FGFR1-induced soluble factors promote mammary tumorigenesis and chemoresistance

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Dedication

I would like to dedicate this dissertation to my grandparents,
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who I love very much and without who I would never be where I am today.

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A disintegrin and metalloprotease	ADAM
Acute phase response factor	APRF
Aldehyde dehydrogenase 1	ALDH1
Amphiregulin	AREG
AP20187	AP (renamed B/B)
Atypical ductal hyperplasia	ADH
B cell CLL/lymphoma-2	BCL-2
Basic FGF	bFGF
BCL-2-related gene, long isoform	BCL-xL
Chromatin immunoprecipitation	ChIP
Cyclic AMP	cAMP
Cyclooxygenase 2	COX2
Ductal carcinoma in situ	DCIS
Epidermal growth factor	EGF
Epidermal growth factor receptor	EGFR
Epiregulin	EREG
Epithelial-to-mesenchymal transition	EMT
Estrogen receptor α	ERα
Extracellular matrix	ECM
Extracellular signal-regulated kinase 1/2	ERK1/2
FGFR substrate 2	FRS2
Fibroblast growth factor	FGF
Fibroblast growth factor receptor 1	FGFR1

Glycoprotein-130	gp130
Growth hormone	GH
Hematoxylin and eosin	H&E
Hepatocyte growth factor	HGF
Hormone receptors	HRs
Hyperplastic enlarged lobular units	HELUs
Immunofluorescence	IF
Immunoglobulin	Ig
Immunohistochemistry	IHC
Inducible FGFR1	iFGFR1
Insulin-like growth factor 1	IGF-1
Insulin-like growth factor-binding protein-5	IGFBP-5
Interleukin 6	IL-6
Intraperitoneal	i.p.
Invasive ductal carcinoma, not otherwise specified	IDC NOS
Invasive lobular carcinoma	ILC
Janus kinase	JAK
Knockout	KO
Leukemia inhibitory factor	LIF
Matrix metalloproteases	MMPs
Mouse mammary tumor virus	MMTV
Myeloid cell leukemia 1	MCL-1
Non-small cell lung cancers	NSCLCs
Oncostatin M	OSM
Parathyroid hormone-related protein	PTHrP

Pathological complete response	pCR
Phorbol 12-myristate 13-acetate	PMA
Phosphoinositide-3 kinase	PI3K
Phospholipase Cγ	PLCγ
Phospho-tyrosine binding	PTB
Phosphorylated	p
Platelet-derived growth factor	PDGF
Platelet-derived growth factor receptor	PDGFR
Progesterone	P
Progesterone receptor	PR
Prolactin	Prl
Prolactin receptor	PrlR
Protein inhibitor of activated STAT	PIAS
Protein kinase C	PKC
Quantitative reverse transcription-PCR	qRT-PCR
Recombinant mouse	rm
Serum free	SF
Signal transducer and activator of transcription 3	STAT3
Src-homology domain 2	SH2
Suppressors of cytokine signaling	SOCS
Terminal duct lobular units	TDLUs
Terminal end buds	TEBs
Transactivation domain	TAD
Transforming growth factor α	TGFα
Triple negative	TN

Triple negative breast cancers	TNBCs
TNFα converting enzyme	TACE
Tumor necrosis factor α	TNFα
Wildtype	WT

Chapter 1: Introduction

1.1 Normal Mammary Gland Development

1.1.A Overview of the mammary gland.

The mammary gland is a complex secretory organ comprised mainly of epithelial cells, adipocytes, and stroma. In a normal, mature mammary gland, epithelial cells make up the branching network of ducts that ultimately functions in the delivery of milk to young. This ductal network is embedded within the fat pad. Surrounding each ductal branch is a thin basal lamina and a fibroblast-rich stromal compartment. Additionally interspersed throughout the mammary gland are blood and lymph vessels and an assortment of immune cells. Development of the mammary gland has been well studied predominantly through the use of mouse and rat models. While there are some differences between human and rodent mammary gland development, the most notable example being the number of mammary gland pairs that develop, animal models have provided vast insight into the intricate events that must take place in the life of a mammary gland and will be the focus of this section. The mature mammary gland develops through a tightly coordinated series of events that occur in three distinct stages: during embryogenesis, at puberty, and throughout adulthood.

1.1.B Embryonic development.

At E10.5 in the mouse embryo, formation of a mammary (or milk) line on each side of the body in an anteroposterior direction between the limb buds marks the first step in mammary gland development [1, 2]. Along each mammary line, five mammary placodes, thick plate-like structures which are commonly found in development as the origins of specialized organs or tissues, begin to form at E11.5 [2-4]. Placode formation occurs in a tightly regulated and ordered fashion, such that the third thoracic placode appears first followed by the fourth inguinal placode before simultaneous formation of the first cervical and fifth inguinal placodes and finally the second thoracic placode appears. Studies suggest that these placodes arise from ectodermal cells migrating along the mammary lines [1, 2]. By E13.5, these placodes become the mammary buds as the epithelial cells of the placodes rapidly proliferate and invade into the underlying mesenchyme. The mammary buds continue to elongate into what are termed the mammary sprouts and signal to the surrounding mesenchyme via parathyroid hormone-related protein (PTHrP) to become mammary mesenchyme, without which mammary bud elongation into the sprout structure and further proper mammary gland development does not occur [5-7]. Within the mammary sprouts, epithelial cell apoptosis produces a hollow lumen around E15.5 that is connected to the developing nipple. By E18.5, the mammary sprouts have branched into rudimentary ductal trees containing between 10 and 20 small branches. Each branch consists of a single layer of epithelial cells

surrounding a lumen. This ductal structure grows into white adipose tissue that has started to accumulate, which functions to support and eventually restrict the growth and branching of the ductal epithelial cells [8, 9]. This simple mammary gland consisting of a small epithelial ductal tree embedded within adipose tissue will cease to undergo further development until puberty. However, it will increase in size to keep pace with the body as it grows in size.

1.1.C Pubertal development.

At puberty, the mammary gland ductal network undergoes rapid, extensive growth and expansion. In mice, club shaped structures known as the terminal end buds (TEBs), appear at the tips of the primary epithelial ducts. In humans, these structures are called terminal duct lobular units (TDLUs). The TEBs are comprised of an outer layer of cap cells and multiple inner layers of body cells. Studies have proposed that the cap cells at the tip of the TEB are the undifferentiated mammary stem cells as they undergo both symmetric division to regenerate cap cells and asymmetric division to give rise to transit cells and have also been seen to migrate into the central layers of cells [10, 11]. Transit cells are bipotent progenitor cells. The outer layer of transit cells will differentiate into a myoepithelial lineage, while the inner transit cells (the body cells) will first transition into early luminal progenitors, then to late luminal progenitors, and then finally become the differentiated luminal epithelial cells. As the TEB progresses through the fat pad, the bulk of the luminal epithelial cells in the TEB will undergo

apoptosis [12]. The resultant ducts have a single layer of myoepithelial cells encompassing a single layer of luminal epithelial cells which are surrounding a hollow lumen.

Expansion of the ducts is driven by the TEBs and ceases when the TEBs reach the outer edges of the mammary fat pad and regress into a mitotically inactive state. Along with ductal expansion, primary bifurcation occurs at the TEBs to give rise to new primary ducts. Along the primary ducts, secondary branches will sprout off laterally to fill in the fat pad.

Unlike embryonic mammary ductal branching, which is apparently hormone independent, branching of the adolescent mammary gland transpires due to the production of hormonal signals from the ovaries and pituitary gland. Estrogen, estrogen receptor α (ERα), and growth hormone (GH) are all required for proper mammary ductal morphogenesis [13-17]. In addition to hormonal cues, localized growth factor signaling is also necessary for proper mammary ductal morphogenesis. Several growth factors or growth factor receptors, including epidermal growth factor receptor (EGFR, also known as ErbB1), amphiregulin (AREG), ErbB2 (also known as HER2 or neu), insulin-like growth factor 1 (IGF-1), transforming growth factor α (TGFα), and numerous members of the fibroblast growth factor (FGF) family, have been shown to play important roles in proper mammary gland ductal morphogenesis at puberty [18-25].

1.1.D Development during adulthood/pregnancy.

During adulthood, the mammary gland undergoes tertiary branching and the formation of alveolar buds at the ends of these branches in response to the cyclical release of ovarian and pituitary gland hormones progesterone (P) and prolactin (Prl), respectively. The alveolar buds will eventually become differentiated alveoli, which are the sites of milk production and secretion, but this only occurs during pregnancy-induced growth of the mammary gland [26]. Studies have also demonstrated the requirement of both the P receptor (PR) and the Prl receptor (PrlR) in the formation of the tertiary branches and alveoli [27-29]. During pregnancy, the mammary gland undergoes extensive ductal epithelial cell proliferation and increased tertiary branching to prepare for lactation. Additionally, alveologenesis occurs, such that the alveolar buds differentiate into individual alveoli. Within each alveolus, a single layer of alveolar epithelial cells form a spherical structure around a hollow lumen. While the luminal epithelial cells of ducts are encompassed within a continuous layer of myoepithelial cells, the myoepithelium of the alveoli is discontinuous, leaving some luminal cells in direct contact with the basement membrane. It is thought that this is important for successful milk secretion [30, 31]. Late in pregnancy, the mammary gland is almost completely filled with alveoli and milk production begins. Parturition marks the beginning of lactation, which will continue until weaning. Following weaning, massive involution of the differentiated alveoli via apoptosis and restructuring of the gland back to its pre-pregnant state occurs, signaled in part via signal

transducer and activator of transcription 3 (STAT3) [32-34]. Upon completion of the involution process, the mammary gland resumes responding to cyclical steroid hormone signals and may recommence lobuloalveologenesis at any time.

1.2 Breast Cancer

1.2.A An overview of breast cancer.

Breast cancer is a complex and heterogeneous disease. In humans, it is thought that the conventional site of breast cancer initiation is within the TDLUs when abnormal enlargement of TDLUs progresses to hyperplastic enlarged lobular units (HELUs), which are the first histologically identifiable premalignant lesions in breast tissue [35, 36]. It has been proposed that HELUs have the ability to advance into many other lesions, most commonly the atypical ductal hyperplasia (ADH), which is generally thought to be the precursor of ductal carcinoma in situ (DCIS) [36-38]. Malignant disease arises from DCIS when the basement membrane surrounding each duct is breached, thereby becoming an invasive ductal carcinoma [35, 36, 38]. Invasive ductal carcinoma, not otherwise specified (IDC NOS) is the most frequently observed pathologically classified type of breast cancer, accounting for about 80% of all breast cancer cases [39, 40]. The second largest pathological category of breast cancer is invasive lobular carcinoma (ILC), which only represents approximately 10% of all breast cancers [39, 40]. The remaining 10% of breast cancers are clinically classified into many rarely observed types [40].

In the clinic, breast cancers are usually characterized for treatment by the presence or absence of the hormone receptors (HRs), ER and PR, and the growth factor receptor HER2, three of the most well-known drivers of breast cancer. Tumors that are found to be HER2+ are treated with therapies that

specifically target HER2, such as the HER2 monoclonal antibody trastuzumab [41-44]. Tumors that express ER are treated with the ER-specific inhibitor Tamoxifen [45-47]. Additionally, aromatase inhibitors, which indirectly inhibit ER function by blocking the production of the ER ligand estrogen, are used to treat patients that are ER+ and ER+/PR+ [45, 48, 49]. However, the subset of breast cancers that are HR-/HER2- (known as triple negative (TN)) do not have any currently available and widely used targeted therapies. Instead, women with TN-designated tumors usually receive chemotherapy, such as Doxorubicin, Cyclophosphamide, Paclitaxel, Capecitabine, 5-fluorouracil, and Gemcitabine [50, 51]. However, not all patients respond well to therapies when given based solely on the presence or absence of the HRs and HER2, and the complexity and heterogeneity of breast cancer is not completely mimicked with these markers alone. Since the pioneering study by Perou and colleagues in 2000, much research has been done using microarray-based global gene expression profiling and has resulted in the identification of several molecular intrinsic subtypes of breast cancer [52-57]. Currently, there are six distinct breast cancer intrinsic subtypes based on molecular profiling: claudin-low, basal-like, HER2-enriched, normal breast-like, luminal A, and luminal B. Each subtype can be distinguished from the others based on gene cluster expression patterns. Additionally, it has been proposed that each subtype arises from distinct progenitors.

1.2.B The luminal A and luminal B subtypes.

The luminal A and B tumors are thought to both arise from differentiated luminal cells of the mammary ducts and were originally characterized by and named for the high expression of genes commonly expressed in luminal cells [53, 54, 58, 59]. They are further closely associated because they are both generally HR+ but HER2- [53, 55, 57]. However, this is not an efficient method for identification of the luminal tumors as at least 20% and approximately 10% of all luminal B and luminal A tumors, respectively, do express HER2 [60]. Moreover, at least 5% of all luminal tumors are HR-, strengthening the need for classification of breast cancers based on gene cluster analysis instead of via individual biomarkers [60]. Indeed, using gene cluster signatures, distinction between the two luminal subtypes can be identified. Luminal A tumors have high expression of ER and ER signaling-related molecules, which might be why they respond so well to the standard endocrine therapy treatment of luminal tumors, and are also enriched for genes involved in fatty acid metabolism [55, 57]. Patients with luminal A tumors have a good overall survival prognosis and much longer metastasis-free survival than any other subtype [56, 61]. The luminal subtypes can also be distinguished based on their proliferation profiles. Luminal B tumors have high expression of genes involved in proliferation, including Ki-67 and Cyclin B1, while luminal A tumors have low levels of the proliferation gene cluster [62, 63]. Importantly, as there is a need for biomarkers in the clinic, luminal B tumors can generally be identified by high Ki-67 and as HR+/HER2-,

while luminal A tumors are usually HR+/HER2- and low for Ki-67 [60, 62]. These data suggest that luminal B tumors are rapidly proliferating, which may account for the poor prognosis associated with this subtype [56, 62, 64].

1.2.C The HER2-enriched subtype.

HER2 overexpression occurs in 15-30% of all breast cancers [65, 66]. The HER2-enriched subtype of breast cancers is therefore justly named because of *HER2* gene amplification as well as gene enrichment for HER2 downstream signaling molecules [53]. Interestingly, most of the genes within the expression profile for this subtype are located near *HER2* on chromosome 17 and are also amplified [53, 67]. However, around 35% of tumors in this category do not actually contain HER2 overexpression, and it has been proposed that the HER2-enriched tumors that are HER2- might have a mutation that phenotypically mimics HER2-amplification/signaling [60]. Surprisingly, although there is a clear target for directed therapy in this subtype, not all patients respond well to trastuzumab treatment. In fact, the HER2-enriched subtype consistently shows the worst overall and disease-free survival of all the intrinsic subtypes [55, 56, 60, 68]. On the other hand, trastuzumab in conjunction with chemotherapy has yielded better overall and recurrence-free survival, perhaps owing to the fact that HER2-enriched tumors are usually highly proliferative [69, 70]. The HER2-enriched intrinsic subtype appears to arise from a late luminal progenitor [59].

1.2.D The basal-like subtype.

The basal-like subtype comprises 15-25% of all breast cancers and largely consists of TN breast cancers (TNBCs) [53, 60, 71, 72]. Because of a lack of conventional targets in this subset, basal-like tumors are usually treated with chemotherapies [50, 51]. However, approximately 20% of all basal-like tumors remain ER+ and/or HER2+ [60]. Therefore, the use of only ER/PR/HER2 negative status is not entirely consistent with the basal-like tumor subset, and some studies have suggested a five gene signature for better identification of this subtype in the clinic. The five marker method, or Core Basal group, includes ER-, PR-, and HER2- in addition to EGFR and cytokeratin 5/6 as positive markers and has been shown to more accurately identify a tumor as basal-like [68, 73]. Interestingly, results using the five marker method have demonstrated that basal-like tumors that are TN, EGFR+, and cytokeratin 5/6+ (Core Basal) have worse prognosis than TN, EGFR-, and cytokeratin 5/6- tumors (5 Negative Profile) [68]. Basal-like tumors are highly mitotically active, often aggressive tumors that are most frequently found in younger, premenopausal patients [71, 72, 74-76]. In general, basal-like tumors initially respond well to chemotherapeutic treatment, but most women do not have complete pathological response, ultimately resulting in high early relapse rates and low overall survival [50, 51, 56, 77-79]. It is thought that basal-like tumors arise from luminal progenitor cells as they have the highest expression of a characteristic set of genes expressed in the luminal progenitor population [58, 59]. Additionally, gene profiling of basal-like tumors

shows that they also highly express genes involved in angiogenesis, cell cycle regulation, proliferation, and cell motility [57, 80]. Interestingly, breast cancers with the hereditary *BRCA1* gene mutation are usually found in the basal-like subset of breast cancers [56, 58, 81].

1.2.E The claudin-low subtype.

The most recently identified breast cancer subtype is the claudin-low tumors [52, 54]. As indicated by the name, one of the characteristic gene signatures of the claudin-low tumors is relatively low expression of a series of proteins known for their role in cell-cell adhesion, including claudin 3, claudin 4, and claudin 7 [52, 54]. Alternatively, claudin-low tumors express high levels of a gene cluster consisting of mesenchymal/extracellular matrix genes and another gene cluster composed mainly of immune system response genes [54, 82, 83]. It is not yet known to what extent the actual tumor cells express the immune system response genes versus the possibility that there are simply high levels of immune cells infiltrating this tumor subtype. High expression of mesenchymal-related genes has been proposed to be characteristic of the mammary stem cell population, and it is thought that the claudin-low tumors arise from a stem cell or stem cell-like population of cells [58, 59, 84, 85]. In favor of this notion, claudin-low tumors express high levels of the mesenchymal proteins vimentin, N-cadherin, and TWIST1 and the stem cell(-like) marker aldehyde dehydrogenase 1 (ALDH1) [54]. Additionally, claudin-low is the only breast cancer intrinsic

subtype to express a signature gene expression profile previously implicated to specify breast cancer stem cells [82, 86]. Several studies have suggested that stem cells are chemoresistant and therefore relapse occurs because, while the bulk of the tumor responds to chemotherapy, the end result is enrichment of the cancer stem cells and an ultimately more aggressive disease [84, 86-88]. As the majority of claudin-low tumors are TN tumors, the current standard treatment plan for these patients is chemotherapy [50, 51, 54]. Indeed, claudin-low tumors treated with chemotherapy show low pathological complete response as compared to basal-like tumors, suggesting that they are relatively chemo-insensitive [60]. In addition, patients with claudin-low tumors have poor overall and relapse-free survival, further supporting that claudin-low tumors arise from a stem cell(-like) progenitor [54, 59, 60].

As evidenced by the data collected in just the last decade, breast cancer is a complicated and intricate set of diseases. Clearly numerous pathways and molecules are involved in the initiation and progression of breast cancers. Much work is still needed to better understand which factors might represent valuable targets for new therapies, to develop new therapies, and to determine when such new therapies will be of benefit to patients. The following sections will introduce the proteins that have been the focus of my thesis research.

1.3 Fibroblast Growth Factor Receptor 1

1.3.A The fibroblast growth factor family.

The fibroblast growth factor (FGF) family is comprised of 22 structurally similar ligands and four membrane-bound receptor tyrosine kinases. Each FGF receptor (FGFR) contains three extracellular immunoglobulin (Ig)-like domains, an acidic box, a transmembrane domain, and a split intracellular tyrosine kinase domain. Additionally, heparin or heparin sulfate proteoglycans have been shown to stabilize FGF to FGFR binding, and each receptor has an extracellular heparin-binding site [89, 90]. Complexity of the FGF family arises not only because each ligand can bind multiple receptors but also because *FGFR1-3* undergo alternative splicing. This alternative splicing usually occurs from the differential usage of two exons both coding for the C-terminal region of the third Ig-like domain resulting in either the IIIb or IIIc isoforms. These splice variants are expressed in different tissues and have different ligand binding specificities. Ligand binding to an FGFR monomer induces dimerization and subsequent transphosphorylation of tyrosine residues within the kinase domains. Adaptor molecules, such as FGFR substrate 2 (FRS2) or phospholipase Cγ (PLCγ), bound to the activated receptor dimer can then themselves be phosphorylated and activated and subsequently act to transmit the FGFR activation signal through a variety of downstream molecules, including phosphoinositide-3 kinase (PI3K), extracellular signal-regulated kinase 1/2 (ERK1/2), various signal transducer and activator of transcription (STAT) proteins, and protein kinase C

(PKC), to stimulate cellular processes such as proliferation, survival, migration, and angiogenesis. The FGF family has been widely studied and reviewed in normal and diseased processes [91-96].

1.3.B The FGF family in mammary gland development.

FGF signaling has been linked to many developmental processes, including the formation of limb buds, stimulation of angiogenesis, and induction of branching morphogenesis in organs such as the kidneys, lungs, prostate, and mammary glands [97-104]. During embryonic mammary gland development, FGF10 and its receptor FGFR2-IIIb are essential for proper mammary placode formation [3, 105]. FGFR2 expression is also required within the mammary epithelium during pubertal ductal morphogenesis, as Cre-mediated removal of FGFR2-IIIb within the epithelium results in reduced ductal morphogenesis and a lack of TEBs [106]. Interestingly, while FGFR2 is expressed in the epithelial cells, its ligand, FGF10 is highly expressed in the mammary fat pad, suggesting a paracrine signaling axis [106]. Additional FGF ligands, FGF2 and FGF7, have also been shown to stimulate ductal morphogenesis in EGFR deficient mammary organoids grown in culture [107]. FGFR2-IIIb and EGFR are expressed and required in separate compartments in the mammary gland, suggesting that there may be crosstalk between the two signaling pathways and mammary gland compartments during proper mammary gland development.

1.3.C FGFR1 in breast cancer.

Dysregulation of FGF signaling has been implicated in mammary tumorigenesis as well. Mammary tumor samples from mice harboring the mouse mammary tumor virus (*MMTV*) often show high rates of *MMTV* insertion upstream of *FGF* genes [108-110]. Aberrant expression of FGF3 in the mouse mammary gland also leads to hyperplastic lesions [111]. Overexpression of FGFR2 or FGFR4 is observed in a portion of breast cancer patients as well [112, 113]. Additionally, amplification of the genomic region 8p11-12, which contains the *FGFR1* gene, occurs in approximately 10% of breast cancers [114-117]. Much debate has ensued regarding whether FGFR1 is actually the oncogenic driver of the 8p11-12 amplification as some studies have found that *FGFR1* is not always contained within the amplified region [112, 118]. Work done by Gelsi-Boyer and colleagues revealed that this debate has probably resulted because instead of one homogeneous amplicon, at least four different, only partially overlapping amplicons arise from this region. However, *FGFR1* is indeed encoded within at least one of these amplicons [119]. Notably, patients harboring the *FGFR1*-containing amplicon have significantly worse metastasis-free survival, an increase in the development of distant metastases, shorter disease-free survival, and decreased overall survival when compared to patients without the *FGFR1* amplicon [116, 119-121]. Importantly, *FGFR1* amplification has been associated with increased gene expression and protein overexpression [114, 116, 119, 121, 122]. In breast cancer cell lines, amplification of *FGFR1* promotes

anchorage-independent growth, and inhibition of *FGFR1* with siRNA decreases cell viability, suggesting that aberrant FGFR1 signaling promotes tumorigenic phenotypes [121, 122]. *FGFR1* amplification in primary breast tumors correlates with ER+ but PR- status, and 8p11-12 amplification is often found in the ER+ luminal A and luminal B type breast cancers [116, 120, 121]. Since luminal B tumors are highly proliferative, poor-prognostic tumors, these results indicate *FGFR1* amplification might be an important occurrence of more aggressive breast cancers. In line with such a notion, breast cancer cell lines with *FGFR1* amplification display increased resistance to endocrine therapies [121]. Furthermore, recent work has revealed that FGFR signaling occurs in TN and basal-like breast cancers and inhibition of FGFR both *in vitro* and *in vivo* inhibits cell survival [123]. Some studies have also indicated that the 8p11-12 amplification cooperates with other amplifications, such as 11q13, to promote tumorigenesis [122, 124-126].

1.3.D The inducible FGFR1 system.

While the work described above demonstrates the importance of FGFR1 in breast tumorigenesis, especially because *FGFR1* amplification occurs in patients with poor prognosis, there is no direct insight into how aberrant FGFR1 expression promotes tumorigenesis in those studies. However, it is difficult to specifically study endogenous FGFR1 signaling because, while multiple FGF ligands can activate FGFR1, no FGF ligand exclusively binds to and only

activates FGFR1. Therefore, the Rosen laboratory engineered an inducible FGFR1 (*iFGFR1*) construct, which allows for the exclusive study of FGFR1 signaling [127]. In this construct, the FGFR1 kinase domain is tethered to the plasma membrane via a myristylation sequence that replaces the extracellular ligand binding and transmembrane domains. Removal of the extracellular ligand binding domain eliminates interactions between endogenous FGF ligands and the iFGFR1 construct and allows for complete control over activation of iFGFR1. Dimerization and subsequent activation of the kinase domain is instead stimulated through a synthetic dimerization system affixed to the kinase domain. Thus, dimerization occurs upon treatment with the synthetic molecule, AP20187 (AP, renamed B/B when bought by Clontech, Mountain View, CA, USA), which traverses the plasma membrane and binds the intracellular dimerization sequences of two iFGFR1 monomeric constructs, allowing for transphosphorylation of kinase domain tyrosine residues.

To ensure that the iFGFR1 construct correctly mimicked endogenous FGFR1, the mouse mammary epithelial cell line, HC11, was retrovirally transduced to express *iFGFR1*. Analysis of the cells stably expressing the *iFGFR1* construct, named HC11/R1 cells, confirmed that the iFGFR1 kinase domain, docking protein FRS2, and downstream signaling proteins Akt and ERK1/2 were all phosphorylated following treatment with AP [127]. These results demonstrate that iFGFR1 activation results in activation of endogenous signaling pathways. Functional studies showed that HC11/R1 cells treated with AP

displayed increased motility and proliferation and decreased apoptosis when grown in a monolayer as compared to controls [127, 128]. Furthermore, AP treatment of the HC11/R1 cells grown in three dimensional culture resulted in increased acinar size, increased proliferation, acquired invasiveness, disrupted cellular polarity, and decreased apoptosis as compared to controls [128]. iFGFR1 activation also resulted in increased expression of matrix metalloprotease 3 (MMP-3) and E-cadherin cleavage, both of which are required for epithelial-to-mesenchymal transition (EMT) to occur [128]. All together, these results indicate that activation of iFGFR1 *in vitro* promotes several cellular processes that are characteristically co-opted in tumorigenesis.

In addition to the creation of the *in vitro* HC11/R1 cell line, the Rosen laboratory also generated iFGFR1 transgenic mice using *MMTV* to drive expression of the *iFGFR1* construct in mammary epithelial cells [127]. While activation of iFGFR1 with intraperitoneal injections of AP did not result in gross morphological changes in ductal morphogenesis as compared to age-matched nontransgenic control mice, there was aberrant formation of lateral buds along primary, secondary, and tertiary ducts after only 48 hours of AP treatment [127, 129]. Prolonged AP treatment led to more dense epithelial lateral budding and eventually to invasive, preneoplastic lesions most likely due to the detectable increase in proliferation in these mammary glands [127]. Similar to *in vitro* results, activation of iFGFR1 *in vivo* induced activity of MMP-2 and MMP-9, suggesting that extended iFGFR1 activation leads to increasingly invasive

properties [127]. Interestingly, it was also found that the iFGFR1-stimulated lateral budding and epithelial proliferation required the presence of macrophages [129]. The idea that recruitment of immune cells may be crucial for iFGFR1-induced tumorigenic phenotypes is currently under further investigation.

1.4 Amphiregulin

1.4.A Discovery and characterization of Amphiregulin.

Amphiregulin (AREG) was originally discovered by Shoyab and colleagues attempting to identify proteins produced in response to the tumor promoting phorbol ester, phorbol 12-myristate 13-acetate (PMA), treatment of the human breast cancer cell line MCF7 [130]. Characterization of AREG revealed that it contains a characteristic set of six spatially conserved cysteine residues that interact and form disulfide bonds resulting in three structural loops essential for interaction with EGFR, thus identifying it as another member of the EGF family of ligands [131, 132]. AREG was confirmed as an EGFR ligand through competitive binding studies with ^{125}I-EGF, where it was found to be able to partially compete with ^{125}I-EGF for binding to EGFR [131]. Other studies demonstrated that AREG actually exclusively binds to and activates EGFR, although it can also activate the other ErbB receptors, ErbB2/HER2/neu, ErbB3, and ErbB4, in an EGFR-dependent manner, perhaps by promoting receptor heterodimerization [133-135]. In addition to its conserved EGF motif in the N-terminal domain, AREG also contains a transmembrane domain characteristic of the EGF family of ligands and is initially synthesized as a 252 amino acid transmembrane precursor protein [132]. This precursor protein is usually cleaved from the membrane by a sheddase and released as a soluble, mature protein, whereby it exerts its activity in both autocrine and paracrine fashions, but it is thought that AREG may also be able to act in a juxtracrine manner [136-138].

There are several isoforms of AREG due to post-translational modifications, however it is not known whether all of the isoforms contain biological activity or not [131, 139].

In humans, the gene encoding *AREG* is located on the long arm of chromosome 4 at 4q13-4q21 [132]. Within the *AREG* promoter, there are several possible transcription factor docking sites and responsive elements, including cyclic AMP (cAMP) responsive element, estrogen responsive element, serum responsive element, specificity protein 1 element, and Wilms' tumor suppressor responsive element, suggesting that there may be multiple avenues of AREG expression regulation [132, 140-145]. The *AREG* transcript is 1.4kb and contains six exons. Exons one through three possess the 5'UTR, two putative nuclear localization signals, and two of the three disulfide loops. The third disulfide loop is located within the fourth exon, as is the transmembrane domain. The fifth exon contains the cytoplasmic domain, and the sixth exon includes the 3'UTR. The putative nuclear localization signals appear to be real, as AREG has been found via immunohistochemistry both in the cytoplasm and in the nucleus of some cell types, but it is yet to be determined whether AREG exhibits any nuclear biological function [131, 146, 147]. The C-terminal domain is essential for proper AREG membrane localization of the transmembrane precursor protein [148]. *AREG* transcript is expressed in many different tissues, including skin, ovaries, placenta, gastrointestinal tract, pancreas, kidney, lung, and mammary gland [132]. Within these tissues, AREG is involved in many different processes such

as epidermal hyperplasia, blastocyst implantation, nerve regeneration, and mammary gland development [19, 20, 149-153]. The role AREG plays in normal mammary gland development has been the most extensively studied.

1.4.B AREG and its regulation in mammary gland development.

Areg knockout (KO) mice have provided valuable insight into the role of AREG in the developing mammary gland. Luetteke and colleagues generated *Areg*, *Egf*, and *Tgfα* individual and combinatorial KO mice [20]. While triple KO animals were viable, healthy, and fertile, pups born to triple KO mothers were often runted or died shortly after birth with little or no milk spots, suggesting that the triple KO mothers possessed some sort of mammary gland defect. Upon comparison of pubertal triple KO mammary glands to wildtype (WT) mammary glands, it was found that the ductal trees of the triple KO glands had not progressed past the small embryonic ductal structures, while the WT glands had clearly undergone normal pubescent ductal elongation and branching. Additionally, WT glands contained many TEBs, but only a few TEBs were visible in the mammary glands of the triple KO animals. Furthermore, glands from double KO mice of both *Egf* and *Tgfα*, but retaining at least one copy of *Areg*, were comparable to glands from WT mice. However, while triple KO females also exhibited severe lobuloalveologenesis defects, *Areg*-only KO females were capable of undergoing lobuloalveolar proliferation and differentiation. In corroboration with the findings of Luetteke and collegues, Ciarloni and colleagues

also found that mammary gland development was similar in *Areg*$^{-/-}$ and WT mice up to puberty at which time WT glands underwent ductal morphogenesis but *Areg*$^{-/-}$ glands did not [154]. It was also found that TEB formation and ductal epithelial cell proliferation were abolished in *Areg*$^{-/-}$ animals, but that AREG depletion did not significantly affect lobuloalveologenesis. Moreover, chimeric transplantation of both WT and *Areg*$^{-/-}$ mammary epithelial cells into cleared mammary fat pads demonstrated the ability of AREG to act in a paracrine manner as *Areg*$^{-/-}$ cells were still able to proliferate as long as they were within close proximity to WT cells. Together these results suggest that AREG is the primary EGFR ligand responsible for normal ductal elongation but is not necessary for lobuloalveologenesis. Instead, other EGFR ligands, such as EGF and TGFα are important for lobuloalveologenesis. Work done by Kenney and colleagues supports the paracrine function of AREG in the mammary gland as exogenous AREG, administered by implantation of slow-release pellets, was able to reestablish ductal morphogenesis in ovariectomized mice [19]. To establish whether AREG is required in the epithelial or stromal compartment of the mammary gland, Sternlicht and colleagues performed mammary transplants using *Areg*$^{-/-}$ epithelial cells embedded in either WT or *Areg*$^{-/-}$ fat pads or WT epithelial cells embedded in either WT or *Areg*$^{-/-}$ fat pads [107]. While the WT epithelial cells were able to grow regardless of the stromal compartment genotype, the *Areg*$^{-/-}$ epithelial cells were unable to grow in either stromal compartment, suggesting that AREG is required in the mammary epithelium for

normal ductal morphogenesis. Recent studies have suggested that AREG might be such a potent growth factor in the mammary gland because it might mediate the growth and self-renewing capacity of a subset of mammary epithelial progenitor cells [155].

In the normal developing mammary gland of the mouse, AREG is the only EGFR ligand to be highly expressed from the beginning of puberty at about four weeks to around 12 weeks of age, which coincides with the phase of highest mammary gland ductal morphogenesis [156]. At this time, estrogen production has also begun in the ovaries, suggesting that circulating estrogen may mediate AREG expression in the mammary gland. This notion was strengthened by the discovery of estrogen response elements within the *Areg* promoter region and by chromatin immunoprecipitation (ChIP) analysis showing direct ERα binding of the *Areg* gene [145, 157]. Additionally, estrogen treatment of the ERα-positive breast cancer cell line MCF7 stimulated increased *AREG* transcript and protein expression [158]. *In vivo* studies using *Erα* KO mice revealed that ductal morphogenesis of the mammary gland was severely stunted but could be rescued by exogenous AREG [15]. This impairment of ductal morphogenesis is mimicked in *Areg* KO mice [154]. Furthermore, it was found that *Erα* was required in the mammary epithelium, but dispensable in the stroma, for proper ductal morphogenesis [15]. Not only does the epithelial requirement of ERα correspond with *Areg* expression occurring exclusively in the mammary epithelium, but the ER transcriptional co-activator, CITED1, also has the same

expression pattern as *Areg* in the developing mammary gland [20, 157, 159, 160]. Perhaps not surprisingly, *Cited1* KO mice display the same ductal morphogenesis impairment as *Erα* KO and *Areg* KO mice, and AREG expression in the mammary gland is reduced in these animals [161]. It has also been shown that $Er\alpha^{-/-}$ cells can proliferate when in close proximity to $Er\alpha^{+/+}$ cells much like $Areg^{-/-}$ mammary epithelial cells can proliferate if in close proximity to $Areg^{+/+}$ cells, signifying that epithelial cell proliferation in the mammary gland is regulated in a paracrine fashion [15, 154].

As previously stated, AREG initially exists as a transmembrane precursor protein that is usually subsequently cleaved from the membrane to exert its activity in an autocrine or paracrine manner. This is common amongst all EGF family ligands, and much work has been done to identify the enzymes responsible for this cleavage [162-164]. Because of their membrane bound nature, the a disintegrin and metalloprotease (ADAM) enzymes were attractive candidates for EGF family ligand release, and studies have demonstrated that ADAM17 (also known as tumor necrosis factor α (TNFα) converting enzyme (TACE) because it was originally discovered to cleave TNFα) is the primary AREG sheddase [107, 164-166]. Several lines of evidence give credence to the idea that ADAM17 is also required for proper mammary gland development. $Adam17^{-/-}$ pups have reduced numbers of ductal branches that are also shorter than their WT littermates, a similar phenotype to that observed in $Egfr^{-/-}$ mammary glands grown under the renal capsules of immuno-compromised host

animals [107, 167]. Additionally, like *Areg*, *Adam17* expression was required in the mammary epithelial compartment, but not in the stroma, for proper mammary gland development [107]. Therefore, ADAM17 and AREG are expressed and essential in the same cells of the developing mammary gland, which is a vital feature for the membrane bound ADAM17 to be able to cleave the also membrane bound AREG precursor protein. Furthermore, a requirement for stromal EGFR, but not epithelial EGFR, has been identified in the mammary gland [107, 167]. As EGFR is the only AREG receptor, these findings augment the necessity for ADAM17 to cleave AREG from the mammary epithelial cells because the ductal epithelium and the stroma are separated from one another by a basement membrane in the mammary gland.

In combination, these studies demonstrate that ERα is a direct mediator of AREG expression in mammary epithelial cells during the period of pubertal ductal growth and expansion. ADAM17 sheds AREG from mammary epithelial cells so that it can act in a paracrine fashion to induce ductal morphogenesis by activating its receptor, EGFR, in the stroma. It is not yet clear how EGFR activation in the stroma then regulates epithelial cell proliferation.

1.4.C AREG in breast cancer.

Because of its significant role in normal mammary gland development, AREG has attracted a lot of attention in the breast cancer field. In the normal developing mammary gland, *Areg* is expressed in the epithelial compartment

[107]. AREG has been found to remain localized to the epithelial tumor cells in breast cancers and to be upregulated in the preneoplastic HELU lesions relative to the normal TDLUs [168, 169]. Estrogen treatment of breast cancer cell lines can induce AREG transcript and protein expression in breast cancer cell lines, suggesting that ERα continues to be a mediator of *AREG* expression in breast cancers [158, 170, 171]. In line with such results, AREG often correlates with ERα and PR positivity in breast cancers [172, 173]. Interestingly, it has been shown that parity, a well-established protector of young women from breast cancer, reduces AREG expression [159]. As AREG is a growth factor, it is not surprising that it has been shown to stimulate proliferation of breast cancer cell lines [138, 174, 175]. Further demonstrating AREG's ability to stimulate tumor growth, it was found that knockdown of *AREG* in the human breast cancer cell line NS2T2A1 resulted in significantly reduced tumor burden in xenograft mice [176]. Additionally, treatment of breast cancer cells with AREG induces increased expression of numerous genes involved in metastasis and invasion, most notably MMP-9 [138, 174, 177]. Furthermore, shRNA knockdown of *AREG* in human breast cancer cells reduced the ability of these cells to invade into a Matrigel matrix [178]. These results implicate AREG expression in more advanced or aggressive breast cancers, and indeed high AREG expression is commonly found in lymph node positive breast cancers and in invasive breast carcinomas [169, 179, 180]. Studies have also revealed that AREG appears to be involved in resistance to targeted therapies and chemotherapies in breast cancer, further

supporting the findings that AREG is linked to a more advanced disease state [145, 181, 182].

1.5 Epiregulin

Epiregulin (EREG) was originally discovered in the conditioned media of the mouse tumorigenic cell line, NIH3T3/clone T7 [183]. Upon investigation into the sequence, a set of six spatially conserved cysteine residues, as well as a key leucine residue, were identified that marked EREG as the newest member of the EGF family of ligands [183]. In humans, the *EREG* gene is located on the long arm of chromosome 4 near the genes for AREG and betacellulin. Because of the close proximity of these genes, it has been suggested that gene duplication occurred at some point in evolutionary history [184]. Perhaps in favor of such an occurrence, characterization of EREG uncovered an N-terminal localization sequence, followed by the EGF domain containing the three loop structures that are the hallmark of the EGF family of ligands, followed by a transmembrane domain [185]. Thus, like all other EGF family ligands, EREG is initially synthesized as a transmembrane precursor protein. However, due to its discovery as a biologically active and mature protein in conditioned media, EREG must be shed from the plasma membrane. As other members of the EGF family of ligands are cleaved by ADAMs, the putative role of ADAMs in releasing EREG was determined [162, 166]. It is thought that ADAM17 might be the major EREG sheddase, although ADAMs 9, 12, and/or 15 also appear to be able to cleave EREG [164]. In its soluble form, EREG can bind to and activate both EGFR and ErbB4 [186, 187]. Even though it does not directly bind to HER2 and ErbB3, EREG does stimulate their phosphorylation, suggesting that EREG can promote

ErbB receptor heterodimerization [186, 187]. Interestingly, although structurally similar to other EGF family ligands, the expression profile of *Ereg* is not very comparable to the other members of the EGF family of ligands. While most EGF family ligands are fairly ubiquitously expressed, *Ereg* transcript expression is much more limited, detectable only in macrophages, the placenta, and during early embryogenesis [185, 188]. Because of its expression in the early mouse embryo, it was originally hypothesized that EREG might be a potent developmental regulator [185].

In spite of its limited expression in normal tissues, EREG has been shown to stimulate proliferation in a variety of cell types, including primary hepatocytes, mouse fibroblasts, keratinocytes, corneal epithelial cells, and vascular smooth muscle cells [183, 189-192]. It also promotes proliferation of pancreatic carcinomas, and *EREG* knockdown in conjunction with knockdown of *N-RAS* confers a decreased growth potential in human hepatoma cells [193, 194]. Furthermore, expression of EREG in oral squamous cell carcinomas has been linked to poor prognosis, and in non-small cell lung cancers (NSCLCs), EREG expression appears to be a marker for advanced disease state [195, 196]. While EREG has not been extensively studied in breast cancer, EREG expression has been identified as an indicator of poor prognosis for inflammatory breast cancer patients and to negatively correlate with HR+ breast cancers [172, 197]. Studies have also demonstrated that EREG can promote not only the proliferation of breast cancer cells but also their ability to metastasize to the lung when

expressed with cyclooxygenase 2 (COX2), MMP-1, and MMP-2 [198, 199]. EREG also conferred an increased invasive potential in NSCLCs [195]. Together, these findings suggest involvement of EREG in cellular processes of proliferation, migration, and invasion.

1.6 Epidermal Growth Factor Receptor

1.6.A The epidermal growth factor receptor protein.

Epidermal growth factor receptor (EGFR) was originally discovered in the early 1980's as a result of the ability of EGF to bind and initiate phosphorylation of EGFR [200, 201]. Since its discovery, it has been determined that EGFR is member of the ErbB family of receptor tyrosine kinases, consisting of ErbB1/EGFR, ErbB2/HER2/neu, ErbB3, and ErbB4. Each receptor contains an extracellular ligand binding domain, a transmembrane domain, and a tyrosine-rich intracellular kinase domain. The extracellular ligand binding domain can be subdivided into four distinct regions: two large EGF binding domains and two cysteine-rich domains. Ligand binding induces a conformational change of the extracellular domain into what is called the extended confirmation. In the extended confirmation, the dimerization domain is exposed and both homodimerization and heterodimerization can occur. Dimerization allows the intracellular kinase domains of each monomer to stabilize and phosphorylate the other monomer's kinase domain. Which tyrosine residues are phosphorylated is determined by which ligand induces dimerization and which monomers dimerize. Subsequent to autophosphorylation, downstream effector molecules containing either a Src-homology domain 2 (SH2) or a phospho-tyrosine binding (PTB) motif bind the intracellular domain. There are several such binding proteins, including Ras, Src, PI3K, Grb2, Cbl, PLCγ, which then further activate molecules like the STAT3 or STAT5 transcription factors, ERK1/2, or Akt. Through such signaling

pathways, EGFR activation mediates many different cellular processes, such as survival, migration, and proliferation, or events like receptor recycling and degradation. EGFR structure, activation, and signaling in normal and disease models has been extensively studied and reviewed [202-209].

1.6.B EGFR in normal mammary gland development.

EGFR has long been known to play a role in normal mammary gland development. Characterization of *waved-2* mice, which exhibit skin, hair, and eye abnormalities, led to the identification of a single nucleotide transversion within the kinase domain of EGFR, which resulted in reduced EGFR kinase activity [210, 211]. In addition to the obvious external phenotypes, *waved-2* mice were also found to have impaired mammary gland development [211]. Moreover, whole mammary glands taken from *Egfr*$^{-/-}$ neonates and grown under the renal capsules of immuno-compromised mice displayed impaired mammary gland development [167]. In these glands, ductal growth and branching was severely reduced compared to WT glands, but lobuloalveolar development still occurred, suggesting that EGFR is only required during pubertal mammary gland development [167]. This is consistent with the necessity of the EGFR ligand, AREG, in the pubertal mammary gland [20, 154]. Although AREG is expressed and essential in mammary epithelial cells, much work has been done that demonstrates a stromal EGFR requirement. Mammary glands deficient for the AREG sheddase ADAM17 display stunted mammary ductal morphogenesis

similar to that seen in *Egfr*$^{-/-}$ animals, suggesting that AREG must be released from the epithelium to exert its activity [107]. Together with fact that the mammary epithelium and stromal compartments are separated by a basement membrane, these results are indicative of different compartmental requirements of AREG and EGFR. Additionally, EGFR is abundantly expressed in the mammary gland stroma, phosphorylated EGFR can be seen in the mammary stroma, and exogenous EGF can stimulate EGFR activation in epithelial cell-free mammary gland fat pads [18, 20, 212]. It has also been shown that EGFR is activated most highly in the stroma directly surrounding the TEBs, where ductal morphogenesis ensues [213]. While these studies imply that stromal EGFR is important, they do not show that EGFR is required in the stroma. However, studies performed using recombinant transplants of combinations of *Egfr*$^{-/-}$ and WT epithelium and stroma do provide direct evidence of a stromal EGFR requirement. While both *Egfr*$^{-/-}$ and WT mammary epithelium develop normal mammary gland ductal structures in WT fat pads, neither epithelial genotype undergoes ductal morphogenesis when transplanted into *Egfr*$^{-/-}$ fat pads [107, 167]. Therefore, stromal EGFR is essential for mammary gland ductal morphogenesis, but is dispensable during lobuloalveolar development, and epithelial EGFR is not required for proper mammary gland development. How stromal EGFR regulates epithelial cell growth is still currently unknown, although it has been proposed that because growth factors such as hepatocyte growth factor (HGF), IGF-1, and FGF10 are all expressed in the stroma during ductal

morphogeneis, they may represent good candidates of EGFR-induced growth factor-mediated epithelial cell growth [154].

1.6.C EGFR in breast cancer.

As a regulator of many cellular processes, including proliferation, survival, migration, invasion, and apoptosis, which are often dysregulated in the initiation and progression of cancer, an expansive amount of research has been done to examine EGFR in tumorigenesis, resulting in the identification of EGFR as a key factor in many types of cancer. Most notably, many gliomas present with EGFR overexpression and a mutant, constitutively active form of EGFR is often found in NSCLCs. EGFR has also been extensively investigated in breast cancer. Using transgenic animals with *Egfr* under the control of either the *MMTV* or the *β-lactoglobulin* promoter, it has been shown that overexpression of EGFR in the mammary gland leads to epithelial hyperplasias in virgin mice which progressively worsen to dysplasias and tubular adenocarcinomas in mice that are lactating, with highest EGFR expression in the adenocarcinomas [214]. However, unlike in gliomas and NSCLCs, EGFR is rarely overexpressed or mutated in breast cancers, although it does appear to be expressed in 18-36% of all breast cancers [215-219]. Interestingly, owing to the fact that the EGFR ligand AREG is regulated by ERα in the mammary gland, many studies have found that EGFR positivity in breast cancer samples is inversely correlated with ER positivity [215, 216, 218, 220-222]. Alternatively, EGFR and HER2 expression

and phosphorylation appear to overlap in many primary breast tumors [223, 224]. While several studies have tried to determine whether EGFR could be used as a prognostic factor, the results have varied. Most studies found that EGFR is indicative of poor prognosis, and correlates with more aggressive or advanced disease [215, 216, 218, 220, 225-227]. In support of these findings, EGFR expression and activation has been linked to increased migration, which is a defining feature of more advanced disease [228-230]. Other studies have identified no link between EGFR status and prognosis or even favorable prognosis with EGFR expression [217, 223, 231]. The discrepancies of these studies may be the result of the various techniques or antibodies used to determine EGFR expression or perhaps because of having been done before the relatively recent categorization of breast cancer into several subtypes based on molecular profiling. Since the advent of breast cancer as multiple distinct diseases, EGFR expression has been indicated to occur in 50-70% of basal-like and/or TNBCs [72, 232]. In fact, EGFR has been proposed to be a marker of basal-like breast cancer, and studies have indicated that basal-like tumors that are EGFR+ have a worse prognosis than TN basal-like tumors [68, 73]. The connection of EGFR with basal-like/TNBCs is important as these cancers currently lack targeted therapies but often do not respond well to chemotherapy regimens, and clinical trials have commenced to determine whether EGFR inhibitors are of benefit to patients undergoing chemotherapy [50, 51].

1.7 Signal Transducer and Activator of Transcription 3

1.7.A Signal transducer and activator of transcription 3 discovery, structure, and activation.

Signal transducer and activator of transcription 3 (STAT3) was originally discovered almost 20 years ago now as an acute phase response factor (and thusly originally named APRF) following interleukin 6 (IL-6) stimulation of hepatoma cells [233]. It was found that APRF binds IL-6 response elements on DNA and regulates transcription of several IL-6 target genes [233, 234]. Cloning of *APRF* identified it as a STAT protein due to high sequence and domain homology with and a similar expression pattern to STAT1 [235, 236]. The STAT family of proteins is now comprised of seven factors that, like the name suggests, relay signals through their transcription factor activity. Sequence comparisons, mutagenesis studies, and crystallography data have together established the structure and modularity of the STAT proteins [237-240]. The amino terminus of each STAT appears to function in stabilizing dimer:dimer interactions on DNA for transcriptional activation [241]. Tetrameric formation of the STATs on adjacent STAT-binding sites strengthens STAT:DNA binding and subsequent transcription [242-244]. Other studies have found additional roles of the amino terminus, including regulation of nuclear transport and subsequent dephosphorylation [245, 246]. Following the amino terminus, there is a coiled-coil domain that appears to be involved in protein:protein interactions. The DNA binding domain resides in the middle of each STAT molecule followed by a linker domain that possibly

reinforces STAT:DNA binding [247]. The SH2 domain of each STAT protein then precedes the transactivation domain (TAD) at the C-terminus. The SH2 domain is required for STATs to interact with activated receptors for successive activation and for successful STAT dimerization. Between the SH2 domain and the TAD, lies the critical tyrosine residue of each STAT protein. This is tyrosine 705 in STAT3 molecules. Additional phosphorylation at a serine residue (serine 727) may be important for maximal transcriptional activity [248]. The TAD interacts with co-activators such as p300/CBP (CREB-binding protein) and is therefore also imperative for proper transcriptional activity [249].

The STAT proteins are predominantly found as latent, unphosphorylated monomers within the cytoplasm, although some work has shown that they can also exist as preformed dimers and/or within larger protein complexes that may possibly act to facilitate STAT redistribution to activated receptors [250-254]. Interestingly, studies have indicated that instead of being entirely cytoplasmic, STAT3 might continuously shuttle back and forth from the nucleus to the cytoplasm [255, 256]. Several cytokines and their receptors, growth factors and their receptors, and non-receptor tyrosine kinases are known to activate the STAT proteins by tyrosine phosphorylation-induced dimerization. For example, it is well known that STAT3 can be activated by EGF binding to EGFR, platelet-derived growth factor (PDGF) binding to the PDGF receptor (PDGFR), and Src [235, 257-260]. Canonical activation of STAT3 occurs via a Janus kinase (JAK) that is non-covalently bound to a cytokine receptor unit, such as glycoprotein-130

(gp130) [234]. Because cytokine receptors lack enzymatic activity, they rely on intracellular non-receptor kinases (i.e. JAKs) to propagate cytokine binding signals. Several cytokines, including IL-6, IL-10, leukemia inhibitory factor (LIF), and oncostatin M (OSM), can bind gp130 and induce either homodimerization with another gp130 receptor unit or heterodimerization with a different cytokine receptor unit, such as the IL-6 receptor [234, 236, 261-263]. Receptor dimerization initiates JAK transphosphorylation, and the now activated JAKs can then phosphorylate the receptor at up to six intracellular tyrosine residues. These phosphorylated tyrosines now act as docking sites for the STAT proteins via their SH2 domains. Docked STAT monomers are then phosphorylated by the JAKs on their highly conserved tyrosine residue leading to rapid dimerization of two STAT molecules via their phosphorylated-SH2 domains. All STAT proteins homodimerize, although STAT1:STAT2 and STAT1:STAT3 heterodimers have also been observed. In their now active dimer state, the STATs translocate to the nucleus, where they undergo facilitated transport into the nucleus. In the nucleus, STATs bind to both DNA and co-activators and mediate transcription of many target genes. STAT3 is known to target genes involved in basically all cellular processes, including proliferation, angiogenesis, apoptosis, inflammatory response, and migration, and dysregulation of STAT3 is associated with many types of cancer.

1.7.B STAT3 in breast cancer and the normal mammary gland.

Evidence from primary breast cancer samples and breast cancer cell lines indicates that STAT3 is constitutively activated in 50-60% of all breast cancers regardless of HR or HER2 status [264-270]. Data demonstrate that the primary mode of STAT3 activation in breast cancer occurs via IL-6 signaling through its gp130 receptor to activate JAKs in both autocrine and paracrine fashions [270-272]. Interestingly, STAT3 activation appears to be dispensable for breast tumor initiation [273, 274]. However, STAT3 activation has been linked to increased proliferation of breast cancer cells, potentially through its concurrent upregulation of genes such as *Cyclin D1* and *c-myc* [269, 271, 275, 276]. Strengthening these results, it has been shown that blocking STAT3 either directly or indirectly leads to attenuated breast cancer cell growth [266, 272, 277]. Furthermore, constitutive activation of STAT3 has been shown to enhance migration and metastasis of breast cancer cells, while inhibition of STAT3 leads to decreased anchorage-independent growth and metastatic potential of mammary tumor cells both *in vitro* and *in vivo* [273-275, 278, 279]. These effects are most likely mediated via the STAT3-induced expression of multiple *MMPs* [269].

Because of its aberrant activation in cancer, the potential of pSTAT3 to be a prognostic factor of breast cancer has been investigated. Immunohistochemistry (IHC) analysis of pSTAT3 demonstrates that nuclear localization of pSTAT3 correlates with increased short- and long-term survival of both node-negative and node-positive primary breast cancers and can be used

as an independent prognostic marker of improved survival [265, 280]. However, it was alternatively found that pSTAT3 correlates with incomplete pathological response of patients to chemotherapy, indicating that STAT3 activation is inversely correlated with chemosensitivity [264]. In fact, many studies have demonstrated that STAT3 activation confers breast cancer cell survival by directly activating Survivin (a member of the Inhibitor of Apoptosis family of proteins) and several anti-apoptotic members of the B cell CLL/lymphoma-2 (BCL-2) family, including BCL-2, myeloid cell leukemia 1 (MCL-1), and BCL-2-related gene, long isoform (BCL-xL) [264, 269, 275, 281, 282]. Moreover, STAT3 activation correlates with expression of these molecules in invasive breast cancers and in patients that do not respond well to chemotherapy, suggesting that STAT3 activation is a common occurrence in chemoresistance [264, 269]. Indeed, several studies have demonstrated that either direct or indirect inhibition of STAT3 restores or increases chemoresponsiveness of breast cancer cells and increases breast cancer cell apoptosis [277, 279, 281-284].

It is interesting that STAT3 activation inhibits apoptosis of breast cancer cells because STAT3 in the normal mammary gland is required to initiate apoptosis of epithelial cells. Following weaning, STAT3 activation is increased dramatically and is imperative for the subsequent process of involution that occurs when milk production is no longer necessary. Cre-mediated *Stat3* removal in the mammary gland via β-lactoglobulin Cre results in a severe delay of involution and a decrease in apoptosis [34, 285]. It is thought that this STAT3-

induced involution of the mammary gland proceeds initially through LIF and is sustained via STAT3-mediated autocrine OSM signaling [262, 263]. Interestingly, even though STAT3 is a known activator of the anti-apoptotic protein, BCL-xL, removal of STAT3 in the mammary gland does not appear to greatly alter the levels of BCL-xL [34, 269, 285]. Instead, there is decrease in insulin-like growth factor-binding protein-5 (IGFBP-5), which acts to inhibit survival signaling through IGF-1 by sequestering IGF-1 during involution, in STAT3 depleted mammary glands [34].

1.8 Thesis Goal

The results of the studies from published literature described thus far demonstrate how the controlled regulation of several factors is essential for proper mammary gland formation and how the dysregulation of even just one factor can easily elicit a chain reaction of dysregulation that consequentially results in breast cancer. It is the goal of my research to gain a better understanding of how amplification of FGFR1 ultimately mediates breast cancer progression. As there are no currently available inhibitors that are specific for FGFR1, identification and examination of FGFR1-induced genes and how these molecules all interact could direct us to novel or more effective targets for therapies. This area of investigation is fundamental because of the clear need for more targeted therapies, especially for patients with TNBCs.

Chapter 2: Fibroblast growth factor receptor-1 induced mammary tumorigenesis requires activation of the epidermal growth factor receptor

2.1 Introduction

The fibroblast growth factor receptor (FGFR) family consists of four receptor tyrosine kinases that are known regulators of cellular processes such as proliferation, migration, survival, and angiogenesis [91, 96, 286, 287]. Anomalous expression or uncontrolled activation of these receptors or their ligands has been correlated with progression of various types of cancer, including breast cancer [113, 288, 289]. Specifically, the chromosomal locus of *FGFR1*, 8p11-12, is found to be aberrantly amplified in approximately 10% of patients diagnosed with breast cancer [116, 119, 290, 291]. Patients who harbor the *FGFR1* amplification do not respond well to current therapies and develop resistance to hormone-based therapies [120, 121]. Therefore, understanding the molecular mechanisms of how FGFR1 overexpression promotes tumorigenesis may provide insights into better targets for novel, more effective therapies.

Because of the lack of binding specificity amongst the FGF ligands and their receptors, it is difficult to specifically activate FGFR1. Therefore, an inducible FGFR1 (iFGFR1) system was previously engineered to mimic endogenous FGFR1 signaling [127]. Because the extracellular ligand-binding

domain was removed, dimerization and activation of iFGFR1 is stimulated not by FGF ligands but by the synthetic molecule AP20187 (AP). Binding of AP to iFGFR1 results in homodimerization and subsequent activation of the same signaling pathways as endogenous FGFR1. Extensive characterization of the iFGFR1 system *in vitro* found that, following AP treatment of mouse mammary epithelial cells expressing iFGFR1, there was an increase in cellular migration, proliferation, and signaling through the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway, similar to endogenous FGFR1 activation [127, 128, 292]. The inducible system was also analyzed *in vivo*. For these studies, a transgenic mouse line was created in which the *iFGFR1* construct was placed under the control of the mouse mammary tumor virus (*MMTV*) promoter, thereby restricting *iFGFR1* expression to mammary epithelial cells [127]. MMTV-iFGFR1 transgenic animals developed neoplasias within as little as three days of AP treatment, which were characterized by aberrant budding of the mammary gland ductal epithelial cells due to increased cellular proliferation. Extended AP treatment led to progressively more severe phenotypes, and by four weeks of iFGFR1 activation, the mammary glands of transgenic animals contained large, locally invasive growths. These results verify that iFGFR1 activation promotes tumorigenic phenotypes both *in vivo* and *in vitro*. To identify mechanisms by which FGFR1 promotes tumorigenesis, previously published studies have described the results of microarray studies that were performed on mammary glands from MMTV-iFGFR1 transgenic mice following iFGFR1 activation [129]. In

the current study, we explore the ability of two targets identified in the screen, amphiregulin (AREG) and epiregulin (EREG), to promote FGFR1-induced mammary tumorigenesis.

AREG and EREG are both members of the epidermal growth factor (EGF) family of ligands [reviewed in 293]. These ligands are originally synthesized as transmembrane proteins and are thought to be cleaved from the plasma membrane by a disintegrin and metalloproteinase (ADAM) sheddases [107, 132, 136, 162, 164, 183, 185]. Furthermore, both ligands are known activators of the EGF receptor (EGFR); AREG exclusively binds to and signals through EGFR [131], whereas EREG can also bind to and signal through ErbB4 [186, 187]. AREG is critical for normal ductal morphogenesis in the mammary gland [20, 154] and has also been linked to breast cancer progression [174, 176, 294, 295]. Studies examining AREG expression in human breast cancers have found AREG expression to significantly correlate with regional lymph node metastases, large tumor size, and high-grade tumors [169, 180, 296]. While EREG promotes proliferation of several normal and cancerous cell types [188, 190, 192, 193], the role of EREG has not been extensively characterized in the mammary gland. However, recent studies have demonstrated that EREG is a potent mediator of metastasis of breast cancer cells to the lung and that overexpression of EREG is an indicator of poor prognosis for inflammatory breast cancer patients [197-199].

EGFR, a member of the ErbB receptor tyrosine kinase family, has been well studied in numerous systems [203, 297]. In the mammary gland, it is known

that EGFR is required for normal mammary gland ductal morphogenesis [167, 298]. However, overexpression or constitutive activation of EGFR in the mammary gland has been linked to mammary tumorigenesis [214, 299, 300]. Additionally, overexpression of EGFR in the breast is associated with recurrence of earlier stage breast cancers and decreased disease-free and overall survival in later stage breast cancer patients [301-305]. Therefore, together with the fact that iFGFR1 activation in the mouse mammary gland of MMTV-iFGFR1 transgenic animals leads to upregulation of the EGFR ligands AREG and EREG, these data strongly support a model in which aberrant FGFR1 signaling requires EGFR activation for mammary tumor formation, a link that has not been previously established. Moreover, the EGFR kinase inhibitor erlotinib is currently an approved therapeutic for both non-small cell lung cancer (NSCLC) patients and advanced pancreatic cancer patients [reviewed in: 306, 307]. However, erlotinib has not been fully tested as a functional therapy for breast cancer patients.

Our current studies focus on identifying downstream mediators of iFGFR1-induced mammary tumorigenesis. We have found that treatment of HC11 mouse mammary epithelial cells with either AREG or EREG promotes increased migration, proliferation, and ERK1/2 activation *in vitro*. Importantly, the ability of these ligands to stimulate migration and proliferation *in vitro* requires activation of EGFR, as inhibiting the EGFR kinase blocks these processes. We also show that inhibiting EGFR *in vivo* blocks iFGFR1-mediated cellular proliferation of mouse

mammary epithelial cells. These studies demonstrate that activation of EGFR is important for effective FGFR1-induced mammary tumorigenesis. Because there are currently no FGFR1-specific drugs that are used clinically, our studies suggest that inhibition of EGFR might represent a useful strategy for targeting breast cancers harboring *FGFR1* amplifications.

2.2 Materials and Methods

2.2.A Cell culture and treatment.

Generation of HC11 cells stably expressing the *iFGFR1* construct (HC11/R1 cells) was described previously [128], and the cells were obtained from Dr. Jeff Rosen (Baylor College of Medicine, Houston, TX, USA). Cells were maintained in HC11/R1 complete media [serum free (SF)-RPMI (Invitrogen, Carlsbad, CA, USA) supplemented with final percentages/concentrations of 10% FBS (Invitrogen), 1% Pen Strep (Invitrogen), 5 µg/mL Insulin (Sigma-Aldrich Corporation, St. Louis, MO, USA), 10 ng/mL EGF (Invitrogen), and 0.7 µg/mL Puromycin (Sigma-Aldrich Corporation)]. HC11 cells were maintained in HC11 complete media (HC11/R1 complete media without Puromycin). Prior to treatment with 30 nM AP (Ariad Pharmaceuticals, Cambridge, MA, USA) or its ethanol solvent, confluent cells were rinsed twice with 1xPBS (Cellgro, Manassas, VA, USA) and incubated in SF-RPMI overnight. Two µM erlotinib (Boynton Pharmacy, University of Minnesota, Minneapolis, MN, USA), 1 µM erlotinib, 0.5 µM erlotinib, or DMSO (Sigma-Aldrich Corporation) as a solvent control, was added to the SF media as indicated. MCF7 cells were previously obtained from the American Type Culture Collection (ATCC**,** Manassas, VA, USA) and maintained in MCF7 complete media [SF-DMEM (Invitrogen) supplemented with final percentages/concentrations of 10% FBS, 1% Pen Strep, and 5 µg/mL Insulin]. Prior to treatment with basic (b)FGF (Invitrogen), confluent MCF7 cells were rinsed twice with 1xPBS and starved overnight in SF-DMEM.

For bFGF treatment, cells were incubated for the indicated times in bFGF diluted in SF media to 50 ng/mL. All cells were grown and maintained at 37°C in 5% CO_2.

2.2.B RNA isolation and qRT-PCR analysis.

RNA was isolated from HC11/R1 and MCF7 cells and from the fourth inguinal mammary glands of MMTV-iFGFR1 transgenic mice using TRIzol® (Invitrogen) as described previously [129, 308]. For quantitative reverse transcription-PCR (qRT-PCR) analysis, cDNA was generated using the Quantitect Reverse Transcription kit (Qiagen, Valencia, CA, USA). Between one-tenth and one-sixteenth of the final reaction volume was used in quantitative SYBR green RT-PCR reactions as described previously [309] using the Bio-Rad iQ5 system (Bio-Rad, Hercules, CA, USA). Relative quantification of the expression of each gene was calculated and normalized to averaged *cyclophilin B* expression levels using the $2^{-\Delta\Delta Ct}$ method [310]. The following mouse primer sequences were used: *Areg*: 5'-GGGGACTACGACTACTCAGAG-3' and 5'-TCTTGGGCTTAATCACCTGTTC-3', *Ereg*: 5'-TCCGAGGATAACTGTACCGC-3' and 5'-CTCTCATGTCCACCAGGTAGAT-3', and *cyclophilin B*: 5'-TGAGCACTGGGGAGAAAGG-3' and 5'-TTGCCATCCAGCCACTCAG-3'. For experiments with MCF7 cells, the following human primers were used: *AREG*: 5'-GTGGTGCTGTCGCTCTTGATA-3' and 5'-ACTCACAGGGGAAATCTCACT-3', *EREG*: 5'-CTGCCTGGGTTTCCATCTTCT-3' and 5'-

GCCATTCATGTCAGAGCTACACT-3', and *cyclophilin B*: 5'-GAAAGAGCATCTACGGTGAGC-3' and 5'-GTCTTGACTGTCGTGATGAAGAA-3'. Experiments were performed in biological triplicates. Statistical analysis was performed using the unpaired student's t-test to compare two means (GraphPad QuickCalcs, GraphPad Software, Inc., CA, USA).

2.2.C Immunohistochemistry.

The following antibodies were used for immunohistochemistry (IHC): anti-AREG (AF989) and anti-EREG (AF1068) (R&D Systems, Minneapolis, MN, USA). For both antibodies, sodium citrate antigen retrieval was used as described previously [311] and primary antibodies were used at a concentration of 15 μg/mL. IHC analysis was performed on mammary gland sections from a minimum of three mice per treatment and genotype.

2.2.D ELISAs.

HC11/R1 cells were plated in six well plates in complete media, grown to confluence, serum starved, and then treated with either 30 nM AP or its ethanol solvent overnight. MCF7 cells were also plated in six well plates in complete media, grown to confluence, serum starved, and then treated with 50 ng/mL bFGF for 4, 6, or 24 hours. Conditioned media were collected and used to quantify soluble ligand concentration of AREG and/or EREG using ELISA kits (R&D Systems) and performed according to the company's protocol.

Experiments were performed in biological triplicates. Statistical analysis was performed using the unpaired student's t-test to compare two means (GraphPad QuickCalcs).

2.2.E Migration assays.

For AREG and EREG overexpression, HC11 cells were plated in six well plates in complete media, grown to confluence, and then starved in SF-RPMI. The next day, a p10 pipette tip was used to make a scratch down the center of each well. Cells were then treated with either 20 ng/mL recombinant mouse (rm)AREG (R&D Systems) or 10 ng/mL rmEREG (R&D Systems) in the presence or absence of either 1 μM or 0.5 μM erlotinib or an equivalent amount of DMSO. To test iFGFR1-induced migration, HC11/R1 cells were plated in six well plates in complete media, grown to confluence, and then starved in SF-RPMI overnight. Serum starved cells were treated with 30 nM AP or an equivalent amount of ethanol overnight. Additionally, the HC11/R1 cells were treated with 1 μM erlotinib or an equivalent amount of DMSO. All treatments were done in biological triplicates. Five representative pictures of each scratch were taken at the original time of treatment and 18 hours later. Areas of the open space in each picture were determined using the Leica LAS software, and percent wound closure was calculated for each treatment. Statistical analysis was performed using the unpaired student's t-test to compare two means (GraphPad QuickCalcs).

2.2.F MTT assays.

HC11 or HC11/R1 cells were plated on day 0 in complete medium into 24 well tissue culture plates at 20,000 cells/well. Cells were grown overnight and then starved in SF-RPMI. The next day, cells were activated with 100 ng/mL rmAREG (HC11 cells), 10 ng/mL rmEREG (HC11 cells), or 30 nM AP (HC11/R1 cells) in the presence or absence of 1 μM erlotinib or an equivalent amount of DMSO. After 48 (HC11 cells) or 72 (HC11/R1 cells) hours, Thiazolyl Blue Tetrazolium Bromide (Sigma-Aldrich Corporation) was added to each well at a final concentration of 0.3 mg/mL for 2 hours. Medium was then removed from wells, and the cells were solubilized in 95%DMSO/5%1xPBS. Solubilized dye was detected on a plate reader at an OD of 570 nm with background fluorescence at 650 nm subtracted from each value. Experiments were performed in biological triplicates. Statistical analysis was performed using the unpaired student's t-test to compare two means (GraphPad QuickCalcs).

2.2.G Immunoblot analysis.

HC11 or HC11/R1 cells were plated in six well plates in complete media, grown to confluence, and then starved in SF-RPMI overnight. The next day, each well of HC11 cells was treated with 20 ng/mL rmAREG or 10 ng/mL rmEREG for the indicated time (Fig. 3E) to overnight (Fig. 3F) in the presence or absence of either 1 μM or 0.5 μM erlotinib or an equivalent amount of DMSO. HC11/R1 cells

were treated with either 30 nM AP or ethanol in the presence of either 1 μM erlotinib or an equivalent amount of DMSO for 7 or 18 hours (Fig. 4A and 4E). Alternatively, HC11/R1 cells were treated with 30 nM AP with 2 μM erlotinib or an equivalent amount of DMSO for the times indicated (Fig. 4D). Following all treatments, lysates were collected in RIPA buffer, and total protein concentration was determined using a Bradford assay. Thirty μg protein was analyzed on an SDS-PAGE gel, and immunoblotting for phosphorylated (p)Akt (1:2000, 9271, Cell Signaling, Danvers, MA, USA), Cyclin D1 (1:2000, 2926, Cell Signaling), β-tubulin (1:1000, 2146, Cell Signaling), EGFR (1:100,000, ab52894, Abcam, Cambridge, MA, USA), pEGFR at Y845 (1:1000, 2231, Cell Signaling), pEGFR at Y1068 (1:1000, 3777, Cell Signaling), pEGFR at Y1173 (1:1000, ab5652, Abcam), or pERK1/2 (1:500, 9101, Cell Signaling) was performed. Additionally, 5 μg protein was analyzed on an SDS-PAGE gel, and immunoblotting for total Akt (1:1000, 9272, Cell Signaling) or ERK1/2 (1:2000, sc-94, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was performed. Experiments were performed at least three separate times.

2.2.H Animals.

Generation of MMTV-iFGFR1 transgenic mice has been described previously [127] and the mice were obtained from Dr. Jeff Rosen. Animal care and procedures were approved by the Institutional Animal Care and Use

Committee of the University of Minnesota and were in accordance with the procedures detailed in the Guide for Care and Use of Laboratory Animals.

2.2.I Treatment of mice.

For the first study, six week old MMTV-iFGFR1 female mice were administered 25 mg/kg/day erlotinib or an equivalent amount of the DMSO solvent via oral gavage once daily for 3 days. On the second day of oral gavage treatment, the mice were also given intraperitoneal (i.p.) injections of 1 mg/kg AP to activate iFGFR1. Twenty-four hours after the third oral gavage, the mice were given i.p. injections of 0.3 mg/kg BrdU (GE Healthcare Life Sciences, Piscataway, NJ, USA). For the second study, six week old MMTV-iFGFR1 female mice were given i.p. injections of 1 mg/kg/day AP to activate iFGFR1 on days 1, 5, and 9. The mice were then treated with 25 mg/kg/day erlotinib or an equivalent amount of the DMSO solvent via oral gavage once daily for five consecutive days (days 10-14). On day 15, mice were given i.p. injections of 0.3 mg/kg BrdU. All mice were sacrificed 2 hours post-BrdU injections, and their fourth inguinal mammary glands were harvested for further analysis.

2.2.J Mammary gland histology and measurement of epithelial budding.

Glands were fixed in fresh 4% PFA for 2 hours on ice and then stored in 70% ethanol. Whole mounts were prepared as previously described [129]. For histological and immunofluorescence (IF) analysis, glands were embedded in

paraffin and sectioned at 5 μm. To calculate percent budding structures, 5 μm sections were stained with hematoxylin and eosin (H&E) according to standard protocols. Each epithelial structure distal to the lymph node was counted and grouped into either no budding, moderate budding (1-4 buds), or severe budding (>4 buds). Percent budding structures was calculated by comparing all budding structures to all structures for each category of mice. At least three sections were analyzed per mouse. For IF analysis, 5 μm sections were stained with anti-BrdU (1:300, ab6326, Abcam, Cambridge, MA, USA) according to standard IF protocols. Sections were mounted in ProLong® Gold antifade reagent with DAPI (Invitrogen) to visualize the nuclei. Ten epithelial ductal structures distal to the lymph node were imaged per mammary gland section. Based on DAPI staining, all epithelial cells within each structure were counted and all BrdU positive epithelial cells of the same structure were counted to determine percent BrdU positive cells. A minimum of 1500 epithelial cells were counted per treatment group, and all studies were performed in a blinded manner. Statistical analysis was performed using the unpaired student's t-test to compare two means (GraphPad QuickCalcs).

2.3 Results

2.3.A Activation of iFGFR1 in the mouse mammary gland results in increased expression of AREG and EREG.

Due to the prevalence of human breast cancer patients harboring aberrant *FGFR1* amplification, it is necessary to determine the downstream molecular effects of FGFR1-induced signaling pathways. In order to specifically study FGFR1 signaling, an inducible FGFR1 system previously engineered to mimic endogenous FGFR1 signaling was developed [127]. Activation of iFGFR1, by treatment with AP, within the mammary epithelial cells of MMTV-iFGFR1 transgenic mice results in the formation of hyperplastic lesions within 3 days, which progress to mammary tumors upon prolonged AP treatment. To identify novel mediators of such FGFR1-induced tumorigenesis, previously published studies described the results of microarray studies that were performed on RNA isolated from the mammary glands of MMTV-iFGFR1 transgenic animals following a AP stimulation [129]. Of the numerous genes induced by iFGFR1, two EGF family ligands, *Areg* and *Ereg*, were significantly upregulated following iFGFR1 activation (Fig. 1A and 1B). *Areg* was significantly induced within 8 hours of treatment, while *Ereg* was significantly induced following 16 hours of treatment. To validate increased expression of *Areg* and *Ereg*, IHC analysis of MMTV-iFGFR1 mammary gland sections was performed. As shown in Figure 1C, both AREG and EREG proteins were produced at elevated levels following

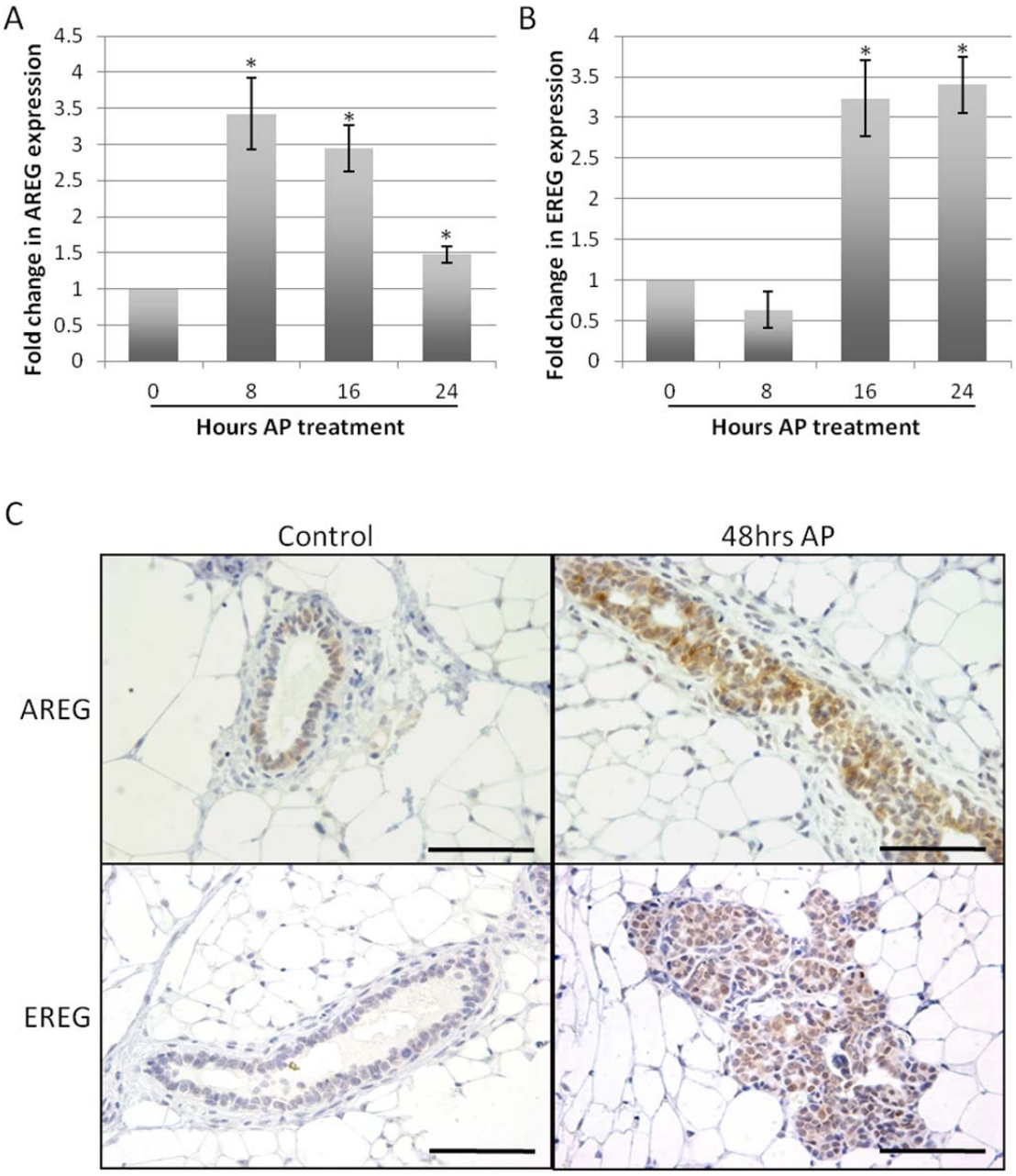

Figure 1: AREG and EREG are induced following FGFR1 activation *in vivo*.
A and B) MMTV-iFGFR1 animals were administered i.p. injections of 1 mg/kg AP for the times indicated. Following AP treatment, the animals were sacrificed, and their fourth inguinal mammary glands were removed for either isolation of RNA or paraffin embedding and subsequent tissue analysis. RNA samples were subjected to microarray analysis in previously published studies [129]. Analysis of the data from these experiments demonstrated that *Areg* (A) and *Ereg* (B) were both significantly upregulated at the indicated timepoints. Error bars represent s.e.m. (*P<0.05) C) Paraffin-embedded mammary glands from either wildtype (WT) or MMTV-iFGFR1 mice treated with AP for 48 hours were

sectioned. IHC was performed on sections to detect either AREG or EREG. Images are representative of at least three mice per treatment group. Scale bars = 50 μm.

iFGFR1 activation. These studies identify two novel targets of FGFR1 activation in the mammary gland *in vivo*.

2.3.B Activation of iFGFR1 in HC11/R1 cells induces expression of AREG and EREG.

We next determined whether iFGFR1 was capable of inducing AREG and EREG in mouse mammary epithelial cells. For these studies, HC11/R1 cells, an immortalized, non-transformed mammary epithelial cell line stably expressing *iFGFR1*, were used. Previous studies of HC11/R1 cells have demonstrated that activation of iFGFR1 via treatment with AP promotes cell survival, proliferation, migration, invasion, and epithelial-to-mesenchymal transition (EMT) [127, 128, 292, 312]. qRT-PCR was performed on RNA collected from HC11/R1 cells treated with AP for 0, 0.5, 1, 2, and 4 hours. As shown in Figures 2A and 2B, both *Areg* and *Ereg* transcript levels significantly increased following iFGFR1 activation *in vitro*. *Areg* transcript levels rapidly increased with AP treatment, peaking at 1 hour of AP treatment, and then decreased with prolonged AP treatment. *Ereg* transcript levels rose more slowly to peak at 2 hours post-AP treatment and then, like *Areg*, decreased with continued AP treatment. These data are consistent with the *in vivo* analysis of *Areg* and *Ereg* transcripts in that an increase in *Areg* mRNA is detectable earlier than an increase in *Ereg* mRNA and demonstrate that *Areg* and *Ereg* are induced in mouse mammary epithelial cells following iFGFR1 activation.

To verify that *Areg* and *Ereg* transcripts are indeed translated into mature AREG and EREG proteins *in vitro*, AREG and EREG protein levels were quantified. Because it is known that EGF family ligands are shed from their membrane-bound precursors into the extracellular matrix (ECM) [162, 164], soluble AREG and EREG protein concentration was measured by ELISA from the conditioned media of HC11/R1 cells treated overnight with either AP or its solvent, ethanol. Compared to the ethanol controls, HC11/R1 cells treated with AP had significantly increased levels of soluble AREG and EREG (Fig. 2C and 2D). These data suggest that iFGFR1 activation induces both gene and protein expression of AREG and EREG.

2.3.C AREG and EREG expression are upregulated following bFGF treatment in MCF7 cells.

Further studies were performed to confirm that AREG and EREG are regulated by FGF signaling in a breast cancer model. The MCF7 human breast cancer cell line has been previously used to study mechanisms of FGF-induced tumorigenesis [313, 314] and was used to verify that FGF treatment leads to increased expression of AREG and EREG. As shown in Figure 2, qRT-PCR performed on RNA isolated from MCF7 cells treated with 50 ng/mL bFGF for 4, 6, and 24 hours displayed a significantly increased fold change in both *AREG* and *EREG* mRNA as compared to the no treatment control samples (Fig. 2F and 2G). As in the mouse, human AREG and EREG are shed from the cell

membrane. Thus, conditioned media were collected to detect AREG protein levels via ELISA (Fig. 2E). Compared to the no treatment control, 50 ng/mL bFGF treatment of MCF7 cells for 4, 6, and 24 hours significantly induced soluble human AREG protein. These results demonstrate that activation of the endogenous FGF pathway by treating cells with ligand also increases AREG and EREG expression. Furthermore, as MCF7 cells are a human-derived breast cancer cell line, these studies suggest that activation of the FGF pathway signals an increase in both AREG and EREG expression in human cells as well as mouse cells.

2.3.D. AREG and EREG both individually promote cellular migration and proliferation of mammary epithelial cells *in vitro*.

Based on the fact that both AREG and EREG are upregulated following iFGFR1 activation and that iFGFR1 activation induces numerous tumorigenic phenotypes including migration and proliferation, we tested whether AREG and/or EREG affects these processes. To study the effects of AREG and EREG on migration, rmAREG or rmEREG was added to freshly wounded serum starved HC11 mouse mammary epithelial cells, which do not express iFGFR1. Percent wound closure was calculated from the original time 0 to 18 hours post-recombinant protein treatment. Compared to controls treated only with DMSO, addition of either rmAREG or rmEREG significantly increased HC11 migration

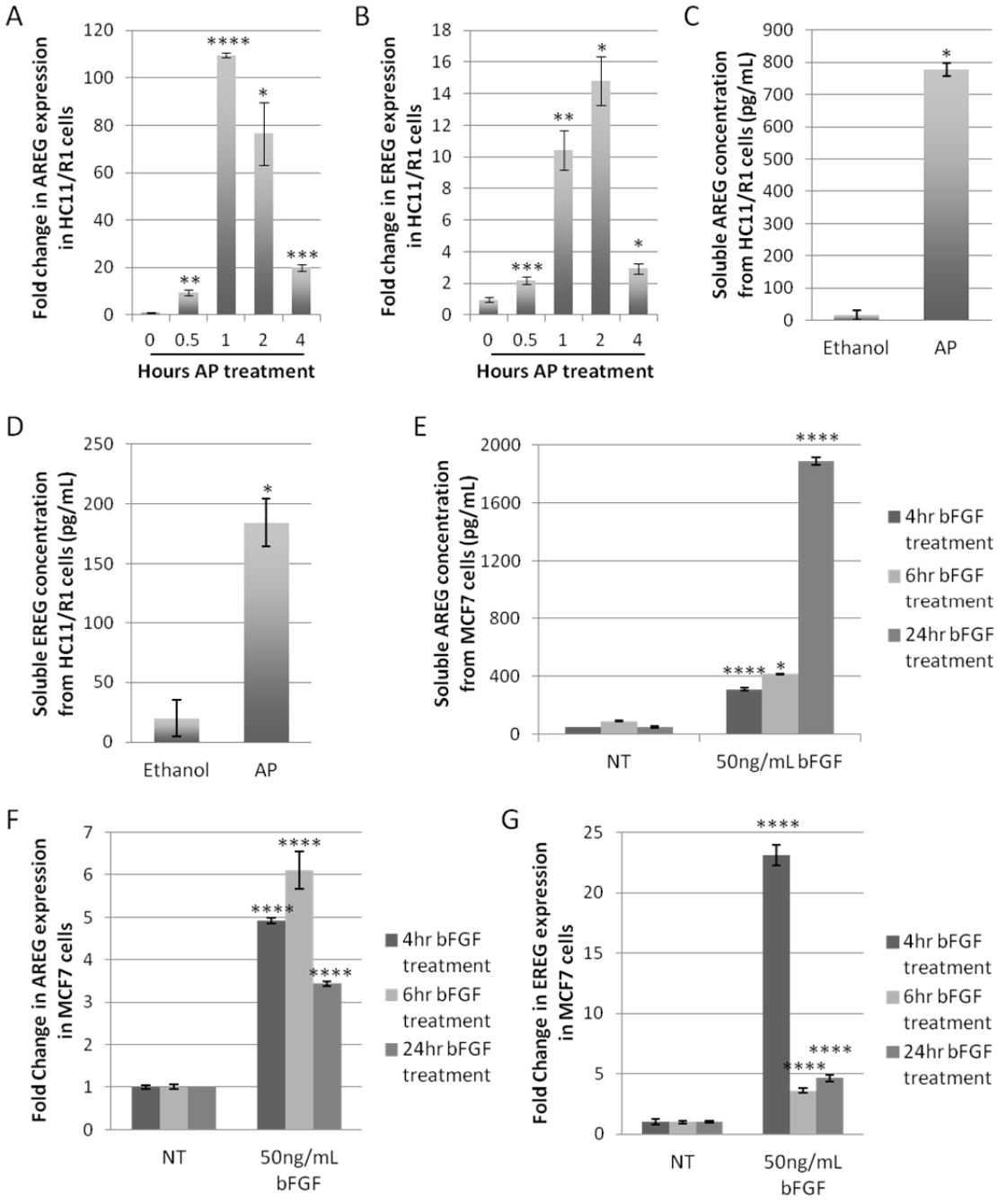

Figure 2: FGFR1 activation in mammary epithelial cells *in vitro* induces expression of AREG and EREG.

A and B) Mouse mammary epithelial HC11/R1 cells were treated with 30 nM AP for the indicated times. Following AP treatment, qRT-PCR analysis was performed on RNA isolated at each timepoint for both *Areg* transcript (A) and *Ereg* transcript (B) and normalized to mouse *cyclophilin B*. Experiments were performed in biological triplicates. Error bars represent s.e.m. (*P<0.05, **P<0.01, ***P<0.005, ****P<0.001) C and D) To detect soluble

AREG (C) and EREG (D) ligands, ELISA analysis was performed on conditioned HC11/R1 media. HC11/R1 cells were treated with either 30 nM AP or its ethanol solvent overnight to condition the media. Experiments were performed in biological triplicates. Error bars represent s.e.m. (*P<0.05) E) The conditioned media of MCF7 human breast cancer cells treated with 50 ng/mL bFGF for 4, 6, or 24 hours were collected and soluble AREG was detected via ELISA. Experiments were performed in biological triplicates. Error bars represent s.e.m. (*P<0.05, ****P<0.001) F and G) MCF7 cells were treated with 50 ng/mL bFGF for 4, 6, or 24 hours. Following bFGF treatment, qRT-PCR analysis was performed on RNA isolated at each timepoint for both *AREG* transcript (F) and *EREG* transcript (G) and normalized to human *cyclophilin B*. NT=no treatment. Experiments were performed in biological triplicates. Error bars represent s.e.m. (****P<0.001)

(Fig. 3A and 3B). Because AREG and EREG both activate EGFR [131, 186, 187], we examined the effects of blocking signaling through EGFR on the migratory potential of the rmAREG- or rmEREG-treated cells. For these studies, we used the EGFR inhibitor erlotinib, which exerts its effects by blocking EGFR kinase function. Cells treated with either rmAREG or rmEREG and erlotinib had significantly decreased migration compared to the rmAREG- or rmEREG-treated cells, similar to the DMSO-treated control levels (Fig. 3A and 3B). Moreover, erlotinib alone did not affect the migration of the HC11 cells as compared to the DMSO-treated controls (Fig. 3A and 3B), suggesting that the reduction in migration is not due to erlotinib-induced off-target effects but is due to an inhibition of EGFR signaling. These data suggest that treatment of cells with AREG or EREG alone is enough to promote increased mammary epithelial cell migration, a hallmark of tumor progression. Furthermore, this migration is promoted at least in part through EGFR, as blocking EGFR activity by addition of erlotinib significantly impairs the ability of these epithelial cells to migrate into the open space of the wound.

Previous studies have also demonstrated that activation of iFGFR1 induces proliferation of mammary epithelial cells [127, 128, 292]. Because EGFR activation has also been linked to increased proliferation, we hypothesized that the upregulation of EGF ligands, such as AREG and EREG, might promote proliferation. Therefore, initial studies were performed to determine the ability of AREG and EREG to stimulate mammary epithelial cell proliferation. For these

studies, HC11 cells were treated with rmAREG or rmEREG and either DMSO or erlotinib for 48 hours prior to performing an MTT assay. Treatment of HC11 cells with either rmAREG (Fig. 3C) or rmEREG (Fig. 3D) significantly promoted proliferation compared to DMSO-treated controls and this proliferation was significantly blocked with erlotinib treatment. Taken together, these data demonstrate that treatment of mammary epithelial cells with recombinant AREG or EREG promotes EGFR-dependent migration and stimulates EGFR-dependent mammary epithelial cell proliferation.

2.3.E AREG and EREG stimulate ERK activation *in vitro*, which is inhibited by erlotinib.

The results described above, in which AREG and EREG treatment stimulates migration and proliferation of HC11 mammary epithelial cells that is blocked by addition of the EGFR kinase inhibitor erlotinib, suggest that EGFR activation is required for AREG and EREG to exert their signaling effects. Because a known target of EGFR activation is ERK1/2, we next examined the activation profile of ERK1/2 following rmAREG or rmEREG treatment. HC11 cells were treated with either rmAREG or rmEREG for various times up to 1 hour and immunoblot analysis was performed to examine pERK1/2 and total ERK1/2. Both rmAREG and rmEREG treatments led to increased ERK1/2 activation by 5 minutes that was largely diminished within 15 minutes (Fig. 3E). These results

suggest that AREG or EREG treatment induces rapid and transient ERK1/2 activation.

To confirm that the detected pERK1/2 is regulated by EGFR, HC11 cells were treated with rmAREG or rmEREG in the presence or absence of erlotinib for 10 minutes. Compared to DMSO-treated controls, rmAREG- or rmEREG-only treated cells showed increased phosphorylation of ERK1/2 (Fig. 3F), as expected based on our above results. Notably, treatment of cells with erlotinib at either 1 μM or 0.5 μM, in addition to rmAREG or rmEREG, blocked ERK1/2 phosphorylation (Fig. 3F), suggesting that blocking EGFR activation, and thus signaling through EGFR, blocks activation of ERK1/2 in mammary epithelial cells. These data imply that EGFR must be activated in order for AREG and EREG to exert their effects and that blocking EGFR signaling effectively inhibits the effects of AREG and EREG stimulation.

2.3.F Activation of iFGFR1 in mammary epithelial cells induces migration, proliferation, and ERK1/2 activation *in vitro*, which are inhibited by erlotinib.

As shown thus far, iFGFR1 activation upregulates expression of the EGFR ligands AREG and EREG and the EGFR-specific inhibitor erlotinib blocks AREG- and EREG-induced cellular processes and signaling. Therefore, we next assessed whether EGFR is activated following iFGFR1 activation in HC11/R1 cells. Serum-starved HC11/R1 cells were treated with AP or ethanol for 18 hours

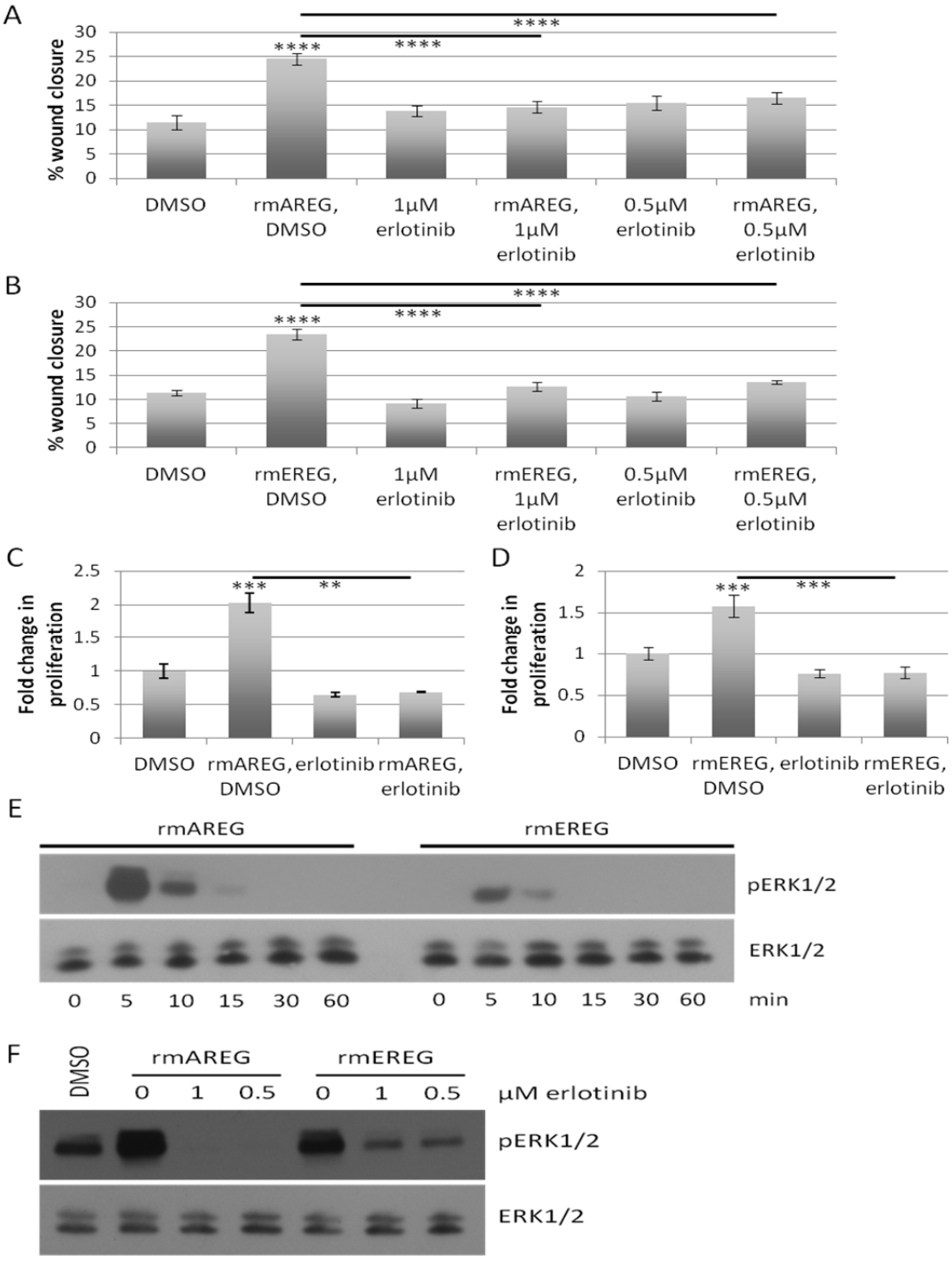

Figure 3: Treatment of HC11 cells with either AREG or EREG *in vitro* stimulates cellular migration, cellular proliferation, and ERK activation.

A) Freshly wounded mouse mammary epithelial HC11 cells were treated with either DMSO, 20 ng/mL rmAREG+DMSO, 1 μM erlotinib, 20 ng/mL rmAREG+1 μM erlotinib, 0.5 μM

erlotinib, or 20 ng/mL rmAREG+0.5 μM erlotinib for 18 hours. Five representative images were taken per well at 0 and 18 hours, and the area of the open space of the wound was determined using Leica LAS Software. Migration was measured as percent wound closure. Each treatment was performed in biological triplicates. Error bars represent s.e.m. (****P<0.001) B) Migration assay was performed as in (A), with the exception that HC11 cells were treated with 10 ng/mL rmEREG instead of rmAREG. Each treatment was performed in biological triplicates. Error bars represent s.e.m. (****P<0.001) C) HC11 cells were treated with DMSO, 100 ng/mL rmAREG, 1 μM erlotinib, or 100 ng/mL rmAREG+1 μM erlotinib for 48 hours. Proliferation was measured via MTT assay and normalized to the levels at treatment day 0. Each treatment was performed in biological triplicates. Error bars represent s.e.m. (**P<0.01, ***P<0.005) D) HC11 cells were treated as in (C), but with 10 ng/mL rmEREG in place of rmAREG. Proliferation was measured via MTT assay at 48 hours and normalized to the levels at day 0 of treatment. Each treatment was performed in biological triplicates. Error bars represent s.e.m. (***P<0.005) E) HC11 cells were treated with either 20 ng/mL rmAREG or 10 ng/mL rmEREG for the indicated times. Following treatment, whole cell lysates were collected and analyzed by immunoblot for pERK1/2 and total ERK1/2. Experiment shown is representative of three individual experiments. F) HC11 cells were treated with combinations of 20 ng/mL rmAREG or 10 ng/mL rmEREG and DMSO, 1 μM erlotinib, or 0.5 μM erlotinib as designated for 10 minutes. Whole cell lysates were collected and analyzed by immunoblot for pERK1/2 and total ERK1/2. Experiment shown is representative of three individual experiments.

before the phosphorylation status of three critical tyrosine residues within the EGFR kinase domain was evaluated. As shown in Figure 4A, AP treatment stimulated phosphorylation of residues Y845, Y1068, and Y1173 as compared to ethanol treatment, suggesting that activation of iFGFR1 does subsequently signal EGFR activation.

Because treatment of cells with recombinant AREG and EREG protein stimulates migration and proliferation of HC11 cells, and because activating iFGFR1 results in EGFR phosphorylation, we next tested whether iFGFR1-induced cellular processes were dependent upon EGFR activity. Freshly wounded serum-starved HC11/R1 cells were treated overnight with either AP and DMSO or ethanol and DMSO. Percent wound closure was calculated from the original time of treatment to 18 hours post-treatment. As expected based on previous studies [128], AP and DMSO treatment significantly stimulated migration of the HC11/R1 cells compared to ethanol and DMSO-treated controls (Fig. 4B). To determine whether EGFR activation is required for the increased iFGFR1-induced migration of HC11/R1 cells, we also added erlotinib to AP- or ethanol-treated cells. As shown in Figure 4B, addition of erlotinib to AP-treated cells partially, but significantly decreased migration to levels close to the ethanol and DMSO-treated control. Erlotinib treatment of the ethanol control cells did not inhibit migration as compared to the ethanol and DMSO-treated control cells, demonstrating that the decreased migration of the AP and erlotinib-treated HC11/R1 cells is not due to non-specific off-target effects of erlotinib. These

results suggest that iFGFR1 activation signals through EGFR to promote migration *in vitro*.

To determine whether iFGFR1-mediated cellular proliferation could also be blocked by erlotinib treatment, an MTT assay was performed. As shown in Figure 4C, AP and DMSO treatment of HC11/R1 cells induced greater cellular proliferation than ethanol and DMSO-treated controls. This proliferation was partially, but significantly, inhibited by erlotinib treatment, suggesting that iFGFR1 activation, at least in part, signals through EGFR to promote cellular proliferation *in vitro*. Moreover, erlotinib treatment of the ethanol control cells did not significantly block proliferation as compared to the ethanol and DMSO-treated control cells, again demonstrating that the reduction in proliferation of the AP and erlotinib-treated HC11/R1 cells is not due to off-target effects of erlotinib.

To be certain that erlotinib does not inhibit iFGFR1-driven migration and proliferation by blocking iFGFR1 activity in a non-specific manner, HC11/R1 cells were treated with AP in the presence of erlotinib and phosphorylation of ERK1/2, a well-established downstream target of FGFR1 signaling, was determined. For these studies, serum-starved cells were treated for 0, 5, 10, 15, 30 and 60 minutes with AP in the presence of either erlotinib or DMSO. As shown in Figure 4D, erlotinib did not block iFGFR1-induced activation of ERK1/2 during this time-course. These results demonstrate that erlotinib is not blocking migration and proliferation by non-specifically inhibiting iFGFR1 activity.

Based on these results, we predicted that the accumulation of AREG and EREG ligands over time leads to activation of EGFR and its downstream signaling pathways and that this autocrine stimulation of EGFR contributes to FGFR1-induced migration and proliferation. To address this prediction, immunoblot analysis of signaling molecules downstream of EGFR was performed on HC11/R1 cells treated with AP or ethanol in the presence or absence of erlotinib or DMSO for 7 or 18 hours. As expected, phosphorylation of Akt and ERK1/2 was higher in the 18 hour AP-treated samples as compared to ethanol-treated samples (Fig. 4E). Moreover, cell cycle regulator Cyclin D1 expression was upregulated following 7 hours of AP treatment as compared to 7 hours of ethanol treatment. Importantly, erlotinib largely inhibited both Akt and ERK1/2 phosphorylation and Cyclin D1 expression (Fig. 4E), suggesting that EGFR activation in HC11/R1 cells results in subsequent activation of several downstream signaling pathways.

2.3.G Activation of iFGFR1 *in vivo* induces cellular proliferation of mouse mammary epithelial cells, which is inhibited by erlotinib.

Activating iFGFR1 *in vivo* is known to stimulate aberrant epithelial cell budding due to increased proliferation of the epithelial cells [127]. Based on our above results in which AREG and EREG promote cellular proliferation *in vitro* in an erlotinib-dependent manner, we wanted to determine whether erlotinib could also inhibit aberrant cellular proliferation *in vivo*. For these studies, MMTV-

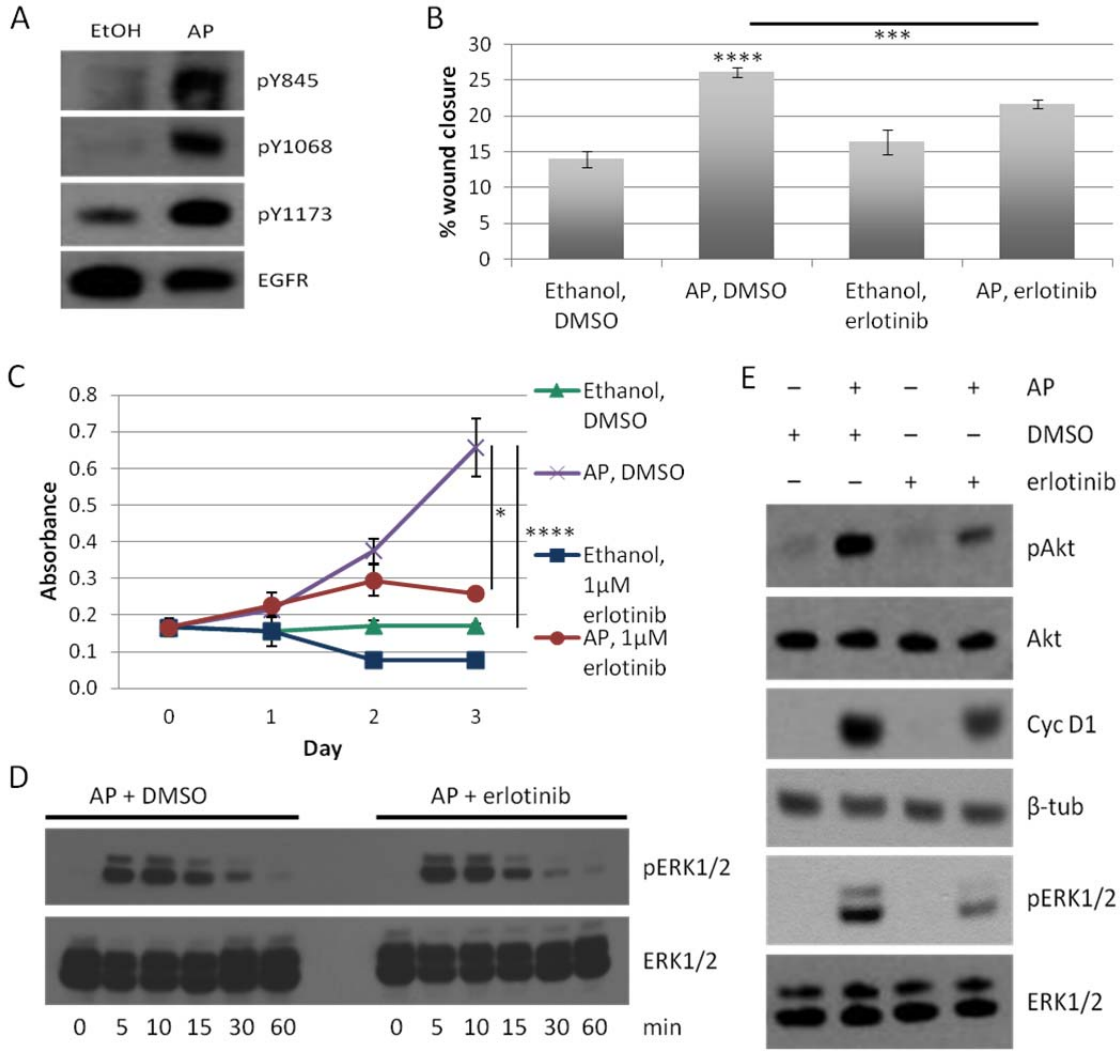

Figure 4: Erlotinib inhibits iFGFR1-induced migration, proliferation, and ERK1/2 activation *in vitro*.

A) HC11/R1 cells were treated with either ethanol or 30 nM AP for 18 hours. Whole cell lysates were collected and analyzed by immunoblot for total EGFR and EGFR phosphorylated at Y845, Y1068, and Y1173. Experiments shown are representative of three individual experiments. B) Freshly wounded mouse mammary epithelial HC11/R1 cells were treated with ethanol+DMSO, 30 nM AP+DMSO, ethanol+1 μM erlotinib, or 30 nM AP+1 μM erlotinib for 18 hours. Five representative images were taken per well at 0 and 18 hours, and the area of the open space of the wound was determined using Leica LAS Software. Migration was measured as percent wound closure. Each treatment was performed in biological triplicates. Error bars represent s.e.m. (***P<0.005, ****P<0.001) C) HC11/R1 cells were treated as in (B) for 72 hours. Proliferation was then measured via MTT assay and normalized to levels at day 0 of treatment. Each treatment was performed in biological triplicates. Error bars represent s.e.m. (*P<0.05, ****P<0.001) D) HC11/R1 cells were treated with 30 nM AP in the presence of either DMSO or 2 μM erlotinib for the times indicated. Whole cell lysates were collected and analyzed by immunoblot for pERK1/2 and total ERK1/2. Experiment shown is representative of three individual experiments. E) HC11/R1

cells were treated with either 30 nM AP or ethanol and either DMSO or 1 μM erlotinib for 7 (Cyc D1 and β-tub) or 18 (Akt and ERK1/2) hours. Whole cell lysates were collected and analyzed by immunoblot for pAkt, total Akt, Cyc D1, β-tub, pERK1/2, and total ERK1/2. Experiments shown are representative of three individual experiments.

iFGFR1 transgenic mice were treated with erlotinib daily via oral gavage for 3 days. To activate iFGFR1, the same mice were given intraperitoneal (i.p.) injections of AP on the second day of erlotinib treatment. Two days after iFGFR1 activation, the mice were injected with BrdU 2 hours prior to sacrifice. The fourth inguinal mammary glands were harvested and either whole mounts or tissue sections were stained for further analysis. Using H&E staining, it was found that, compared to WT controls, mammary glands from mice in which iFGFR1 was activated exhibited more aberrant epithelial budding around the ductal lumens (Fig. 5A-D). This aberrant budding significantly decreased when the transgenic animals were treated with erlotinib in conjunction with iFGFR1 activation, suggesting that activation of EGFR is at least in part required for the anomalous mammary epithelial cell budding (Fig. 5A-D).

The above described study indicates that preventive inhibition of EGFR might be a beneficial therapy; however, preventive treatment is not common in the clinic setting. Therefore, we performed a second *in vivo* study again using the MMTV-iFGFR1 transgenic mice. This time, iFGFR1 was activated with i.p. injections of AP on days 1, 5, and 9 prior to once daily treatment with erlotinib via oral gavage for 5 consecutive days (days 10-14). Mice were sacrificed on day 15 2 hours after being injected with BrdU. Once again, the fourth inguinal mammary glands were harvested and whole mounts and tissue sections were analyzed. Mammary glands from iFGFR1-activated mice contained significantly more lateral epithelial budding as visualized using H&E staining than glands from WT

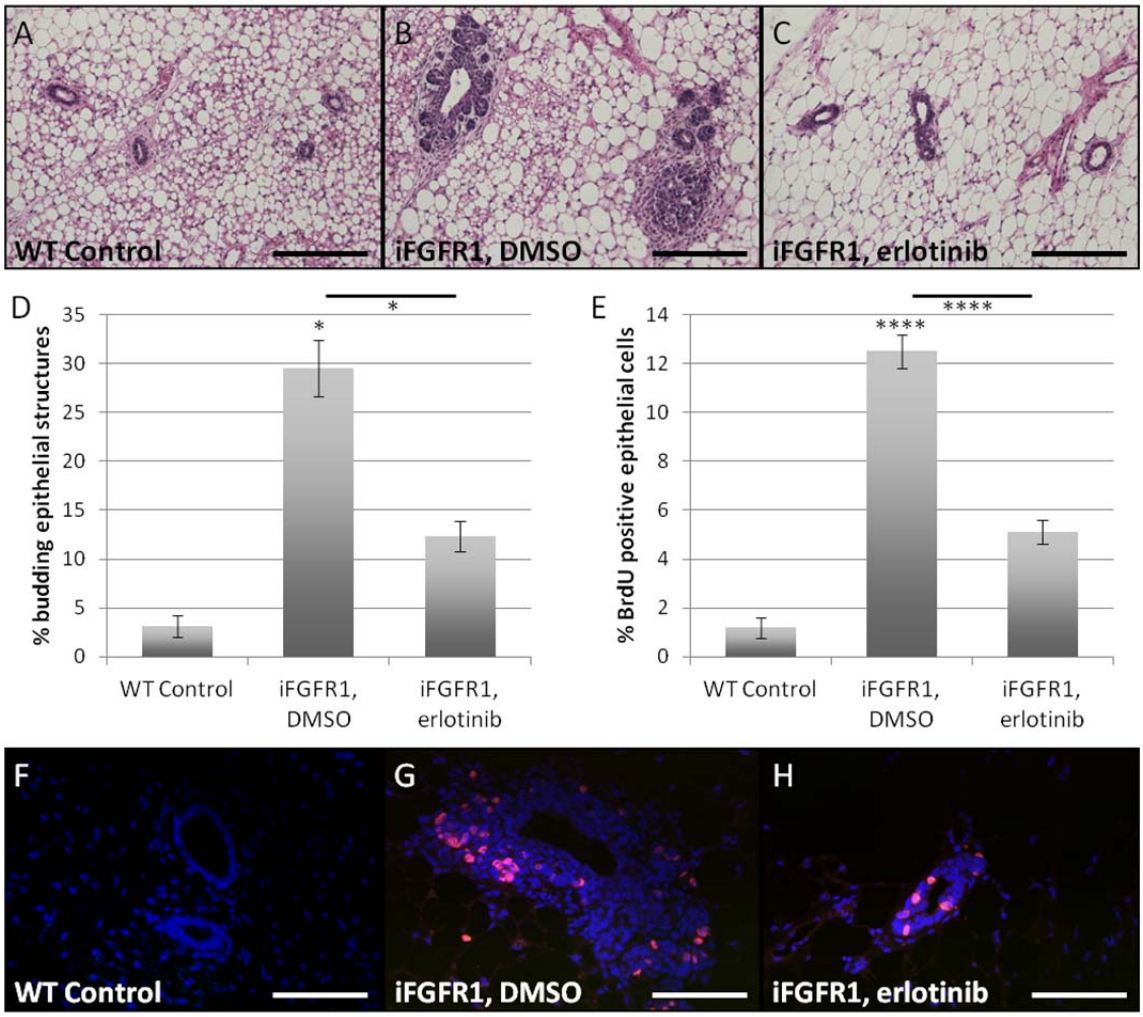

Figure 5: Erlotinib prevents FGFR1-induced mammary epithelial cell budding and proliferation *in vivo*.

A to C) Representative images of H&E stained sections of paraffin-embedded mammary glands from WT (A), MMTV-iFGFR1 transgenic mice treated with 1 mg/kg AP via i.p. injections and either DMSO (B) or 25 mg/kg/day erlotinib (C) via oral gavage. Scale bars = 50 μm. D) Quantification of the number of budding ductal structures. Every ductal structure distal to the lymph node was counted in H&E stained sections of the three classes of mice and then grouped into budding or no budding structures. At least three sections were analyzed per mouse. Error bars represent s.e.m. (*P<0.05) E) Cellular proliferation was measured by IF BrdU staining. All epithelial cells were counted in ten ductal structures per section by DAPI staining, and the percent BrdU-positive epithelial cells was determined. A minimum of 1500 cells were counted per treatment group. Error bars represent s.e.m. (****P<0.001) F to H) Representative images of BrdU (red) stained sections of paraffin-embedded mammary glands from WT (F), MMTV-iFGFR1 transgenic mice treated with 1 mg/kg AP via i.p. injections and either DMSO (G) or 25 mg/kg/day erlotinib (H) via oral gavage. The sections were also stained with DAPI (blue) to visualize all cells. Scale bars = 50 μm.

control animals (Fig. 6A-D). Erlotinib treatment significantly reduced this phenotype in iFGFR1-activated mammary glands (Fig. 6A-D). Moreover, the severity of the aberrant budding was significantly decreased in mammary glands of erlotinib-treated iFGFR1-activated animals as compared to mammary glands of iFGFR1-activated animals not treated with erlotinib (Fig. 6D). These results further support that EGFR activation is required to promote iFGFR1-stimulated tumorigenic phenotypes and notably, that inhibition of EGFR is sufficient to reduce established iFGFR1-stimulated tumorigenic phenotypes. Together, these data indicate that EGFR might represent a novel therapeutic target in FGFR1-amplified breast tumors.

To ensure that the observed aberrant lateral budding is due to aberrant cellular proliferation and not just a rearrangement of the cellular structure of the ductal tree, histological sections were stained with an anti-BrdU antibody. As shown in Figures 5E-H and 6E-H, iFGFR1 activation in transgenic animals significantly increased cellular proliferation of the ductal epithelial cells as compared to WT controls, while erlotinib treatment of iFGFR1 transgenic mice significantly reduced the number of BrdU positive epithelial cells. These data suggest that iFGFR1-induced cellular proliferation *in vivo* requires activation of EGFR, as inhibiting the EGFR kinase domain with erlotinib treatment blocks the ability of the epithelial cells to proliferate.

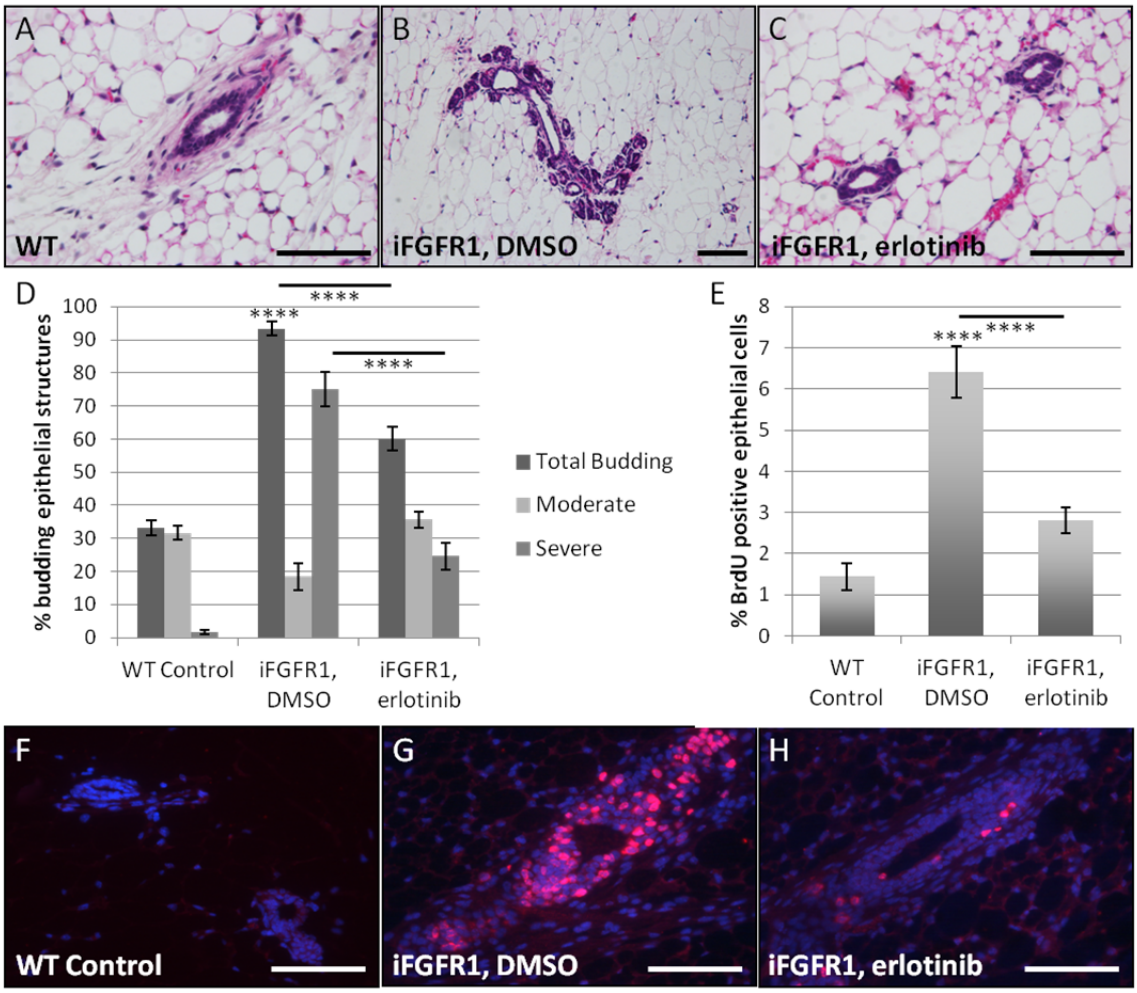

Figure 6: Erlotinib reduces established FGFR1-induced mammary epithelial cell budding and proliferation *in vivo*.

A to C) Representative images of sectioned, paraffin-embedded mammary glands stained with H&E. (A) WT control mice. (B) MMTV-iFGFR1 transgenic mice treated with 1mg/kg/day AP via i.p. injections on days 1, 5, and 9 and DMSO via oral gavage on days 10-14. (C) MMTV-iFGFR1 transgenic mice treated with 1mg/kg/day AP via i.p. injections on days 1, 5, and 9 and 25 mg/kg/day erlotinib via oral gavage on days 10-14. Scale bars = 50 μm. D) Quantification of the severity and total number of budding ductal structures in each treatment group. All ductal structures distal to the lymph node were counted in each H&E stained section. Severe budding structures were denoted by five or more buds. Moderate budding structures contained one to four buds. At least three sections were analyzed per mouse. Error bars represent s.e.m. (****P<0.001) E) Quantification of epithelial cell proliferation measured by DAPI staining to visualize all cells and IF staining of BrdU to visualize proliferating cells. Percent BrdU-positive epithelial cells was determined by counting all epithelial cells (by DAPI) and all BrdU-positive cells (red) in each of ten ductal structures per section. A minimum of 1500 cells were counted per treatment group. Error bars represent s.e.m. (****P<0.001) F to H) Representative images of sectioned, paraffin-embedded mammary glands stained with DAPI (blue) and for BrdU (red). (F) WT control mice. (G) MMTV-iFGFR1 transgenic mice treated with 1mg/kg/day AP via i.p. injections on days 1, 5,

and 9 and DMSO via oral gavage on days 10-14. (H) MMTV-iFGFR1 transgenic mice treated with 1mg/kg/day AP via i.p. injections on days 1, 5, and 9 and 25 mg/kg/day erlotinib via oral gavage on days 10-14. Scale bars = 50 μm.

2.4 Discussion

FGFR1 is located on human chromosome 8p11-12, which is a common site of amplification in human breast cancers [117, 119, 125, 315]. While it is still not clear whether *FGFR1* is the driving oncogene in this region, studies that focused on identification of smaller segments within the amplicon have found that the region containing *FGFR1* is linked to poor prognosis [119]. Furthermore, recent studies have demonstrated that while FGFR1 may not be sufficient to drive tumor formation on its own, it can act in concert with genes in other co-amplified regions, such as *myc* on 11q13, to promote tumorigenesis [125]. In agreement with this hypothesis, studies using mouse models have demonstrated that FGFR1 activation, in conjunction with another oncogenic signal, such as Wnt-1, can dramatically decrease tumor latency [314]. Moreover, recent studies have implicated FGFR1 in breast cancer, particularly in the resistance of breast cancer cells to endocrine- and chemotherapy-based treatments [120, 121]. Therefore, FGFR1 may represent a novel therapeutic target in breast cancer patients, particularly in patients that do not respond well to standard therapies.

Based on the potential contributions of FGFR1 to breast tumorigenesis, we have utilized both *in vitro* and *in vivo* models to better understand the mechanisms by which FGFR1 promotes mammary tumor formation. Previous studies have demonstrated that activation of iFGFR1 in mammary epithelial cells *in vitro* results in increased proliferation, survival, migration, invasion, and EMT [127, 128, 312]. Furthermore, activation of iFGFR1 in mammary epithelial cells *in*

vivo leads to the formation of alveolar hyperplasias, ultimately resulting in the formation of tumors with both adenocarcinoma and squamous characteristics [127, 129]. We have further utilized these cell culture and transgenic mouse models to delineate the mechanisms by which FGFR1 promotes mammary tumor formation. Obtaining a better understanding of these mechanisms will ultimately lead to the development of therapeutic strategies to target tumors in breast cancer patients with high levels of FGFR1 and increased resistance to conventional therapies.

Previous gene expression studies using microarray analysis led to the identification of numerous potential genes involved in promoting FGFR1-driven tumorigenesis [129]. Our recent studies have focused primarily on secreted factors regulated by FGFR1 because of the potential ability of secreted factors to regulate both epithelial and stromal cells. During the analysis of these studies, we found a significant induction of two EGF ligands, AREG and EREG, following iFGFR1 activation. This finding was further verified in our iFGFR1 *in vitro* system. Importantly, AREG and EREG expression was increased following iFGFR1 activation at both the transcript and protein levels. This observation was also confirmed in the MCF7 human breast cancer cell line, which has been previously used to study FGF signaling in breast cancer cells. We chose to further evaluate the ability of these ligands to act through the EGFR pathway to promote FGFR1-driven mammary tumorigenesis.

Both AREG and EREG have been implicated in breast cancer [19, 174, 176, 197-199, 295]. High AREG expression has been identified in cancerous lesions but not in neighboring, non-cancerous tissue [169, 180, 316]. This expression has also been associated with large, high-grade tumors and in metastases found in lymph nodes, suggesting that high AREG expression is a marker for aggressive, invasive breast cancers [169, 180, 296]. Interestingly, EREG expression has also been found to correlate with breast cancer metastasis to the lung, suggesting that it too promotes invasive breast cancer progression [198, 199]. AREG is known to exclusively bind and activate EGFR [131], while EREG acts through both EGFR and ErbB4 [186, 187]. In these studies, we found that AREG and EREG were both able to induce proliferation, migration and activation of the ERK1/2 pathway in HC11 cells. Furthermore, AREG and EREG both act primarily through EGFR, as demonstrated by the inhibition of these phenotypes by the EGFR-specific inhibitor, erlotinib.

Because AREG and EREG, which are both EGFR ligands, are both induced by iFGFR1 activation and are capable of promoting both migration and proliferation of the HC11 cells, we hypothesized that activation of EGFR could be a mechanism by which iFGFR1 promotes these phenotypes. In agreement with this hypothesis, our studies demonstrate that iFGFR1 activation stimulated phosphorylation of EGFR at tyrosine residues 845, 1068, and 1173 and that blocking EGFR activity inhibited iFGFR1-induced migration and proliferation. Moreover, investigation of different downstream molecules demonstrated that AP

treatment of HC11/R1 cells stimulated activation of Akt and ERK1/2 and expression of Cyclin D1 that was inhibited upon erlotinib treatment. However, analysis of other signaling pathways revealed that some signaling molecules, including p38 and phospholipase Cγ (PLCγ), were regulated solely by iFGFR1 and not by EGFR (data not shown). Although erlotinib treatment significantly inhibited iFGFR1-driven proliferation and migration, this inhibition was not complete, suggesting that EGFR-independent pathways also contribute to these phenotypes. Therefore, it is possible that using a combination of erlotinib and additional inhibitors that specifically target these pathways will lead to a more complete inhibition of FGFR1-driven phenotypes. Overall, the results presented here support the hypothesis that iFGFR1 leads to activation of EGFR, which then contributes to the regulation of signaling pathways that promote iFGFR1-induced tumorigenic phenotypes.

Because the concentration of AREG in the media was consistently higher than that of EREG, we predicted that AREG may be the dominant ligand responsible for EGFR activation. However, knockdown of *Areg* in the HC11/R1 cells using shRNA strategies did not reveal any differences in iFGFR1-induced migration or proliferation (data not shown). Therefore, it is likely that EREG is able to compensate for loss of AREG and that targeting single ligands may not be an effective therapeutic strategy when multiple EGFR ligands are present. Current studies are focusing on understanding the regulation of the sheddase ADAM17 by FGFR1 and the ability of this protease to release of both AREG and

EREG into the media. Targeting the regulation of AREG and EREG release into the media might represent a better strategy than targeting the ligands themselves.

Using a mouse model of mammary tumorigenesis, we demonstrate that treatment of mice with erlotinib inhibits epithelial proliferation and subsequent formation of early-stage hyperplastic lesions following iFGFR1-activation *in vivo*. Although our cell culture studies focused on the autocrine effects of iFGFR1-induced activation of EGFR, it is likely that paracrine stimulation of EGFR is also involved in regulating the tumorigenic phenotype *in vivo*. For example, transplantation studies using different combinations of *Areg* and *Egfr* null and positive epithelial and stromal cell types found that, for proper mammary gland development, *Areg* expression is required in the mammary epithelial cells, while *Egfr* is required in the stromal, and not the epithelial, compartment [107, 167]. These data indicate that AREG can act in a paracrine manner through stromal EGFR to stimulate expression of growth factor(s) that can act to drive epithelial cell proliferation, which is necessary for proper ductal network formation within the mammary gland. Further studies are required to better understand the effects of AREG and EREG on cells within the stroma and how these interactions might promote tumorigenesis.

Together, our results reveal the complex interactions between various signaling pathways that can regulate tumorigenesis and suggest that targeted therapies may need to take into account more than one pathway. To date,

FGFR1 inhibitors have not yet been tested in patients, although there are reports of FGFR1 inhibitors in preparation [317]. However, EGFR inhibitors have been successfully utilized in patients with certain types of cancers, including lung cancers [306, 307]. Therefore, inhibition of the EGFR pathway may be an alternative targeting strategy in breast cancer patients with high levels of FGFR1. Further studies are required to determine the link between FGFR1 expression and EGFR activation in human breast cancer tissue samples.

In summary, we have demonstrated that the EGFR pathway is an important downstream regulator of FGFR1-induced mammary tumor formation. Growth factor receptors and their downstream signaling pathways in breast cancer have been the focus of numerous studies. However, these studies have generally focused on single pathways at a time. It is becoming clear that these pathways likely interact and that understanding the key points at which these pathways communicate is important for generating novel therapies that most efficiently inhibit tumor formation and progression.

Chapter 3: FGFR1 mediates Doxorubicin chemoresistance through activation of STAT3

3.1 Introduction

Breast cancer is the second leading cause of cancer-related deaths in women and the most frequently diagnosed type of cancer in women (American Cancer Society. Cancer Facts & Figures 2012. Atlanta: American Cancer Society; 2012.). Much work has been done to better understand the complexity and heterogeneity of breast cancer, and in the past decade, breast cancer has been categorized into six intrinsically distinct molecular subtypes [52-55]. While these subtypes have greatly improved our understanding of the disease, they have not yet been translated into functionally useful contexts in the clinical setting. Instead, in the clinic, treatment plans are determined based on expression of the hormone receptors (HRs), estrogen receptor (ER) and progesterone receptor (PR), and the growth factor receptor, HER2/ErbB2/neu. Breast cancers that do not express any of these receptors are termed triple negative (TN). While targeted therapies against the HRs and HER2 are used for patients with cancers expressing these receptors, the lack of these targets precludes their use in TN breast cancers (TNBCs). Alternatively, TNBCs are treated with systemic chemotherapies, which function by inducing apoptosis in a non-directed manner [50, 51]. Notably, while some patients do respond well to

chemotherapy, most do not achieve pathologic complete response (pCR), and eventually stop responding to chemotherapy. Therefore, identification of alternative targets commonly expressed in TNBCs is a current focus of study.

The genomic locus of fibroblast growth factor receptor 1 (*FGFR1*), 8p11-12, is amplified in approximately 10% of all breast cancers [114-117]. Studies have demonstrated that *FGFR1*-containing 8p11-12 amplification correlates with poor patient prognosis and increased metastatic development [116, 119-121]. Moreover, patients harboring an 8p11-12 amplification and who had undergone aggressive treatment plans had poorer outcome than patients that had undergone aggressive treatment plans but did not harbor 8p11-12 amplification, suggesting that FGFR1 might represent a good therapeutic target for patients with drug resistant breast cancers [120]. Interestingly, *FGFR1* amplification is commonly found in luminal A and luminal B breast cancer subtypes, which are largely HR+ breast cancers, and studies using *FGFR1*-amplified breast cancer cell lines demonstrate that *FGFR1* amplification mediates endocrine therapy resistance that can be reduced by using siRNA against *FGFR1* [120, 121]. Furthermore, a number of TNBC cell lines appear to be dependent on FGFR signaling, as inhibition of FGFR signaling with the small molecule inhibitor, PD173074, or siRNAs results in decreased cell growth *in vitro* and *in vivo* [123]. Studies using a previously engineered inducible FGFR1 (iFGFR1) system have further ascertained that iFGFR1 activation in mouse mammary epithelial cells results in decreased apoptosis and increased cell growth, proliferation, and

survival [127, 128]. Taken together, these studies suggest that FGFR1 is involved in mediating therapeutic resistance by promoting cell survival and inhibiting apoptosis, and led us to hypothesize that FGFR1 signaling mediates chemoresistance.

Previously, we have shown that iFGFR1 signaling requires epidermal growth factor receptor (EGFR) activation to stimulate increased proliferation, migration, and extracellular signal-regulated kinase 1/2 (ERK1/2) activation in mammary epithelial cells [318]. EGFR is expressed in 50-70% of basal-like and/or TNBCs, and patients with TN tumors that do express EGFR have worse prognosis than those who do not [68, 72, 73, 232]. Additionally, clinical trials to determine if there is a benefit to adding EGFR inhibitors with chemotherapy regimens have commenced [50]. Together, these studies indicate that EGFR might be a valuable target downstream of FGFR1 in mediating TNBC chemoresistance.

Another factor that has been indicated to mediate breast cancer chemoresistance is signal transducer and activator of transcription 3 (STAT3). STAT3 has been reported to be constitutively activated in a number of cancers, including 50-60% of all breast cancers [264-270]. Several studies have found that STAT3 constitutive activation occurs in chemoresistant tumors and that inhibition of STAT3 re-establishes chemosensitivity of breast cancer cell lines [269, 282-284]. Because of its role as a transcription factor, aberrant activation of STAT3 leads to increased expression of many genes that act to promote tumorigenesis,

such as *Cyclin D1*, *c-myc*, and several matrix metalloproteases (*MMPs*) [269, 276]. In breast cancer cell lines, STAT3 activation has been shown to increase proliferation, migration, and invasion [266, 271, 275, 278]. Alternatively, inhibition of STAT3 leads to decreased growth and metastatic ability of breast cancer cell lines [272, 273, 277]. Studies have also demonstrated that inhibition of STAT3 increases apoptosis [281, 283]. Notably, inhibition of Janus kinases (JAKs) or glycoprotein-130 (gp130) can also block STAT3 activation and induce chemosensitivity and apoptosis, suggesting that STAT3 activation in breast cancers is stimulated via cytokine signaling through gp130/JAK [270-272, 277, 282, 283].

In the present study, we demonstrate that FGFR1 activation decreases responsiveness of mammary epithelial and breast cancer cells to the chemotherapeutic agent, Doxorubicin. Inhibiting FGFR1 with the small molecule inhibitor, PD173074, both *in vitro* and *in vivo* results in increased chemosensitivity and apoptosis. We also show that FGFR1 and EGFR are co-expressed in a panel of TNBC cell lines. Interestingly, while our data suggest that inhibiting EGFR increases chemosensitivity, it appears that FGFR1-induced chemoresistance is only partially mediated through EGFR signaling. Conversely, we demonstrate that FGFR1 signaling indirectly activates STAT3 through iFGFR1-induced expression of leukemia inhibitory factor (LIF), which subsequently signals through gp130. Furthermore, we show that inhibition of

STAT3 with the small molecule inhibitor, Stattic, restores mouse mammary epithelial cell chemosensitivity to Doxorubicin *in vitro*.

3.2 Materials and Methods

3.2.A Cell culture.

Generation of HC11/R1 cells has been described previously [128], and HC11/R1 and HC11 cells were obtained from Dr. Jeff Rosen (Baylor College of Medicine, Houston, TX, USA). HC11/R1 cells were maintained in HC11/R1 complete media [serum free (SF)-RPMI (Invitrogen, Carlsbad, CA, USA) supplemented with final percentages/concentrations of 10% FBS (Invitrogen), 1% Pen Strep (Invitrogen), 5 µg/mL Insulin (Sigma-Aldrich Corporation, St. Louis, MO, USA), 10 ng/mL EGF (Invitrogen), and 0.7 µg/mL Puromycin (Sigma-Aldrich Corporation)]. HC11 cells were maintained in HC11 complete media (HC11/R1 complete media without Puromycin). Hs578T cells were obtained from the American Type Culture Collection (ATCC**,** Manassas, VA, USA) and maintained in Hs578T complete media [SF-DMEM (Invitrogen) supplemented with final percentages of 10% FBS and 1% Pen Strep]. MCF10A cells were obtained from ATCC and maintained in DMEM/F12 (Lonza, Allendale, NJ, USA), supplemented with final percentages/concentrations of 5% horse serum (Invitrogen), 1% Pen Strep, 10 µg/mL Insulin, 0.5 µg/mL Hydrocortisone (Sigma-Alrich Corporation), 100 ng/mL Cholera toxin (Sigma-Alrich Corporation), 20 ng/mL EGF. MDA-MB-231 cells were obtained from ATCC and maintained in SF-DMEM/F12 supplemented with final percentages of 10% FBS and 1% Pen Strep**.** MDA-MB-468 cells were obtained from ATCC and maintained in SF-DMEM supplemented

with final percentages of 10% FBS and 1% Pen Strep. All cells were grown and maintained at 37°C in 5% CO_2.

3.2.B ApoTox-Glo assays.

HC11/R1 or HC11 cells were plated in 96 well white-wall, clear-bottom plates at a concentration of 5000 cells in 100 µL complete media per well. Two days later, the complete media was removed, each well was rinsed with 1xPBS (Cellgro, Manassas, VA, USA), and the cells were starved overnight in SF-RPMI. The next day, 30 nM B/B (Clontech, Mountain View, CA, USA) or its ethanol solvent was added to each well of HC11/R1 cells. HC11 cells were treated with fresh HC11/R1 conditioned media or 20 ng/mL recombinant mouse (rm)AREG (R&D Systems, Minneapolis, MN, USA) or its solvent control 1xPBS following overnight starvation. To obtain HC11/R1 conditioned media, HC11/R1 cells were plated in 10 cm dishes and grown to confluence. Confluent cells were rinsed twice with 1xPBS and starved overnight in SF-RPMI. Starved cells were treated with either 30 nM B/B or its ethanol solvent overnight to condition the media. At the time of B/B, HC11/R1 conditioned media, or rmAREG treatment, 2 µM Doxorubicin (Boynton Pharmacy, University of Minnesota, Minneapolis, MN, USA) or saline (0.9% sodium chloride (Amresco, Solon, OH, USA) in milliQ-water and then filter sterilized) as a solvent control and/or 1 µM erlotinib (Boynton Pharmacy) or DMSO (Sigma-Aldrich Corporation) as a solvent control was added as indicated. All treatments were done in at least biological triplicates. Using the

ApoTox-Glo assay from Promega (Madison, WI, USA) according to the manufacturer's protocol, apoptosis and viability were assessed 20 hours after treatment. Results are displayed as the average fold change in apoptosis relative to viability. Experiments were performed at least three separate times. Statistical analysis was performed using the unpaired student's t-test to compare two means (GraphPad QuickCalcs, GraphPad Software, Inc., CA, USA).

3.2.C TUNEL assays.

Cells were plated on poly-L-ornithine hydrobromide-coated (at 500 µg/mL, Sigma-Aldrich Corporation) 12 mm circular glass cover slips placed one per well in 24 well plates. Cells were allowed to grow to 80-90% confluency before rinsing twice with 1xPBS and then starving overnight in SF-media. Starved HC11/R1 cells were treated with either 30 nM B/B or ethanol and either 2 µM Doxorubicin or saline for 20 hours. Where indicated, 4 µM Stattic (Sigma-Aldrich Corporation) or its DMSO solvent was also added to the media for the same 20 hours. Starved HC11 cells were treated with fresh HC11/R1 conditioned media (acquired as described in the previous section) and either 2 µM Doxorubicin or saline for 20 hours. Starved Hs578T cells were treated for 20 hours with 50 ng/mL basic (b)FGF (Invitrogen) and either 2 µM Doxorubicin or saline. Alternatively, Hs578T cells grown to 80-90% confluency were treated with 1 µM PD173074 or its DMSO solvent in the complete Hs578T media overnight. The next day, the media was replaced with fresh complete Hs578T media supplemented with 1 µM

PD173074 or DMSO and either 2 μM Doxorubicin or saline for 20 hours. At 20 hours post treatment, the media was removed and replaced with 300 μL 4% PFA for 25 minutes at 4°C. Fixed cells were then stained using the DeadEnd Fluorometric TUNEL System (Promega) according to the manufacturer's protocol with the minor change that the staining protocol was carried out while the cells/coverslips were still in the wells of the 24 well plate because the cells were grown on small coverslips instead of slides. The cells/coverslips were mounted onto slides using ProLong® Gold antifade reagent with DAPI (Invitrogen) to visualize the nuclei. Five representative pictures were taken of each treatment using Leica LAS software, and all cells (nuclei) and TUNEL-positive cells were counted in a blinded manner for each of the five pictures per treatment to obtain percent TUNEL positive cells. Experiments were performed at least three separate times. Statistical analysis was performed using the unpaired student's t-test to compare two means (GraphPad QuickCalcs).

3.2.D Transplant studies.

Three to four week old Balb/c female mice were purchased from Harlan Laboratories (Indianapolis, IN, USA). 200,000 HC11/R1 cells in a total volume of 10 μL 50% 1xPBS:50% Matrigel (BD Biosciences, San Jose, CA, USA) were injected into the cleared fat pads of the fourth inguinal mammary gland of anesthetized mice. 200,000 HC11 cells in a total volume of 10 μL 50% 1xPBS:50% Matrigel were injected into the contralateral cleared fat pads of the

fourth inguinal mammary gland of anesthetized mice as a control. Four days post-transplant surgeries, all mice were started on twice weekly intraperitoneal (i.p.) injections of 1 mg/kg/day B/B to activate iFGFR1. Mice were palpated daily for the presence of tumor formation, and once tumors became measurable, they were measured daily using a caliper. When tumors reached at least 100mm^3, mice were administered 10 mg/kg/day Doxorubicin or an equivalent volume of saline via tail vein injection once weekly. Additionally, mice were treated with 25 mg/kg/day PD173074 or an equivalent volume of the 50 mM Lactate Buffer solvent via i.p. injections five out of seven days of the week. Three mice were in each treatment group. Mice were sacrificed at two weeks, when tumors reached a volume of 1cm^3, or when tumors became too small to measure. Two hours before sacrificing, mice were given i.p. injections of 0.3 mg/kg BrdU (GE Healthcare Life Sciences, Piscataway, NJ, USA). At the time of sacrifice, the fourth inguinal mammary glands/tumors were harvested for further analysis. All animal care and procedures were approved by the Institutional Animal Care and Use Committee of the University of Minnesota and were in accordance with the procedures detailed in the Guide for Care and Use of Laboratory Animals.

3.2.E TUNEL *in vivo* staining.

Glands/tumors were fixed in fresh 4% PFA for 2 hours on ice and then stored in 70% ethanol at 4°C until embedded in paraffin and sectioned at 5 μm. For apoptosis analysis, 5 μm sections were stained using the DeadEnd

Flourometric TUNEL System (Promega) according to the manufacturer's protocol. All sections were mounted in ProLong® Gold antifade reagent with DAPI to visualize the nuclei. Three sections per mouse were TUNEL stained. Five representative pictures were taken of each section. Based on DAPI staining, all cells within each picture were counted (using ImageJ software) and all TUNEL positive cells of the same picture were counted (using Leica LAS software) to determine percent TUNEL positive cells. A minimum of 33,000 cells were counted per treatment group, and all studies were performed in a blinded manner. Statistical analysis was performed using the unpaired student's t-test to compare two means (GraphPad QuickCalcs).

3.2.F Immunoblot analysis.

HC11/R1 or Hs578T cells were plated in six well plates in complete media, grown to confluence, and then starved overnight in SF-RPMI or SF-DMEM, respectively. The next day, HC11/R1 cells were treated with either 30 nM B/B or ethanol for the indicated times (Fig. 11A) or for 6 hours (Fig. 11D). Where designated, α-LIF (at 4 ng/mL, 40 ng/mL, and 0.4 μg/mL) or α-gp130 (at 50 ng/mL, 0.5 μg/mL, and 5 μg/mL) neutralizing antibodies (R&D Systems, Minneapolis, MN, USA) were added to the media at the time of B/B treatment. Starved Hs578T cells were treated with 50 ng/mL bFGF for the indicated times. Additionally, MCF10A, Hs578T, MDA-MB-231, and MDA-MB-468 cells were grown to confluence in 10 cm dishes in complete media before collecting lysates.

Following all treatments, lysates were collected in RIPA buffer, and total protein concentration was determined using a D/C Protein Assay (BioRad, Hercules, CA, USA) assay. Thirty µg protein was analyzed on an SDS-PAGE gel, and immunoblotting for FGFR1 (1:1000, 3472, Cell Signaling Technology, Danvers, MA, USA), EGFR (1:100,000, ab52894, Abcam, Cambridge, MA, USA), or phosphorylated (p)STAT3 at Y705 (1:1000, 9131, Cell Signaling Technology) was performed. Additionally, 5 µg protein was analyzed on an SDS-PAGE gel, and immunoblotting for total STAT3 (1:1000, 9132, Cell Signaling Technology) was performed. Before immunotblotting for FGFR1 and EGFR, Ponceau S (MP Biomedicals, LLC, Solon, OH, USA) staining was performed according to standard protocols on the membranes to ensure equal loading.

3.2.G ELISA analysis.

HC11/R1 cells were plated in six well plates in complete media, grown to confluence, and then serum-starved overnight. Following serum starvation, cells were treated with either 30 nM B/B or an equivalent amount of ethanol for 24 hours. Conditioned media were collected and used to quantify LIF protein concentration using an ELISA kit (R&D Systems) performed according to the manufacturer's protocol. Experiments were performed in biological duplicates. Statistical analysis was performed using the unpaired student's t-test to compare two means (GraphPad QuickCalcs).

3.3 Results

3.3.A iFGFR1 activation inhibits apoptosis of mouse mammary epithelial cells *in vitro*

Previously, it was shown that activation of iFGFR1 in the mouse mammary epithelial cell line HC11/R1 reduces the ability of these cells to undergo cell death as compared to controls with no iFGFR1 activation [128]. We verified these results using an ApoTox-Glo assay from Promega, which measures apoptosis, viability, and cytotoxicity all within the sample. As expected, after 20 hours of B/B treatment following overnight serum starvation, HC11/R1 cells undergo significantly less apoptosis than HC11/R1 cells treated with ethanol, the B/B solvent control (Fig. 7A). Because inhibition of FGFR signaling has recently been shown to inhibit survival of basal-like and TNBCs and FGFR1 has been associated with resistance to available breast cancer therapies, we hypothesized that FGFR1 activation promotes chemoresistance [121, 123]. Doxorubicin is a canonical anthracycline-based chemotherapy frequently used in the clinic to treat women with TNBCs [50, 51]. Therefore, we treated HC11/R1 cells with Doxorubicin in conjunction with either B/B or ethanol treatment for 20 hours and again performed an ApoTox-Glo assay. As seen in Figure 7A, while Doxorubicin significantly increases the amount of apoptosis of ethanol-treated HC11/R1 cells, there is still a significant reduction in iFGFR1-activated HC11/R1 cell death even with Doxorubicin treatment. These results were confirmed using a second method to detect cells undergoing apoptosis, TUNEL (Fig. 7C and 7E). Together,

these data suggest that FGFR1 signaling confers mammary epithelial cells a protection from programmed cell death.

As we have previously shown that iFGFR1 activation results in increased secretion of proteins such as the growth factor ligands AREG and EREG, we wanted to examine whether the FGFR1-mediated inhibition of apoptosis occurred through secreted factors [318]. For that reason, we incubated HC11 cells, which do not express the *iFGFR1* construct, with fresh conditioned media from HC11/R1 cells treated overnight with either B/B or ethanol. Figure 7B shows that HC11 cells treated with conditioned media from iFGFR1-activated HC11/R1 cells still undergo significantly less apoptosis than HC11 cells incubated in conditioned media from ethanol-treated HC11/R1 cells. Interestingly, HC11 cells incubated in conditioned media from ethanol-treated HC11/R1 cells underwent significantly more apoptosis when Doxorubicin was added to the HC11 cells as compared to no Doxorubicin treatment (Fig. 7B). However, HC11 cells incubated in iFGFR1-activated HC11/R1 conditioned media were significantly protected from Doxorubicin-induced apoptosis as compared to HC11 cells incubated in the ethanol-treated conditioned media and treated with Doxorubicin (Fig. 7B). These results were also confirmed using TUNEL staining (Fig. 7D and 7F). These data suggest that activating iFGFR1 results in the secretion of factors that confer protection of these cells from apoptosis in an autocrine manner.

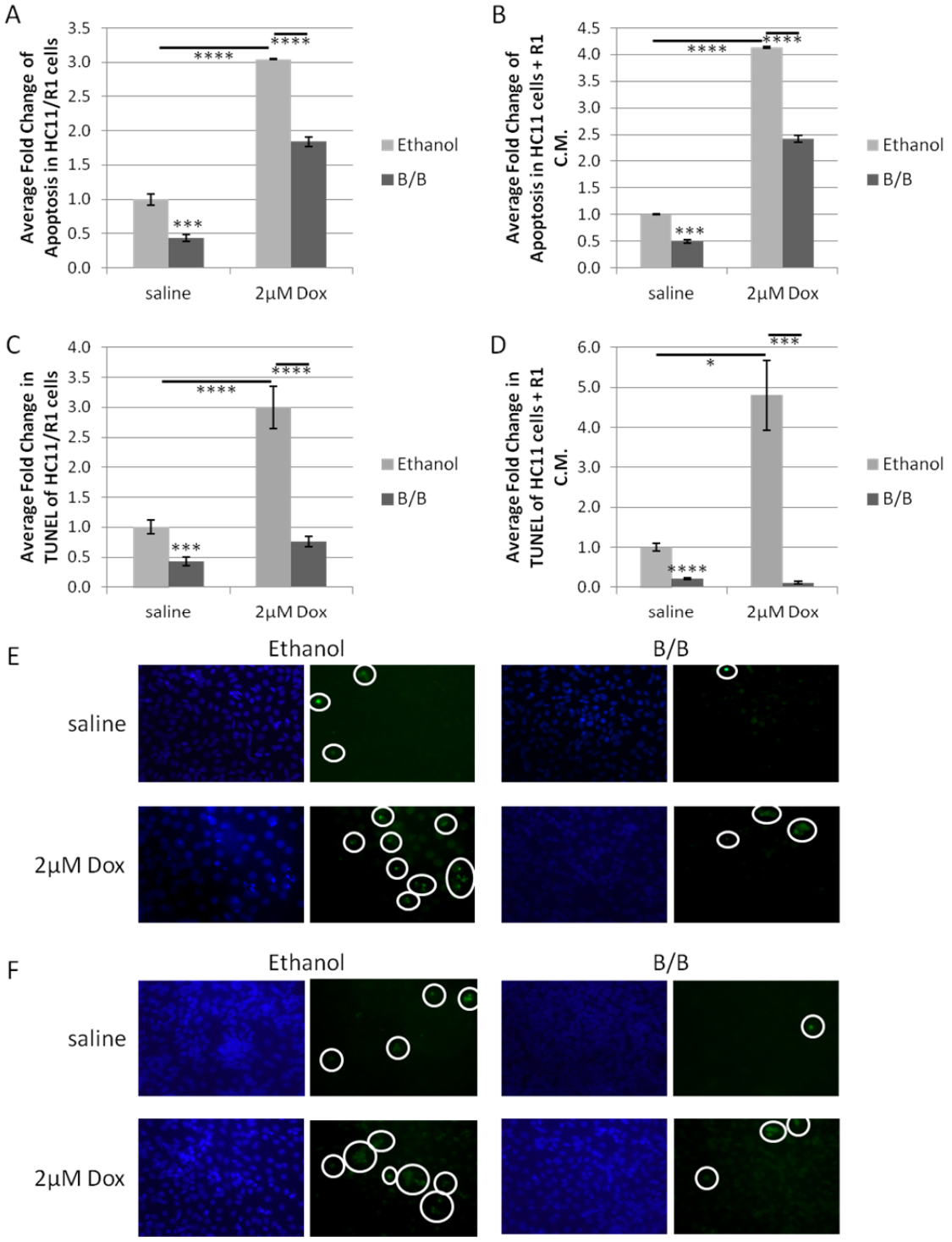

Figure 7: iFGFR1 activation reduces apoptosis in mouse mammary epithelial cells. A) Quantification of apoptosis as detected by ApoTox-Glo assay of serum-starved HC11/R1 cells following 20 hours of treatment with 30 nM B/B or its ethanol solvent and either 2 µM Doxorubicin or its saline solvent. Results are shown as average fold change in apoptosis

normalized to viability. Experiments were conducted in biological triplicate and performed three individual times. Error bars represent s.e.m. (***P<0.005, ****P<0.001) B) Quantification of apoptosis as detected by ApoTox-Glo assay of serum-starved HC11 cells incubated in HC11/R1 conditioned media for 20 hours. Conditioned media was obtained by treating serum-starved HC11/R1 cells with 30 nM B/B or ethanol overnight. Additionally, either 2 μM Doxorubicin or saline was added to the HC11 cells at the time of conditioned media treatment. Results are shown as average fold change in apoptosis normalized to viability. Experiments were conducted in biological triplicate and performed three individual times. Error bars represent s.e.m. (***P<0.005, ****P<0.001) C) Quantification of apoptosis as detected by TUNEL positive staining in serum-starved HC11/R1 cells after 20 hours of treatment with 30 nM B/B or ethanol and either 2 μM Doxorubicin or saline. Five representative images of each treatment were taken, and all cells were counted using DAPI to identify nuclei. TUNEL positive cells in each picture were also counted, and percent TUNEL positive cells was determined. Results are shown as average fold change of TUNEL positive cells relative to total cell number. Experiments were performed three individual times, and cells were counted in a blinded manner. Error bars represent s.e.m. (***P<0.005, ****P<0.001) D) Quantification of apoptosis as detected by TUNEL positive staining in serum-starved HC11 cells after 20 hours of treatment with HC11/R1 conditioned media. HC11/R1 conditioned media was obtained as in (B). At the time of conditioned media treatment, HC11 cells were also given either 2 μM Doxorubicin or saline. Five representative images of each treatment were taken, and all cells were counted using DAPI to identify nuclei. TUNEL positive cells in each picture were also counted, and percent TUNEL positive cells was determined. Results are shown as average fold change of TUNEL positive cells relative to total cell number. Experiments were performed three individual times, and cells were counted in a blinded manner. Error bars represent s.e.m. (*P<0.05, ***P<0.005, ****P<0.001) E) Representative images of TUNEL staining in serum-starved HC11/R1 cells following 20 hours of treatment with 30 nM B/B or ethanol and either 2 μM Doxorubicin or saline. TUNEL positive cells are circled. DAPI = blue, TUNEL = green. All pictures were taken at 40x magnification. F) Representative images of TUNEL staining in serum-starved HC11 cells following 20 hours of treatment with HC11/R1 conditioned media and either 2 μM Doxorubicin or saline. TUNEL positive cells are circled. DAPI = blue, TUNEL = green. All pictures were taken at 40x magnification.

3.3.B FGFR signaling inhibits apoptosis of a TNBC cell line

To validate the importance of FGFR1 signaling in chemoresistance, we used the human TNBC cell line Hs578T. This cell line is dependent on FGFR signaling, and it has previously been shown that the FGFR inhibitor, PD173074, reduces cell growth in these cells [123]. Treatment of Hs578T cells with PD173074 resulted in an increase in apoptosis as compared to cells treated with the DMSO solvent control as detected by TUNEL staining (Fig. 8A and 8C). Additionally, while Hs578T cells growing in complete media had a partial response to Doxorubicin treatment, PD173074 treatment significantly increased Hs578T cell Doxorubicin-induced apoptosis (Fig. 8A and 8C). Furthermore, when we first starved the Hs578T cells and then subsequently treated with bFGF to activate FGFR signaling, we observed a significant decrease in apoptosis of these cells as compared to no treatment control cells (Fig. 8B and 8D). These results are in line with iFGFR1 activation in HC11/R1 cells as described above. Whereas Doxorubicin significantly increased apoptosis in the no treatment starved cells, FGFR signaling activation with bFGF significantly reduced the ability of Hs578T cells to undergo apoptosis as detected by TUNEL staining (Fig. 8B and 8D). These results demonstrate a role of FGFR1 in the inhibition of TNBC cell death, and furthermore, they provide evidence that FGFR1 signaling promotes chemoresistance that can be reversed by inhibiting FGFR1.

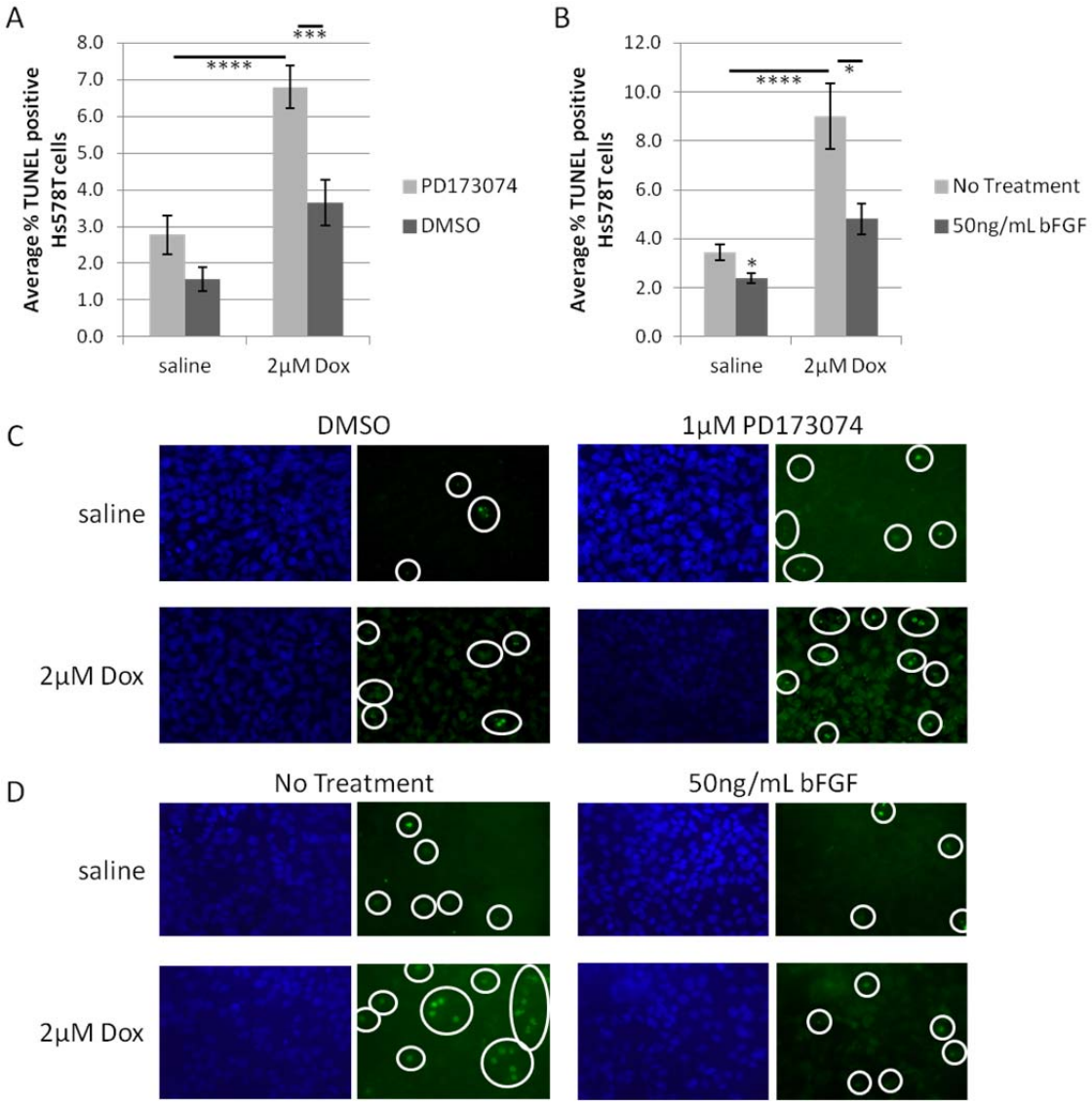

Figure 8: FGFR1 signaling reduces apoptosis in a TNBC cell line.
A) Quantification of apoptosis as detected by TUNEL positive staining in Hs578T cells in complete media after 20 hours of treatment with 1 μM PD173074 or its DMSO solvent and either 2 μM Doxorubicin or saline. Five representative images of each treatment were taken, and all cells were counted using DAPI to identify nuclei. TUNEL positive cells in each picture were also counted, and percent TUNEL positive cells was determined. Results are shown as average percent TUNEL positive cells relative to total cell number. Experiments were performed three individual times, and cells were counted in a blinded manner. Error bars represent s.e.m. (***P<0.005, ****P<0.001) B) Quantification of apoptosis as detected by TUNEL positive staining in serum-starved Hs578T cells after 20 hours of treatment with 50 ng/mL bFGF and either 2 μM Doxorubicin or its saline solvent. Five representative images of each treatment were taken, and all cells were counted using DAPI to identify nuclei. TUNEL positive cells in each picture were also counted, and percent TUNEL positive cells was determined. Results are shown as average percent TUNEL positive cells relative to total cell

number. Experiments were performed three individual times, and cells were counted in a blinded manner. Error bars represent s.e.m. (*P<0.05, ****P<0.001) C) Representative images of TUNEL staining in Hs578T cells in complete media following 20 hours of treatment with 1 μM PD173074 or its DMSO solvent and either 2 μM Doxorubicin or saline. TUNEL positive cells are circled. DAPI = blue, TUNEL = green. All pictures were taken at 40x magnification. D) Representative images of TUNEL staining in serum-starved Hs578T cells following 20 hours of treatment with 50 ng/mL bFGF and either 2 μM Doxorubicin or saline. TUNEL positive cells are circled. DAPI = blue, TUNEL = green. All pictures were taken at 40x magnification.

3.3.C Inhibition of iFGFR1 *in vivo* increases Doxorubicin-induced apoptosis

Since the complexity of tumor formation and chemoresistance is not fully recapitulated *in vitro*, we examined whether inhibition of FGFR1 *in vivo* resulted in increased chemosensitivity. Three to four week old Balb/c female mice were transplanted with HC11/R1 cells in the cleared fat pads of the fourth inguinal mammary glands and subsequently injected with B/B twice weekly to activate iFGFR1. As a control, HC11 cells were injected into the cleared fat pads of the fourth inguinal mammary glands on the contralateral side. Mice were monitored for tumor formation and growth, and once tumors reached at least 100mm^3, mice were treated with either Doxorubicin or saline as well as with either PD173074 or 50 mM Lactate Buffer. Control animals (treated with saline and Lactate Buffer), had steady tumor growth (Fig. 9A). Notably, mice treated with both Doxorubicin and PD173074 had significantly less tumor burden than control animals. Combined treatment resulted in immediate response and ultimately a decrease in tumor burden in these animals. While mice given Doxorubicin and Lactate Buffer or mice given saline and PD173074 did eventually start responding to the individual treatments, there was not a significant difference in tumor burden in these mice as compared to control animals.

Because chemotherapies act by inducing cell death, we investigated the levels of apoptosis in the collected tumors. As shown in Figure 9B, mice treated with both Doxorubicin and PD173074 had significantly more cells undergoing apoptosis as assessed via TUNEL staining than control or individually treated

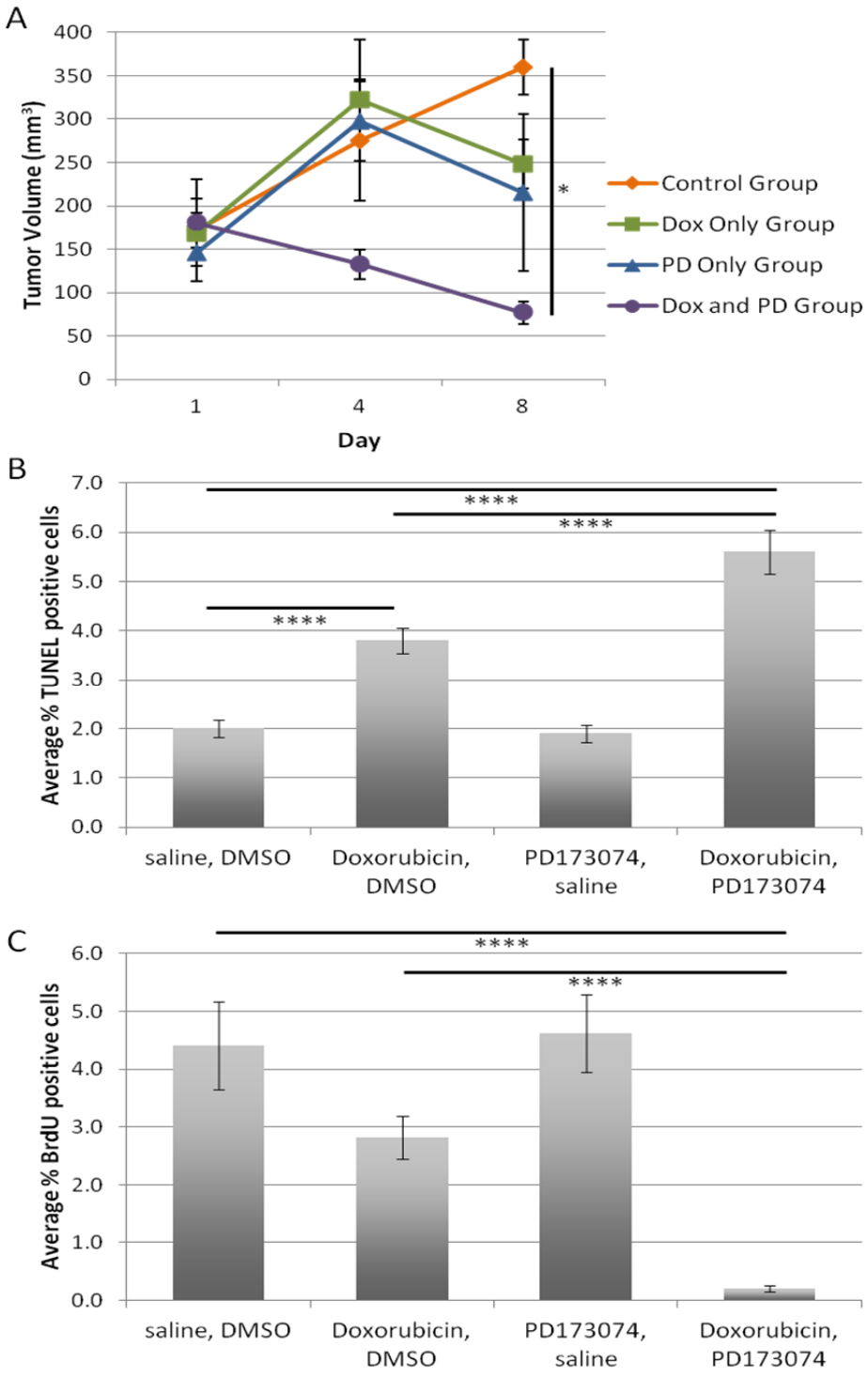

Figure 9: Inhibition of iFGFR1 increases Doxorubicin chemosensitivity *in vivo*.
A) Three to 4 week old Balb/c female mice were injected with 200,000 HC11/R1 cells into the cleared fat pads of the fourth inguinal mammary gland. Mice were given twice weekly

injections of 1mg/kg/day B/B to activate iFGFR1 and palpated daily for tumor formation. Once tumors reached at least 100mm^3, mice were treated with either 10 mg/kg/day Doxorubicin or an equivalent volume of saline via tail vein injection once weekly and either 25 mg/kg/day PD173074 or an equivalent volume of the 50 mM Lactate Buffer solvent via i.p. injections five out of seven days of the week. Graph depicts average tumor volume for each treatment group at days 1, 4, and 8 of treatment. Each treatment group contained three mice. Error bars represent s.e.m. (*P<0.05) B) Quantification of TUNEL staining of tumor sections for each treatment group. Tumors were sectioned, and three sections of each mouse were stained with TUNEL and DAPI. Five representative pictures of each section were taken. Total cell number was determined for each picture using ImageJ software, and the number of TUNEL positive cells was also counted for each picture. Graph shows average percent TUNEL positive cells for each treatment group. Error bars represent s.e.m. (****P<0.001) C) Quantification of BrdU IF staining of tumor sections for each treatment group. Tumors were sectioned, and three sections of each mouse were stained for BrdU and DAPI. Five representative pictures of each section were taken. Total cell number was determined for each picture using ImageJ software, and the number of BrdU positive cells was also counted for each picture. Graph shows average percent BrdU positive cells for each treatment group. Error bars represent s.e.m. (****P<0.001)

animals, suggesting that inhibition of FGFR1 signaling confers increased chemosensitivity.

3.3.D Inhibiting EGFR partially restores Doxorubicin-induced apoptosis of mouse mammary epithelial cells

Like FGFR1, EGFR has recently been identified as a potential druggable target of basal-like/TNBCs. Accordingly, we wanted to test whether EGFR is also involved in acquisition of chemoresistance in FGFR1-activated mammary epithelial cells, so we first examined FGFR1 and EGFR expression in a small panel of TNBC cell lines. As shown in Figure 10A, FGFR1 and EGFR are both expressed in all TN cell lines tested, indicating that FGFR1 and EGFR signaling may cooperate in the attainment of chemoresistance.

Previously, we demonstrated that activation of FGFR1 leads to increased expression of the EGFR ligands AREG and EREG [318]. These ligands then signal in an autocrine manner through EGFR to promote the FGFR1-induced increase in mammary epithelial cell proliferation and migration. The EGFR kinase inhibitor, erlotinib, could significantly reduce the aberrant proliferation and migration stimulated upon iFGFR1 activation, providing evidence that EGFR signaling is required for FGFR1-mediated tumorigenic processes [318]. To determine the effect of EGFR activation in mediating chemoresistance, we used rmAREG to stimulate EGFR activation in HC11 cells. While rmAREG treatment reduced the amount of HC11 cell death detected by ApoTox-Glo assay as

compared to PBS-treated control cells, erlotinib treatment completely abolished this apoptosis reduction (Fig. 10B). Additionally, Doxorubicin in combination with erlotinib increases apoptosis in both rmAREG- and PBS-treated HC11 cells. However, EGFR activation could block Doxorubicin-induced apoptosis as compared to PBS-treated controls. These results suggest that EGFR may be an important target for increasing sensitivity to chemotherapies.

To explore this further, we used ApoTox-Glo assays to assess whether inhibiting EGFR could reverse the reduction in apoptosis seen in iFGFR1-activated mammary epithelial cells. As expected, HC11/R1 cells treated with B/B underwent significantly less apoptosis as compared to ethanol-treated controls, even in the presence of Doxorubicin (Fig. 10C). Erlotinib treatment alone did not significantly induce apoptosis in ethanol-treated control cells, nor did it eradicate the reduced levels of apoptosis in B/B-treated HC11/R1 cells. Interestingly though, combined Doxorubicin and erlotinib treatment induced more cell death in HC11/R1 cells treated with ethanol and modestly increased apoptosis in iFGFR1-activated HC11/R1 cells as compared to cells treated with Doxorubicin only. Similar results were obtained when HC11 cells were incubated in HC11/R1 conditioned media. B/B-treated HC11/R1 conditioned media protected HC11 cells from Doxorubicin-induced apoptosis, suggesting that FGFR1 mediates chemoresistance through the secretion of soluble factors (Fig. 10D). Erlotinib treatment conferred only a slight increase in apoptosis in cells incubated in conditioned media from iFGFR1-activated HC11/R1 cells. All together, these

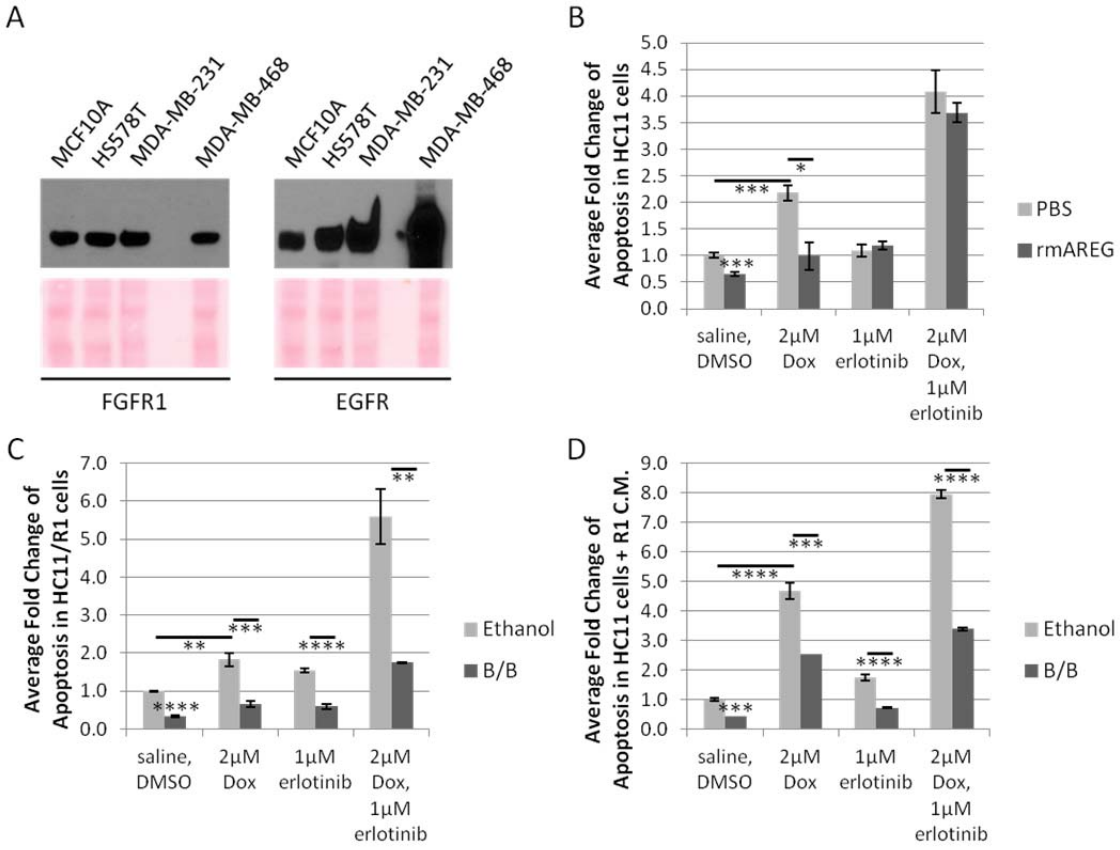

Figure 10: EGFR is partially responsible for FGFR1-mediated chemoresistance.
A) Cells were grown to confluence in complete media and whole cell lysates were collected and analyzed by immunoblot for expression of FGFR1 and EGFR. Bottom panels show Ponceau S staining of the membrane for loading control. B) Quantification of apoptosis as detected by ApoTox-Glo assay of serum-starved HC11 cells treated with 20 ng/mL rmAREG or its PBS solvent control for 20 hours. Cells were also treated with either 2 μM Doxorubicin or saline and either 1 μM erlotinib or its solvent control DMSO. Results are shown as average fold change in apoptosis normalized to viability. Experiments were conducted in biological triplicate and performed three individual times. Error bars represent s.e.m. (*P<0.05, ***P<0.005, ****P<0.001) C) Quantification of apoptosis as detected by ApoTox-Glo assay of serum-starved HC11/R1 cells treated with 30 nM B/B or ethanol for 20 hours. Cells were also treated with either 2 μM Doxorubicin or saline and either 1 μM erlotinib or DMSO. Results are shown as average fold change in apoptosis normalized to viability. Experiments were conducted in biological triplicate and performed three individual times. Error bars represent s.e.m. (**P<0.01, ***P<0.005, ****P<0.001) D) Quantification of apoptosis as detected by ApoTox-Glo assay of serum-starved HC11 cells incubated in HC11/R1 conditioned media for 20 hours. Conditioned media was obtained by treating serum-starved HC11/R1 cells with 30 nM B/B or ethanol overnight. At time of conditioned media treatment, HC11 cells were also treated with either 2 μM Doxorubicin or saline and either 1 μM erlotinib or its solvent control DMSO. Results are shown as average fold change in apoptosis normalized to viability. Experiments were conducted in biological triplicate and performed three individual times. Error bars represent s.e.m. (***P<0.005, ****P<0.001)

results suggest that, in cells that do not have FGFR1 activation, EGFR may be an important target for increasing sensitivity to chemotherapies but provide evidence against strong cooperation between FGFR1 and EGFR signaling in chemoresistance.

3.3.E FGFR1 induces activation of STAT3

The results described above indicate that FGFR1-mediated chemoresistance is not fully dependent on EGFR activation. Therefore, FGFR1 must activate other signaling molecules whose induction interferes with chemotherapy-induced cell death. Recently, STAT3 has been implicated in chemoresistance and reduced apoptosis in a number of breast cancer cell lines [281-284]. Consequently, we next determined whether FGFR1 signaling regulates STAT3 activation. HC11/R1 cells were treated with B/B or ethanol over a 24 hour time course. Whole cell lysates were collected at regular intervals, and immunoblotting for pSTAT3 was performed. As seen in Figure 11A, iFGFR1 activation results in increased pSTAT3 by 2 hours that is sustained at least 24 hours. This same STAT3 activation pattern is detected in the human TNBC cells, Hs578T, treated with bFGF to activate FGFR signaling (Fig. 11B). Because STAT3 activation is not stimulated immediately following FGFR1 activation (data not shown) as would be expected if STAT3 directly binds to FGFR1, we next sought to ascertain what the STAT3 activation signaling axis is in our system. Canonical STAT3 activation occurs via cytokine signaling through gp130/JAK. In

the mammary gland, LIF and oncostatin M (OSM) are known cytokine regulators of STAT3 activation [262, 263]. Furthermore, LIF expression is upregulated in HC11/R1 cells following iFGFR1 activation as assessed via ELISA (Fig. 11C). Therefore, we used neutralizing antibodies against LIF and gp130 to determine whether cytokine signaling through gp130 induces pSTAT3 in our system. As seen in Figure 11D, HC11/R1 cells treated with B/B and increasing amounts of α-LIF or α-gp130 for 6 hours, have reduced pSTAT3 levels in a dose-dependent fashion as compared to B/B treatment alone. To examine if STAT3 activation confers chemoresistance, we used the STAT3 inhibitor, Stattic, and performed TUNEL analysis on HC11/R1 cells. Interestingly, inhibition of STAT3 alone is not sufficient to induce increased apoptosis, but blocking STAT3 in Doxorubicin-treated iFGFR1-activated HC11/R1 cells significantly abolishes the block in apoptosis seen in Doxorubicin-treated iFGFR1-activated HC11/R1 cells (Fig. 11E). These results suggest that FGFR1 indirectly mediates STAT3 activation which then ultimately confers chemoresistance in FGFR1-activated mammary epithelial cells.

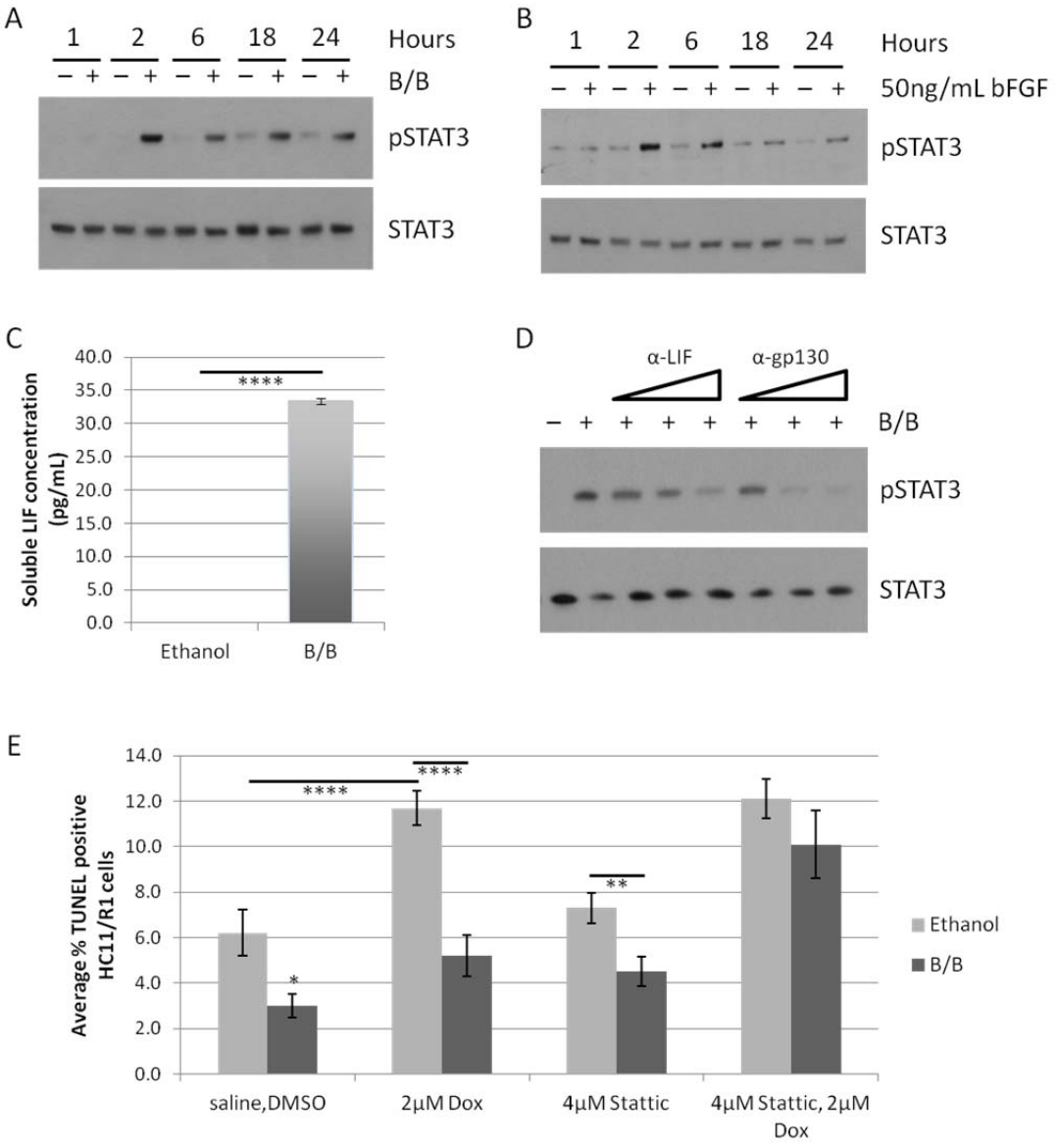

Figure 11: FGFR1 mediates Doxorubicin chemoresistance by activating STAT3.
A) Serum-starved HC11/R1 cells were treated with either 30 nM B/B or ethanol for the indicated times. Following treatment, whole cell lysates were collected and analyzed by immunoblot for pSTAT3 and total STAT3. B) Serum-starved Hs578T cells were treated with 50 ng/mL bFGF or not treated for the indicated times. Following treatment, whole cell lysates were collected and analyzed by immunoblot for pSTAT3 and total STAT3. C) To detect secreted LIF protein, ELISA analysis was performed on conditioned HC11/R1 media. Serum-starved HC11/R1 cells were treated with either 30 nM AP or its ethanol solvent for 24 hours to condition the media. Experiments were performed in biological duplicates. Error bars represent s.e.m. (****P<0.001) D) Serum-starved HC11/R1 cells were treated with either 30 nM B/B or ethanol for 6 hours. Additionally, cells were treated with either α-LIF (at 4 ng/mL, 40 ng/mL, and 0.4 μg/mL) or α-gp130 (at 50 ng/mL, 0.5 μg/mL, and 5 μg/mL) neutralizing antibodies as indicated. Following treatment, whole cell lysates were collected and analyzed

by immunoblot for pSTAT3 and total STAT3. E) Quantification of apoptosis as detected by TUNEL positive staining in serum-starved HC11/R1 cells after 20 hours of treatment with 30 nM B/B or ethanol. Cells were also treated with either 2 μM Doxorubicin or saline and either 4 μM Stattic or its DMSO solvent. Five representative images of each treatment were taken, and all cells were counted using DAPI to identify nuclei. TUNEL positive cells in each picture were also counted, and percent TUNEL positive cells was determined. Results are shown as average percent TUNEL positive cells relative to total cell number. Experiments were performed three individual times, and cells were counted in a blinded manner. Error bars represent s.e.m. (**P<0.01, ****P<0.001)

3.4 Discussion

While TNBCs represent only approximately 20% of all breast cancers, they account for a disproportionate number of breast cancer-related deaths [50, 319]. Because they do not express HRs or HER2, they do not respond to endocrine therapy or HER2-based therapies. Instead, the typical course of treatment for TNBCs involves various regimens of chemotherapies, which non-specifically induce apoptosis. There are several currently used chemotherapeutic agents [50, 51, 320, 321]. In the present study, we concentrated on the anthracycline-based chemotherapy drug, Doxorubicin, which is often used in both adjuvant and neoadjuvant settings [51, 322-324]. Intercalation of Doxorubicin into DNA induces double-stranded breaks, which when not properly repaired ultimately results in cell death. Interestingly, while patients harboring TNBCs do seem to respond relatively well to chemotherapies, this group of tumors continues to have the worst prognosis of all breast cancers [78, 325]. Patients with TNBCs have significantly worse disease-free survival than patients with non-TNBCs, and following disease recurrence, patients with TNBCs have significantly worse overall survival than patients without TNBCs with shorter time from recurrence to death [325-327]. Additionally, TNBC recurrence often occurs at distant rather than local sites [328]. Because recurrent metastatic TNBCs result from not achieving pCR and correspond to resistance of chemotherapeutic treatment, much work is being done to identify alternative targets in TNBCs that

will either increase the number of patients achieving pCR or decrease chemoresistance.

Recently, FGFR1 has been implicated as a potential target in TNBCs. Work has been done demonstrating that inhibition of FGFR signaling via the small molecule inhibitor, PD173074, in a number of TNBC cell lines sensitizes them to decreased cell survival and increased apoptosis [123]. Using siRNA directed against FGFR1 also resulted in decreased survival of a TNBC cell line [123]. Furthermore, activation of iFGFR1 *in vitro* has previously been shown to reduce the number of cells undergoing apoptosis [128]. We hypothesized that FGFR1 activation promotes chemoresistance of TNBCs. In support of this hypothesis, it has recently been demonstrated that FGFR1 signaling appears to drive endocrine resistance in *FGFR1*-amplified, ER+ breast cancer cell lines [121]. Additionally, another FGFR, FGFR4, has been shown to be upregulated in Doxorubicin-treated breast cancer cell lines, whereby it acts to promote chemoresistance [329]. We presently verify that iFGFR1 activation in the mouse mammary epithelial cell line HC11/R1 decreases apoptosis and further demonstrate that iFGFR1 activation confers significant inhibition of apoptosis compared to ethanol-treated controls when these cells are treated with the chemotherapy Doxorubicin. This effect is at least in part driven by FGFR1-induced expression of secreted factors because HC11 cells incubated in conditioned media from iFGFR1-activated HC11/R1 cells are also protected from Doxorubicin-mediated cell death. Moreover, the FGFR-dependent TNBC cell line,

Hs578T, undergoes significantly more Doxorubicin-stimulated apoptosis when also treated with the FGFR small molecule inhibitor, PD173074. Alternatively, bFGF treatment of serum-starved Hs578T cells significantly inhibits Doxorubicin-induced cell death. We also find that mice transplanted with HC11/R1 cells into cleared mammary fat pads and treated with B/B to activate iFGFR1 have significantly smaller tumor burden when given both Doxorubicin and PD173074 as compared to control mice. Tumors in control mice exhibit continual growth, while the tumors in mice treated with both Doxorubicin and PD173074 shrink in size. Furthermore, these combined treatment tumors have significantly more cells undergoing apoptosis than control tumors. Mice treated with only Doxorubicin have intermediate tumor size and number of cells undergoing apoptosis compared to control or Doxorubicin/PD173074 combination-treated mice. Interestingly, inhibition of iFGFR1 with PD173074 alone did not increase levels of apoptosis as compared to control treatment animals, although they had tumor burden similar to Doxorubicin-only treated animals. It is possible that the level of proliferation or angiogenesis differs between the treatment groups as well and are currently under investigation. Together these results indicate that FGFR1 signaling can promote chemoresistance and that inhibiting FGFR signaling can increase chemoresponsiveness and apoptosis both *in vitro* and *in vivo*.

Acquisition of chemoresistance is thought to arise through the stem cell population. Although the vast majority of tumor cells are in a differentiated state, it has been proposed that there exists within the tumor a small number of

undifferentiated cancer stem cells or cancer-initiating cells [330, 331]. Studies have demonstrated that cancer stem cells are insensitive to chemotherapies and that chemotherapy treatment ultimately enriches for the cancer stem cell population while the bulk of the tumor responds to chemotherapy and undergoes apoptosis [84, 86-88]. Recently, the EGFR ligand AREG has been implicated in mammary stem cell maintenance and was shown to be essential for mammosphere formation of the mammary epithelial cell line, COMMA-D β-geo [155]. We have previously shown that AREG is significantly upregulated following iFGFR1-activation and subsequently activates EGFR [318]. In the present study, we show that treatment of HC11 cells with rmAREG decreases Doxorubicin-mediated apoptosis as compared to PBS-treated control cells. Inhibition of EGFR with the small molecule inhibitor erlotinib, however, abolishes this chemoresistance. Studies attempting to identify factors that are consistently expressed in TNBCs have demonstrated that EGFR and cytokeratin 5/6 are positive markers that reliably distinguish the set of basal-like TNBCs. This set of five markers (EGFR+, cytokeratin 5/6+, ER-, PR-, HER2-) has been designated the Core Basal group. The Core Basal group of TNBCs has been found to have poorer prognosis than TN basal-like breast cancers that do not express EGFR [68, 73]. Our results suggest that EGFR signaling might also mediate chemoresistance, thereby resulting in worse patient prognosis in EGFR+ TNBCs, and strengthen the hypothesis that EGFR represents a good therapeutic target for TNBCs.

We previously demonstrated that FGFR1-induced tumorigenic phenotypes, such as increased proliferation and migration, require autocrine EGFR activation [318]. In this study, we verified that FGFR1 and EGFR are expressed in the same TNBC cell lines as detected via immunoblotting of a small panel of TNBC cell lines. In iFGFR1-activated HC11/R1 cells treated with Doxorubicin, we found that also inhibiting EGFR with erlotinib resulted in a significant, but moderate, increase in apoptosis. Because EGFR is activated via soluble ligands, we also incubated HC11 cells with HC11/R1 conditioned media. HC11 cells incubated in iFGFR1-activated conditioned media and treated with Doxorubicin also had a limited increase in apoptosis when erlotinib was added to inhibit EGFR signaling. Together, these data suggest that FGFR1 does, at least in part, signal through EGFR to stimulate chemoresistance. However, these results also indicate that FGFR1 most likely mediates chemoresistance by activating multiple downstream effector molecules. Therefore, while EGFR appears to be an important mediator of chemoresistance, these results suggest that in TNBCs that express both FGFR1 and EGFR, inhibition of FGFR1 might be the more beneficial therapeutic strategy. Further studies will need to be conducted to fully understand the signaling axis between FGFR1 and EGFR in TNBCs.

Because FGFR1 appears to activate more than one factor to mediate chemoresistance, we also investigated the role of STAT3 in FGFR1-stimulated chemoresistance. STAT3 has previously been identified to mediate

chemoresistance in breast cancer cell lines [282-284]. Notably, STAT3 is activated in the TNBC cell line, MDA-MB-231, and inhibition of STAT3 in these cells results in increased sensitivity to Doxorubicin [283]. While STAT3 is constitutively activated in 50-60% of all breast cancers, it has not yet been specifically linked to breast cancers harboring an *FGFR1* amplification. In the present study, we demonstrate that iFGFR1 activation in HC11/R1 cells results in activated STAT3 as detected via immunoblot. Moreover, we show that activating FGFR signaling in the TNBC cell line, Hs578T, also stimulates STAT3 activation. Notably, STAT3 activation in both cell lines first occurs at the same time point of 2 hours post-FGFR signaling activation. Interestingly, we did not detect phosphorylated STAT3 at 5, 10, 15, 30, or 60 minutes post-FGFR signaling activation (data not shown), suggesting that STAT3 is not directly activated by binding to FGFR1. This result is in stark contrast to another study demonstrating that STAT3 directly binds to and is activated by FGFRs [332]. One possible explanation for this discrepancy is that activated FGFR1 does directly induce STAT3 activation, but at very low levels in the cell lines we used. It is also possible that FGFR1 mediates STAT3 activation in multiple ways, and direct activation is not the predominant mode in the cell lines used in this study.

In support of indirect activation of STAT3 by FGFR1, we show that inhibiting either LIF or gp130 with neutralizing antibodies blocks phosphorylation of STAT3 in a dose dependent manner in iFGFR1-activated HC11/R1 cells. STAT3 was originally discovered as a downstream effector molecule of IL-6

signaling, and subsequent studies have demonstrated that multiple cytokines, including IL-6, LIF, and OSM can activate gp130/JAK and ultimately stimulate STAT3 activation in the normal mammary gland or in breast cancer cell lines [233, 262, 263, 270-272]. We show here that iFGFR1 activation significantly induces expression of LIF, but LIF is also secreted by immune cells such as macrophages. It has previously been shown that iFGFR1 activation in the mammary gland recruits macrophages to the sites of prolonged iFGFR1 signaling [129]. While most TNBCs fall into the basal-like subtype, a number of TNBCs can be classified as claudin-low tumors. One proposed feature of claudin-low tumors is high levels of infiltrating immune cells [60]. It would be interesting to determine if FGFR1-stimulated activation of STAT3 also occurs in claudin-low tumors and whether activation of STAT3 in epithelial cells is also mediated by infiltrating macrophages.

We also show that direct inhibition of STAT3 via the small molecule inhibitor, Stattic, abolishes the block in Doxorubicin-treated, iFGFR1-activated HC11/R1 cells *in vitro*. These data suggest that FGFR1-stimulated chemoresistance is largely mediated through STAT3 activation. As STAT3 is a transcription factor, further studies will need to be conducted to determine which genes STAT3 regulates in FGFR1-amplified breast cancers. Studies have shown that STAT3 directly induces increased *Survivin* expression, which is a strong mediator of cell survival [264, 269, 281]. Other well-known targets of STAT3 activation include cell cycle regulators, like Cyclin D1 and c-myc, and anti-

apoptosis factors such as B cell CLL/lymphoma-2 (BCL-2), BCL-2-related gene, long isoform (BCL-xL), and myeloid cell leukemia 1 (MCL-1) [269, 275, 276, 282]. Moreover, while we directly inhibited STAT3, other studies have demonstrated that indirect inhibition of STAT3, by inhibiting JAKs for example, results in analogous phenotypes as direct inhibition of STAT3 [266, 272, 277, 282, 283]. Further studies might focus on whether gp130 or JAKs represent good targets for therapeutic intervention in FGFR1-amplified breast cancers.

We demonstrate in this study that FGFR1 signaling stimulates chemoresistance of Doxorubicin-treated TNBCs. While STAT3 activation appears to be the predominant mediator of FGFR1-induced chemoresistance, EGFR activation is also involved in this process. Further studies will need to be conducted to examine whether inhibiting STAT3 *in vivo* increases Doxorubicin chemosensitivity and to what extent STAT3 activation and FGFR1 amplification correlate in human breast cancer tumor samples.

Chapter 4: FGFR1-induced soluble factors in mammary gland tumorigenesis and chemoresistance: What we know and where we could go.

4.1 Discussion and Future Directions

Amplification of the genomic locus of fibroblast growth factor receptor 1 (*FGFR1*) occurs in approximately 10% of all breast cancers [114-117]. While multiple amplicons have been identified from this region, the amplicon containing *FGFR1* correlates with increased *FGFR1* gene and protein expression [114, 116, 119, 121, 122]. Moreover, this *FGFR1*-containing amplicon is found in patients with decreased overall and metastasis-free survival and increased development of distant metastases, indicating that FGFR1 might prove to be a valuable therapeutic target for these patients [116, 119-121]. As such, an early phase clinical trial is currently underway to examine the benefits of adding the FGFR inhibitor AZD4547 to exemestane treatment of patients with *FGFR1* amplification in ER+ breast cancers (Safety and Efficacy of AZD4547 in Combination With Exemestane Versus Exemestane Alone in ER+ Breast Cancer Patients, NCI Clinical Trial Protocol ID: D2610C00003 2010-021220-10, NCT01202591, NCI Clinical Trials Database). Further examination of the downstream effects of

amplification and overexpression of FGFR1 could lead to identification of novel factors for targeted therapies for patients with the *FGFR1* amplification, as there are currently no FGFR1 specific therapies. Difficulty in specifically targeting FGFR1 stems from the complex nature of the FGF family. There are four FGFRs, and alternative splicing ultimately generates seven FGFRs that are highly homologous within the kinase domains [91, 93-96]. Additionally, there are 22 FGF ligands, none of which exclusively activates FGFR1 [92, 93]. Therefore, to investigate FGFR1 signaling, we use an inducible FGFR1 (iFGFR1) system in our studies.

While the FGFR1 kinase domain was retained in the iFGFR1 system, the extracellular ligand binding and transmembrane domains were replaced with a myristylation sequence to tether the kinase domain to the plasma membrane [127]. Without the ligand binding domain, endogenous FGF ligands cannot bind or induce dimerization of the receptor. Instead, dimerization of the iFGFR1 occurs via treatment with the synthetic molecule originally named AP20187 (AP) and then renamed B/B, which binds to an intracellular dimerization domain affixed to the kinase domain [127]. HC11 mouse mammary epithelial cells were retrovirally transduced to stably express the iFGFR1 construct (called HC11/R1 cells), and extensive characterization of the iFGFR1 system was performed to verify that iFGFR1 behaves in a similar manner to endogenous FGFR1 [127, 128]. In addition to the HC11/R1 *in vitro* system, a transgenic mouse line was created in which the *iFGFR1* construct is under the control of the mouse

mammary tumor virus (*MMTV*) promoter [127]. Therefore, the MMTV-iFGFR1 transgenic animals express *iFGFR1* primarily in mammary epithelial cells. To examine FGFR1 signaling on a global scale, microarrays were previously performed on RNA isolated from HC11/R1 cells (KLS unpublished data) or from mammary glands taken from MMTV-iFGFR1 mice [129]. Numerous genes were found to be dysregulated in the iFGFR-activated samples as compared to the ethanol-treated control samples, and studies are currently focused on investigating several of these factors.

Two of the most highly upregulated molecules in the iFGFR1-activated samples are amphiregulin (AREG) and epiregulin (EREG). These ligands were of great interest to us because both AREG and EREG belong to the EGFR family of ligands and, in their mature protein state, are soluble factors. Moreover, both ligands have been identified in human breast cancers prior to our studies. Notably, both ligands appear to correlate with advanced disease state or more aggressive breast tumors [169, 179, 180, 197]. Studies using mouse models and human breast cancer cell lines have shown that both AREG and EREG stimulate mammary tumor invasion and that AREG expression further promotes proliferation and tumor growth [138, 174-178, 198, 199].

In the present work, we confirm that iFGFR1 activation significantly increases AREG and EREG expression. Using quantitative reverse transcription-PCR (qRT-PCR), significant induction of both *Areg* and *Ereg* transcripts is detected within as little as 30 minutes of AP treatment of serum-starved HC11/R1

cells, and increased transcript levels are sustained for at least four hours after iFGFR1-activation. *Areg* and *Ereg* transcripts are also upregulated in MMTV-iFGFR1 mouse mammary glands following activation of iFGFR1 via AP intraperitoneal (i.p.) injections. Interestingly, significant induction of *Areg* transcripts occurs before significant induction of *Ereg* transcripts *in vivo*, suggesting that there may be some sort of positive feedback loop in the mammary gland. Because much less is known about *EREG* gene regulation, future studies could focus on examining if *Areg* and *Ereg* are regulated by the same factors and if not, what factors do regulate *EREG* expression. Immunohistochemistry (IHC) analysis of the fourth inguinal mammary glands of iFGFR1-activated MMTV-iFGFR1 animals shows that AREG and EREG are also upregulated at the protein level. Because AREG and EREG exist as transmembrane precursor proteins, we further demonstrate that both ligands are cleaved into their mature, soluble forms via ELISA analysis performed using conditioned media from serum-starved HC11/R1 cells treated with AP. These results are important because they demonstrate that AREG and EREG are not just upregulated at the transcript level and then degraded nor are they perpetually expressed in their precursor forms. These results also indicate that the enzymes responsible for releasing AREG and EREG from the plasma membrane are expressed in the HC11/R1 cells. The a disintegrin and metalloprotease (ADAM) enzymes have previously been shown to cleave AREG and EREG sheddase [107, 164-166]. Future studies could focus on verifying that

ADAM proteins are responsible for cleaving AREG and EREG in our system and examining how inhibition of the AREG and EREG sheddase(s) affects FGFR1-mediated tumorigenesis.

Additionally, we show that upregulation of AREG and EREG occurs in a human breast cancer cell line. *AREG* and *EREG* transcripts are detectable 4 hours after basic (b)FGF treatment of serum-starved MCF7 cells, and transcript levels remain increased for at least 24 hours after FGFR signaling is stimulated with bFGF. Using ELISA analysis, we find that soluble AREG is significantly upregulated in the conditioned media of bFGF-treated serum-starved MCF7 cells as compared to the no treatment control conditioned media. These results confirm that AREG is translated into mature protein that is cleaved from the plasma membrane in a human breast cancer cell line. It is of importance to note that MCF7 cells are ER+ and PR+ [333]. In the normal developing mammary gland, *AREG* expression is directly mediated by estrogen receptor α (ERα), and it has been found that AREG expression often correlates with ER+ and progesterone receptor (PR)+ status in breast cancers [145, 157, 172, 173]. Because hormone receptor (HR)+ breast cancers generally fall into the luminal A and luminal B breast cancer subtypes, it would be interesting to determine if AREG is expressed in multiple breast cancer subtypes and whether AREG is more highly expressed in luminal A and/or B tumors than in other breast cancer subtypes [55, 57, 60]. Moreover, if AREG is found to be most highly expressed in the luminal tumors, future studies could be focused on determining if AREG can

be used as an indicator of outcome for patients with luminal tumors or as an indicator of response to endocrine therapies, which is the common treatment for HR+ breast cancers. However, while MCF7 cells are ER+ and PR+, the HC11/R1 cells are ER- and PR-. Therefore, *AREG* expression could be mediated by different factors between these two cell lines. Other factors known to regulate *AREG* expression include cyclic AMP (cAMP), specificity protein 1, and Wilms' tumor suppressor [132, 140-144]. Future studies could focus on determining if any of these factors regulate *AREG* in ER- breast cancers or, if they do not, on identifying which factors do regulate *AREG* expression in ER- breast cancer. While AREG usually positively correlates with HR expression in breast cancers, it has been shown that EREG is typically associated with HR- breast cancers [172]. Therefore, future studies could examine if AREG and EREG are downstream targets of FGFR1 in different types of breast cancers or if they are always co-expressed in *FGFR1*-amplified tumors. Alternatively, they may be differentially regulated, with AREG more highly upregulated in ER+ *FGFR1*-amplifed breast cancers while EREG is more highly expressed in ER- *FGFR1*-amplified breast cancers.

Because of their nature as EGFR ligands, upregulation of AREG and EREG suggests that EGFR signaling is stimulated downstream of FGFR1 activation. Immunoblot analysis of multiple tyrosine residues within the EGFR kinase domain confirmed that in HC11/R1 cells treated with B/B to activate iFGFR1, EGFR phosphorylation is increased as compared to ethanol-treated

HC11/R1 control cells. While the residues we assessed are commonly phosphorylated when EGFR is activated, there are several other EGFR kinase domain tyrosine residues [334, 335]. Studies have found that different ligands can induce different phosphorylation patterns of the various tyrosines [334]. Future studies aimed at determining whether AREG stimulates a different EGFR phosphorylation signature than EREG or other EGFR ligands and whether these different phosphorylation patterns result in activation of different downstream signaling molecules could result in the development of more specific and less toxic EGFR inhibitors. Moreover, these results indicate that EGFR is activated in mammary epithelial cells subsequent to FGFR1 activation. Conversely, in the normal developing mammary gland, epithelial EGFR is dispensable, while stromal EGFR is required, for proper mammary gland development [107, 167]. Studies using *in vitro* co-culture experiments or *in vivo* tumor models could examine whether stromal EGFR is activated and involved in promoting *FGFR1*-amplified mammary tumorigenesis. Furthermore, if EGFR is activated in tumor stromal cells, it would be important to understand how EGFR activation then mediates signaling back to the epithelial cells of the tumors.

We also show that treatment of serum-starved HC11 cells with either recombinant mouse (rm)AREG or rmEREG stimulates ERK1/2 activation and significantly increases migration and proliferation. Addition of the EGFR kinase inhibitor, erlotinib, to rmAREG- or rmEREG-treated HC11 cells reduces ERK1/2 activation and significantly inhibits migration and proliferation back to levels

comparable to control cells. Additionally, while iFGFR1 activation of HC11/R1 cells also promotes increased ERK1/2 activation, migration, and proliferation, we demonstrate that these processes are partially but significantly inhibited when AP-treated HC11/R1 cells are also treated with erlotinib. Taken together, these results indicate that FGFR1-induced cellular migration and proliferation and ERK1/2 activation are at least in part mediated through AREG- and/or EREG-stimulated EGFR signaling. Because erlotinib treatment cannot completely inhibit the FGFR1-induced cellular migration and proliferation and ERK1/2 activation, studies are currently focused on determining what other pathways, besides EGFR signaling, are involved in promoting FGFR1-induced tumorigenesis. Additionally, while uncontrolled proliferation and an ability to migrate are hallmarks of cancer, there are other cellular processes, which AREG and/or EREG have been shown to mediate, that are required for tumor progression. For example, breast cancer cell lines treated with exogenous AREG have increased expression of genes known to promote invasion, such as matrix metalloprotease 9 (*MMP-9*), and other studies have shown that using shRNA directed against *AREG* in breast cancer cells reduces invasion into a Matrigel matrix *in vitro* [138, 174, 177, 178]. EREG has also been shown to be essential for breast cancer cells to intravasate, extravasate, and colonize in the lung, and thus, like AREG, is a regulator of invasion [198]. Accordingly, studies examining the role of AREG- and/or EREG-stimulated EGFR activation in promoting processes such as

invasion of or angiogenesis in *FGFR1*-amplified tumors should be performed to more thoroughly understand the FGFR1/EGFR signaling axis.

While inhibition of EGFR ultimately abrogates the effects of AREG upregulation, we also wanted to determine if directly targeting AREG was a valuable therapeutic strategy. We focused on AREG because other studies have successfully used shRNA directed against *AREG* to inhibit processes like breast cancer invasion and we consistently see much higher levels of AREG protein in iFGFR1-activated HC11/R1 conditioned media than EREG [176, 178]. Therefore, HC11/R1 cells were retrovirally transduced to stably express shRNA directed against *Areg*. However, knockdown of *Areg* did not inhibit the ability of these cells to undergo increased proliferation or migration when treated with B/B (data not shown). These results may be a consequence of incomplete *Areg* knockdown or redundancy amongst the EGFR ligands. While these data do not support the hypothesis that AREG would be a good target for directed therapies in *FGFR1*-amplified breast cancer, these cells could be used to evaluate other potential roles of AREG in *FGFR1*-amplified breast cancers. For instance, studies have shown that AREG overexpression in mammary epithelial cells leads to altered EGFR regulation [336]. While EGFR was rapidly internalized and degraded following EGF stimulation, there was prolonged EGFR localization at the plasma membrane and decreased phosphorylation at tyrosine 1045 following AREG stimulation [336]. Phosphorylation of EGFR tyrosine residue 1045 marks EGFR for degradation as it is the binding site for c-Cbl, the E3 ubiquitin ligase

responsible for targeting EGFR for degradation [337, 338]. Future work could use the HC11/R1 cells expressing *Areg* shRNA to examine whether EGFR signaling is aberrantly prolonged following FGFR1 activation or to study the rate of EGFR degradation in our system. Furthermore, AREG activation of EGFR appears to stimulate NFkB, which in turn induces expression of *IL-1* in human breast cancer cell lines [339]. Because IL-1 is a cytokine, these results imply that AREG could be involved in mediating immune cell function or recruitment in mammary tumors. Investigation of these alternative functions of AREG would ultimately result in a better understanding of AREG signaling in *FGFR1*-amplified breast cancers.

To expand upon our *in vitro* results showing that FGFR1 activation requires EGFR signaling to promote cell migration and proliferation, we performed two *in vivo* studies using the MMTV-iFGFR1 transgenic mice. In the first study, six week old MMTV-iFGFR1 mice were given erlotinib via oral gavage for three consecutive days and AP i.p. injections on the second day of erlotinib treatment to activate iFGFR1. This method of concurrent iFGFR1 activation and erlotinib treatment allows us to analyze the role of EGFR signaling in FGFR1-mediated tumor initiation. In the second study, we activated iFGFR1 in six week old MMTV-iFGFR1 mice prior to five consecutive days of erlotinib oral gavage treatment. By activating iFGFR1 before erlotinib treatment, we can analyze how inhibiting EGFR effects established aberrant FGFR1 signaling in the mammary gland. At the end of each study, mice were injected with BrdU and sacrificed, and the fourth inguinal mammary glands were harvested, fixed, and sectioned for

further investigation. In line with previous observations from studies using MMTV-iFGFR1 transgenic mice, we observed a significant increase in aberrant ductal epithelial cell lateral budding in AP-treated animals from both studies as detected by hematoxylin and eosin (H&E) staining [127, 129]. Importantly, erlotinib treatment significantly reduced the number of ductal structures exhibiting this lateral budding, and in the second study, erlotinib treatment also significantly reduced the severity (as determined by number of buds) of the budding phenotype in the ducts that do exhibit aberrant budding. Furthermore, immunofluorescence (IF) staining to detect BrdU demonstrated that iFGFR activation significantly increases proliferation, while erlotinib treatment of AP-treated mice significantly reduces proliferation. Taken together, these results demonstrate that EGFR signaling is required to promote FGFR1-mediated tumorigenic phenotypes *in vivo*. Although the MMTV-iFGFR1 mice provide a good model for studying tumor initiation, future studies should be directed at determining the benefit of erlotinib treatment in *FGFR1*-amplified mammary tumors. While the data presented thus far have concentrated on proliferation and migration in FGFR1-activated cells, many other cellular processes are co-opted during tumor progression [340]. Therefore, it would be of value to examine the effects of EGFR signaling on other tumorigenic pathways, such as angiogenesis and metastasis, of *FGFR1*-amplified tumors. Additionally, previous work has shown that EGFR mediates mammary tumor cell metastasis to the bone and that *FGFR1*-amplification correlates with increased distant metastasis [119, 120, 205,

341]. It would be interesting to investigate whether *FGFR1*-amplified tumors most commonly metastasize to the bone. Moreover, studies using human breast cancer specimen will need to be performed to determine whether co-expression of FGFR1, AREG, EREG, and EGFR occurs in human breast cancers.

The data described thus far demonstrate that FGFR1 signaling promotes mammary tumorigenesis by upregulating AREG and EREG which then activate EGFR signaling. These studies have primarily focused on FGFR1 signaling during initial stages of tumor development, but FGFR1 signaling is likely involved in all stages of mammary tumorigenesis. Other studies investigating the role of FGFR1 in breast cancers have determined that FGFR1 signaling can mediate endocrine therapy resistance and is also crucial for survival of several triple negative breast cancer (TNBC) cell lines both *in vitro* and *in vivo* [121, 123]. In line with these results, it has also been shown that AREG and EGFR are involved in therapeutic resistance of breast cancers [145, 181, 182]. These results, along with the fact that TNBCs are currently treated with chemotherapies, such as the anthracycline-based chemotherapy Doxorubicin, led us to hypothesize that FGFR1 signaling mediates chemoresistance [50, 51, 320, 321]. In support of this hypothesis, it has previously been shown that iFGFR1 activation decreases the number of cells undergoing apoptosis [128].

In the present study, we confirm that activation of iFGFR1 in serum-starved HC11/R1 cells significantly reduces apoptosis compared to ethanol-treated control cells as assessed by ApoTox-Glo and TUNEL assays. Moreover,

while Doxorubicin significantly increases apoptosis of serum-starved ethanol-treated HC11/R1 cells, there is significantly less Doxorubicin-induced apoptosis in serum-starved B/B-treated HC11/R1 cells. These results demonstrate that iFGFR1 signaling confers resistance to a commonly used chemotherapeutic. We further demonstrate that FGFR1-mediated chemoresistance involves the secretion of soluble factors because serum-starved Doxorubicin-treated HC11 cells incubated in conditioned media from B/B-treated HC11/R1 cells undergo significantly less apoptosis than serum-starved Doxorubicin-treated HC11 cells incubated in conditioned media from ethanol-treated HC11/R1 cells. Moreover, these results are consistent with results from the human TNBC cell line, Hs578T. Serum-starved Hs578T cells treated with bFGF to stimulate FGFR signaling undergo significantly less apoptosis than no treatment control serum-starved Hs578T cells. Like the HC11/R1 and HC11 control cells, serum-starved Hs578T control cells undergo significantly more apoptosis when given Doxorubicin, while bFGF-induced FGFR signaling reduces the amount of apoptosis detected in Doxorubicin-treated serum-starved Hs578T cells. Conversely, Hs578T cells treated with the FGFR inhibitor PD173074 undergo significantly more apoptosis than Hs578T cells treated with the PD173074 solvent control, DMSO. Hs578T cells treated with both PD173074 and Doxorubicin have a significant increase in apoptosis. However, Hs578T cells treated only with Doxorubicin undergo significantly less apoptosis than Hs578T cells treated with both Doxorubicin and PD173074. In line with these *in vitro* results, we show that inhibition of FGFR

signaling in an orthotopic transplant model of iFGFR1-activated mammary tumorigenesis also sensitizes tumor cells to Doxorubicin-induced apoptosis. Mice treated with both Doxorubicin and PD173074 have significantly less tumor burden than control mice. Moreover, tumors actually shrink in size once the combination therapy is started, while control tumors undergo continual growth. Based on TUNEL analysis of tumor sections, we observe a significant increase in apoptosis in tumors treated with both Doxorubicin and PD173074 as compared to control treatment tumors. Taken together, these data strongly support the hypothesis that FGFR1 signaling prevents chemotherapy-induced apoptosis. It would be interesting to determine whether FGFR1-mediated chemoresistance is restricted to the class of anthracycline chemotherapies or if FGFR1 universally confers chemoresistance. Moreover, because chemotherapy regimens often involve multiple chemotherapy drugs, future studies could be directed at determining if FGFR1-mediated chemoresistance can be overridden in a multi-drug treatment plan.

Due to our earlier results showing that EGFR is required downstream of FGFR1 to promote tumorigenic phenotypes and other reports demonstrating that EGFR is involved in mediating chemoresistance, we investigated the involvement of EGFR signaling in FGFR1-mediated chemoresistance by first examining whether FGFR1 and EGFR are expressed in the same TNBC cell lines. Immunoblot analysis of a small panel of TNBC cell lines demonstrated that FGFR1 and EGFR are co-expressed in the cell lines tested. Clearly a more

extensive examination will need to be conducted to discern whether FGFR1 and EGFR co-expression is a common occurrence in TNBCs. Furthermore, although the focus of the current study is TNBCs because these tumors are treated with chemotherapy regimens, future work could explore whether FGFR1 and EGFR co-expression is confined to TNBCs. We further show that serum-starved HC11 cells treated with rmAREG to stimulate EGFR activation undergo significantly less apoptosis than serum-starved PBS-treated control HC11 cells. EGFR activation significantly inhibits Doxorubicin-induced apoptosis of serum-starved HC11 cells although Doxorubicin significantly increases apoptosis of serum-starved PBS-treated HC11 cells. Interestingly, in serum-starved PBS-treated HC11 cells treated with both erlotinib and Doxorubicin, there is a significant increase in apoptosis. Because EGFR is not activated in these cells, future studies will need to be conducted to determine why erlotinib confers increased cell death when given in conjunction with Doxorubicin when erlotinib treatment alone does not increase apoptosis of PBS-treated serum-starved HC11 cells. Regardless, addition of erlotinib to serum-starved HC11 cells treated with both rmAREG and Doxorubicin abolishes the apoptosis inhibition seen in serum-starved HC11 cells treated with both rmAREG and Doxorubicin. Taken together, these results indicate that EGFR signaling can mediate chemoresistance and that inhibiting EGFR with erlotinib can sensitize cells to Doxorubicin-induced cell death. Additionally, because FGFR1 and EGFR are co-expressed in the TNBC cell lines tested, these results suggest that FGFR1 might mediate

chemoresistance by activating EGFR. Using ApoTox-Glo assay, we find that HC11/R1 cells treated with B/B, Doxorubicin, and erlotinib have a moderate increase in apoptosis as compared to HC11/R1 cells treated with B/B and Doxorubicin. Analogous results were obtained using serum-starved HC11 cells incubated in HC11/R1 conditioned media. HC11 cells incubated in conditioned media from B/B-treated cells undergo a slight increase in apoptosis when further treated with Doxorubicin and erlotinib as compared to only Doxorubicin treatment. Future studies should be focused on verifying these results in human breast cancer cell lines and with different chemotherapies or combinations of chemotherapies. Together, these data suggest that in breast cancers without *FGFR1* amplification, EGFR signaling can mediate chemoresistance and should be further evaluated as a target for increasing chemosensitivity, but in *FGFR1*-amplified breast cancers, EGFR is one of multiple downstream factors mediating chemoresistance.

Activation of signal transducer and activator of transcription 3 (STAT3) has been shown to correlate with incomplete pathological complete response of patients treated with chemotherapies and, thus, correlates with acquisition of chemoresistance [264]. Additionally, STAT3 has been identified to promote chemoresistance of human breast cancer cell lines, the most notable example being STAT3-mediated Doxorubicin chemoresistance of MDA-MA-231 cells [282-284]. Consequently, in the present study, we investigate STAT3 involvement in regulating FGFR1-mediated chemoresistance. Immunoblot analysis of serum-

starved HC11/R1 cells revealed that in B/B-treated cells, phosphorylation of STAT3 is stimulated after 2 hours of iFGFR1 activation but is not detectable in ethanol-treated control samples. This iFGFR1-induced activation of STAT3 continues to occur for at least 24 hours. These results demonstrate that STAT3 is regulated by iFGFR1 signaling. Furthermore, these results were verified in the Hs578T human TNBC cell line. Activation of STAT3 occurs by 2 hours, and is still detectable 24 hours, after bFGF treatment of serum starved Hs578T cells. Interestingly, although work from another lab shows that STAT3 directly binds to and is activated by FGFRs, the delay from FGFR activation to STAT3 activation implies that STAT3 is not directly activated by FGFR1 in our system [332]. Supporting our results of indirect activation of STAT3 by FGFR1, several reports investigating STAT3 activation in breast cancer cell lines show that STAT3 is activated by glycoprotein-130/Janus kinase (gp130/JAK) signaling stimulated by cytokine binding to gp130 [270-272]. Additionally, we show that soluble factors in conditioned media from iFGFR1-activated HC11/R1 cells can protect HC11 cells from Doxorubicin-induced apoptosis. Together, these data suggest that iFGFR1-induced cytokines might be stimulating activation of STAT3 in our system. In the present study, we verify using ELISA analysis that a well-known cytokine inducer of STAT3 in the mammary gland, leukemia inhibitory factor (LIF), is significantly upregulated in B/B-treated HC11/R1 conditioned media as compared to in ethanol-treated HC11/R1 conditioned media [262]. Furthermore, we show that inhibition of either LIF or gp130 with neutralizing antibodies effectively blocks

STAT3 activation in iFGFR1-activated HC11/R1 cells. Together, these data suggest that STAT3 activation is regulated by iFGFR1 signaling through iFGFR1-induced expression of LIF, which activates gp130. Future studies will need to be conducted to determine what other cytokines are induced following iFGFR1 activation and if other iFGFR1-induced cytokines can activate STAT3. Moreover, cytokines are traditionally secreted by immune cells such as macrophages, and previous work using MMTV-iFGFR1 transgenic mice has shown that iFGFR1 activation recruits macrophages to the sites of prolonged iFGFR1 signaling in the mammary glands of these mice [129]. Therefore, it would be interesting to determine if FGFR1-stimulated activation of STAT3 is also mediated by infiltrating macrophages *in vivo*. Additionally, STAT3 is negatively regulated by several factors, including the cytoplasmic suppressors of cytokine signaling (SOCS) proteins and the nuclear protein inhibitor of activated STAT (PIAS) [342, 343]. It would be interesting to examine how expression of these negative regulators changes when iFGFR1 is activated. Further work will also need to be done to determine what factors are regulated by STAT3 following FGFR1 activation. Other studies have reported that STAT3 can induce expression of several genes, including multiple *MMPs*, *Cyclin D1*, and *c-myc* [269, 276]. Most notably for our studies, upregulation of several anti-apoptotic factors, such as B cell CLL/lymphoma-2 (BCL-2), myeloid cell leukemia 1 (MCL-1), BCL-2-related gene, long isoform (BCL-xL), and Survivin (a member of the Inhibitor of Apoptosis family of proteins) has been detected in breast cancer cell lines

following STAT3 activation, making them attractive candidates for mediating FGFR1-induced chemoresistance [264, 269, 275, 281, 282].

Importantly, in the present study, we demonstrate that STAT3 activation confers Doxorubicin chemoresistance in iFGFR1-activated cells. As we have shown before in this work, serum-starved B/B-treated HC11/R1 cells undergo significantly less apoptosis when given Doxorubicin than serum-starved ethanol-treated HC11/R1 cells given Doxorubicin as detected by TUNEL staining. Alternatively, we observe that directly inhibiting STAT3 with the small molecule inhibitor Stattic in Doxorubicin-treated serum-starved iFGFR1-activated HC11/R1 cells abolishes this reduction in apoptosis, and these cells undergo apoptosis at levels comparable to serum-starved ethanol-treated HC11/R1 cells given Doxorubicin with or without Stattic treatment. Future studies will need to be directed at determining if these results are consistent in human breast cancer cells as well as in an *in vivo* mouse model of mammary tumorigenesis. Human breast cancer samples will also need used to see if FGFR1 and activated STAT3 are co-expressed in breast cancers, especially in TNBCs. Additionally, while clinical trials are currently underway to examine the effects of inhibiting STAT3 in other types of cancer, there are currently no clinical trials using STAT3 inhibitors in breast cancer patients. The data presented here give credence to the need for clinical trials using STAT3 inhibitors in breast cancer patients, especially for patients with *FGFR1*-amplified TNBCs. Moreover, EGFR is a known activator of STAT3 in breast cancer cell lines [257, 266, 344]. While we have preliminary

data suggesting that EGFR does not mediate activation of STAT3 in iFGFR1-activated HC11/R1 cells (data not shown), it would be interesting to see if STAT3 is activated by EGFR in non-*FGFR1*-amplified breast cancers.

4.2 Conclusions

As summarized in Figure 12, the work presented here shows that FGFR1 activation significantly upregulates expression of the EGFR ligands AREG and EREG at the transcript and protein levels both *in vitro* and *in vivo*. AREG and EREG then activate EGFR signaling. Notably, EGFR activation is at least in part required for FGFR1-induced proliferation and migration and ERK1/2 activation, as inhibition of EGFR with the small molecule kinase inhibitor erlotinib significantly blocks these processes. Moreover, we show that FGFR1 and EGFR are co-expressed in TNBC cell lines and that both FGFR1 and EGFR can mediate Doxorubicin chemoresistance. We further show that FGFR1 upregulates expression of the cytokine LIF, which then signals through gp130/JAK to activate STAT3 *in vitro*. Directly inhibiting either FGFR1 or STAT3 significantly reduces chemoresistance and increases apoptosis *in vitro*. Furthermore, inhibition of FGFR1 with the small molecule inhibitor PD173074 results in increased chemosensitivity and apoptosis in a mouse model of mammary tumorigenesis. These results are significant because they are the first to show that FGFR1 signals through EGFR and that FGFR1 mediates chemoresistance through activation of STAT3. This study furthers our understanding of *FGFR1*-amplified mammary tumorigenesis and presents alternative factors for targeted therapies for patients with *FGFR1*-amplified breast cancers.

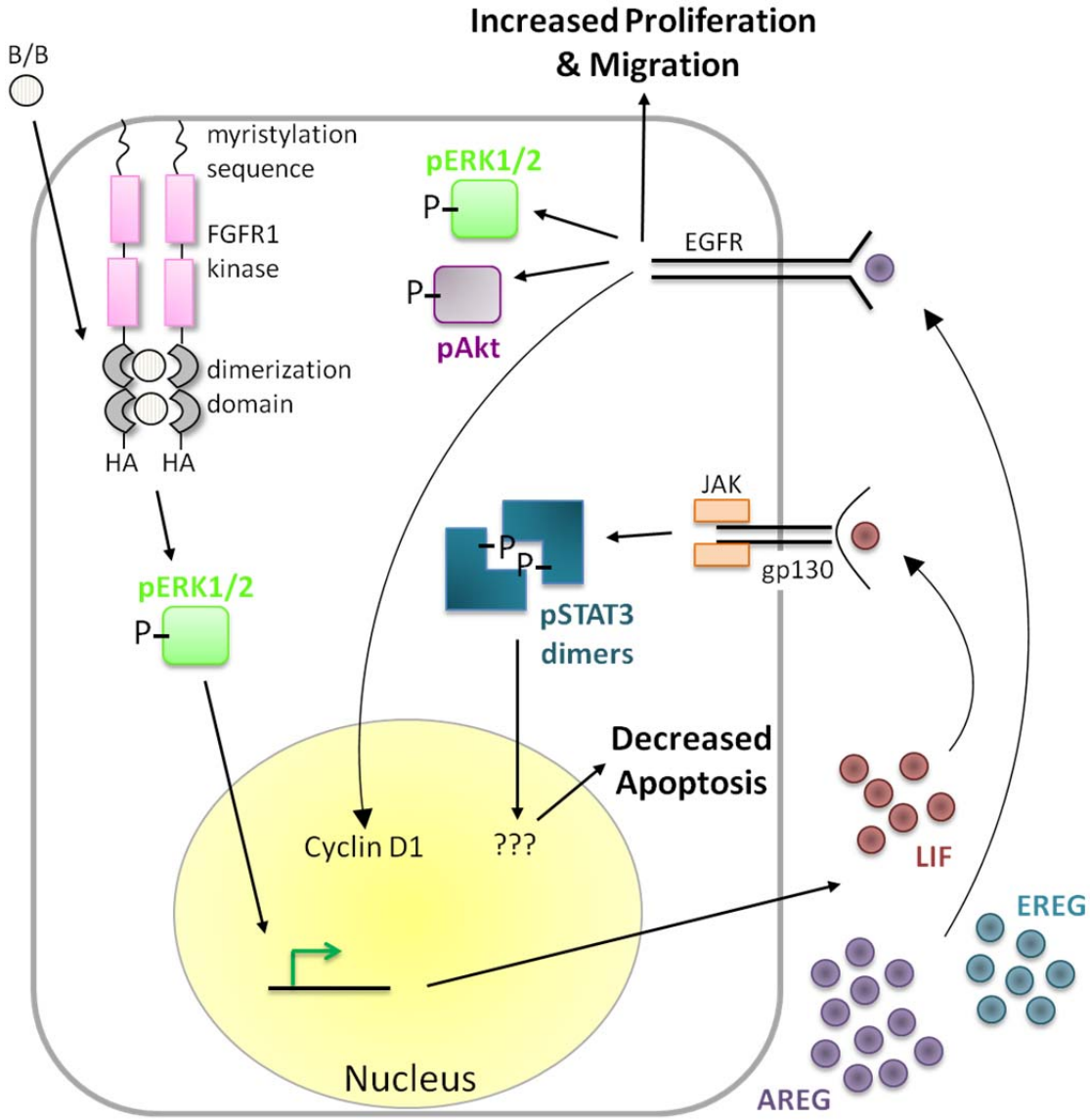

Figure 12: Final Model Figure.
Activation of iFGFR1 via treatment with the B/B homodimerizer results in phosphorylated ERK1/2 and ultimately in increased expression of the EGFR ligands, AREG and EREG, and the cytokine, LIF. Soluble AREG and EREG can then bind and activate EGFR, leading to phosphorylation of ERK1/2 and Akt and expression of Cyclin D1. Functional studies demonstrate that activation of EGFR downstream of iFGFR activation results in increased cellular proliferation and migration. Alternatively, LIF binding to its cytokine receptor results in phosphorylation of STAT3, which subsequently decreases Doxorubicin-induced apoptosis.

Chapter 5: References

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