The Role of Alternatively spliced Fibroblast Growth Factor Receptor 2 Isoforms in Breast Cancer

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Institute of Medical Science
University of Toronto

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I. Abstract

Recent genome-wide association studies identified FGFR2 as one of breast cancer susceptibility genes. FGFR2 expression was down-regulated in breast carcinomas when compared with paired normal epithelium. Stable retroviral transduction of FGFR2-IIIb and its alternatively spliced FGFR2-IIIc variants was achieved in breast cancer MDA-MB-231, T47D and near normal MCF-10A cells. Our findings revealed a direct reduction of breast cancer cell growth and motility, a significant arrest of transformed morphogenetic changes including the Epithelial to Mesenchymal transition (EMT), anchorage independent growth, and the formation of growth-arrested 3D acinar architectures, and suppressive actions on orthotopically xenografted epithelial neoplasms and surrounding tumor stroma. These tumor protective effects were concordant with physical interactions between the two FGFR2 isoforms and IKKβ. Consistent with these interactions we noted FGFR2 to inhibit NF-κB signaling, including decreased nuclear RelA/p65 NF-κB localization, down-regulation of a transfected NF-κB luciferase reporter, reduced production of NF-κB-dependent transcripts, Interleukin-6 and p-STAT3.
II. Acknowledgement

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University of Toronto’s staples of cultural diversity, creative thinking and academic freedom enormously benefitted me. I am grateful to have had the opportunity to meet such great professors, colleagues and friends within the Institute of Medical Science. Finally, I would like to express my deep appreciation for my beloved family members.
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<td>atypical ductal hyperplasia</td>
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<tr>
<td>ALH</td>
<td>atypical lobular hyperplasia</td>
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<tr>
<td>BAD</td>
<td>Bcl2 antagonist of cell death</td>
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<tr>
<td>CDK4</td>
<td>cyclin dependent kinase 4</td>
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<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
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<tr>
<td>DCIS</td>
<td>ductal carcinoma in situ</td>
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<tr>
<td>DAG</td>
<td>diacylglycerol</td>
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<tr>
<td>EGF/EGFR</td>
<td>epidermal growth factor (receptor)</td>
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<td>FRS2</td>
<td>FGFR substrate 2</td>
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<tr>
<td>EMT</td>
<td>Epithelial to Mesenchymal Transition</td>
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<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assays</td>
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<td>ERK</td>
<td>extracellular regulated kinase</td>
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<tr>
<td>ER</td>
<td>estrogen receptor</td>
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<tr>
<td>ERE</td>
<td>ER Element</td>
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<tr>
<td>FGFR/FGFR</td>
<td>Fibroblast growth factors (receptor)</td>
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<tr>
<td>FKBP52</td>
<td>immunophilin-FK-binding protein52</td>
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<tr>
<td>FRK</td>
<td>Fyn-related kinase</td>
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<td>GH</td>
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<td>Growth factor receptor-bound protein 2</td>
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<td>GSK-3</td>
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<td>HATs</td>
<td>histone acetyltransferases</td>
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<td>Human Epidermal growth factor Receptor 2</td>
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<td>HIF-1α</td>
<td>Hypoxia-inducible factor 1-alpha</td>
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<td>Hsp90</td>
<td>heat-shock protein 90</td>
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<td>HSPG</td>
<td>Heparan-sulfate proteoglycan</td>
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<td>IDC</td>
<td>invasive ductal carcinoma</td>
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<tr>
<td>IGF-1(R)</td>
<td>Insulin-like growth factor–I (receptor)</td>
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<tr>
<td>IkB</td>
<td>Inhibitor of κB</td>
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<tr>
<td>IKK</td>
<td>IkB kinase</td>
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<tr>
<td>ILC</td>
<td>invasive lobular carcinoma</td>
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<td>IL-6</td>
<td>interleukin 6</td>
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<tr>
<td>IR</td>
<td>insulin receptor</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<td>LBD</td>
<td>ligand-binding domain</td>
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<td>LCIS</td>
<td>lobular carcinoma in situ</td>
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LEF/TCF  lymphoid enhancer factor/T cell factor
LTR     long terminal repeat
MAPK    Mitogen-activated protein kinases
MEFs    mouse embryo fibroblast
MMLV    Moloney murine leukemia virus
MMP     Matrix metalloproteinases
MMTV    Mouse Mammary Tumor Virus
MSCV    Murine Stem Cell Virus
NEMO    NF-κB essential modulator
PDGF    platelet-derived growth factor
PDK1    phosphoinositide-dependent kinase 1
PKC     protein kinase C
PI3K    phosphatidylinositol 3-kinase
PIP2    phosphatidyl inositol 4,5 diphosphate
PIP3    phosphatidyl inositol-3,4,5-trisphosphate
PH      plextrin homology
PLCγ    phospholipase Cγ
PR      progesterone receptor
PTB     phosphotyrosine binding
PyMT    polyoma middle T antigen
PTHrP   parathyroid hormone-related protein
Rb      retinoblastoma
rTA     tetR/VP16 transactivator
RTK     receptor tyrosine kinases
SCID    Severe Combined Immune Deficiency
SH2     Src-homology 2
SHP2    SH2 domain-containing protein tyrosine phosphatase-2
SV40    Simian Virus 40
SMA     smooth muscle action
SNP     Single Nucleotide Polymorphisms
SPTY    Sprouty
SRC-1   steroid receptor coactivator-1
STAT    signal transducer and activator of transcription
TAM     tumor-associated macrophages
TEB     Terminal End Buds
TGF-β   transforming growth factor
TNF-α   Tumor Necrosis Factor Alpha
uPA     Urokinase-type plasminogen activator
VEGF    vascular endothelial growth factor
VSV-G   Vesicular Stomatitis Virus Glycoprotein
1. Introduction

1.1 Rationale

As well as the wealth of evidence that links fibroblast growth factor (FGF) signals with transforming function via gene amplification and/or mutations, several studies of mouse models, human tumors or cancer cell lines potentially support tumor suppressive functions of FGFR2 in some contexts [1]. The recent genome-wide association findings have highlighted FGFR2 as an important breast cancer candidate gene. An increased risk of developing invasive breast cancer was significantly linked to 8 single nucleotide polymorphisms (SNP) in intron 2 of the FGFR2 gene [2, 3]. We have reported that FGFR2 mRNA and protein levels were down-regulated in micro-dissected primary cancer cells when compared with paired normal breast epithelium [4]. However, there is limited knowledge of the role of FGFR2 in breast cancer initiation and/or progression, the mechanisms underlying the context-dependent tumor promoting or tumor suppressive effect of FGFR2 isoforms, and if the splicing of the extracellular domain affects the intracellular signalling.

1.2 Hypothesis

The hypothesis is that alternatively spliced FGFR2 isoforms play important roles in breast cancer initiation and/or progression, through driving the signaling pathways responsible for cancer-related biological responses.

1.3 Objectives

1. To elucidate the function of FGFR2 isoforms in breast cancer by examining the following cancer hallmarks:

   a. The effect on breast cancer cell growth and proliferation
b. The effect on breast cancer cell invasion and motility

c. The effect on tumor cell differentiation

d. The effect on tumor cell survival

e. The effect on mammary gland acinar morphology

2. To identify the potential mechanism underlying the action of FGFR2 isoforms and the key signal transduction paths implicated in FGFR2-driven tumorigenesis.

3. To investigate the potential relationship between FGFR2-IIIb and FGFR2-IIIc.
2. Literature Review

2.1 The Mammary Gland

2.1.1 Mammary Gland Structure, Development and Hormone Control

The mammary gland is a milk-producing gland characteristic of all female mammals and present in a rudimentary and generally nonfunctional form in males. Mammary glands are regulated by the endocrine system and become functional in response to hormonal changes [5]. The basic components of a mature mammary gland are the alveoli lined with milk-secreting cells and surrounded by myoepithelial cells. Groups of alveoli form lobules, and each lobule has a separate lactiferous duct that converges into openings in the nipple. The myoepithelial cells contract under the stimulation of oxytocin thereby milk is secreted from alveolar units into the lobular lumen toward the nipple [5].

Mammary glands develop to form a rudimentary duct tree at birth. In this stage, the development depends on systemic hormones, and is also regulated by paracrine communication between neighboring epithelial and mesenchymal cells by parathyroid hormone-related protein (PTHrP) [6]. Mammary bud epithelial cells proliferate and sprout down into the mesenchymal layer to begin the first round of branching in the fat pad. Meanwhile, the embryonic mesenchymal cells around the epithelial bud transform into a dense, mammary-specific mesenchyme, which later develop into connective tissue, forming blood vessels and the lymph system. Basement membrane, mainly containing laminin and collagen, formed thereafter by differentiated myoepithelial cells keeps the polarized morphology of the primary lactiferous duct tree [7].

Under the influence of estrogens from the maturing ovary at puberty, a mature duct tree comes into being by bifurcation of duct terminal end buds (TEB) in the fat pad of the mammary gland. After ovulation, progesterone from the corpus luteum causes the terminal ductal cells to differentiate into the milk-producing cells, which form acini [8, 9]. In pregnancy and lactation, rising levels of prolactin, estrogen and progesterone cause further branching until the ducts reach
the limits of the fat pad, together with an increase in adipose tissue and blood flow. The secretion of milk is induced by prolactin from the pituitary and the release of oxytocin stimulates the contraction of the myoepithelial cells in basement membrane. During weaning, decreased prolactin, missing mechanical stimulation (baby suckling) and changes in osmotic balance cause cessation of milk production [10].

### 2.1.2. Epithelial-Stromal Crosstalk in Mammary Gland

The mammary gland comprises stromal and epithelial cells, and crosstalk between the mammary epithelium and stroma is crucial for the proper patterning and function of the normal mammary gland. Disruption of communication can induce breast tumorigenesis [8]. The mammary stroma is composed of adipocytes, pre-adipocytes, fibroblasts, blood vessels, inflammatory cells, and extracellular matrix (ECM). Interaction between the epithelium and the stroma plays a major role in mammary gland branching morphogenesis. A candidate factor postulated to directly mediate the crosstalk between the stroma and epithelium during mammary gland development is parathyroid hormone-related protein (PTHRP). It is produced by mammary epithelial cells, whereas, its receptor is expressed by stromal cells. PTHR P is involved in supporting the initiation of branching growth that is responsible for transforming the mammary bud into the rudimentary mammary duct system. Loss of PTHR P or its receptors cause the mammary bud cells to change back into epidermis [11, 12], and provides a direct epithelial-to-mesenchymal communication which is essential for mammary gland development. Also, Insulin-like growth factor–I (IGF-I) is induced by and mediates the function of growth hormone (GH) and the GH receptor that are required for mammary ductal development [13]. IGF-I receptor (IGF-IR) is expressed in mammary epithelium for proper ductal development, and GHR functions in the mammary stroma [14, 15]. GH activates GHR in the stroma, thereby inducing stromal expression of IGF-I, which then acts on its receptor in the epithelium. Such factors implicated in mammary development are also vital for breast cancer. Understanding how these factors function in normal development may help us to better understand how tumors initiate and thrive.
2.2 Breast cancer

Breast cancer is one of the leading causes of cancer-related deaths in women worldwide, it is estimated that there are 57320 new breast cancer cases each year [16]. Although risk factors such as abnormal expression level of circulating hormones [17], mechanical changes in the tension of mammary stroma [18], inherited or somatic defects in high-penetrance cancer susceptibility genes including BRCA1, BRCA2 and p53, are associated with breast cancer initiation and progression [19], tumors of the mammary tissues have an almost individually unpredictable behavior and aggressiveness. Therefore, a better insight in the genetic and epigenetic changes, which are responsible for of breast cancer development, is necessary.

2.2.1. Histological and Molecular Types of Breast Cancer

Breast cancer commonly originates from epithelial cells of the terminal ductal or lobular units in the mammary glands, starting with benign lesions including atypical ductal hyperplasia (ADH), atypical lobular hyperplasia (ALH), ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS), and with subsequent evolution into invasive metastatic types including invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) [20, 21]. Women with atypical hyperplasia (ADH and ALH) and in situ carcinomas (IDC and ILC) have approximately fivefold and tenfold increased risks, respectively, of eventually developing invasive breast cancer [20]. The existence of at least 18 distinct histological types of invasive breast cancers is recognized by the WHO, and the large majority (50-80%) of them are IDC [22].

Histological grading is a powerful indicator of prognosis in breast cancer, combining the degree of differentiation (e.g. tubule formation and nuclear pleomorphism) and proliferative activity (e.g. mitotic count) of the tumors (Figure 1). The highest possible score (3+3+3=9) is employed for the poorly differentiated tumor with a high mitotic rate [23].
Figure 1. Breast Cancer Histological Grading System

Low grade (I): The cancer cells are well-differentiated, lower metastatic risk and potentially better prognosis. Intermediate grade (II): The cancer cells have features between grade 1 and 3 and moderately-differentiated. High grade (III): The cancer cells are poorly-differentiated, high metastatic risk and poor prognosis.

The microarray-based gene-expression profiling studies have led to the development of a molecular classification of breast cancer that comprised four biologically distinct subtypes [24]: (a) normal-like phenotype, which gene-expression profile is similar to non-cancerous breast tissue; (b) luminal phenotype, which is generally estrogen receptor (ER)-positive with expression of epithelial markers, e.g. E-cadherin, and cytokeratin 8 and 18; (c) Her2-positive phenotype, which show \textit{ERBB2} over-expression/ gene amplification; (d) basal-like phenotype, which is characterized by lack of estrogen receptor, progesterone receptor and Her2, and by consistent expression of markers usually found in normal basal or the myoepithelial cells of the mammary gland such as p63, and basal cytokeratins CK5, 6, 14, 17. The malignant phenotypes, especially basal-like, show unfavorable prognosis and/or resistance to chemotherapy, and have a tendency to distant metastasis [25, 26].

2.2.2. The Heterogenity of Breast Cancer

Human breast cancer represents a group of highly heterogeneous lesions with substantially different molecular and biochemical signatures. Several potential mechanisms, including cancer stem cells, genetic instability due to mutations, deletion or amplifications, and breast tumor susceptibility genes have provided some plausible explanations [27].
Increasing evidence indicates that breast tumors are driven by a population of cancer stem cells (CSCs), and tumor heterogeneity is a reflection of the number of CSC-like cells in the tumors [27]. In addition to the BRCA1 and BRCA2 genes, in which mutations confer a high risk of susceptibility to breast cancer, a number of low-risk variants [2] that have been recently identified by genome-wide association studies [19], might also introduce some levels of heterogeneity.

2.2.3. Key Signaling modules in Mammary Gland Development and Cancer

2.2.3.1 Snail1-Twist1, the key EMT molecules, in malignant breast cancer progression

Similarities have been observed between the invasive and metastatic behaviors of cancer cells and the long-distance migration of cells during development [28]. Epithelial cells are tightly connected each other by adhesion proteins and form a protective barrier on basement membrane. They express epithelial markers (e.g. cytokeratins). Whereas mesenchymal cells lack cell-cell contacts, express mesenchymal markers (e.g. N-cadherin and vimentin) and exhibit migratory behavior [28]. During Epithelial-to-Mesenchymal Transition (EMT), epithelial cells lose their epithelial features and acquire a fibroblast-like morphology with cytoskeletal reorganization, up-regulation of mesenchymal markers, and enhancement of motility, invasiveness and metastatic capabilities [29]. The occurrence of EMT in breast cancer in vivo was illustrated by immunohistochemical analysis of human invasive breast carcinomas and carcinosarcomas [30]. Simultaneous up-regulation of mesenchymal markers together with down-regulation of epithelial markers such as E-cadherin, were predominant in breast tumors with a basal-like phenotype [30].

Snail1 (encoded by SNAI1) is a zinc-finger transcription factor belonging to the Snail super family and characterized by a strongly conserved carboxy-terminal region containing four to six C2H2-zinc fingers. Snail1 and Snail2 (Slug) act as transcriptional repressors when their zinc fingers bind to E-box motifs (5’-CANNTG-3’) in target promoters including the E-cadherin gene promoter [31]. The two mammalian Twist proteins (Twist 1 and Twist 2) possess a strong
structural homology with a conserved basic helix-loop-helix domain [32]. Snail1 controls expression of aromatase that converts androgens to estrogens, indicating that it has a role in the ductal network development [33]. During malignant cancer progression, Snail1 plays a role in initiating the EMT steps and enables epithelial cancer cell invasive and migratory capacities. Twist1 plays a role in the development of distant metastases by facilitating cancer cells to enter the bloodstream [34]. At distant organs, these cells undergo a mesenchymal-to-epithelial transition (MET) [28].

A key molecular change in EMT is E-cadherin down-regulation, which results in reduction of cell-cell adhesion and destabilization of the epithelial architecture. Several signaling cascades including transforming growth factor (TGF)-β and Wnt, lead to transcriptional repressors of E-cadherin [35]. TGF-β is a potential inducer of EMT, leading to translocation of Snail1 to the nucleus, where it interacts with activated Smad3/4. This complex binds and represses E-cadherin promoter. In vivo, the Snail1-Smad3/4 complex was found in the nucleus of tumor cells at the invasive front [28, 36]. Wnt proteins bind to cell-surface receptors of the Frizzled family and inhibit the axin/GSK-3/APC complex, and then inhibit the phosphorylation and proteolytic degradation of β-catenin. Thus, cytoplasmic β-catenin is stabilized and permitted to build up in the cytosol to a significant level, and subsequently translocates into the nucleus to interact with TCF/LEF family transcription promote specific gene expression including Snail1 [28]. Also, some novel inducers were demonstrated such as LBX1, Src1 and HIF-1α. A schematic overview of upstream regulators of Snail1 and Twist1, and their corresponding downstream effects is summarized in Figure 2 [28].

In addition to the regulators and their signaling pathways, the EMT mechanisms in breast cancer also involve tumor micro-environment. There is a strong association between inflammation and tumorigenesis. The inflammatory microenvironment drives tumor progression, with infiltration of immune cells and activation of the inflammatory responses [28]. Inflammatory cells, particularly tumor-associated macrophages (TAMs), are usually found at the tumor invasive front [37]. TAMs facilitate angiogenesis, ECM breakdown and tissue remodeling, and also
secrete pro-inflammatory cytokines, such as TNF-α [37]. Snail1 can be stabilized by TNF-α through the activation of the NF-κB pathway which induces a wide range of pro-inflammatory cytokines, chemokines and growth factors [38]. NF-κB can also induce IL-6, a pleiotropic cytokine that participates in acute inflammation [39]. Elevation of serum IL-6 has been shown to be correlated with advanced breast tumor stage, metastasis and poor prognosis due to aberrant activation of STAT3 [28, 40-41]. MCF7 cells constitutively expressing IL-6 exhibit an EMT phenotype characterized by up-regulation of Snail1 and Twist1 [42]. Besides inflammatory cytokines, matrix metalloproteinases (MMPs) are also important participants in tumor progression because they degrade structural components of the ECM, which allow tumor invasion and metastasis. In breast tumors, MMP-3 is frequently up-regulated and induces Snail1 expression and EMT through increased production of cellular reactive oxygen species [43].

Figure 2. A schematic overview of upstream regulators of Snail1 and Twist1, and their corresponding downstream effects in mammary gland development and cancer related progression. Snail1 and Twist1 contribute to different developmental and pathological outcomes in the mammary gland. Examples of effectors or direct target genes that are regulated by Snail1 and Twist1 to produce the indicated outcomes are shown.
2.2.3.2. Signaling downstream of PI3 kinase and breast cancer

The PI3K/Akt pathway is frequently dysregulated in breast cancer as a result of mutation, amplification, or deletion of different components of the pathway [44]. There are three principal components of the pathway: phosphatidylinositol 3-kinase (PI3K), its antagonist PTEN and the serine/threonine kinase Akt which is expressed as three different isoforms, Akt1, Akt2, and Akt3 [45]. PI3K is a family member of intracellular lipid kinases that are activated by growth factor receptor tyrosine kinases (RTKs) such as insulin receptor (IR), insulin-like growth factor 1 receptor (IGF-1R), and members of the epidermal growth factor (EGF)/ErbB family in addition to G protein-coupled receptors and oncogenes such as Ras. The 3’-hydroxyl group of phosphatidyl inositol 4,5 diphosphate (PIP2) is phosphorylated by PI3K to generate phosphatidyl inositol-3,4,5-trisphosphate (PIP3), a lipid second messenger that binds to the downstream 3-phosphoinositide-dependent kinase 1 (PDK1). The subsequent phosphorylation of PDK1 at Ser241 results in a trans-phosphorylation of Akt at threonine 308. Also, phosphorylation on Ser 473 by the mTOR-rictor kinase enables a full activation of Akt with kinase activity [44, 45], which leads, in turn, to phosphorylation of Akt downstream targets such as the FOXO family of transcription factors. PTEN is a phosphatase that reverses the action of PI3K by dephosphorylating PIP3, thereby regulating Akt activity and its downstream responses [46]. Several kinases have been reported to phosphorylate PTEN [47] including the Fyn-related kinase (FRK).

The functions of PI3K-AKT pathway include the regulation of glucose homeostasis and metabolism, particularly in muscle and fat [44]. AKT activates glycogen synthase and ATP citrate lyase by inhibiting GSK3, thereby regulating fatty acid synthesis. Given the importance of adipose tissue and its remodeling during a pregnancy/lactation/involution cycle, this signaling pathway could have a critical role in mammary gland development and function [44]. Activated AKT protein modulates the function of numerous substrates related to the regulation of cell proliferation such as P21\textsuperscript{Waf1/Cip1} and P27\textsuperscript{Kip1}. They are phosphorylated by AKT and retained in the cytoplasm. Thus, their anti-proliferative effects and inhibitory effects on Cyclin D1/ CDK4/6 are limited [48, 49]. AKT is a survival factor, regulating the inactivation of the pro-apoptotic
protein Bcl2 antagonist of cell death (BAD) by direct phosphorylation. It also leads to p53 degradation by phosphorylating the p53 regulator MDM2 [44]. Additionally, PI3K regulates cell polarity and motility in concert with the small GTPases Rac, Rho, and Cdc42 by controlling actin dynamics in motile cells [50]. Over-expression of PTEN, the predominant negative regulator of PI3K, would promote apoptosis. PTEN-null mammary epithelial cells were hyper-proliferative, resulting in the early development of mammary tumors [44].

Given the importance of the PI3K/Akt pathway in regulating proliferation, differentiation, and apoptosis of mammary epithelial cells, inappropriate activation of this pathway such as genetic alterations of the key signaling components, could result in tumorigenesis [44]. The types of mutations and their frequencies are listed in Table 1.

Table 1. The type and frequency of mutations in PI3K-AKT pathway components in breast cancer

<table>
<thead>
<tr>
<th>PI3K component</th>
<th>Type of mutation</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p110</td>
<td>Mutation</td>
<td>27%</td>
</tr>
<tr>
<td>p110</td>
<td>Amplification</td>
<td>8.7%</td>
</tr>
<tr>
<td>PDKP1</td>
<td>Amplification and overexpression</td>
<td>20%</td>
</tr>
<tr>
<td>AKT1</td>
<td>Mutation (E17K)</td>
<td>3.7%</td>
</tr>
<tr>
<td>AKT2</td>
<td>Amplification</td>
<td>3%</td>
</tr>
<tr>
<td>PTEN</td>
<td>Loss of heterozygosity</td>
<td>24.9%</td>
</tr>
<tr>
<td>PTEN</td>
<td>Mutation</td>
<td>6%</td>
</tr>
</tbody>
</table>

2.2.3.3. Mitogen-activated protein kinases signaling in breast cancer

Four dominant mitogen-activated protein kinase (MAPK) signaling cascades are implicated in breast diseases and function in breast epithelial cells: the extracellular regulated kinase (ERK) ½ pathway, c-Jun N-terminal kinase (JNK) pathway, the p38 pathway and the ERK5 pathway [51]. Signaling through each pathway involves sequential activation of a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and the MAPK. Considering the ERK1/2 pathway, the
primary activator Ras, a small GTPase, activates Raf1 (MAPKKK), which then phosphorylates and activates MEK1/2 (MAPKK), which finally activates ERK1/2 [51]. The activated ERK1/2 phosphorylates a wide range of protein substrates (on serine or threonine) including transcriptional regulators, apoptosis regulators and steroid hormone receptors (e.g. oestrogen receptor α). The biological consequences include pro-proliferative, pro-differentiation, pro-survival [52], pro-angiogenic [53] and pro-motility [54, 55] effects.

In approximately 30% of human breast cancers, mutations are found in the ERK1/2 MAPK pathway. Dysregulated ERK1/2 signaling alone is usually not sufficient to cause cancer, and additional genetic alterations are commonly observed as well (e.g. ErbB2 receptor, c-myc and p53) [56]. ERK1/2 is hyper-activated in a large subset of mammary tumors especially in the cases with ‘node positive’ metastasis. A survival analysis demonstrated potential prognostic value of ERK1/2 for poor disease-free survival of breast cancer cases [55, 56] and impact on patients’ response to the treatments [56, 58].

Primary cells and various phenotypically normal mammary epithelial cell lines, when supported on a reconstituted basement membrane (extracellular matrix), undergo a proliferative-arrested phase and an apoptotic phase resulting in formation of three-dimensional spherical acini [59]. However, transformed cells do not develop into this normal structure, in particular, when co-expression of an oncogene in parallel ERK1/2 signaling [60, 61].

ERK1/2 signaling induces MMP expression/activity, which degrade type IV collagen and other matrix components, and confer on cells the ability to migrate and invade neighbouring tissues [62]. The serine protease uPA (urokinase-type Plasminogen activator) and its receptor (uPAR) also play a crucial role in breast cancer cell invasion and metastasis. uPA induces cell proliferation and prevent apoptosis in MDA-MB-231 breast cancer cells via ERK1/2 activation [63, 64].
In breast cancer, there is a functional link between EGFR/ErbB receptor activation, induction of ERK1/2 pathway signaling and relevant outcomes such as increased proliferation, cell survival, and induction of motility and invasiveness [56, 65].

Anti-hormone treatment with selective estrogen receptor modulators, such as tamoxifen, acts to inhibit ER activity in breast cancer cells and their metastases. ERK1/2 signaling contributes significantly to tamoxifen resistance [56].

2.2.3.4. The Insulin Growth Factor (IGF) system in Breast Cancer

The IGF system includes at least three ligands - insulin and insulin like growth factor I and II (IGF I and IGF II) and multiple receptors- the IGF-I receptor (IGF-IR), the insulin receptor (IR) and the insulin receptor- related receptor (IRR), which belong to the tyrosine-kinase superfamily, and binding proteins such as insulin receptor substrates (IRSs) [66]. They act as critical mediators both in normal mammary epithelium development and tumorigenesis [67].

An essential role for IGF-I in pubertal mammary growth has been established [67], whereas, elevated IGF-I plasma concentration is linked to a higher risk for breast and other malignancies [68]. A reduced occurrence of breast tumors was implicated in IGF-I-deficient mice [69]. IGF-I has been shown to induce cell-cycle regulatory changes including up-regulation of cyclin D1 and cyclin dependent kinase 4 (CDK4), retinoblastoma (RB) phosphorylation, activation of cyclin E and down-regulation of p27KIP1 [70, 71]. IGF-I also plays a role in enhancing expression of MMPs, such as MMP2 and MMP9, which are of importance in tumor invasion and metastases [72] and synergizing with HIF1 in promoting tumor cell replication [73]. Another major action of IGF-I on tumor cells is to inhibit apoptosis and confer resistance to cancer therapies [74]. IGF-IR is important in promoting oncogenic transformation, growth and the survival of cancer cells, and influences other growth factors or receptors such as vascular endothelial growth factor (VEGF) and EGFR [66, 75]. In addition, Insulin also contributes to breast tumor growth via the mediation of IR, not IGF-IR [66].
IRS-1 and IRS-2 are adaptor proteins in IGF system. Both contain a highly conserved N terminus composed of a plextrin homology (PH) and phosphotyrosine binding (PTB) domains, which mediates the binding to IR and IGF-IR [76], as well as the C-termini that serve as docking sites for Src-homology 2 (SH2) containing proteins. Upon activation, the downstream adaptor proteins are recruited and activated [77, 78]. Among them, the well-known downstream effectors are PI3K and ERK1/2 [79-82]. In addition to binding to IGF-IR/IR, IRSs also drive cytokine (e.g. interleukins) or hormone (e.g. prolactin and growth hormone)-induced signaling pathways [83, 84]. A transforming potential of IRSs was firstly found in mouse embryo fibroblast (MEFs) [85]. Over-expression of IRSs was then found to induce extensive proliferation and disrupted polarized acini in MCF-10A cells grown in 3D culture [86]. The transgenic mice with overexpressed IRSs in the mammary gland developed mammary gland hyperplasia, tumors, and metastases [87].

Studies have shown the synergistic effect of the crosstalk between steroid hormones and IGF-IR in breast cancer cells. Upon estrogen stimulation, IRS-1 was found to interact with ERα, followed by a nuclear translocation of the complex [88, 89]. The IRS-1/ERα complex then bound to the ER Elements of ER responsive genes and regulated their transcription [88]. In addition, a progesterone-induced IRS-2 activation was found to promote cell migration [89].

2.2.4. Breast Cancer Metastasis

Most of the complications associated with breast cancer are due to metastatic spread to regional lymph nodes or distant organs, including bone, lung, liver and brain [90, 91]. Specific molecular changes occurring within both the tumor cells and the tumor microenvironment contribute to the detachment of the tumor cells from the primary tumor, invasion into the tumor stroma, intravasation into nearby blood vessels or lymphatics, transportation by the circulation, extravasation and colonization of the target organs and, finally metastatic outgrowth [92, 93].

Several molecular processes contribute to mammary cancer metastasis: Mammary gland morphogenesis and branching involve the regulatory function of several signaling pathways,
including signaling by Wnt family members [94], TGF-β [95], IGF-I [96], EGF and others [97]. These pathways are frequently activated during malignant transformation by mutation or gene amplification; the cross-talk between tumor cells and surrounding stroma, via soluble growth factors, cytokines and chemokines are constantly modulating tumor development [91]; altered expression and/or function of the adhesion molecules responsible for the adhesion of breast cancer cells to themselves or to the tumor stroma, including integrin family members, immunoglobulin-domain cell adhesion molecules, cadherin family members, or other cell surface receptors, contributes to late stage tumor progression and metastasis [98, 99]; angiogenesis is crucial for growth and persistence of primary solid tumors and their metastases. An increase in vascular density facilitates intravasation of tumor cells. VEGFs and MMPs are all major contributors to angiogenesis [91].

A large number of established or putative prognostic markers have been demonstrated in the literature. A large-sized primary tumor (tumors over 2 cm in diameter have a higher risk of metastasis), the presence of axillary lymph node metastasis (the presence of over 4 lymph-node metastases is associated with high metastasis risk), loss of histological differentiation, ERBB2 gene amplification and/or protein overexpression are established breast cancer prognostic markers [90].

Several promoters can be used to drive the expression of transgenes in the mammary epithelium, and many known oncogenes have been expressed under their control to initiate or modulate breast carcinogenesis and metastasis in mice, including ErbB2/Neu, polyoma middle T antigen (PyMT), simian virus 40 (SV40) T antigen, Ha-Ras, Wnt-1, c-myc [90]. MMTV-Neu transgenic mice, in which the expression of ErbB2 oncogene is driven by the Mouse Mammary Tumor Virus (MMTV) promoter, develop multifocal adenocarcinomas with lung metastases after pregnancy [100]; Mammary epithelium specific expression of PyMT under the control of the MMTV promoter in transgenic mice results in the development of multifocal mammary adenocarcinomas and metastatic lesions in the lymph nodes and in the lungs [101]. Tumor formation and progression in this mouse model includes four stages: hyperplasia,
adenoma/mammary intra-epithelial neoplasia, and early and late carcinoma [102]. A gradual loss of steroid hormone receptors (estrogen and progesterone) associated with over-expression of ErbB2 and cyclin D1 was observed in late-stage metastatic cancer, which shows a high similarity to human breast cancer. Furthermore, the MMTV-PyMT mouse model is characterized by short latency, high penetrance and high incidence of lung metastasis occurring independent of pregnancy [90]. However, it appears that additional genetic alterations are required to unmask a more malignant phenotype in this mouse model [90].

2.2.5. The Estrogen Receptors

Breast cancer is frequently hormone-dependent. The growth of the tumor cells can be negatively regulated by anti-hormones. Therefore, a hormone therapy is possible as an adjuvant treatment on breast cancer as well as with metastases [103].

Estrogen receptor, progesterone receptor and Her2 are clinically significant biomarkers. Usually, the triple negative breast cancer is more aggressive, less responsive to standard hormone treatment and associated with a poor prognosis [104]. Here, Estrogen receptor plays a central role in the hormone actions. 17β –estradiol (E2) plays a prominent role in mediating the maturation, proliferation, differentiation, apoptosis, inflammation, metabolism and homeostasis, and also influences the growth and development of breast cancer [105]. Because of its numerous involvement and particular importance, the deficiency of E2 in healthy women may be associated with an increased risk of various diseases. On the contrary, the existence of E2 in women with hormone-positive breast cancer may worsen the disease [103].

The biological effects of estrogens are mostly mediated through the Estrogen Receptor (ER), which acts as hormone-activated transcription factor belonging to the nuclear receptor superfamily. Mammalian ERs are encoded by two genes: ERα and ERβ. Both receptors stimulate transcription of an ER-responsive gene containing an ER Element (ERE) in an E2-dependent manner. Human ER has a multi-domain structure consisting of six functional regions, from the N- terminal A/B domain to the C-terminal F domain: the poorly conserved combined A/B region contains the autonomous transactivation function AF-1; the highly conserved C region harboring the DNA-binding domain (DBD) and the E region containing the ligand-binding domain (LBD) as well as the transactivation function AF-2 (Figure 3) [103].
Figure 3. Structural domains of the human ERα and ERβ isoforms

In the non-active state, the ER exists as a hetero-complex consisting of the heat-shock protein 90 (HSP90) and immunophilin-FK-binding protein52 (FKBP52) [106]. This inactive ER complex continually shuttles between the nucleus and cytoplasm. Once E2 binds to the LBD domain of the ER in the nucleus, the ER becomes activated through a process that involves dissociation from the protein chaperons HSP90 and FKBP52, conformational change, dimerization and binding to the ERE of target gene, and recruits co-regulators that stimulate the ER-responsive gene transcription (Figure 4) [103, 106, 107]. Co-regulators can be broadly divided into co-activators and co-repressors, and many co-activators of ER activity are histone acetyltransferases (HATs) such as CBP/p300 [108]. Transcriptional activation involves alterations in chromatin structure mediated by ATP-dependent chromatin remodeling enzymes in conjunction with factors that contain HAT activity [103]. ERα and ERβ have similar DNA-binding affinity and specificity when respond to E2 individually. However, ERα tends to act predominantly as an activator whereas ERβ functions more as an attenuator of Erα. Both isoforms are typically co-expressed in tissues [103].
2.3 Fibroblast Growth Factor Receptor (FGFR)

Fibroblast growth factors (FGFs) represent one of the largest families of growth and differentiation factors known to regulate many biological functions in a spectrum of organisms. The 22 human members of FGFs mediate a variety of cellular responses including cell proliferation, differentiation and survival [109-112]. The activities of FGFs are mediated through their binding to four receptor tyrosine kinases (RTK), designated FGFR1-4 [112]. Structural features shared by the FGFR family include three glycosylated immunoglobulin-like extracellular domain, a single trans-membrane domain, and a conserved intracellular flanking tyrosine kinase domain [113]. A common “acidic box” of eight acidic residues situated between the first and the second immunoglobulin-like loop, which is important for ligand-receptor interaction [114]. The C-terminal tails contain a number of phosphorylatable tyrosine residues which are located at identical positions in the receptors. Ligand-dependent receptor dimerization leads to phosphorylation of these sites, which creates docking sites for downstream signal transduction molecules, resulting in biological responses [115].

2.3.1. FGF/FGFR signalling
The interaction of FGFs and FGFRs is highly dependent on the availability of Heparan-sulfate proteoglycan (HSPG), a key scaffolding protein of their signaling action [116]. The ternary complex leads to the docking of adaptor proteins and activation of multiple signal transduction pathways including RAS-RAF-MAPK, PI3K-AKT, signal transducer and activator of transcription (STAT) and phospholipase Cγ (PLC-γ) [116].

Key adaptor protein FGFR substrate 2 (FRS2) includes two members, FRS2α and FRS2β [117] (Figure 5). FRS2β was reported to negatively regulate EGFR signaling, and might have a tumor suppressive role [117]. FRS2α acts as a control centre for FGFR signaling, which binds to the juxtamembrane region of FGFRs through the PTB domain and becomes phosphorylated on multiple tyrosine residues [117]. This allows the recruitment of the downstream adaptor protein Growth factor receptor-bound protein 2 (Grb2) to activate the guanine nucleotide exchange factor (SOS) and the downstream RAS and MAPK pathways [1]. Following the tyrosine phosphorylation of FRS2α, an assembly of Grb2-associated binding protein 1 (Gab1) in the complex of Grb2/FRS2α initiates the activation of PI3K-AKT anti-apoptotic signaling cascade [116].

Independent of FRS2 binding, the SH2 domain of phospholipase Cγ (PLCγ) binds to the carboxyl terminus of FGFRs. After activation, PLCγ hydrolyses Phosphatidylinositol 4, 5-bisphosphate (PIP2) to phosphatidylinositol 3, 4, 5triphosphate (PIP3) and diacylglycerol (DAG), activating protein kinase C (PKC), which partly reinforces the activation of the MAPK pathway by phosphorylating RAF [1].
Both proteins have a myristylation sequence at the N-terminus (N), followed by a PTB domain and multiple tyrosine phosphorylation sites (Y) at the C-terminus (C). FRS2β contains ERK binding sites. The PTBs of FRS2α and FRS2β have 72% identity and 93% similarity in amino acids. Six tyrosine phosphorylation sites were found at C-terminus of FRS2α and five at FRS2β. Grb2 binding sites activate Ras/ERK, ubiquination/degradation, and PI-3 kinase pathways, while the Ras/ERK pathway is stronger activated by SH2 domain-containing protein tyrosine phosphatase-2 (Shp2) binding sites than the Grb2 binding site.

FGF signaling can be negatively controlled at several levels via the activation of FRS2α including Cbl-mediated ubiquitination, which is regulated by the complex FRS2α–Grb2–Cbl resulting in degradation of FRS2α and its upstream receptors [118]; negative regulator Sprouty (SPTY), which competes for Grb2 binding, thus preventing SOS-mediated RAS activation, or blocks MAPK signaling by directly binding to RAF [1]. ERK-mediated negative feedback is a process activated by MAPK phosphorylation of multiple threonine residues on FRS2α in response to a variety of ligands (e.g. FGF, insulin, EGF and PDGF). This serves to interrupt tyrosine phosphorylation of FRS2 [118] thereby disrupting FGF signaling.

Although four FGFRs signal through a similar network of downstream pathways in general, the consequences of activation of different FGFRs are often distinct and the effects during tumorigenesis are context-specific. There is some evidence that the FGFR1 and FGFR2 kinase domains show differential responses to the extracellular signals [119], and the kinase domain of FGFR1 drives stronger downstream activation than FGFR4 [120].

**2.3.2. Alternatively Spliced FGFR2 Isoforms**
FGFR2 gene is located at human chromosome 10q26 which is one of cancer-related recombination hot spots. Alternative splicing of the third Ig-like extracellular domain of FGFR2 generates two major transcripts referred to as FGFR2-IIIb and FGFR2-IIIc due to inclusion of exon 7 or 8 respectively (Figure 6) [121, 122]. These two isoforms display distinct domain structure and ligand-binding specificity. FGFR2-IIIb tends to be expressed in epithelium, and is a high affinity receptor for FGF1, FGF3, FGF7, FGF10 and FGF22, while FGFR2-IIIc is mainly expressed in mesenchymal elements where it shows a high affinity to FGF1, FGF2, FGF4, FGF6, FGF9, FGF16 and FGF20 [123, 124].

![Figure 6. Alternatively spliced FGFR2 isoforms](image)

**2.3.3 Deregulation of FGFR2 signalling in Cancer**

The underlying mechanisms driving FGF signalling is largely tumor specific, and can be broken down into genomic alterations that drive ligand-independent signalling as well as ligand-dependent alterations. The genetic alterations in FGFR2 related to cancers are: 1) activating mutations: missense mutations of FGFR2 have been described in lung squamous cell carcinoma, diffuse-type gastric and endometrial cancers [125, 126]. 2) Gene amplifications: Approximately 10% of gastric cancers show FGFR2 gene amplification which is associated with poor prognosis [127, 128]. The amplification of FGFR2 is accompanied by a C-terminal deletion and truncation...
[129], which interferes with receptor internalization thus preventing wild-type signalling and contributing to constitutive ligand-independent activation [1, 130]. 3) Germline single nucleotide polymorphism (SNP): Recent genome-wide association studies identified FGFR2 as one of the breast cancer susceptibility genes. The minor disease-predisposing allele of FGFR2 is inherited as a haplotype of eight single nucleotide polymorphosis (SNPs) covering a region of 7.5 kb within intron 2 of the gene. FGFR2 is expressed at higher levels by tumors that are homozygous or the minor alleles than by those with the common alleles. These functional SNPs increase signaling activity including FRS2α and ERK1/2 phosphorylation, activate paracrine signaling, and alter transcriptional factor (TF)-binding sites by creating new binding sites for some TFs including ER and Oct1/RunX2, [2, 3]

Other than the genomic aberrations, ligand-dependent alterations and the resultant constitutive activation have a similarly important role in the pathogenesis of cancer, through either autocrine production of ligand in cancer cells or paracrine production of ligand from tumor stromal cells [1]. Additionally, the released FGFs from stromal cells or cancer cells can act on endothelial cells to promote angiogenesis [1].

2.3.4 Tumour Suppressive Effects in Cancer

As well as the wealth of evidence that links fibroblast growth factor (FGF) signals with transforming function which can promote tumor development by directly driving cancer cell proliferation and survival, and by supporting tumor angiogenesis, several studies of mouse models, human tumors or cancer cell lines potentially support tumor suppressive functions of FGFR2 in some contexts [1]. For example, mice that specifically lack FGFR2-IIIb in keratinocytes were sensitive to carcinogenesis [131]; FGFR2-IIIb was down-regulated in bladder cancer, prostate cancer, and salivary adenocarcinoma [132-134]; FGFR2-IIIb was down-regulated in neoplastic thyroid cell line and restoration of FGFR2-IIIb impeded signaling upstream of the RAS/BRAF/MAPK pathway through competing with FGFR1 for FRS2 activation [135]. We have reported that FGFR2 mRNA and protein levels are down-regulated in micro-dissected primary breast cancer cells when compared with paired normal epithelium [4].
Some mechanisms have been proposed for the tumor suppressive function of FGFR2-IIIb. One possibility is to induce cytoprotective pathways in epithelial cells, helping to maintain genomic stability [136]. Another is that FGFR2-IIIb may play a potential role in promoting immune surveillance [137]. Current studies are underway to distinguish the mechanisms underlying the role of FGFR2 between genuine tumor suppressive effects and oncogene induced senescence [1].

### 2.3.5. Distinct Effects of FGFRs

Despite a highly structural homology in the kinase domains, FGFRs display distinct effects during carcinogenesis [116], especially between FGFR1 and FGFR2. Although FGFR1 and 2 induce similar ERK 1/2 activation, the activation by FGFR1 is sustained, and the sustained signal is associated with translocation of ERK1/2 to the nucleus. In contrast, FGFR2 induces transit increases of pERK1/2 that are insufficient to lead to nuclear translocation [116, 138].

FGFR1 and FGFR2 differentially regulate cell apoptosis in 3D cultures of primary mammary epithelial cells [119]. Cancer cells can be protected from apoptosis through the survival signaling mediated by the integrin receptors, such as β1-integrin [42]. FGFR1 activation induces cell survival, and results in a dramatic increase of β1-integrin expression. However, FGFR2 observed a significant down-regulation of β1-integrin which correlated with increased apoptosis and lack of cell invasion [139].
3. Materials and Methods

3.1 Experimental Models

The *in vitro* work in this study was carried out using three breast epithelial cell lines:

(1) Human MDA-MB-231 cells

(2) Human T47D cells

(3) Human MCF-10A cells

And mouse fibroblast NIH 3T3 cells.

*In vivo* experiments on the effect of FGFR2 isoforms in breast cancer cell growth and metastasis were performed using severe combined immune deficiency (SCID) mice;

3.1.1 Cell line background

MDA-MB-231 is typical of triple negative (ER-negative, PR-negative and Her2-negative) breast cancer cell line that is derived from invasive human breast adenocarcinoma. With an epithelial morphology and spindle-shaped phenotype, MDA-MB-231 show tumourigenic properties including high anchorage independent growth efficiency in soft agar, abundant activity in invasion in vitro and the ability to form mammary fat pad tumors and metastases in nude mice [140].

T47D is a human mammary epithelial tumor cell line derived from pleural effusion of invasive ductal carcinoma. These cells express receptors for a variety of steroids and calcitonin, and express PR and ER under normal culture condition, but lose ER under long-term estrogen deprivation [141]. T47 D cells are less malignant than ER-negative cancer cells.

MCF-10A is a near normal mammary epithelial cell line derived from the mammary tissue of a cystic fibrosis patient [142]. These cells exhibit numerous features of normal breast epithelium, including lack of tumorigenicity in nude mice, lack of anchorage-independent growth, and dependence on growth factors and hormones for proliferation and survival [142]. In confluent,
three-dimensional culture of MCF-10A reconstituted matrix results in formation of polarized, growth-arrested acini-like structures that resemble mammary glandular architectures in vivo [143]. The proliferation-arrested MCF-10A morphogenesis is of extreme experimental value, representing an important parameter to evaluate oncogenic effects. Oncogenes introduced into MCF-10A cells disrupt normal 3D structure, and elicit aberrant morphological phenotypes [143]. Additionally, NIH 3T3 is a mouse fibroblast cell line established from disaggregated Swiss mouse embryos [144].

### 3.1.2 Culture conditions

MDA-MB-231 (kindly provided by Dr. F. Liu’s lab) and T47D cells (kindly provided by Dr. S. Muthuswamy’s lab) were cultured in RPMI 1640 (100 IU/ml penicillin, 100 IU/ml streptomycin) supplemented with 10% fetal bovine serum (FBS). MCF10A cells (kindly provided by Dr. S. Muthuswamy’s lab) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplement with 5% horse serum, 20 ug/ml EGF, 0.5 ug/ml Hydrocortisone, 0.1 ug/ml cholera toxin, 10 ug/ml insulin, and 100 IU/ml penicillin/streptomycin. NIH 3T3 cells (ATCC) were cultured in DMEM supplement with 10% FBS. These cells were incubated at 37°C with 5% CO₂. For long-term storage, cells were frozen in their growth media supplemented with 10% DMSO.

### 3.2 Retroviral transduction of FGFR2 isoforms

#### 3.2.1 Retroviral expression vector construction

The MSCV (Murine Stem Cell Virus) –PIG (Puro-IRES-GFP) vector (Clontech, Cat# 634401) can express the gene of interest as well as a transcript containing the viral packaging signal Ψ+, which are driven by the promoter CMV of 5’ long terminal repeat. The coding regions of FGFR2-IIIb and its alternatively spliced FGFR2-IIIc variant were amplified by using the full-length cDNA as templates (pcDNA1 vector containing FGFR2-IIIb and pcDNA3.1-V5-Topo vector containing FGFR2-IIIc) and the following PCR primers (F: 5’- AAG ACT CGA GAT GGT CAG CTG GGG TTT CA-3’   R: 5’- AGC CTC GAG TCA TGT TTT AAC ACT GCC G-3’). After optimization, the PCR reaction was performed in 50 ul volume containing 200 ng PCR template, 1x PCR buffer, 2mM Mg²⁺, 0.2mM dNTP, 0.4 uM of each
primer and 5U of AmpliTaq polymerase (Applied Biosystems, Cat# K2020-20). The reaction condition included initial denaturation at 95°C for 2mins, followed by 34 cycles at 94°C for 30 sec, annealing at 50.5°C for 30sec and extension at 68°C for 1min and 72°C for 3mins, then followed by a 10-min final extension at 72°C. The purified PCR products were cloned into the PCR2.1 vector using the TA cloning Kit (Invitrogen, Cat#450046) and fragmented by digestion with Xhol I (Roche). The MSCV-PIG empty vector was also linearized by the same enzyme, and both DNA fragments were run on 1% agarose gel and purified using the Gel purification Kit (QIAGEN Cat#28706). The ligation of the blunt ends was performed using 50 ng of linearlized vector DNA, 150 ng of insert DNA, DNA ligation buffer and 5U of T4 DNA ligase (Roche, Cat# 11635379001). Then the plasmids are incorporated into bacterial (DH5α E. coli) host cells by transformation and produce detectable quantities. Positive clones were identified by restriction enzyme digestion and DNA sequencing.

3.2.2 Retrovirus production in 293GPG packaging cell line

A human 293-derived retroviral packaging cell line (kindly provided by Dr. S. Muthuswamy’s lab) was used to produce integrated transcripts containing our target genes, in response to the viral packaging signal, they can be packaged into a replication-incompetent envelope glycoprotein VSV-G encoded by the Vesicular Stomatitis Virus. VSV-G enables viral entry through lipid binding and plasma membrane fusion because of its putative fusagenic domain [145]. Because the constitutive high-level expression of VSV-G is toxic to most of cells, a tetracycline-mediated inducible system (Tet-Off system) was used to regulate VSV-G expression in the 293 GPG packaging cells. The tetR/VP16 transactivator (tTA) binds to the Tet operator sequences (TRE) and activates transcription of VSV-G in the absence of tetracycline [146].

The 293 GPG packaging cells were plated in the growth medium (DMEM medium supplement with 10% heat-inactivated FBS, 50 IU/ml penicillin/streptomycin, 2mM /L L-Glutamine, 1ug/ml Tetracyclin, 2ug/ml puromycin and 300ug/ml G418) and reached a 50-80% confluency at the time of transfection. These cells were transiently transfected with the retroviral expression vector (MSCV-PIG-FGFR2IIb or MSCV-PIG-FGFR2IIc) or a MSCV-PIG empty vector. Transfections were performed in Opti-MEM media using Genejuice Transfection Reagent (Novagen Cat#70967) according to the manufacturer’s protocol. After 24-hour transfection, the Opti-MEM media was changed for the collection medium (DMEM medium supplement with
10% heat-inactivated FBS, 50 IU/ml penicillin/streptomycin) to turn on VSV-G expression. Viral supernatant from the packaging cells, after 4-day incubation, started to be harvested in 24 hr intervals until cells were no longer viable. Cell debris was removed by centrifugation at 10000g for 5mins and the cleared supernatant was aliquoted and stored at -70°C.

### 3.2.3. Target cells infection

After the viral titer optimization, the breast epithelial cells were plated one day before infection at a density of 2x 10⁵ cells per 6cm plate, and incubated with viral supernatants (3ml/plate) containing 8 ug/ml polybrene (Sigma) for 24hrs. Then fresh growth media were changed. For some cells that were difficult to infect, multiple rounds of infection were used to increase the number of infected cells as well as increasing the copy number per cell. To determine the efficiency of the transduction, the infected cells were exposed to 3ug/ml puromycin for selection 48-hour after infection. The survival cells were subjected to screening by western blotting.

### 3.3 Analysis of Gene Expression

#### 3.3.1 Protein extraction

Cells were washed twice with cold PBS and lysed in buffer (20mM Tris-Hcl pH=8.8, 137mM Nacl, 10% Glycerol, 1% Triton X 100 and 2mM EDTA) containing aprotinin (Sigma, Cat#A6279) and proteinase inhibitors (Sigma, Cat#P1860). Total cell lysates were incubated on ice for 30 mins, followed by micro-centrifugation at 10,000 g for 10 min at 4°C. The protein concentrations of the supernatants were determined by the Bradford method using the Bio-Rad Protein Assay Kit (Bio-Rad, Cat#500-0002).

#### 3.3.2 Western Blotting

Equal amounts of protein (50 µg) were mixed with 2x Sample Buffer ( 0.5M Tris-Hcl, pH6.8, 25% Glycerol, 2%SDS, 0.001% bromothymol blue, 5% β-mercaptoethanol), boiled for 5mins and separated by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes (0.45um, Bio-Rad laboratories Cat#1620094).
Nonspecific binding was blocked with 5% nonfat milk (w/v) in 1x TBST (Tris-buffered saline with 0.1% Tween-20). The membranes were incubated with primary antibodies shaking at 4°C overnight. All primary antibodies are listed in Table 2. After washing 4 times each for 10mins in 1X TBST, blots were exposed to the secondary antibody (anti-mouse or anti-rabbit IgG-horseradish peroxidise linked (HRP) at a dilution of 1:2000 for 1 hour at RT and washed again in 1x TBST for 4 times, protein expression was visualized using chemiluminescent HRP detection reagent (Denville Scientific, Cat# E2500) and autoradiography. Band intensities were quantified by normalization to β-actin controls.

Table 2. List of Primary antibodies used:

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3.3.3. RNA extraction and Reverse Transcription (RT)-PCR

3.3.3.1 RNA extraction

RNA was isolated from cells using an RNeasy Mini Kit (Qiagen, Cat # 74104), according to the manufacturer’s protocol. Cells were lysed, collected in RNase-free eppendorf tubes and homogenized by passage through a blunt 20-gauge needle 5 times. The nucleic acids were precipitated by the addition of 70% ethanol. The samples were then transferred to RNeasy spin columns and centrifuged at 8,000 x g to discard flow-through during each subsequent step. On-column DNase treatments were performed using the RNase-Free DNase Set (Qiagen, Cat#79254) for DNase removal. Samples were washed with the provided buffers, and eluted by the addition of 30 ul of RNase-free water. RNA concentration and purity was determined by UV spectrophotometry showing an $A_{260} : A_{230}$ ratio>1.7, $A_{260} : A_{280}$ ratio between 1.8 and 2.0. The integrity of RNA was verified by electrophoresis on agarose gel yielding a sharp distinction of the 18S and 28S bands. RNA was stored at -70ºC.

3.3.3.2 Reverse Transcription (RT) and PCR

A high-capacity cDNA Reverse Transcription Kit (SABiosciences, Cat# C-03) was used to prepare cDNA according to the manufacturer’s protocol. 0.5ug total RNA per sample was mixed with Genomic DNA Elimination Buffer to achieve a final volume of 10ul. The mixture was incubated at 42 ºC for 5 mins, combined with RT cocktail in a 1:1 ratio, which includes 5x RT buffer, Primer & External Control Mix and RT Enzyme Mix. The thermal conditions were as follows: 42 ºC for 5mins and 95 ºC for 5mins. The 20-ul of cDNA synthesis product was achieved and the isoform examination was performed. Primers were designed to span exons 6 to 9 of FGFR2, the region that contains exon 7 (encoding the FGFR2-IIIb isoform) and exon 8 (encoding the FGFR2-IIIc isoform) as described previously [147].

3.4 In vitro studies of FGFR2 function

3.4.1. Proliferation Assay (Cell counting)
One x10^5 MDA-MB-231 or T47D breast cancer cells were seeded in 35-mm standard culture plates (Falcon™ Cat#353046). The cells were cultured in the growth media, and the growth rates were determined by direct counting 2.5 and 4 days after seeding. The viable adherent cells were trypsinized and collected, followed by centrifugation and addition of 1 ml PBS to the cell pellet. The cells were directly counted using the Vi-CELL XR 2.03 program of the BECKMAN COULTER system. Three independent experiments were repeated and each was performed in triplicate. The mean and standard deviation (SD) of each group were compared to analyze the growth rates of the different cells.

### 3.4.2. Soft Agar Assay

3% agar stock was prepared by boiling for 15mins, and cooled to 50°C in a water bath. The agar was plated in two layers. The bottom agar (0.4%) was composed of 73.3% growth medium, 10% FBS and 13.3% agar stock, and was added 1ml per 35mm plate. While the bottom agar was solidifying, cells were suspended in top agar (0.3%) containing 80% growth medium, 10% FBS and 10% agar stock. 1ml of the cell suspensions were plated on the solidified bottom layers to achieve a final concentration of 4500 cells/plate. After solidification, 0.5ml of the cell culture media was added to the surface. The cells were grown at 37°C in humidified incubator for 20 days, and fed with fresh cell culture media every week. Colonies were stained with 0.4% Trypan Blue (GIBCO) and the total colonies of each plate were counted using a Leica MZFLIII Stereo Microscope and photographed. The average counts achieved from three experiments (each performed in triplicate) were used for the analysis.

### 3.4.3. Migration and Invasion Assay

Cell motility was examined by a transwell assay using 24-well plates with uncoated inserts (BD Bioscience, Cat#354578) for migration or Matrigel-coated inserts (BD Bioscience, Cat#354480) for invasion. The upper and lower culture compartments were separated by polyethylene terephthalate (PET) membranes with a pore size of 8 um. After trypsinization, 8 x 10^4 cells suspended in 0.5ml serum-free culture media containing 0.2% BSA were plated in each insert.
10% FBS was used as a chemoattractant in the bottom well. After 20-24 hrs of incubation, cells on the upper surface were removed by scrubbing with a cotton swab. Cells on the lower surface of the membrane were stained with Diff-Quik stain (DADE Behring, Cat# B4132-1A). Then the membranes were removed from the inserts, photographed and quantified using ImagePro software. The average cell number of three experiments (each performed in triplicate) in each group was used to compare to the abilities of cell migration and invasiveness.

3.4.4. Three-Dimensional culture of mammary epithelial cell

An overlay method [148] was used for three dimensional culture of MCF10A in which single cells were seeded on a solidified layer of growth factor-reduced Matrigel (BD Biosciences, Cat# 354230) measuring approximately 1–2mm in thickness.

The Matrigel was thawed on ice and plated on each well of the 8-well chamber slide and incubated for 20mins to solidify in 37°C culture incubator. The stock assay medium (DMEM supplemented with 5% horse serum, 20ug/ml EGF, 0.5ug/ml Hydrocortizone, 0.1ug/ml cholera toxin, 10ug/ml insulin and 100IU/ml penicillin/streptomycin) was prepared containing 5% Matrigel and 10ng/ml EGF. 200 ul of this stock was used for each well of the chamber slide. MCF-10A cells were suspended in the assay medium to achieve a final concentration of 25,000 cells / ml. Then the cell suspension and the Matrigel solution were mixed in a 1:1 ratio and 400 µl of the mixture were added to each well of the solidified pre-coated chamber slide. This corresponded to a final overlay solution of 5000 cells/well in medium containing 2.5% Matrigel and 5ng/ml EGF. The chamber slides were incubated in a cell culture incubator for 16 days, and the media (assay media containing 2.5ng/ml Matrigel and 5ng/mL EGF) were changed every 4 days. The cell images were photographed and 3D acini size was quantitated by the Imagepro program, and the average size was calculated based on 500 of cells of each group.

3.5. In vivo studies of FGFR2 function

3.5.1. Xenografted mouse model of breast cancer
MDA-MB-231 cells stably expressing FGFR2-IIIb, FGFR2-IIIc or pMSCV empty vector (Control) were washed twice with PBS, trypsinized and harvested by centrifugation at 10000rpm for 5mins. 10⁷ of the cells suspended in 150ul PBS were orthotopically implanted into the second lower mammary gland fat pad of 6-week-old female severe combined immune deficiency (SCID) mouse for the assessment of tumor growth and metastasis. Tumor volume was determined every 6 days by measuring in two dimensions (length and width) and calculating volume (mm³) as tumor width x tumor length x tumor length/2. Mice were sacrificed after monitored for 84 days and tumors were weighed. Excised tissues including lungs, liver and bone were fixed in 10% formalin and embedded in paraffin for immunohistochemical staining.

3.5.2. Heterologous Xenografts Establishment

After trypsinization and PBS wash, 7.5x 10⁶ MDA-MB-231 cells expressing FGFR2IIIb, FGFR2-IIIc or pMSCV empty vector (Control) were mixed with 2.5x 10⁶ mouse fibroblast 3T3 expressing FGFR2c or pMSCV empty vector (Control) in order to investigate the action of FGFR2-IIIc on tumor-stroma interactions. The combined cells were suspended in 150ul PBS and implanted into the second upper mammary gland fat pad of 6-week-old female SCID mice. Tumor volume was measured every 3 days and calculated in (mm³) = tumor width x tumor length x tumor length/2. Mice were sacrificed after monitored for 12 days and tumors were weighed. Excised tissues including lungs and liver were fixed in 10% formalin and embedded in paraffin for immunohistochemical staining.

3.5.3. Tissue Immunohistochemistry

This work was done by PRP research lab. Four micrometer formalin-fixed paraffin-embedded sections from animal tissues were de-waxed in five changes of xylene and rehydrated through graded alcohols. Tissue sections were then heat-treated in 10 mmol/L citrate buffer at pH 6.0 or Tris-EDTA buffer at PH 9.0 for antigen retrieval. Endogenous peroxidase and biotin activities were blocked, respectively, using 3% hydrogen peroxide and avidin/biotin blocking kit (Lab Vision Cat#TA-015-BB). After blocking for 10mins with 10% normal serum from the species
where the secondary antibody is obtained, sections were then incubated with the appropriate primary antibody as listed in Table 1 followed by incubation with biotinylated secondary antibody for 30mins and horseradish peroxidase-conjugated ultrastreptavidin labeling reagent for 30mins. After TBS wash, color development was done with freshly prepared NovaRed solution (Vector Lab Cat#SK-4800). Finally, sections were counterstained lightly with Mayer’s Hematoxylin, dehydrated in alcohols, cleared in xylene and mounted in Permount (Fisher, Cat# SP15-500).

3.6 Studies of the mechanism underlying FGFR2 action

3.6.1. FGF ligand-stimulation condition

As previous optimization [134], following a 24-hour serum deprivation, cells were treated with 25 ng/ml various FGF ligand for 10mins at 37°C in the presence of 10 units/ml Heparin (Sigma, Cat# H3393). Human FGF-1 (Sigma Cat#F5542), FGF-7 (Sigma, Cat#K1757) and FGF-4 (Sigma Cat# F8424) have been shown to interact with FGFRs, FGFR2IIIb and FGFR2IIIc, respectively. Identical volume of vehicle served as control. The cells were lysed using the lysis buffer containing proteinase inhibitors and phosphatase inhibitors (Sigma Cat# P5726), and the total cell lysates were subjected to western blotting.

3.6.2. Treatment condition of Tumor Necrosis Factor Alpha (TNFα)

Upon reaching 80% confluency, MDA-MB-231 cells expressing FGFR2IIIb, -IIIc or pMSCV empty vector (Control) were starved for 24 hours, and treated with 10ng/ml TNFα (R&D System, Cat# 210TA) for 30 min, or exposed to 25ng/ml FGF-1, FGF-4 or FGF-7 in the presence of 10 units/ml heparin for 10 min prior to the addition of 10ng/ml TNFα for 30 min. Identical volume of vehicle was used as control.

3.6.3. Monoclonal Antibodies for blocking FGFR2 signaling

GAL-FR21, -22 and -23 (Galaxy Biotech, LLC) are monoclonal antibodies that are specific against FGFR2 isoforms. GAL-FR21 only binds to FGFR2-IIIb, whereas GAL-FR22 and -23
bind to both isoforms [149]. After 24hrs starvation in serum-free medium, cells were treated with the mAbs (10ug/ml) for 20hr at 37°C and then exposed to FGF ligands with heparin as described above. Identical volume of vehicle served as control.

### 3.6.4. Immunofluorescent Staining

Cells cultured on sterile cover slip were washed by ice cold PBS twice, fixed with 4% paraformaldehyde for 20-mins at RT, and then permeabilized with 0.3% Triton X-100 in PBS for 15mins followed by blocking in 3% bovine serum albumin (BSA) for 30-mins at RT. Then the cells were incubated with the primary antibody(s) at a dilution of 1:100 in 3% BSA for 2hrs at RT. The cover slips were washed by PBS for 3x 5-mins, and then exposed to the secondary antibody in a dilution of 1:200 for 1 hour [goat anti-rabbit cy3-conjugated IgG (Millipore Cat# AP132C), goat anti-mouse cy3-conjugated IgG (Millipore Cat# AP124C) or goat anti-rabbit Alexa Fluor 647-conjugated IgG (Invitrogen, Cat#A-21245)]. After 3 times wash in PBS, the cover slips were incubated with DAPI (1:1000) for 15-mins and rinsed in distilled water to remove excess salts, mounted with fluorescent mounting media (Southern Biotech Cat#17984-25) onto microscope slides, sealed and stored at 4°C in the dark. The images were visualized using an inverted Olympus FluoView FV1000 Confocal Laser Scanning Microscope (Olympus).

### 3.6.5. Co-Immunoprecipitation

Co-Immunoprecipitation (Co-IP) experiments were performed using Protein A (GE Healthcare, 17-0780-01) or Protein G (GE Healthcare, 17-0618-01) sepharose beads for immunoprecipitation with polyclonal and monoclonal antibodies, respectively. The beads were washed 3 times in wash buffer (20mM TrisHcl pH=8.8, 137mM Nacl, 10% Glycerol, 1% Triton X100 and 2mM EDTA), diluted to 50% working concentration. The pre-cleared protein lysates were incubated with 1ug of antibody for 2-3 hours, shaking at 4°C. The antibodies used are detailed in Table 2. Then, the beads were added to the antibody-bound complex, shaking overnight at 4°C. Unbound protein was removed by a final wash step.
3.6.6. Luciferase Assay

A Gaussia Luciferase assay was performed to determine the binding activity of NF-κB transcription factor. Upon reaching 70-80% confluency, MDA-MB-231 cells expressing FGFR2IIIb, FGFR2IIIc or pMSCV empty vector (Control) were transiently transfected with a NF-κB Luciferase reporter that was cloned in the PRR-High reporter vector (kindly provided by Dr. A Schimmer, Ontario Cancer Institute). After 24 hour transfection, the cells were treated with vehicle, TNFα or TNFα in combination with FGF ligands as described above, then lysed in Gaussia lysis buffer (Active Motif, Cat#33001), shaking at RT for 30mins. The flash luminescence was measured using an injecting luminometer that was set to inject 25ul per well (96 well) of 1x Assay Buffer with the substrate. Each sample was duplicated and two independent experiments were performed.

3.6.7. Cytoplasmic-Nuclear Fractionation

Whole cell lysates were extracted using the protocol described above. Cytoplasmic-nuclear fractionation was achieved using the ProteoJET Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas Life Sciences, Cat# K0311). Protease inhibitors and DTT were added to the Cell Lysis buffer, Nuclear Lysis Reagent and all the washing or storage buffers. The protein concentration was determined by the Bradford method. Western blotting was performed using Histone H3 and β-Tubulin as nuclear and cytoplasmic loading control, respectively. Antibody concentrations were detailed in Table 2.

3.6.8. Enzyme Linked Immunosorbent Assays (ELISA)

MDA-MB-231 cells expressing FGFR2-IIIb, -IIIc or control (empty vector) were treated with vehicle, TNFα, TNFα combined with FGFs or FGFR2 blocking antibodies as described above. Total cell lysates and cell culture media were collected for accurate measurement of human Interleukin-6 protein levels by ELISA. This experiment was performed using the Human Interleukin-6 ELISA reagent set (eBioscience, Cat#88-7066). The standard curve range is 2-200 pg/ml. The absorbance at 450nm was measured by spectrophotometry.
3.7 Statistical Analysis

The results were based on at least two independent experiments and data are presented as mean ± SD. Difference was assessed by Microsoft Excel Student’s t-test. Significance level was assigned at $p<0.05$. 
4. Results

4.1 Validation of FGFR2 Isoforms Expression in Breast Epithelial Cells

Based on previous research for endogenous expression of FGFR2 in a variety of breast cancer cell lines [150], FGFR2-deficient MDA-MB-231, MCF-10A and T47D cells were selected to examine the properties of FGFR2 isoforms and their contributions to breast cancers initiation and/or progression using gain-of-function approaches.

Stable cell clones of these cells expressing FGFR2-IIIb, FGFR2-IIIc or pMSCV empty vector (control) were established, and the screening for FGFR2 by Western blotting and cell immunofluorescent staining was shown in Figure 7 A and B. Western blotting identified strong FGFR2 protein levels in transduced cells compared to their control cells. To confirm the activities of the transduced FGFR2 isoforms, the ligand-dependent receptor phosphorylation at tyrosines 653 and 654 was detected, in response to selective ligands. We used FGF7 and FGF4 which are specific to FGFR2-IIIb and FGFR2-IIIc, respectively. These phosphorylation sites are important for catalytic activity of activated FGFRs and their signaling [151]. The activation of FGFR2 isoforms can be successfully induced by their specific ligands. Consistent with variable N-terminal glycosylation [134], both FGFR2-IIIb and FGFR2-IIIc migrate as doublets, a fully glycosylated mature form (120-KD) and a partially glycosylated immature form (110-KD). Similarly, cell immunofluorescence confirmed the stable FGFR2 over-expression, and these two isoforms express throughout the cells including cell membrane and nucleus. FGFR2 expression was confirmed before each experiment.

4.2 The Role of FGFR2 Isoforms in Breast Cancer Cells in vitro

4.2.1 FGFR2 Isoforms Impair MDA-MB-231 and T47D Cancer Cell Growth
### A

#### MDA-MB-231

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### B

#### MDA-MB-231

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#### T47D

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Figure 7. Retroviral-mediated stable transduction of FGFR2 isoforms in breast epithelial cells.

Forced expression of FGFR2-IIIb, FGFR2-IIIc and pMSCV empty vector (control) was established in FGFR2-deficient MD-MB-231, T47D and MCF-10A cells. Western blotting (A) and cell immunofluorescent staining (Cy3-conjugated) (B) confirmed the effectiveness of the transduction. The activation of FGFR2 isoforms (p-FGFR) can be induced by FGF-7 and FGF-4, which are specific ligands to FGFR2-IIIb and FGFR2-IIIc, respectively. Actin was detected as a loading control.

To assess the effect of FGFR2 up-regulation on breast cancer cell growth, we performed a cell-counting assay on MDA-MB-231 and T47D cells (Figure 8A). The cell growth rates were determined by directly counting the number of cells on day 2.5 and 4 after seeding 1x10^5 cells. For MDA-MB-231, after 4-day growth, there was an average of 25% reduction in FGFR2-IIIb-expressing cells and an average of 35% reduction in those expressing FGFR2-IIIc, when compared to their FGFR2-deficient controls. This result was highly significant (P_{Control: FGFR2IIIb}=0.000049, P_{Control: FGFR2IIIc}=0.000033) according to a Students’ paired t-test analysis. The 4-day duration was used for the comparison because the control cells grow to confluence after 4 days resulting in overcrowding and detachment from the culture dish. The significant reduction in cell proliferation was noted as early as day 2.5 (P_{Control: FGFR2IIIb}=0.018, P_{Control: FGFR2IIIc}=0.0033).

Similarly, after 4-days of growth, T47D cells were on average 30% and 40% fewer when expressing FGFR2-IIIb or FGFR2-IIIc, respectively compared to their control cells. Although, the reduction on day 4 was significant (P_{Control: FGFR2IIIb}=0.02, P_{Control: FGFR2IIIc}=0.011), there was no significant difference in proliferation on day 2.5 due to a relatively lower growth rate of this cell line compared to MDA-MB-231 cells.
4.2.2 FGFR2 Isoforms Inhibit Anchorage-Independent Growth of MDA-MB-231 Cells in Soft Agar

Cellular transformation is associated with some phenotypic changes such as anchorage-independent colony formation as measured by the soft agar assay. To evaluate the effect of FGFR2 isoforms on breast cancer cell transformation, MDA-MB-231 cells were used to perform this assay due to their high anchorage independent growth efficiency in soft agar. As shown in Figure 8B, both FGFR2 isoforms inhibit colony formation by MDA-MB-231 cells. After 20-days of growth, there was an average of 60% and 75% colony number decrease in the cells expressing FGFR2-IIIb and FGFR2-IIIc, respectively, in comparison with the number of the control cells. This result was highly significant ($P_{\text{Control: FGFR2IIIb}} = 0.0015$, $P_{\text{Control: FGFR2IIIc}} = 0.000064$) according to a Students’ paired t-test analysis. T47D cells were also tested for their potential to grow independently in soft agar, but they didn’t show a satisfactory ability to form colonies when forced to express FGFR2-IIIb or FGFR2-IIIc even after 20 days of culture.
Figure 8. FGFR2 isoforms impair MDA-MB-231 and T47D cancer cell proliferation.

A. MDA-MB-231 (upper) and T47D (lower) cell growth was monitored by counting the cells on days 2.5 and 4 after seeding $0.1 \times 10^6$ cells. 10% FBS in the culture media was used as a source of multiple ligands for the activation of FGFR2 isoforms. Bar graphs indicate the average number of the cells based on 3 experiments (each performed in triplicate).

B. FGFR2 isoforms inhibit anchorage-independent growth of MDA-MB-231 cells in agar. The soft agar consisted of two layers, both of which were prepared from 3% stock agar. A 0.5% agar concentration was achieved in the bottom layer. For the top layer, cell suspension (4,500 cells/plate) was mixed with the stock agar to achieve a 0.3% agar concentration. Colonies were visualized after 20 days of culture. Bar graphs represent the average colony counts of two experiments, each performed in triplicate. Error bars depict standard deviation. Significance (the Student’s paired t-test) is indicated.

4.2.3 FGFR2 Isoforms Block MDA-MB-231 Cell Invasiveness in Matrigel

The polyethylene terephthalate (PET) membrane coated with Matrigel mimics the basement membrane in vivo, which can block non-invasive cells from migrating through it, but invasive cells are able to detach and invade through the pores. We used the trans-well matrigel chambers to examine the ability of FGFR2 isoforms to influence of breast cancer cell invasiveness. MDA-MB-231 cells are an ideal cell model for this assay because of their established abundant activity in invasion *in vitro*. After a 24-hour period, we found the average invasive cell number of MDA-MB-231 was significantly decreased in the FGFR2-IIIb and FGFR2-IIIc expressing cells, when compared to the controls (Figure 9A). We calculated the invasion index of FGFR2-IIIb to be 56.44% and that of FGFR2-IIIc to be 47.27%. This was statistically significant ($P_{\text{Control}}$...
FGFR2IIIb = 0.00051, P_{Control: FGFR2IIIb} = 0.00011) as indicated. In contrast, T47D and MCF10A cells failed to pass through the membranes even when forced to express FGFR2-IIIb or FGFR2-IIIc presumably due to their lack of invasive ability.

### 4.2.4 Influence of FGFR2 Isoforms on Breast Cancer Cell Migration

The effect of FGFR2 isoforms on cell migration was examined by a transwell assay using 24-well plates with uncoated inserts. As shown in Figure 9B, the average number of T47D cells, expressing FGFR2-IIIb or FGFR2-IIIc that migrated through the membrane was significantly decreased when compared to their control cells. The migration index of FGFR2-IIIb was 46.5% and that of FGFR2-IIIc was 60.4%. This result was highly significant (P_{Control: FGFR2IIIb} = 0.0057, P_{Control: FGFR2IIIc} = 0.0033) according to a Students’ paired t-test analysis. However, as shown in Figure 9C no significant difference (p>0.05) was detected in MDA-MB-231 expressing FGFR2-IIIb (migration index of 100.02%) or FGFR2-IIIc (migration index of 102.78%). Similar results were obtained in MCF-10A cells which showed no difference in migration when forced to express FGFR2-IIIb (96.85%) or FGFR2-IIIc (100.01%) (Figure 9D).
Figure 9. The Influence of FGFR2 Isoforms on Breast Cancer Cell Motility

A. FGFR2 up-regulation significantly suppresses MDA-MB-231 cell invasion, when compared with the controls. $8 \times 10^4$ of MDA-MB-231 cells expressing FGFR2-IIIb and FGFR2-IIIc and pMSCV empty vector (Control) were allowed to pass through Matrigel-coated filters with 8-um pores. The bar graphs indicate the mean of the invasive cell number in each group based on 3 experiments.

B-D. FGFR2 up-regulation significantly suppresses T47D, but not MDA-MB-231 and MCF-10A, cell migration, when compared with the controls. T47D (B), MDA-MB-231 (C) and MCF-10A (D) cells expressing FGFR2-IIIb and FGFR2-IIIc or pMSCV empty vector (Control) were allowed to pass through the uncoated migration filters in the presence of 10% FBS. The bar graphs indicate the mean of the migrating cell number in each group based on 3 experiments. SD and Significance (paired t-test) is indicated.

4.2.5 Influence of FGFR2 Isoforms on Indicators of Epithelial-Mesenchymal Transition (EMT) in Breast Cancer Cells

Progression of breast cancer is accompanied by the existence of epithelial to mesenchymal transition (EMT) - like changes which are characterized by loss of epithelial features and gain of mesenchymal properties. During EMT, cells often lose their epithelial molecular markers such as E-cadherin [152]. A major regulator of EMT during tumor progression is the Snail family of zinc finger transcription factors, of which Snail (Snail1) and Slug (Snail2) are the most intensively studied [153]. EMT is often induced via up-regulation of Snail1, which is a repressor of E-
cadherin gene expression in epithelial tumor cells [154]. We thus examined the expression of E-
cadherin and Snail1 to determine whether FGFR2 up-regulation is associated with an EMT
event. Endogenously, E-cadherin was highly detectable in normal epithelial MCF-10A cells, but
was down-regulated in malignant MDA-MB-231 and T47D breast cancer cells. In response to
FGF-1, the overexpressed FGFR2 isoforms did not affect E-cadherin expression level in MCF-
10A cells, but was associated with increased E-cadherin in MDA-MB-231 and T47D cells. The
protective restoration of E-cadherin is associated with the down-regulation of Snail1 in MDA-
MB-231 cells, which may indicate an EMT-arrest process. Endogenous Snail1 levels were
nearly undetectable in T47D cells. All the expression levels were normalized to Actin.

Figure 10. Influence of FGFR2 isoforms on key indicators of Epithelial-Mesenchymal
Transition (EMT) in breast cancer cells
E-cadherin levels in MCF10A (upper), MDA-MB-231 (Lower Left) and T47D (Lower Right)
cells were detected. The cells were treated with FGF1, and Actin was used as an endogenous
loading control. Endogenous Snail1 levels remained undetectable in T47D cells.

4.2.6. FGFR2 isoforms do not interrupt three-dimensional (3D)
mammary epithelial acini
Breast cancers originate from epithelial cells of the terminal ductal or lobular units (TDLU) in the breast. Each TDLU has multiple small units referred to as acini that consist of a single polarized layer of luminal epithelial cells surrounding a hollow lumen [155]. The establishment and maintenance of polarized organization is critical for normal function of mammary epithelial cells in vivo. During initiation and progression of carcinoma, epithelial cells lose their ability to maintain a normal polarized organization, resulting in aberrant cell architecture [155, 156]. MCF-10A cells, when cultured in 3D matrix, undergo morphogenesis that involves a proliferative phase (days 1-8) and an apoptotic phase (days 6-9) resulting in formation of growth-arrested architectures that resemble breast acini in vivo [155]. To investigate the cellular effects of FGFR2 up-regulation during acinar morphogenesis of mammary epithelial cells, MCF-10A cells expressing FGFR2-IIIb, FGFR2-IIIc, and pMSCV empty vector (Control) were used for a 16-day 3D morphogenesis assay. From the phase images of day 10 and 16 (Figure 11), FGFR2 did not interfere with normal acinar growth. FGFR2-expressing cells continue to develop into their normal 3D structures similar to their wild-type controls. Further, the average acini size of these cells was comparable to that of their control cells (p>0.05).
Figure 11. FGFR2 isoforms do not interrupt 3D mammary epithelial acinar growth
Shown above are phase contrast images (10X and 40X) at day 10 and 16 demonstrating that FGFR2 does not interfere with normal acinar growth. The bar graph shown below is acini size (um$^2$) at Day 16. The average size of FGFR2 expressing cells is comparable to the wild-type MCF-10A controls (p>0.05).

4.3 The Role of FGFR2 Isoforms in Breast Cancer Cells in vivo
4.3.1 FGFR2 isoforms reduce MDA-MB-231 xenografted tumor growth

For assessment of tumor growth in vivo, studies using xenografts in SCID mice confirmed a consistent lower tumor volume of MDA-MB-231 cells with forced FGFR2-IIIb and FGFR2-IIIc expression compared with control tumors (Day 48- Control: 209.66±112.1mm$^3$, FGFR2-IIIb: 45.75±22.05mm$^3$ FGFR2-IIIc=22.49± 13.95mm$^3$, P$_{control}$ vs FGFR2-IIIb =0.0001 , P$_{control}$ vs FGFR-2IIIc = 0.000056, n=16 Day 60- Control: 379.97±217.58mm$^3$, FGFR2-IIIb: 109.1±62.71mm$^3$ FGFR2-IIIc=44.67±28.36mm$^3$, P$_{control}$ vs FGFR2-IIIb =0.0008 , P$_{control}$ vs FGFR2-IIIc =0.000055, n=16; Day 72- Control: 701.96±217.58mm$^3$, FGFR2-IIIb: 197.2±90.58mm$^3$, FGFR2-IIIc=58.95±44.62mm$^3$, P$_{control}$ vs FGFR2-IIIb =0.0004 , P$_{control}$ vs FGFR2-IIIc =0.000024, P$_{FGFR2-IIIb}$ vs FGFR2-IIIc = 0.0002, n=16; Day 84- Control: 957.1±253.8 mm$^3$, FGFR2-IIIb: 332.6±149.7mm$^3$, FGFR2-IIIc: 84.45±52.54 mm3, P$_{control}$ vs FGFR2-IIIb = 0.00000062, P$_{control}$ vs FGFR2-IIIc =0.00000059, P$_{FGFR2-IIIb}$ vs FGFR2-IIIc = 0.000029, n=16) (Figure 12A). The formation of orthotopic tumors were monitored on a 6-day basis.

Tumor weight (Figure 12B) was measured when the mice were sacrificed. There was a significant reduction of weight of MDA-MB-231 tumors expressing FGFR2-IIIb or FGFR2-IIIc compared with control tumors (Control: 0.78±0.36g, FGFR2-IIIb: 0.41±0.21g, FGFR2-IIIc: 0.11±0.07g, P$_{control}$ vs FGFR2-IIIb =0.0039, P$_{control}$ vs FGFR2-IIIc =0.000055, P$_{FGFR2-IIIb}$ vs FGFR2-IIIc =0.000029).

4.3.2. Lung metastasis

Excised tissues including lung, liver and bone were examined for metastasis development. Lung metastases were only observed in the control group (1 of 16) and no metastatic lesions were found in mice with FGFR2-IIIb or FGFR2-IIIc xenografts (data was not shown). Additionally, some local skeletal muscle invasion was found in the control and FGFR2-IIIb groups. However, no metastatic spread was observed in any of the organs examined including liver, lung, and bone.
(A) Tumor Growth of orthotopic xenografts (n=16; 2 independent experiments)

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(B) Tumor Weight (g)

- Control: n=16, P=0.0039
- FGFR2b: n=18
- FGFR2c: n=18, P=0.0000055
Figure 12. FGFR2 isoforms reduce MDA-MB-231 xenografted tumor growth
10^7 of MDA-MB-231 cells expressing FGFR2-IIIb, FGFR2-IIIc or pMSCV empty vector (control) were orthotopically implanted into the second lower mammary gland fat pad of each 6-week-old female SCID mouse for the assessment of tumor growth. (A) Tumor growth curve of the orthotopic xenografts were determined every 6 days by measuring in two dimensions (tumor length and tumor width) and calculating volume (mm^3) as width x length^2/2. (B) Tumor weight was measured when the mice were sacrificed. Bar graph represents the mean ± SD of 16 mice in each group. Significance (Students’ paired T-Test) is indicated.

4.3.3. Ki67 staining of xenografted MDA-MB-231 breast tumors: FGFR2-mediated reduction of cancer cell proliferation

Ki67 is a cellular marker for proliferation because it is present in the nuclei of cells in all active phases of the cell cycle (G_1, S, G_2, and mitosis), but is absent from quiescent or resting cells in the G_0 phase [157]. Immunostaining for Ki67 was performed on the MDA-MB-231 xenografted breast tumors to evaluate cell proliferation. We observed a lower percentage of Ki67-positive cells in the xenografts of FGFR2-IIIb and FGFR2-IIIc when compared with the controls, 100 cells in 3 areas of each slide were counted, and the Ki67 proliferative index (Control 83.3%; FGFR2-IIIb 64.3% and FGFR2-IIIc 39.8%) was shown in Figure 13. The significance is as follows: P_{control vs FGFR2b} = 0.0093, P_{control vs FGFR2c} = 0.0014).
4.3.4. FGFR2 Do Not Influence Xenografted MDA-MB-231 Breast Tumors Cell Differentiation

To investigate the effect of FGFR2 isoforms on breast cancer cell differentiation, immunostaining for Cytokeratin 8/18, Cytokeratin 14 and alpha smooth muscle action (α-SMA) was performed on the MDA-MB-231 xenografted breast tumors.

Cytokeratin 18, together with its filament partner keratin 8, is one of the keratins that are responsible for the structural integrity of epithelial cells, and mainly expressed in many single epithelia [158]; Cytokeratin 14, is mainly expressed in the basal layer of stratified squamous epithelia, glandular epithelia and myoepithelium [159]. In normal breast tissue, α-SMA is only present in myoepithelial cells and smooth muscle cells of small vessels, and is not expressed in the various cellular components of the stroma [160]. However, in breast malignancies, an exaggerated tissue remodeling is characterized by proliferation of fibroblasts with smooth-muscle morphologic features, and α-SMA is expressed in the large majority of stromal cells constituting the desmoplastic reaction [160].

Although MDA-MB-231 cells are characterized by loss of epithelial properties and gain of mesenchymal properties, all mouse xenografts showed strong epithelial Cytokeratin (8/18)
staining. However, the expression of the myoepithelial and stromal markers was comparable in all three types of xenografts (data was not shown). These results indicated that FGFR2 isoforms have little effect on xenografted MDA-MB-231 breast tumor differentiation markers.

### 4.3.5. Protective Effect of FGFR2-IIIc on tumor-associated fibroblasts

The cancer microenvironment plays important roles in tumor development and progression via cross-talk between neoplastic epithelial cells and stromal cells. Tumor stroma can stimulate neoplastic cell growth, facilitate tumour cell invasion and metastasis and provide a suitable environment for tumor progression [161].

In order to study the effect of FGFR2 on tumor stroma, we used mouse fibroblast NIH 3T3 cells as tumor-associated fibroblast combined with epithelial MDA-MB-231 cells (in a 1:3 ratio) to establish heterologous xenografts. Endogenous FGFR2-IIIc can be detected in 3T3 cells by RT-PCR. To avoid the interference between the two FGFR2 isoforms, we over-expressed FGFR2-IIIc in these fibroblasts as this is the dominant isoform encoded by mesenchymal cells. The transduction effectiveness was detected by Western-blotting for FGFR2 and normalized to actin. In order to confirm the activity of FGFR2-IIIc in NIH 3T3 cells, ligand-dependent receptor phosphorylation at tyrosines 653 and 654 was monitored in response to specific FGF4 stimulation (Figure 14A).

To assess the effect of FGFR2-IIIc up-regulation on NIH 3T3 cell growth in vitro, a proliferation assay was performed (Figure 14B). The cell growth rates were determined by directly counting cells on day 3 and 4 after seeding $1 \times 10^5$ cells. There was an average of 35% cell number reduction in the cells expressing FGFR2-IIIc after 4-day growth, when compared to their empty vector control. This result was significant ($P= 0.012$) according to a Students’ paired t-test analysis.

To study the effect in vivo, $7.5 \times 10^6$ epithelial MDA-MB-231 cells expressing FGFR2-IIib, FGFR2-IIIc or pMSCV empty vector (control) combined with $2.5 \times 10^6$ fibroblast 3T3 cells expressing FGFR2-IIIc or pMSCV empty vector (control) were co-injected in different
combinations. These heterologous xenografts revealed a significant reduction of tumor volume when comparing the combination of MDA-MB-231/control & 3T3 /FGFR2-IIIc to their control combination of MDA-MB-231/control & NIH-3T3/control (622.92±330.49mm\(^3\) n=9 versus 1916.88±575.3mm\(^3\) n=5 P=0.0044) (Figure 14C). Compared to the tumor growth rate of the homologous xenografts, the heterologous xenografts as shown in Figure 12 were larger at the same time point consistent with the the relatively higher growth rate of NIH/3T3 fibroblasts.

Figure 14. Protective Effect of FGFR2-IIIc on tumor-associated fibroblasts
(A) Forced expression of FGFR2-IIIc and pMSCV empty vector (control) was established in NIH 3T3 fibroblast cells. Western blotting confirmed the effectiveness of the transduction. The activation of FGFR2-IIIc (p-FGFR) can be induced by FGF4, which is a specific ligand to FGFR2-IIIc. Actin was detected as an endogenous control. (B) NIH 3T3 cell growth was monitored by counting the cells on day 3 and 4 after seeding 0.1 x 10\(^6\) cells. Bar graphs indicate the average number of the cells based on 3 experiments (each performed in triplicate). (C) 7.5x10\(^6\) epithelial MDA-MB-231 cells combined with 2.5x10\(^6\) fibroblast 3T3 cells in differential
combinations were orthotopically implanted into the second upper mammary gland fat pad of each 6-week-old female SCID mouse for the assessment of tumor growth. Tumor growth curve of heterologous orthotopic xenografts were determined every 3 days by measuring in two dimensions (tumor length and tumor width) and calculating volume (mm$^3$) as width x length$^2$/2. Bar graph represents the mean $\pm$ SD. Significance (Students’ paired T-Test) is indicated.

These results indicated that the reduced tumor growth was not only due to the growth suppressive effect of FGFR2 on autonomous neoplastic epithelial cells as described above, but also due to the protective actions of FGFR2-IIIc on tumor-associated fibroblasts.

4.4. A Putative Mechanism for FGFR2 action

4.4.1. Interaction of FGFR2 and IkappaB kinase (IKK$\beta$)

The interaction of FGFR2 isoforms and IKK$\beta$ was examined by co-immunoprecipitation using endogenous IKK$\beta$ and overexpressed FGFR2 isoforms in MDA-MB-231 cells. All cells were exposed to TNF$\alpha$ to induce endogenous IKK$\beta$, and FGF1 was used to activate both FGFR2 isoforms. FGFR2 isoforms was recovered in IKK$\beta$ immunoprecipitants, and these interactions were further confirmed in the opposite direction. Although a relatively stronger binding was achieved upon a 10-min stimulation of FGF-1 (Figure 15), at present, we cannot exclude that this is a constitutive interaction between the intracellular domain of FGFR2 and IKK$\beta$, an important regulatory protein in NF-$\kappa$B signaling. The work to further identify whether the interaction is FGF-ligand dependent is still an ongoing area of our research.
Figure 15. FGFR2 interacts with IKKβ

TNFα-stimulated MDA-MB-231 cells were immunoprecipitated with IKKβ, and probed with FGFR2 by western blotting. The same membrane was stripped and re-probed with IKKβ. Shown immediately below are FGFR2 immunoprecipitants of the same cells immunoprecipitated with FGFR2 and probed with IKKβ. The membrane was subsequently stripped and re-probed with FGFR2. The expression of FGFR2 and IKKβ was detected in whole lysates.

4.4.2. FGFR2 isoforms negatively mediate nuclear translocation of NF-κB

To further examine the biological significance of FGFR2-IKKβ interactions, we further monitored the localization of NF-κB in MDA-MB-231 cells expressing FGFR2 isoforms using immunofluorescence. Cells were co-stained with Cy3-conjugated NF-κB and Alexa Fluor 647-conjugated FGFR2. The changes in NF-κB translocation to the nucleus were monitored. In starved unstimulated cells, NF-κB was observed to be predominantly cytoplasmic, presumably due to the inhibitory action of IκB [162-164]. In contrast, NF-κB was translocated to the nucleus in response to TNFα stimulation. IKK-mediated phosphorylation induces proteasomal degradation of the IκB inhibitor, allowing the released NF-κB to dimerize and translocate to the nucleus where it induces target gene expression [162]. Significantly, when cells expressing
FGFR2 isoforms were stimulated with FGF-1 prior to the addition of TNFα, we observed an approximately 50% decrease in NF-kB nuclear localization when compared to cells exposed only to TNFα (Figure 16). To further quantify this response, 100 FGFR2-positively stained cells were analyzed for each sample in two independent experiments, and the percentage of the positive NF-kB nuclear-stained cells were shown in Table 3. Of note, this significant reduction was not observed in the FGFR2-negative control when counting the same number of the cells. These results indicated that activation of FGFR2 isoforms results in a significant decrease in TNFα-induced NF-kB nuclear localization.

Table 3. Proportion of breast cancer MDA-231 cells demonstrating nuclear NF-kB translocation

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| FGFR2c             |       |           |       |      |
Figure 16. FGFR2 isoforms negatively mediate nuclear translocation of NF-κB

MDA-MB-231 cells expressing FGFR2-IIIb, FGFR2-IIIc or pMSCV empty vector (control) were seeded onto glass cover slips and the cells were co-stained with cy3-conjugated NF-κB and Alexa Fluor 647-conjugated FGFR2. The localization of endogenous NF-κB was detected in starved un-stimulated cells (top lanes), TNFα-stimulated cells (middle lanes) and the cells stimulated with FGF1 prior to the addition of TNFα (lower lanes). The nuclei were visualized with DAPI. 100 of these cells were counted for each sample in two independent experiments. The percentage of the positive NF-κB nuclear-stained cells, the error bars with standard deviation and the significance is shown.

4.4.3. FGFR2 isoforms down-regulate NF-κB Luciferase reporter activity

To further examine the effect of FGFR2 expression on NF-κB reporter activity was monitored by a Gaussia Luciferase assay. MDA-MB-231 cells expressing FGFR2-IIIb, FGFR2-IIIc or pMSCV empty vector (control) were transiently transfected with NF-κB reporter that was cloned in the PRR-High Reporter vector. Again, cells were treated with TNFα in the absence or presence of FGF ligand stimulation. The vehicle-treated cells in each group were used as a baseline, and luciferase reporter activities of other ligand-treated groups were normalized to their vehicle controls. The normalized fold changes are shown in Figure 17. Treatment with TNFα resulted in the expected increase in the NF-κB reporter activity, compared to their un-stimulated cells (Lane 2 versus Lane 1 of each group). Cells treated with FGF-1, a ligand that binds equally to FGFR2-IIIb and FGFR2-IIIc, exhibited a reduction in NF-κB activation (Lane 3 of each
group), which was further confirmed by stimulation with FGF-4 to specifically activate FGFR2-IIIc, but not FGFR2-IIIb (Lane 4 of each group). Similarly, we used FGF-7, to specifically activate FGFR2-IIIb, but not FGFR2-IIIc (Lane 5 of each group). The significance in responses are as follows: FGFR2-IIIb: \( P (\text{TNF}\alpha \text{v.s. TNF}\alpha +\text{FGF-7}) = 0.024; \) \( P (\text{TNF}\alpha \text{v.s. TNF}\alpha +\text{FGF-4}) = 0.145; \) FGFR2-IIIc: \( P (\text{TNF}\alpha \text{v.s. TNF}\alpha +\text{FGF-4}) = 0.00032; \) \( P (\text{TNF}\alpha \text{v.s. TNF}\alpha +\text{FGF-7}) = 0.802. \) However, this reduction was not significant in the FGFR2-negative group. Control: \( P (\text{TNF}\alpha \text{v.s. TNF}\alpha +\text{FGF7}) = 0.4732; \) \( P (\text{TNF}\alpha \text{v.s. TNF}\alpha +\text{FGF4}) = 0.6533. \) These results indicated that activation of FGFR2 isoforms expression leads to down-regulated TNF-\( \alpha \) mediated NF-\( \kappa \)B reporter activity.

**Figure 17. FGFR2 isoforms down-regulate TNF-mediated activation of transcription factor NF-\( \kappa \)B**

MDA-MB-231 cells expressing FGFR2-IIIb, FGFR2-IIIc or pMSCV empty vector (Control) were transiently transfected with NF-\( \kappa \)B Luciferase reporter that was cloned in PRR-High reporter vector. A Gaussia Luciferase assay was performed to determine the activation of NF-\( \kappa \)B. The cells were treated with vehicle, TNF\( \alpha \) or TNF\( \alpha \) the presence of FGF ligand stimulation. The vehicle-treated cells in each group were used as a baseline, and the other ligand-treated groups were normalized to their vehicle controls. The normalized fold changes, the error bars with standard deviation and the significance is shown.

**4.4.4. FGFR2 isoforms attenuate STAT3 tyrosyl phosphorylation**
STAT3 is a key signaling molecule for many cytokines and growth-factor receptors, and is constitutively activated in a number of human tumors [165] and possesses oncogenic potential and anti-apoptotic activities [165, 166]. Upon phosphorylation at Tyr705, STAT3 induces dimerization, nuclear translocation and STAT-related DNA binding [167]. Its phosphorylation level can be induced after NF-κB activation, and it is also one of the classical FGFR downstream signaling targets.

After 18hrs of 10% FBS stimulation, phosphorylated levels of pY705-STAT3 were down-regulated in FGFR2 expressing cells when compared to their control cells. However, the decrease in pY-STAT3 levels was not observed in response to 10 min of 10%FBS stimulation (Figure 18A). To further confirm this FGFR2-mediated STAT3 down-regulation, TNFα was used to induce the activation of NF-κB signaling, and FGF-7 or FGF-4 was used as specific stimuli to activate FGFR2-IIIb and FGFR2-IIIc, respectively. Upon 30-min stimulation of TNFα, as noted in the control/MDA-MB-231 cells, the Tyr 705 phosphorylation of STAT3 was effectively induced but the levels were comparable in absence and presence of FGF ligands. In contrast, MDA-MB-231 cells expressing FGFR2-IIIb or -IIIc showed a significant decrease in STAT3 phosphorylation when compared to control cells exposed to the FGF ligands FGF-7 and FGF-4. Of note, this decrease was not observed upon the non-specific ligand stimulation (Figure 18B). GAL-FR22, a highly specific monoclonal antibody (mAb) against FGFR2, was used to target FGFR2 signaling by blocking the binding of ligands and ligand-induced phosphorylation [149]. MDA-MB-231 cells were treated with GAL-FR22 or identical volume of vehicle for 20 hrs, followed by the addition of FGF ligands and TNFα. We found that the phosphorylated levels of STAT3 in response to TNFα were restored when FGFR2 signaling was blocked by GAL-FR22.
Figure 18. FGFR2 isoforms attenuate STAT3 phosphorylation

(A) MDA-MB-231 cells expressing FGFR2-IIIb, FGFR2-IIIc or pMSCV empty vector (Control) were treated with 10% FBS for short-term (10mins) or long-term (18hrs) stimulation, and immunoblotted with p-STAT3 (Tyr705), and normalized to t-STAT3. Densitometric quantification of the p-STAT3/t-STAT3 upon 18-hour treatment is shown to the right. SD of two experiments and significance are indicated. The cells were then treated with TNFα, FGF7 (B), FGF4 (C) or GAL-FR22, immunoblotted with p-STAT3 (Tyr705), t-STAT3, FGFR2 and actin as indicated.

4.4.5. FGFR2 Down-regulate Interleukin-6 Expression in MDA-MB-231 Cell.
Interleukin-6 (IL-6) acts as both a pro-inflammatory and anti-inflammatory cytokine, and has been demonstrated to be of importance for cellular transformation. It is up-regulated in epithelial cancers such as breast and prostate [168], and is also a principal mediator to the activation of the STAT cascades [169].

To determine the effect of FGFR2 isoforms on IL-6 expression, endogenous IL-6 levels were examined by multiple methods. We found that IL-6 levels were down-regulated in MDA-MB-231 cells expressing FGFR2-IIIb and FGFR2-IIIc when compared to their controls by Western blotting (Figure 19A) and densitometric analysis. This down-regulation, at both the intracellular and secreted levels was further confirmed by ELISA (Figure 19B). These results indicated that the protective effect of FGFR2 isoforms on breast cancer MDA-MB-231 cell progression is, at least partially, through the negative impact on IL-6/STAT3 signaling.
Figure 19. FGFR2 Down-regulate Interleukin-6 Expression in MDA-MB-231 Cell

(A) Interleukin 6 was down-regulated in MDA-MB-231 cells expressing FGFR2IIIb, FGFR2IIIc vs pMSCV empty vector (Control). Actin was detected as an endogenous control. Densitometric quantification of the IL-6/actin is shown. SD of two experiments and significance are indicated. The down-regulation of IL-6 was also demonstrated in both cellular levels (B) and cultured cell supernatants (C) as determined by ELISA. SD and significance are shown.
5. Discussion

Gain-of-function studies are powerful approaches to gene functional characterization, leading to a further understanding of cellular responses in mammalian systems. Further they allow deeper insight into the control of cell proliferation and survival, and their alterations during cancer progression. Retroviral-mediated gene transduction has particular advantages in effectively introducing stable gene expression into the genome of any dividing cells and organisms for long-term analyses [170]. The MSCV vectors used here contain a retroviral packaging signal, $\Psi^+$, which promotes high-titer virus production that is controlled by the CMV promoter in a specifically designed long terminal repeat (LTR), which differs from other Moloney murine leukemia virus (MMLV) LTRs by several point mutations and a deletion. These changes enhance transcriptional activation and drive high-level constitutive expression of a target gene in stem cells and other mammalian cell lines [171]. The human-derived packaging cell line-293GPG is capable of producing recombinant MMLV virions that have incorporated the envelope glycoprotein from the vesicular stomatitis virus G (VSV-G) [146]. The viral gag and pol genes that are necessary for particle formation and replication are stably integrated into the genome of the packaging cell line. However, constitutive expression of the VSV-G envelope protein is toxic, and thereby a tetracycline-mediated inducible system was used to regulate VSV-G expression in the 293 GPG cells [146]. Transfection of the MSCV retroviral expression vector into 293GPG packaging cell line produces high-titer, replication-incompetent virus which lacks majority of the genes for virion synthesis and propagation, but retains cis-regulatory elements necessary for packaging, reverse transcription and integration, allowing the desired gene expression in the target cells [172]. Therefore, this coordinated retroviral gene transfer system greatly creates a high-level safety, and promotes the effectiveness of the transduction.

Utilizing this approach, the role of FGFR2-IIIb and its alternatively spliced FGFR2-IIIc variant in breast cancer initiation/progression were examined in malignant MDA-MB-231, T47D and near normal MCF-10A cells. These cell lines were selected on the basis of their endogenous
FGFR2 expression [151]. The effectiveness of the retrovirus-mediated transduction was confirmed by multiple methods. Here, MDA-MB-231 is an invasive human breast adenocarcinoma cell line. It is typical of triple negative (ER-negative, PR-negative and Her2-negative). With an epithelial morphology and spindle-shaped phenotype, MDA-MB-231 cells show tumourigenic properties including high anchorage independent growth efficiency in soft agar, abundant activity in invasion in vitro and the ability to form mammary fat pad tumors and metastases in immunodeficient mice [140]. T47D cells express receptors for a variety of steroids and calcitonin and express PR and ER under normal culture condition [141]. They are less malignant than ER-negative cancer cells. MCF-10A is a near normal mammary epithelial cell line. These cells exhibit numerous features of normal breast epithelium, including lack of tumorigenicity in nude mice, lack of anchorage-independent growth, and dependence on growth factors and hormones for proliferation and survival [142]. Due to unknown function of FGFR2 isoforms in breast cancer previously, these three cell lines were selected as cell models for the study of their possible context-dependent tumor promoting or tumor suppressive effects.

The fibroblast growth factors (FGFs) exert their diverse biological responses through the binding and activation of specific cell surface receptors. FGFRs initiate intracellular signaling through receptor tyrosine phosphorylation. To confirm the activities of the induced FGFR2 isoforms, the ligand-dependent receptor phosphorylation at tyrosines 653 and 654 was detected, in response to selective ligands (Figure 7). These phosphorylation sites are important for catalytic activity of activated FGFRs and their signaling [151]. The activation of FGFR2 isoforms can be successfully induced by their specific ligands.

Due to variable N-terminal glycosylation [134], both FGFR2-IIIb and FGFR2-IIIc migrate as doublets, a fully glycosylated 120-kDa form and a partially glycosylated 110-kDa form. FGFR2 was expressed throughout the cell and clearly revealed a cellular outline as shown in Figure 7. Several studies suggested that N-Glycosylation is critical for normal intracellular trafficking of this receptor [173]. The fully glycosylated form is trafficked to the cell surface for ligand-
dependent signaling, while the partially glycosylated receptor is trafficked from the ER/Golgi to the proteasome for degradation. Inhibition of N-glycosylation of FGFR2 prevents its cell surface trafficking, causes dramatically increased association with FRS2 to promote ligand-independent activation of the receptor. The importance of protein glycosylation is becoming widely recognized through studies on the folding, trafficking, and functions of proteins whereas the mechanism by which N-glycosylation controls receptor functions is unclear [173].

Usually, five cellular functions tend to be inappropriately regulated in a neoplasm: ineffective constraints on cellular proliferation; blocked or distorted differentiation into an abnormal type; destabilized chromosomal and genetic organization; increased cell motility or enzyme production that permits invasion and metastases and dysregulated cell death program (apoptosis) [174]. The effect of FGFR2 isoforms on these cancer hallmarks was examined.

Proliferation is a key feature of tumor progression. Most cancer therapies are aiming to reduce the number of tumor cells and to prevent their further accumulation. A direct reduction of MDA-MB-231 and T47D breast cancer cell number (Figure 8A), reduced tumor growth in SCID mice (Figure 12) and down-regulated Ki67 expression level (Figure 13) supported that FGFR2 isoforms impair breast cancer cell proliferation both in vitro and in vivo. The suppressive effect was associated with decreased nuclear RelA/p65 NF-κB localization (Figure 16), down-regulation of a transfected NF-κB luciferase reporter (Figure 17), reduced production of NF-κB-dependent transcripts, Interleukin-6 and p-STAT3 (Figure 18-19). These indicated that FGFR2 isoforms perform their tumor protective function presumably via the regulation of NF-kappa B signaling.

NF-κB is a transcription factor of pivotal importance as a regulator of genes that control cell proliferation, differentiation, survival, and inflammatory responses in mammalian cells [175]. Members of the NF-κB family share a Rel homology domain (RHD) in the N-terminus, which serves as an IkBα binding site as well as a dimerization interface for other NF-κB transcription factors [176]. In mammals, the NF-κB family is composed of five members: p50/p105,
p52/p100, RelA (p65), c-Rel and RelB. The precursor proteins p100 and p105 function as both \(\text{IkB}\) and, when processed by the proteasome, NF-\(\kappa B\) family members. Due to lack of C-terminal transcriptional activation domains, p50 and p52 homodimers cannot activate target gene expression unless they are bound to one of the transactivation domain-containing subunits like RelA, c-Rel or RelB to form heterodimers [177]. These dimers can bind to a variety of related target DNA sequences called \(\kappa B\) sites to modulate gene expression.

In unstimulated cells, the NF-\(\kappa B\) dimers are sequestered in the cytoplasm by a family of inhibitors called \(\text{IkB}\)s (Inhibitor of \(\kappa B\)). Due to the presence of the ankyrin repeat domains, the \(\text{IkB}\) proteins block the nuclear localization signals of NF-\(\kappa B\) proteins and keep them sequestered in an inactive state in the cytoplasm [178]. Activation of the NF-\(\kappa B\) complex is initiated by the signal-induced degradation of \(\text{IkB}\) proteins. This occurs primarily via activation of the \(\text{IkB}\) kinase (IKK). IKK complex is composed of a heterodimer of the catalytic IKK\(\alpha\) and IKK\(\beta\) subunits and a regulatory scaffold protein called NF-\(\kappa B\) essential modulator (NEMO, also designated IKK gamma) [179]. Upon activation, the IKK complex phosphorylates two serine residues located in an \(\text{IkB}\) regulatory domain (e.g. serines 32 and 36 in human \(\text{IkB}\alpha\)) and induces proteasomal degradation of the \(\text{IkB}\). Due to loss of inhibition, the activated NF-\(\kappa B\) dimers primarily comprising of RelA and p50, translocate to the nucleus, and induce target gene expression [180].

NF-\(\kappa B\) activation subsequently turns on the expression of its own repressor - \(\text{IkB}\alpha\), and the resulting \(\text{IkB}\alpha\) then inhibits transcriptional activity of NF-\(\kappa B\) and forms an auto feedback loop unless a persistent activation signal is present [181]. Changes that lead to inactivation of \(\text{IkB}\) proteins, deregulated amplification and rearrangement of the genes encoding the NF-\(\kappa B\) transcription factor subunits contribute to constitutive activation of the NF-\(\kappa B\) pathway. However, more commonly, it has been thought that changes in the upstream pathways lead to NF-\(\kappa B\) aberrant action in cancer [182].
Besides the canonical pathway described above, there is a non-canonical pathway that is responsible for the activation of p52/RelB complexes. In this pathway, ligand-induced activation of NF-κB -inducing kinase (NIK) phosphorylates the IKK complex, which comprises two IKKα subunits, but not NEMO, and in turn leads to the processing of p100 and liberation of the p52/RelB active heterodimer [180].

Many signaling pathways involved in cancer cell biology have an intersection with NF-κB. For example, EGF/EGFR enhances the degradation of IκBα and results in NF-κB activation in mouse embryo fibroblasts [183], non-small cell lung adenocarcinoma cells [184] and ER-negative breast cancer cells [185]. Over-expression of ErbB2, in MCF-7 breast carcinoma cells results in enhanced NF-κB activation in response to ionizing radiation [186]. A prostate cancer tissue microarray documented a significant role of ErbB/PI3K/AKT/ NF-κB signaling in the progression of prostate cancer [187]. However, there was a limited knowledge of a direct relationship between FGFR2 signaling and NF-κB activation.

A publication from Donoghue’s group [175] recently identified the negative regulation of FGFR4 in NF-κB signaling through a key regulatory protein-IKKβ which is the core of the classical NF-κB activation pathway. Interestingly, they showed these interactions existed constitutively and independent of receptor kinase activation. Based on a highly structural homology of FGFR family members, we hypothesized that a potential interaction may also exist between our receptor tyrosine kinase, FGFR2 and IKKβ. This hypothesis was identified by co-immunoprecipitation, and this physical interaction was confirmed in both directions in MDA-MB-231 cells (Figure 15). Although it is possible that the interaction between these proteins was due to a direct association, at present, we cannot exclude that some other proteins are involved in mediating this interaction. This study is still an ongoing area of our research, and some current work indicated that the binding of FGFR2 isoforms leads to an increase in tyrosine phosphorylation of IKKβ, which may be due to a direct activity of FGFR2 on IKKβ phosphorylation or due to the involvement of other signaling proteins into the FGFR2-IKKβ...
complex, e.g. some key adaptor or regulatory proteins in FGFR family such as FRS2α, Grb2-SOS [188], Pyk2 [189] and others that might mediate the effects as well through interaction with the NF-κB transcription factors. It has been well-known that IKKβ regulation is mainly through the phosphorylation of serine residues [175, 190]. Tyrosine phosphorylation of IKKβ in response to growth factor receptor activation was only reported by Donoghue [175] in their FGFR4 study. Here, the binding of FGFR2 isoforms and the increase in tyrosine phosphorylation may directly modulate the activity and/or stability of IKKβ, or perform an inhibitory effect on the serine activation and its subsequent action on proteasomal degradation of the IκB. The work to identify FGFR2 binding site(s) to IKKβ and the understanding of the role of these multiple phosphorylation sites in IKKβ will be one of our future directions.

To elucidate the biological significance of FGFR2-IKKβ interactions, we further monitored the localization of NF-κB in MDA-MB-231 cells in response to differential treatments. In starved un-stimulated cells, inactive NF-κB was predominantly cytoplasmic, presumably due to the inhibitory action of IκB as describe above. In contrast, TNFα effectively induced the activation and nuclear translocation of NF-κB complex presumably due to IKK-mediated phosphorylation and the proteasomal degradation of the IκB inhibitor [162]. In contrast, when FGFR2-expressing cells were exposed to FGF ligands prior to the addition of TNFα, we observed a significant decrease in NF-kB nuclear localization. This effect was not observed in FGFR2-negative control cells. These results indicated that activated FGFR2 isoforms lead to a reduction in TNFα-stimulated NF-kB nuclear localization (Figure 16). In addition, we also examined the activation of NF-κB reporter by Gaussia Luciferase assay. Upon FGF/FGFR2 activation, a similar effect was observed with regard to the down-regulation of NF-κB activity (Figure 17).

Altogether, based on these findings, we elucidate that the binding of FGFR2 isoforms directly or indirectly modulates the activity and/or stability of IKKβ thereby preventing phosphorylation of inhibitory IκB proteins and subsequently their proteasomal degradation. This we believe leads to down-regulated NF-κB activity, arrested nuclear translocation, and diminished downstream gene
activation. Therefore, FGFR2 isoforms exert an inhibitory effect on the activation of NF-κB signaling. Currently, we are further investigating the interaction of FGFR2 isoforms and other key components of NF-κB signaling, and we anticipate that the interactions that lead to negative regulation of NF-κB activation will not be limited to IKKβ alone.

There is a strong association between inflammation and tumorigenesis. The inflammatory microenvironment drives tumors progression, with infiltration of immune cells and activation of the inflammatory responses. NF-κB is a key molecular connection between inflammation and cancer, and regulates the expression of multiple anti-apoptotic genes and activates different pro-inflammatory cytokines and chemokines. These inflammatory molecules can provide growth signals that promote the proliferation of malignant cells, and mediate cell transformation [168].

Cellular transformation is associated with morphogenetic changes such as anchorage-independent colony formation. Based on the soft agar assay, we found FGFR2 isoforms significantly reduced the colony formation of breast cancer MDA-MB-231 cells (Figure 8B). In addition, the introduction of FGFR2 into the immortalized mammary epithelial MCF-10A cells did not interfere with normal acinar growth (Figure 11). MCF-10A cells were used to perform the 3D morphogenesis assay because they can undergo morphogenesis, when cultured in 3D matrix, that involves a proliferative phase (days 1-8) and an apoptotic phase (days 6-9) resulting in formation of growth-arrested architectures that resemble breast acini in vivo [155].

During oncogenic transformation, mammary glandular epithelial cells lose their ability to maintain polarized hollow acini, resulting in aberrant changes in cell architecture [155, 156]. However, MCF-10A cells expressing FGFR2 isoforms continue to develop into normal 3D structures similar to wild-type MCF-10A control cells. These findings argue strongly against a role for FGFR2 as an oncogenic signal in breast epithelial cell transformation.

To better understand the mechanisms underlying the protective effects of FGFR2 isoforms on breast cancer cell transformation, we further investigated another important linker between FGFR2 signaling and NF-κB, inflammation, and cancer. Among these inflammatory molecules
that are activated by NF-κB signaling. Interleukin 6 (IL-6) has been demonstrated to be of importance for cellular transformation and is up-regulated in epithelial cancers such as breast and prostate [168]. This cytokine is also a prominent mediator of activation of the STAT signaling cascades in breast cancer [169]. IL-6 is involved in cancer-related inflammation and promotes angiogenesis, metastasis, and resistance to hormones and chemotherapeutics [192]. IL-6 signals through a cell-surface cytokine receptor complex consisting of the ligand-binding subunit IL-6 Receptor (IL-6R) and the signal transducer component gp130 (glycoprotein 130) [192] and activates the JAK tyrosine kinase family members, leading to the activation of STAT family [192].

STATs are latent transcription factors that mediate cytokine- and growth factor–directed transcription. Through tyrosine phosphorylation (Tyr705), active STAT3 induces dimerization, nuclear translocation, and STAT-related DNA binding, leading to the transcriptional activation of specific genes, such as myc, Bcl-XL, and cyclin D. STAT3 can both interfere with synthesis of the tumor suppressor p53 and attenuate p53-mediated genomic surveillance [193, 194]. In normal cells, STAT3 tyrosine phosphorylation is transient, whereas it is constitutively activated in numerous human tumors and transformed cell lines, and possesses oncogenic potential and anti-apoptotic activities [165, 166]. It is persistently tyrosine phosphorylated in >50% of breast cancer- derived cell lines and in >30% of breast adenocarcinomas [195], and may be a poor prognostic indicator [196, 197]. Constitutive activation of STAT3 is frequently due to aberrant autocrine or paracrine IL-6 signaling. Inhibition of STAT3 activity in tumor-derived cell lines by the introduction of small interfering RNA has been associated with blocked cell transformation and apoptosis, and decreased angiogenesis and invasion [198].

The IL-6/STAT3 signaling pathway has become increasingly well-established in mediating cell transformation [168]. In our study, we sought to determine if the protective effect of FGFR2 isoforms on breast cancer cell transformation is through the negative mediation of IL-6/STAT3 signaling. Upon extended (18 hrs) multiple ligands stimulation, the phosphorylated levels of
STAT3 were down-regulated in FGFR2-expressing cells when compared to their controls. However, the decrease in p-STAT3 levels was not observed in response to shorter 10min 10% FBS stimulation (Figure 18A). This latent response indicated that the activation of STAT3 might not be direct, but through a sequential signaling cascade. Furthermore, we examined our cells’ responses to TNFα in the absence or presence of FGF ligands. As anticipated, STAT3 was phosphorylated in a TNFα-dependent way, and this TNFα-induced activation was significantly inhibited when FGFR2 isoforms were activated by their selective ligands (Figure 18B). The decrease in p-STAT3 levels by Western blotting corresponded with a decrease in IL-6 expression in both intracellular and secreted levels as determined by ELISA (Figure 19).

Progression of breast cancer is accompanied with the existence of epithelial-mesenchymal transition (EMT) - like changes which are characterized by loss of epithelial features and gain of mesenchymal properties. Mesenchymal transformation correlates with loss of epithelial cell adhesion molecules [152]. Reduced expression of E-cadherin is emerging as one of the most common indicators of EMT onset, resulting in the release of β-catenin and its translocation from the cytoplasm to the nucleus where it can activate LEF/TCF (lymphoid enhancer factor/T cell factor) transcription, and increase the production of transcription factors, such as Snail, Slug, Twist, which are central mediators of EMT [152, 199]. Snail 1 is a repressor of E-cadherin, and high Snail1 expression correlated with an increased risk of tumor relapse and poor survival in breast cancer [28]. E-cadherin and Snail1 levels were detected to determine whether FGFR2 up-regulation is associated with an EMT event. We found that FGFR2 isoforms promote increased E-cadherin expression levels associated with the down-regulation of Snail1 (Figure 10), which may indicate an EMT-arrest process. Consistently, the breast cancer cell invasiveness and migration were blocked by FGFR2 isoforms (Figure 9).

In addition to the regulators in EMT, Snail1 can be stabilized by TNF-α through the activation of the NF-κB pathway which induces a wide range of pro-inflammatory cytokines, chemokines and growth factors [37]. A dependency on IL-6/STAT3 signaling for cell motility was previously
demonstrated, MCF-7 cells constitutively expressing IL-6 exhibit an EMT phenotype characterized by up-regulation of Snail1 [41]. Additionally, the IL-6/STAT3 axis represents an essential effector in Rho family GTPases-mediated cancer cell invasiveness and migration [200]. Rho family GTPases switch between the active, GTP-bound state and the inactive, GDP-bound form [201]. Upon activation, the Rho GTPases are able to bind to a distinct panel of effectors, initiate downstream signaling and regulate cellular functions. The Rho family members RhoA, Rac1 and Cdc42 are well-known as regulators of actin cytoskeleton and increase cell motility [201]. Deregulation of these Rho GTPases members has been shown in multiple types of cancers [202]. A signaling model was demonstrated by Raptis et al [200] that constitutive Rac1/Cdc42 expression leads to STAT3 activation through a signaling cascade of NF-κB, IL-6/gp130 and JAK, and activated STAT3 mediates motility of cancer cells by regulating genes such as integrin β6 and/or interacting with focal adhesion kinase (FAK), which results in abrogated cell adhesion associated with reduced E-cadherin expression and increased cell invasion and migration [200]. Based on these studies, we predict that the negative regulation of FGFR2 on the activation of NF-κB / IL-6 / STAT3 axis may be a blockade to the signaling transduction of Rho family GTPases to their downstream effectors in regulating intracellular actin dynamics and cell motility. Consistent with this prediction, we observed EMT-arrest evidenced by enhanced E-cadherin and down-regulation of Snail1 associated with inhibited MDA-231 cancer cell motility in the face of forced FGFR2 expression.

Increasing evidence suggests that the cancer microenvironment plays important roles in tumor development and progression via cross-talk between neoplastic and stromal cells [161]. Tumor-stroma interactions influence tumor growth, and facilitate cell invasion and metastasis by regulating angiogenesis, secreting signaling molecules and modulating extracellular matrix which contributes to tumor rigidity. The tumor stroma includes fibroblasts, endothelial cells and immune cells [161]. Tumor-associated fibroblasts have been suggested to play an essential role in the tumor-stroma interactions and provide a suitable environment for tumor progression by
secreting cytokines, growth factors and other tumor promoting effectos, such as TGF-β, matrix degrading enzymes MMPs, angiogenic factors VEGF and IL-6 [161].

To further study the effect of FGFR2 isoforms in tumor-associated fibroblast, mouse fibroblast NIH-3T3 cells were used as tumor-associated fibroblast combined with epithelial MDA-MB-231 cells to establish heterologous xenografts. Endogenous FGFR2-IIIc can be detected in 3T3 cells by RT-PCR. To avoid the interference between the two FGFR2 isoforms, we only overexpressed FGFR2-IIIc because this is the dominant isoform encoded in mesenchemal cells. We found that the enhanced expression of FGFR2-IIIc in fibroblasts was associated with a significant reduction of xenografted tumor volume, which was consistently shown in differential cell combinations (Figure 15). These results indicated that the reduced tumor growth was not only due to the growth suppressive effect of FGFR2 on autonomous neoplastic epithelial cells, but also due to the suppressive actions of FGFR2-IIIc on tumor-associated fibroblasts.

A principle mechanism of STAT3 activation in breast cancer is through autocrine production of IL-6 [197]. The tumor microenvironment is critical for the expression of IL-6 ligands. Some work has demonstrated that IL-6 that was secreted by stromal cells in a paracrine manner can induce epithelial cells to produce IL-6 in an autocrine manner [203, 204]. Thus, we hypothesized that FGFR2-IIIc may inhibit the expression of IL-6 in the tumor-associated fibroblasts, and subsequently through tumor-stroma interactions, the IL-6 levels in epithelial neoplastic cells were down-regulated as well. Based on this hypothesis, we will further investigate the mechanisms underlying the protective effect of FGFR2-IIIc on tumor stroma.
6. Summary and Conclusion

In order to investigate the roles of FGFR2-IIIb and its alternatively spliced FGFR2-IIIc variants in breast cancer, stable retroviral transduction of these two isoforms was achieved in breast cancer MDA-MB-231, T47D and near normal MCF-10A cells. These FGFR2 over-expressed cells exhibited impaired cell growth by direct cell counting, and were found to significantly reduce anchorage-independent colony formation in soft agar which is used to monitor the morphogenetic changes during cell transformation. FGFR2 blocked breast cancer cell motility by transwell assays with matrigel-coated inserts for invasion and uncoated insert for migration. Markers of epithelial to mesenchymal transition (EMT) including up-regulation of E-cadherin and down-regulation of Snail1 supported this growth arrest in response to FGFR2. We observed MCF-10A cells to undergo proliferation-arrest with development of 3D structures resembling breast acini in the presence of FGFR2.

4-6 week SCID mice were injected orthotopically for the assessment of the tumor growth. Reduced tumor volume and down-regulated Ki67 expression level supported an inhibitory effect of FGFR2 isoforms on tumor cell proliferation. But they do not influence xenografted breast tumors cell differentiation. As tumor stroma facilitates growth progression through crosstalk with neoplastic epithelial cells, we performed heterologous orthotopic xenografts, and found that reduced tumor growth was not only due to the growth suppressive effect of FGFR2 on neoplastic epithelial cells but also due to the protective actions of FGFR2-IIIc on tumor-associated fibroblasts.

Altogether, based on these observations, it is reasonable to propose a tumor-suppressive role for FGFR2. To better understand the underlying mechanisms, immunoprecipitates from FGFR2-IIIb and IIIc-expressing cells were shown to interact with IKKβ, a key regulatory protein in NF-κB signaling. This was identified by co-immunoprecipitation at both directions in MDA-MB-231 cells. To determine the biological significance of FGFR2-IKKβ interactions, we monitored the localization of NF-κB in response to differential treatments, and found that activated FGFR2
isoforms lead to a reduction in TNFα-stimulated NF-κB nuclear localization. This was concordant with down-regulation of NF-κB reporter by assays. Meanwhile, the decrease in p-STAT3 levels by Western blotting corresponded with a decrease in IL-6 expression in both cellular levels and cultured cell supernatants as determined by ELISA. Therefore, we elucidate that FGFR2 isoforms negatively regulate NF-κB signalling through an interaction with IKKβ, and perform their tumour suppressive effects, at least partially, by down-regulation of Interleukin-6 and p-STAT3.
7. Future Directions

The work presented in this thesis provides strong evidence of FGFR2 function in the breast cancer cells, and link the tumor suppressive activity of this receptor tyrosine kinase with negative regulation of NF-κB signalling. This offers a putative mechanism underlying FGFR2 function. This work will serve as a basis for the future studies described below.

1. To further identify the interaction between FGFR2 and NF-κB transcription factors

In the present work, we have identified an interaction between FGFR2 and IKKβ by co-immunoprecipitation in both directions in MDA-MB-231 cells. Although it is possible that the interaction between FGFR2 and IKKβ is due to a direct association, at present, we cannot exclude the involvement of other proteins in mediating this interaction. Therefore, we are going to identify this uncertainty, and to find out FGFR2 binding site(s) to IKKβ.

Although a relatively stronger FGFR2-IKKβ binding was achieved upon FGF1 stimulation according to the densitometric analysis, at present, it is still not quite certain if this interaction is FGF-ligand dependent. Thus, we will compare the binding affinity in response to differential specific-ligands.

Some current work indicated that the binding of FGFR2 isoforms leads to an increase in tyrosine phosphorylation of IKKβ, which may be due to a direct activity of FGFR2 on IKKβ phosphorylation or due to the involvement of other signaling proteins into the FGFR2-IKKβ complex. Thus, it will be essential to understand the role of these multiple phosphorylation sites in IKKβ.

In addition to IKKβ, we cannot exclude that there are alternative interactions of FGFR2 isoforms with other NF-κB signal regulatory proteins, which may also regulate NF-κB and its downstream signal transduction.

2. To further investigate the mechanism underlying the protective action of FGFR2
An epigenetic mechanism demonstrated by Struhl K et al involves the regulatory circuit including NF-κB transcription factor, Let-7 microRNA, the microRNA processing inhibitor Lin28, and IL-6 [168]. Micro RNAs play a critical role in many biological processes including cancers, by directly interacting with specific mRNAs through base pairing and then inhibiting expression of the target genes through a variety of molecular mechanisms [203]. Let-7, an important tumor suppressor microRNA that promotes cell differentiation, directly inhibits expression of IL-6 through binding its 3’ UTR and also inhibits the phosphorylation of STAT3 [168]. However, Let-7 is down-regulated at an early stage of cellular transformation by NF-κB activation of Lin28 [168]. Lin28 acts as a negative regulator of micro RNA biogenesis and plays a central role in blocking microRNA-mediated cell differentiation [204]. It directly binds to the Let-7 pre-micro RNA, and blocks the microRNA processing and the production of the mature let-7 posttranscriptionally in embryonic stem cells, embryonic carcinoma cells, and primary tumors [204]. In short, the repression of Let-7 through NF-κB activation of Lin28 results in a dramatic increase in IL-6 and p-STAT3 levels, which is necessary for tumor progression.

Based on these findings, we will examine if NF-κB signaling is negatively regulated by FGFR2 isoforms, which might be associated with the down-regulation of Lin28, and the restoration of Let-7 microRNA dramatically inhibited IL-6 expression and the activation of downstream STAT3 signaling cascades. Thus, the inhibition of NF-κB, Lin28, IL-6 and p-STAT3 may lead to loss of tumorigenicity and transformed cellular phenotype.

Another principle mechanism of STAT3 activation in breast cancer is through autocrine production of IL-6 [197]. Some work has demonstrated that IL-6 that was secreted by stromal cells in a paracrine manner can induce epithelial cells to produce IL-6 in an autocrine manner [203, 204]. Thus, we hypothesized that FGFR2-IIIc may inhibit the expression of IL-6 in the tumor-associated fibroblasts, and subsequently through tumor-stroma interactions. IL-6 levels in epithelial neoplastic cells were down-regulated as well. Based on this hypothesis, we will further investigate the mechanisms underlying the protective effect of FGFR2-IIIc on tumor stroma.

3. To validate other partners engaged by FGFR2 isoforms
Immunoprecipitates from FGFR2-IIIb and FGFR2-IIIc-expressing cells will be subjected to Mass Spectrometry for identification of novel interacting partners. Current studies are underway to validate other differential proteins. Some of these targets e.g. Pyruvate kinase M1/M2, Peroxiredoxin 1, are also providing new insights into the signaling networks of FGFR2 isoforms, and will likely prove critical in deciphering FGFR2 gene’s functions in breast cancer initiation and progression.
8. References


115. Klint P and Claesson-Welsh L. Signal transduction by fibroblast growth factor receptors Front Biosci.1999; 4: p165-77


