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The intersection of NFkappaB and fibroblast growth factor receptor signaling pathways

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The Intersection of NFkappaB and Fibroblast Growth Factor Receptor Signaling Pathways

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Chemistry

by

Kristine A. Drafahl

Committee in charge:

Professor Daniel J. Donoghue, Chair
Professor Kim Barrett
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Professor Patricia Jennings
Professor Andrew Kummel

2009
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Chair

University of California, San Diego

2009
I dedicate this dissertation to all my friends and family: without their continued support and encouragement, this dissertation would not be possible.
# TABLE OF CONTENTS

Signature Page .............................................................................................................. iii

Dedication.................................................................................................................... iv

Table of Contents .......................................................................................................... v

List of Figures.............................................................................................................. vii

List of Tables ................................................................................................................ ix

Acknowledgements ....................................................................................................... x

Vita .............................................................................................................................. xii

Abstract of the Dissertation ........................................................................................ xiii

Chapter 1  

**Signaling from Fibroblast Growth Factor Receptors in Development and Disease** .......................................................... 1

Abstract.................................................................................................................... 2

Introduction .................................................................................................................. 2

FGFR Expression and Role During Development ............................................. 3

Signaling Pathways Mediated by FGFRS ........................................................... 4

FGFRs and Developmental Disorders ................................................................. 9

Role of FGFRs in Human Cancer .............................................................. 11

Acknowledgments ................................................................................................. 14

References ................................................................................................................. 14

Chapter 2  

**FGFR2 as a Mediator of Opposing Forces: Bringing Balance to Inflammatory Signaling in Breast Cancer** ........................................... 26

Abstract.................................................................................................................... 27

Introduction .................................................................................................................. 27

FGFR2 in Estrogen-Mediated Cancer Progression ........................................... 28

FGFR2 Decreases Inflammation and Plays a Protective Role Against Injury and Cellular Damage ........................................... 31

FGFR2 as Tumor Promoter or Tumor Suppressor: Could NFkB Play a Part? .......................................................... 34

Acknowledgements ................................................................................................. 35

References ................................................................................................................. 36

Chapter 3  

**The Novel Interaction of FGFR4 and IKKβ Negatively Regulates NFkB Activity in DU145 Prostate Cancer Cells** ......................... 38
<table>
<thead>
<tr>
<th>Chapter 4</th>
<th>FGFR2 Interaction and Tyrosine Phosphorylation of IKKβ Negatively Regulates NFκB in T47D Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td></td>
</tr>
<tr>
<td>Results</td>
<td></td>
</tr>
<tr>
<td>Discussion</td>
<td></td>
</tr>
<tr>
<td>Materials and Methods</td>
<td></td>
</tr>
<tr>
<td>Acknowledgements</td>
<td></td>
</tr>
<tr>
<td>References</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 5</th>
<th>E681K in FGFR4 Acts as a Dominant Negative Mutation and is Unable to Decrease NFκB Activity in Lung Adenocarcinoma Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td></td>
</tr>
<tr>
<td>Results</td>
<td></td>
</tr>
<tr>
<td>Discussion</td>
<td></td>
</tr>
<tr>
<td>Materials and Methods</td>
<td></td>
</tr>
<tr>
<td>Acknowledgements</td>
<td></td>
</tr>
<tr>
<td>References</td>
<td></td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Chapter 1

Figure 1-1: Signaling pathways activated by FGFRs ............................................... 8
Figure 1-2: FGFR mutations associated with human cancer ......................... 13

Chapter 3

Figure 3-1: Novel interaction of IKKβ with FGFR4 ............................................. 44
Figure 3-2: Tyrosine phosphorylation of IKKβ by FGFR4 ............................ 48
Figure 3-3: Re-localization of NFκB with FGFR4 expression ................. 51
Figure 3-4: FGFR4 expression and/or FGF19 stimulation inhibits endogenous IKKβ activity ................................................................. 55
Figure 3-5: Endogenous interaction and effect on downstream signaling in DU145 prostate cancer cells .............................................................. 59

Chapter 4

Figure 4-1: Novel interaction of IKKβ with FGFR2 ............................................ 76
Figure 4-2: Tyrosine phosphorylation of IKKβ by FGFR2 ............................ 78
Figure 4-3: FGFR2 expression inhibits TNFα-induced IKKβ activity ............. 80
Figure 4-4: Inhibition of NFκB activity by FGFR2 ....................................... 82
Figure 4-5: Stimulation of T47D cells with FGF8b leads to a decrease in NFκB activity ................................................................. 83

Chapter 5

Figure 5-1: FGFR4 E681K is a dominant-negative mutation and has no kinase activity ................................................................. 98
Figure 5-2: FGFR4 interaction and tyrosine phosphorylation of IKKβ ............ 99
Figure 5-3: E681K FGFR4 has no effect on TNFα-induced IKK activity in A549 cells ................................................................. 100
Figure 5-4: The E681K mutation in FGFR4 is less effective at altering NFκB nuclear localization than a kinase-active FGFR4.
LIST OF TABLES

Chapter 1

Table 1-1: FGFR mutations associated with developmental disorders................. 10
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possible to succeed in life and without them, I would not be here today. And lastly, I would like to thank my loving husband Tommy, who stood by me through all the years and faithfully supported me through both my disappointment in failure and elation in success. With him by my side, I knew I could reach my goals.

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Major Field: Chemistry and Biochemistry

  Studies in Biochemistry, Cellular, and Molecular biology
  Professor Daniel J. Donoghue
ABSTRACT OF THE DISSERTATION

The Intersection of NFκB and Fibroblast Growth Factor Receptor Signaling Pathways

by

Kristine A. Drafahl

Doctor of Philosophy in Chemistry

University of California, San Diego, 2009

Professor Daniel J. Donoghue, Chair

Misregulation of FGFR signaling, or overexpression of the receptors, can lead to uncontrolled downstream signaling associated with a range of developmental syndromes and various forms of cancer. Given the growing evidence on the importance of FGFR4 in cancer progression, we sought to better understand FGFR4 downstream signaling by searching for novel proteins that interact directly with the receptor. Using the intracellular domain of FGFR4 as bait, a yeast two-hybrid assay was performed against a mouse cDNA library. Interestingly, one of the interacting proteins identified was IKKβ, a critical component of NFκB signaling. Research has suggested a link between growth factor signaling pathways and activation of NFκB.
transcriptional activity. The role of NFκB in regulating apoptosis and proliferation in a variety of human cancers, as well as understanding the significance of inflammatory responses in the development of cancer, has been a topic of increasing interest in recent years.

In this work, we show tyrosine phosphorylation of IKKβ resulting from FGFR4 expression, and demonstrate a decrease in NFκB signaling upon FGFR4 activation, which is dependent upon FGFR4 kinase activity. Our research identifies a novel and compelling link between FGFR4 signaling and the NFκB pathway, providing a unique model of NFκB inactivation and implicating FGFR4 as a tumor suppressor in prostate cancer.

Evidence exists to implicate FGFR2 signaling in cancer progression as well as protection from inflammatory insults, indicating FGFR2 may play dual roles as a tumor promoter and a tumor suppressor. We found that similar to FGFR4, FGFR2 is also able to interact with IKKβ and lead to its tyrosine phosphorylation. FGFR2 kinase activity was essential for a decrease in IKK and NFκB activity in breast cancer cells, implicating FGFR2 signaling in the inflammatory response.

A somatic mutation was recently identified in the FGFR4 kinase domain in a screen of lung adenocarcinomas. We determined that this mutation is dominant negative and eliminates all kinase activity of the receptor. Additionally, we found that E681K renders FGFR4 incapable of phosphorylating IKKβ on tyrosine, decreasing IKK activity, and decreasing NFκB activity. This research potentially identifies one mechanism of lung carcinogenesis through inhibition of FGFR4 kinase activity.
Chapter 1

Signaling from Fibroblast Growth Factor Receptors in Development and Disease
Abstract

FGFRs play an important role in cell growth, development, differentiation, and migration and are expressed in a multitude of tissues, indicating their importance in development. The large family of FGFs, as well as the various splice forms of the receptors, allows for specific activation of a variety of intracellular pathways depending on the type of receptor or ligand expressed. Misregulation of FGFR signaling, or overexpression of the receptors, can lead to uncontrolled downstream signaling associated with a range of developmental syndromes and diseases. Much is still unknown about the diverse nature of FGFR signaling, and expanding our understanding of this family of receptors will aid in the development of treatments for the many diseases and cancers linked to misregulation of FGFR signaling.

Introduction

Fibroblast growth factor receptors (FGFRs) constitute a family of four (FGFR1-4) \(^1^3\) structurally related, cell surface receptor tyrosine kinases (RTKs), with 55-72% homology \(^4\). FGFRs are involved in a variety of biological processes including cell growth, migration, differentiation, survival, and apoptosis and are essential for embryonic and neural development, skeletal and organ formation, and adult tissue homoeostasis \(^5,^6\). Alternative splicing of Fgfr transcripts generates up to 15 isoforms, which transmit the signals of at least 22 fibroblast growth factors (FGF1-22) \(^7\). Each receptor is comprised of an extracellular ligand binding domain consisting of three immunoglobulin (Ig)-like domains, an acidic box between IgI and IgII \(^4\), a transmembrane domain, and a split intracellular tyrosine kinase domain composed of
an ATP binding site and catalytic site. FGFR activation is achieved upon ligand binding\textsuperscript{8, 9}, resulting in receptor dimerization and transautophosphorylation of multiple conserved intracellular tyrosine residues\textsuperscript{10}, which stimulate the receptor’s intrinsic kinase activity and recruit downstream adaptor and signaling proteins\textsuperscript{11-13}. Heparan sulfate proteoglycans (HSPGs) facilitate ligand binding and are obligate cofactors for FGFR activation by FGFs\textsuperscript{14-17}. The three main signaling pathways associated with FGFR activation include the Ras/MAPK, PI3-Kinase, and PLC\textsubscript{\gamma} pathways. All but one of the mutations known for the \textit{Fgfr} genes are gain-of-function mutations, and activation of these receptors is associated with many developmental and skeletal disorders\textsuperscript{18, 19}. Additionally, FGFR and FGF overexpression has been observed in many tumor samples, and mutations are also likely to be involved in carcinogenesis.

**FGFR Expression and Role During Development**

During embryonic development, FGFR signaling is essential for organ growth and patterning of the embryo. All FGFRs are widely expressed in distinct spatial patterns during development and in adult tissues\textsuperscript{20-24}. FGFR1 expression is found mainly in the mesenchyme in the central nervous system and limbs, and targeted inactivation of \textit{Fgfr1} in mice severely impairs growth and results in recessive embryonic lethality\textsuperscript{25}. During early neurogenesis, FGFR1 expression is upregulated in the ventricular zone of the neural tube and mesenchyme of developing limbs\textsuperscript{26, 27}, and at later stages is expressed in spinal cord motor neurons and maturing neurons in the brain\textsuperscript{26, 28}. Although required for correct axial organization and embryonic cell
proliferation, FGFR1 is not directly required for mesoderm formation. FGFR1 was also shown to play a role in neurulation, as chimeric mouse embryos, created by injection of FGFR1 deficient (R1-/−) embryonic stem (ES) cells into wild type blastocyes, showed limb bud and tail distortion, partial neural tube duplication, and spina bifida. FGFR2 is highly expressed in epithelial lineages during early gastrulation, and in both epithelial and mesenchymal cells during later development and organogenesis. Like Fgfr1, targeted disruption of Fgfr2 results in an embryonic lethal phenotype. Its expression is essential for limb outgrowth, mammalian lung branching morphogenesis, and keratinocyte differentiation. FGFR3 expression primarily occurs in the central nervous system and bone rudiments, specifically the developing brain, spinal cord, cochlea, and hypertrophic zone of the growth plate. Targeted disruption of Fgfr3 in mice is not embryonic lethal, but leads to severe skeletal and inner ear defects, and mouse models indicate FGFR3 negatively regulates bone growth and development. FGFR3 also cooperates with FGFR4 to mediate liver functions and lung development. FGFR4 expression occurs in the definitive endoderm, somatic myotome, and the ventricular zone of developing dorsal root ganglia and spinal cord. Although, Fgfr4 null mice appear normal, they exhibit elevated liver bile acids, enhanced cholesterol biosynthesis, and depleted gall bladders.

**Signaling Pathways Mediated by FGFRs**

Activation of FGFRs can result in a variety of outcomes by initiating various intracellular signaling pathways. In many cases, the pathways activated depend on the
cell type or stage of differentiation, leading to specific activation of downstream targets. Specificity is also achieved through the binding of different FGFs of which many have unique and cell-specific roles. Splice variants of FGFRs also contribute to diverse cell signaling. Despite the varied outcomes of FGFR signaling, several key pathways are commonly activated in most cell types. FGFR activation results in tyrosine autophosphorylation, and these phosphorylated tyrosines serve as high-affinity binding sites for proteins containing Src-homology 2 (SH2) domains or phosphotyrosine binding (PTB) domains. These intracellular proteins then transduce the activation signal from the receptor through signaling cascades which eventually lead to changes in gene transcription and a biological response.

The membrane-associated docking protein FGF receptor substrate 2 (FRS2) binds to the FGFR juxtamembrane domain (JM) through its PTB domain and is phosphorylated by the receptor. This leads to recruitment of a variety of adaptor proteins, including growth factor receptor bound protein 2 (Grb2), which then binds the guanine nucleotide exchange factor son of sevenless (Sos). Recruitment of this complex to the plasma membrane activates the G-protein Ras, which stimulates the mitogen-activated protein kinase (MAPK) pathway. MAPK pathway activation results in a variety of outcomes depending on cell type or state, including DNA synthesis, proliferation, and/or differentiation. The adaptor molecule Shc is also phosphorylated by FGFR, leading to Grb2 recruitment and activation of the Ras/MAPK pathway.

FRS2 activation also signals through the PI3-Kinase pathway. The SH2 domain of Grb2 binds to a phosphorylated tyrosine residue on FRS2 while the C-
terminal SH3 domain of Grb2 forms a complex with the proline-rich region of Grb2 associated binding protein 1 (Gab1). Gab1 recruitment in close proximity to the receptor results in its tyrosine phosphorylation. Recruitment of PI3-Kinase and activation of AKT follows, leading to cell survival. FGFR binding and phosphorylation of FRS2 is essential for Gab1 recruitment and eventual activation of the PI3-Kinase cascade indicating FGFR activation of FRS2 plays a prominent role in promoting cell survival. The N-terminal SH2 domain of SH2 tyrosine phosphatase 2 (Shp2) interacts with a phosphotyrosine on FRS2 and leads to phosphorylation of Shp2 itself. Phosphorylated Shp2 interacts with the Grb2/Sos complex and forms a ternary complex with FRS2. Shb also interacts with Shp2, and potentiates its FGF-mediated phosphorylation and FRS2 interaction. Interaction of phosphorylated Shp2 with FRS2 is essential for MAPK activation, indicating an important role for the adaptor Shb. FRS2 has also been shown to associate with Src, a non-receptor tyrosine kinase, which phosphorylates cortactin to affect cell migration.

Autophosphorylation of Tyr766 in the carboxy-terminal tail of FGFR1 creates a specific binding site for the SH2 domain of PLCγ. Activation of PLCγ by tyrosine phosphorylation results in hydrolysis of phosphatidylinositol, generating diacylglycerol (DAG) and Ins (1,4,5) P3 (IP3). Generation of these second messengers results in Ca^{2+} release and activation of PKC. Shb also interact with FGFR1 through Y766, although it does not seem to compete for binding with PLCγ.

Other adaptor molecules link FGFR activation to various biological activities. Crk interacts with Tyr463 on FGFR1 and results in cellular proliferation in certain cell types. The adaptor protein Nck also binds to phosphorylated FGFR, facilitating...
the interaction between Pak and Rac, and may link FGFR signaling to the actin cytoskeleton\textsuperscript{56}. Activated FGFR1, 3, and 4 also promote Stat1 and Stat3 activation\textsuperscript{57} and FGFR3 can activate STAT5 through the adaptor protein SH2-B\textsuperscript{58}. Many of the interactions and signaling pathways activated by FGFRs described above are shown in Figure 1-1.
FGFRs dimerize and undergo autophosphorylation upon ligand stimulation, creating docking sites for various signaling molecules. Additional proteins are recruited to the membrane through modular domain interactions involving SH2, PTB, and other domains. Once at the membrane, these proteins activate multiple cellular signaling pathways, most notably the MAPK, PI3-K, and PLCγ pathways.

**Figure 1-1: Signaling Pathways Activated by FGFRs.**
FGFRs and Developmental Disorders

Specific mutations in the *Fgfr1-3* genes lead to congenital bone diseases classified as chondrodysplasia and craniosynostosis syndromes, which cause dwarfism, deafness, and abnormalities of the skeleton, skin and eye. Almost all of these are activating, gain-of-function mutations, and many occur in the IgII and IgIII domains, which mediate FGF binding. Over sixty mutations have been found to be associated with craniosynostosis syndromes, with a majority in FGFR2, including Antley-Bixler-like syndrome (ABS), Apert syndrome (AS), Beare-Stevenson syndrome (BSS), Crouzon syndrome (CS), Jackson-Weiss syndrome (JWS), Muenke-like syndrome (MS), Saethre-Chotzen syndrome (SCS), as well as the FGFR1 associated craniofacial dysplasia with hypophosphatemia (CFDH) and Pfeiffer syndrome (PS). All of these mutations are dominant, and craniofacial abnormalities varying in severity result from these syndromes. Missense mutations in FGFR3 result in skeletal dysplasia syndromes and short-limbed dwarfisms, including achondroplasia (ACH), Crouzon syndrome with acanthosis nigricans (CAN), hypochondroplasia (HCH), severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN), and the platyspondylic lethal skeletal dysplasias (PLSDs), including thanatophoric dysplasia (TD) types I and II. Additionally, two syndromes caused by loss-of-function mutations in FGFRs have been described, including the FGFR1 associated type 2 Kallmann syndrome (KS) and the FGFR3 associated camptodactyly, tall stature, and hearing loss (CATSHL) syndrome. To date, no mutations in FGFR4 are associated with any known chondrodysplasia.
or craniosynostosis syndromes. A list of the mutations and syndromes associated with their respective FGFR can be seen in Table 1-1.

Table 1-1: FGFR mutations associated with developmental disorders

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Missense Mutations</th>
<th>Receptor (Domain)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACH</td>
<td>Y278C, S279C, G346E, G375C, G380R</td>
<td>FGFR3 (IgIII and TM)</td>
</tr>
<tr>
<td>ABS</td>
<td>S267P, W290C, C342R/S, S351C</td>
<td>FGFR2 (IgIII)</td>
</tr>
<tr>
<td>AS</td>
<td>S252W/F, P253R</td>
<td>FGFR2 (IgII-IgIII linker)</td>
</tr>
<tr>
<td>BSS</td>
<td>S372C, Y375C</td>
<td>FGFR2 (JM)</td>
</tr>
<tr>
<td>CFDH</td>
<td>Y372C</td>
<td>FGFR1 (TM)</td>
</tr>
<tr>
<td>CAN</td>
<td>A391E</td>
<td>FGFR3 (TM)</td>
</tr>
<tr>
<td>CATSHL</td>
<td>R621H</td>
<td>FGFR3 (KD)</td>
</tr>
<tr>
<td>HCH</td>
<td>S84L, G268C, R200C, N262H, V381E, I538V, N540K/T/S, K650N/Q</td>
<td>FGFR3 (KD)</td>
</tr>
<tr>
<td>JWS</td>
<td>C278F, Q289P, C342S/R, A344G</td>
<td>FGFR2 (IgII)</td>
</tr>
<tr>
<td>MS</td>
<td>P250R</td>
<td>FGFR3 (IgII-IgIII linker)</td>
</tr>
<tr>
<td>PS</td>
<td>P252R</td>
<td>FGFR2 (IgII-IgIII linker)</td>
</tr>
<tr>
<td>TDI</td>
<td>R248C, S249C, G370C, S371C, Y373C, x807L/G/C/R/W</td>
<td>FGFR3 (IgII-IgIII linker and IgIII-TM linker)</td>
</tr>
<tr>
<td>TDII</td>
<td>K650E</td>
<td>FGFR3 (KD)</td>
</tr>
<tr>
<td>SCS</td>
<td>Q289P</td>
<td>FGFR2 (IgII-IgIII linker)</td>
</tr>
<tr>
<td>SADDAN</td>
<td>K650M</td>
<td>FGFR3 (KD)</td>
</tr>
</tbody>
</table>
Role of FGFRs in Human Cancer

All four members of the FGFR family and many of their ligands have been implicated in human cancers as well. They play roles in cancer progression by inducing angiogenesis, changes in cell morphology, increased motility, and tumor cell proliferation. FGFRs are overexpressed or have altered activity in cancers of the colon, prostate, breast, kidneys, ovaries, central nervous system, gastrointestinal system, thyroid, pituitary, brain, liver, pancreas, skin, and lung as well as in leukemia, multiple myeloma, urological cancers, soft tissue sarcomas, head and neck squamous cell carcinoma, and lymphoma. Recent evidence indicates FGFRs may be used to target tumors for growth inhibition and targeted inhibition of FGFRs may provide a therapeutic approach in the fight against cancer.

FGFR1 was recently found to be amplified in a small percentage of breast cancers and contributes to the survival of lobular breast carcinomas. In estrogen-receptor positive breast cancer cells, FGFR1 amplification is a prognostic of poor outcome. Recent research found that activation of FGFR1 plays a role in the initiation of angiogenesis in prostate cancer and may be a new marker for prostate cancer progression, as it was shown to be upregulated in late stage prostate tumors. The role of FGFR1 is most widely described in chronic myeloproliferative disorders (CMPDs). One rare CMPD, known as 8p11 myeloproliferative syndrome (EMS) or stem cell leukemia lymphoma (SCLL), is caused by an 8p11 translocation of Fgfr1. This leads to fusion of Fgfr1 to other genes and constitutive activation of the receptor.
The first fusion identified was to a zinc finger gene, \textit{ZNF198}, and subsequently many \textit{Fgfr1} rearrangements involved with a variety of partners have been demonstrated \cite{119}.

In two recent genome-wide association studies, FGFR2 was implicated with increased susceptibility to breast cancer \cite{120, 121}. It is believed that a splice variant of FGFR2 or possibly an unwarranted estrogen receptor binding site may be the cause for the associated risk of breast cancer \cite{121}. Also, certain types of gastric cancers overexpress FGFR2 and recent research has discovered that an inhibitor, AZD2171, exerts potent antitumor activity against gastric cancer xenografts overexpressing FGFR2 \cite{122}.

A frequent translocation observed in multiple myeloma, t(4;14)(p16.3;q32.3), involves the \textit{Fgfr3} gene, and results in increased expression of FGFR3 alleles that contain activating mutations \cite{123, 124}, including Y373C and K650E, which cause the lethal skeletal syndromes TDI and TDII \cite{72, 79}. The splice variant FGFRIIIb is expressed in a wide range of bladder and cervical carcinoma cell lines \cite{125}, and these cancers exhibited expression of mutant alleles of FGFRIIIb, including R248C, S249C, G372C and K652E \cite{125}. These and other FGFR mutations are shown in Figure 1-2.
Figure 1-2: FGFR mutations associated with human cancer. FGFR mutations associated with various human cancers are indicated. The abbreviations are as follows: bladder cancer (B) (135-138), cervical cancer (C) (125), seborrheic keratoses (SK) (139), colorectal cancer (CR) (140), multiple myeloma (MM) (123, 124, 141, 142), glioblastoma (Gl) (143), and gastric cancer (Ga) (140). The mutations are placed at their approximate location in the FGF receptor.
Although FGFR4 is not associated with any known syndromes, it is associated with the widest range of cancers. Of recent debate is the significance of the G388R polymorphism. This polymorphism exists in approximately half of the population and appears to have no affect on cancer susceptibility. However, evidence suggests that the polymorphism leads to reduced disease-free survival in cancer patients and correlates with a poor prognosis compared to the Gly388 allele in head and neck squamous cell carcinoma, breast cancer, melanoma, lung adenocarcinoma, prostate cancer, and high grade soft tissue sarcomas. Opposing evidence suggests there is no correlation between the G388R polymorphism and cancer prognosis. Continued research into the significance of this polymorphism is needed to conclude if it is a valuable marker for cancer prognosis.

Acknowledgements

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References


16. Pantoliano, M.W. *et al.* Multivalent ligand-receptor binding interactions in the fibroblast growth factor system produce a cooperative growth factor and


Chapter 2

FGFR2 as a Mediator of Opposing Forces: Bringing Balance to Inflammatory Signaling in Breast Cancer
Abstract

Though FGFR signaling is most commonly associated with components of increased tumor growth and cancer progression such as cell proliferation and migration, recent evidence indicates FGFR signaling may also play a protective role against inflammatory insults in some cellular contexts. Inhibitors targeting aberrant FGFR signaling in cancer cells may prove beneficial to treatment of these cancers, but understanding how FGFR signaling may also protect cells from injury is important to take into consideration. In this review, we discuss evidence which suggests FGFR2 signaling may be tumor-promoting in some contexts, while tumor-suppressive in other instances, and hypothesize a mechanism for this dual role in breast cancer.

Introduction

FGFR2 is one member of a family of four fibroblast growth factor receptors. The FGFRs are transmembrane receptor tyrosine kinases which are high affinity receptors for a large family of FGF ligands. Activating mutations in FGFR2 have been associated gastric \(^1\), bladder \(^2\), and endometrial cancers \(^3\). FGFR2 has several splice variants which alter the receptor activity. Alternative splicing in the 3\(^{rd}\) IG-like domain creates the FGFR2IIIb or IIIc isoforms. The FGFR2IIIb isoform (FGFR2b, also known as the keratinocyte growth factor receptor KGFR) is expressed specifically in epithelial cells, while the FGFR2IIIc isoform is expressed in mesenchymal cells. Many of the FGF ligands bind to all four FGFRs; however, FGF7 (also known as KGF-1), binds specifically to FGFR2b. FGF7 is secreted from mesenchymal cells to
act in a paracrine fashion to activate the FGFR2b receptor on neighboring epithelial cells.

**FGFR2 in Estrogen-Mediated Cancer Progression**

Breast cancer is the most common cancer women face today (aside from nonmelanoma skin cancer), with the American Cancer Society estimating 182,460 new cases of and 40,480 deaths from breast cancer in US women during 2008. A large percentage of breast cancers are dependent upon estrogen to grow. Targeted therapies such as tamoxifen exploit this trait and deprive cancer cells of the estrogen needed to grow. However, many breast cancers become resistant to this treatment and are no longer dependent upon estrogen, leading to increased aggressive and metastatic growth. Understanding how these cancer cells become resistant to treatment is currently of great interest.

In a study aimed at analyzing FGF7/FGFR2b expression in relation to estrogen receptor status, a correlation was found between breast cancer cases positive for ERα and FGF7 expression. Additionally, it was hypothesized that FGF7 expression may be regulated by ERα in a ligand-independent manner, as addition of 17-β estradiol did not increase expression of FGF-7. Cases positive for both FGF7 and FGFR2b showed decreased TUNEL staining, indicating that co-expression of the ligand and receptor is important for decreasing apoptosis. Interestingly, when MCF-7 cells were treated with chemotherapeutic drugs cyclophosphamide or 5-fluorauracil, FGF7-treatment decreased apoptosis indicating that FGF7 may protect cells from chemotherapeutic-induced apoptosis. A correlation was also found between FGFR2b
expression and Bcl-2, indicating ERα-induced expression of FGF7 may allow activation of FGFR2b, leading to decreased Bcl-2 expression and apoptosis in breast cancer cells. These results seem to implicate FGFR2b signaling in breast cancer progression through an anti-apoptotic effect. In a separate study, treatment with 17β-estradiol has been shown to decrease FGFR2b expression at the transcriptional level, while treatment with progesterone caused an increase in FGFR2b mRNA levels, indicating the synthesis of FGFR2b may be dependent on the ratio of estradiol and progesterone. Alterations in ER or PR expression may lead to abnormal growth control in breast cells and implicate FGFR2b in the pathogenesis of breast cancer. A recent study found that in cells treated with tamoxifen, FGF7-treatment abolished a tamoxifen-induced decrease in proliferation, while FGFR2b silencing was able to restore the antiproliferative effect of tamoxifen on ER-positive breast cancer cells, indicating the FGFR2b signaling pathway might be a valuable target for combating tamoxifen resistance.

Isoform switching has also been implicated in the role of FGFR2 in breast cancer progression. The FGFR2b isoform has been found to be expressed in untransformed MCF-10A cells as well as noninvasive breast cancer cell lines, while the FGFR2c isoform has been detected in invasive breast carcinoma cell lines. In addition to splice variants in the third IG loop of FGFR2, there are carboxy-terminal splice variants termed C1, C2, and C3 which vary in the number of amino acids which are removed from the C-terminus, with C3 removing the largest number of amino acids. The C2 and C3 splice variants have been found to have increased transforming potential in NIH3T3 cells. Cha et al. pose an interesting hypothesis that breast cancer
progression may involve two alternative splicing events whereby expression of the
FGFR2b C2 or C3 splice forms may be associated with progression from normal breast
epithelia to noninvasive breast cancer, while conversion from epithelial FGFR2b to
mesenchymal FGFR2c is associated with transition to an invasive breast cancer\(^9\).

Similar to breast cancers, endometrial cancers may also be dependent upon
estrogen to grow. The American Cancer Society estimated 51,170 new uterine cancer
cases and 11,340 deaths in 2008\(^4\). Endometrial stromal fibroblasts (ESFs) are known
to increase anchorage independent growth with chronic treatment with a model
estrogen DES (diethylstilbestrol), and long term treatment of ESFs was found to
increase expression of FGF7\(^10\). Endometrial stromal fibroblasts stably transfected
with IκB\(\alpha\) to inhibit the endogenous NFκB pathway, had no effect on long-term DES-
induced expression of FGF7, indicating long-term estrogen treatment increases FGF7
expression in an NFκB-independent manner\(^10\). FGF7 expression increased the cell
number and capacity for anchorage independent proliferation of fetal endometrial
epithelial cells, indicating estrogen-induced increases in FGF7-expression may lead to
tumorigenesis in the human endometrium, independent of NFκB\(^10\). In a more recent
study, FGF7 was found to induce NFκB activation in immortalized human pancreatic
ductal epithelial cells, resulting in increased expression of MMP9 and VEGF, and
FGFR2b was required for the observed decrease in NFκB activity\(^11\). It seems possible
that the role of FGFR2b in breast or endometrial cancer progression may or may not
be dependent on NFκB, and may vary in different cellular contexts.
FGFR2 Decreases Inflammation and Plays a Protective Role Against Injury and Cellular Damage

Although there is strong evidence to indicate FGFR2 acts as a tumor promoter, there is equally convincing evidence to suggest it acts as a tumor suppressor. A question remains whether this tumor suppressive and protective effect is due to an FGFR-mediated increase in mitogenicity or another mechanism, such as an FGFR2-mediated decrease in inflammation. It is also possible that FGFR2 has various mechanisms for decreasing cellular injury depending on the circumstance. A large body of evidence suggests FGFR2b is a suppressor of inflammation and tumors in the lung, bowel, and the skin.

The barrier function of airway epithelial cells is important in preventing airborne bacteria, viruses, as well as other substances from entering the body. Inflammatory diseases and air pollution expose the airway epithelium to reactive oxygen species (ROS) for extended periods of time. Among patients with acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) which is common among medical and postsurgical patients, the mortality rate is high. A recent study used adenovirus-mediated transfer of KGF-cDNA to airway epithelial cells, which was found to raise lung epithelial cell proliferation, increase arterial blood gas, and increase the survival rate of mice with hyperoxia-induced lung injury, suggesting that KGF gene transduction into the lungs may provide a promising new treatment for acute lung injury

Pulmonary emphysema is characterized by persistent inflammation and progressive alveolar destruction. Elastase treatment of mice induces pulmonary
emphysema, while pre-treatment with FGF7 reduced the influx of elastase-induced BALF (bronchoalveolar lavage fluid) proteins (which are a measure of injury in the lung), prevented an influx of inflammatory cells, and decreased expression of ICAM-1, a protein implicated in the recruitment of macrophages and neutrophils to the site of injury in the lung. In this study, treatment with FGF7 after elastase treatment did not seem to have any effect on elastase-induced emphysema, indicating FGF7 may have a protective effect rather than a healing effect toward pulmonary emphysema in mice.

Asthma is one of the fastest growing health problems today and is also caused by increased airway epithelial permeability. In a recent study, ovalbumin treatment of brown Norway rats was found to induce chronic asthma, while FGF7 pre-treatment seemed to improve alterations in permeability. FGF7 pre-treatment also inhibited lymphocyte and neutrophil recruitment in BALF, limited epithelial alterations and restored epithelial integrity, and also increased the levels of β-catenin and ZO-1 protein expression, which are intercellular junction proteins important in maintaining cell-cell adhesions. This evidence implicates FGFR2b signaling in the maintenance of cell-cell adhesions in the airway epithelium, thereby decreasing injury-induced cell permeability. In an earlier study, airway epithelial cell monolayers continually treated with H₂O₂ to induce ROS, resulted in decreased trans-epithelial electrical resistance (TER) which is a measure of permeability in the monolayer. When these monolayers were pretreated with FGF7, the TER decreases caused by continuous exposure to H₂O₂ were delayed, and FGF7 treatment also helped to maintain localization of these tight junction proteins at the cell border. Additionally, FGF7 pretreatment of cells prior to H₂O₂ increased the rate of TER recovery, indicating
FGF7 pre-treatment protects airway epithelial cells against injury as well as promoting recovery following injury.\textsuperscript{15}

Efficient epithelial repair is also an essential component in the resolution of intestinal inflammation. Indomethacin treatment disrupts the mucosal barrier function and leads to reduced weight and intestinal inflammation in rats. Continuous treatment with FGF10, another ligand for the FGFR2b isoform, reduces the indomethacin-induced injury by decreasing weight loss, preventing ulcer formation and reducing acute and chronic histological inflammatory scores.\textsuperscript{16} FGF7 pre-treatment prior to indomethacin had a protective effect up to 5 days prior to injury, but not at 7 days indicating FGF7 has a limited protective capacity.\textsuperscript{16} FGF7 also increased the expression and activity of COX-2 which stimulates the production of protective prostaglandins such as PGE2, which inhibits macrophage activation and proinflammatory cytokine production.\textsuperscript{16} The results of this study indicate FGFR2b activity may be protective toward intestinal inflammation, further implicating FGFR2 signaling in the inflammatory pathway.

Exposure of skin to UV is known to induce damaging effects such as inflammation and tumorigenesis, and the cellular responses to UV appear to be mostly dependent upon ROS production.\textsuperscript{17} Expression of FGFR2 and activation by FGF7 have been found to down-regulate the generation of ROS following UVB irradiation.\textsuperscript{17} Oxidant-stressed cells undergo changes in their actin structure and pre-treatment with FGF7 before UVB prevents cytoskeletal destabilization induced after UVB, supporting a protective role of FGF7 against oxidative damages following UVB exposure.\textsuperscript{17} As further evidence of a protective role for FGFR signaling in
inflammation, knockout of FGFR2b in mice results in increased macrophage recruitment in the skin\(^ {18}\). Also, mice lacking FGFR2b have a greater incidence of papilloma formation, an enhanced sensitivity to DMBA-induced skin carcinogenesis, and showed increased progression of skin lesions to squamous cell carcinoma, indicating FGFR2b is important for inhibiting the progression of benign papillomas to a more aggressive and malignant cancer\(^ {18}\). Melanoma is the most aggressive form of skin cancer with 62,480 estimated cases and 8,420 estimated deaths in 2008\(^ {4}\). During a screen of melanoma cell lines and uncultured melanoma tumors, a large percentage contained mutations in FGFR2, many of which were predicted to be loss-of-function mutations\(^ {19}\). Reintroduction of FGFR2 into these melanoma cell lines, however, did not lead to a decrease in cellular proliferation, indicating that the cells may have acquired additional mutations that allow increased proliferation in the absence of FGFR2, or that FGFR2 expression inhibits other factors of tumorigenesis\(^ {19}\). The results of these studies strongly implicate FGFR2b signaling in the protection of lung, bowel, and skin against inflammatory insults, indicating under these circumstances, FGFR2b acts as a tumor suppressor.

**FGFR2 as Tumor Promoter or Tumor Suppressor: Could NFκB Play a Part?**

FGFR2 signaling may play dual roles as tumor promoter and tumor suppressor depending on cellular circumstances. For instance, in epithelial disorders of the skin, bowel, and lung, FGFR2b seems to play a strong role as tumor suppressor and inhibitor of inflammation. In these cells NFκB is most likely very active and damaged
cells may even depend on its activity to grow. What if FGFR2 is able to inhibit NFκB activity, leading to a decrease in inflammatory signaling?

In estrogen-dependent cancers, such as breast and endometrial cancers, FGFR2 signaling appears to act as a tumor promoter. These cells may have minimal NFκB signaling and may use FGFR2 signaling to promote growth, migration, and invasion. NFκB has been implicated in the pathogenesis of cancer, and the relationship between inflammation and cancer has been thoroughly investigated. Some breast cancers signal through the NFκB pathway to survive, and inhibitors toward NFκB signaling components have been shown to reduce breast cancer growth. Additionally, it is thought signaling through the NFκB pathway contributes to tamoxifen-resistance, further indicating the importance of NFκB signaling in certain types of breast cancer. It is possible that the protective role of FGFR2b against inflammation may be through inhibition of NFκB signaling, though further research into the specific mechanism of the FGFR2-decrease in inflammatory response is needed to make any conclusions. It is clear, however, that FGFR2 signaling is complex and far from fully understood.

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References


Chapter 3

The Novel Interaction of FGFR4 and IKKβ Negatively Regulates NFκB Activity in DU145 Prostate Cancer Cells
Abstract

The role of NFκB in regulating apoptosis and proliferation in a variety of human cancers, as well as understanding the significance of inflammatory responses in the development of cancer, has been a topic of increasing interest in recent years. Research has suggested a link between growth factor signaling pathways and activation of NFκB transcriptional activity. In this work, we show a novel direct interaction between FGFR4 and IKKβ, an essential component in the NFκB pathway, as well as tyrosine phosphorylation of IKKβ resulting from FGFR4 expression. Furthermore, we demonstrate a decrease in NFκB signaling upon FGFR4 activation, which is dependent upon FGFR4 kinase activity. In the absence of TNFα stimulation, FGFR4 activation exhibits no discernible effect on NFκB signaling. Following TNFα stimulation, however, FGFR4 activation results in significant inhibition of NFκB signaling as measured by decreased nuclear NFκB localization, reduced NFκB transcriptional activation, and inhibition of IKKβ kinase activity. FGF19 stimulation of endogenous FGFR4 in TNFα-treated DU145 prostate cancer cells also leads to a decrease in IKKβ activity and concomitant reduction in NFκB nuclear localization. Our research identifies a novel and compelling link between FGFR4 signaling and the NFκB pathway, providing a unique model of NFκB inactivation and implicating FGFR4 as a tumor suppressor in prostate cancer.
**Introduction**

NFκB is a transcription factor widely used as a regulator of genes that control cell proliferation and survival in mammalian cells. Numerous human cancers exhibit misregulation of NFκB signaling, in which NFκB is constitutively active as a transcription factor. Thus, NFκB has been the subject of intense research to identify clinically useful inhibitors, and to understand the intersection of NFκB signaling with other regulatory and signaling pathways that are important in cancer cell biology.

IKKβ is a critical component in the regulation of NFκB activity and has been extensively studied. Upon activation with TNFα, IKKβ phosphorylates IκB, the inhibitor of NFκB, which targets it for proteasomal degradation. Subsequently, NFκB is released from sequestration in the cytoplasm, permitting translocation of NFκB dimers into the nucleus where they activate the transcription of target genes. Signaling by FGF2 has been shown to be important for inhibition of apoptosis through PI3K/AKT and IKKβ, both in cancer cell lines and radiation-induced apoptosis. FGF signaling has also been shown to decrease TNFα-induced apoptosis through activation of the p44/42 MAPK pathway.

Members of the FGFR family of receptor tyrosine kinases, particularly FGFR4, have been strongly implicated in a variety of human cancers, including prostate cancer, though their role in cancer progression is not yet fully understood. FGFR4 was found to be highly expressed in 83% of grade 5 prostate cancers and in 25% of grade 1-3 cancers. Furthermore, suppression of FGFR4 expression by RNA interference effectively blocked prostate cancer cell proliferation and invasion.
Another study reported on the importance of FGFR4 in prostate cancer, demonstrating that FGFR4 expression was significantly upregulated in prostate cancer compared to benign prostatic hyperplasia, and FGFR4 overexpression was associated with an unfavorable outcome with decreased disease-specific survival \(^{14}\).

**Results**

Given the growing evidence on the importance of FGFR4 in cancer progression, we sought to better understand the downstream signaling of FGFR4 by searching for novel proteins that interact directly with the receptor. Using the intracellular domain of FGFR4 as bait, a yeast two-hybrid assay was performed against a mouse cDNA library. Interestingly, one of the interacting proteins identified was IKK\(\beta\). This novel interaction was first confirmed with a \(\beta\)-galactosidase filter lift assay (Figure 3-1a, left panel), where a positive interaction is indicated by a blue colony. Lamin bait was used as a negative control to confirm that the interaction with IKK\(\beta\) was specific for FGFR4. To further confirm this interaction, the co-transformed yeast were plated on selective media that allows only yeast displaying positive interactions to grow. Again, lamin was used as the negative control while PLC\(\gamma\) bait was used as a positive control (Figure 3-1a, right panel).

To confirm this novel interaction using full-length proteins, human IKK\(\beta\) was co-expressed with human FGFR4 in HEK293 cells. IKK\(\beta\) was able to interact with wild-type FGFR4 (FGFR4 WT), a kinase-dead FGFR4 (FGFR4 KD) as well as an activated mutant of the receptor (FGFR4 K645E) (Figure 3-1b), indicating the kinase activity of the receptor is not essential for the interaction with IKK\(\beta\). These
interactions were further confirmed in the opposite direction by immunoprecipitation of each FGFR4 construct. As before, IKKβ was able to bind to all the FGFR4 derivatives (Figure 3-1c).
Figure 3-1: Novel interaction of IKKβ with FGFR4. (a) Confirmation of yeast two-hybrid assay with the intracellular domain of FGFR4 bait protein and IKKβ clone isolated by b-gal filter lift assay (left panel) and growth on selective media (right panel). (b) Full-length IKKβ and full-length FGFR4 derivatives were transfected in HEK293 cells to examine in vivo association. Cells were lysed in 1% NP-40 lysis buffer and immunoprecipitated with IKKβ (H-4) antibody. Immunoblot analysis was performed with FGFR4 (C-16) antibody (top panel). The membrane was stripped and reprobed with anti- IKKβ (middle panel). The expression of the FGFR4 derivatives in the whole cell lysate is shown in lower panel. (c) Cells were transfected and lysed as in (b) then immunoprecipitated with FGFR4 (C-16) antibody. Immunoblot analysis was performed with IKKβ (H-4) antibody (top panel). The membrane was stripped and reprobed with anti-FGFR4 (second panel). The expression of IKKβ and FGFR4 in the whole cell lysate are shown in the two lower panels.
a. Bait
   FGFR4 Lamin
   Prey
   IKKβ

b. FGFR4:  IKKβ:
   WT    KD    K645E
   -     -    -
   +     +    +

   FGFR4
   IKKβ
   IP: IKKβ
   FGFR4
   Lysates

Cc. FGFR4:
   IKKβ:
   WT    KD    K645E
   -     -    -
   +     +    +

   IKKβ
   FGFR4
   IP: FGFR4
   IKKβ
   FGFR4
   Lysates
In addition to activating IKKβ by phosphorylation on serine residues 177 and 181, serine phosphorylation of C-terminal residues leads to inhibition of IKKβ autophosphorylation. Recent research, however, has shown that IKKβ may be phosphorylated on tyrosine residues 188 and 199, which are believed to be important for NFκB-induced Cox-2 and ICAM-1 expression. We investigated IKKβ tyrosine phosphorylation in HEK293 cells overexpressing both IKKβ and the various derivatives of FGFR4. IKKβ was immunoprecipitated, separated by SDS-PAGE, and immunoblotted using a phosphotyrosine-specific antibody. IKKβ was indeed tyrosine phosphorylated in the presence of FGFR4 (Figure 3-2). Overexpression of FGFR4 WT led to an increase in tyrosine phosphorylation of IKKβ, while the kinase-dead mutant of FGFR4 did not result in tyrosine phosphorylation of IKKβ, indicating the kinase activity of FGFR4 is essential for tyrosine phosphorylation of IKKβ. Additionally, the activated mutant of FGFR4 led to a dramatic increase in tyrosine phosphorylation of IKKβ (Figure 3-2a).

Importantly, all experiments were performed using a non-epitope-tagged IKKβ. In initial control experiments, we determined that the presence of the 3x-HA epitope tag (YPYDVPDYA) at the N-terminus of IKKβ resulted in a significant increase in the extent of tyrosine phosphorylation in response to FGFR4 activation (data not shown), presumably due to phosphorylation at some of the 9 Tyr residues contained within the 3x-HA-tag. Indeed, tyrosine phosphorylation of 3x-HA-IKKβ may have been previously observed by other investigators, yet ignored due to its nonphysiological origin.
The IKKβ protein is predicted to be 87 kDa while the lower, unmodified band of FGFR4 appears to co-migrate at about 85 kDa by SDS-PAGE. To ensure that the tyrosine phosphorylation observed was on IKKβ and not autophosphorylation of FGFR4, the cells in figure 3-2a were lysed in RIPA buffer and immunoprecipitations were washed over 10% sucrose to eliminate protein-protein interactions. In addition, we examined the tyrosine phosphorylation of IKKβ when co-transfected with myristylated FGFR4 constructs, containing only the intracellular domain of FGFR4 with a myristylation signal to target them to the membrane. Using these shorter FGFR4 constructs allowed separation from IKKβ, and revealed that tyrosine phosphorylation of IKKβ was still present (Figure 3-2b). Furthermore, we examined the interaction of these proteins and, as expected, they still interacted in co-immunoprecipitations (Figure 3-2b).
Figure 3-2: Tyrosine phosphorylation of IKKβ by FGFR4. (a) HEK293 cells were transfected with IKKβ and FGFR4 derivatives. Cells were lysed in RIPA and immunoprecipitated with IKKβ (H-4) antibody. Immunoblot analysis was performed with the phosphotyrosine-specific antibody 4G10 (top panel). The membrane was stripped and reprobed with IKKβ (H-4) antibody (second panel). The expression of the FGFR4 derivatives in the lysate is shown (lower panel). (b) HEK293 cells were transfected with IKKβ and FGFR4 derivatives that lack their extracellular domain and are targeted to the membrane with a myristylation signal (myr-FGFR4). Cells were lysed in 1% NP-40 lysis buffer and immunoprecipitated with IKKβ (H-4) antibody. After the proteins were transferred, the membrane was cut in half and the upper part was immunoblotted with the phosphotyrosine-specific antibody 4G10 (top panel). It was stripped and reprobed with IKKβ (H-4) antibody (second panel). The lower half of the membrane was immunoblotted with FGFR4 (C-16) antibody (third panel). The lysates were examined for the expression of IKKβ and myr-FGFR4 derivatives (bottom panels).
When IKKβ is activated by serine phosphorylation, it leads to release of NFκB dimers from IκB, allowing movement to the nucleus and increased NFκB transcriptional activity. Utilizing indirect immunofluorescence, we monitored changes in NFκB translocation to the nucleus in TNFα stimulated cells expressing FGFR4 constructs. HeLa cells were plated on glass coverslips, transfected with various mutants of FGFR4, and stained using an antibody to the p65 subunit of NFκB. As seen in Figure 3-3a, IκB primarily sequestered NFκB in the cytoplasm when starved cells were left untreated. As expected, in TNFα stimulated cells NFκB was primarily located in the nucleus. Representative cells are shown in Figure 3-3a. In TNFα treated cells expressing FGFR4 WT, there was a 40% decrease in cells with NFκB nuclear localization compared to mock-transfected cells. Expression of the activated mutant of FGFR4 led to a 65% decrease in cells with nuclear localization of NFκB, while FGFR4 KD led to only a 30% decrease in NFκB nuclear localization. The percentage of FGFR4-expressing cells appearing to have NFκB nuclear localization is shown in Figure 3-3b. This data indicates that not only the association with IKKβ, but also the kinase activity of FGFR4 are important for this observed affect on NFκB signaling, revealing a novel mechanism for NFκB inactivation by FGFR4 expression. We also performed experiments in these, and subsequent experiments in the paper, utilizing FGFR4 expression or FGF19 treatment of cells in the absence of TNFα stimulation and found no detectable effect on the NFκB signaling pathway (data not shown).
Figure 3-3: Re-localization of NFκB with FGFR4 expression. HeLa cells were seeded onto glass coverslips and transfected with FGFR4 derivatives. The cells were treated with TNFα for 30 min. Indirect immunofluorescence was performed. The localization of endogenous NFκB was detected with NFκB (F-6). Cells expressing the FGFR4 derivatives were stained with anti-FGFR4 (C-16) and Rh-conjugated anti-rabbit secondary antibody. The nuclei were visualized with Hoechst dye. (a) The endogenous localization of NFκB is shown in non-transfected cells +/- TNFα treatment (top panels). The altered localization of NFκB in a cell expressing FGFR4 WT with TNFα treatment is shown in lower panels. (b) Cells expressing FGFR4 derivatives were scored for the localization of NFκB. 100 cells were counted for each sample in three independent experiments. The error bars represent the standard deviation.
In order to further determine the effects of FGFR4 on the downstream signaling of the NFκB pathway, changes in endogenous IKKβ activity were monitored in HEK293 cells overexpressing FGFR4 and/or treated with the FGFR4-specific ligand FGF19. FGFR4 WT, FGFR4 K645E, and FGFR4 KD were transfected into HEK293 cells, followed by stimulation with TNFα. Equal amounts of total protein were subjected to immunoprecipitation with an IKKγ antibody to obtain the active IKK complex. The resulting immunoprecipitates were subjected to in vitro kinase assays utilizing GST-IκB\textsuperscript{(1-54)} as the substrate. The reactions were separated by SDS-PAGE, and phosphorylation of GST-IκB\textsuperscript{(1-54)} was visualized using a Phosphorimager and quantified (Figure 3-4a and b). As expected, treatment with TNFα resulted in an almost ten-fold increase in the IKK complex activity, compared to unstimulated cells. However, in cells overexpressing FGFR4 WT, there was a 30% reduction in the IKK complex activity and stimulation of FGFR4 WT expressing cells with FGF19 potentiated this reduction. The expression of the activated FGFR4 K645E mutant resulted in a 45% reduction of IKK activity. The activity of the IKK complex was restored in cells expressing the inactive FGFR4 KD mutant. The reduction in activity was also observed in mock transfected cells treated with FGF19, indicating that activation of the endogenous FGFR4 pathway, and not just overexpression of the FGFR4 protein, is sufficient to reduce endogenous IKK activity.

Since previous research has implicated FGFR4 in prostate cancer progression, we sought to examine the effect of FGFR4 activation on NFκB signaling in DU145 cells, as these prostate cancer cells are known to have high levels of FGFR4.
As described above for HEK293 cells, TNFα-treated DU145 cells stimulated with FGF19 also exhibit a decrease in IKKβ activity, compared to TNFα-stimulation alone, as measured in a IKK kinase assay using GST-IκB^{(1-54)} as a substrate (Figure 3-4c and d). This indicates that activation of endogenous FGFR4 in these prostate cancer cells can decrease TNFα-stimulated activity of the IKK complex.
Figure 3-4: FGFR4 expression and/or FGF19 stimulation inhibits endogenous IKKβ activity. (a) HEK293 cells were transfected with empty vector or the indicated FGFR4 constructs, then starved for 16 h. Cells were then either stimulated with vehicle for 10 min or FGF19 for 10 min prior to the addition of TNFα for an additional 10 min. The IKK complex was then immunoprecipitated from cytoplasmic extracts and subjected to an in vitro kinase assay utilizing GST-IκB(1-54) as substrate. The top panel shows $^{32}$P incorporation on GST-IκB(1-54) while the second panel shows coomassie-staining as a loading control. Lysates were separated by SDS-PAGE, transferred to Immobilon-P, and probed with the indicated antibodies. (b) Kinase reactions described in (a) were exposed to a Phosphorimager (Bio-Rad). Quantification of $^{32}$P incorporation into GST-IκB was performed using the Quantity One software (Bio-Rad). The average $^{32}$P incorporation from three independent experiments, normalized to mock transfected cells stimulated with TNFα, is shown +/- std. dev. (c) DU145 cells were starved for 24 hr prior to stimulation as described in (a). Kinase assays and western blots were performed as in (a). (d) Quantification of $^{32}$P incorporation into GST-IκB(1-54) was performed as in (b). The average $^{32}$P incorporation from three independent experiments, normalized to mock transfected cells stimulated with TNFα, is shown +/- std. dev.
We also examined the interaction of endogenous IKKβ and FGFR4 in DU145 cells by immunoprecipitating IKKβ, and immunoblotting for FGFR4. As a control, identical lysates were included where no antibody was added. We were able to detect endogenous FGFR4 interacting with endogenous IKKβ in the DU145 cells (Figure 3-5a). In addition, we examined NFκB localization by cell fractionation and found that activation of endogenous FGFR4 with FGF19 led to a decrease in NFκB translocation to the nucleus when treated with TNFα (Figure 3-5b). FGF19 treatment of DU145 cells also decreased TNFα-induced NFκB transcriptional activity as measured by Electrophoretic Mobility Shift Assay (EMSA) (Figure 3-5c, d). The fact that FGF19 inhibition of TNFα-induced NFκB signaling occurs in multiple cell lines indicates that activated FGFR4 may have therapeutic applications in cancer cells exhibiting a hyperactive NFκB pathway. As stated above, FGFR4 expression has been associated with increased proliferation, invasion, and decreased disease-free survival in prostate cancer. It’s possible that our observed FGFR4-dependent decrease in NFκB activity may be important for decreasing NFκB-induced inflammatory responses.

Our data suggest that overexpression of FGFR4 as well as activation of FGFR4 by FGF19 inhibits IKKβ activity, which prevents IκB degradation and results in downregulation of NFκB-mediated transcription. Since the NFκB pathway is involved in both the pathogenesis of the inflammatory response and in cellular growth control, this pathway also represents a potential target for inhibition by FGFR4, and possibly by other FGFR family members. TNFα leads to apoptosis through FADD and downstream caspases while, at the same time, exerting anti-apoptotic effects.
through NFκB transcriptional activation. Our results clearly show FGF19 activation of FGFR4 leads to a decrease in NFκB activity; however, given the number and complexity of genes transcribed by NFκB, it is yet unclear what effect FGFR4 has on specific cellular outcomes such as apoptosis. FGF has also been shown to be anti-apoptotic through activation of MAPK, PI3K, and up-regulation of Bcl-2. Similar to the dual roles played by TNF, we hypothesize that FGF19 activation of FGFR4 may also lead to apoptosis, through inhibition of IKKβ activity as described in Figure 3-5e.
Figure 3-5: Endogenous interaction and effect on downstream signaling in DU145 prostate cancer cells. (a) Approximately 400mg of total lysate was immunoprecipitated with IKKβ (H-4) antibody in 1% NP-40 lysis buffer. Immunoblot analysis was performed with FGFR4 (C-16) antibody (top panel). The membrane was stripped and reprobed with anti- IKKβ (lower panel). No IKKβ (H-4) antibody was added during the immunoprecipitation for the control lane. (b) DU145 cells were treated with TNFα or TNFα plus FGF19. Cells were fractionated and the cytoplasmic and nuclear fractions were immunoblotted with NFκB (F-6) antibody (top panel). The membranes were stripped and reprobed with β-tubulin and mSin3a antibodies to confirm cytoplasmic and nuclear fractions (lower panels). (c) DU145 cells were stimulated with vehicle for 30 min, TNFα for 30 min, or FGF19 for 10 min prior to the addition of TNFα for an additional 30 min. Nuclear extracts were prepared and equal amounts of protein (2 µg) were subjected to an electrophoretic mobility shift assay with 32P-labeled 30bp double-stranded oligonucleotide containing a consensus κB-site. (d) Samples from (c) were exposed to a phosphorimager (Bio-Rad). Quantification of NFκB binding to the probe was performed using the Quantity One software (Bio-Rad). The average NFκB binding from three independent experiments, normalized to mock transfected cells stimulated with TNFα, is shown +/- std. dev. (e) A schematic of possible FGFR4 involvements in the NFκB pathway.
a. No antibody
anti-IKKβ

FGFR4
IKKβ

IP: IKKβ

b. FGF19:

- - +

TNFα:

- + +

NFκB
mSin3a
β-Tubulin

Cytoplasmic
Nuclear
c. FGF19:

- - +

TNFα:

- + +

NFκB

d. Fold NFκB transcriptional activity

1.2
1.0
0.8
0.6
0.4
0.2
0

TNFα:

- + +

FGF19:

- - +

e. TNFα

FGFR4

FADD Caspases

IKK activation

IκB degradation

apoptosis

NFκB transcriptional activation of inflammation

MAPK Bcl-2 PI3-K

anti-apoptosis
Discussion

FGFRs are overexpressed or have altered activity in a variety of human diseases, including cancers of the prostate\textsuperscript{24}, breast\textsuperscript{25}, and lung\textsuperscript{26} and recent evidence indicates FGFRs may be used to target tumors for growth inhibition\textsuperscript{27, 28}. Our novel findings indicate a substantially expanded role for FGFR4, not only as a regulator of cellular proliferative pathways, but as an important regulator of NF\kappa B inflammatory pathways previously viewed as distinct. These results implicate FGFR4 as a potential tumor suppressor in prostate cancer. FGF2 stimulation of cells has been previously shown to protect cells from TNF\alpha-induced apoptosis\textsuperscript{11}, which may lead to uncontrolled cell growth, while we show that in cells that have constitutively active NF\kappa B, FGF19 stimulation leads to a decrease in TNF\alpha-induced NF\kappa B nuclear localization and transcription. It is possible that specific activation of FGFR4 using FGF19 may have a distinct function in the cell by decreasing NF\kappa B activity, and other FGFRs, or activation with other FGF family members, may have separate and distinct roles in NF\kappa B activation. Cells that overexpress FGFR4 may do so in order to circumvent NF\kappa B inflammatory pathways; this predicts that cancers exhibiting overexpressed FGFR4 may actually have acquired a kinase-inactivating mutation that eliminates the tumor-suppressive effect seen with FGFR4 WT. In a screen of lung adenocarcinoma cells, an E681K mutation in FGFR4 was discovered\textsuperscript{29}. This mutation was proposed to lead to altered FGFR4 activity; possibly, this mutation may decrease the ability of FGFR4 to inhibit NF\kappa B signaling. While the role of FGFR4 in cancer is still not fully understood, we have presented a novel mechanism by which FGFR4
functions as a tumor suppressor to inhibit IKKβ activity, resulting in decreased NFκB signaling in DU145 prostate cancer cells.

Materials and Methods

Cell culture:

HeLa and HEK293 cells were grown in DMEM supplemented with 10% FBS and 1% Pen/strep; DU145 cells were grown in RPMI supplemented with 10% FBS and 1% Pen/strep. HeLa and DU145 cells were kept in a humid atmosphere of 5% CO₂; HEK293 cells were kept in a humid atmosphere of 10% CO₂.

Plasmid constructs:

The full-length FGFR4 wild-type, kinase active (K645E), and myristylated forms of FGFR4 have been described previously\textsuperscript{18}. The kinase dead (K504M) form of full-length FGFR4 was generated by QuikChange site directed mutagenesis (Stratagene). The HA-IKKβ clone was received from Dr. Mark Hannink (University of Missouri). The HA-tag was removed from this clone by QuikChange site-directed mutagenesis in which a HindIII site was created along with an ATG start site downstream of the HA-tag. The parental clone contained a HindIII site upstream of the HA-tag. The plasmid containing the new HindIII site was digested with HindIII and re-ligated to generate the untagged IKKβ expression clone. It was necessary to delete this tag as it contains numerous tyrosine sites that are phosphorylated by activated FGFR4. Derivatives were confirmed by DNA sequencing at the UCSD Moores Cancer Center Shared Resource facility. The GST-IκB\textsuperscript{(1-54)} plasmid was a gift from the Hoffmann Lab (UCSD).
Antibodies and Reagents:

Antibodies were obtained from the following sources: FGFR4 (C-16), IKKβ (H-4), NFκB (F-6), β-tubulin (H-235), IKKγ (FL-419) from Santa Cruz Biotechnology; 4G10 (antiphosphotyrosine) from Upstate Biotechnology; Phospho-p44/42 MAPK (Thr202/Tyr204; E-10) from Cell Signaling; MAPK (ERK1+ERK2) from Zymed; horseradish peroxidase (HRP) anti-mouse, HRP anti-rabbit from GE Healthcare; fluorescein-conjugated anti-mouse from Sigma and rhodamine-conjugated anti-rabbit from Boehringer-Mannheim. FGF19 and TNFα were obtained from R&D. mSin3a antibody was a gift from Dr. Alex Hoffmann.

Immunoprecipitation and Immunoblot:

HEK293 cells (1X10^6) were plated on 10 cm dishes 1 day prior to transfection with 4-6µg of total DNA by calcium phosphate precipitation at 3% CO₂. After 18 to 20 h, cultures were moved back to 10% CO₂ for 4-6 h before starving overnight in DMEM lacking FBS. Cells were harvested, washed once in PBS, and lysed in 1% NP-40 Lysis Buffer [20mM Tris-HCl, pH 7.5, 137mM NaCl, 1% Nonidet P-40, 5mM EDTA, 50mM NaF, 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride (PMSF), 10µg/ml aprotinin] or radioimmunoprecipitation assay buffer [RIPA; 50mM Tris-HCl, pH 7.5; 150mM NaCl; 0.1% SDS; 1% Triton-X 100; 1% DOC; 50mM NaF; 1mM sodium orthovanadate; 1mM phenylmethylsulfonyl fluoride (PMSF); 10µg/ml aprotinin]. Total protein concentrations were measured by Bradford Assay or Lowry Assay (Bio-Rad). Immunoprecipitations were performed overnight at 4°C and collected by Protein A-Sepharose (Sigma). Samples were washed three times with Lysis Buffer, boiled for 4 min in sample buffer and separated by SDS-PAGE. Proteins
were transferred to Immobilon-P membranes and blocked in 3% or 5% milk/TBS/0.05% Tween 20 or 3% bovine serum albumin (BSA)/TBS/0.05% Tween 20 (for anti-phosphotyrosine blots). Membranes were immunoblotted with antibodies for 2 h at room temperature or overnight at 4°C. After primary incubations, membranes were washed with TBS/0.05% Tween 20 and incubated with HRP-conjugated secondary antibodies. Proteins were detected by enhanced chemiluminescence (ECL) (GE Healthcare) or (Millipore). To reprobe with subsequent antibodies, membranes were incubated in stripping buffer [100mM β-mercaptoethanol; 2% SDS; 62.5 mM Tris-HCl, pH 6.8] at 80°C for 1 h to remove bound antibodies. To detect the endogenous interaction between IKKβ and FGFR4 in the DU145 cells, 400µg of total lysate from starved cells was immunoprecipitated with IKKβ antibody in 1% NP-40 lysis buffer and analyzed by SDS-PAGE and Western Blot to detect the interaction of endogenous FGFR4.

**Yeast two-hybrid assay:**

The Saccharomyces cerevisiae strain L40 generated by Dr. Stan Hollenberg was transformed with derivatives of pBTM116 (constructed by Dr. Paul Bartel and Dr. Stan Fields). The derivatives constructed for this work encoded a LexA fusion protein containing the juxtamembrane and intracellular region of FGFR4. Briefly, an MluI site and an XhoI site were created in the multiple cloning region of pBTM116. Using these sites, amino acids 373-803 of FGFR4 were moved to the pBTM116 vector in frame with LexA. This bait was screened against a 9.5 d.p.c. mouse embryonic cDNA library encoding fusion proteins with the transactivation domain of pVP16, kindly provided
by Dr. Stan Hollenberg. The two-hybrid screen and His± minimal media assays were performed as described previously. The ability to activate the lacZ reporter was also confirmed by β-galactosidase filter assay as described.

**Indirect Immunofluorescence:**

HeLa cells were plated on glass coverslips at a density of 75,000 cells/35mm plate. The next day, cells were transfected using Fugene 6 transfection reagent (Roche). The following day, cells were starved in DMEM containing no serum for 24 h. 50ng/ml FGF19 plus 1µg/ml heparin was added for 10 min prior to adding 10ng/ml TNFα for 30 min. Coverslips were washed in PBS, fixed in 3% paraformaldehyde in PBS for 15 min, washed 3 times with PBS, and permeabilized for 10 min with 0.5% Triton-X 100 in PBS. Coverslips were again washed 3 times with PBS before blocking with 3% BSA in PBS for 1h. Coverslips were incubated with FGFR4 (C-16) at 1:1000 and NFκB (F-6) at 1:250 for 1 h. Coverslips were washed 3 times with PBS and labeled with secondary antibodies for 1 h at room temperature: Rhodamine-Rabbit, 1:1000; FITC-mouse, 1:1000; and hoechst, 1:1000. Coverslips were washed 3 times with PBS before mounting in 90% glycerol, 10% 1M Tris pH 8.5, containing p-phenylenediamine. Cells were photographed using a Nikon Microphot-FXA with a cooled CCD camera (Hamamatsu C5810).

**In vitro kinase assays:**

1X10⁶ HEK293 or DU145 cells were plated on 10cm dishes. HEK293 cells were transfected as described above. Cells were thenstarved in DMEM lacking FBS overnight, prior to being treated with 25ng/mL FGF19 for 10 min. Subsequently, cells were stimulated with 10ng/mL TNFα for 10min. Cells were then harvested and
washed once in PBS + 1mM EDTA. Cells were then lysed in Cytoplasmic Extract Buffer (CEB) [10mM HEPES-KOH (pH 7.9), 250mM NaCl, 1mM EDTA, 0.5% Nonidet P-40, 0.2% Tween 20, 20mM β-glycerophosphate, 2mM DTT, 10mM NaF, 1mM sodium orthovanadate, 1mM PMSF, 10µg/mL aprotinin]. Protein concentrations were determined by Bradford Assay. 200µg lysates were immunoprecipitated with IKKγ antibody for 2 h at 4°C. Protein A-Sepharose beads were added and incubated for 1 h at 4°C. The immunoprecipitated samples were washed twice with CEB and twice with Kinase Buffer (KB) [20mM HEPES (pH 7.7), 100mM NaCl, 10mM MgCl₂, 20mM β-glycerophosphate, 2mM DTT, 10mM NaF, 0.1mM sodium orthovanadate, 1mM PMSF]. Samples were then resuspended in 2X KB with 20µM ATP. 1µCi [γ-32P]-ATP and 0.5µg GST-IκB(1-54) bacterially expressed purified protein were added to each reaction and incubated at 30°C for 30 min. Proteins were separated by 10% SDS-PAGE, Coomassie stained, dried, and exposed to film or a phosphorimager (BioRad) screen directly. Band intensities were quantified using Quantity One Software.

**Electrophoretic Mobility Shift Assay (EMSA):**

Cells were collected in PBS, 1mM EDTA, pelleted at 2000 g, resuspended in 200µl CE buffer (10mM Hepes-KOH pH7.9, 60mM KCl, 1mM EDTA, 0.5% NP-40, 1mM DTT, 1mM PMSF) and vortexed. Nuclei were pelleted at 4000 g, and the supernatant was removed. Nuclei were resuspended in 50µl NE buffer (250mM Tris pH7.8, 60mM KCl, 1mM EDTA, 1mM DTT, 1mM PMSF) and lysed by 3 freeze-thaw cycles. Nuclear lysates were cleared by centrifugation and normalized to a
concentration of 1µg/µl following Bradford assay. 2µg of total nuclear protein was reacted at room temperature for 15 min with excess $^{32}$P-labeled 30 bp double-stranded oligonucleotide containing a consensus κB-site (AGCTTGCTACAAGGGACTTTCCGCTGTCTACTTT) in 6µl binding buffer (10mM Tris-HCl pH 7.5, 50mM NaCl, 10% glycerol, 1% NP-40, 1mM EDTA, 0.1µg/µl polydIdC). Complexes were resolved on a non-denaturing 5% acrylamide (30:0.8) gel containing 5% glycerol and 1x TGE (24.8mM Tris-HCl, 190mM glycine, 1mM EDTA) and visualized by autoradiography and/or quantified using a Phosphorimager (Bio-Rad).

**NFκB localization by cell fractionation:**

DU145 cells were plated on 10cm dishes. Upon reaching 80% confluency, cells were starved overnight and treated the next day with 50ng/ml FGF19 and 1µg/ml Heparin for 10 min prior to the addition of 10ng/ml TNFα for 30 min. Cell lysates were fractioned as in the EMSA.

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Chapter 3, in full, has been submitted for publication as it may appear in EMBO Reports, 2009, Drafahl, Kristine A.; McAndrew, Christopher W.; Meyer, April N.; Haas, Martin; Donoghue, Daniel J. The dissertation author was the primary investigator and author of this paper.

References


Chapter 4

FGFR2 Interaction and Tyrosine Phosphorylation of IKKβ Negatively Regulates NFκB in T47D Cells
Abstract

The role of FGFR2 in the pathogenesis of cancer has been widely researched. Evidence exists to implicate FGFR2 signaling in cancer progression as well as protection from inflammatory insults \(^1\text{-}^8\), indicating FGFR2 may play dual roles as a tumor promoter and a tumor suppressor. We have previously identified the novel interaction of FGFR4 and IKK\(\beta\) and have shown this interaction leads to a decrease in NF\(\kappa\)B activity\(^9\). In this work, we show a direct interaction between FGFR2 and IKK\(\beta\), as well as tyrosine phosphorylation of IKK\(\beta\) resulting from FGFR2 expression. Additionally, we show that kinase activation of FGFR2 prior to TNF\(\alpha\) treatment leads to a decrease in IKK activity as measured by a kinase assay using GST-I\(\kappa\)B as a substrate. Furthermore, we demonstrate a decrease in NF\(\kappa\)B signaling upon FGFR2 activation, which is dependent upon FGFR2 kinase activity. FGF8b stimulation of endogenous FGFR2b in TNF\(\alpha\)-treated T47D breast cancer cells also leads to a decrease in NF\(\kappa\)B activity. Our research further implicates FGFR2 as a tumor suppressor and suggests a mechanism through which FGFR2 kinase activity leads to inactivation of IKK and NF\(\kappa\)B signaling in breast cancer.

Introduction

FGFR2 is a receptor tyrosine kinase (RTK) belonging to the Fibroblast Growth Factor Receptor (FGFR) family. Like all RTKs, FGFR2 has an extracellular ligand binding domain, a single-pass transmembrane domain, and an intracellular kinase domain that phosphorylates tyrosine residues when activated. FGFR2 mutations are
responsible for developmental syndromes, including Antley-Bixler-like syndrome (ABS), Apert syndrome (AS), Beare-Stevenson syndrome (BSS), Crouzon syndrome (CS), Jackson-Weiss syndrome (JWS), Saethre-Chotzen syndrome (SCS), and Pfeiffer syndrome (PS) \(^{10-17}\). We were the first to show that mutations in the FGFR2 extracellular domain result in constitutive kinase activation, providing a mechanistic understanding \(^{18, 19}\). Also, certain types of gastric cancers overexpress FGFR2 and recent research has discovered that an inhibitor, AZD2171, exerts potent antitumor activity against gastric cancer xenografts overexpressing FGFR2 \(^{20}\).

There are many isoforms of FGFRs due to splice variants. Two splice forms of FGFR2 in the third IG-like domain result in two separate isoforms, FGFR2b (also known as KGFR) and FGFR2c. FGFR2b is specifically expressed in epithelial cells, while FGFR2c is expressed in mesenchymal cells. FGF7 (or KGF) binds specifically to the FGFR2b isoform and is secreted from mesenchymal cells, which leads to a paracrine mode of receptor activation. The FGFR2b isoform and its ligand, FGF7, have been extensively implicated in the protection of cells from inflammatory insults in the lung, skin, and bowel \(^{1-8}\), while also implicated in the progression of breast and ovarian cancers \(^{21-27}\). It is possible that FGFR2 may serve as a tumor suppressor as well as tumor promoter under certain conditions and cell types.

We have recently reported the novel interaction of FGFR4 with IKK\(\beta\). IKK\(\beta\) is an important component of the NF\(\kappa\)B signaling pathway. Activation of IKK\(\beta\) through phosphorylation on serine residues leads to phosphorylation and degradation of I\(\kappa\)B \(^{28}\). I\(\kappa\)B inhibits NF\(\kappa\)B by sequestering it in the cytoplasm, but upon degradation of I\(\kappa\)B, the nuclear localization signal on NF\(\kappa\)B is exposed, allowing movement to the nucleus
and transcription of a variety of genes which are important in cell survival as well as inflammation \(^{29}\). The NFκB inflammatory pathway has been widely studied in its role in cancer progression \(^{29}\), and targeted inhibition of this pathway is of significant interest. We have shown previously that IKKβ is tyrosine phosphorylated when FGFR4 is co-expressed. We have also shown that interaction with IKKβ as well as FGFR4 kinase activity leads to decreased IKK activity as measured in a kinase assay using GST-IκB as a substrate. Additionally, we have shown decreased NFκB nuclear localization through indirect immunofluorescence and decreased NFκB transcriptional activity by EMSA \(^9\). This discovery has illuminated an alternate pathway for NFκB inhibition, through FGFR4 signaling, implicating FGFR4 as a tumor suppressor.

We were interested in determining whether FGFR2 may also interact with IKKβ and inhibit NFκB activity. As mentioned above, the FGFR2b isoform has been extensively implicated in the inflammatory pathway. We hypothesized that FGFR2 may also interact with IKKβ to inhibit NFκB activity, and this may be one mechanism for how it is able to decrease inflammation under certain conditions. We were also interested in determining which specific tyrosines on IKKβ were phosphorylated in response to FGFR activation. One group has suggested that Src-phosphorylation of Tyr188 and Tyr199 is important for activation of the NFκB pathway \(^{30, 31}\), although this initial observation has not been further studied.
Results

Although all four members of the FGFR family are similar in structure, FGFR4 and FGFR2 share only 57% amino acid identity\(^{32}\) and are expressed in distinct patterns in various cell types. Therefore, we sought to determine if FGFR2 was able to interact with IKK\(\beta\) as we have previously shown for FGFR4. HEK293 cells were transfected with FGFR2 and IKK\(\beta\) and immunoprecipitated for IKK\(\beta\). FGFR2 WT as well as FGFR2 KD were able to interact with IKK\(\beta\), indicating as with FGFR4, the kinase activity of the receptor is not important for the interaction (Figure 4-1a). We confirmed this interaction in the opposite direction by immunoprecipitating FGFR2 and immunoblotting for IKK\(\beta\) (Figure 4-1b). IKK\(\beta\) forms an active complex with IKK\(\alpha\) and IKK\(\gamma\) in order to function in the cell. Of interest was determining whether FGFR2 interacts with free IKK\(\beta\) or with the IKK complex. HEK293 cells were transfected with IKK\(\beta\) or FGFR2 and lysed in NP-40 lysis buffer. Lysates were immunoprecipitated for IKK\(\gamma\) and immunoblotted for either FGFR2 or IKK\(\beta\). As seen in Figure 4-1c, FGFR2 co-immunoprecipitates with IKK\(\gamma\), and this interaction increases with overexpression of IKK\(\beta\). Similarly, IKK\(\beta\) co-immunoprecipitates with IKK\(\gamma\), and this association increases with overexpressed FGFR2. These results indicate that FGFR2 does interact with the IKK complex through IKK\(\beta\) and that overexpression of either protein leads to increased interaction with the IKK complex.
Figure 4-1: Novel interaction of IKKβ with FGFR2. (a) Full-length IKKβ and full-length FGFR2 derivatives were transfected in HEK293 cells to examine in vivo association. Cells were lysed in 1% NP-40 lysis buffer and immunoprecipitated with IKKβ (H-4) antibody. Immunoblot analysis was performed with FGFR2 (C-17) antibody (top panel). The membrane was stripped and reprobed with anti-IKKβ (bottom panel). (b) Cells were transfected and lysed as in (a) then immunoprecipitated with FGFR2 (C-17) antibody. Immunoblot analysis was performed with IKKβ (H-4) antibody (top panel). The membrane was stripped and reprobed with anti-FGFR2 (bottom panel). (c) Cells were transfected and lysed as in (a), then immunoprecipitated with IKKγ antibody. Immunoblot analysis was performed with FGFR2 (C-17) antibody (top panel). The membrane was stripped and reprobed with anti-IKKβ (second panel). Expression of FGFR2 and IKKβ was detected by immunoblot of lysates (lower two panels).
IKKβ is activated by serine phosphorylation, which leads to phosphorylation and degradation of IκB. This releases NFκB to translocate to the nucleus and transcribe a variety of genes important for the inflammatory pathway. One group has shown that IKKβ may be tyrosine phosphorylated by Src, and provide evidence that this leads to an increase in NFκB activity\textsuperscript{30, 31}. We have recently shown that FGFR4 activation leads to tyrosine phosphorylation of IKKβ, but this leads to inactivation of NFκB\textsuperscript{9}. We sought to determine whether the tyrosine phosphorylation of IKKβ is specific to FGFR4 or if FGFR2 is also able to phosphorylate IKKβ. HEK293 cells were transfected with FGFR2 WT, FGFR2 KD, and IKKβ. Cells were lysed with RIPA lysis buffer, immunoprecipitated using an IKKβ antibody, and immunoblotted with an anti-phosphotyrosine antibody. FGFR2 WT was able to tyrosine phosphorylate IKKβ, while FGFR2 KD was not, indicating that the kinase activity of FGFR2 is essential for the tyrosine phosphorylation of IKKβ and that this activity is not specific to FGFR4 (Figure 4-2, top panel). The membrane was stripped and re-probed for IKKβ to show equal amounts of protein were immunoprecipitated (second panel). Lysates were immunoblotted for FGFR2 and IKKβ to show equal protein expression (bottom two panels).
Figure 4-2: Tyrosine phosphorylation of IKKβ by FGFR2. HEK293 cells were transfected with IKKβ and FGFR2 derivatives. Cells were lysed in RIPA and immunoprecipitated with IKKβ (H-4) antibody. Immunoblot analysis was performed with the phosphotyrosine-specific antibody 4G10 (top panel). The membrane was stripped and reprobed with IKKβ (H-4) antibody (second panel). Immunoblot analysis of lysates shows protein expression of FGFR2 and IKKβ (lower two panels).

We have previously shown that FGFR4 interaction with IKKβ leads to a decrease in IKK activity and that the kinase activity of the receptor is important for this effect. To determine whether FGFR2 kinase activity has the same effect on IKK activity, we performed a kinase assay using GST-IκB as a substrate. FGFR2 WT, FGFR2 C278F (a kinase active mutation)\(^3\)\(^3\), and FGFR2 KD were transfected into HEK293 cells followed by stimulation with TNFα. Equal amounts of total protein were subjected to immunoprecipitation with an IKKγ antibody to obtain an active IKK complex. The resulting immunoprecipitates were subjected to in vitro kinase assays utilizing GST-IκB\(^{(1-54)}\) as the substrate\(^3\)\(^4\). The reactions were separated by SDS PAGE, and phosphorylation of GST-IκB\(^{(1-54)}\) was visualized using a Phosphorimager and quantified (Figure 4-3a and b). Treatment with TNFα led to a dramatic increase in
IKK activity, and quantification of TNFα-induced IKK activity was set at a value of 1 with all other samples were relative to this sample (Figure 4-3b). Expression of FGFR2 WT decreased IKK activity by about 35%, while expression of the kinase active FGFR2 C278F led to a 50% decrease in IKK activity. Expression of the kinase-dead FGFR2 caused only a minor decrease in IKK activity, indicating the kinase activity of FGFR2 is important for the observed decrease in IKK activity (Figure 4-3b).
Figure 4-3: FGFR2 expression inhibits TNFα-induced IKKβ activity. (a) HEK293 cells were transfected with empty vector or the indicated FGFR2 constructs, then starved for 16 h. Cells were then stimulated with TNFα for 10 min. The IKK complex was immunoprecipitated from cytoplasmic extracts and subjected to an in vitro kinase assay utilizing GST-IκB(1-54) as substrate. The top panel shows $^{32}$P incorporation on GST-IκB(1-54) while the second panel shows FGFR2 expression in the lysates. (b) Kinase reactions described in (a) were exposed to a Phosphorimager (Bio-Rad). Quantification of $^{32}$P incorporation into GST-IκB was performed using the Quantity One software (Bio-Rad). The average $^{32}$P incorporation from three independent experiments, normalized to mock transfected cells stimulated with TNFα, is shown +/- std. dev.
After observing that FGFR2 kinase activity leads to a decrease in IKK activity, we sought to examine downstream NFκB activity by EMSA. HEK293 cells overexpressing various constructs of FGFR2 were stimulated with TNFα, lysed, and nuclear extracts were collected. Equal amounts of protein were subjected to an EMSA using a $^{32}$P-labeled probe containing a consensus kB-site. TNFα treatment alone led to a dramatic increase in NFκB activity as measured by a shift in the probe (Figure 4-4a). Overexpression of FGFR2 decreased the TNFα-induced NFκB activity by 25%, while overexpression of the kinase active C278F FGFR2 mutant decreased NFκB activity even further. The kinase-dead mutant of FGFR2 was unable to decrease the TNFα-induced NFκB activity, indicating the kinase activity of the receptor is essential for the observed decrease in NFκB activity (Figure 4-4a). Quantification of these results is shown in Figure 4-4b and represent three independent experiments.
Figure 4-4: Inhibition of NFκB activity by FGFR2. (a) HEK293 cells were stimulated with vehicle or TNFα for 30 min. Nuclear extracts were prepared and equal amounts of protein (2 µg) were subjected to an electrophoretic mobility shift assay with $^{32}$P-labeled 30bp double-stranded oligonucleotide containing a consensus kB-site. (b) Samples from (a) were exposed to a phosphorimager (Bio-Rad). Quantification of NF-kB binding to the probe was performed using the Quantity One software (Bio-Rad). The average NF-kB binding from three independent experiments, normalized to mock transfected cells stimulated with TNFα, is shown +/- std. dev.

As described earlier, FGFR2 expression has been implicated in the progression and pathogenesis of breast cancer and endometrial cancer $^{21-27}$. Research has found that under certain conditions in specific cell types, FGFR2 may protect cells from inflammation and may be considered a tumor suppressor $^{1-8}$. We sought to understand the role of FGFR2 activation in T47D breast cancer cells, which express high levels of the FGFR2b isoform $^{35}$. Of all the FGFR2 isoforms, FGFR2b has been most widely associated with a protective effect against inflammatory insults, and we hypothesized that activation of the endogenous receptor would lead to a decrease in NFκB signaling.
in these breast cancer cells. TNFα treatment alone led to a strong activation of NFκB, while FGF8b pre-treatment decreased this effect (Figure 4-5a). These results are quantified in Figure 4-5b and indicate that FGF8b pre-treatment decreases NFκB activity by 40%.

**Figure 4-5: Stimulation of T47D cells with FGF8b leads to a decrease in NFκB activity.** (a) T47D cells were stimulated with vehicle for 30 min, TNFα for 30 min, or FGF8b for 10 min prior to the addition of TNFα for an additional 30 min. Nuclear extracts were prepared and equal amounts of protein (2 µg) were subjected to an electrophoretic mobility shift assay with 32P-labeled 30bp double-stranded oligonucleotide containing a consensus kB-site. (b) Samples from (a) were exposed to a phosphorimager (Bio-Rad). Quantification of NF-kB binding to the probe was performed using the Quantity One software (Bio-Rad).
Discussion

We have previously identified the novel interaction of FGFR4 with IKKβ and shown that this interaction leads to a decrease in IKK and NFκB activity. In this paper, we show that FGFR2 interacts with IKKβ as well. Additionally, FGFR2 kinase activity is essential for tyrosine phosphorylation of IKKβ. The kinase activity of the receptor is also important for a decrease in IKK activity as measured in a kinase assay using IκB as a substrate, as well as NFκB activity as measured in an EMSA. Taken together, these results implicate FGFR2 in the inflammatory pathway and indicate the activation of this receptor may play a protective role against inflammatory stimuli.

FGFR2 kinase activity is important for tyrosine phosphorylation of IKKβ. However, it is yet to be determined whether the tyrosine phosphorylation of IKKβ is essential for the observed decrease in activity. We are currently working on mutation of the various tyrosine residues in IKKβ as well as determination of phosphotyrosine-peptides by mass-spectrometry analysis. We are also currently working on determining which domain or domains of IKKβ interact with FGFR2 by in vitro binding assays using GST-fused constructs.

Evidence exists to suggest that FGFR2 may play a role as a tumor suppressor in some circumstances and a tumor promoter in other circumstances. Previous research has identified FGFR2 in the pathogenesis of breast cancer. Additionally, the FGFR2b isoform has been shown to decrease inflammation and play a protective role in the skin, lung, and bowel. Our results indicate that in breast cancer cells expressing high levels of the FGFR2b isoform, activation of this receptor leads to a
decrease in the activation of the inflammatory pathway. We have also shown in HEK293 cells, expression of FGFR2c leads to a decrease in NFκB activity, indicating both FGFR2c and the FGFR2b isoform are able to inhibit NFκB activity. It’s possible that expression of various FGF ligands in different types of cells or cancers may play a role in dictating how FGFR2 functions. Additionally, dependence on NFκB activity may determine a specific cell’s dependence on FGFR2 as a tumor suppressor. Further research into the various roles of FGFR2 in cancer progression is needed to fully understand how the activity of this signaling pathway might be exploited in the fight against cancer.

**Materials and Methods**

**Cell culture:**

T47D and HEK293 cells were grown in DMEM supplemented with 10% FBS and 1% Pen/strep. T47D cells were kept in a humid atmosphere of 5% CO₂; HEK293 cells were kept in a humid atmosphere of 10% CO₂.

**Plasmid constructs:**

Full-length FGFR2 wild-type has been described previously. The HA-IKKβ clone was received from Dr. Mark Hannink (University of Missouri). The HA-tag was removed from this clone by QuikChange site-directed mutagenesis in which a HindIII site was created along with an ATG start site downstream of the HA-tag. The parental clone contained a HindIII site upstream of the HA-tag. The plasmid containing the new HindIII site was digested with HindIII and re-ligated to generate the untagged IKKβ expression clone. It was necessary to delete this tag as it contains numerous
tyrosine sites that are phosphorylated by activated FGFR2. Derivatives were confirmed by DNA sequencing at the UCSD Moores Cancer Center Shared Resource facility. The GST-IkB\(^{(1-54)}\) plasmid was a gift from the Hoffmann Lab (UCSD).

**Antibodies and Reagents:**

Antibodies were obtained from the following sources: FGFR2 (C-17), IKK\(\beta\) (H-4), NF\(\kappa\)B (F-6), \(\beta\)-tubulin (H-235), IKK\(\gamma\) (FL-419) from Santa Cruz Biotechnology; 4G10 (antiphosphotyrosine) from Upstate Biotechnology; horseradish peroxidase (HRP) anti-mouse, HRP anti-rabbit from GE Healthcare. FGF19 and TNF\(\alpha\) were obtained from R&D.

**Immunoprecipitation and Immunoblot:**

HEK293 cells (1X10\(^6\)) were plated on 10 cm dishes 1 day prior to transfection with 4-6\(\mu\)g of total DNA by calcium phosphate precipitation at 3% CO\(_2\). After 18 to 20 h, cultures were moved back to 10% CO\(_2\) for 4-6 h before starving overnight in DMEM lacking FBS. Cells were harvested, washed once in PBS, and lysed in 1% NP-40 Lysis Buffer [20mM Tris-HCl, pH 7.5, 137mM NaCl, 1% Nonidet P-40, 5mM EDTA, 50mM NaF, 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride (PMSF), 10ug/ml aprotinin] or radioimmunoprecipitation assay buffer [RIPA; 50mM Tris-HCl, pH 7.5; 150mM NaCl; 0.1% SDS; 1% Triton-X 100; 1% DOC; 50mM NaF; 1mM sodium orthovanadate; 1mM phenylmethylsulfonyl fluoride (PMSF); 10ug/ml aprotinin]. Total protein was measured by Bradford Assay or Lowry Assay (Bio-Rad). Immunoprecipitations were performed overnight at 4\(^\circ\)C and collected by Protein A-Sepharose (Sigma). Samples were washed three times with Lysis Buffer, boiled for 4
min in sample buffer and separated by SDS-PAGE. Proteins were transferred to Immobilon-P membranes and blocked in 3% or 5% milk/TBS/0.05% Tween 20 or 3% bovine serum albumin (BSA)/TBS/0.05% Tween 20 (for anti-phosphotyrosine blots). Membranes were immunoblotted with antibodies for 2 h at room temperature or overnight at 4°C. After primary incubations, membranes were washed with TBS/0.05% Tween 20 and incubated with HRP-conjugated secondary antibodies. Proteins were detected by enhanced chemiluminescence (ECL) (GE Healthcare) or (Millipore). To reprobe with subsequent antibodies, membranes were incubated in stripping buffer [100mM β-mercaptoethanol; 2% SDS; 62.5 mM Tris-HCl, pH 6.8] to remove bound antibodies.

**In vitro kinase assays**

1X10^6 HEK293 cells were plated on 10cm dishes. HEK293 cells were transfected as described above. Cells were then starved in DMEM lacking FBS overnight, prior to being treated with 10ng/mL TNFα for 10min. Cells were then harvested and washed once in PBS + 1mM EDTA. Cells were then lysed in Cytoplasmic Extract Buffer (CEB) [10mM HEPES-KOH (pH 7.9), 250mM NaCl, 1mM EDTA, 0.5% Nonidet P-40, 0.2% Tween 20, 20mM β-glycerophosphate, 2mM DTT, 10mM NaF, 1mM sodium orthovanadate, 1mM PMSF, 10µg/mL aprotinin]. Protein concentrations were determined by Bradford Assay. 200µg lysates were immunoprecipitated with IKKγ antibody for 2 h at 4°C. Protein A-Sepharose beads were added and incubated for 1 h at 4°C. The immunoprecipitated samples were washed twice with CEB and twice with Kinase Buffer (KB) [20mM HEPES (pH 7.7),
100mM NaCl, 10mM MgCl₂, 20mM β-glycerophosphate, 2mM DTT, 10mM NaF, 0.1mM sodium orthovanadate, 1mM PMSF]. Samples were then resuspended in 2X KB with 20µM ATP. 1µCi [γ⁻³²P]-ATP and 0.5µg GST-IkB⁽¹⁻⁵⁴⁾ bacterially expressed purified protein were added to each reaction and incubated at 30°C for 30 min. Proteins were separated by 10% SDS-PAGE, Coomassie stained, dried, and exposed to film or a phosphorimager (BioRad) screen directly. Band intensities were quantified using Quantity One Software.

**Electrophoretic Mobility Shift Assay (EMSA):**

T47D cells were collected in PBS, 1mM EDTA, pelleted at 2000 g, resuspended in 200µl CE buffer (10mM Hepes-KOH pH7.9, 60mM KCl, 1mM EDTA, 0.5% NP-40, 1mM DTT, 1mM PMSF) and vortexed. Nuclei were pelleted at 4000 g, and the supernatant was removed. Nuclei were resuspended in 50µl NE buffer (250mM Tris pH7.8, 60mM KCl, 1mM EDTA, 1mM DTT, 1mM PMSF) and lysed by 3 freeze-thaw cycles. Nuclear lysates were cleared by centrifugation and normalized to a concentration of 1µg/µl following Bradford assay. 2µg of total nuclear protein was reacted at room temperature for 15 min. with excess ³²P-labeled 30 bp double-stranded oligonucleotide containing a consensus κB-site (AGCTTGCTACAAAGGGACTTTCCGCTGTCTACTTT) in 6µl binding buffer (10mM Tri.Cl pH7.5, 50mM NaCl, 10% glycerol, 1% NP-40, 1mM EDTA, 0.1µg/µl polydIdC). Complexes were resolved on a non-denaturing 5% acrylamide (30:0.8) gel containing 5% glycerol and 1x TGE (24.8mM Tris, 190mM glycine, 1mM EDTA) and visualized by autoradiography and/or quantified using a Phosphorimager (Bio-Rad).
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Chapter 4, in full, is material that is currently being prepared for submission for publication. Drafall, Kristine A.; McAndrew, Christopher W., Meyer, April N., Donoghue, Daniel J. 2009. The dissertation author was the primary investigator and author of this paper.

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Chapter 5

E681K in FGFR4 Acts as a Dominant Negative Mutation and is Unable to Decrease NFκB Activity in Lung Adenocarcinoma Cells
Abstract
We have previously identified a novel interaction between FGFR4 and IKKβ and shown that this interaction leads to a decrease in NFκB signaling. Recent research has identified the E681K somatic mutation in FGFR4 in a screen for lung adenocarcinomas. In this paper, we have determined that this mutation is a dominant negative mutation of FGFR4 leading to inhibition of all kinase activity. We have also shown that FGFR4 E681K still interacts with IKKβ, but is unable to tyrosine phosphorylate IKKβ. Additionally, we show that the mutation of E681K leads to a decreased ability of FGFR4 to inhibit IKK activity or NFκB nuclear localization in A549 lung adenocarcinoma cells. Our data further implicate FGFR4 as a tumor suppressor in lung adenocarcinoma cells and describes a mechanism by which this dominant negative mutation may promote lung carcinogenesis through NFκB signaling.

Introduction
FGFR4 is one member of a family of four fibroblast growth factor receptors (FGFRs). This group of receptor tyrosine kinases (RTKs) are high-affinity receptors for the family of fibroblast growth factors (FGFs) of which there are more than 20 known members. Each FGFR has different affinities for the FGFs as well as expression in various tissues and cell types. FGF signaling is important for many cellular processes, including wound healing, migration, survival, and motility.

FGFR signaling has been implicated in a variety of different diseases, including cancer of the breast, lung, and skin among others. Mutations have
been identified in FGFR1, 2, and 3 that are implicated in many of these cancers. There has been significant research into the Gly388Arg polymorphism that exists in the transmembrane of FGFR4. This polymorphism is homologous to the FGFR3 Gly380R mutation that has been shown to lead to increased kinase activity through stabilization of the FGFR dimer. The FGFR4 Gly388Arg polymorphism exists in approximately 50% of the population and it is believed to be a possible prognostic factor in determining disease free survival, though research has led to opposing views on the importance of this polymorphism in cancer progression, with almost an equal number of publications supporting or refuting the importance of the polymorphism as a prognostic factor. A mutation in the kinase domain of FGFR4 at Pro712Thr has been identified in a lung adenocarcinoma, though further research into the relevance of this mutation has yet to be undertaken.

Recently, during a screen for mutations in a group of patients with lung adenocarcinomas, a somatic mutation was identified in FGFR4. This mutation, E681K, is in the kinase domain of FGFR4. Using structural modeling, the authors proposed that this mutation may lead to altered activity of FGFR4 due to the charge of the amino acids, possibly through disruption of an ionic bond with Arg650. In this paper, we sought to understand the importance of this mutation on the kinase activity of the receptor and to determine the relevance in lung adenocarcinoma.

We recently identified the novel interaction of FGFR4 with IKKβ and showed that the kinase activity of FGFR4 is important for a decrease in NFκB activity, implicating FGFR4 as a tumor suppressor. We sought to determine whether this mutation decreases the interaction of FGFR4 with IKKβ or if it alters the ability of
FGFR4 to decrease NFκB activity. The results presented here identify a possible mechanism for NFκB-related cancer progression in the lung.

**Results**

We initially sought to characterize the kinase activity of the E681K mutation of FGFR4 as it was believed to lead to altered kinase activity\(^1\). HEK293 cells were transfected with various constructs of FGFR4. Lysates were subjected to immunoprecipitation using an antibody for FGFR4, separated by SDS-PAGE, and immunoblotted with a phosphotyrosine-specific antibody. Overexpression of FGFR4 WT led to a high level of autophosphorylation, with the FGFR4 K645E kinase active mutation\(^2\) showing even higher levels of autophosphorylation. The FGFR4 kinase dead (KD) mutation showed no tyrosine phosphorylation. Similarly, the FGFR4 E681K mutation showed was not autophosphorylated, indicating this mutation leads to a kinase-dead receptor (Figure 5-1a, top panel). The membrane was stripped and re-probed for FGFR4 to show equal protein expression (second panel). We next examined activation of downstream components of the FGFR signaling pathway. Lysates were separated by SDS-PAGE and immunoblotted for phospho-MAPK. While FGFR4 WT and K645E led to increased phosphorylation of MAPK, FGFR4 KD and E681K were unable to phosphorylate MAPK (fourth panel). Lysates were probed for MAPK to show equal sample loading (fifth panel). We also examined phosphorylation of STAT1, and found that although the kinase active mutant K645E led to an increase in tyrosine phosphorylation of STAT1, the E681K mutant was
unable to activate STAT1 (Figure 5-1a, panel six). Total expression of STAT1 is shown in the bottom panel.

To determine whether the E681K mutant acts as a dominant negative mutation, we co-transfected HEK293 cells with an equal amount of FGFR4 K645E and varied the amount of WT or E681K transfected to obtain a decreasing ratio of WT:E681K. Samples were immunoprecipitated with an antibody for FGFR4 and immunoblotted using a phosphotyrosine specific antibody. As the ratio of E681K to WT increased, the tyrosine phosphorylation on the receptors decreased, indicating the E681K mutation serves as a dominant negative mutation and can decrease the autophosphorylation of the kinase active K645E mutant (Figure 5-1b).

We next confirmed that the kinase activity of E681K was also absent by using poly-glu-tyr as a substrate in a kinase assay. HEK293 cells were transfected with the various constructs of FGFR4 and subjected to immunoprecipitation using an FGFR4 antibody. Samples were separated by SDS-PAGE and exposed to a phosphoimager (Figure 5-1c). Both WT and K645E FGFR4 showed high levels of kinase activity toward poly-glu-tyr, while KD and E681K FGFR4 showed no detectable kinase activity, further confirming that the E681K mutant of FGFR4 has no kinase activity. Lysates were immunoblotted using an FGFR4 antibody to show equal protein expression (Figure 5-1c, bottom panel). These results confirm the hypothesis by Marks, et al. that the change from E to K at amino acid 681 would alter the kinase activity of the receptor.
Figure 5-1: FGFR4 E681K is a dominant-negative mutation and has no kinase activity. (a) HEK293 cells were transfected with FGFR4 wild-type (WT), kinase-dead (KD), E681K, or a kinase active mutation (K645E). Samples were immunoprecipitated using a FGFR4 antibody and immunoblotted with anti-phosphotyrosine (top); then the membrane was stripped and re-probed for FGFR4 (second panel). Lysates were immunoblotted for FGFR4, phospho-MAPK, MAPK, phospho-STAT1, or STAT1. (b) HEK293 cells were transfected with equal amounts of K645E and varying ratios of WT or E681K FGFR4 plasmid DNA. Samples were immunoprecipitated for FGFR4 and immunoblotted with anti-phosphotyrosine (top). The membrane was stripped and re-probed with anti-FGFR4 (bottom). (c) Kinase assay using poly-glu-tyr as a substrate. HEK293 cells were transfected with FGFR4 constructs, immunoprecipitated with anti-FGFR4, and subjected to kinase assay. The gel was exposed to phosphoimager to analyze $^{32}$P incorporation onto poly-glu-tyr.
We have previously shown that FGFR4 can interact with, and tyrosine phosphorylate IKKβ to lead to decreased NFκB activity\(^{25}\). Of interest was understanding if the FGFR4 E681K mutation is still able to interact with FGFR4 or if it has altered binding capacity compared to FGFR4 WT. HEK293 cells were co-transfected with various constructs of FGFR4 as well as IKKβ. Lysates were immunoprecipitated for IKKβ and immunoblotted for FGFR4. The E681K FGFR4 is still able to interact with IKKβ, though to a slightly lesser extent (Figure 5-2a). Since the E681K mutation has no kinase activity, we hypothesized that expression of this mutant would not lead to tyrosine phosphorylation on IKKβ. Indeed, the E681K mutation as well as the KD mutation were unable to tyrosine phosphorylate IKKβ (Figure 5-2b).

**Figure 5-2: FGFR4 interaction and tyrosine phosphorylation of IKKβ.** (a) HEK293 cells were co-transfected with FGFR4 constructs plus IKKβ. Samples were lysed in NP-40 lysis buffer and immunoprecipitated using an IKKβ antibody, and immunoblotted for FGFR4 (top). The membrane was stripped and re-probed for IKKβ (second panel). Lysates were immunoblotted for FGFR4 to show equal expression (bottom). (b) HEK293 cells were transfected as in (a), lysed in RIPA lysis buffer, and immunoprecipitated for IKKβ. The membrane was immunoblotted using anti-phosphotyrosine (top), or stripped and re-probed for IKKβ (bottom).
The E681K mutation was identified during a screen of lung adenocarcinomas\(^1\). Inflammation in the lung has been associated with lung carcinogenesis\(^27\), and recent research has implicated NFκB activation in the progression and metastasis of lung carcinomas\(^28\). We have previously shown that FGFR4 kinase activity leads to a decrease in NFκB activation\(^25\). We hypothesized that if the E681K mutation has no kinase activity, it will no longer be capable of altering NFκB signaling. In order to understand the effect that the FGFR4 E681K mutation has on lung adenocarcinoma cells, we used the A549 lung adenocarcinoma cell line. We investigated the effect the E681K mutation has on the FGFR4-mediated decreased in TNFα-induced NFκB activity by performing a GST-IκB kinase assay to measure IKK activity. A549 cells were transfected with the various constructs of FGFR4 and treated with TNFα. Samples were separated by SDS-PAGE and exposed to a phosphoimager to analyze incorporation of \(^{32}\)P. Expression of FGFR4 WT and K645E led to a decrease in IKK activity as seen previously, while the KD and E681K mutations were unable to inhibit TNFα-induced IKK activity (Figure 5-3).

\[\text{Figure 5-3: E681K FGFR4 has no effect on TNFα-induced IKK activity in A549 cells. A549 cells were transfected with the various constructs of FGFR4. Lysates were subjected to an in vitro kinase assay using GST-IκB as a substrate.}\]
In order to characterize the effect the E681K mutation had on downstream NFκB activity, we measured nuclear localization by indirect immunofluorescence. A549 cells were transfected with the FGFR4 constructs and treated with TNFα. Cells expressing FGFR4 were counted based on NFκB nuclear localization. Representative cells are shown in Figure 5-4a and cell counts are shown in Figure 5-4b. FGFR4 K645E was able to decrease TNF-induced NFκB nuclear localization by 80%, while FGFR4 KD and E681K showed a modest decrease of 40% and 35% respectively (Figure 5-4b), indicating the interaction with IKKβ leads to inhibition of NFκB activity, but the lack of kinase activity of FGFR4 E681K decreases this effect. These results indicate that the E681K mutation decreases the ability of FGFR4 to inhibit NFκB activity and may be one mechanism for cell survival in lung adenocarcinomas.
Figure 5-4: The E681K mutation in FGFR4 is less effective at altering NFκB nuclear localization than a kinase-active FGFR4. (a) A549 cells were transfected with various constructs of FGFR4 and treated with TNFα for 30 min. Coverslips were fixed and stained for NFκB (FITC) or FGFR4 (Rhod). (b) 100 cells each sample were counted in three independent experiments. Error bars represent +/- st. dev. Cells were considered to have nuclear NFκB if the FITC staining was predominantly in the nucleus.

Discussion

The E681K mutation in FGFR4 was identified in a screen of lung adenocarcinoma cells. This somatic mutation was the only mutation identified in the adenocarcinoma, indicating this mutation may be important for the progression of lung adenocarcinomas. It was hypothesized using molecular modeling that this mutation would lead to altered FGFR4 kinase activity due to the change in charge of the amino acids. We have determined that this mutation is dominant negative and eliminates the kinase activity of FGFR4. We have also shown that this mutation does not alter...
FGFR4 interaction with IKKβ substantially, and causes FGFR4 to be unable to tyrosine phosphorylate IKKβ. While expression of WT FGFR4 in A549 lung adenocarcinoma cells leads to a decrease in IKK activity as measured by a GST-IκB kinase assay, or a decrease in NFκB nuclear localization as measured by indirect immunofluorescence, FGFR4 E681K has diminished capacity to alter IKK or NFκB activity.

Given the recent evidence to support the importance of NFκB activity in the progression of lung cancer,28-31 it is possible that activation of FGFR4 may serve as a tumor-suppressive role by decreasing NFκB signaling. Mutations such as E681K, which are dominant negative and eliminate the kinase activity of FGFR4 may be important in the progression of cancers which rely on NFκB signaling. We have identified a novel mechanism of NFκB inhibition by FGFR4 signaling and have shown here that altered FGFR4 activity may lead to carcinogenesis of the lung.

Materials and Methods

Cell culture:

A549 and HEK293 cells were grown in DMEM supplemented with 10% FBS and 1% Pen/strep; A549 cells were kept in a humid atmosphere of 5% CO₂; HEK293 cells were kept in a humid atmosphere of 10% CO₂.

Plasmid constructs:

The full-length FGFR4 wild-type, kinase active (K645E), and myristylated forms of FGFR4 have been described previously.26 The kinase dead (K504M) and
E681K forms of full-length FGFR4 were generated by QuikChange site directed mutagenesis (Stratagene). The HA-IKKβ clone was received from Dr. Mark Hannink (University of Missouri). The HA-tag was removed from this clone by QuikChange site-directed mutagenesis in which a HindIII site was created along with an ATG start site downstream of the HA-tag. The parental clone contained a HindIII site upstream of the HA-tag. The plasmid containing the new HindIII site was digested with HindIII and re-ligated to generate the untagged IKKβ expression clone. It was necessary to delete this tag as it contains numerous tyrosine sites that are phosphorylated by activated FGFR4. Derivatives were confirmed by DNA sequencing at the UCSD Moores Cancer Center Shared Resource facility. The GST-IκB$^{(1-54)}$ plasmid was a gift from the Hoffmann Lab (UCSD).

**Antibodies and Reagents:**

Antibodies were obtained from the following sources: FGFR4 (C-16), IKKβ (H-4), NFκB (F-6), IKKγ (FL-419) from Santa Cruz Biotechnology; 4G10 (antiphosphotyrosine) from Upstate Biotechnology; Phospho-p44/42 MAPK (Thr202/Tyr204; E-10), phospho-STAT1 and STAT1 from Cell Signaling; MAPK (ERK1+ERK2) from Zymed; horseradish peroxidase (HRP) anti-mouse, HRP anti-rabbit from GE Healthcare; fluorescein-conjugated anti-mouse from Sigma and rhodamine-conjugated anti-rabbit from Boehringer-Mannheim. TNFα was obtained from R&D. Poly-glu-tyr was obtained from Sigma.
**Immunoprecipitation and Immunoblot:**

HEK293 or A549 cells (1X10^6) were plated on 10 cm dishes 1 day prior to transfection with 4-6µg of total DNA by calcium phosphate precipitation at 3% CO2. After 18 to 20 h, cultures were moved back to 10% CO2 for 4-6 h before starving overnight in DMEM lacking FBS. Cells were harvested, washed once in PBS, and lysed in 1% NP-40 Lysis Buffer [20mM Tris-HCl, pH 7.5, 137mM NaCl, 1% Nonidet P-40, 5mM EDTA, 50mM NaF, 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride (PMSF), 10ug/ml aprotinin] or radioimmunoprecipitation assay buffer [RIPA; 50mM Tris-HCl, pH 7.5; 150mM NaCl; 0.1% SDS; 1% Triton-X 100; 1% DOC; 50mM NaF; 1mM sodium orthovanadate; 1mM phenylmethylsulfonyl fluoride (PMSF); 10ug/ml aprotinin]. Total protein concentrations were measured by Bradford Assay or Lowry Assay (Bio-Rad). Immunoprecipitations were performed overnight at 4°C and collected by Protein A-Sepharose (Sigma). Samples were washed three times with Lysis Buffer, boiled for 4 min in sample buffer and separated by SDS-PAGE. Proteins were transferred to Immobilon-P membranes and blocked in 3% or 5% milk/TBS/0.05% Tween 20 or 3% bovine serum albumin (BSA)/TBS/0.05% Tween 20 (for anti-phosphotyrosine blots). Membranes were immunoblotted with antibodies for 2 h at room temperature or overnight at 4°C. After primary incubations, membranes were washed with TBS/0.05% Tween 20 and incubated with HRP-conjugated secondary antibodies. Proteins were detected by enhanced chemiluminescence (ECL) (GE Healthcare) or (Millipore). To reprobe with subsequent antibodies, membranes were incubated in
stripping buffer [100mM β-mercaptoethanol; 2% SDS; 62.5 mM Tris-HCl, pH 6.8] at 80°C for 1 h to remove bound antibodies.

**Indirect Immunofluorescence:**

A549 cells were plated on glass coverslips at a density of 1,000,000/10cm plate. The next day, cells were transfected using calcium phosphate precipitation. The following day, cells were starved in DMEM containing no serum for 24 h. 10ng/ml TNFα was added for 30 min. Coverslips were washed in PBS, fixed in 3% paraformaldehyde in PBS for 15 min, washed 3 times with PBS, and permeabilized for 10 min with 0.5% Triton-X 100 in PBS. Coverslips were again washed 3 times with PBS before blocking with 3% BSA in PBS for 1h. Coverslips were incubated with FGFR4 (C-16) at 1:1000 and NFκB (F-6) at 1:250 for 1 h. Coverslips were washed 3 times with PBS and labeled with secondary antibodies for 1 h at room temperature: Rhodamine-Rabbit, 1:1000; FITC-mouse, 1:1000; and hoechst, 1:1000. Coverslips were washed 3 times with PBS before mounting in 90% glycerol, 10% 1M Tris pH 8.5, containing p-phenylenediamine. Cells were photographed using a Nikon Microphot-FXA with a cooled CCD camera (Hamamatsu C5810).

**In vitro kinase assays:**

1X10^6 HEK293 or A549 cells were plated on 10cm dishes. Cells were transfected as described above. Cells were then starved in DMEM lacking FBS overnight, prior to being treated with 10ng/mL TNFα for 10min. Cells were then harvested and washed once in PBS + 1mM EDTA. Cells were then lysed in Cytoplasmic Extract Buffer (CEB) [10mM HEPES-KOH (pH 7.9), 250mM NaCl,
1mM EDTA, 0.5% Nonidet P-40, 0.2% Tween 20, 20mM β-glycerophosphate, 2mM DTT, 10mM NaF, 1mM sodium orthovanadate, 1mM PMSF, 10µg/mL aprotinin]. Protein concentrations were determined by Bradford Assay. 200µg lysates were immunoprecipitated with IKKγ antibody (for the IKK kinase assay) or FGFR4 antibody (for the poly-glu-tyr assay) for 2 h at 4°C. Protein A-Sepharose beads were added and incubated for 1 h at 4°C. The immunoprecipitated samples were washed twice with CEB and twice with Kinase Buffer (KB) [20mM HEPES (pH 7.7), 100mM NaCl, 10mM MgCl₂, 20mM β-glycerophosphate, 2mM DTT, 10mM NaF, 0.1mM sodium orthovanadate, 1mM PMSF]. Samples were then resuspended in 2X KB with 20µM ATP. 1µCi [γ-32P]-ATP and 0.5µg GST-IκB(1-54) bacterially expressed purified protein or poly-glu-tyr were added to each reaction and incubated at 30°C for 30 min. Proteins were separated by 10% SDS-PAGE, Coomaisse stained, dried, and exposed to film or a phosphorimager (BioRad) screen directly. Band intensities were quantified using Quantity One Software.

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