Estrogen expands breast cancer stem-like cells through paracrine FGF/Tbx3 signaling

The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>As Published</td>
<td><a href="http://dx.doi.org/10.1073/pnas.1007863107">http://dx.doi.org/10.1073/pnas.1007863107</a></td>
</tr>
<tr>
<td>Publisher</td>
<td>National Academy of Sciences (U.S.)</td>
</tr>
<tr>
<td>Version</td>
<td>Final published version</td>
</tr>
<tr>
<td>Accessed</td>
<td>Sun Dec 16 10:09:31 EST 2018</td>
</tr>
<tr>
<td>Citable Link</td>
<td><a href="http://hdl.handle.net/1721.1/84629">http://hdl.handle.net/1721.1/84629</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>Article is made available in accordance with the publisher's policy and may be subject to US copyright law. Please refer to the publisher's site for terms of use.</td>
</tr>
<tr>
<td>Detailed Terms</td>
<td>atra is made available in accordance with the publisher's policy and may be subject to US copyright law. Please refer to the publisher's site for terms of use.</td>
</tr>
</tbody>
</table>
Estrogen expands breast cancer stem-like cells through paracrine FGF/Tbx3 signaling

Christine M. Fillmore\textsuperscript{a,b}, Piyush B. Gupta\textsuperscript{a,1}, Jenny A. Rudnick\textsuperscript{b,d}, Silvia Caballer\textsuperscript{o,d}, Patricia J. Keller\textsuperscript{b,d}, Eric S. Lander\textsuperscript{c,e}, and Charlotte Kuperwasser\textsuperscript{a,b,d,2}

\textsuperscript{a}Genetics Program, Sackler School of Graduate Biomedical Sciences, Tufts University School of Medicine, Boston, MA 02111; \textsuperscript{b}Molecular Oncology Research Institute, Tufts Medical Center, Boston, MA 02111; \textsuperscript{c}Broad Institute of MIT/Harvard and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142; \textsuperscript{d}Department of Anatomy and Cellular Biology, Sackler School of Graduate Biomedical Sciences, Tufts University School of Medicine, Boston, MA 02111; and \textsuperscript{e}Department of Systems Biology, Harvard Medical School, Boston, MA 02115

Edited by Geoffrey M. Wahl, Salk Institute for Biological Studies, La Jolla, CA, and accepted by the Editorial Board October 26, 2010 (received for review June 17, 2010)

Many tumors contain heterogeneous populations of cells, only some of which exhibit increased tumorigenicity and resistance to anticancer therapies. Evidence suggests that these aggressive cancer cells, often termed “cancer stem cells” or “cancer stem-like cells” (CSCs), rely upon developmental signaling pathways that are important for survival and expansion of normal stem cells. Here we report that, in analogy to embryonic mammary epithelial biology, estrogen signaling expands the pool of functional breast CSCs through a paracrine FGF/FGFR/Tbx3 signaling pathway. Estrogen or FGFR pretreatment induced CSC properties of breast cancer cell lines and freshly isolated breast cancer cells, whereas cotreatment of cells with tamoxifen or a small molecule inhibitor of FGFR signaling was sufficient to prevent the estrogen-induced expansion of CSCs. Furthermore, reduction of FGFR or Tbx3 gene expression was able to abrogate tumourosphere formation, whereas ectopic Tbx3 expression increased tumor seeding potential by 100-fold. These findings demonstrate that breast CSCs are stimulated by estrogen through a signaling pathway that similarly controls normal mammary epithelial stem cell biology.

More than 70% of breast cancers express high levels of the estrogen receptor (ER\textsubscript{a}), and many of these tumors require estrogen for sustained growth and progression. In recent years, multiple reports have shown that subpopulations of so-called cancer stem cells (CSCs; also called stem-like cells or tumor-initiating cells) are also required for sustained tumor growth and progression, and may be responsible for cancer recurrence and metastasis (1). Whether such CSCs in ER\textsubscript{a}+ breast cancers are sensitive to estrogen is currently unknown.

Breast CSCs, which are operationally defined based on the number of self-renewing cells required to initiate a tumor and drive long-term tumor growth when transplanted into mice, can be isolated from primary tumor tissue or cultured cells lines (2–7). In human breast cancers, CSCs appear to be enriched within cell subpopulations with a CD44\textsuperscript{+}/CD24\textsuperscript{−}/ESA\textsuperscript{+} surface marker profile, are better able to form colonies, or “tumourospheres,” under low-adherance conditions, and display increased resistance to chemotherapeutic compounds (2–7).

The molecular mechanisms that regulate breast CSC frequency, localization, and maintenance remain poorly understood. However, a fair amount is known about the spatio-temporal signaling dynamics that govern the specification and maintenance of normal mammalian gland stem cells. Embryonic development of the mouse mammary gland begins when Wnt and FGF signaling proteins, which are secreted by the underlying mesenchyme, induce placode formation and localize mammary epithelial fate specification (8). FGF ligands, acting through cognate receptors, activate the Tbx3 transcription factor in both the mesenchymal and mammary placodes. Tbx3, in a positive-feedback loop, activates additional FGF secretion and also Wnt signaling (9–12).

During puberty, estrogen is responsible for maturation of the mammalian gland by mediating ductal elongation (9–13). Interestingly, there is significant evidence to suggest that estrogen signaling does not act directly on adult mammary epithelial stem cells but, rather, activates their proliferation through paracrine signaling (14, 15). These data imply a two-component mammary stem cell niche in which estrogen signaling in the ERF\textsuperscript{a} non-stem cell compartment stimulates the proliferation of cells within the ERF\textsuperscript{−} stem cell compartment.

In breast cancer, it is unclear whether stem-like cells are also regulated by specific hormone-growth factor paracrine signaling pathways. In this study, we discovered that estrogen regulates breast CSC numbers through the FGF/Tbx3 signaling pathway, which happens also to regulate normal embryonic breast stem cells.

Results

Estrogen Stimulation Induces Expansion of Breast Cancer Stem-Like Cell Subpopulations. To study the signaling pathways that regulate breast CSC expansion and maintenance, we needed an experimental system that allowed for consistent modulation of breast CSC numbers through defined signaling perturbations. Tumor initiation by the MCF7 cell line appears to rely on estrogen signaling; these cells are very poor at forming tumors in ovariectomized mice (16). However, we and others have found that MCF7 cells can proliferate in vitro in the absence of estrogen (E2) if serum (even charcoal-stripped serum) is supplemented in high enough concentrations (Fig. S14). MCF7 cells grown under these conditions maintain a low percentage of CSCs as gauged by flow cytometry (Fig. S1B) and are likewise poor at forming tumors in ovariectomized mice. Yet, intact ovaries or estrogen supplementation allows even an estrogen-deprived MCF7 line to form tumors, suggesting that estrogen induces the survival or expansion of MCF7 CSCs.

To determine whether estrogen could indeed induce CSC expansion, we treated MCF7 cells as well as other estrogen receptor-positive (ER\textsuperscript{+}) cell lines (T47D, HCC1428) with 1 nM 17β-estradiol or ethanol (vehicle control) for 6 d, and evaluated the proportion of stem-like cells by flow cytometry and sphere formation assays. We found that after estrogen stimulation, the proportion of CD44\textsuperscript{+}/CD24\textsuperscript{−}/ESA\textsuperscript{+} stem-like cells was nearly eightfold higher in ER\textsuperscript{a} cultures, whereas no significant change in the proportion of CD44\textsuperscript{−}/CD24\textsuperscript{−}/ESA\textsuperscript{−} cells was observed when the same culture conditions were imposed on cells that lacked ER expression (Fig. 1A and Fig. S1C). When we challenged...
these estrogen pretreated cultures formed sevenfold more tumorspheres than the ethanol-pretreated cultures (Fig. 1B). Moreover, we observed that the addition of the potent estrogen antagonist, 4-hydroxy tamoxifen (4OHT), could prevent the 17β-estradiol induced expansion of CD44+/CD24−/ESA+ cells and sphere formation, indicating that these changes in marker expression and sphere formation were mediated through ER signaling.

We next evaluated the ability of estrogen-pretreated MCF7 cultures to form tumors by injecting cells pretreated with estrogen in vitro for 6 d into the mammary glands of ovariectomized NOD/SCID mice in dilution series. Estrogen-pretreated cells were able to form tumors in mice 100-fold more efficiently when compared with the vehicle (EtOH+DMSO) treated cells (P = 0.001, Fig. 1C). Histological examination of tissue sections revealed that MCF7 cells pretreated in vitro with estrogen formed invasive ductal carcinomas (Fig. 1D). We also examined the injection sites of MCF7 cells pretreated with ethanol that had not formed tumors and observed viable cells within the mammary glands that formed only benign epithelial structures, suggesting that lack of tumor growth was not due to immune clearance of cells or increased cell death. These results indicate that estrogen-induced expansion of cancer stem-like cells in vitro leads to a functional increase in breast CSCs and tumorigenic phenotypes in vivo.

**Estrogen Expands Breast CSCs via Paracrine-Acting Protein Factors**

Because ER activity appeared necessary for the expansion of breast CSCs in response to estrogen, we next examined the levels of ERα expression in the CD44+/CD24−/ESA+ stem-like MCF7 subpopulation. Using both immunofluorescence on freshly sorted cytospun cells and four-color flow cytometry, we found that ≥70% of the cells in the bulk fraction (CD44+/CD24−/ESA+) were strongly ERα+, whereas only 20–25% of the CD44+/CD24−/ESA+ stem-like cells had detectable nuclear ERα staining (Fig. 2A and B and Fig. S2A). We also observed that ERα+ cells in the CD44+/CD24−/ESA+ population had much lower levels of staining than ERα− cells from the bulk fraction (Fig. 2B, histogram).

Given these results, we hypothesized that in analogy to the normal mammary gland, paracrine factors released by the ERα+ cells in response to estrogen stimulation might induce the expansion of CD44+/CD24−/ESA+ stem-like cells. To evaluate this hypothesis, we harvested conditioned media from MCF7 cells that were pretreated with either vehicle (EtOH) or 1 nM 17β-estradiol. We observed that MCF7 cultures fed estrogen-conditioned media for 6 d contained 20-fold more CD44+/CD24−/ESA+ cells than matched cultures fed vehicle conditioned media (Fig. S2B, P < 0.002). In addition, expansion of this subpopulation was significantly attenuated if the conditioned medium was boiled before treatment of recipient lines (P < 0.05), indicating that the factors promoting stem-like cell expansion were heat labile and thus likely to be secreted proteins.

We tested whether conditioned media from estrogen-pretreated MCF7 cells could increase CSC numbers in three ERα− breast cancer lines, SUM149, SUM159, and BT-20. We observed that exposure to conditioned media from estrogen-pretreated MCF7 cells induced a statistically significant expansion of the CD44+/CD24−/ESA+ stem-like cells in all three cell lines, yielding cultures that were more efficient at forming tumorspheres (Fig. 2 C and D). In sum, these data suggest that estrogen acts to induce secretion of paracrine acting proteins, which in turn increase percentages of CD44+/CD24−/ESA+ populations and corresponding cancer stem-like cell properties in many breast cancer cell lines.

**Estrogen Induces FGF9/FGFR3 Signaling to Increase Cancer Stem-Like Numbers.** To identify the secreted proteins mediating breast cancer stem-like cell expansion following estrogen treatment, we examined the conditioned media from either 17β-estradiol-treated or vehicle-treated MCF7 cells and quantitatively assessed for 164 secreted growth factors and cytokines using an antibody-based protein array. In addition to known estrogen-induced factors, we observed that the secretion of every assayed FGF family member (FGF2, FGF4, FGF6, FGF7, and FGF9) was increased at least twofold upon estrogen treatment compared with ethanol-treated controls (Fig. S2C). Notably, FGF9, which is induced by estrogen in endometriosis and during embryonic mammary placode formation (17), was increased 14-fold following estrogen treatment of MCF7 cells.

We next tested whether FGF signaling was necessary for the estrogen-induced expansion of the breast CSC-enriched subpopulation. Accordingly, we treated MCF7 cells with an FGFR signaling inhibitor, FGF9, and estrogen to serum-free cultures was sufficient to increase the CD44+/CD24−/ESA+ subpopulation (Fig. S2D) and to promote tumor sphere formation to levels comparable to those in estrogen-treated sphere cultures (Fig. 3B). FGF9 and estrogen appeared to have a synergistic effect on increasing MCF7 CSCs (Fig. 3A). When we tested two other ligands from the FGF family, FG2F2 and FG2F10, we saw that although these factors did not increase the basal levels of CD44+/CD24−/ESA+ cells in the cell line, they were able to slightly increase the effect of estrogen (Fig. S2E). In contrast, feeding candidate growth factors, including EGF, HRG, IGFII, BMP6, and SDF1β, failed to increase the proportion of CSCs in the presence or absence of estrogen supplementation (Fig. S2E).

There are four FGF receptors, and MCF7 cells express high levels of FGF3R (Fig. S3A), which binds with high affinity to FGF9 (17). To rule out a nonspecific effect of the PD173074 compound, we examined whether the knockdown of FGF3R in MCF7 cells might also abolish estrogen-induced expansion of the breast cancer stem-like cell populations. Accordingly, we inhibited FGF3R expression using lentiviral infection with targeted shRNAs. We observed a 76% reduction in FGF3R protein...
expression in MCF7 cultures transduced with shFGFR3 (Fig. S3B). Similar to treatment with PD173074, inhibition of FGFR3 expression in MCF7 cells led to a fourfold reduction in the proportion of CD44+/CD24-/ESA+ cells and a twofold reduction in sphere formation in response to estrogen treatment (Fig. S3C and Fig. 3C) without reducing estrogen-induced proliferation in adherent cultures (Fig. S3D).

To functionally assess whether inhibition of FGF signaling in the presence of estrogen affected tumor formation, MCF7 cells were pretreated for 6 d in vitro with estrogen in the presence of PD173074 and injected into mice. Tumor-initiating potential conferred by 17β-estradiol pretreatment alone was abolished in the presence of FGFR inhibition (Fig. 1C, \( P = 0.001 \)). These data indicate that estrogen expands breast cancer stem cell numbers at least in part through the FGF/FGFR signaling pathway.

To determine whether the FGF signaling pathway also regulates stem-like cell populations in ERα− breast cancer cell lines, we added either recombinant FGF9 or PD173074 to SUM149, SUM159, and BT-20 cultures. Treatment with FGF9 induced an average 2.5-fold expansion of the stem-like cells and enhanced tumor sphere formation, whereas inhibition of FGFR signaling with PD173074 decreased the proportion of CD44+/CD24-/ESA+ stem-like cells by eightfold and reduced sphere formation (Fig. 3D and Fig. S3E). In addition, SUM159 cells pretreated in vitro with PD173074 or FGF9 were injected orthotopically into immunocompromised mice to evaluate tumor initiation. Indeed, PD173074 pretreatment significantly inhibited SUM159-derived tumor growth in vivo (\( P < 0.02, \) Fig. 3E).

We also isolated patient-derived breast carcinoma cells, and treated these cells with either FGF9 or PD173074 in sphere culture. We observed a modest 1.2-fold increase in tumorsphere formation in response to treatment with FGF9 but a statistically significant twofold reduction in sphere formation in the presence of PD173074 (\( P < 0.01, \) Fig. 3F). Similarly, when we dissociated freshly isolated tumors from human-in-mouse tumor generated tissues (SI Materials and Methods), we found that these cells grew significantly fewer sphere colonies in the presence of PD173074 than in the presence of FGF9 (Fig. S3F). Collectively, these data demonstrate that FGF/FGFR signaling is an important regulator of breast cancer stem-like cells.

**Estrogen and FGF Signaling Induce Tbx3 Expression.** The Tbx3 transcription factor has been reported to activate FGF signaling but also act downstream of FGF signaling, where it is required for propagation of FGF and Wnt signals in the rudimentary mammary epithelium (8–10). Therefore, we wanted to determine whether levels of Tbx3 correlated with estrogen or FGF signaling. Accordingly, we examined Tbx3 expression in MCF7 cultures treated with combinations of estrogen, tamoxifen, FGF9, or PD173074. Indeed, Tbx3 mRNA and protein expression were increased in MCF7 cells treated with estrogen and further increased by FGF9 stimulation (Fig. 4A and B). This induction was effectively inhibited by 4OHT or PD173074. Tbx3 protein was also visualized by immuno-fluorescence, revealing nuclear localization for the Tbx3 transcription factor in 60% of the MCF7 culture following estrogen treatment (Fig. 4C).

We also examined the FGF-Tbx3 signaling axis in ERα− SUM149, SUM159, and BT-20 breast cancer cells treated with recombinant FGF9 or PD173074. Consistent with the findings in MCF7 cells, Tbx3 mRNA and protein expression was induced in response to FGF9 treatment (Fig. 4D and E). Although treatment of the cultures with PD173074 did not affect the basal Tbx3 mRNA levels, protein levels appeared to be modestly decreased. Taken together, these data indicate that: (i) estrogen stimulates expansion of tumorigenic breast cancer cells in part through FGF signaling, (ii) inhibition of FGF/FGFR signaling decreases tumorigenic breast stem-like cells, and (iii) estrogen causes induction of Tbx3 expression breast cancer cells and is a likely mechanism through which FGF signaling is perpetuated.

**Tbx3 Expression Is Sufficient for Breast CSC Expansion.** Because Tbx3 is known to be necessary for the specification and expansion of normal mammary stem cells, we next examined whether Tbx3 might also be necessary for the expansion of breast cancer stem-like cells. Using an RNAi knock-down approach in three different breast cancer cell lines (MCF7, SUM149, and SUM159), we were able to reduce endogenous Tbx3 mRNA and protein levels 60–85% (Fig. S4A and B). It is known that the DUSP6 phosphatase is activated following FGF signaling, and that the spatiotemporal expression pattern of DUSP6 in the developing mammary gland is similar to that of Tbx3 (10). Therefore, we also assayed expression of DUSP6 and found that DUSP6 mRNA expression was reduced an average of 2.4-fold in the shTbx3 transduced cultures. These results are analogous to observations in embryonic mammary epithelial cells showing that FGF signaling is required for Tbx3 and DUSP6 expression and that Tbx3 expression is important for further FGF production and signal propagation (8–10).

To assess the role of Tbx3 in breast CSC maintenance, we performed flow cytometry and tumorsphere assays on cells exhibiting the greatest inhibition of Tbx3 expression. We found no significant difference in the proliferation rates of SUM149, SUM159, or MCF7 breast cancer cells upon Tbx3 knockdown (Fig. S4 C and D). However, the ability of MCF7 cells transduced with shTbx3 to increase the proportion of CD44+/CD24-/ESA+...
cells or increase sphere formation following estrogen was significantly attenuated (Fig. 5 A and B). Likewise, a 20–50% reduction in cancer stem-like cells and tumoursphere formation was observed in shTbx3 transduced SUM149 and SUM159 lines (Fig. 5 A and B). Furthermore, Tbx3 was inhibited using two different hairpins in patient-derived cancer cells and also resulted in a significant reduction in sphere formation (Fig. 5C).

Notably, we were unable to maintain efficient knockdown of Tbx3 in any cell line for more than two passages following selection; therefore, we could not assess in vivo tumor seeding ability of shTbx3 cells. Consequently, we took an alternative approach and ectopically overexpressed Tbx3 in normal human mammary epithelial cells (HMECs) and MCF7 cancer cells to determine whether Tbx3 expression would suffice to promote stem-like cell behavior. Indeed, expression of Tbx3 resulted in a twofold increase in the number of spheres formed by HMEC cells and increased the proportion CD44+/CD24−/ESA+ cancer stem-like cells in MCF7 cells by ninefold (Fig. 5D and Fig. S4E). Furthermore, overexpression of Tbx3 in MCF7 cells led to a robust twofold increase in tumoursphere formation (Fig. 5D). Consistent with the expansion of cancer stem-like cells, overexpression of Tbx3 in MCF7 cells resulted in a 100-fold increase in tumour-initiation compared with control cells (Fig. 5E, P = 0.001). Collectively, these findings indicate that Tbx3 is sufficient to promote normal and cancer stem-like cell phenotypes.

Expression of FGFR3 and Tbx3 in Human Breast Cancers. Our results suggest that paracrine FGFR signaling mediated through Tbx3 is important in regulating the proportion of CSCs within cultured breast cancer populations. To determine whether this mechanism might also operate within the context of primary human breast cancers, we queried a gene expression database that encompasses more than 18,000 human cancer gene expression microarrays (18, 19) for FGFR3 and Tbx3 expression. We found that Tbx3 was highly expressed in many subtypes of breast cancer when compared with normal tissue, and that Tbx3 expression correlated with ER-positive tumors. Furthermore, Tbx3 expression was highly correlated with metastatic recurrence at both 3 and 5 y, whereas Stage III tumors had a high correlation with genomic amplification of the Tbx3 locus (Figs. S5 and S6 A and B). These data are consistent with and support other recent findings that Tbx3 is up-regulated in human breast cancers (16). In addition, we found that breast tumors that responded to chemotherapy expressed significantly lower levels of Tbx3 than nonresponders, and that cell lines that are sensitive to chemotherapies likewise have much lower Tbx3 expression relative to chemotherapy-resistant cell lines (Fig. S6 C and D). Furthermore, ERα expression levels were strongly correlated with FGFR3 expression in a majority of primary tumor samples (P = 0.001, Fig. S5). These data are compatible with the notion that the E2/FGF/Tbx3 signaling axis is activated in many primary breast cancers.

Discussion

Here, we identify the estrogen/FGF/Tbx3 signaling axis as an important modulator of CSC properties both in vitro and in vivo.
While much of our data were collected using the experimental system of the ERα+ MCF7 cell line, we were able to observe up-regulation of Tbx3 in many different primary human tumor datasets, suggesting the relevance of this pathway in primary tumor samples. In addition, we observed conservation of the FGF/FGFR3/Tbx3 signaling pathway in basal-type ERα− cell lines, as well as in freshly dissociated patient tissue, indicating that this pathway may be important for growth of many subtypes of breast cancers other than the common ERα+ subtype.

The experiments described here also demonstrate that the regulation of breast CSCs is influenced by the same regulatory pathways that control stem cells in the developing mammary gland. Although the underlying basis for this connection is unclear, several other groups have observed a conservation of expression of developmental signaling programs in cancers and cancer stem cells (20–22). Based on the results described here, we propose a model in which Tbx3 expressing cancer cells promote the expansion of CSCs through paracrine FGF signaling (Fig. S7). In ERα+ breast cancer cell lines, estrogen (E2) binds to the estrogen receptor to induce FGF9 secretion and Tbx3 expression in the non-CSC compartment. Expression of Tbx3 leads to further expression of Wnts and FGFs to perpetuate expression in the non-CSC compartment. Expression of Tbx3 contributes to breast CSC expansion. Nevertheless, an important prediction of our model is that the acquisition of resistance to anti-hormone therapies might be accompanied by an increase in FGF/FGFR/Tbx3 signaling and a concomitant increase in the proportion of CSCs. Therefore, targeting the FGF/FGFR/Tbx3 pathway may be a useful therapeutic strategy for hormone-therapy refractory luminal (ERα+) breast cancers.

The experiments described here as well as in other studies have demonstrated that normal and cancer breast stem cell pools lack abundant ERα+ expression (24–26, 12). This suggests that the successes of tamoxifen and aromatase inhibitors, such as letrozole, for the treatment estrogen-sensitive breast tumors may be attributed to the inhibition of paracrine factors released by ERα+ breast cancer cells but not to the eradication of ER-negative CSCs. Indeed, residual breast cancer cells in tumor tissues treated with letrozole exhibited a pronounced enrichment of cells exhibiting CSC phenotypes (27). This observation, combined with our findings, suggests that resistance to anti-estrogen therapies and recurrence of ERα+ breast cancers could arise from genetic or epigenetic alterations that allow for the acquisition of FGF/Tbx3 activity in the absence of continued estrogen stimulation. In support of this notion, tamoxifen resistance by breast cancer cells is accompanied by increases in DUSP6 expression (28), as well as mesenchymal transdifferentiation (29). Furthermore, studies have shown that overexpression of FGF ligands subverts the requirement for estrogen to drive tumor formation (30–32).

Although our experiments focused on breast cancer CSC expansion stimulated by FGF9, we found that other FGF ligands are also capable of influencing CSC numbers. We did not address here whether other FGFRs in addition to FGFR3 can contribute to breast CSC expansion. Nevertheless, an important prediction of our model is that the acquisition of resistance to anti-hormone therapies might be accompanied by an increase in FGF/FGFR/Tbx3 signaling and a concomitant increase in the proportion of CSCs. Therefore, targeting the FGF/FGFR/Tbx3 pathway may be a useful therapeutic strategy for hormone-therapy refractory luminal (ERα+) breast cancers.

Materials and Methods

Detailed methods are described in SI Materials and Methods.

Cells and Tissue Culture. Cell line procurement and culture is described in SI Materials and Methods.

All human breast tissue was obtained in compliance with the laws and institutional guidelines, as approved by the institutional institutional review board committee from Tufts University School of Medicine. An ERα+, Her2− tumor was obtained from discarded material, and noncancerous breast tissue was obtained from breast reduction surgery. All tissues were harvested with the approval of the institutional review board committee from Tufts University School of Medicine.
was obtained from patients undergoing elective reduction mammoplasty at Tufts Medical Center. Cells were manipulated as described in SI Materials and Methods.

**Flow Cytometry.** Antibodies used were EpCAM (ESA-FITC) (clone VU-ID9, AbD Serotec), CD24-PE (clone ML5, BD Pharmingen), and CD44-APC (clone G44-26, BD Pharmingen). When staining for ERα-FITC (clone SP1, Abcam) cells were stained sequentially with EpCAM (clone VU-ID9, Abcam), rat-anti-mouse PerCP (BD Pharmingen) and CD24-PE/CD44-APC (BD Pharmingen) before cells were fixed in 4% paraformaldehyde and 0.1% Saponin and incubated with ERα-FITC.

**Tumorsphere Assays.** Cells were trypsinized and mechanically separated and, when necessary, passed through 40-μm filters to obtain single cell suspensions that were plated at less than 10,000 cells per mL in super-low-attachment plates in normal growth media (with supplements where indicated). Quantification of mammosphere and tumorsphere numbers was accomplished using a Multisizer 3 Coulter Counter (Beckman-Coulter) that provided number and size distributions of particles between 40 μm and 336 μm.

**Conditioned Medium Experiments.** Subconfluent MCF7 cultures grown in standard phenol red containing DMEM with 10% FBS were washed and switched to phenol-red-free DMEM + 10% charcoal-dextran stripped FBS supplemented with 1 nM 17-β-estradiol or E2OH for 6 d. Cultures were then washed five times with PBS and incubated with fresh serum-free phenol-red-free DMEM. Conditioned medium (CM) was harvested 72 h later, passed through a 0.2-μm filter, and frozen at −80°C. For each experiment, at least three distinct batches of CM were combined and supplemented with 2 mM L-glutamine and 10% charcoal-dextran–stripped FBS and fed to naïve cells for a total of 6 d, with media changed every 2 d, after boiling for 5 min where specified.

**Western Blot and Immunofluorescence.** Antibodies used for IF were ERα-FITC (clone SP1, Abcam), EpCAM (clone B29.1, Abcam), and Tbx3 (rabbit, Aviva). Antibodies used for Western blotting were Tbx3 (mouse, Abcam), FGFR3 (rabbit, Sigma), and β-actin (clone mAbcam 8226, Abcam).

**Isolation of RNA, Microarray, and Quantitative RT-PCR.** Cells were harvested by trypsinization of fluorescence-activated cell sorting and pelleted by centrifugation, and RNA isolation was performed using the RNeasy kit (Qiagen) in accordance with the manufacturer's protocol. The RNA samples were then reverse transcribed using the Script cDNA kit (Bio-Rad), and quantitative PCR was performed with Sybr green (Bio-Rad) on a Bio-Rad iCycler. Primers are listed in SI Materials and Methods.

**ACKNOWLEDGMENTS.** We thank Ima Kieble and Dr. Lisa Arendt for surgical assistance and maintenance of the animal colony. We thank Allen Parmelee and Steve Kwok for expert technical assistance with cell sorting. We thank Josh LaBaeer at Harvard Medical School (Boston, MA) for generously providing us with human Tbx3 cDNA. This work was supported by grants from the American Cancer Society–New England Division–Broadway on Beachside Postdoctoral Fellowship PF-08-101-01-CSM (to P.J.K.), Breast Cancer Research Foundation (to C.K. and C.M.F.), Raymond and Beverly Sackler Foundation–New England Division (to C.K.), and National Institutes of Health/National Cancer Institute Grant R01CA125554 (to C.K.). C.K. is a Raymond and Beverly Sackler Foundation Scholar.

Supporting Information

Fillmore et al. 10.1073/pnas.1007863107

SI Materials and Methods

**Cells and Tissue Culture.** SUM cell lines were obtained from Dr. Stephen Ethier (Karmanos Institute, Detroit) and are commercially available (Astarand). MCF7, HCC1428, T47D, and BT-20 cell lines were purchased from ATCC. MCF7, HCC1428, T47D, and BT-20 cells were cultured in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco, Invitrogen). Cultures were washed twice with PBS and incubated at 37°C in a 5% CO2 incubator. Estrogen (17β-estradiol) was dissolved in ethanol to a stock concentration of 1 mM, PD173074 (Sigma) was dissolved in DMSO to a stock concentration of 10 mM. All treatments, including those with conditioned media, lasted 6 d.

All human breast tissue procurement for these experiments was obtained in compliance with the laws and institutional guidelines, as approved by the institutional institutional review board committee from Tufts University School of Medicine. An ER+ Her2- tumor was obtained from discarded material, and noncancerous breast tissue was obtained from patients undergoing elective reduction mammoplasty at Tufts Medical Center. Breast tissues were minced and enzymatically digested overnight with a mixture of collagenase and hyaluronidase as previously described (1, 2). Digested cells were plated briefly in serum (1–2 h) to deplete mammary fibroblasts from the organoid fraction of mammary fibroblasts. The organoids were dissociated to a single cell suspension by trypsinization and filtered with a 40-μm filter (BD Biosciences) to remove residual clustered cells. Immediately after dissociation, cells were assayed for mammospheres formation or were infected with lentivirus and then assayed. For the human-in-mouse tumors, mammary epithelial cells from three different patient samples were spin infected with lentivirus-encoding MyrP110x, KrasG12V, p53R175H, and CCND1 and implanted into humanized mouse mammary glands (1).

**Flow Cytometry.** Subconfluent cultures were trypsinized into single cell suspension, counted, washed with PBS, and stained with antibodies specific for the following human cell-surface markers: EpCAM (ES-A)-FITC (clone VU-ID9, AbD Serotec), CD24-PE (clone ML5, BD Pharmingen); and CD24-APC (clone G44-26, BD Pharmingen). For each staining reaction, 100,000 cells were incubated with 4 μL of each antibody for 15 min at room temperature. Unbound antibody was washed off and cells were analyzed on a BD FACSCaliber no more than 1 h poststaining. Isotype controls included mouse IgG1-FITC, mouse IgG2a-PE, and mouse IgG2a-APC (BD Pharmingen). For staining for ERα-FITC (clone SP1, Abcam) cells were stained sequentially with EpCAM (clone VU-ID9, Abcam), rat–anti-mouse PerCP (BD Pharmingen), and CD24-PE/CD44-APC (BD Pharmingen) before cells were fixed in 4% paraformaldehyde and 0.1% saponin and incubated with ERα-FITC.

**Animals and Surgery.** All animal procedures were conducted in accordance with relevant national and international guidelines and according to the animal protocol approved by the Tufts University Institutional Animal Care and Use Committee. NOD/SCID mice were purchased from Jackson Labs. Female mice 5–7 wk of age were ovarioectomized and allowed to recover for 4 wk before tumor cell injection. For tumor-seeding studies, the indicated numbers of MCF7 cells pretreated for 6 d with vehicle (EtOH), 1 nM 17-β-estradiol (E2), or 1 nM 17-β-estradiol and the FGFR inhibitor PD173074 (E2+ PD) were suspended in 1:1 (vol/vol) culture medium: Matrigel (BD Biosciences) mixture and injected into the fourth inguinal mammary gland. For SUM159 pretreatment experiments, intact 8-wk-old female NOD/SCID mice were injected into the fourth inguinal mammary gland (n = 12 for each group) with 10,000 cells pretreated for 6 d with PD173074 or with FGFR.

**Tumorsphere Assays.** Viable dissociated single cells (~30,000/mL) were plated in 6-cm ultra-low-attachment plates (Corning) in the indicated media. Tumorspheres and mammospheres were allowed to form for 5 or 8 d, respectively, after which spheres were collected for analysis. Quantification of mammosphere and tumorsphere numbers was accomplished using a Multisizer 3 Coulter Counter (Beckman-Coulter) that provided number and size distributions with an overall sizing range of 40 μm to 336 μm. Tumorspheres and mammospheres were collected and pelleted at 800 rpm for 5 min and resuspended in 1 mL freshly filtered growth media, diluted in 20 mL 6:4 isoton II:glycerol diluent (Beckman-Coulter), and run in triplicate on the Multisizer 3.

**Conditioned Medium Experiments.** Subconfluent MCF7 cultures were treated with 1 nM 17-β-estradiol or EtOH for 6 d in phenol-red-free DMEM and 10% charcoal-dextran–stripped FBS (In-vitrogen). Cultures were washed five times with PBS and incubated with fresh serum-free, phenol-red-free DMEM. Conditioned medium (CM) was harvested 72 h later, passed through a 0.2-μm filter, and frozen at −80°C. For each experiment, at least three distinct batches of CM were combined and supplemented with 2 mM l-glutamine and 10% charcoal-dextran-stripped FBS and fed to naive cells for a total of 6 d, with media changed every 2 d, after boiling for 5 min where specified.

**Cytokine Array and Quantification.** Serum-free CM was collected as described above. Human cytokine arrays (2000 series, RayBio-tech) were exposed to conditioned medium from MCF7 cultures pretreated with either ethanol (vehicle) or estrogen and processed in accordance with the manufacturer’s protocols. Exposed films of chemiluminescence signal obtained from dot blots were scanned, and the pixel intensity for each cytokine was quantified and normalized to IgG loading controls using ImageJ software (National Institutes of Health).

**Western Blot and Immunofluorescence.** For immunofluorescence (IF), cells were fixed in 4% paraformaldehyde and 0.1% saponin and permeabilized with 0.1% BSA and 0.25% Triton-X, both in PBS. Coverslips were mounted with Vectashield mounting medium plus DAPI (Vector Labs). Antibodies used for IF were ERα-FITC (clone SP1, Abcam), DUSP6 (clone 3G2, Novus), EpCAM (clone B29.1, Abcam), and Tbx3 (rabbit, Aviva). For Western blotting, 25 μg protein extract per sample denatured with heat and reducing agents, separated on a 4–12% acrylamide gel, and transferred to nitrocellulose. Antibodies used for Western blotting were Tbx3 (mouse, Abcam), FGFR3 (rabbit, Sigma), and β-actin (clone mAbcam 8226, Abcam).

**Isolation of RNA and Quantitative RT-PCR.** Cells were harvested by trypsinization, pelleted by centrifugation, and RNA isolation was performed using the RNAeasy kit (Qiagen) in accordance with the manufacturer’s protocol. The RNA samples were then reverse transcribed using the iScript cDNA kit (Bio-Rad), and quantitative PCR was performed with Sybr green (Bio-Rad) on a Bio-Rad iCycler. Primers used are: GAPDH F-GAGTCAAC-CATGCCACGACGTTCC, RPL13A R-ATCCGCCTCGTGAAGACAT, ERα F-ACAACCCACCTGGCTTGTAC, and R-ACGCGGTTCACTGGTCTCT.
GGATTTGGTCGT R-GACAAGCTTCCCGTTCTCAG, Tbx3
F-TGGGGACCTCTGATGAGTCCT R-CCATGCTCCTCTTGCTCTGAGC, Wnt5a
F-GGGAGGTTGGCTTTGAACATA R-GAATGGCACGCAATTACCTT, ERα
F-ATTTGAAGTGGGCAGAGAACAT R-CAATACCAACATCAGCCAGAAA, FGFR3
F-ACTGGGGAACAGTGGATGTC R-GGATGCCTGCATACACACTG, FGF9
F-TTTCTGGTGCCTGTTAGTCC R-GACTACCTGCTGGGCATCAA, Vimentin
F-AGATGGCCCTTGACATTGAG R-GGTCATCGTGATGCTGAGAA, N-Cadherin
F-ACAGTGGCCACCTACAAAGG R-CCGAGATGGGGTTGATAATG, E-Cadherin
F-TGCCCAAGAAAATGAAAAAGG R-GGATGACACAGCGTGAGAGA, Zeb-1
F-GATCAACCACCAATGGTTCC R-TTGCGCAAGACTGGAATCAAG.

Lentiviral Constructs and Infection. Bacterial glycerol stocks of
MISSION shRNA were obtained (Sigma), and plasmid DNA was
isolated by miniprep (Qiagen). Lentiviral expression construct
for Tbx3 gene transduction was created using standard Gateway
cloning techniques into the self-inactivating pLenti6.2/V5-DEST Gateway vector (Invitrogen). A WT human Tbx3 cDNA
clone (NM_016569.2–443) was generously provided by Josh
LaBaer (Harvard Institute of Proteomics, Harvard Medical
School, Boston, MA). The VSV-G-pseudotyped lentiviral vec-
tors were generated by transient cotransfection of the above
vectors with the VSV-G-expressing construct pCMV-VSV-G
and the packaging construct pCMV DR8.2Dvpr (3), both gener-
ously provided by Inder Verma (Salk Institute), into 293T cells
with the FuGENE 6 transfection reagent (Roche). Viral super-
natant was collected and introduced to subconfluent SUM149,
SUM159, MCF7, and HMEC cultures, or to patient-derived
breast cancer cells. Lentiviral integration was selected with 1 μg/
ml puromycin (for shRNAs), or with 10 μg/ml blasticidin (Tbx3)
for 7 d.

a tissue transgenic model of human breast cancer in mice. Proc Natl Acad Sci USA 106:
7022–7027.

Fig. S1. (A) MCF7 cells were seeded in six-well plates at 100,000 cells per well. The next day, the cultures were switched to phenol-red–free DMEM with 10%
charcoal–dextran FBS and either 1 nM estrogen or vehicle (EtOH). Each day, two wells per condition were trypsinized and counted. Average cell number per
well per day is shown. (B) Cells described in A were assayed daily for percentage of CD44+/CD24+/ESA+ cells by flow cytometry. (C) Average percentage of
CD44+/CD24+/ESA+ cells as measured by flow cytometry in the ERα+ cell lines T47D (*P < 0.01) and HCC1428 (*P < 0.0001), or in the ERα– cell lines SUM159 and
SUM1315, following 6-d treatment with either 1 nM 17-β-estradiol (E2) or vehicle (EtOH). Data are mean ± SEM; n = 5 biological replicates.
Fig. S2. (A) Quantification of ERα immunofluorescence staining on sorted and cytospun MCF7 cells. Graph represents percentage of ERα+ cells for from seven fields per sort with an average of 56 nuclei per field ± SEM. (B) Average percentage of CD44+/CD24-/ESA+ cells as assayed by flow cytometry in MCF7 cultures following 6-d treatment with fresh estrogen, vehicle (EtOH), or conditioned media from EtOH- or E2-pretreated MCF7 cells. *P < 0.001 E2 CM vs. EtOH CM. Where indicated, unconditioned or conditioned media was boiled before feeding the cells. For unconditioned media containing fresh 1 nM 17-β-estradiol, the estrogen was added before boiling to show its relative heat stability. *P < 0.04 E2 CM vs. E2 CM boiled. Data are mean ± SEM; n = 4 biological replicates. (C) Cytokine array quantification of proteins secreted by MCF7 cells in response to estrogen (E2). All data are normalized to the respective IgG controls, and the fold increase in secretion is shown as the E2 pixel value divided by the EtOH pixel value for matched exposure lengths. (D) Average percentage of CD44+/CD24-/ESA+ cells in MCF7 cultures treated with 1 nM 17-β-estradiol (E2), recombinant human FGF9 (100 ng/mL), or FGF9 and E2 in the absence of serum. *P < 0.05 E2+FGF9 vs. E2; **P < 0.001 E2 vs. E2+PD. Data are mean ± SEM; n = 6 biological replicates. (E) Average percentage of CD44+/CD24-/ESA+ cells in MCF7 cultures following 6-d treatment with EtOH vehicle, 1 nM 17-β-estradiol (E2), fibroblast growth factor 2 (FGF2), fibroblast growth factor 10 (FGF10), human epidermal growth factor (EGF), recombinant human heregulin (HRG), insulin-like growth factor 2 (IGFII), bone morphogenic protein 6 (BMP6), or stromal-derived factor 1-β (SDF1β). Data are mean ± SEM.
Fig. S3.  (A) Immunofluorescence of FGFR3 (red) and DAPI (blue) of untreated MCF7 cultures. (B) Western blot of FGFR3 expression in MCF7 cells transduced lentivirus containing a small-hairpin directed to FGFR3. (C) Average percentage of CD44+/CD24−/ESA+ cells in MCF7 cultures treated for 6 d with 1 nM 17-β-estradiol (E2) in cultures transduced with indicated small hairpins. *P = 0.001 shFGFR3+E2 vs. shCntrl+E2. (D) Proliferation rates of FGFR3 knockdown MCF7 cell lines in response to 1 nM 17-β-estradiol (E2). Data are shown as total cell number in E2-treated cultures normalized to cell number in matched EtOH-treated cultures; n = 4. (E) Tumorsphere formation of SUM159 or SUM149 cells pretreated with recombinant human FGF9 or FGFR inhibitor PD173074, or treated while making spheres with FGF9 or PD173074. Data collected at 6 d after seeding, *P < 0.002 FGF9 vs. vehicle. (F) Three different tumors derived from primary mammary epithelial cells transformed with MyrP110α, kRasG12V, p53R175H, and CCND1 and implanted into humanized mouse mammary glands were dissociated into single cells and plated at low dilution on super–low-attachment plates in filtered MEGM ± DMSO, 100 ng/mL FGF9, or 10 µM PD173074. Spheres were quantified on a Becton Dickonson Multisizer 8 d after seeding, and sphere formation was normalized to the DMSO condition; n = 12.
Fig. S4. RNAi-mediated knock down of Tbx3. (A) RT-PCR of Tbx3 and DUSP6 expression (relative to shCntrl). (B) Western blot of Tbx3 expression in MCF7, SUM159, and SUM149 cultures transduced with lentivirus containing short hairpins targeting a scrambled sequence (Cntrl), GFP, or Tbx3. (C) Proliferation rates of Tbx3 knockdown MCF7 cell lines in response to 1 nM 17-β-estradiol (E2). Data are shown as total cell number in E2-treated cultures normalized to the cell number in matched EtOH-treated cultures; n = 4. (D) Proliferation rates of SUM149 and SUM159 Tbx3 knockdown cultures as measured by Crystal Violet staining. Cells were stained 6 d after being equally plated, and OD595 was measured on a spectrophotometer. Data are mean ± SEM. (E) Representative flow cytometric dot plots of CD44 and CD24 expression in MCF7 cultures over-expressing Tbx3. Empty vector control-transduced cultures are shown for comparison.
TBX3 expression in human breast cancers. (A) Oncomine (Compendia Bioscience) was used for analysis and visualization of TBX3 expression in published microarray data sets. TBX3 overexpression was observed in many invasive breast cancer subtypes when compared with normal breast tissue, and correlates very highly with ER expression. DCIS, ductal breast carcinoma in situ; IDBC, invasive ductal breast carcinoma; ILBC, invasive lobular breast carcinoma; IMBC, invasive mixed breast carcinoma; NB, normal breast; NV, no value. (B) Gene expression correlation analysis in a data set shows that a large group of primary breast tumors coexpress high levels of ERα and FGFR3.
TBX3 expression in tumors following response to therapy. Oncomine (Compendia Bioscience) was used for analysis and visualization of TBX3 expression in published microarray data sets grouped as follows: (A) metastatic recurrence at 3 and 5 y; (B) genomic amplification of Tbx3 locus and tumor stage; (C) pathological complete response to therapy; and (D) sensitivity of breast cancer cells to common breast cancer therapeutics in vitro.
Fig. S7. Proposed model of paracrine signaling within breast cancer cell lines. In ERα+ tumors, estrogen (E2) binds to the estrogen receptor in the non-CSC compartment to induce paracrine FGF9 secretion. FGF9 then binds to FGFR3 and induces Tbx3 expression. Expression of Tbx3 leads to further expression of Wnts and FGF/FGF signaling which promote CSC phenotypes. In ERα− tumors, it appears that FGFR/Tbx3 is active in the EMT-like cells, leading to stabilized paracrine signaling that regulates cancer stem cell (CSC) subpopulations.