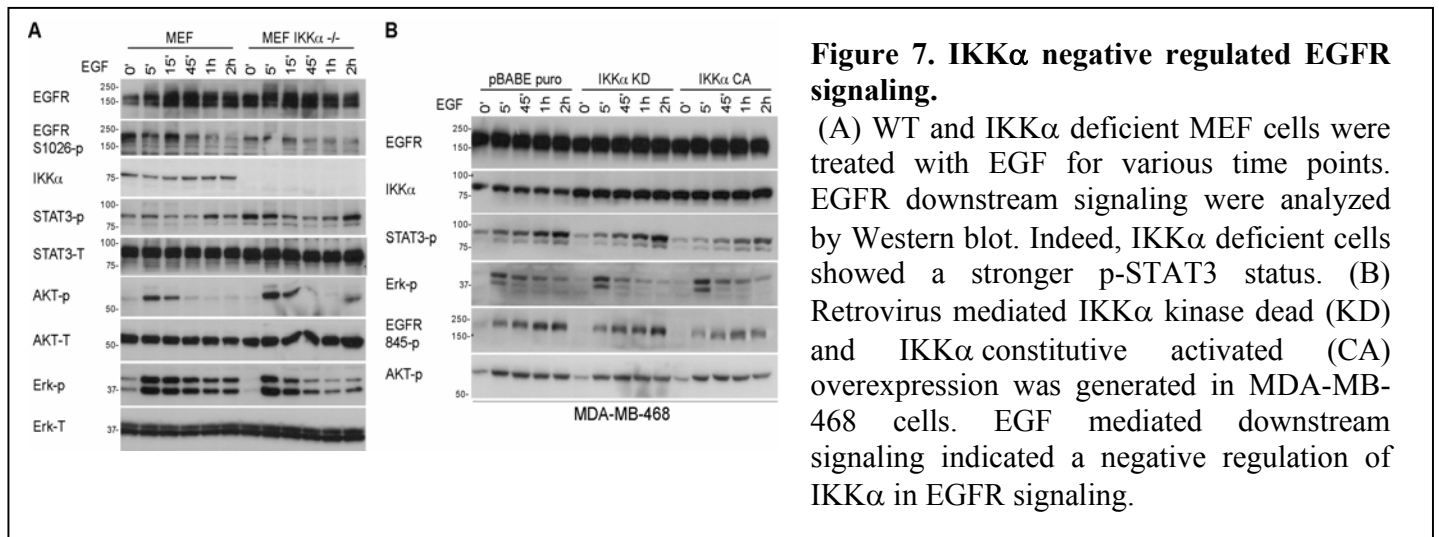


Figure 6. EGFR S1026A associates with more aggressive phenotype.

In vitro cell proliferation rate were assayed using MTT colorimetric method. Compared with Empty vector, EGFR WT and dominant negative (DN), EGFR S1026A showed a enhanced cell proliferation.

Since EGFR also regulates cellular migration and invasion, we also observed a significant increase of cell migration in MCF7-EGFR S1026A in compared with MCF7-EGFR WT (data not shown). As we proposed in the sub-aim 1, orthotropic breast cancer mouse model analyzing tumorigenesis of EGFR S1026A has recently been initiated. A total of 80 mice carrying MCF7-EV, MCF7-EGFR WT, EGFR DN and EGFR (S1026A) were established via mammary fat pad injection to measure the impact of EGFR S1026A on cells proliferation *in vivo*. This part of study is currently ongoing and the result will be presented in the next annual report.

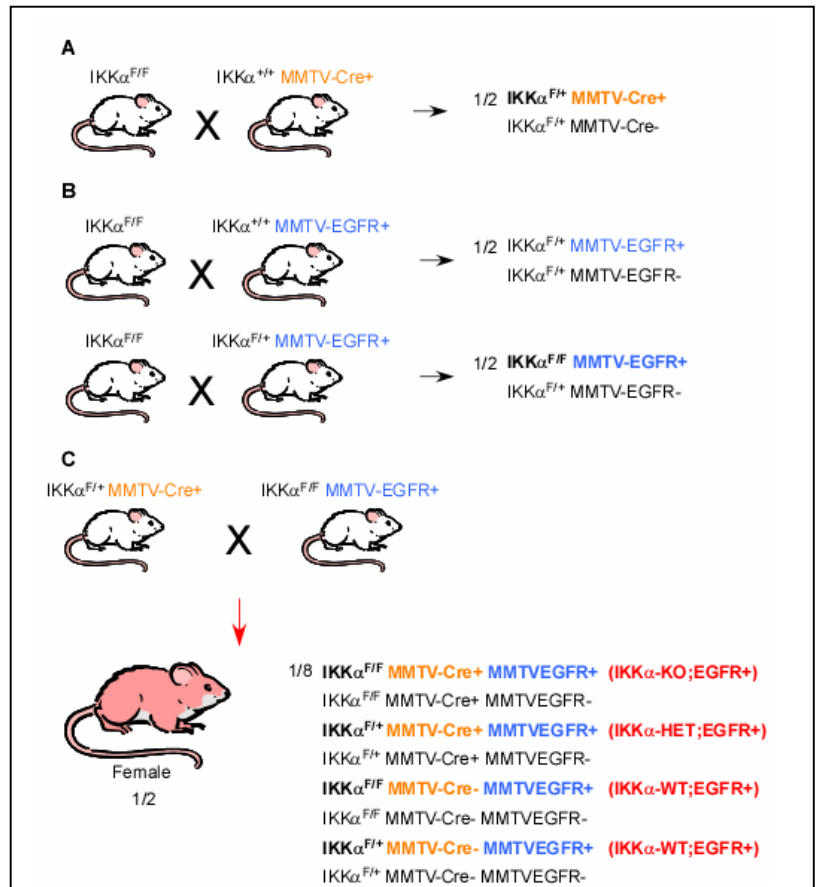
Consistently, IKK α deficient MEF cells specifically upregulated EGF-induced Tyr 845 phosphorylation and p-STAT3 status (Fig. 7A). In addition, stably overexpression of IKK α KD (Kinase dead) and IKK α CA (constitutive activated) in MDA-MB-468 cells showed lower EGFR p-Y845 and p-STAT3 status (Fig. 7B). These results indicate that IKK α inhibits EGFR activity by suppressing EGFR Y845 phosphorylation and STAT3 activity.



3. Future Works

To determine this IKK α /EGFR/STAT3 signaling axis occur *in vivo*, we proposed to generate transgenic mice using current available strain. In the following year, we will generate a conditional knockout mouse model of IKK α in the mammary gland (IKK α ^{F/F} MMTV-Cre, deleted in mammary gland) by crossing IKK α -floxed mice with the MMTV-Cre mice that express Cre recombinase under the control of the mouse mammary tumor virus promoter (MMTV-Cre), which is exclusively expressed in the mammary gland. The IKK α ^{F/F} MMTV-Cre mammary gland specific knockout mice may not develop physically palpable or microscopically observable mammary tumors. Because the tumor suppressor function of IKK α is exclusively via its phosphorylation of EGFR, we will cross IKK α ^{F/+} MMTV-Cre mice with IKK α ^{F/+} MMTV-EGFR to see if the phenotype could be enhanced in the conditional knockout-transgenic mice.

The MMTV-hEGFR transgenic mice developed mammary epithelial hyperplasias, hypertrophy, or slight dysplasias in about 55% of mammary glands of animals examined. Since the inhibition of IKK α results in hyperactivation of EGFR to provide a survival advantage for cancer cells, we would anticipate that co-expression of EGFR in IKK α deleted background may dramatically accelerate the tumor formation in the tri-transgenic mice. 30 IKK $\alpha^{F/F}$ MMTV-Cre MMTV-EGFR conditional knockout-transgenic mice and the same number of their littermate controls will be monitored twice per week for tumor formation. The age of the mouse in which mammary tumor is first palpable will be recorded and tumor size will be measured. Biopsies of tumor tissue will be obtained. To see if IKK α mediated EGFR phosphorylation is important in enhancing the malignant phenotype of EGFR induced tumor progression. Mice tumor section will be stained with EGFR S1026 antibody. Downstream signaling such as p-EGFR 845 and p-STAT3 will also be included to test our hypothesis.



4. Key Research Accomplishments 2010-2011

Characterize the biological activity of EGFR S1026A and S1026D

a) Generate EGFR WT, DN, S1026A, S1026D constructs in the retroviral vector (pBABE) to produce viruses in HEK293 phoenix cells. (All constructs have been restriction mapped and confirmed by full-length sequencing)

b) Establish EGFR S1026A stable cell line in breast cancer cells that harbor low or no EGFR expression. To achieve this aim, NIH3T3, Cho and MCF7 cells were selected as recipients for retrovirus infection. Cells are now stabilized in 2 μ g/ μ l puromycin with equal level of EGFR expression (Fig. 5). NIH3T3 and Cho stable cells are used for signaling and molecular studies, whereas MCF7 cells are used for tumorigenesis for mammary fat pad injection.

c) Investigate the impact of EGFR S1026A in regulating EGFR Y845 and p-STAT3 phosphorylation. As shown in Figure 4, EGFR S1026A show an elevated phosphorylation of p-Y845 and p-STAT3.

d) Investigate the biological function of EGFR S1026 *in vitro* using soft agar assay and MTT cell proliferation assay. EGFR S1026A shows more malignant nature in cell proliferation assay and anchorage independent assay.

e) Investigate the biological function of EGFR S1026 *in vivo* using orthotopic mammary mouse model. The tumorigenesis assay via mammary fat pad injection have been initiated, the result will be presented in the next progress report.

f) Consolidate IKK α in EGFR signaling regulation. We found higher EGFR p-Y845 and p-STAT3 in IKK α deficient MEF cells. Overexpression IKK α using retrovirus inhibits EGF signaling.

5. Reportable Outcomes

Ding Q, Chang, C. J., Xie, X., Xia, W., Yang, J. Y., Wang, S. C., Wang, Y., Xia, J., Chen, L., Cai, C., Li, H., Yen, C. J., Kuo, H. P., Lee, D. F., Lang, J., Huo, L., Cheng, X., Chen, Y. J., **Li, C. W.**, Jeng, L. B., Hsu, J. L., Li, L. Y., Tan, A., Curley, S. A., Ellis, L. M., Dubois, R. N., and Hung, M. C. (2011) APOBEC3G Promotes Liver Metastasis in Orthotopic Colorectal Cancer Mouse Model and Predicts Human Hepatic Metastasis, **J Clin Invest**, in press.

6. Conclusions

The link between inflammation and tumor progression has long been suspected, and accumulating evidence supports a tumor-promoting role of inflammation. For example, the proinflammatory cytokines such as TNF α , IL-1, IL-6, and IL-8 produced in the tumor microenvironment, enhance cell proliferation, cell survival, cell migration, and tumor angiogenesis, thereby promoting tumor development. Until recently, deletion of IKK α in keratinocytes causes skin defects in conditional knockout mice. For the first time, this result suggests that IKK α may acts as a tumor suppressor in preventing skin cancer. Similarly, we found that IKK α interacts with and phosphorylates EGFR at S1026 in the breast cancer cells. Inhibition of IKK α activity through chemical inhibitor or shRNA enhances EGFR Y845 phosphorylation and subsequently activates its downstream target, STAT3. Moreover, EGFR S1026A which failed to be phosphorylated by IKK α , showed higher activity of EGFR pY845 and p-STAT3 compared to EGFR wild type stable clone. In agree with earlier observation, this proposal will establish a novel molecular signaling from IKK α to EGFR and STAT3 regulation.

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