

**FGFR4 OVEREXPRESSION AND HOTSPOT MUTATIONS ARE DRUGGABLE
TARGETS FOR ENDOCRINE-RESISTANT LOBULAR BREAST CANCER**

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Invasive lobular carcinoma (ILC) is an understudied subtype of breast cancer that requires novel therapies in the advanced setting. Because most ILC tumors are estrogen receptor positive (ER+), first-line therapies for patients with ILC often include drugs that block estrogen signaling. Although these treatments have a high rate of initial success, *de novo* and acquired resistance remain major clinical problems. To study the mechanisms of resistance to endocrine therapy in ILC, we performed several transcriptomic studies. First, RNA-Sequencing of cell line models identified overexpression of fibroblast growth factor receptor 4 (FGFR4) as a top druggable target for estrogen-independent ILC. To test the clinical relevance of this finding, we next profiled treatment-naïve primary ER+ ILC tumors. In this setting, elevated FGFR4 expression was poorly prognostic for distant recurrences, suggesting that FGFR4 may play a role in *de novo* resistance to endocrine therapy. To study acquired resistance, we next sequenced matched, metachronous primary and metastatic tumors. This study showed that FGFR4 expression increases dramatically in distant metastases of ER+ ILC treated with endocrine therapy. With recent publication of mutational profiles of metastatic breast cancer, we next queried datasets for DNA-level FGFR4 alterations. We found FGFR4 hotspot mutations were uniquely enriched in metastatic ILC, suggesting a multimodal selection of FGFR4 activation in advanced lobular carcinoma. Because of the consistent results pointing to FGFR4 as a key mediator of resistance in ILC, we next tested the effects of FGFR4 inhibition *in vitro*. Although blockade of FGFR4 had minimal effects on short-term growth, FGFR4 inhibition via shRNA or small molecules drastically decreased colony

formation. Initial results from signaling studies show minimal effects of FGFR4 inhibition on estrogen signaling and cell cycle progression. Lastly, FGFR4 overexpression was not sufficient to drive *in vitro* breast cancer growth, suggesting additional ligands and/or co-receptors may be needed to fully activate signaling. These data collectively support the notion that FGFR4 is an important mediator of endocrine resistance in ILC, warranting further characterization of phenotypic effects and mechanistic studies of signaling alterations.

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1.0 INTRODUCTION

1.1 BREAST CANCER

Breast cancer remains a major clinical problem worldwide, accounting for an estimated 2 million new cases and 600,000 deaths in 2018¹. The United States is no exception to these sobering statistics, with breast cancer cases expected to total ~269,000 this year, and deaths reaching more than 40,000, with the vast majority of these occurring in women². For women in the United States, breast cancer is the most common cancer type and the second most common cause of cancer death². Thankfully, there have been gains in treatment outcomes for breast cancer in the past few decades, in large part due to our ability to classify breast cancer into subtypes³. This subtyping is a form of precision medicine, where the goal is to allow for the correct treatment to be given to each individual patient, allowing for maximal benefit while avoiding unnecessary harm. The first kind of subtyping for breast cancers come from receptor status, identified by expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). These receptors act as both prognostic and predictive markers for therapy. The second main way to subtype breast cancer is based on the PAM50 expression status. PAM50 subtyping is based on seminal work showing that RNA expression can identify clusters of patients, and this subtyping has been proven useful as a prognostic tool to assess treatment needs⁴⁻⁷. Lastly is histological subtyping of breast cancer, which identifies most

tumors as invasive ductal carcinoma (IDC) or invasive lobular carcinoma (ILC). Although IDC and ILC tumors look different under the microscope and on radiographic imaging, behave differently in response to therapy, and have a different preference for site of metastasis, histological subtype is not currently a routinely used variable in making treatment decisions⁸⁻¹⁰.

1.1.1 Receptor subtypes

By 1973, it was understood that receptor status is not a binary variable, but in fact the expression of a receptor can vary over a wide range in primary and metastatic tumors¹¹. Even so, binary classification of three receptors: ER, PR, and HER2 has been shown to be a useful tool for prognostic and predictive purposes. In 2010, the American Society of Clinical Oncology and College of American Pathologists (ASCO/CAP) published guidelines for assigning ER and PR status based on a minimum of 1% of tumor nuclei staining positive by immunohistochemistry (IHC)¹². In 2018, ASCO/CAP published updated guidelines for HER2, with a minimum threshold for positivity defined as weak to moderate complete membrane staining observed in 10% of tumor cells by IHC, plus in situ hybridization showing a copy number gain of the DNA locus¹³. Although ER-/PR+ tumors have been identified, it was shown recently that this classification is not reproducible finding, so to simplify matters, ER+ tumors are often classified being hormone receptor positive (HR+)¹⁴. This allows for 4 main receptor subtypes: HR+/HER2+, HR+/HER2-, HR-/HER2+, and HR-/HER2- (triple negative). In total, ~80% of breast cancers are ER+, and patients with ER+ tumors will most often receive endocrine therapy¹⁵.

1.1.1.1 Endocrine therapy for ER+ breast cancer

The theory for the use of endocrine therapy in breast cancer is to block signaling from estrogen receptor. This can be accomplished by decreasing the amount of circulating estrogen or by decreasing the amount of functioning estrogen receptor. In premenopausal women, the primary source of estrogen is the ovaries, while in postmenopausal women, the primary source are the adrenal glands, which produce androgen that can then be converted to estrogen via a process called aromatization¹⁶.

Evidence for the benefit of endocrine therapy in breast cancer dates back to at least 1896, when ovariectomy showed promising results in one woman with metastatic disease¹⁷. In 1952, bilateral adrenalectomy was performed for seven patients with advanced breast cancer¹⁸. By 1971, a technique for measuring estrogen receptor status in human tissue samples could be performed, and lack of estrogen receptor expression was shown to have negative predictive value for adrenalectomy¹⁹.

Today, these aggressive surgeries are no longer routinely performed for breast cancer. Instead, there are three main classes of endocrine therapy given for breast cancer: aromatase inhibitors (AIs), selective estrogen receptor modulators (SERMs), and selective estrogen receptor degraders (SERDs).

SERMs, such as tamoxifen, act as a partial agonist for estrogen receptor, and so they can be given to either premenopausal or postmenopausal women²⁰. Tamoxifen was first developed in the late 1960s, with benefit for metastatic disease proven in the 1970s, and FDA approval in 1977²¹. Starting in 1981, the National Surgical Adjuvant Breast and Bowel Project (NSABP) began reporting groundbreaking clinical trials of tamoxifen for women with ER+ tumors, with results showing : 1) tamoxifen adds benefit to chemotherapy regimens, 2) expression of PR adds

predictive value for tamoxifen, and 3) when given alone to node-negative women, tamoxifen increases disease-free survival^{22–24}. Based on the continued positive trials of tamoxifen in the adjuvant setting, 5-10 years of tamoxifen and/or AI treatment is recommended following primary surgery^{25–27}.

Aromatase inhibitors are drugs that block aromatase (*CYP19A1*), the enzyme responsible for the peripheral conversion of androgens to estrogen, leading to much decreased levels of circulating estrogen²⁸. AIs can be given to postmenopausal women, and recent guidelines now also suggest that AI treatment is a reasonable option for premenopausal women, in combination with ovarian function suppression^{29–31}. There is some debate about the preferred treatment in the premenopausal setting, with questions including whether tamoxifen should be always be supplemented with ovarian function suppression, and whether AIs or tamoxifen is the best choice^{32–35}.

After progression of disease on an AI or tamoxifen, endocrine therapy has still proven to be a beneficial treatment. In particular, SERDs, such as fulvestrant (ICI 182,780) were first shown in clinical trials in 2002 to provide benefit in this setting^{36,37}. SERDs act as “pure” antiestrogen compounds, which block receptor dimerization, prevent nuclear entry, and cause receptor degradation^{38–41}. More recently, fulvestrant was shown to be the preferred treatment option for patients with ER+ locally advanced or metastatic breast cancer who have not received previous endocrine therapy⁴².

There are now two additional FDA-approved therapies for advanced breast cancer, often given in combination with endocrine therapy: mTOR inhibitors and CDK4/6 inhibitors. In 2012, everolimus, an mTOR inhibitor, was shown to improve progression-free survival (PFS) for advanced ER+ breast cancer, when combined with an AI^{43,44}. Since 2015, there have a plethora

of studies of CDK4/6 inhibitors, showing improved PFS for advanced ER+ breast cancer when combined with either an AI or fulvestrant^{45–49}. These CDK4/6 inhibitors significantly increase PFS for advanced ER+ disease, and more clinical trials are currently underway to assess the best timing and combination of treatments. In fact, there are at least 12 clinical trials testing the triple combination of an mTOR inhibitor, CDK4/6 inhibitor, and endocrine therapy⁵⁰.

Even given these recent advances, the mechanisms of resistance to endocrine therapy remain incompletely understood^{51,52}. Currently supported and proposed mechanisms include: changes in of ER or ER cofactor expression^{53,54}, alterations in extracellular matrix expression^{55,56}, gains in cell cycle promotion^{57,58}, increases in growth factor signaling^{59,60}, and more recently identified, mutations in ER^{61–64}. The goal of this thesis work was to identify additional mechanisms of resistance for endocrine therapy based on expression changes. One main method to study this resistance is through the use of long-term estrogen deprived (LTED) cell lines, which because of the loss of estrogen, model AI resistance^{65–68}.

1.1.2 PAM50 subtypes

The second major way to classify breast cancer is based on the PAM50 subtypes, which allows for four main classes of tumors: luminal A, luminal B, HER2-enriched, and basal^{4,5}. These subtypes are based on hierarchical clustering using expression profiles, which were found to be robust indicators of intrinsic signaling and were prognostic for treatment outcome. Specifically, ER+ breast cancer could be subtyped into luminal A and luminal B tumors, with the latter having worse survival. Based on this subtyping, there is now an FDA-approved test (Prosigna™) to assess risk of distant recurrence in ER+ disease^{6,7}. This subtyping allows for additional

prognostic information not provided by IHC analyses alone, particular for women with ER+ node-negative disease^{69,70}.

1.1.3 Histological subtypes

Breast cancer can also be subtyped by a pathologist, based on the histological appearance of the cells and the manner in which the cells grow. The two main histological subtypes of invasive breast cancer are ductal (IDC) and lobular (ILC). The molecular basis for ILC is a loss of membranous E-cadherin expression, which disrupts the adherens junctions and therefore cell-to-cell contact⁷¹. This loss has many downstream effects, including the characteristic presentation of ILCs growing in a single-file invasion pattern⁷². The loss of E-cadherin results in aberrant cytoplasmic localization of p120 catenin, and the recent development of dual-staining for E-cadherin and p120 has facilitated the diagnosis of ILC^{73,74} (Figure 1).

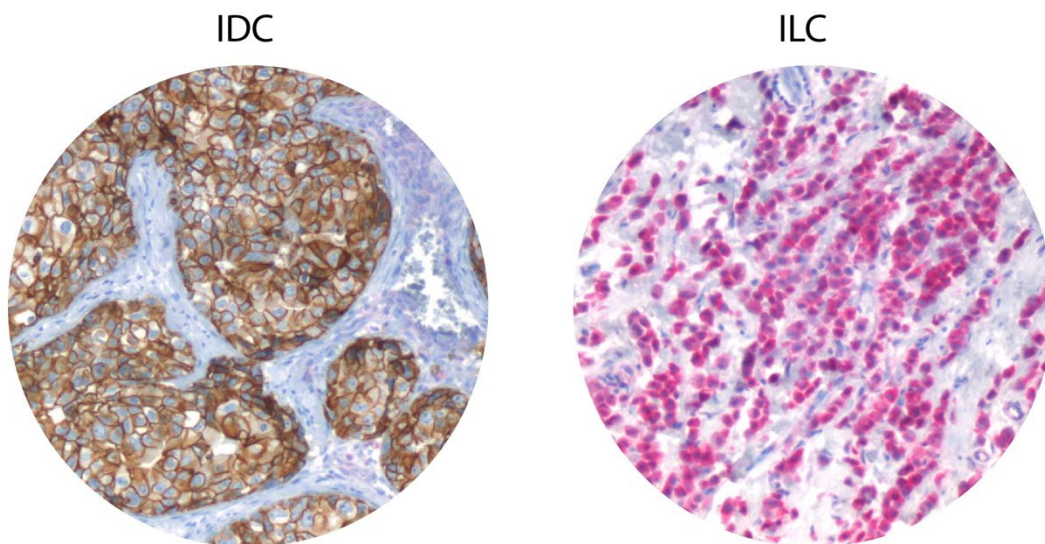


Figure 1. E-cadherin/p120 staining of an IDC and ILC tumor

Representative E-cadherin and p120 dual staining of an IDC (left) and ILC (right) tumor from a tissue microarray. IDC tumor cells, with membranous E-cadherin expression (brown), typically grow in large clumps of cells, while ILC tumor cells, with cytoplasmic p120 expression (pink), characteristically invade in a single-file pattern.

ILCs account for 10-15% of the breast carcinomas seen in the US, or in other terms, roughly 25,000 new cases per year^{9,75}. This incidence rate would place ILC in the top ten most common cancers for women if it were counted as a separate disease². The unique growth patterns (single-file invasion), metastatic behavior (preference for the GI tract and ovaries in comparison to IDCs), receptor status (almost universally estrogen receptor positive), and survival outcomes of ILC suggest that it should be considered a separate disease^{8,72,76,77}. For survival outcome, ILC patients with estrogen receptor (ER) positive disease have lower long-term survival than ER+ IDC patients⁷⁸. Recent studies have shown that when controlling for tumor grade, ILC patients have lower breast cancer specific survival, and when controlling for luminal tumors, ILC patients have lower disease-free survival^{79,80}. Recently the BIG 1-98 trial showed that tamoxifen treatment is not as effective for ILC tumors as compared with IDC⁸¹. Also noteworthy is the steady rise of ILC incidence in recent years, although this seems to have been related to the use of exogenous estrogen as menopausal hormone therapy^{75,82}.

Because biomarkers for breast cancer outcomes were originally designed using an IDC:ILC ratio of ~6:1, perhaps we should not be too surprised that they may not be as predictive for ILC outcome⁸³. Only in recent years has there been an appreciation for the need to use histological subtype to inform therapy, with preclinical data showing potential therapeutic vulnerabilities of ILC, and the opening of four ILC-specific clinical trials since 2014⁸⁴⁻⁹¹ (Table 1). This thesis work aims to continue this research by identifying biomarkers and therapeutic targets that can account for the unique behavior of ILC. Because the standard treatment regimen for lobular cancers includes endocrine therapy, the main goal of this thesis was to answer why some ILC patients have a worse outcome than expected on endocrine therapy, and for those patients, what additional treatments can we provide?

Table 1. Current clinical trials specific for patients with ILC

ClinicalTrials.gov Identifier	Clinical Trial Title	Treatment Intervention	Phase	Year First Posted to ClinicalTrials.gov
NCT02206984	Endocrine Response in Women with ILC	Window trial of Tamoxifen, AI, Fulvestrant	0	2014
NCT03147040	GELATO	Carboplatin and Atezolizumab	2	2017
NCT03620643	ROLo	Crizotinib and Fulvestrant	2	2018

1.2 FGFR SIGNALING AND ALTERATIONS IN CANCER

The fibroblast growth factor receptor (FGFR) signaling pathway has long been known to cancer researchers because of its role in cell survival, proliferation, migration, and angiogenesis^{92–95}.

Yet, most basic and translational studies on FGFRs focus exclusively on FGFR1 when studying the effect of gene amplification, or FGFR2-3 when studying gene fusions. Likewise, current clinical trials of drugs that target FGFRs, often ignore FGFR4 as a biomarker for entry^{96–99}. Only recently has FGFR4 gained attention as a putative target in cancer, including in colorectal, hepatic, and ovarian cancers^{100–102}. These studies have shown a functional role of FGFR4 in cancer survival, but the limited mechanistic studies to date have unclear conservation across different cancer types.

1.2.1 FGFR signaling and role in development

The active mammalian FGF signaling pathways consist of 18 ligands and 4 receptors (FGFR1, FGFR2, FGFR3, and FGFR4)¹⁰³. The FGF ligands are secreted glycoproteins that bind to the

membranous receptors with the help of heparan sulfate proteoglycans (HSPGs)^{104–106}. Specificity of ligand-receptor binding is determined by many factors, including the differential heparin-binding domains of ligands¹⁰⁷, differential isoform usage (of FGFR1-3)⁹⁵, and differential co-receptor expression¹⁰⁸. Of particular importance to FGFR4 is expression of β Klotho (*KLB*), which is required for the liver-specific function of FGF19 to maintain bile acid homeostasis¹⁰⁹.

The downstream signaling of FGFR pathways is frequently studied in the context of development¹¹⁰. Knockout mouse models of FGFR1 or FGFR2 are embryonically lethal, with blockade of mesodermal differentiation and inner cell mass growth, respectively^{111,112}. FGFR4 knockout mouse models are viable, and display no obvious developmental abnormalities¹¹³. This basic science research has laid the groundwork for translational and clinical research to be done, particularly in the context of cancer, where there are now a plethora of downstream signaling effects known following FGFR activation (reviewed recently¹⁰³). Briefly, ligand-binding leads to receptor dimerization and activation of the tyrosine kinase domains, which can phosphorylate FRS2, PLC γ , PI3K, and JAK/STAT signaling^{114,115} (Figure 2). Negative regulation of FGFR signaling can come from SEF, SPRY, and MKP3 (*DUSP6*) feedback^{116–118}.

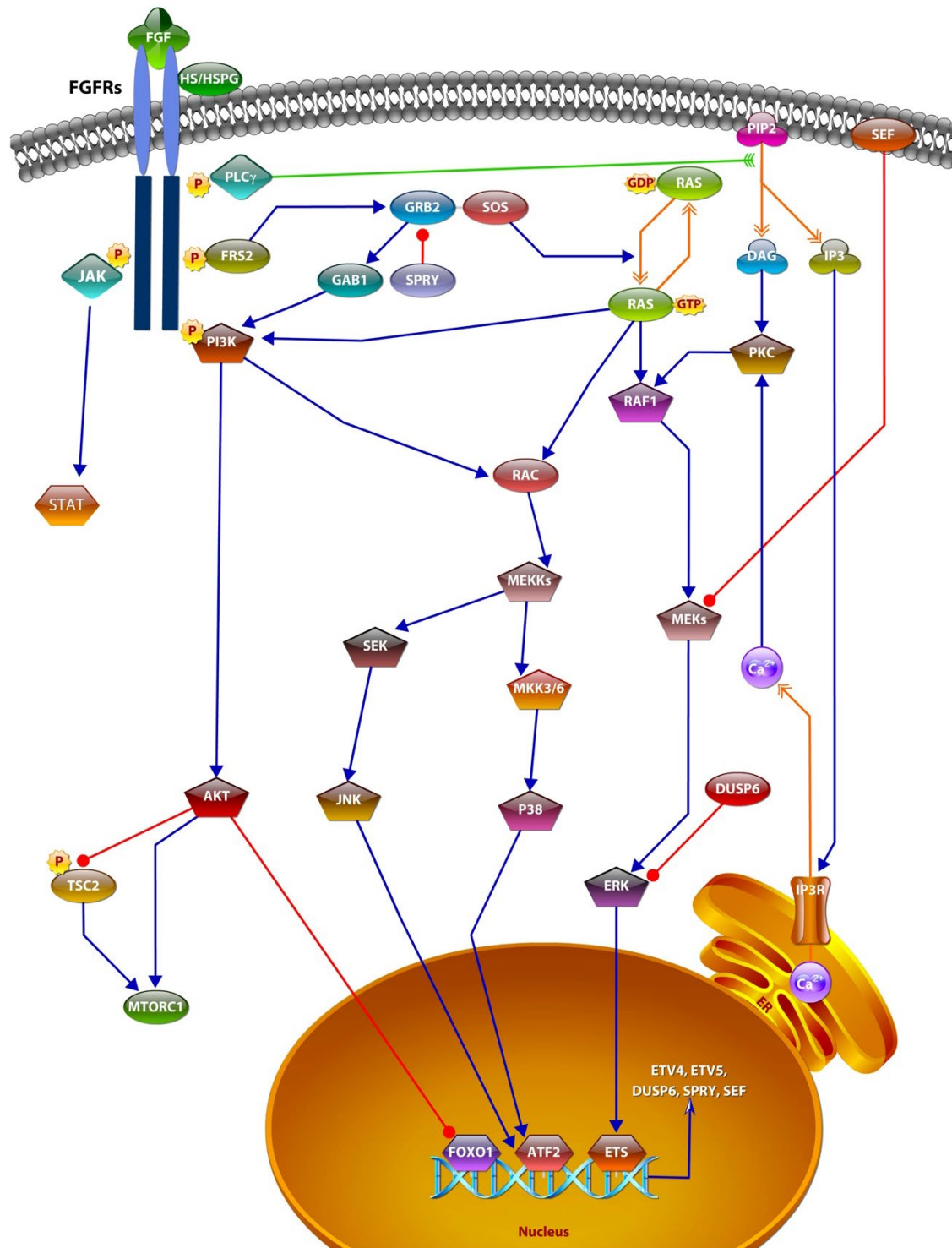


Figure 2. Schematic overview of FGFR signaling pathways

FGF ligand binding to FGFRs activates several main intracellular signaling pathways, including PLCγ, MEK/ERK, PI3K/AKT, and JAK/STAT. When activated aberrantly, these pathways lead to proliferation, growth, and survival. Figure was generated by modifying an existing template from ProteinLounge (Copyright©), access via University of Pittsburgh HSLs Library.

1.2.2 DNA-level alterations in FGFRs in cancer

In the context of cancer, FGFRs have been frequently studied because of the propensity of DNA amplifications, fusions, and mutations. FGFR1 copy number gains are particularly common, with a rate exceeding 5% in TCGA studies of lung squamous (19%), breast (12%), bladder (12%), uterine (9%), head and neck (8%), esophagus (8%), prostate (7%), colorectal (7%), sarcoma (6%), and ovarian (6%) cancers^{119,120}. FGFR1 amplification allows for increased ligand binding and activation of downstream signaling, and in breast cancer, leads to endocrine therapy resistance^{121–124}.

Amplification of the FGF ligands FGF3, FGF4, and FGF19, on the 11q13 amplicon, shares a very similar cancer-type specificity as FGFR1 amplification, with frequent gains seen in lung squamous (14%), breast (14%), bladder (10%), head and neck (24%), and esophagus (34%) cancers^{119,125}. As mentioned, the FGF19-FGFR4 interaction is important for normal liver function, but this axis also plays a role in hepatocellular carcinoma (HCC). FGF19 is amplified in ~7% of HCC cases, and FGF19 signaling increases tumor cell survival, xenograft tumor growth, and sorafenib resistance^{126–128}.

FGFR2 and FGFR3 alterations are not so common in breast cancer, but these two receptors are often involved in fusion events in other cancer types. A recent pan-cancer TCGA analysis found that FGFR fusions are the most frequent 5'-kinase fusions, with recurrent *FGFR3–TACC3* fusions in glioblastoma and bladder cancer, and *FGFR2–BICC1* fusions in cholangiocarcinoma¹²⁹. These fusions lead to constitutive activation of the FGFR tyrosine kinase domain, and so far, there has been promising results from clinical trials targeting these fusions^{130,131}.

On a pan-cancer scale, somatic single nucleotide variants are relatively rare in FGFRs, at least in treatment-naïve patients. Figure 3 shows all mutated sites appearing in the Catalogue of Somatic Mutations in Cancer (COSMIC database) at least 10 times¹³². The most frequently mutated site is FGFR3 at position S294C, which is present in ~10% of urothelial carcinomas^{133–136}.

Although somatic FGFR4 mutations are exceedingly rare (Figure 3), two hotspot mutations (N535 and V550) are common in rhabdomyosarcomas, accounting for ~8% of cases^{137,138}. Analogous mutations at these sites in FGFR1, FGFR2, and FGFR3 have been identified, and acquired FGFR mutations at these amino acids have been shown to be a mechanism of resistance to one FGFR inhibitor (AZD4547)¹³⁹. Because of the different structural designs of the FGFR inhibitors in clinical trials, some inhibitors actually do maintain activity against these mutations, including LY2874455¹⁴⁰. The FGFR4 N535 and V550 mutations have been shown to promote tumor progression in rhabdomyosarcoma mouse models through activation of pSTAT3¹⁴¹.

Finally, there is one frequent germline mutation in FGFR4, at position G388R (rs351855) in the transmembrane domain¹⁴². In the EXaC database, this single nucleotide polymorphism (SNP) has an allele frequency of 13% in the African population, 30% in the European population, and 45% in the Latino and East Asian populations¹⁴³. This SNP confers a poor prognosis for a variety of cancer types, including lung, melanoma, and breast^{144–146}. Recent data has shown that this SNP alters an important motif within the transmembrane domain, such that STAT3 binding and phosphorylation are increased, leading to tumor cell invasion^{147,148}.

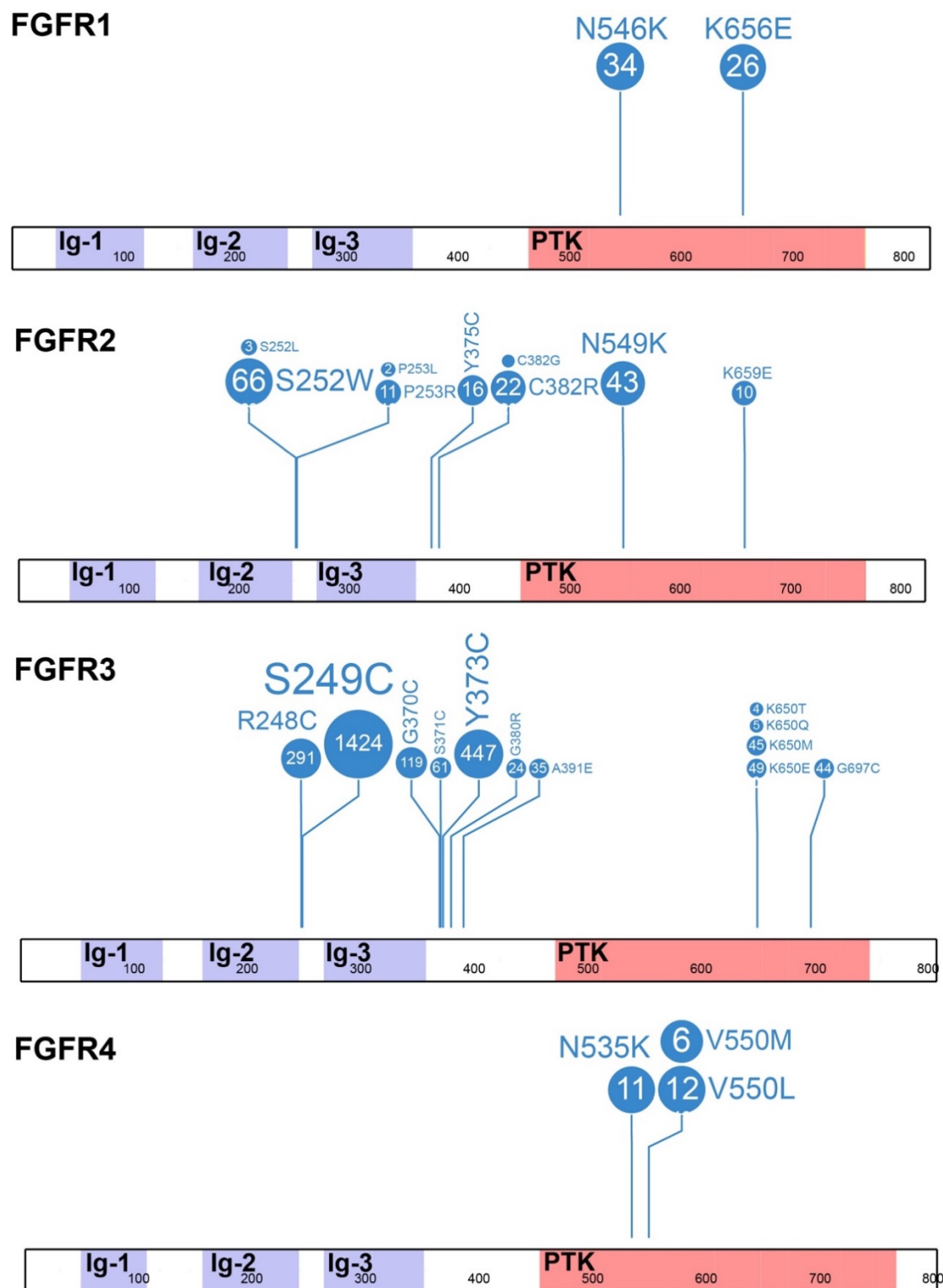


Figure 3. Missense FGFR mutations annotated in COSMIC database

Lollipop plots of somatic missense FGFR mutations that are annotated in COSMIC database (version 83)¹³². Only the amino acid positions with at least 10 alterations are shown. Amino acid position numbered with respect to human genome assembly GRCh37 (hg19). Plot generated using ProteinPaint software¹⁴⁹. Immunoglobulin-like (Ig) and protein tyrosine kinase (PTK) domains were identified from ProteinPaint using NCBI CDD software¹⁵⁰.

1.2.3 RNA expression of FGFR4 in breast cancer

FGFR4 has long been known to be overexpressed in a subset of breast cancer, occurring at a rate of ~30%¹⁵¹. The cause of this overexpression is yet to be determined, with copy number gains occurring in only ~2% of breast cancer cases, and almost no correlation of DNA and RNA levels^{4,152}. The overexpression of FGFR4 does correlate very well with expression of HER2, so much so, that FGFR4 is actually one of the genes on the PAM50 array encouraging classification of tumors into the HER2-enriched subtype¹⁵³. FGFR4 and HER2 are also frequently overexpressed together in cell lines, and targeting the combination of receptors had additive effects on inhibiting cell growth¹⁵⁴.

1.3 CLINICAL TRIALS WITH FGFR INHIBITORS

1.3.1 Results of previous clinical trials of FGFR inhibition

Previous clinical trials of FGFR inhibitors have largely included nonselective tyrosine kinase inhibitors (e.g., dovitinib and ponatinib) and more recently, selective FGFR1-3 inhibitors (e.g., AZD4547)¹⁵⁵. The nonselective inhibitors had a major toxicity profile related to inhibition of VEGFR, making it difficult to interpret the full potential of FGFR inhibition¹⁵⁶. In early phase trials reported thus far, the toxicity profile of FGFR selective inhibitors include hyperphosphatemia, stomatitis, and decreased appetite^{156,157}. The success of these trials has been limited, with a clear need for better biomarkers, including the possibility of RNA and protein expression¹⁵⁸.

1.3.2 Current clinical trials targeting FGFRs

Current clinical trials include agents that selectively target FGFR1/2/3 (AZD4547⁹⁸, Debio1347^{159,160}, E7090¹⁶¹, HMPL-453, Pemigatinib¹⁶²), pan FGFR inhibitors (BGJ398^{99,163}, Erdafitinib¹⁶⁴, PRN1371¹⁶⁵, Rogaratinib¹⁶⁶, TAS120¹⁶⁷), and antibodies directed to FGFR2 (Bemarituzumab¹⁶⁸) or FGFR3 (Vofatamab¹⁶⁹). See Table 2 for a detailed list of active trials as of December 2018. Of note, 3 of these studies are focused on ER+ breast cancer, including AZD4547 in combination with an aromatase inhibitor, Debio1347 in combination with fulvestrant, and erdafitinib in combination with palbociclib and fulvestrant. Of these, erdafitinib has the highest affinity for FGFR4, so results of this trial will be particularly interesting to follow. Additionally, there are now FGFR4-specific inhibitors in clinical trials, mostly designed for patients with hepatocellular carcinoma (Table 3). These inhibitors gain their specificity by targeting a cysteine residue within the ATP binding pocket (Cys552) that is unique to FGFR4¹²⁸. Based on the results of the aforementioned trials, specific inhibition of FGFR4 and ER ($\pm$ CDK4/6 inhibition) may soon be a novel clinical trial design option for metastatic breast cancer patients. Thus far, one of the main adverse effects seen with FGFR4-specific inhibition is increase in liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT)^{170,171}. Since these trials are focused on patients with liver cancer, it is not clear if the same adverse effects would be seen in patients with breast cancer.

Table 2. Current clinical trials with selective FGFR inhibitors

Drug name (company)	ClinicalTrials.gov Identifiers	Targets	Phase	Conditions
AZD4547 (AstraZeneca)	NCT02965378 NCT01791985 NCT02546661 NCT02664935	FGFR1/2/3	2/3 1/2 1 2	Lung Cancer Breast Cancer Bladder Cancer Lung
Debio1347 (Debiopharm)	NCT01948297 NCT03344536	FGFR1/2/3	1 1/2	Solid Tumors Breast Cancer
E7090 (Eisai)	NCT02275910	FGFR1/2/3	1	Solid Tumors
HMPL-453 (Hutchison)	NCT02966171 NCT03160833	FGFR1/2/3	1 1/2	Solid tumor Solid Tumor
Pemigatinib (Incyte)	NCT03011372 NCT02872714 NCT03235570 NCT02924376 NCT02393248	FGFR1/2/3	2 2 1 2 1/2	Myeloproliferative Neoplasms Urothelial Cancer Solid Tumors Cholangiocarcinoma Solid Tumors
BGJ398 (Novartis)	NCT02706691 NCT01697605 NCT02150967 NCT01975701	FGFR1-4	2 1 2 2	Head and Neck Cancer Solid Tumors Cholangiocarcinoma Gliomas
Erdafitinib (Janssen)	NCT03238196 NCT03210714 NCT03390504 NCT02699606 NCT02365597 NCT03473743 NCT02952573 NCT02421185	FGFR1-4	1 2 3 2 2 1/2 2 1	Metastatic Breast Cancer Pediatric Tumors Urothelial Cancer Solid Tumors Urothelial Cancer Urothelial Cancer Multiple Myeloma Hepatocellular Carcinoma
PRN1371 (Principia)	NCT02608125	FGFR1-4	1	Solid Tumors
Rogaratinib (Bayer)	NCT03517956 NCT01976741 NCT03410693 NCT03473756	FGFR1-4	1 1 2/3 1/2	Solid Tumors Solid Tumors Urothelial Cancer Urothelial Cancer
TAS120 (Taiho)	NCT02052778	FGFR1-4	1/2	Solid Tumors
Bemarituzumab (Five Prime)	NCT02318329 NCT03694522 NCT03343301	FGFR2b	1 3 1	Solid Tumors Gastric Cancer GI Tumors
Vofatamab (BioClin)	NCT03123055 NCT02401542	FGFR3	1/2 1/2	Urothelial Cancer Urothelial Cancer

Table 3. Current clinical trials with FGFR4-specific inhibitors

Drug name (company)	ClinicalTrials.gov Identifier	Phase	Conditions
FGF401 (Novartis)	NCT02325739	1/2	HCC and advanced solid tumors
Blu-554 (Blueprint Medicines)	NCT02508467	1	HCC
H3B-6527 (H3 Biomedicine)	NCT02834780	1	HCC or intrahepatic cholangiocarcinoma
INCB062079 (Incyte)	NCT03144661	1	HCC and advanced solid tumors

2.0 IDENTIFICATION OF FGFR4 OVEREXPRESSION AND HOTSPOT MUTATIONS AS DRUGGABLE TARGETS FOR ENDOCRINE-RESISTANT LOBULAR BREAST CANCER

Contributors to study: Kevin M. Levine^{1,2,3,4}, Nolan Priedigkeit^{1,2,3,5}, Ahmed Basudan^{1,2,6}, Nilgun Tasdemir^{1,2,5}, Matthew J. Sikora⁷, Ethan S. Sokol⁸, Ryan J. Hartmaier^{8,9}, Kai Ding¹⁰, Zahra Ahmad¹, Michelle M Boisen¹¹, Esther Elishaev⁴, Peter C. Lucas^{1,2,4}, Adrian V. Lee^{1,2,5}, Steffi Oesterreich^{1,2,5}

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2.1 INTRODUCTION

Invasive lobular carcinoma (ILC) accounts for 10-15% of all breast cancer diagnoses. Since most of these tumors are estrogen receptor positive (ER+), patients with ILC are often treated with endocrine therapy. Although these treatments are highly efficacious, long-term recurrences remain a major clinical problem for ILC^{78,81}. Here, we set out to identify the mechanisms of both acquired and *de novo* resistance to endocrine therapy in ILC.

To model acquired resistance, we first created long-term estrogen deprived (LTED) cell lines and performed RNA-Sequencing^{87,88}. Overlapping our data with other *in vitro* models of acquired endocrine resistance in ILC pointed to overexpression of FGFR4 (fibroblast growth factor receptor 4) as a potential therapeutic target. To further assay mechanisms of acquired resistance, we next performed RNA-Sequencing on primary ER+ tumors and their subsequent recurrences following treatment with endocrine therapy^{172,173}. Again, these results identified FGFR4 overexpression as a key putative driver of endocrine-resistant ILC.

FGFR4 is a tyrosine kinase receptor previously reported to be overexpressed in a variety of cancer types, with specifically-targeted drugs currently in clinical trials^{100,101,128,174–178}. Because of the therapeutic potential of these drugs in ILC, we next asked whether elevated FGFR4 expression may also play a role in *de novo* resistance to endocrine therapy. Lastly, we queried recently published datasets of mutation and copy-number changes in metastatic disease to determine if there are alternate routes of FGFR4 activation in advanced ILC.

2.2 MATERIALS AND METHODS

2.2.1 FGFR4 expression gain in endocrine-resistant cell lines

Microarray data from 3 replicates each of Sum44PE tamoxifen-resistant cells was analyzed using limma¹⁷⁹ from GSE12708¹⁸⁰. Microarray data from 3 replicates each of LTED cells from GSE75971¹⁸¹ were compared with parental cells treated with 1-week estrogen deprivation in charcoal-stripped serum (CSS), and analyzed using lumi¹⁸². RNA-Seq data from LTED cells generated in the Oesterreich lab⁸⁸ (GSE116744⁸⁷) were compared with parental cells treated with 3-day estrogen deprivation in CSS, with reads processed via Salmon¹⁸³ v.0.6.0 and analyzed using DeSeq2¹⁸⁴. In all cases of microarray or RNA-Seq, multiple comparisons testing was done using the Benjamini-Hochberg method of all expressed genes to identify FGFR4 expression as being significantly upregulated¹⁸⁵. qPCR was performed with the following primers: FGFR4: 5'-tgcagaatctcaccttgattaca-3', 5'-ggggttaactgtgcctattcg-3', RPLPO: 5'-taaaccctgcgtggcaatc-3', 5'-ttgtctgctcccacaatgaaa-3'. qPCR results were tested for significance using a two-tailed Student's t-test.

2.2.2 Cell culture reagents

MDA-MB-134VI (MM134) (American Type Culture Collection [ATCC], Manassas, VA, USA) and SUM44F (Asterand Bioscience, Detroit, MI, USA) cells were maintained in 1:1 DMEM (11965; Life Technologies, Carlsbad, CA, USA): L-15 (11415, Life Technologies) + 10% FBS (26140; Life Technologies). LTED cell lines were maintained in IMEM (A10488; Life

Technologies. Richter's modification, no Phenol Red, no Gentamycin) + 10% charcoal-stripped FBS (CSS).

2.2.3 Antibodies

For IHC, FGFR4 antibody MABD120 (EMD Millipore) was used at a 1:250 dilution after antigen retrieval using heated citrate buffer, pH 6.0. Staining was detected using Envision Dual Link+ HRP Polymer and DAB (Dako). For IB, FGFR4 antibody sc-124 (Santa Cruz) was used at a 1:1000 dilution, and beta-actin (Sigma) at 1:10,000. Blots were imaged on the Olympus LI-COR system.

2.2.4 RNA-Sequencing clinical samples

RNA extraction for the brain and bone metastases cohorts have been described in detail previously^{172,173}. RNA extraction for the GI/ovarian metastasis cohort as well as for the local recurrences cohort was performed as for the brain metastasis cohort. Briefly, biospecimens were reviewed by a trained molecular pathologist to confirm pathology, quantify tumor cellularity and to highlight regions of relatively high tumor cellularity for macrodissection. RNA was extracted from FFPE tissue using Qiagen's All-Prep Kit. As with the brain and bone cohorts, library preparation for the GI/ovarian metastasis cohort was performed using Illumina's TruSeq RNA Access Library Preparation protocol. For the local recurrence cohort, Illumina's TruSight RNA Pan-Cancer (1390 targets) protocol was used. Transcript counts from all samples were quantified with Salmon¹⁸³ v.0.7.2 and converted to gene-level counts with biomaRt^{186,187}. The gene-level counts for the overlapping targets were then merged together using TMM-normalization¹⁸⁸ with

the edgeR package¹⁸⁹. Log2 transformed TMM-normalized counts per million: $\log_2(\text{TMM-CPM} + 1)$ expression values were used for the analysis. Michelle M Boisen, Ahmed Basudan, and Esther Elishaev collected the GI and ovarian metastases and performed pathology. Peter Lucas served as the pathologist for the brain, bone, and local recurrences cohorts.

2.2.5 Defining outlier expression gains

Outlier gains were determined for each patient by discretely categorizing all genes into one of 3 categories. If $\log_2\text{FC}$ values (i.e. recurrence $\log_2\text{normCPM}$ - primary $\log_2\text{normCPM}$) for a given gene were less than $Q1 - (1.5 \times \text{IQR})$ using case-specific $\log_2\text{FC}$ values for all genes as the distribution, that gene was deemed an “Outlier Loss”. If $\log_2\text{FC}$ values calculated were greater than $Q3 + (1.5 \times \text{IQR})$, it was deemed an “Outlier Gain”. All other genes for that patient were considered “Stable”.

2.2.6 FGFR4 expression in large cohorts of ER+ and ER- primary and metastatic tumors

Transcript per million (TPM) expression data from The Cancer Genome Atlas (TCGA⁴) was downloaded from the Gene expression Omnibus database (GSE62944¹⁹⁰). Raw microarray data from Molecular Taxonomy of Breast Cancer International Consortium (METABRIC¹⁹¹) was downloaded from Synapse software platform (syn1688369; Sage Bionetworks, Seattle, WA, USA) respectively. For METABRIC, the probe with the largest IQR was chosen to represent FGFR4. The MET500 FASTQ files for hybrid-capture RNA-Seq breast metastatic samples were downloaded via dbGaP (phs000673.v2.p1)¹⁹². Transcript quantification was performed with Salmon v0.8.2 (quasi-mapping mode, 31-kmer index using GRCh38 Ensembl v82 transcript

annotations, seqBias and gcBias corrections), followed by mapping to gene counts via tximport¹⁹³, and normalization via log2 transformed TMM-normalized counts-per million (log2 CPM)¹⁸⁸ with the edgeR package¹⁸⁹. ER-positivity for MET500 was defined as RNA expression of ESR1 greater than or equal to the median.

2.2.7 Nanostring study of *de novo* endocrine therapy resistance

This case-control study design included all female patients over the age of 18 with a diagnosis of primary invasive lobular carcinoma who were treated at Magee-Women's Hospital. A total population of 709 ILC cases was identified, including 85 with recurrences. A simulation study using the observed event rate in the population was performed to determine the cohort size needed for 80% power to detect a hazard ratio of 1.850. Based on the results, a sample of n=200 subjects (including recurrences) was selected. However, tissue acquisition, subject eligibility and verification of recurrences yielded a sample of n=129 total subjects with n=32 distant recurrences. Subjects were excluded for a previous history of breast cancer, metastasis at diagnosis, concurrent non-breast tumor at diagnosis, or ER- disease. Subjects were censored at date of diagnosis of second primary, including contralateral breast cancer, date of death unrelated to breast cancer or at date of last follow-up in the absence of any other qualifying event. Rachel Jankowitz, David Dabbs, and Priscilla McAuliffe, and Louise Mazur helped to identify available patient samples and collect updated clinical information. Kristine Cooper and Daniel Normolle performed the power calculations.

We designed hybridization probes for 695 genes, including genes of interest to the Oesterreich lab (including FGFR4), genes with copy-number alterations in the TCGA ILC cohort, and several housekeeping genes. Sample processing was done using the automated

nCounter Prep Station. Raw counts were then collected from the nCounter Digital Analyzer and transferred to the nSolver™ software (v 2.5) for data analysis. Raw counts were normalized to 5 invariant reference probes (count normalization).

For IHC of FGFR4, antibody MABD120 (EMD Millipore) was used at a 1:250 dilution. Staining was done in a blinded manner and one representative image was captured at 200x magnification. Protein expression in tumor cells was ranked from low to high by 3 blinded observers. Ranks were then averaged across the 3 observers.

2.2.8 RATHER consortium data analysis

FGFR4 RPPA data was downloaded from: GSM1626980 as log(2) transformed and centered around zero. Cell signaling antibody CST-8562 was used. FGFR4 microarray expression data was downloaded from GSE68057 as log2 normalized counts centered around zero.

2.2.9 FGFR4 hotspot mutation rates

The FGFR4 hotspots (N535 and V550), as defined in rhabdomyosarcoma¹³⁸, were queried in MSK-IMPACT and the Lefebvre et al. study¹⁹⁴ using the cBio portal¹¹⁹, and MET500¹⁹² using the MET500 portal (<https://met500.path.med.umich.edu>). Foundation medicine mutation data was analyzed for codon 510 and N495 alterations. MSK-IMPACT contains designations for primary and metastatic tumors, whereas Foundation Medicine contains designations for local (including local recurrences) and metastatic tumors. In all cases, lymph node metastases and distant recurrences were grouped together. For analysis of mutation rate in the Foundation Medicine and Lefebvre et al. studies, tumors of unspecified histology with a CDH1 mutation or

homozygous deletion in CDH1 were classified as ILC. Fisher exact test odds ratios were calculated using R version 3.5.1.

2.2.10 Statistical considerations

GraphPad Prism software version 7, and R version 3.5.1 were used for statistical analysis. All tests were two-tailed, with $p < .05$ considered statistically significant. Paired Wilcoxon rank signed tests were used for expression gains in metastases, not corrected for multiple comparisons testing. Fisher's exact tests were used to quantify odds-ratios and significance for enrichment of FGFR4 hotspot mutations.

2.3 RESULTS

2.3.1 Transcriptome analysis of endocrine-resistant ILC cell lines identifies FGFR4 overexpression as a druggable target

To model acquired resistance to endocrine therapy in ILC, we performed RNA-Sequencing on long-term estrogen deprived (LTED) cell lines (GSE116744⁸⁸) and short-term estrogen deprived parental controls. We overlapped our results with microarray data from a previously published ILC cell line model of tamoxifen resistance (GSE75971¹⁸⁰). From this analysis, we identified eight total genes that were consistently changed across all seven models (Figure 4). FGFR4 was the top overexpressed gene, and the only gene among these that is currently considered druggable¹⁹⁵.

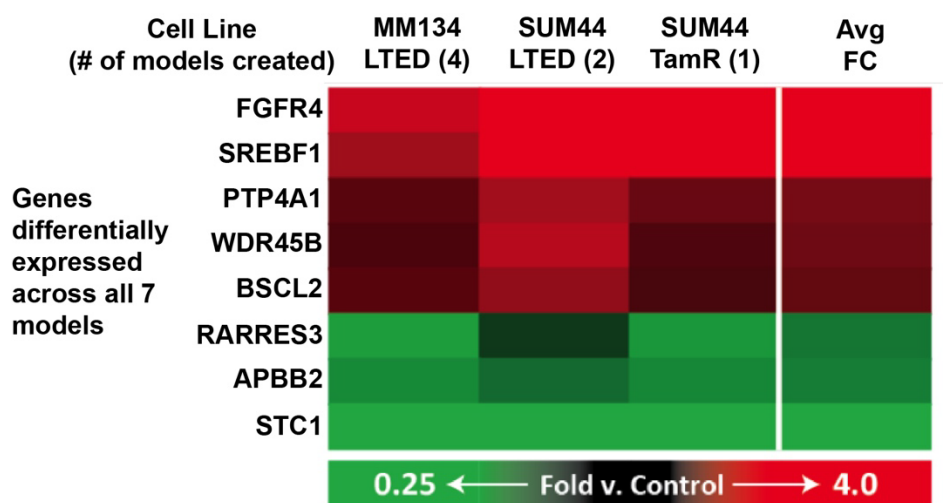


Figure 4. Top differentially expressed genes in endocrine-resistant ILC cell lines

All differentially expressed genes that are shared between 7 endocrine-resistant ILC models. Colors represent the average fold change between the endocrine-resistant cell lines of a particular class versus their parental cells with and short-term estrogen deprivation. Heatmap generated by Matthew Sikora using MeV¹⁹⁶.

To further explore whether FGFR4 may play a role in acquired resistance to endocrine therapy, we queried its expression in a third collection of LTED cell lines (GSE75971¹⁸¹) including IDC cell line models. In total, FGFR4 was overexpressed in 8/8 ILC cell line models and 4/4 IDC cell line models at the RNA level relative to parental cells treated with short-term estrogen deprivation (Figure 5A). Importantly, the FGFR4 overexpression in our ILC LTED cells was also increased relative to parental cells in full serum, at the RNA and protein level (Figure 5B).

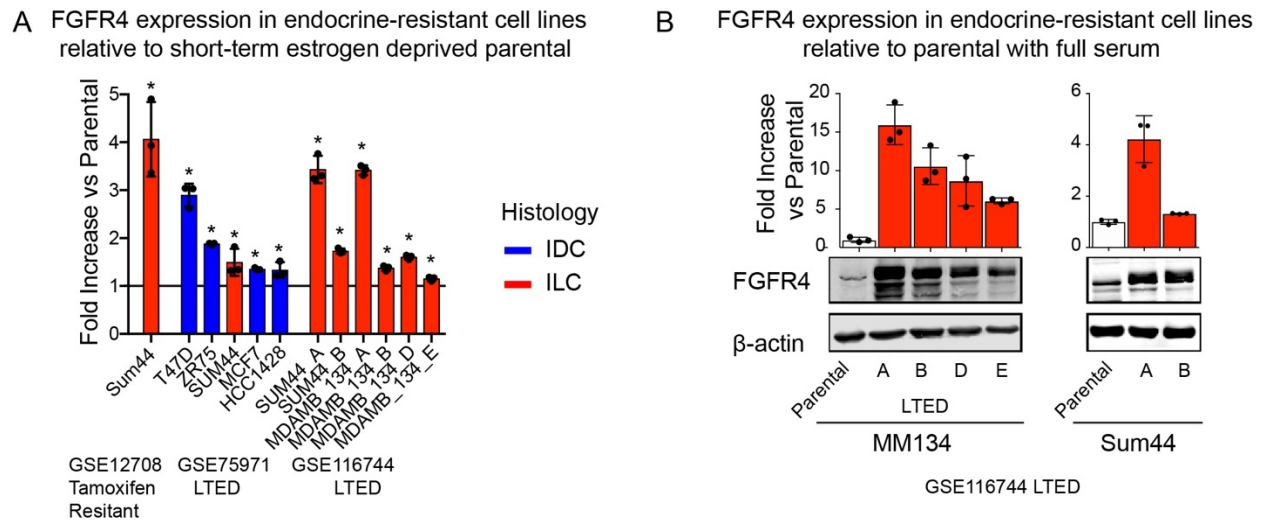


Figure 5. FGFR4 expression is elevated in cell line models of endocrine resistance

A) FGFR4 RNA expression fold-change in long-term endocrine-resistant cell line models of ER+ breast cancer, relative to parental cell lines with short-term estrogen deprivation. From left to right, tamoxifen-resistant cells from GSE12708, long-term estrogen deprived (LTED) cells from GSE75971, LTED cells from GSE116744. * $p < .05$ for differential expression vs parental, corrected for multiple comparisons with Benjamini-Hochberg.

B) qRT-PCR comparison of GSE116744 MM134 and SUM44 LTED to parental cells grown in FBS. Error bars represent $\pm$ SD for 3 biological replicates. Red bars represent ILC and blue bars represent IDC.

Figure B generated in collaboration with Jian Chen.

2.3.2 Transcriptome analysis of paired primary and recurrent tumors identifies FGFR4 overexpression as a druggable target

To explore mechanisms of endocrine resistance in clinical samples, we next collected paired primary and recurrent tumors. In this subset analysis, we focus on the patients with ER+ primary tumors who received endocrine therapy prior to local recurrence or distant metastasis of bone¹⁷², brain¹⁷³, or GI/ovarian tumors (Basudan et al, manuscript in preparation). This dataset consists of 74 tumors: treatment-naïve primary tumors and endocrine-treated paired recurrences from 37 patients, consisting of 11 local recurrences, and 7 bone, 7 brain, 3 GI, and 9 ovarian metastases (Figure 6A). The average time to recurrence was 55 months, suggesting the observed changes in expression likely represent acquired alterations following long-term endocrine therapy. Our

study cohorts were enriched for ILC, and this subset analysis consists of a histological distribution of 22 IDCs, 10 ILCs, and 5 cases of mixed IDC/ILC.

For each pair of tumors, we identified genes with outlier gains in expression, by comparing log₂-normalized fold-changes across the 1390 genes present on Illumina's TruSight RNA Pan-Cancer panel. For example, for ovarian metastasis patient 1 (OV_1), a gene was considered to have an outlier gain if the expression in the metastasis was more than 3-fold higher than in the primary tumor. Figure 6B shows the genes with outlier gains occurring in at least 12/37 patients. NCAM1 and FGFR4 were the two most frequently gained in expression, each occurring in 18/37 patients (49%). The gains in FGFR4 led to a bimodal distribution of expression in the recurrences, with a subset of patients having significantly higher expression than seen in primary tumors (Figure 6C). Figure 6D shows immunohistochemical (IHC) staining of FGFR4 in one patient with a 4-fold gain in FGFR4 expression in a local recurrence.

Overall, 31/37 (84%) recurrences have an increase in FGFR4 RNA relative to their matched primary tumor ($p=41.4e-5$), including 26/37 (70%) with a fold change > 2 (Figure 7). Of note, these large gains in FGFR4 spanned all four distant metastatic sites studied (Figure 7). Given the small sample size for each metastatic site, there is no significant difference for FGFR4 expression gain by tumor site or histological type, nor is there an interaction effect ($p>.05$ for all three tests by two-way ANOVA). However, there is a trend for increased FGFR4 gain in tumors with a lobular component, with a mean increase of 6.3-fold versus 2.5-fold for the pure IDCs.

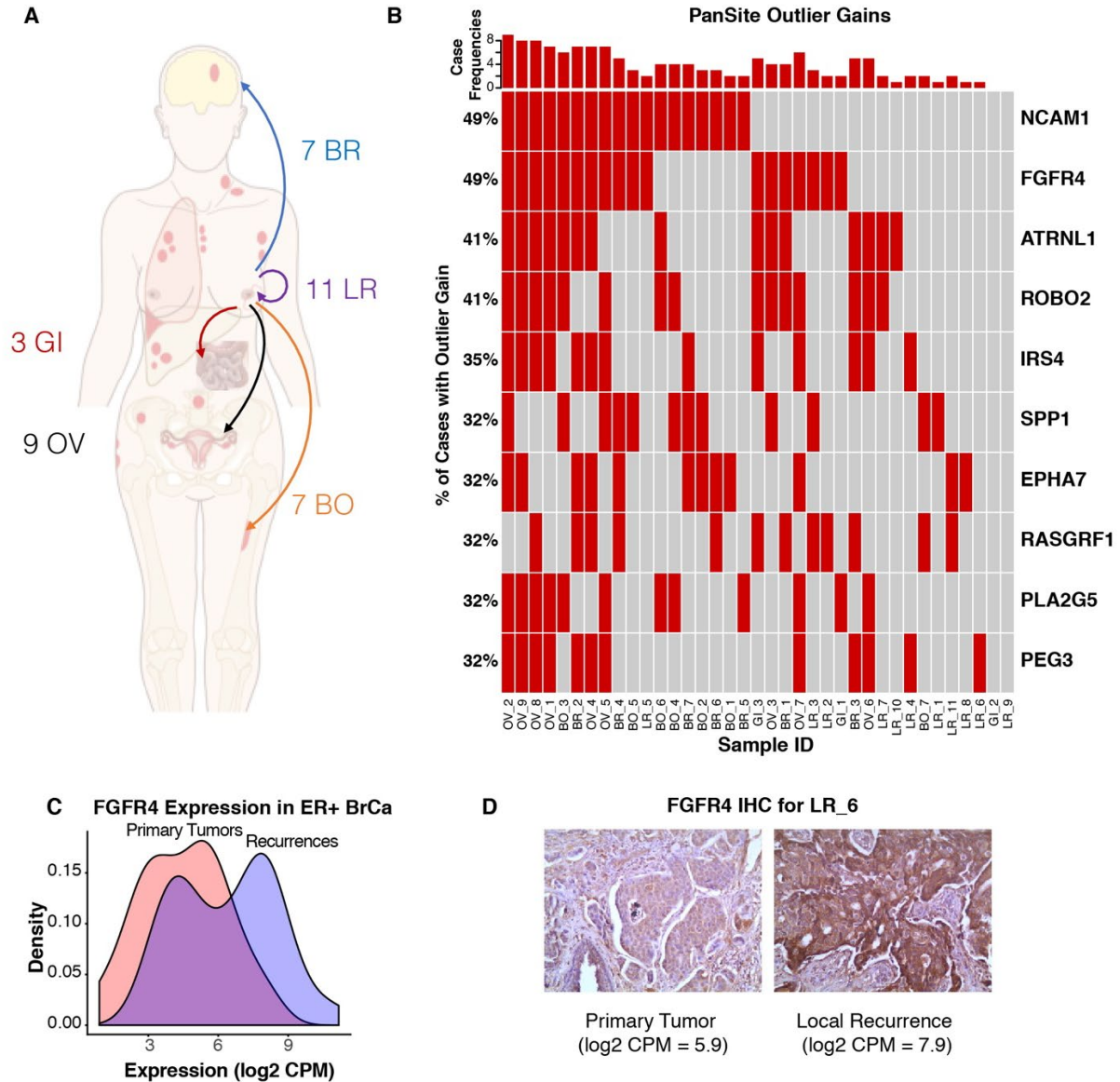


Figure 6. FGFR4 expression increases in recurrent tumors treated with endocrine therapy

A) Diagram showing sites and n of advanced disease samples (GI = gastrointestinal, OV = ovary, BR = brain, LR = local recurrence, BO = bone).

B) OncoPrint of outlier gains seen in at least 12/37 endocrine-treated advanced breast cancers. Genes are sorted by frequency of gain across pairs.

C) FGFR4 expression distribution in recurrent (purple) and primary (salmon) ER+ breast cancers (BrCa).

D) Immunohistochemical staining of FGFR4 protein in patient-matched case LR_6, with log2 normalized counts per million (log2 CPM) primary tumor FGFR4 expression of 5.9 and local recurrent tumor FGFR4 expression of 7.9 (log2 fold change = 2, absolute fold change = 4).

Figure generated in collaboration with Nolan Priedigkeit.

FGFR4 Expression in Paired Endocrine-Treated Breast Recurrences

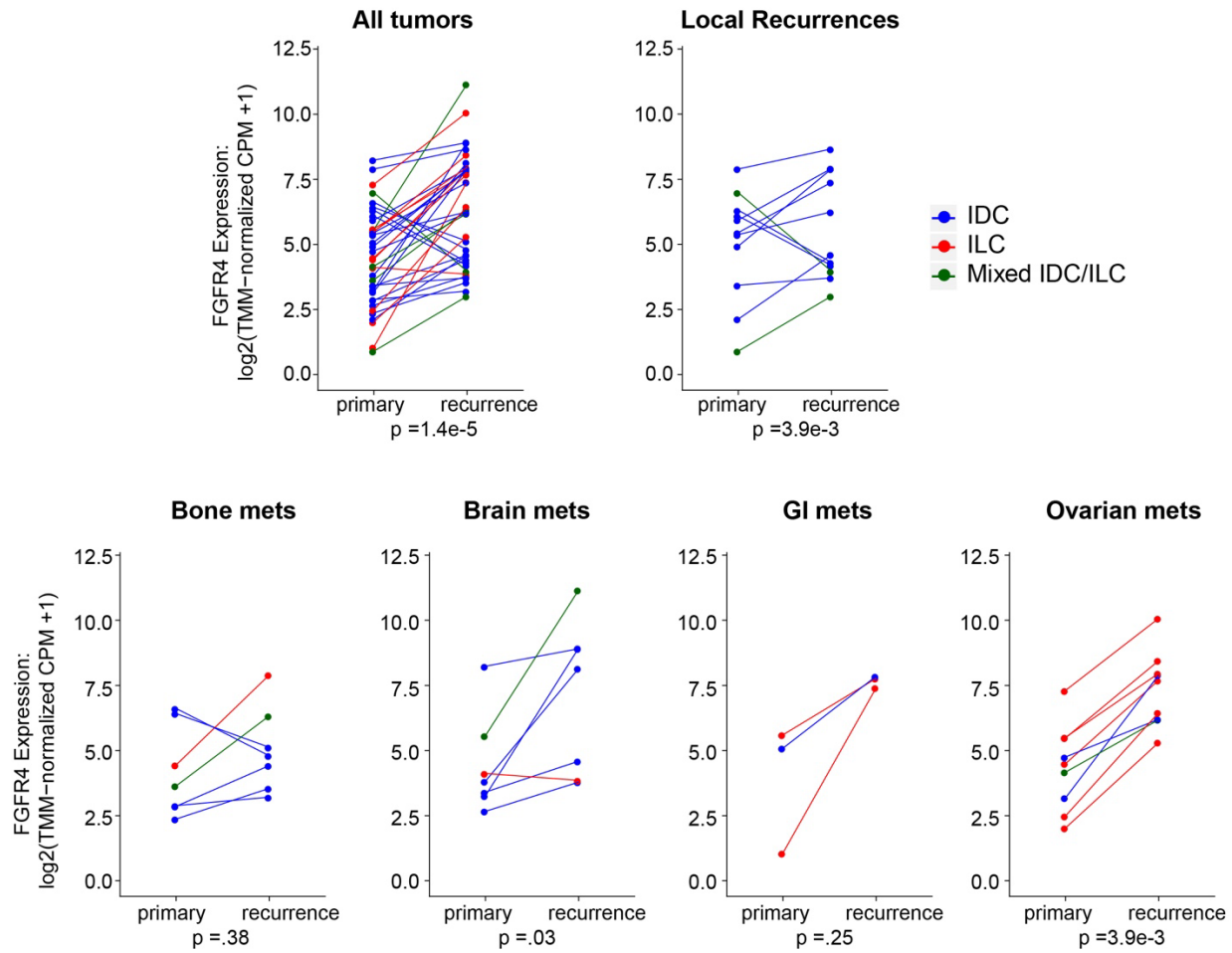


Figure 7. FGFR4 expression gains by histology and tumor site

FGFR4 expression gain in recurrent pairs, separated by primary tumor histology and recurrent tumor site. Red lines represent primary tumor histology of ILC, blue lines represent IDC, and green lines represent mixed IDC/ILC tumors. Two-sided paired Wilcoxon rank tests were used to calculate p-values for FGFR4 gain.

2.3.3 Outlier alterations that co-occur with FGFR4 gains in paired endocrine-treated samples

To identify putative signaling partners with FGFR4, we next queried all outlier gains for significant co-occurrence with FGFR4 gains. Co-occurrence was tested using fisher-exact tests, and genes were filtered using a Benjamini-Hochberg adjusted p-value less than 0.2. Many of these co-occurring gains appeared in ovarian metastases. To ensure that ovarian-specific genes were not chosen, an additional filter was added to ensure outlier gains in at least 2 tissue sites. Figure 8 shows an oncoprint of these outlier gains, which include, *FAM19A2*, *MAP2*, *GATA6*, and *ALDOC*. Likewise, genes with outlier losses that co-occur with FGFR4 were identified, and these include *POSTN* and *TP63* (Figure 9).

Of these, *EPHA5* is particularly interesting, given ephrin receptors have been previously shown to physically interact with FGFRs, including FGFR4^{197,198}. Ephrin receptors are membrane-bound tyrosine kinases with documented pro- and antitumorigenic activity, depending on ligand specificity and interacting molecules¹⁹⁹. *EPHA5* is frequently overexpressed in lung cancer, helping to drive cell cycle progression in the presence of genotoxic stress²⁰⁰, whereas *EPHA4* has been studied in the context of breast cancer and FGFR signaling. Elevated *EPHA4* expression is poorly prognostic for breast cancer^{201,202}, can bind to the juxtamembrane domain of FGFR4¹⁹⁷, and can increase glioblastoma cell proliferation and migration through FGFR1 signaling²⁰³.

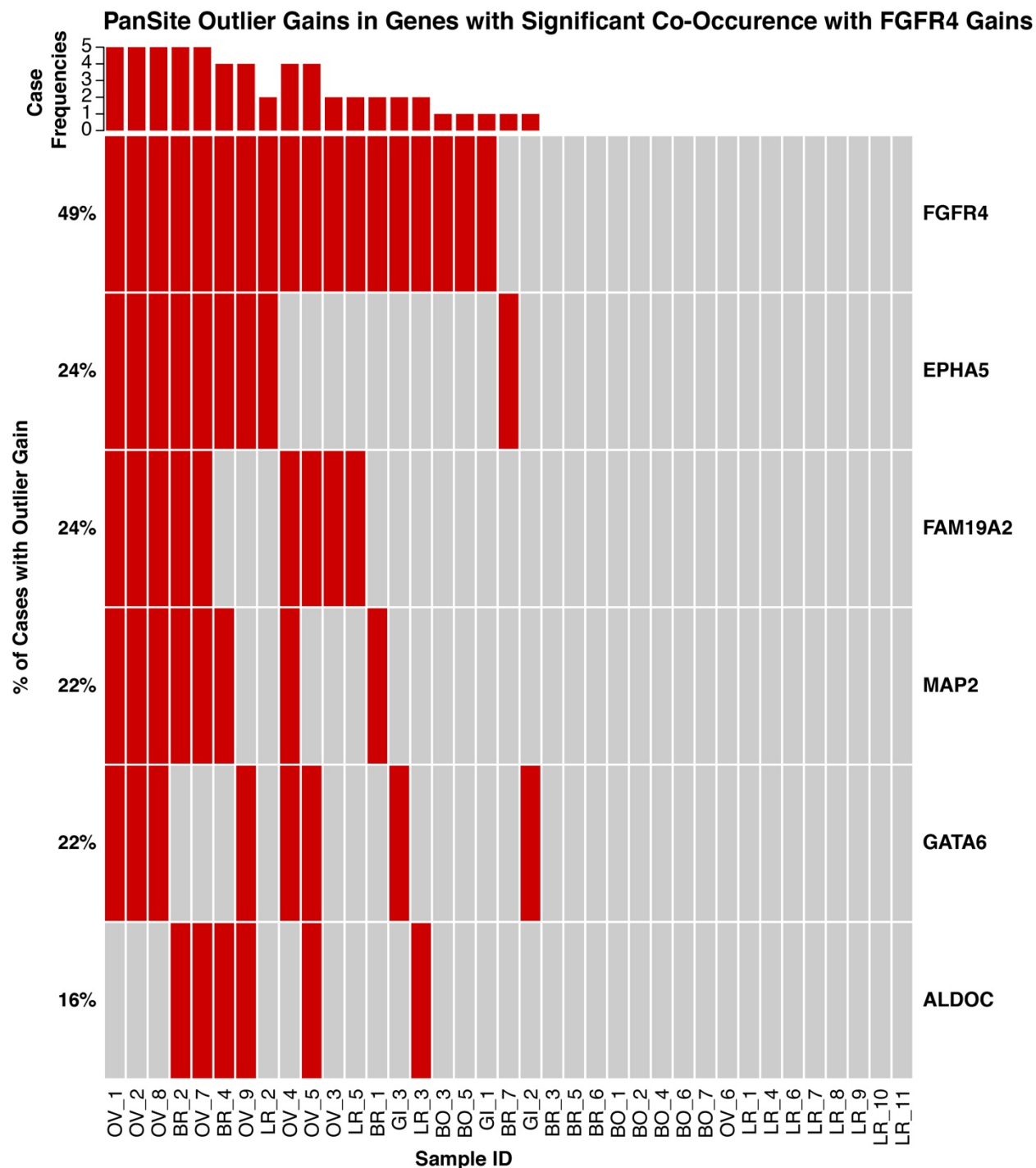


Figure 8. Oncoprint of outlier gains with significant co-occurrence with FGFR4 gains

Oncoprint of outlier gains in genes with co-occurrence with FGFR4 gains. Fisher-exact tests were used to calculate co-occurrence probabilities, and a Benjamini-Hochberg adjusted p-value <0.2 was used as a filter. Only genes with outlier gains in at least 2 tissue sites are shown. (GI = gastrointestinal, OV = ovary, BR = brain, LR = local recurrence, BO = bone).

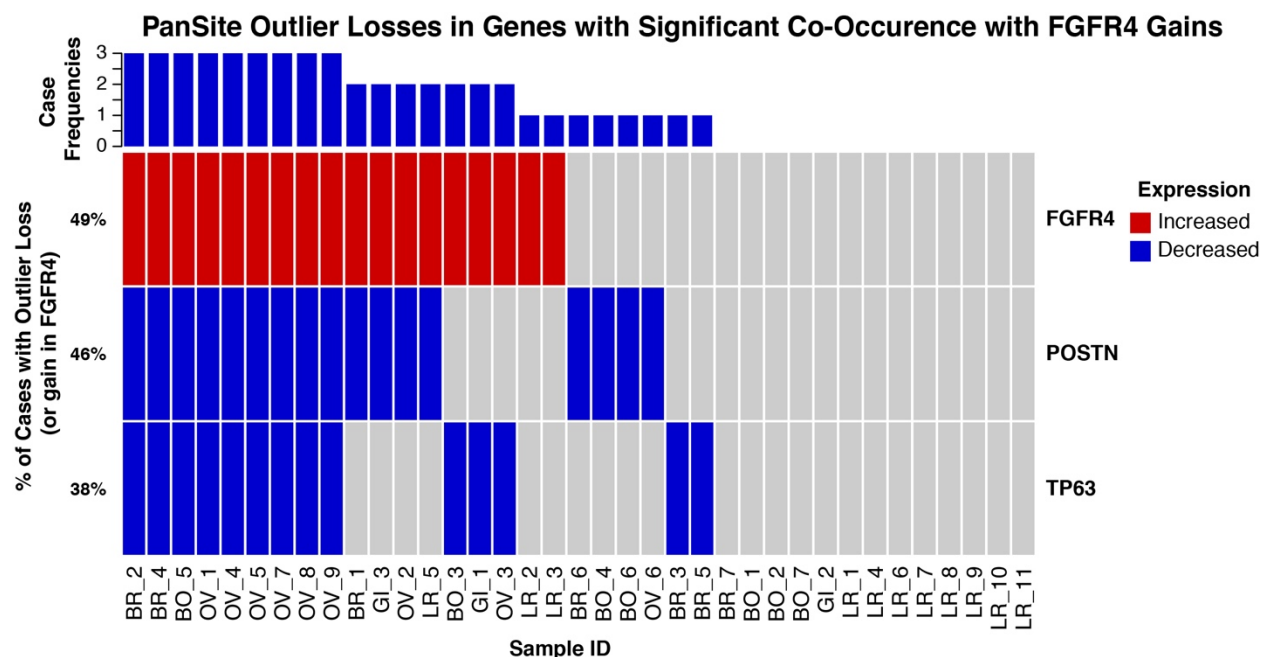


Figure 9. Oncoprint of outlier losses with significant co-occurrence with FGFR4 gains

Oncoprint of outlier losses in genes with co-occurrence with FGFR4 gains. Fisher-exact tests were used to calculate co-occurrence probabilities, and a Benjamini-Hochberg adjusted p-value <0.2 was used as a filter. (GI = gastrointestinal, OV = ovary, BR = brain, LR = local recurrence, BO = bone).

2.3.4 FGFR4 overexpression is enriched in ER+ metastatic tumors

To further assess the role of FGFR4 in acquired endocrine resistance, we next assayed a large cohort of metastatic tumors (MET500)¹⁹² and compared FGFR4 expression with primary tumors present in the TCGA⁴ and METABRIC¹⁹¹ cohorts. Analysis of the MET500 breast cohort revealed that FGFR4 expression is higher in ER+ metastatic tumors than in ER- metastatic tumors, even though the opposite is true in primary disease (Figure 10A). An important limitation of our cohorts of metastases is the absence of metastatic tissue from the lung and liver—two common metastatic sites in breast cancer. FGFR4 expression in the MET500 breast cohort does not seem to be site-specific (Figure 10B), suggesting the increase in FGFR4 expression is a generalizable finding for breast cancer recurrences in ER+ disease.

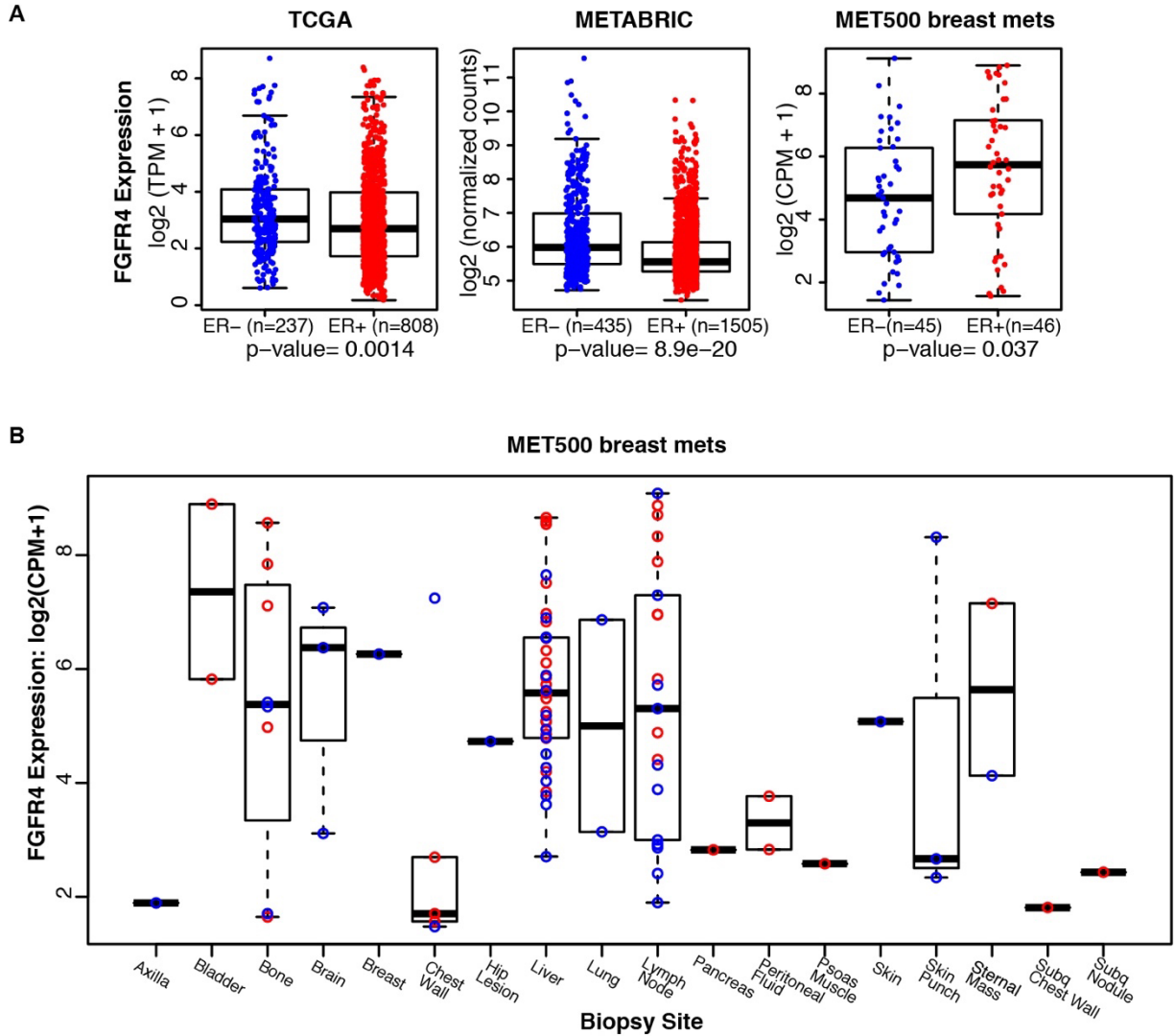


Figure 10. FGFR4 expression in metastatic ER+ and ER- tumors

A) FGFR4 RNA expression in TCGA primary, METABRIC primary, and MET500 metastatic tumors originating in the breast. p-values are from two-tailed Mann-Whitney U-tests.

B) FGFR4 expression in MET500 tumors segregated by biopsy site. Blue dots represent ER- tumors and red dots ER+ tumors.

2.3.5 Nanostring study of ER+ ILC patients suggests FGFR4 expression may play a role in de novo resistance to endocrine therapy

To test if FGFR4 expression may also play a role in *de novo* resistance to endocrine therapy, we next collected 129 treatment-naïve ER+ ILC tumors. We quantified FGFR4 expression using the Nanostring platform²⁰⁴ and defined patients to have high FGFR4 expression using the upper quartile as a threshold. Figure 11 shows that high FGFR4 expression is in fact prognostic for an increased risk of distant recurrence (log-rank p-value <.05).

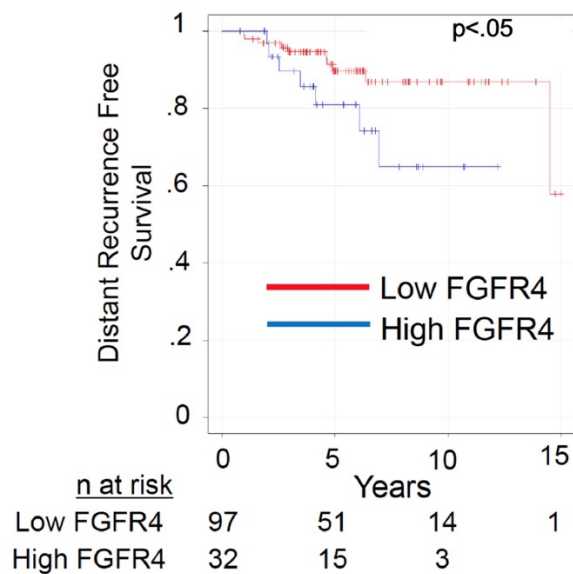


Figure 11. FGFR4 expression is prognostic for poor survival in ER+ ILC
n = 129 ER+ ILC patients treated at the University of Pittsburgh. High FGFR4 is defined as the upper quartile of RNA expression. The number of women at risk for recurrence is shown at each 5-year interval.
Log-rank test: p<0.05.
Nanostring analysis was performed by Matthew Sikora. Rachel Jankowitz, David Dabbs, Priscilla McAuliffe, and Louise Mazur helped to acquire clinical samples and retrieve updated clinical information.

We next asked whether this poor prognosis could also be predicted from protein-level expression of FGFR4. First, we queried the RATHER consortium microarray and RPPA data of ILC primary tumors to assess if high FGFR4 RNA expression is predictive of high protein

expression²⁰⁵. Although FGFR4 RNA was only weakly correlated with protein expression in that study, high RNA expression (upper quartile) was predictive of high protein expression (Figure 12). Given this relationship, we expect high FGFR4 protein expression in our cohort of ER+ ILC to be predictive of distant recurrences. To test this formally, we have begun staining our samples for FGFR4 protein expression by IHC and ranking samples by tumor cell expression of FGFR4 (Figure 13A). From a limited sample size of 22 patients thus far, FGFR4 RNA and protein expression is significantly correlated, and the protein expression itself is predictive of developing a distant recurrence, suggesting potential utility of an IHC-based prognostic assay (Figure 13B-D).

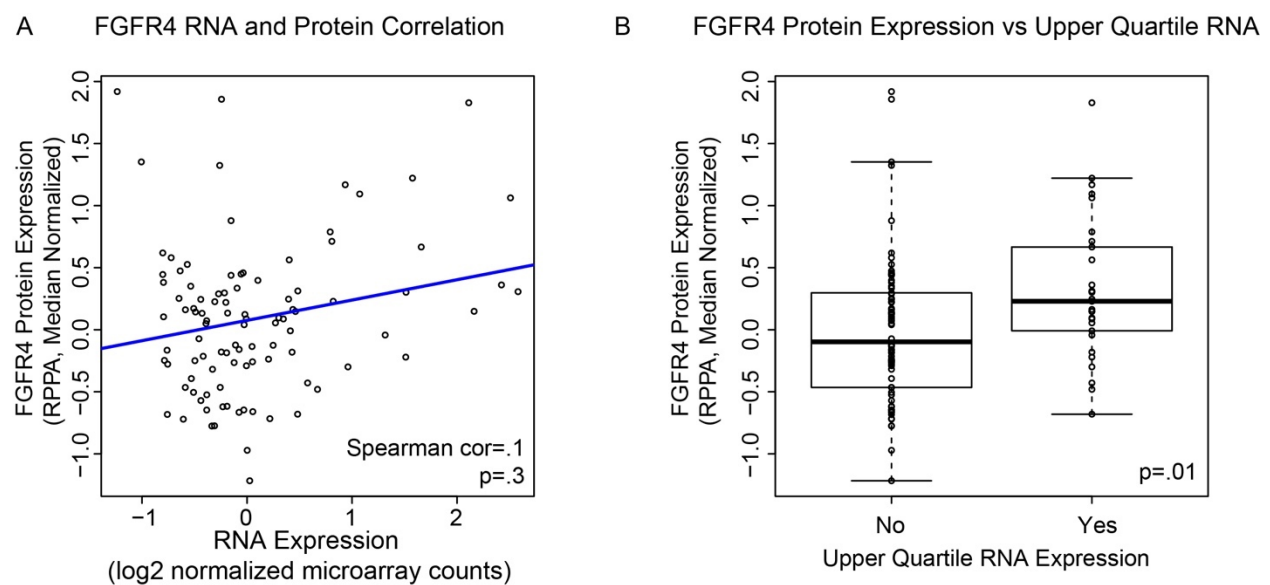


Figure 12. FGFR4 RNA and protein expression in RATHER consortium

A) FGFR4 RNA and protein correlation for 99 primary ILC patients. Spearman correlation = 0.1, $p=0.3$. Blue line represents linear regression fit.

B) FGFR4 protein expression with patients separated by upper quartile FGFR4 RNA expression. Mann-Whitney U $p=0.1$.

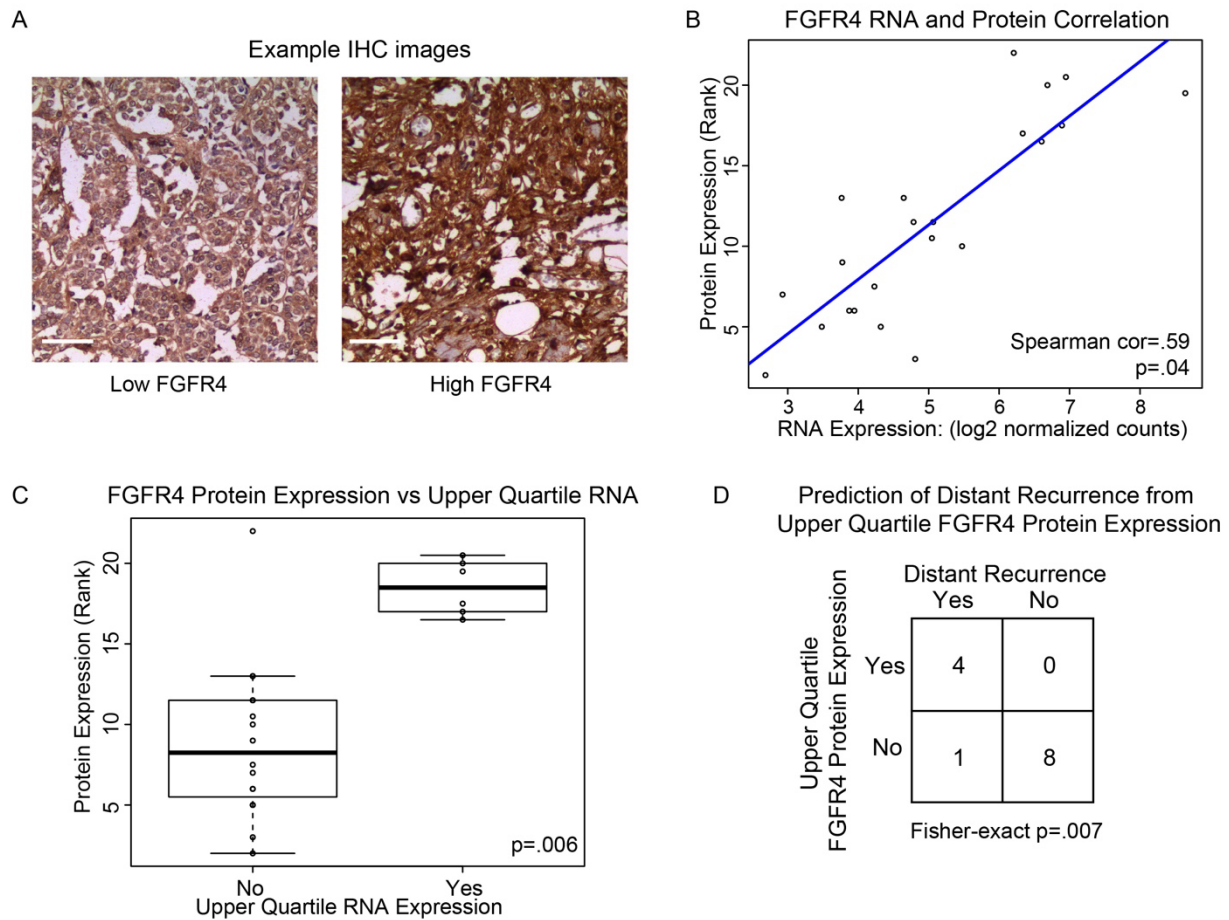


Figure 13. FGFR4 RNA and protein relationship in WCRC primary

A) Example IHC images of FGFR4 staining (Low expression: MJS-120, High Expression: MJS-322). Scale bar=200um.

B) FGFR4 RNA and protein correlation for 22 primary ILC patients. Spearman correlation = 0.59, p=0.04. Blue line represents linear regression fit. Protein expression rank from 1=lowest to 22=highest.

C) FGFR4 protein expression with patients separated by upper quartile FGFR4 RNA expression. Mann-Whitney U p=0.006.

D) Prediction of distant recurrence by upper quartile FGFR4 protein expression (fisher-exact p=.007).

Figure generated in collaboration with Zahra Ahmad.

2.3.6 FGFR4 hotspot mutations in metastatic ER+ breast cancer are enriched in the lobular subtype

Finally, the rate of FGFR4 mutations in metastatic cancer was examined in all patients from three recent sequencing studies: MSK-IMPACT^{206,207}, MET500¹⁹², and Lefebvre et al¹⁹⁴, as well as from sequencing data from Foundation Medicine. Figure 14A shows the distribution of FGFR4 mutations in these studies, with the most frequently mutated sites being the FGFR4 hotspot mutations previously identified in rhabdomyosarcomas (N535, V550)^{137,138,141}. Although FGFR4 hotspot mutations are rarely detected in primary tumors (<.05%), they are present in ~0.5-1% of breast metastases, significantly enriched relative to non-breast metastases (~.02%) (Figure 14B). Strikingly, these mutations are enriched significantly in metastatic ILC relative to metastatic IDC (Figure 14C). Treatment data is only available for the MSK-IMPACT data, which shows that 8/9 patients with FGFR4 hotspot mutations were previously treated with endocrine therapy (Tables 2-3). The total rate of FGFR4 hotspot mutations in endocrine-treated metastases is 3.5% for ILC versus 0.5% for IDC (Figure 14D).

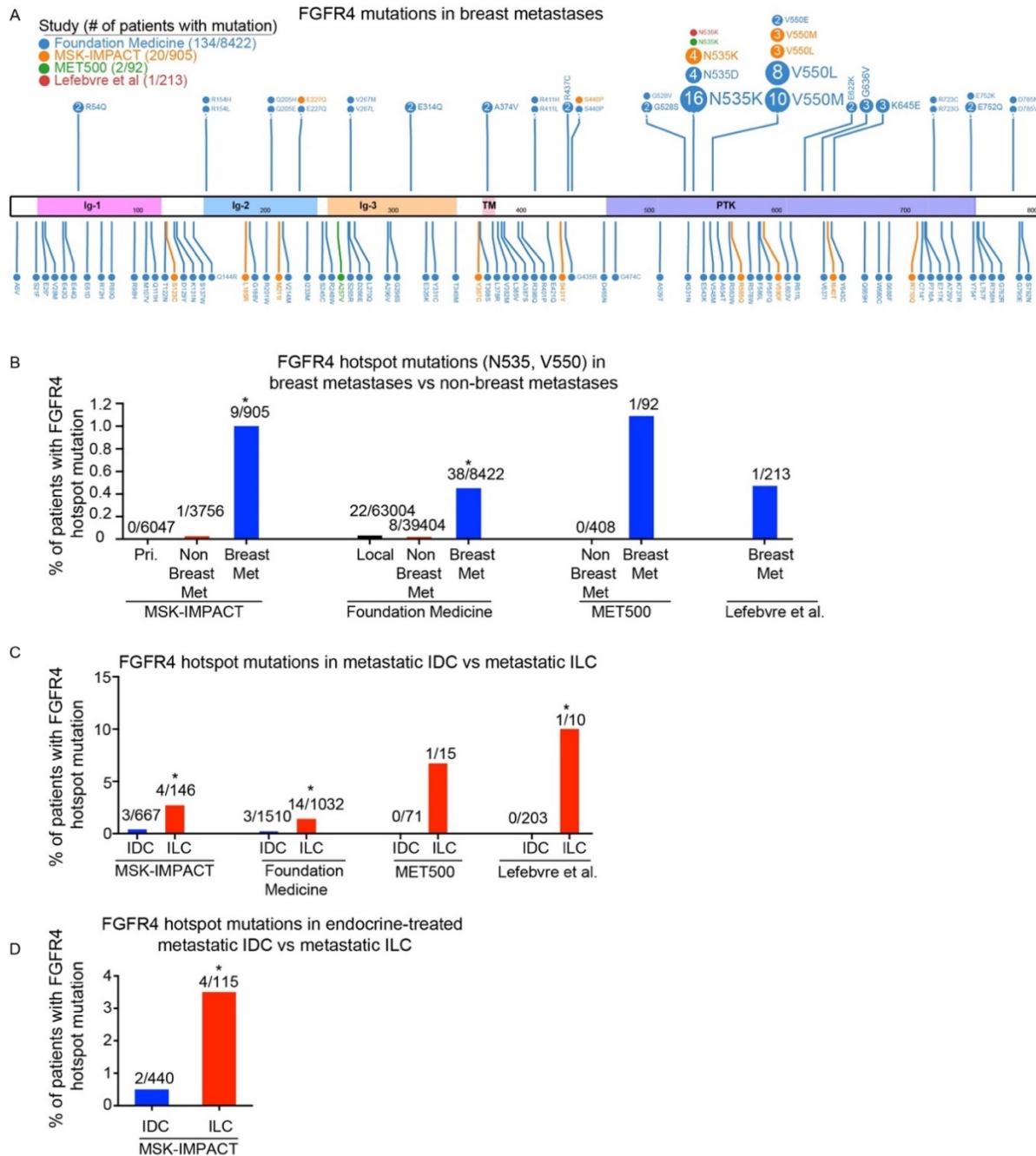


Figure 14. FGFR4 hotspot (N535, V550) mutations are enriched in metastatic ILC

A) Lollipop plot of FGFR4 mutations in breast metastases generated using ProteinPaint¹⁴⁹. Top: all mutations appearing at least twice. B) FGFR4 hotspot (N535, V550) mutations in MSK-IMPACT primary, non-breast metastatic, and breast metastatic tumors, Foundation Medicine local, non-breast metastatic, and breast metastatic tumors, MET500, and Lefebvre et al. tumors. *FGFR4 hotspot mutations are enriched in breast metastatic tumors versus non-breast metastatic tumors (MSK-IMPACT OR: 38.7, fisher exact p=5.8e-6, Foundation Medicine OR: 22.3, fisher exact p <2.2e-16). C) FGFR4 hotspot mutations in all cases of metastatic ILC vs metastatic IDC. *FGFR4 hotspot mutations are enriched in ILC (MSK-IMPACT: OR=6.2, p=.02, Foundation Medicine: OR=6.9, p<.0007, Lefebvre et al: OR=Inf., p=.05). D) FGFR4 hotspot mutations in endocrine-treated metastatic ILC vs metastatic IDC. *FGFR4 hotspot mutations are enriched in ILC (MSK-IMPACT: OR=7.9, p=.02).

Figure A generated in collaboration with Kai Ding.

Table 4. Site and ER status of MSK-IMPACT metastases with FGFR4 hotspot mutations

Sample	Patient	Histology	Metastasis Site	ER Status Primary	ER Status Met	Mutation	Allele Frequency
P-0000138-T01-IM3	P-0000138	Mixed ILC/IDC	Liver	Positive	Positive	V550M	0.18
P-0000138-T02-IM3	P-0000138	Mixed ILC/IDC	Liver	Positive	Positive	V550L	0.04
P-0001491-T01-IM3	P-0001491	IDC	Liver	Positive	Unknown	V550L	0.44
P-0004987-T01-IM5	P-0004987	ILC	Bone	Positive	Positive	N535K	0.14
P-0007282-T01-IM5	P-0007282	ILC	Lymph Node	Positive	Positive	V550M	0.39
P-0009364-T01-IM5	P-0009364	IDC	Lymph Node	Positive	Positive	V550M	0.22
P-0009602-T01-IM5	P-0009602	NOS	Lymph Node	Positive	Positive	N535K	0.23
P-0010245-T01-IM5	P-0010245	ILC	Bone	Positive	Positive	N535K	0.29
P-0010979-T02-IM5	P-0010979	IDC	Liver	Positive	Negative	V550L	0.44
P-0013568-T01-IM5	P-0013568	ILC	Bone	Positive	Negative	N535K	0.83

Table 5. Treatment history of patients in MSK-IMPACT with FGFR4 hotspot mutations

Patient	Prior Endocrine Therapy	Prior HER2 Therapy	Prior Chemotherapy	Prior CDK4/6i Therapy
P-0000138	Yes	No	Yes	No
P-0001491	Yes	No	Yes	No
P-0004987	Yes	No	Yes	No
P-0007282	Yes	No	Yes	No
P-0009364	No	No	Yes	No
P-0009602	Yes	No	Yes	No
P-0010245	Yes	No	Yes	No
P-0010979	Yes	No	No	Yes
P-0013568	Yes	No	Yes	No

2.4 DISCUSSION

From analyzing cell line models of acquired endocrine resistance, as well as clinical samples from pre and post-endocrine treatment, we find FGFR4 overexpression to be a remarkably common phenomenon. FGFR4 overexpression in endocrine-resistant cell lines is seen relative to parental cells treated with short-term estrogen deprivation, suggesting that the FGFR4 gains are not an artifact of estrogen loss. The overexpression is also seen relative to parental cells growing in full serum, suggesting that FGFR4 is not simply a marker of proliferation. Instead, FGFR4 overexpression may represent a long-term signaling adaption in the tumor cells.

From the clinical specimens, the large gains in FGFR4 spanned all four distant metastatic sites and the local recurrences. This data, as well as the fact that the brain, GI, and ovarian metastases underwent macrodissection prior to RNA extraction, suggest that the gains in FGFR4 are a result of overexpression within tumor cells rather than stromal cells in the distant metastatic sites. However, IHC is warranted to confirm tumor-specific expression of FGFR4, as well as to determine if RNA and protein levels are coupled as they are in our cell line models.

Because of the low rate of mutations, copy number amplifications, and fusions of FGFR4 identified in previous studies, FGFR4 has been understudied in clinical trials relative to the other FGFR family members¹⁷⁷. However, there are clinical trials with novel pan-FGFR inhibitors that have high potency for wild-type and mutated FGFR4^{140,164} (NCT03238196), as well as at least four ongoing clinical trials with FGFR4-specific small molecules (NCT02325739, NCT02834780, NCT03144661, NCT02508467). These FGFR4-specific inhibitors gain their specificity by interacting with a cysteine near the hotspot mutations, meaning although they are appropriate for wild-type overexpression of FGFR4, modifications would likely be needed to treat patients with hotspot mutations¹²⁸. Recent studies show that FGFR1 amplification may play

a role in endocrine resistance, and that combined FGFR1 and CDK4/6 inhibition can reverse this phenotype^{122,123}. Future studies of resistance to antiestrogen and CDK4/6 inhibitors should also examine FGFR4 expression and mutations as a potential factor, particularly for patients with lobular carcinoma.

3.0 INVESTIGATING THE ROLE OF FGFR4 INHIBITION AND FGFR4 OVEREXPRESSION ON CELL LINE GROWTH AND SIGNALING

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3.1 INTRODUCTION

In Chapter 2, we show that FGFR4 RNA is frequently upregulated in ILC in the setting of endocrine therapy resistance. Encouraged by the number of clinical cases with FGFR4 upregulation, we set out to test the phenotypic effects of FGFR4 signaling *in vitro*. We begin with assessing endogenous FGFR4 expression in commonly used ILC and IDC cell lines. Using the LTED cell lines as a model, we tested the role of FGFR4 on continued endocrine resistance. We also tested the efficacy of combination FGFR4 and ER-targeted therapy with a parental ER+ cell line. Phenotypic experiments in this chapter were performed with both FGFR4-specific small molecule inhibitors and FGFR4-targeting shRNA.

Next, we examine the ability of FGFR4 to decrease estrogen signaling in ILC cells and perform unbiased studies of downstream signaling. Lastly, we test the effect of FGFR4 overexpression on ILC and IDC cell growth. These data collectively encourage future phenotypic and signaling studies to better determine the mechanism of FGFR4 activity in ILC.

3.2 MATERIALS AND METHODS

3.2.1 FGFR4 expression in primary tumors by histology

Transcript per million (TPM) expression data from The Cancer Genome Atlas (TCGA⁴) was downloaded from the Gene expression Omnibus database (GSE62944¹⁹⁰). PAM50 subtypes for the TCGA tumors were defined using the *genefu* R package²⁰⁸. Briefly, 50:50 distributions of ER+:ER- tumors were sampled 100 times, to calculate gene expression for median centering.

The most frequent assignment was taken for each tumor. Raw microarray data from Molecular Taxonomy of Breast Cancer International Consortium (METABRIC¹⁹¹) was downloaded from Synapse software platform (syn1688369; Sage Bionetworks, Seattle, WA, USA) respectively. For METABRIC, the probe with the largest IQR was chosen to represent FGFR4. Log2 fragments per kilobase of transcript per million mapped reads (FPKM) expression data from SCAN-B was downloaded from the Gene expression Omnibus database (GSE96058²⁰⁹). Binary histology classification was derived from CDH1 expression.

3.2.2 FGFR4 RNA expression in cell lines

For endogenous FGFR4 expression in Rachel Schiff MCF7L cell lines²¹⁰ and ILC/IDC panel, qPCR was performed with the following primers: FGFR4: 5'-tgcagaatctcaccttgattaca-3', 5'-ggggtaactgtgcctattcg-3', RPLPO: 5'-taaaccctgcgtggcaatc-3', 5'-ttgtctgctcccacaatgaaa-3'. qPCR results were tested for significance using a two-tailed Student's t-test. WCRC0025 is a cell line that we recently generated from a pleural effusion from a patient with ER+ ILC (unpublished), using the "Schlegel" method²¹¹. BCK4 and IPH926 lines were kindly provided by Drs. Jacobson²¹² and Lehman²¹³.

3.2.3 Cell culture reagents

MCF7 and MDA-MB-134 (American Type Culture Collection [ATCC]) were cultured in DMEM (11965; Life Technologies) +10%FBS (26140; Life Technologies) and DMEM+L15 (1:1) + 10% FBS, respectively. SUM44PE (Asterand Bioscience) were maintained as described previously²¹⁴. LTED cells were generated by maintaining cells in hormone-deprived conditions

using IMEM + 10 % CSS^{88,210}. Cell lines were routinely tested to be mycoplasma free, authenticated by the University of Arizona Genetics Core by Short Tandem Repeat DNA profiling and kept in continuous culture for <6 months. Blu9931 (S7819, Selleck Chemicals), was dissolved in DMSO at 5mM concentration, ICI-182,780 (Tocris Bioscience) in ethanol at 10mM concentration, and Doxycycline (Sigma-Aldrich) in water.

3.2.4 Antibodies

For IB, FGFR4 antibodies sc-124 (Santa Cruz) and CST-8562 (Cell Signaling) were used at a 1:1000 dilution, and beta-actin (Sigma) at 1:10,000. Blots were imaged on the Olympus LI-COR system. For IHC, FGFR4 antibody MABD120 (EMD Millipore) was used at a 1:250 dilution after antigen retrieval using heated citrate buffer, pH 6.0. Staining was detected using Envision Dual Link+ HRP Polymer and DAB (Dako). For differentiation of human versus mouse cells, MAB1273 (anti-mitochondria antibody, MAB1273, Millipore), was used at a 1:100.

3.2.5 Proliferation Assays

1,000 MCF7L parental and LTED cells (Schiff Lab²¹⁰) and 5,000 MDA-MB-134 parental and LTED cells were plated to each well of a 96-well plate using 5 replicates per experiment. The next day, Blu9931 was added, and the cells were allowed to grow for 8 days. 2,500 Sum44PE cells were plated to each well of a 96-well plate using 6 replicates per experiment. The next day, Blu9931 and ICI were added, and the cells were allowed to grow for 14 days. Quantification of cell number was performed using the FluoReporter™ Blue Fluorometric dsDNA Quantitation Kit (Thermo Scientific). Statistical significance for parental vs. LTED sensitivity was calculated

using a two-way ANOVA in graphpad prism v.7.0. Synergy was calculated using the zero interaction potency (ZIP) model via the SynergyFinder web application²¹⁵.

3.2.6 Colony Formation Assays

15,000 Sum44PE cells were plated to each well of a 6-well plate. The following day, Blu9931, ICI, or doxycycline were added. Media was removed and replaced with new media + drug every 4-6 days for 18-24 days, at which point the cells were stained with crystal violet solution (0.5% crystal violet in 25% methanol). Quantification of well surface area taken by cells was performed with an SZX16 microscope with cellSens Dimension (Olympus) software. Statistical significance versus vehicle treated cells were tested using a one-way ANOVA, followed by Dunnett's post-hoc test, with significance set at $p < .05$. Quantification of the Blu9931 and H3B-6527 comparison experiment was performed by dissolving the crystal violet in 10% acetic acid and measuring absorbance at 560nm.

3.2.7 *In vivo* tumor growth

Julie Scott and Sreeja Sreekumar performed injections of NOD/SCID mice. Five million Sum44 LTED A cells were collected in 50ul of 1:1 matrigel: serum free IMEM and added to individual insulin syringes (Needle Lo-Dose™ Micro-Fine™ 1/2 mL 28 Gauge 1/2 Inch, Becton Dickinson product # 329461). Injections were performed to the 4th inguinal mammary fat pad, bilaterally. A total of 24 mice (48 tumors) were injected, with 12 received cells with doxycycline-inducible shRNA targeting Renilla, and the other half, shRNA targeting FGFR4 (shRNA 1). For each shRNA group, half of the mice were fed a control diet (2014 base diet, Envigo), and the other

half, a doxycycline-supplemented diet (TD.00426, 625mg doxycycline/kg, Envigo). At 22 weeks after injection, mice were sacrificed, and primary tumors were weighed.

3.2.8 Dox-inducible shRNA

Sum44PE cells were first infected with an rtTA construct (a gift from Scott Lowe, PhD; Addgene#18782; modified by Dr. Lowe's laboratory), followed by infection with LT3GEPIR²¹⁶ constructs containing the following sequences within the miR-E backbone targeting: 1) Renilla luciferase control (non-silencing): CAGGAATTATAATGCTTATCTA, 2) FGFR4-1: ACGTCAAGATGCTCAAAGACAA, 3) FGFR4-2: ACATTGACTACTATAAGAAATA. Nilgun Tasdemir helped to design the shRNA sequences using a tool generated by the Lowe Lab.

3.2.9 RPPA and phospho-kinase array

RPPA analysis was performed at MD Anderson. Samples were prepared according to their standard protocol, collected 48 hours after dox treatment. A total of 305 phospho and total protein levels were quantified and normalized using a log₂ calculation of the median-centered data. A Human Phospho-Kinase Antibody Array Kit (R&D Systems) was used to profile 43 phosphorylated and 2 total proteins. The kit was used according to the manufacturer's recommendations, with protein collected at 7 days post dox treatment. The cell lines were serum starved for 48 hours and treated with a 10-minute induction of + 0.5X Sum44 media + 10ng/ml FGF19.

3.2.10 FGFR4 overexpression

FGFR4 wild-type (WT) and mutant (N535K, V550M) constructs were generated by performing site-directed mutagenesis on pENTR221-FGFR4. The plasmid pENTR221 with an FGFR4 mutation, P712T, was a gift from John Brognard (Addgene plasmid # 60531)²¹⁷. Mutagenesis was performed using the QuikChange II system (Agilent) according to the manufacturer's recommendations. Plasmids underwent Sanger Sequencing to confirm full sequence integrity (Genewiz), followed by Gateway cloning to pINDUCER20, a doxycycline-inducible overexpression plasmid. The plasmid pINDUCER20 was a gift from Stephen Elledge (Addgene plasmid # 44012)²¹⁸.

3.3 RESULTS

3.3.1 Cell line expression of FGFR4

When controlled for PAM50 status (luminal A), ILC tumors display a higher FGFR4 expression than IDC tumors (Figure 15). This increase in FGFR4 expression in ILC is also true in cell lines, with Sum44PE (Sum44) and MDA-MB-330 (MM330) having particularly high FGFR4 expression (Figure 16). As with the LTED cell lines shown in Figure 5B, endogenous FGFR4 RNA and protein levels are well correlated.

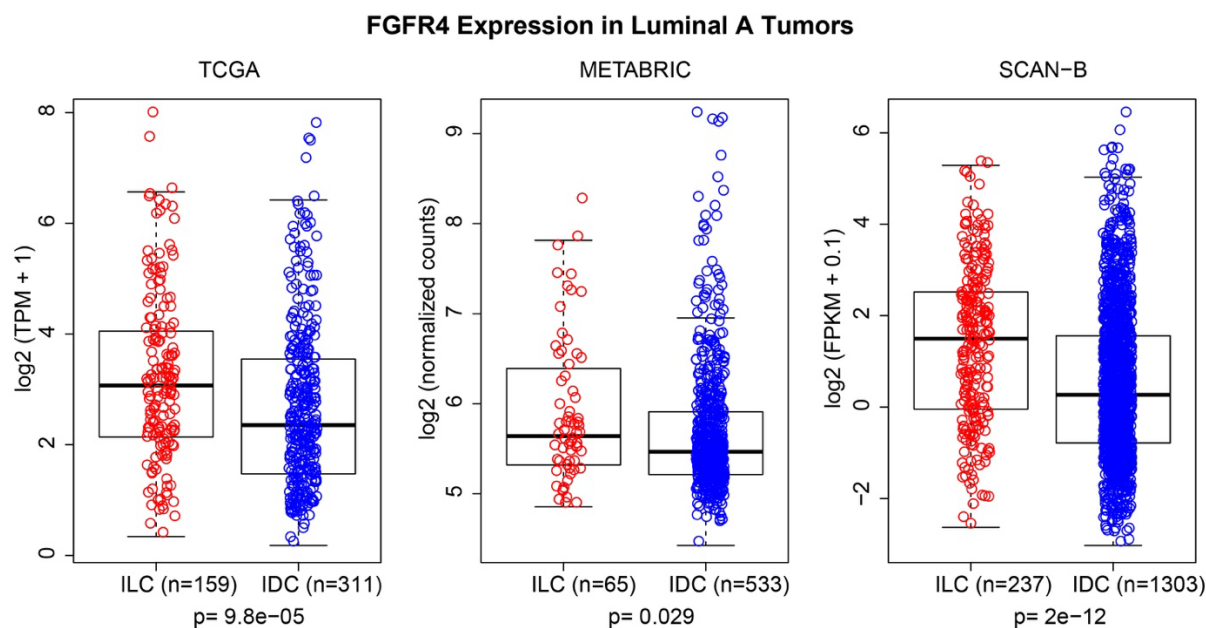


Figure 15. Primary tumor FGFR4 expression by histology

FGFR4 RNA expression of luminal A ILC and IDC tumors in TCGA, METABRIC, and SCAN-B datasets. FGFR4 expression is significantly higher in ILC than IDC tumors ($p=9.8e-5$, $p=.029$, $p=2e-12$). p-values are from Mann-Whitney U tests.

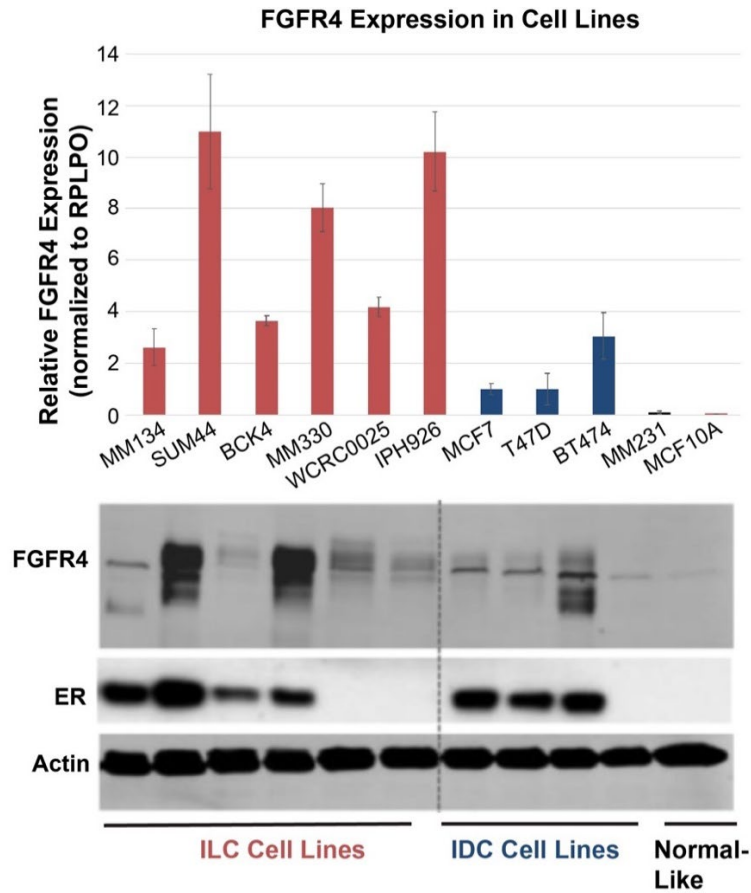


Figure 16. FGFR4 expression in cell lines

FGFR4 RNA and protein expression in a panel of commonly used ILC (red) and IDC (blue) cell lines, as well as the normal-like MCF10A cell line.

Figure generated in collaboration with Tian Du and Jian Chen.

3.3.2 FGFR4 inhibition in LTED cell lines

To begin testing the effect of FGFR4 inhibition *in vitro*, we treated the Oesterreich Lab MM134 LTED cell lines and the Schiff Lab MCF7 LTED cell line (Figures 5 and 17) with the FGFR4-specific inhibitor Blu9931. Over 9 days, FGFR4 inhibition significantly decreased LTED cell growth relative to parentals. However, the magnitude of effect varied over a large range, with the MM134 LTED B cells showing the lowest decrease in growth, even though those cells have a relatively large amount of FGFR4 expression. To learn more about the time-course effect of

FGFR4 inhibition on cell growth, we next treated Sum44 LTED cells with doxycycline-inducible shRNA, targeted to Renilla (negative control) or FGFR4. As quantified from incucyte live-cell imaging, FGFR4 knockdown decreases LTED cell growth over 2 weeks, with initial separation of curves beginning around day 9 (Figure 18).

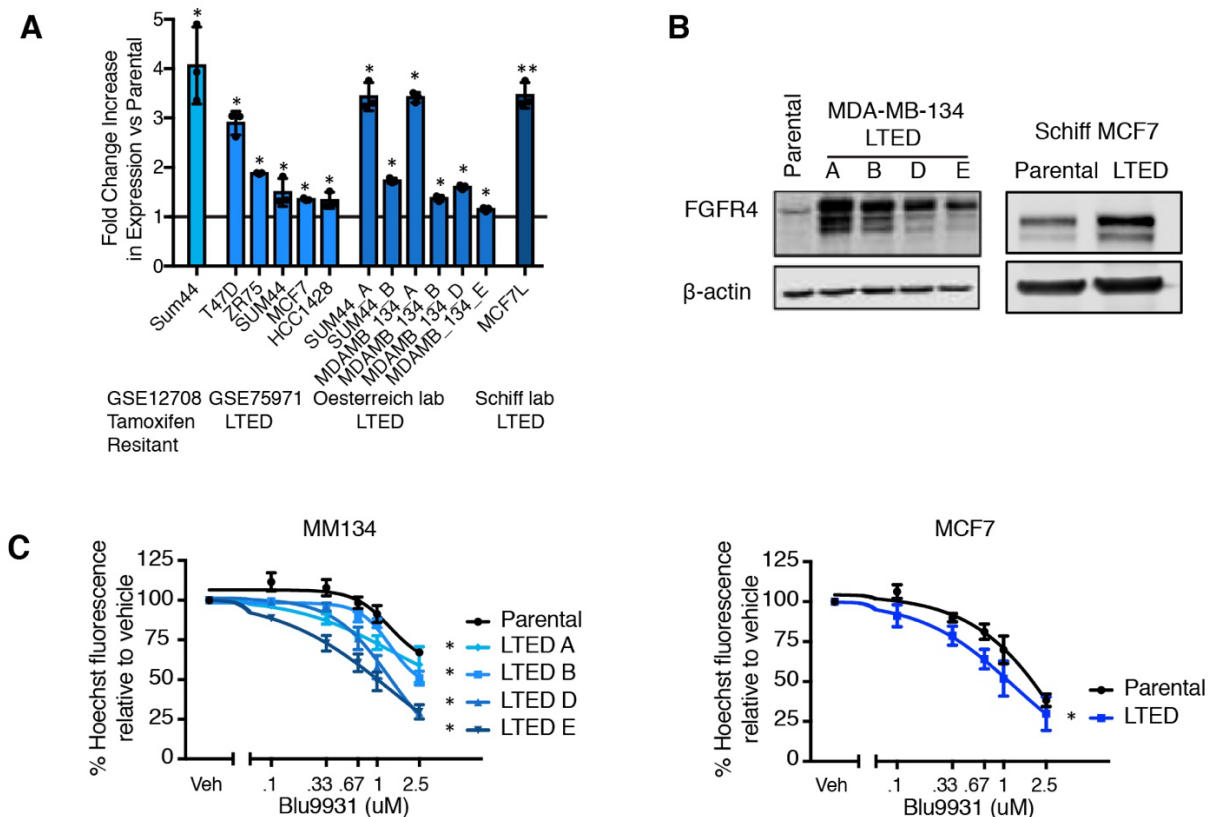


Figure 17. LTED cell line sensitivity to Blu9931

A) Schiff Lab MCF7L LTED cell line FGFR4 RNA expression relative to parental cells. (The other LTED cell lines with available FGFR4 expression are shown for comparison, duplicated from Figure 5). * $p < .05$ for differential expression vs parental, corrected for multiple comparisons with Benjamini-Hochberg, ** $p < .005$ for differential expression via qPCR for (t-test, $t = 15.52$, $df = 4$). Error bars represent $\pm$ SD from 3 replicates.

B) Schiff Lab MCF7L parental and LTED FGFR4 protein expression. (The MM134 LTED cells are shown for comparison and as a reference for figure C).

C) Proliferation studies of MDA-MB-134 and Schiff MCF7 parental and LTED cells, collected 8 days after drug treatment. Error bars represent $\pm$ SEM, $n = 4$ for all cell lines except MDA-MB-134 LTED A ($n = 2$) and LTED D ($n = 3$). * $p < .005$ for cell line effect in two-way ANOVA.

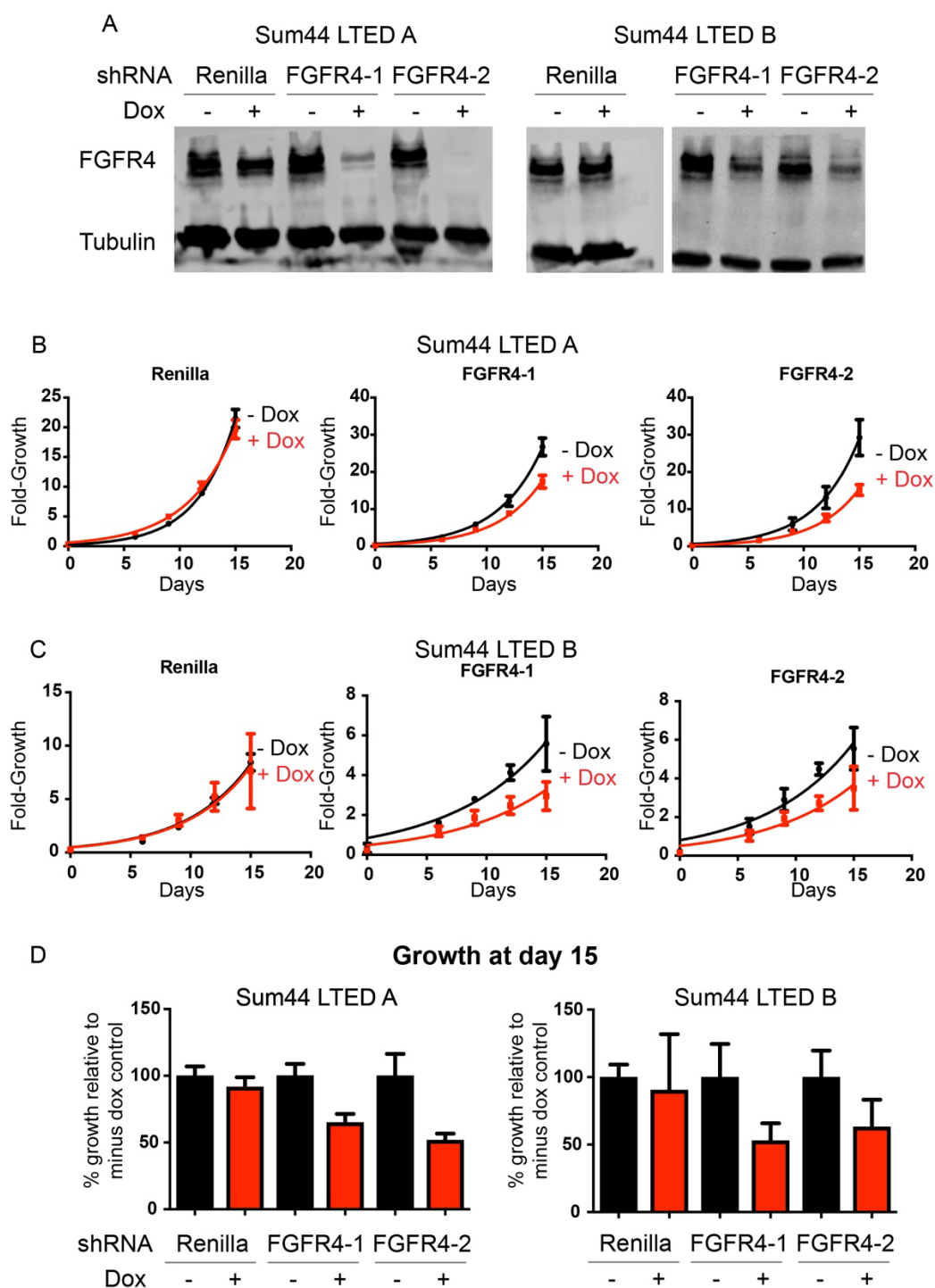


Figure 18. FGFR4 knockdown decreases Sum44 LTED growth at 2 weeks

A) Immunoblot of FGFR4 knockdown with 0 or 750ng/ml dox. Protein collected at day 11 after dox induction. Experiment performed in collaboration with Tianmeng Chen.

B,C) Percent confluency of cell lines $\pm$ 750ng/ml dox, as measured by incuCyte. 2000 cells were plated to 96-well plates in triplicate. Images were taken at days 6, 9, 12, 15. Data represents mean $\pm$ from 1 independent experiment.

D) Percent confluency of cell lines at day 15 from figures B and C.

3.3.3 Synergy of Blu9931 and fulvestrant

The LTED cell lines model a form of acquired endocrine resistance. To test if FGFR4 may also have an effect on cells that maintain estrogen signaling, we next treated the ER+ ILC cell line Sum44PE. Blu9931 has an IC₅₀ of ~500nM on these cells and works synergistically with fulvestrant treatment to block cell growth (Figure 19).

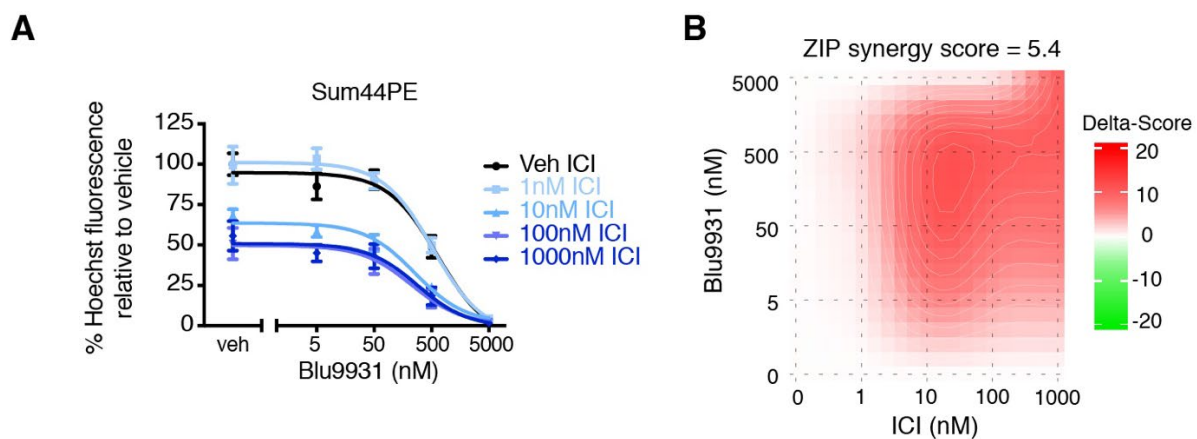


Figure 19. Sum44PE sensitivity to Blu9931 and fulvestrant

A) Proliferation study of Sum44PE cells treated with Blu9931 and fulvestrant (ICI-182,780, ICI), collected 14 days after drug treatment. Error bars represent $\pm$ SD from 6 replicates of one representative experiment.

B) Heatmap showing ZIP synergy scores for experiment on left. Average ZIP synergy score for n=3 independent experiments = 7.36 ± 2.45 (mean $\pm$ SEM).

3.3.4 FGFR4 inhibition decreases colony formation

Given the modest results of FGFR4 inhibition as monotherapy over a short time course, we next tested the effect of FGFR4 inhibition on colony formation. This assay introduces two additional variables to the shorter proliferation experiments: 1) increased time of exposure to FGFR4 inhibition, and 2) decreased cell confluency at the start of the experiment. Both of these variables

may play a role in explaining the striking decrease in colony formation seen in Sum44PE cells, with either Blu9931 treatment or shRNA-mediated inhibition (Figure 20). A very similar effect on colony formation was seen with a second FGFR4-specific inhibitor, H3B-6527 (Figure 21).

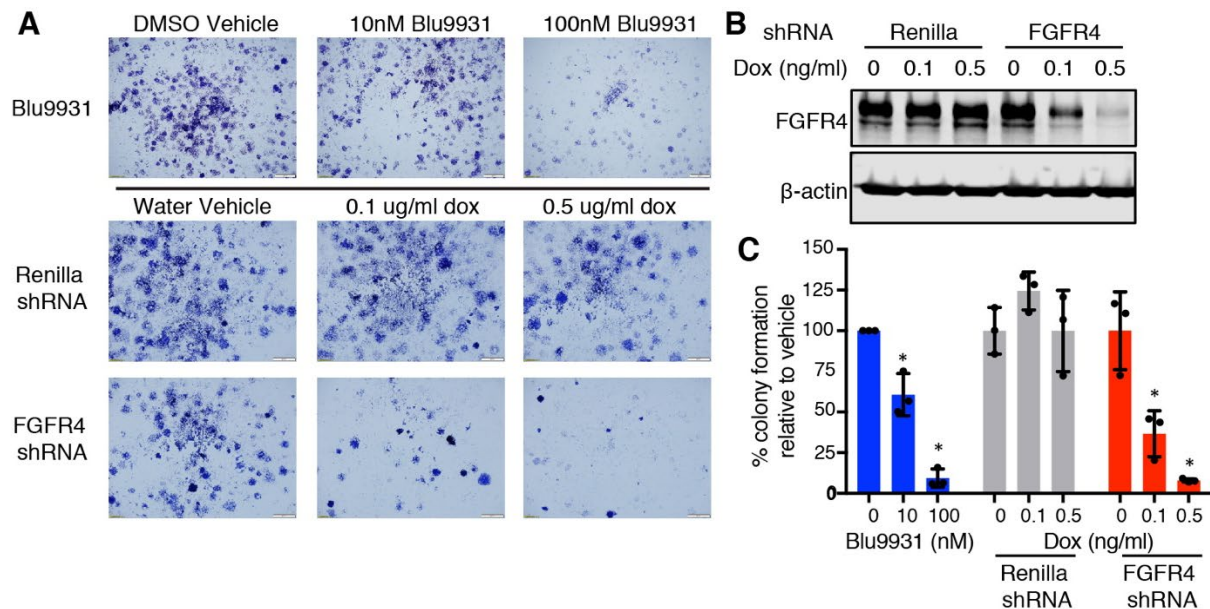


Figure 20. FGFR4 inhibition decreases colony formation ability of Sum44PE cells

A) Representative images of Sum44PE cells stained with crystal violet after 3 weeks of growth in 2D setting.
 B) Immunoblot of FGFR4 protein expression after 48 hours of doxycycline treatment.
 C) Quantification of crystal violet staining. Error bars $\pm$ SEM, $n=3$ for Blu9931 and $\pm$ SD for 3 replicates of $n=1$ independent experiment for shRNA. * $p<.01$ for one-way ANOVA ($df=8$) and Dunnett's test, comparing treatment groups versus vehicle: Blu9931: $F=550.6$, Renilla: $F=1.9$, FGFR4: $F=25.7$)

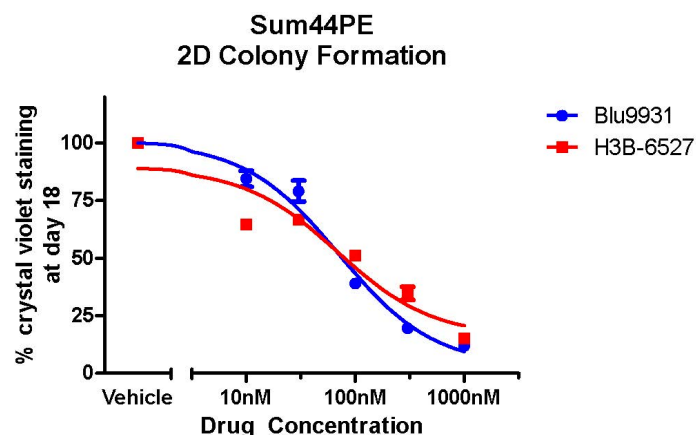


Figure 21. Comparison of two FGFR4 inhibitors on Sum44PE colony formation

Blu9931 and H3B-6527 were used to treat Sum44PE cells in 18-day colony formation experiments. Blu9931 (blue line) had an IC_{50} of 70.7nM and H3B-6527 (red line) had an IC_{50} of 69.3 nM. Error bars represent $\pm$ SEM for $n=2$ independent experiments.

We next tested colony formation in the Sum44 LTED A cell line. With a similar effect size to the 15-day growth experiment, and to 10nM Blu9931 treatment, FGFR4 shRNA decreased colony formation of these cells (Figure 22). FGF401 (Novartis) treatment had a much larger effect on decreasing colony formation at the 10nM concentration, with almost no added benefit to increasing concentration to 1uM. FGF401 binds to the same Cys552 residue as Blu9931 but acts in a reversible manner. The different binding behavior or drug stability may explain the lack of dose-response of FGF401.

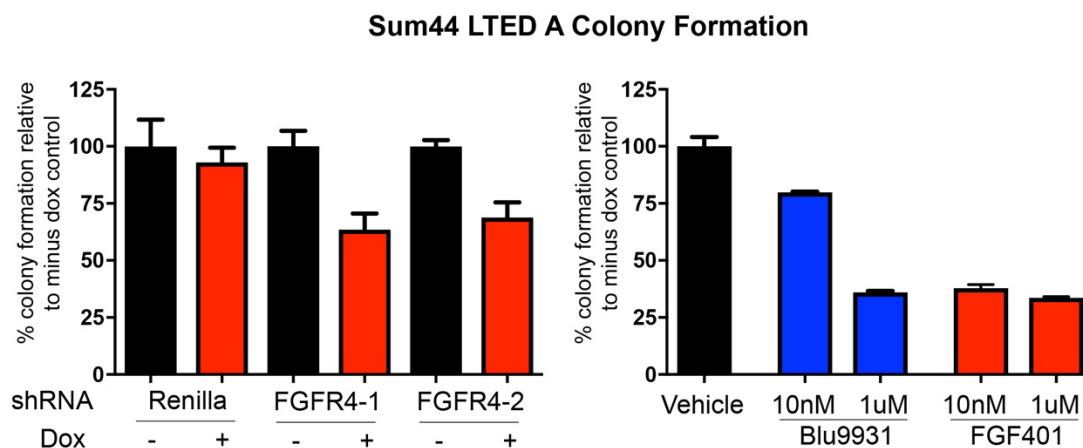


Figure 22. FGFR4 inhibition decreases colony formation ability of Sum44 LTED A cells

Percent colony formation relative to minus dox control or vehicle (DMSO) treatment control. 15,000 Sum44 LTED A cells were plates, and quantification of colonies at 3 weeks was performed using an SZX16 microscope with cellSens Dimension (Olympus) software. Error bars represent $\pm$ SD for 3 biological replicates of doxycycline (1ug/ml) treatment and 2 biological replicates of small molecule treatment.

3.3.5 FGFR4 shRNA has no effect on *in vivo* tumor growth

With the help of Julie Scott and Sreeja Sreekumar, NOD/SCID mice were injected with 5 million Sum44 LTED A cells to the 4th inguinal mammary fat pad, bilaterally. A total of 24 mice (48 tumors) were injected, to study the effect FGFR4 knockdown on *in vivo* tumor growth. Half of the mice received cells with doxycycline-inducible shRNA targeting Renilla, and the other half,

shRNA targeting FGFR4. For each shRNA group, half of the mice were fed a control diet, and the other half, a doxycycline-supplemented diet (625mg/kg, Envigo). At 22 weeks after injection, mice were sacrificed, and primary tumors were weighed. Figure 23A shows no difference in final tumor weight. To test if the doxycycline diet led to shRNA production and subsequent loss of FGFR4 expression, we performed IHC on FFPE sections of primary tumors. Figure 23B shows example staining images, with equivalent FGFR4 expression in the presence or absence of doxycycline. It is possible that the concentration of doxycycline was not sufficient to drive shRNA expression *in vivo*, or that cells with low shRNA expression selectively grew over the 22-week span. To our surprise, some mice appeared to develop lung metastases. Figure 23C shows, by IHC, that lung tissue from one mouse does in fact contain a human-cell derived metastasis. Ongoing work in the Oesterreich lab by Nilgun Tasdemir, shows that ILC cell lines can in fact metastasize *in vivo*, setting the stage for future studies on FGFR4 inhibition in the setting of primary tumor growth and metastasis formation.

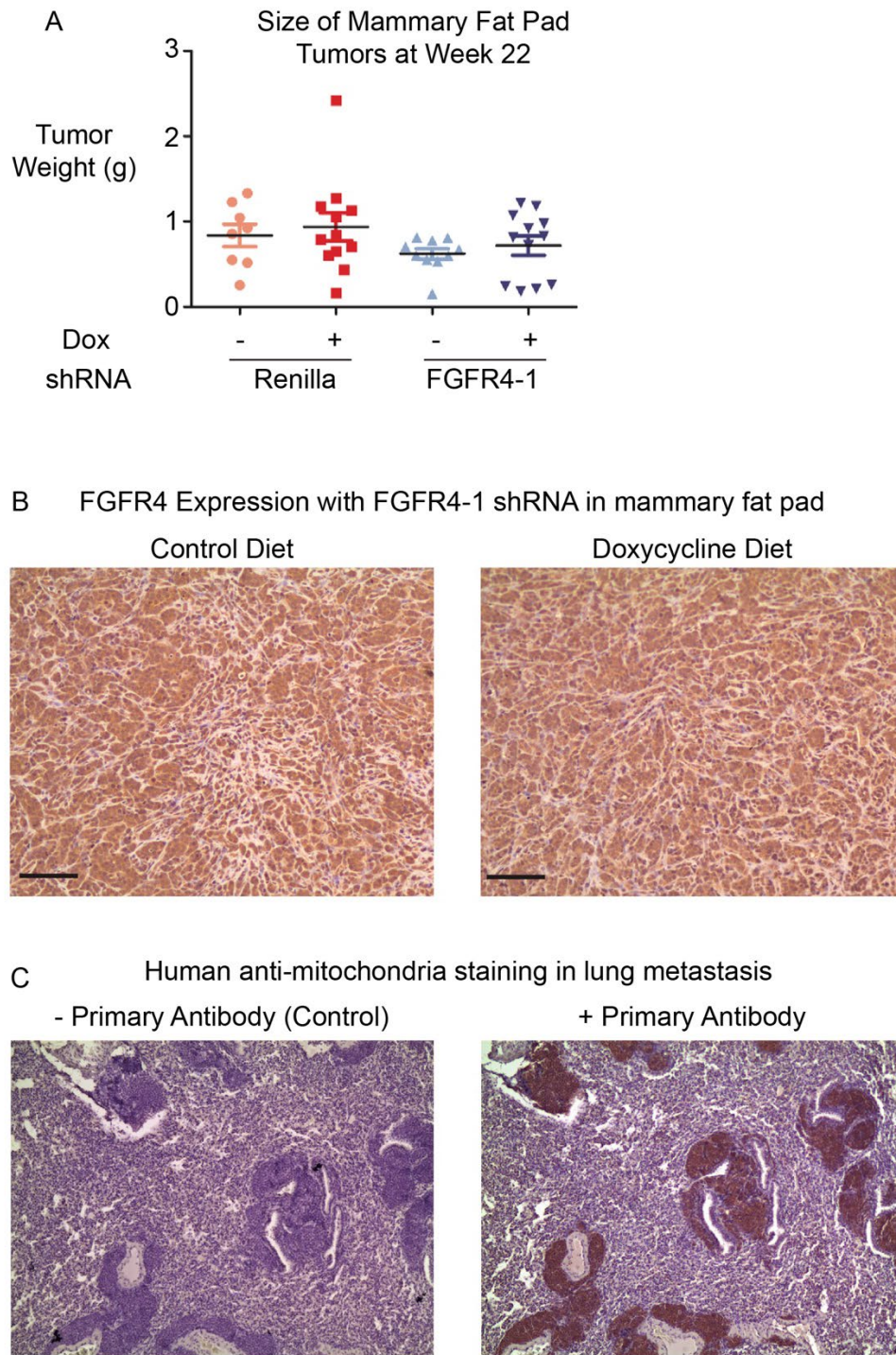


Figure 23. Doxycycline diet does not affect *in vivo* tumor growth

A) Size of primary tumors at week 22 after 5 million cells were injected to the 4th inguinal mammary fat pad.

B) IHC staining of FGFR4 (Millipore, MABD120, 1:250) shows no difference in FGFR4 expression with or without doxycycline diet to induce shRNA. Left: mouse 3R, left mammary fat pad. Right: mouse 4L, left mammary fat pad. Scale bar = 200nm.

C) IHC of putative lung metastasis shows positive staining for human cells (anti-mitochondria antibody, MAB1273, 1:100) in mouse 3N. Left: no primary antibody (negative control), Right: plus primary antibody.

3.3.6 FGFR4 inhibition does not alter Sum44PE cell response to FGF1

We began signaling studies using the Sum44PE cell line, since FGFR4 inhibition via shRNA or small molecules had a large phenotypic effect (Figures 20-21). We decreased FGFR4 expression via doxycycline treatment for 6 days, followed by one day of serum starvation, and 10 minutes of FGF1 treatment (10ng/ml). Although FGFR4 expression was nicely decreased in this study, FGFR4 knockdown had no effect on phosphorylation of FRS2, PLC γ , or MAPK with FGF1 stimulation (Figure 24). While FGF1 is able to increase downstream FGFR signaling in Sum44 cells, this ligand does not act through FGFR4 in this context.

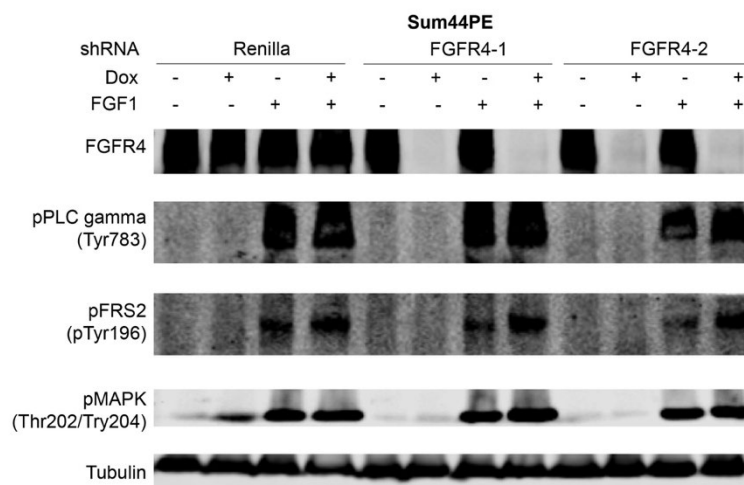


Figure 24. FGFR4 knockdown does not alter FGF1 activation of FRS2, PLC γ , or MAPK

Immunoblots of FGFR4 (CST), pPLC γ , pFRS2, and pMAPK following 10 minutes of FGF1 (10ng/ml) stimulation. Sum44PE cells were treated with 500ng/ml doxycycline for 6 days, followed by a 24-hour serum starvation period.

3.3.7 FGFR4 inhibition has modest effects on estrogen signaling and cell cycle protein phosphorylation

With the positive phenotypic results, especially in the context of combination endocrine therapy in the form of fulvestrant or estrogen deprivation, we next tested if FGFR4 inhibition had any effect on estrogen signaling. We treated MM134 cells with FGFR4 siRNA and measured ILC-specific estrogen targets with a custom Nanostring array. Figure 25 shows that the effects on estrogen signaling were modest. Overall, 6/13 estrogen-regulated genes were significantly altered with FGFR4 siRNA treatment, with 5 showing that FGFR4 signaling decreases estrogenic signaling. This result corresponds well with our clinical data, in which FGFR4 and ER signaling seem to be opposing forces. In our paired, endocrine-treated tumors, the most significant pathways associated with an increase in FGFR4 RNA expression are pathways suggesting decreases in estrogen activity. Thus far, it is not clear if the relationship with FGFR4 and ER is bidirectional, nor is it clear if the relationship is due to direct interaction.

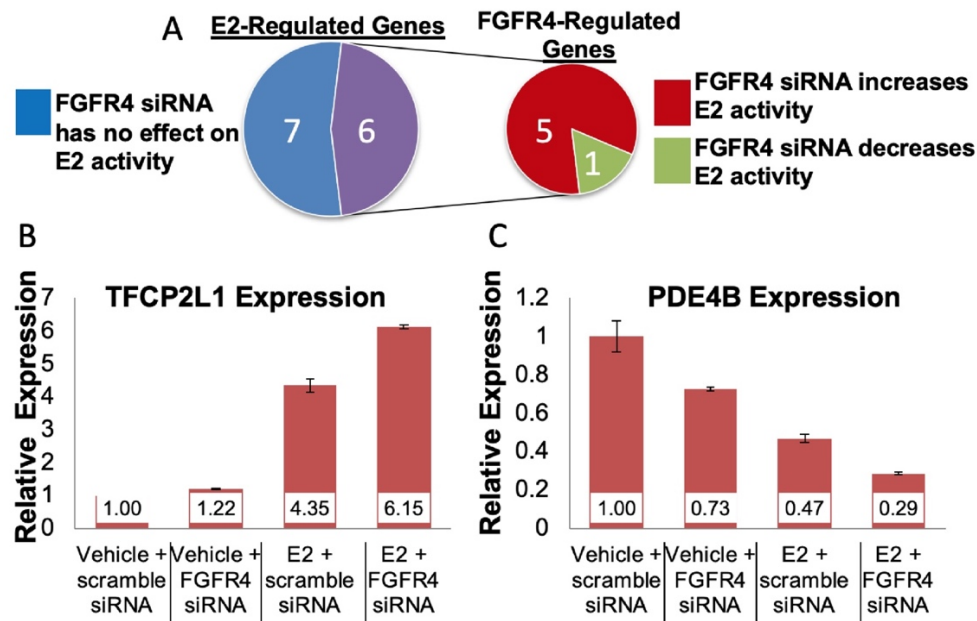


Figure 25. FGFR4 knockdown has modest effects on increasing estrogen-induced signaling

A) 13 genes were defined to be E2 regulated in MM134 cells using a custom nanostring panel (defined as adjusted $p < .01$ and fold change ≥ 2). Expression of 6 (46%, purple pie slice) of these genes is significantly altered by FGFR4 inhibition (adjusted $p < .05$), including 5 that suggest an increase in ER-effect (red pie slice).

B) TFCP2L1 is representative of an E2-activated gene, whose expression is further increased with FGFR4 siRNA.

C) PDE4B expression is representative of an E2-repressed gene, whose expression is further decreased with FGFR4 siRNA. Expression across the four treatment groups for both genes are significantly different from all other groups ($p < .005$, two-way anova followed by Tukey's HSD test). Pie charts showing number of ILC-E2 genes altered by FGFR4 siRNA treatment for 48 hours.

Figure generated in collaboration with Jian Chen.

For a more unbiased assessment of the signaling effect of FGFR4 inhibition, we performed an RPPA analysis and phospho-protein array analysis on shRNA-treated cells (Figure 26). There was not much significant change with FGFR4 shRNA, but the largest magnitude effects included pWEE1 (S642) and pCHK2 (T68) decreases (Figure 27).

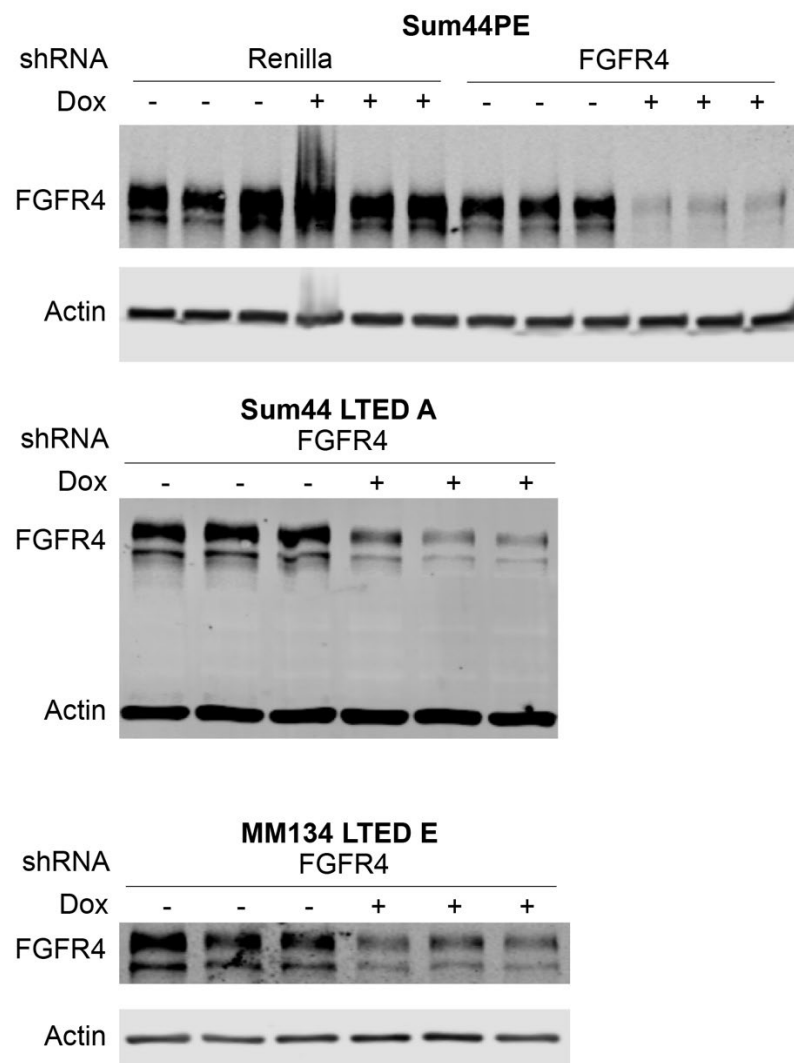


Figure 26. FGFR4 expression in samples sent for RPPA analysis

Top, FGFR4 expression in Sum44PE cells. Middle, FGFR4 expression in Sum44 LTED A cells. Bottom, FGFR4 expression in MM134 LTED E cells. Protein was collected 48 hours after dox treatment (1ug/ml). FGFR4 (Cell Signaling) antibody was used at 1:1000 and actin at 1:10,000.

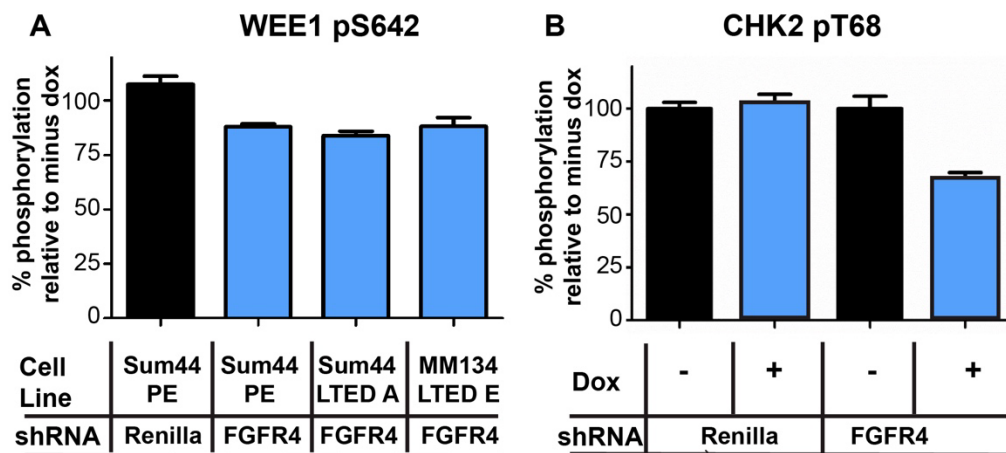


Figure 27. FGFR4 shRNA decreases pWEE1 and pCHK1

A) Percent WEE1 pS462 levels relative to minus dox controls. Samples were treated with dox for 48 hours. Black bar: Renilla shRNA. Blue bars: FGFR4 shRNA.

B) Percent CHK2 pT68 levels in Sum44PE cells relative to minus dox control. Samples were treated with dox for a total of 7 days. During the last 48 hours, the cells were serum starved, followed by a 10-minute induction of 0.5X Sum44 media and 10ng/ml FGF19.

Error bars represent $\pm$ SD for 3 biological replicates

3.3.8 FGFR4 overexpression is not sufficient to drive breast cancer phenotypes

Next, we tested if FGFR4 overexpression is sufficient to drive breast cancer growth and resistance to endocrine therapy. We developed doxycycline-inducible FGFR4 overexpression cell line models of MM134 and MCF7 wild-type (WT), V550M, and N535K mutations using the pInducer system (Figure 28).

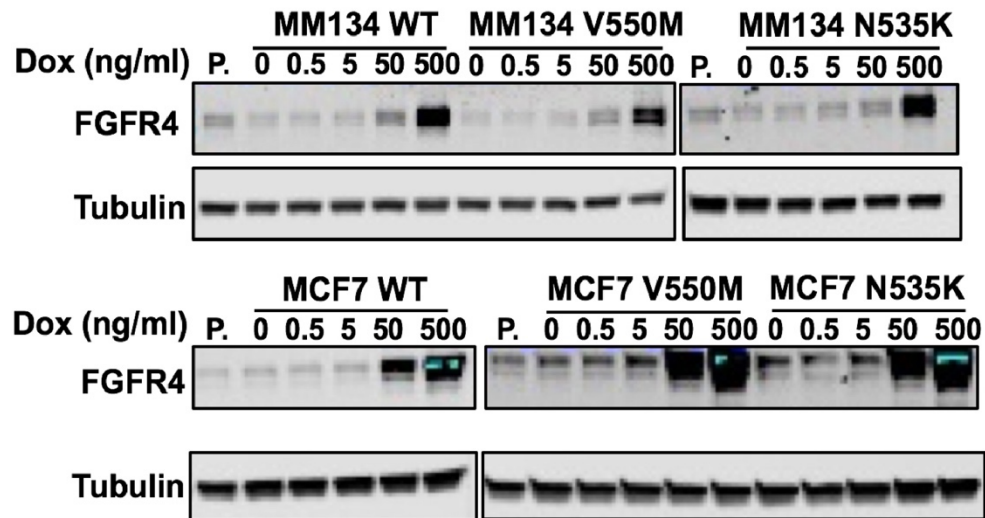


Figure 28. Doxycycline-inducible FGFR4 overexpression cell line models

Immunoblot images of dox-inducible MM134 and MCF7 wild-type (WT), V550M, and N535K FGFR4. Protein was collected 3 days after dox-induction in full serum. Figure generated in collaboration with Kai Ding.

Overexpression of FGFR4 was not sufficient to allow increased cell survival with short-term fulvestrant exposure (Figure 29), nor was it sufficient to drive cell growth following estrogen deprivation over the span of one month (Figure 30). Lastly, FGFR4 overexpression had no effect on short-term chemotaxis or migration ability of cells.

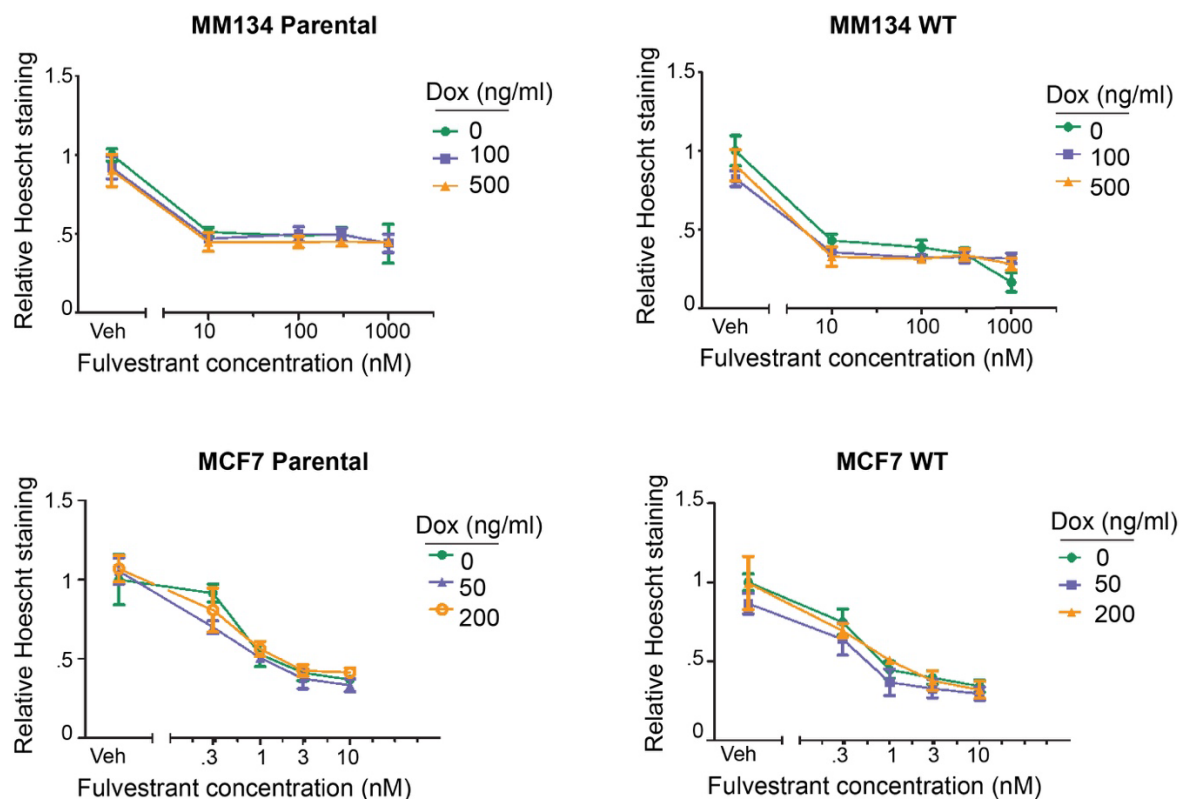


Figure 29. FGFR4 overexpression does not increase cell growth with short-term fulvestrant exposure
 Top, MM134 parental and FGFR4 wild-type (WT) cell models treated with 0, 100, 500 ng/ml doxycycline to induce FGFR4 expression. Bottom, MCF7 parental and FGFR4 wild-type (WT) cell models treated with 0, 50, 200 ng/ml doxycycline to induce FGFR4 expression. Cells were seeded to 96-well plates and cell number measured at day 9 with FlouReporter Hoescht staining. Error bars represent $\pm$ SD for 6 biological replicates
 Figure generated in collaboration with Kai Ding.

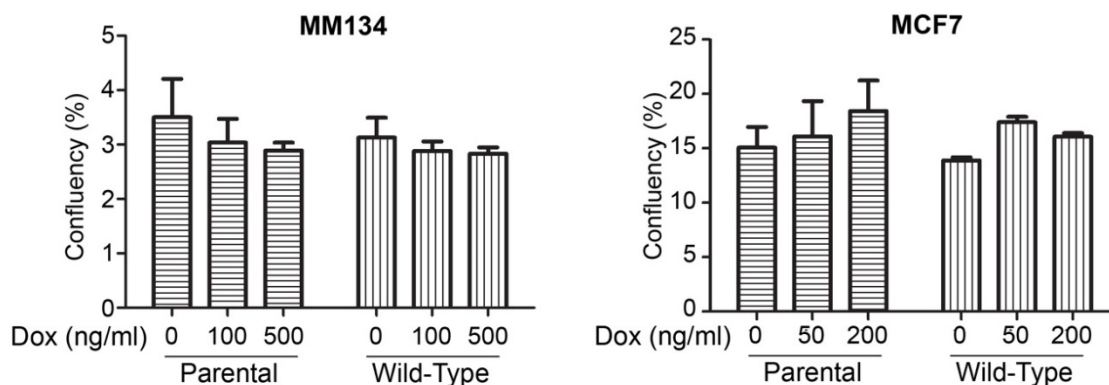


Figure 30. FGFR4 overexpression does not cell growth with long-term estrogen deprivation
 Left, MM134 parental and FGFR4 wild-type (WT) cell models treated with 0, 100, 500 ng/ml doxycycline to induce FGFR4 expression. Right, MCF7 parental and FGFR4 wild-type (WT) cell models treated with 0, 50, 200 ng/ml doxycycline to induce FGFR4 expression. Cells were seeded to 12-well plates and well confluency measured at 1month with incucyte live-cell imaging. Error bars represent $\pm$ SD for 6 biological replicates
 Figure generated in collaboration with Kai Ding.

3.4 DISCUSSION

Treating LTED cell lines with an FGFR4-specific inhibitor significantly decreased *in vitro* cell growth relative to parental cell lines. Surprisingly, the level of FGFR4 expression across the MM134 LTEDs did not correlate well with outcomes, in contrast to previously published data on the level of FGFR1 expression predicting response to FGFR inhibition¹⁵⁸. It is possible that additional FGF ligand treatment would lead to enhanced FGFR signaling, in a manner proportional to the level of FGFR4 expression, thus leading to a better correlation of receptor expression and sensitivity to inhibition. These ligand experiments, particularly with FGF19, may be key to better understanding the downstream signaling effects of FGFR4 activation^{219,220}. To date, we have not seen changes in pFRS2 in our cell lines with FGFR4 manipulation, nor with treatment of FGF19. It may be that a co-receptor is required to activate FGF19-induced signaling in breast cancer cells or that canonical pFRS2 signaling is not activated in ILC. In the absence of the canonical changes, there was minimal effect of FGFR4 inhibition on the cell cycle regulators WEE1 and CHK2. A more detailed analysis of cell cycle regulation, by further protein analysis, as well as flow-cytometry based studies should be performed.

There does seem to be a significant disconnect for the sensitivity of FGFR4 inhibition in Sum44PE cells when treated in a short-term growth assay versus a colony formation assay. The increased amount of time with drug exposure does not likely account for this entire difference. One possible explanation is an increased reliance on FGFR4 in the setting of low confluency because of an increased dependency on autocrine signaling in stem-cell like populations that require FGFR4²²¹. Further growth assays and further signaling experiments performed at low confluency, but at shorter time points, should help tease out this mechanism.

The lack of function or signaling effects from FGFR4 overexpression suggests that additional co-receptors or ligands are needed for full activity. There are several promising membranous proteins to study, based on previous research and our data. First, HER2 overexpression is known to coincide with FGFR4 expression in primary breast tumors, and in our collection of paired breast and brain metastases, FGFR4 and HER2 outlier gains frequently co-occurred²²². Next, N-Cadherin has been shown to potentiate FGFR4 signaling in breast²²³, pituitary²²⁴, and lung cancers²²⁵, and N-cadherin is upregulated in lobular breast cancer relative to ductal because of the loss of E-cadherin expression. NCAM1 is another potential option, since N-cadherin is able to form a complex with FGFR4 and NCAM (NCAM1) in pituitary tumors, FGFRs and NCAM interact in ovarian cancer, and NCAM1 is frequently overexpressed with FGFR4 in our paired, endocrine-treated breast recurrences^{226,227}. Lastly, ephrin receptors (EPHA5 and EPHA4) were shown in Chapter 2 to be often gained together with FGFR4 in endocrine-treated distant metastases or previously shown to increase FGFR signaling.

4.0 CONCLUSIONS AND FUTURE DIRECTIONS

Invasive lobular carcinoma (ILC) is a common histological subtype of breast cancer with limited treatment options in the setting of endocrine resistance. To model acquired resistance *in vitro*, we made several long-term estrogen deprived (LTED) ILC cell lines. Overlapping our RNA-Seq data with previously published transcriptomic studies led to the identification of FGFR4 as a top druggable target. Subsequent unbiased analysis of paired primary and recurrent tumors echoed this result, with FGFR4 overexpression again being a top candidate for driving acquired resistance, particularly true of ILCs. Cell line expression data confirmed that FGFR4 is often highly expressed in ILC cell lines at the RNA and protein level. Analysis of a large cohort of ER+ primary ILC tumors showed that elevated FGFR4 expression may also predict *de novo* resistance to endocrine therapy. Additionally, we report that FGFR4 hotspot mutations are uniquely enriched in metastatic ILC, suggesting a multimodal selection of FGFR4 activation in advanced ILC.

Although phenotypic effects of FGFR4 blockade were minimal for short-term growth assays, they were consistently strong in the colony formation assays. These different outcomes may be reflective of a couple of different variables in the colony formation assay, including the increased time of FGFR4 inhibition (3 weeks versus 1-2 weeks) and the lower confluency of cells at time of initial FGFR4 inhibition (~30x less confluent). To rule out time as the major contributor to the different effect-size, a simple experiment could be performed with serial

growth experiments of FGFR4 inhibition at high cell confluency. Following one week of growth, cells $\pm$ FGFR4 inhibition could be counted with trypan blue and replated at an equal density with one more week $\pm$ FGFR4 inhibition. This could be repeated one more time, with an analysis of growth differences of FGFR4 inhibition at 1, 2, and 3 weeks. If there was a significant difference of growth at three weeks versus one week, it may be reflective of FGFR4 inhibition acting in a weak cytostatic manner, in which differences in cell number become more apparent with more cell doublings in the control group. Effects of FGFR4 inhibition on apoptosis and senescence at the high confluency setting could be assessed with IncuCyte caspase-3/7 live imaging and beta-galactosidase based assays.

If these serial growth experiments still resulted in weak effects of FGFR4 inhibition, it would suggest that the low confluency is the main contributor of the striking results of the colony formation assay. Importantly, this low confluency also mimics the setting in which the LTED cell lines were derived - plating cells at low density in CSS media and waiting for colony outgrowth. Together, this suggests that FGFR4 may be playing an important role in autocrine signaling, rather than juxtacrine signaling. If juxtacrine signaling were important for FGFR4, as in an interaction with heparan sulfate proteoglycans (HSPGs) on the cell surface of neighboring cells, the effect of FGFR4 inhibition should be strongest when there are lots of cell-to-cell contacts^{93,106}. To help rule out juxtacrine signaling, conditioned media from highly confluent cells $\pm$ FGFR4 inhibition could be applied to cells at low confluency, with the expected result of no difference in colony formation ability. On the other hand, if conditioned media from sparsely confluent cells $\pm$ FGFR4 inhibition had an effect on cell growth for highly confluent cells, it would suggest that FGFR4 is playing a role in the secretion of factors important for autocrine signaling. This lack of cell contact and sparse growth does mirror the *in vivo* growth of ILC, and

could explain why the expression of FGFR4, and gain of FGFR4 expression in metastasis, is higher in ILC than IDC. One missing factor in the *in vitro* experiments is the extracellular matrix, which may also play an important role in FGFR4 signaling *in vivo*. Additional mouse experiments could add a lot of useful information for the functional impact of FGFR4 inhibition, where low confluency experiments could be performed in the setting of endogenous FGF ligands (there is cross-species reactivity of FGF ligands), extracellular matrix, and HSPGs.

Given the lack of growth effect of overexpression, additional ligands or co-receptors are likely needed to activate FGFR4. As mentioned in the discussion section of chapter 3, selective overexpression of HER2, N-cadherin, NCAM1, and ephrin receptors could be performed along with FGFR4 to determine co-receptor interaction. To determine ligand reactivity, a panel of FGF ligands could be added *in vitro* $\pm$ additional heparin. For a more unbiased approach, a cDNA library could be used to determine ligands and/or co-receptors sufficient for FGFR4 activation. Lastly, if conditioned media was found to activate FGFR4, mass-spectrometry on the media could be used to identify the relevant secreted factors.

If the low-confluency setting is found to potentiate the importance of FGFR4, future signaling experiments should be performed in this setting. For an unbiased approach, RNA-Sequencing or mass-spectrometry studies could be performed on cell lines $\pm$ FGFR4 inhibition. Ideally, these experiments would include both shRNA and small-molecule inhibition, to rule out off-target effects on downstream signaling. Performing these experiments at high confluency in addition to the low confluency setting could add useful information for why FGFR4 is less important at high confluency. Results of the previous experiments will be necessary to assess which ligands are used to activate FGFR4 for the signaling studies. These findings could be used to generate an FGFR4-signature, and a score of FGFR4 activation could be calculated for TCGA,

METABRIC, MET500, and our WCRC patients. Comparing primary and metastatic FGFR4 activation scores would add important rationale for choosing which patients are most likely to benefit from FGFR4 inhibition. Correlation analysis of FGFR4 activation scores and FGFR4 expression could assess whether FGFR4 RNA and/or protein is a useful biomarker.

Another major line of future studies could focus on the interaction of FGFR4 and CDK4/6 pathways. As described in the introduction, treatment regimens including CDK4/6 inhibitors are becoming standard of care for metastatic ER+ breast cancer, and so any future clinical trials of FGFR4 inhibitors would likely be performed alongside CDK4/6 inhibition, or after progression on those agents. To start, synergism of FGFR4 and CDK4/6 could be assessed in short-term growth experiments. Bioinformatic analysis of CDK4/6 treated cell lines could also be performed to determine if FGFR4 activation is predictive of efficacious CDK4/6 inhibition, or if FGFR4 activation increases following CDK4/6 inhibition.

Overall, the data presented collectively support the notion that FGFR4 is an important mediator of endocrine resistance in ILC. If FGFR4-specific inhibitors are well-tolerated in hepatocellular carcinoma, and if there is promising data from the ongoing FGFR trials in ER+ breast cancer, combination FGFR4 and ER-targeted therapy should be considered for future clinical trials of recurrent and metastatic ILC.

APPENDIX

CO-AUTHORED PUBLICATIONS ON BREAST CANCER

A significant portion of my research was focused on learning to apply the aforementioned bioinformatics techniques to study FGFR4 in lobular breast cancer. This process led me to apply these techniques to other aspects of breast cancer biology. A list of my co-authored publications in chronological order is presented below, followed by a summary of my contributions.

- 1) Sikora MJ, Jacobsen BM, **Levine K**, Chen J, Davidson NE, Lee AV, Alexander CM, Oesterreich S. WNT4 mediates estrogen receptor signaling and endocrine resistance in invasive lobular carcinoma cell lines. *Breast Cancer Res.* 2016 Sep 20;18(1):92. PMCID: PMC5028957
- 2) Bahreini A, **Levine K**, Santana-Santos L, Benos PV, Wang P, Andersen C, Oesterreich S, Lee AV. Non-coding single nucleotide variants affecting estrogen receptor binding and activity. *Genome Med.* 2016 Dec 13;8(1):128. PMCID: PMC5154163
- 3) Hopkins BL, Nadler M, Skoko JJ, Bertomeu T, Pelosi A, Mousavi Shafaei P, **Levine K**, Schempf A, Pennarun B, Yang B, Datta D, Oesterreich S, Yang D, Rozzi M, Khosravi-Far R, Neumann CA. A Peroxidase Peroxiredoxin 1-Specific Redox Regulation of the Novel FOXO3 microRNA Target let-7. *Antioxid Redox Signal.* 2017 May 1;28(1):62–77. PMCID: PMC5695745
- 4) Chen L, Vasilatos SN, Qin Y, Katz TA, Cao C, Wu H, Tasdemir N, **Levine KM**, Oesterreich S, Davidson NE, Huang Y. Functional characterization of lysine-specific demethylase 2 (LSD2/KDM1B) in breast cancer progression. *Oncotarget.* 2017 Jul 19;8(47):81737–81753. PMCID: PMC5669845
- 5) Bahreini A, Li Z, Wang P, **Levine KM**, Tasdemir N, Cao L, Weir HM, Puhalla SL, Davidson NE, Stern AM, Chu D, Park BH, Lee AV, Oesterreich S. Mutation site and context dependent effects of ESR1 mutation in genome-edited breast cancer cell models. *Breast Cancer Res.* 2017 May 23;19(1):60. PMCID: PMC5442865

- 6) Li Z, **Levine KM**, Bahreini A, Wang P, Chu D, Park BH, Oesterreich S, Lee AV. Upregulation of IRS1 enhances IGF1 response in Y537S and D538G ESR1 mutant breast cancer cells. *Endocrinology*. 2018 Jan 1;159(1):285–296. PMCID: PMC5761602
- 7) Du T, Zhu L, **Levine KM**, Tasdemir N, Lee AV, Vignali DAA, Houten BV, Tseng GC, Oesterreich S. Invasive lobular and ductal breast carcinoma differ in immune response, protein translation efficiency and metabolism. *Sci Rep*. 2018 May 8;8(1):7205. PMCID: PMC5940770
- 8) Du T, Sikora MJ, **Levine KM**, Tasdemir N, Riggins RB, Wendell SG, Van Houten B, Oesterreich S. Key regulators of lipid metabolism drive endocrine resistance in invasive lobular breast cancer. *Breast Cancer Res*. 2018 Sep 4;20(1):106. PMCID: PMC6124012
- 9) Nagle AM, **Levine KM**, Tasdemir N, Scott JA, Burlbaugh K, Kehm J, Katz TA, Boone DN, Jacobsen BM, Atkinson JM, Oesterreich S, Lee AV. Loss of E-cadherin Enhances IGF1-IGF1R Pathway Activation and Sensitizes Breast Cancers to Anti-IGF1R/InsR Inhibitors. *Clin Cancer Res*. 2018 Jun 25; PMID: 29941485
- 10) Tasdemir N, Bossart EA, Li Z, Zhu L, Sikora MJ, **Levine KM**, Jacobsen BM, Tseng GC, Davidson NE, Oesterreich S. Comprehensive phenotypic characterization of human invasive lobular carcinoma cell lines in 2D and 3D cultures. *Cancer Res*. 2018 Nov 1;78(21):6209–6222. PMID: 30228172

Much of my work focused on analysis of expression data within the TCGA and METABRIC datasets of primary breast cancer, to identify the clinical relevance of *in vitro* findings. My contribution to reference 1 was analysis of WNT4 gene expression by subtype and the effect of WNT4 expression on survival outcomes⁸⁸. This work showed that WNT4 expression is increased in luminal A tumors relative to other PAM50 subtypes, and consequently, is associated with a good prognosis. In reference 3, I showed that let-7c miRNA is expressed at lower levels in tumors versus adjacent-normal tissue, and that let-7c expression is positively correlated with PRDX1 expression and negatively correlated with FOXO3 expression²²⁸. In reference 4, I showed that LSD2 expression is elevated in tumor versus adjacent normal tissue in several tumor types, including breast cancer²²⁹. Additionally, LSD2 expression is increased in basal versus the other PAM50 subtypes. In reference 10, I showed that higher expression of PPFIBP2 or lower expression of PLOD2 are both associated with poor prognosis

in luminal A ILCs²³⁰. These genes were identified from a differential expression analysis of ER+ ILC and IDC cell lines.

A more detailed analysis of TCGA data was presented in reference 2, in which Dr. Amir Bahreini and I assessed the effect of single nucleotide variants (SNVs) in ER binding sites (regSNVs) on target gene expression²³¹. This analysis identified several regSNVs that were associated with altered tumor expression, including rs36208869 in *GSTM1*. This germline alteration results in increased expression of *GSTM1*, and this increased expression was associated with improved survival of patients with ER+ tumors.

A significant portion of my thesis work was devoted to understanding how to analyze RNA-Sequencing data, from processing the initial fastq output, to performing differential expression analysis. In reference 5, I worked alongside Dr. Amir Bahreini to critically evaluate the transcriptomic effects of *ESR1* mutations in genome-edited cell lines genes²³². This work showed the important context dependency of mutation site and genetic background on downstream signaling. In particular, we identified constitutive activation of both classical E2-target genes and novel target genes of *ESR1* mutants. In reference 8, I worked with Tian Du to analyze the RNA-Sequencing data of our ILC LTED cell lines⁸⁷. This study showed that lipid metabolism can drive estrogen-independent ILC cell growth.

The next category of analysis I performed was in assessing IGF activation in tumors and in cell lines. Previously, the lab of Adrian Lee developed an IGF1-signature based on microarray analysis of ligand-stimulated cell lines²³³. From RNA-Sequencing data, I showed that the IGF pathway is constitutively activated in *ESR1*-mutant cell lines²³⁴ (reference 6). In reference 9, I used TCGA RNA-Sequencing and RPPA data to show that IGF pathway activation is increased in ER+ ILCs relative to ER+ IDCs⁸⁹.

Lastly, my work in reference 7 contributed to a detailed analysis of the different transcriptomic landscapes of ILC and IDC²³⁵. This study resulted from a long-term collaboration of several trainees, including Li Zhu of the George Tseng lab, and Tian Du and Nilgun Tasdemir of the Lee-Oesterreich lab. Together, we analyzed TCGA expression data to show that luminal A ILCs are enriched for immune signaling pathways and have lower protein translation and metabolic rates as compared with luminal A IDCs. My contribution included analysis of the GTEX dataset, to show a higher amount of regulatory T-cells, mast cells, and activated dendritic cells in luminal A tumors versus normal adjacent tissue, proving that differences in ILCs and IDCs were not simply an artifact of differences in tumor purity. Additionally, I showed that luminal A ILCs, in comparison to luminal A IDCs, have markedly higher expression of PD-1 and CTLA4, encouraging more studies to assess the efficacy of immune checkpoint inhibitors in ILC. This study in particular is a good representation of the synergy that results from sharing ideas and data with colleagues, in which any one person's individual contributions can be difficult to tease out. I am proud that the Lee-Oesterreich lab shares data and analysis tools so freely on their website: leeoesterreich.org/resources. I hope my future research continues to reflect this ethos of team science, to more quickly make progress for patients and families affected by breast cancer.

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