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FOXC1 is a critical mediator of EGFR function in human basal-like breast cancer

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Abstract

Background.—Human basal-like breast cancer (BLBC) has a poor prognosis and is often identified by expression of the epidermal growth factor receptor (EGFR). BLBC remains a major clinical challenge because its pathogenesis is not well understood, thus hindering efforts to develop targeted therapies. Recent data implicate the forkhead box C1 (FOXC1) transcription factor as an important prognostic biomarker and functional regulator of BLBC, but its regulatory mechanism and impact on BLBC tumorigenesis remain unclear.

Methods.—The association between FOXC1 and EGFR expression in human breast cancer was examined by immunohistochemistry in formalin-fixed tissues and analysis of the TCGA database. The regulation of FOXC1 by EGFR activation was investigated in MDA-MB-468 cells using immunoblotting, qRT-PCR, and luciferase activity assays. This EGFR effect on FOXC1 expression was confirmed using the MDA-MB-468 xenograft model.

Results.—Both FOXC1 mRNA and protein levels significantly correlated with EGFR expression in human breast tumors. EGFR activation induced FOXC1 transcription through the ERK and Akt pathways in BLBC. EGFR inhibition in vivo reduced FOXC1 expression in

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xenograft tumors. We also found that FOXC1 knockdown impaired the effects of EGF on BLBC cell proliferation, migration, and invasion.

Conclusions.—Our findings uncover a novel EGFR-FOXC1 signaling axis critical for BLBC cell functions, supporting the notion that intervention in the FOXC1 pathway may provide potential modalities for BLBC treatment.

Keywords

Akt; basal-like breast cancer; EGFR; ERK; FOXC1

INTRODUCTION

Basal-like breast cancers (BLBCs), which express genes characteristic of basal/myoepithelial cells in the normal mammary gland, comprise up to 25% of all breast cancers.¹ They under-express estrogen receptor (ER), progesterone receptor,² and HER-2, encompassing 60-90% of ER-/PR-/HER-2-(triple-negative breast cancers). BLBC usually presents with high histologic grade, aggressive clinical features, poor prognosis, and a propensity to metastasize to the brain and lung.³ Chemotherapy is the only modality of systemic therapy for BLBC and shows limited success against this subtype of breast cancer. A better understanding of the molecular mechanisms of BLBC and the development of effective targeted therapy are urgently needed.

Forkhead box (FOX) proteins constitute a family of evolutionarily conserved transcription factors characterized by a common 110-amino acid DNA-binding FOX domain. FOX proteins have been shown to regulate a wide variety of biological processes, including development, differentiation, proliferation, apoptosis, migration, invasion, and tumorigenesis.⁴ FOXC1, a FOX family member, plays important roles in the development of the brain, heart, and eye during the embryonic stage.⁵ In addition to its important role in development, FOXC1 has been recently found to be overexpressed in different types of cancer including breast cancer,⁶⁻⁸ hepatocellular carcinoma,⁹ prostate cancer,¹⁰ pancreatic adenocarcinoma,¹¹ and non-small cell lung cancer.¹² FOXC1 expression is associated with poor clinical outcome in these cancers. Notably, FOXC1 is specifically overexpressed in BLBC,¹³ and its overexpression induces cellular traits commonly associated with this breast cancer subtype, such as epithelial-to-mesenchymal transition (EMT) and increased cell proliferation, and migration.^{6,7} Recently, it has been reported that FOXC1 expression is a hallmark for circulating breast tumor cells with mesenchymal phenotypes.¹⁴ Thus, FOXC1 may serve as a functional regulator of BLBC. A recent study showed that BRCA1 in cooperation with GATA3 represses FOXC1 expression to inhibit the pathogenesis of BLBC.¹⁵ Of note, analysis of an oligonucleotide comparative genomic hybridization array revealed that the *FOXC1* gene is not amplified in basal-like tumors.¹⁶ The mechanism for the exclusive induction of FOXC1 in BLBC is poorly understood.

A commonly accepted surrogate biomarker for BLBC is epidermal growth factor receptor (EGFR), which is abnormally activated by overexpression or constitutive mutation in many epithelial tumors. EGFR is widely used together with several other proteins in immunohistochemical detection of BLBC tumors and its high expression is associated with

poor prognosis.^{17,18} Numerous lines of evidence have shown the critical role of EGFR in cancer cell functions. It is still not clear whether EGFR and other BLBC-related genes form signaling pathways or networks dictating BLBC traits. Because both FOXC1 and EGFR are critical markers and functional regulators for BLBC, we hypothesize that EGFR may crosstalk with FOXC1 and that EGFR/FOXC1 signaling may orchestrate BLBC cellular traits. Our studies corroborate the association of EGFR and FOXC1 in human breast cancers. We demonstrate that EGFR activation can potently increase FOXC1 expression through ERK and Akt pathways in BLBC cells. This mechanism integrates the function of several key molecules that have been implicated in the regulation of human BLBC cells. We also delineate the role of FOXC1 in EGF-elicited cell functions. Taken together, our findings provide insight into the role of a novel EGFR/FOXC1 axis in BLBC pathogenesis.

MATERIALS AND METHODS

Detailed methods for in vitro migration/invasion, in vivo experiments, immunoblotting, and reverse transcription-PCR, and transfection are provided in the supplement.

Cell culture and cell proliferation assays

All cell lines were purchased from American Type Culture Collection. Cell proliferation was assessed by CellTiter-Glo Luminescent cell viability assay (Promega, Madison, WI). The 2-kb FOXC1-promoter from the transcription start site was cloned into the pGL4-luc vector (Promega). Details about the reagents are provided in the supplement.

Immunohistochemistry (IHC)—IHC in formalin-fixed breast cancer tissues was performed as described previously using a generated mouse monoclonal FOXC1 antibody.¹³

In vivo experiments—Animal studies were conducted with the approval of the institutional animal care and use committee. Details are described in the supplement.

Statistical analysis—All experiments were performed 3 times with samples measured in triplicate. Results are expressed as mean \pm standard deviation, unless otherwise stated. GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA) was used for statistical analysis. Correlation analysis between EGFR and FOXC1 expression in human cancer samples was analyzed for significance with Pearson *r* test, $P < 0.05$ was considered statistically significant.

RESULTS

FOXC1 expression correlates with EGFR expression in human BLBC

Because both FOXC1 and EGFR are critical markers and functional regulators of BLBC, we set out to assess the association between EGFR and FOXC1 expression. To this end, we performed IHC of EGFR and FOXC1 in 34 human triple-negative breast tumors. Quantitative IHC scoring showed that FOXC1 protein levels were significantly associated with EGFR protein levels ($P=0.0356$; Fig. 1A). The representative pictures for FOXC1 and EGFR IHC in breast cancer samples are shown in Fig. 1B. Consistent with this result, a

positive correlation between FOXC1 and EGFR mRNA in invasive breast cancers was observed in The Cancer Genome Atlas database (Fig. 1C).¹⁹

EGFR activation up-regulates FOXC1 expression in BLBC cells

Next, we examined whether EGFR activation regulates FOXC1 expression. EGFR-positive MDA-MB-468 and BT-20 cells were treated with EGF for 24 h. Immunoblotting showed that EGF treatment induced FOXC1 protein expression (Fig. 2A). Similar results were found in MCF-10A mammary epithelial cells (Fig. 2A). Transforming growth factor (TGF)- α and heparin-binding EGF-like growth factor (HB-EGF), ligands of the EGF family, also upregulated FOXC1 expression (Fig. 2A). Dose-response and time-course studies using MDA-MB-468 and MCF-10A cells confirmed that EGF is a potent inducer of FOXC1 expression (Fig. 2B). To examine whether EGFR activation is required for EGF-elicited FOXC1 up-regulation, we pretreated MDA-MB-468 cells with the small-molecule EGFR inhibitors AG1478, Lapatinib, or Erlotinib, followed by EGF treatment. Immunoblotting showed that EGFR inhibitors blocked the EGF induction of FOXC1 expression (Fig. 2C).

We then proceeded to investigate the mechanism for the EGF-induced up-regulation of FOXC1 expression. Using qRT-PCR, we found that EGF increased FOXC1 mRNA levels in MDA-MB-468 and MCF-10A cells (Fig. 2D). We then transiently transfected MDA-MB-231 cells, which normally express low levels of EGFR, with an EGFR construct. qRT-PCR analysis showed that EGFR overexpression also increased FOXC1 mRNA levels (Fig. 2E). To test whether EGFR activation induces FOXC1 at the transcriptional level, we pretreated MDA-MB-468 cells with the transcription inhibitor 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), followed by 6 h of EGF treatment. As shown in Fig. 2F, DRB blocked the EGF induction of FOXC1 mRNA levels, suggesting that EGF activates FOXC1 transcription.

To further explore the mechanism whereby EGF induces FOXC1 transcription, we generated a 2-kb FOXC1 promoter reporter construct. Luciferase assays with a panel of human breast cancer cell lines revealed that EGF potentiated FOXC1 promoter activity specifically in BLBC cells (Supplementary Fig. 1A), consistent with the notion that EGFR is preferentially expressed in BLBC cells. Using MDA-MB-468 and MCF-10A cells, we further showed that EGF increased FOXC1 promoter activity, but not pGL4-luc control vector activity (Supplementary Fig. 1B). In agreement with these results, EGFR overexpression in MDA-MB-231 cells also dramatically increased FOXC1 promoter activity (Supplementary Fig. 1C). As expected, the EGFR inhibitor AG1478 suppressed the EGF effect on the FOXC1 promoter (Supplementary Fig. 1D). Taken together, these results establish EGFR as a positive regulator of FOXC1 expression in breast cancer cells.

EGFR inhibition abrogates the growth of MDA-MB-468 xenograft mammary tumors

The antineoplastic and FOXC1-inhibitory activities of the EGFR inhibitor Gefitinib were evaluated in an in vivo xenograft model. The mammary glands of twenty *nu/nu* BALB/c mice were subcutaneously injected with MDA-MB-468 cells. When tumors grew to ~ 150 mm³, the mice were randomized to receive treatment with vehicle or Gefitinib (100 mg/kg/d) for 20 days (n=10). Tumors were harvested 8 days after treatment cessation. As

expected, Gefitinib abrogated the growth of the xenograft tumors (Fig. 3A and B). Immunoblotting of tumor cell lysates showed pronounced decreases in phosphorylated EGFR and FOXC1 levels in the treatment group compared with the control group (Fig. 3C). The body weight, motor activity, and feeding behavior of the mice showed no significant differences between the drug-treated and control groups (data not shown). These results indicate that EGFR inhibition represses FOXC1 expression *in vivo*.

EGFR activation induces FOXC1 through the Ras/ERK and phosphatidylinositol 3-kinase (PI3K)/Akt pathways

It is well-established that Ras/ERK and PI3K/Akt are two major signaling pathways downstream of EGFR.²⁰ Indeed, EGF treatment of MDA-MB-468 cells activated ERK and Akt (Supplementary Fig. 2A), while the EGFR inhibitor AG1478 abolished the effect of EGF (Supplementary Fig. 2A). Immunoblotting showed that inhibition of ERK and PI3K by the respective small-molecule inhibitors U0126 and LY294002 blocked the EGF induction of FOXC1, while JNK inhibition by SP600125 did not (Fig. 4A). Likewise, luciferase reporter assays indicated that ERK and PI3K inhibition, not JNK inhibition, suppressed the EGF-elicited FOXC1 promoter activity (Fig. 4B). A specific Akt inhibitor AI-IV yielded a similar effect (Fig. 4C). Notably, simultaneous blockade of ERK and Akt exerted a more pronounced inhibitory effect on the EGF-mediated increase of FOXC1 promoter activity (Fig. 4C) and mRNA levels (Fig. 4D). The ERK signaling pathway is known to play a critical role in tumor cell function.²¹ BLBC has been shown to harbor high ERK activity and was sensitive to small-molecule MEK inhibitors *in vitro* and *in vivo*.^{22,23} To further elucidate the involvement of ERK in FOXC1 expression, we transfected ERK2 into MDA-MB-231 cells. It was found that ERK2 overexpression markedly increased FOXC1 promoter activity (Supplementary Fig. 2B), mRNA levels (Supplementary Fig. 2C), and protein levels (Supplementary Fig. 2D). In agreement with these results, overexpression of the ERK activator H-Ras induced FOXC1 promoter activation in MDA-MB-231 cells (Supplementary Fig. 2E).

The PI3K/Akt pathway is highly activated in BLBC due to a loss of *PTEN* and inositol polyphosphate-4-phosphatase-II (*INPP4B*) or amplification of *PIK3CA*.¹⁹ Akt3 is reportedly upregulated and hyperactive in ER-negative breast cancers compared with other breast cancers.^{24,25} To further ascertain the role of PI3K/Akt signaling in FOXC1 expression, we overexpressed a constitutively active myristoylated (Myr) PI3K catalytic subunit p110 α in MDA-MB-231 cells. The mutant p110 α dramatically enhanced FOXC1 promoter activity (Supplementary Fig. 2F). Similarly, overexpression of a hyperactive Akt1 or Akt3 (Myr-Akt) containing a myristolation membrane-targeting sequence also induced FOXC1 promoter activity (Supplementary Fig. 2G), mRNA levels (Supplementary Fig. 2H), and protein expression (Supplementary Fig. 2I). Taken together, these data indicate that ERK and Akt activation is sufficient to elicit FOXC1 transcription.

FOXC1 plays an essential role in EGF-mediated cell proliferation, migration, and invasion

To address the question whether FOXC1 plays an important role in EGF-regulated cell function, we used control or FOXC1 siRNA to inhibit endogenous and EGF-induced FOXC1 levels in basal-like MDA-MB-157 and BT-20 breast cancer cells. Immunoblotting

confirmed the knockdown of FOXC1 (Fig. 5A). Control or FOXC1 siRNA-transfected cells were then stimulated with EGF. Cell viability assays showed that FOXC1 knockdown impeded EGF-elicited cell proliferation (Fig. 5B). Given these results, we examined whether FOXC1 is essential for EGF-regulated, cell cycle-associated gene expression.

Immunoblotting showed that overexpression of FOXC1 increased c-Myc and cyclin D1 levels (Supplementary Fig. 3A) while knockdown of FOXC1 impaired the induction of c-Myc, cyclin D1, E2F1, and p-Rb by EGF (Supplementary Fig. 3B). These genes are well-documented to be critically involved in cell cycle progression.²⁶⁻²⁸ Collectively, these data suggest that FOXC1 is essential for EGF-induced cell proliferation.

To study the role of FOXC1 in EGF-elicited cell migration and invasion, we performed FOXC1 knockdown in MDA-MB-468 cells. In these cells, EGF treatment induces EMT and migration.²⁹ As expected, FOXC1 siRNA transfection prevented the EGF induction of FOXC1 in MDA-MB-468 cells (Fig. 5C). Boyden chamber assays showed that FOXC1 knockdown abolished the increase of cell migration and invasion by EGF (Fig. 5D). We next examined the expression of matrix metalloproteinases (MMPs), as they are major proteases involved in cancer cell invasion.³⁰ As shown in Supplementary Fig. 3C, overexpression of FOXC1 increased MMP-2 and MMP-9 levels. Conversely, knockdown of FOXC1 abrogated EGF-induced MMP-2 and MMP-9 expression and activity (Supplementary Fig. 3D, E). Of note, MMP-7 has also been shown to be regulated by FOXC1.³¹ Together, these results indicate that FOXC1 orchestrates EGF-elicited cell migration and invasion.

DISCUSSION

In this study, we show that EGFR activation up-regulates FOXC1 expression via ERK- and PI3K/Akt-mediated activation in BLBC. We demonstrate that FOXC1 is required for EGF-elicited cell proliferation, migration, and invasion. These findings uncover a regulatory mechanism of FOXC1 expression and an important role of the EGFR-FOXC1 signaling axis in BLBC cell function. Although EGFR has been widely accepted as a biomarker for BLBC, it is not well understood how EGFR modulates the basal-like phenotype. The induction of FOXC1 by hyperactive EGFR provides new insight into the aggressive behaviors of these cancers.

A critical issue arising from this study is the specificity of the Ras/ERK and PI3K/Akt pathways in the EGF/EGFR regulation of FOXC1. Many receptor tyrosine kinases (RTKs) such as HER-2 act through these pathways. Interestingly, FOXC1 levels are low in HER-2 (+) and luminal breast cancers, but high in BLBC. This unique expression pattern of FOXC1 may be attributed to the combined effect of several regulatory mechanisms. First, the FOXC1 promoter region is hypermethylated in ER+ breast cancer,³² which may explain why FOXC1 levels are suppressed in non-basal tumors despite ERK and Akt activation. In contrast, basal-like tumors possess lower frequency of aberrant promoter methylation compared to other subtypes, and FOXC1 gene methylation is absent in basal-like tumors.^{6,33} This may lead to chromatin configuration permissive of transcription factor binding elicited by growth factor signaling. Notably, recent studies have shown that a small

subset of breast cancer cells, called luminobasal and basal-HER2 cells,^{34,35} exhibit the basal phenotype and express high levels of FOXC1 (X. Cui, unpublished data).

Second, PI3K/Akt and Ras/ERK activation is more prevalent in ER- /PR- and basal-like tumors.^{14,22,24,27} FOXC1 may serve as an indicator for high PI3K/Akt and Ras/ERK activities in BLBC. Of note, FOXC1 levels were low in some EGFR-positive BLBC cells. This might be due to the inactivation of EGFR in these cells or interrupted signal transduction downstream of EGFR, preventing transmission of signals from EGF/EGFR to the FOXC1 promoter. In addition, activation of ERK and Akt by other RTKs in BLBC may explain why FOXC1 is highly expressed in some EGFR-negative cases. Lastly, EGFR induces NF- κ B and other transcription factors, some of which are preferentially activated in BLBC.^{36,37} Whether these transcription factors dictate the effect of Akt and ERK on FOXC1 in BLBC remains to be determined.

Recent studies show that the PKC and CARMA3 pathways are involved in EGFR-induced effects in cancer cells.^{38,39} It is unknown whether their levels correlate with BLBC. PKC can act downstream or upstream of Ras, inducing ERK and Akt activation. We found that PKC is not involved in the EGF regulation of FOXC1 (Y. Jin, unpublished data).

It is known that cyclin D1 and c-Myc are instrumental in EGF-elicited tumor cell proliferation. Numerous studies suggest that MMPs are critical for EGF-elicited cancer cell invasion and metastasis. Our results implicate FOXC1 as an important mediator for the EGF effect on these genes and BLBC cell functions. EGFR-directed drugs have been evaluated in breast cancer clinical trials; however, the results were disappointing.⁴⁰ There is a need to exploit new predictive biomarkers for EGFR-targeted therapy to identify those patients with “EGFR-addicted” breast cancers dependent on this signaling for growth and progression. Our results might implicate FOXC1 as a read-out of EGFR activity and a useful marker in this regard. We propose that intervention of the EGFR/FOXC1 pathway may provide effective modalities for the treatment of BLBC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Synopsis

FOXC1 expression is regulated by EGFR activation via Akt and ERK signaling, and FOXC1 mediates the effect of EGF/EGFR in human basal-like breast cancer cells.

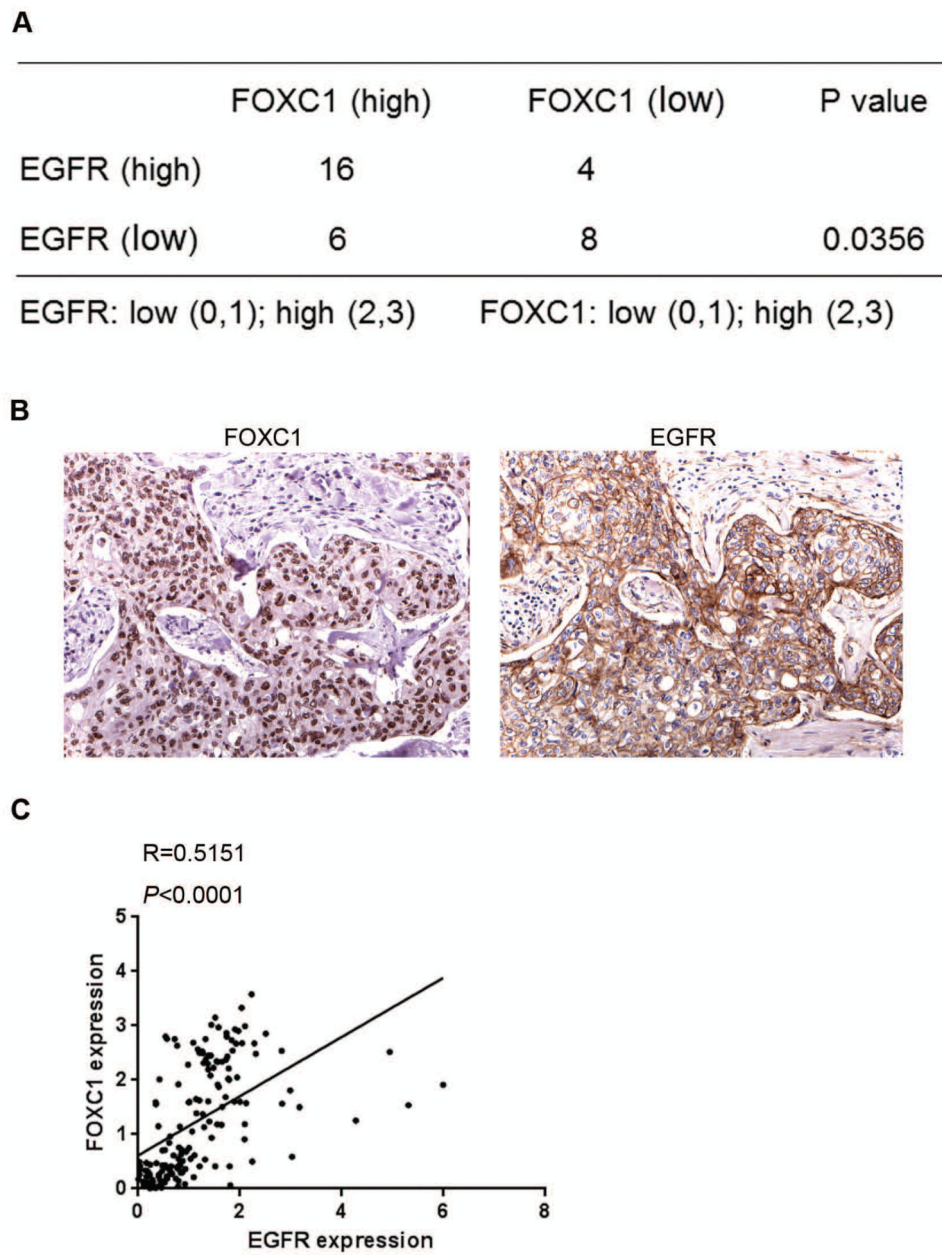


Figure 1. FOXC1 expression significantly correlates with EGFR expression in human BLBC tumors

(A) Distribution of EGFR and FOXC1 by immunohistochemical staining scores in 34 human triple-negative samples ($P=0.0356$). Association was examined using the Fisher's Exact Test. (B) Representative images for FOXC1 and EGFR IHC in a human breast tumor. Magnification: $\times 400$. (C) Correlation between EGFR and FOXC1 mRNA expression in invasive breast cancer samples from the TCGA database ($R=0.5151$, $P<0.0001$).

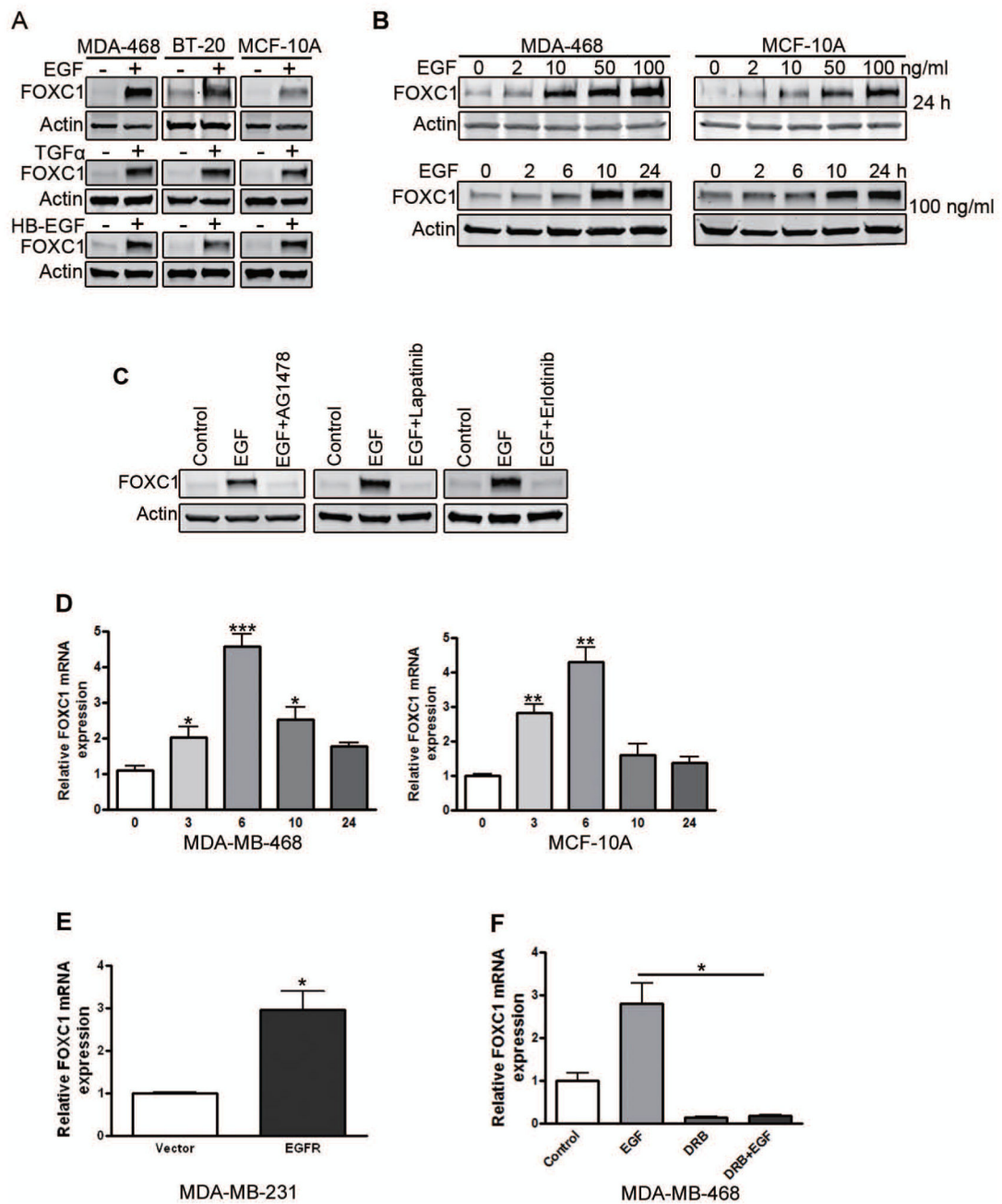


Figure 2. EGFR activation up-regulates FOXC1 expression in BLBC cells

(A) MDA-MB-468 and BT-20 EGFR-positive human breast cancer cells and MCF-10A human mammary epithelial cells were serum-starved overnight and treated with EGF, TGF α , or HB-EGF (50 ng/ml) for 24 h. FOXC1 level was analyzed by immunoblotting. Actin was used as a loading control. (B) Serum-starved MDA-MB-468 cells were treated with increasing concentrations of EGF for 24 h (top) or with EGF (100 ng/ml) for the indicated time periods (bottom), followed by immunoblotting analysis. (C) MDA-MB-468 cells were serum starved overnight. Then cells were treated with EGF for 24 h in the presence or absence of the EGFR inhibitor AG1478 (1 μ M), Lapatinib (1 μ M) or Erlotinib (3

μM). FOXC1 protein levels were measured by immunoblotting. (D) MDA-MB-468 cells (left) and MCF-10A cells (right) were serum-starved overnight and treated with EGF for the indicated time periods. FOXC1 mRNA levels were analyzed by qRT-PCR. *, $P<0.05$; ***, $P<0.0001$; **, $P<0.001$. (E) MDA-MB-231 cells were transfected with pBABE-EGFR construct for 48 h. FOXC1 mRNA levels were detected by qRT-PCR. *, $P<0.05$. (F) MDA-MB-468 cells were serum-starved overnight. Then cells were treated with EGF for 6 h after DRB pretreated for 1 h. FOXC1 mRNA levels were detected by qRT-PCR. *, $P<0.05$. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Data represent mean \pm SD from 3 independent experiments.

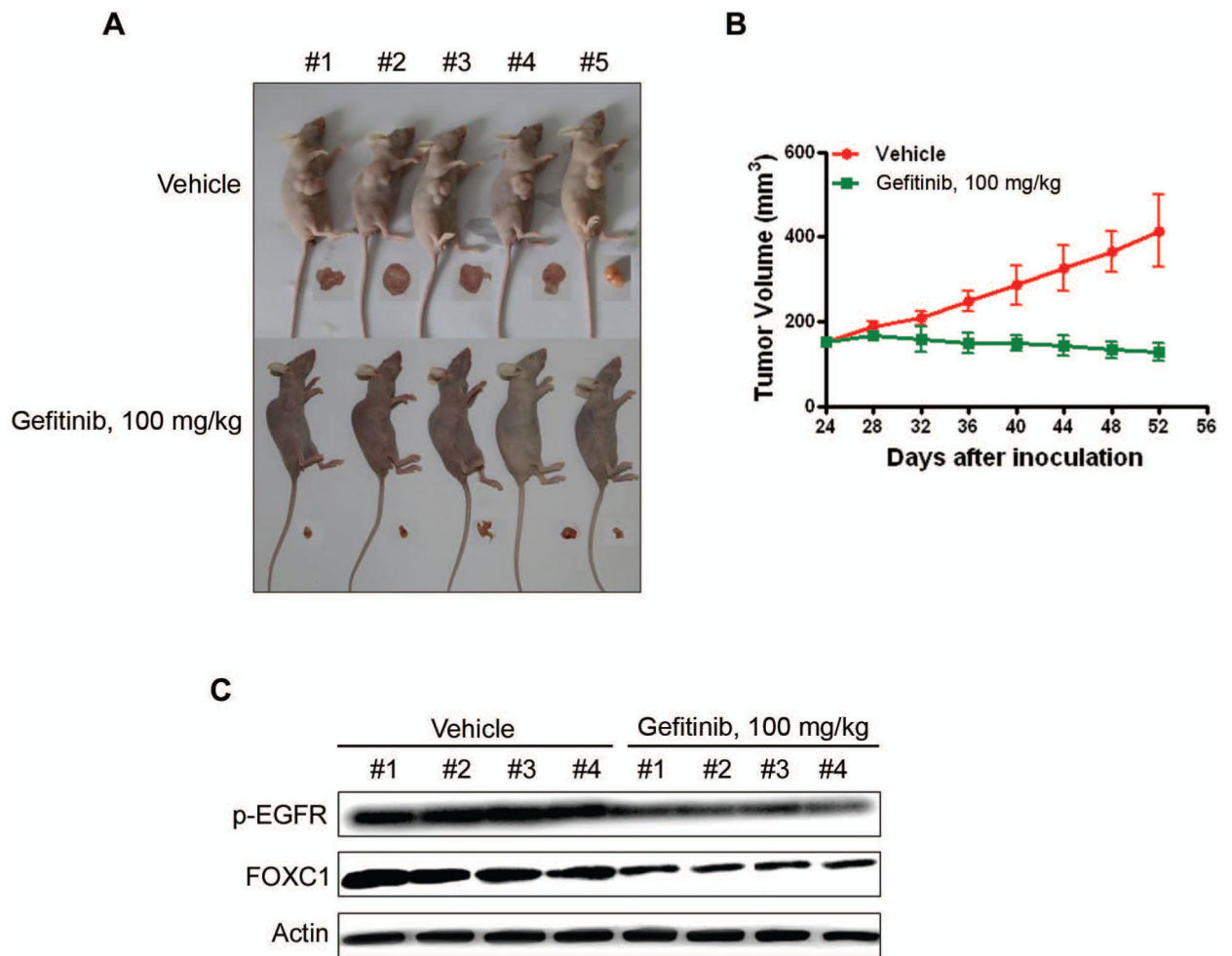


Figure 3. EGFR inhibitor Gefitinib abrogates the growth of xenograft mammary tumor in nude mice

BALB/c *nu/nu* nude mice were subcutaneously inoculated with MDA-MB-468 cells. When tumors reached ~150 mm³, these mice were randomized into 2 groups (n=10) for Iressa treatment. (A) Comparisons of mice and tumors of between the two groups are shown. (B) The tumor growth curves are plotted. Error bars represent 95% confidence intervals, $P < 0.05$. (C) The levels of p-EGFR and FOXC1 in the tumor tissues were detected by immunoblotting with the indicated antibodies.

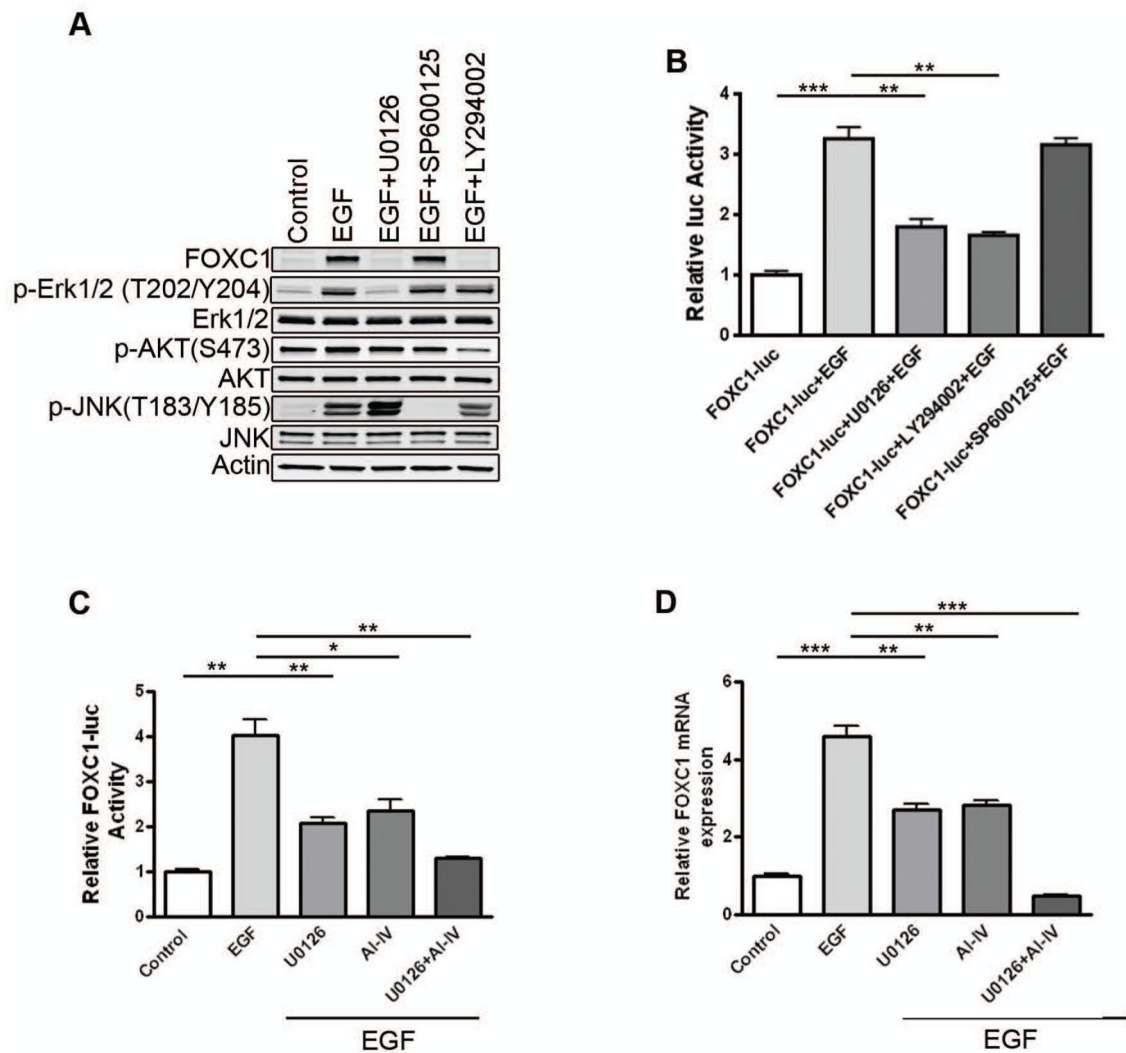


Figure 4. EGFR activation up-regulates FOXC1 expression through ERK and PI3K/Akt pathways

(A) MDA-MB-468 cells were serum starved overnight. Cells were treated with EGF for 24 h in the presence or absence of 5 μ M U0126 (MEK inhibitor), 20 μ M LY294002 (PI3K inhibitor), or 10 μ M SP600125 (JNKII inhibitor) after preincubation with these inhibitors for 30 minutes. p-ERK (T202/Y204), ERK, p-Akt (S473), Akt, p-JNK (T183/Y185), JNK, and FOXC1 levels were analyzed by immunoblotting. (B) MDA-MB-468 cells were transiently transfected with the FOXC1-luc and then pretreated with the inhibitors for 30 minutes followed by EGF treatment for 24 h in the presence or absence of these inhibitors, followed by luciferase assays. **, $P < 0.001$; ***, $P < 0.0001$. (C) MDA-MB-468 cells were transiently transfected with the FOXC1 promoter-luc and then pretreated with 5 μ M U0126, 1 μ M AI-IV (Akt inhibitor) or their combination for 30 minutes followed by EGF treatment for 24 h in the presence or absence of these inhibitors, followed by luciferase assays. *, $P < 0.05$; **, $P < 0.001$. (D) MDA-MB-468 cells were serum-starved overnight, then pretreated with 5 μ M U0126, 1 μ M AI-IV or their combination for 30 minutes followed by EGF treatment for 6 h

in the presence or absence of these inhibitors. FOXC1 mRNA levels were detected by qRT-PCR. **, $P < 0.001$; ***, $P < 0.0001$. Data represent mean \pm SD of 3 independent experiments.

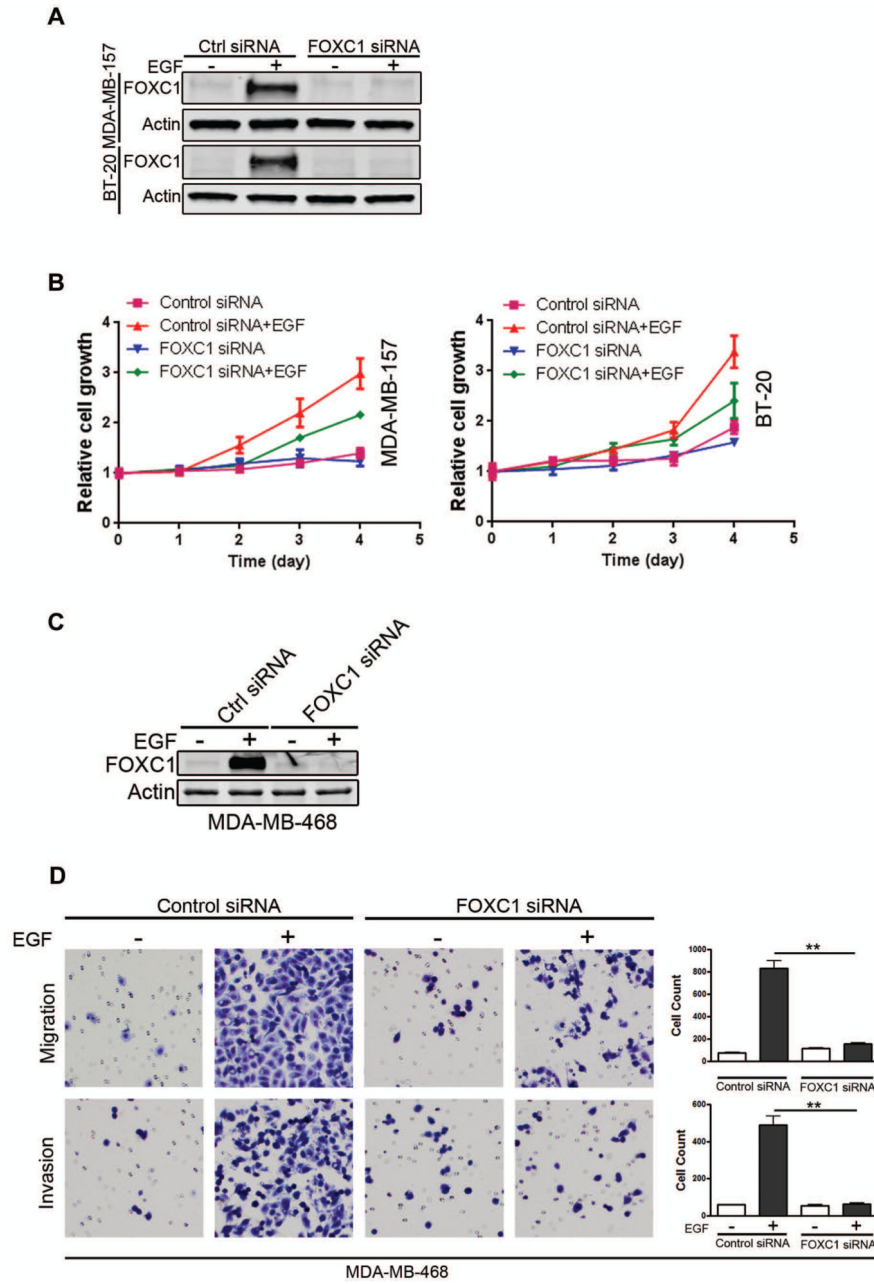


Figure 5. FOXC1 is essential for EGF-mediated cell proliferation, migration, and invasion (A) MDA-MB-157 or BT-20 cells were transiently transfected with control or FOXC1 siRNA for 24 h, and then treated with EGF for another 24 h after starvation overnight. FOXC1 knockdown was confirmed by immunoblotting. (B) Control or FOXC1-knockdown cells were stimulated with EGF and cell proliferation was evaluated by CellTiter-Glo assays at the indicated time points. Data represent 3 independent experiments. (C) MDA-MB-468 cells were transfected with control or FOXC1 siRNA for 24 h and then treated with EGF for 24 h. FOXC1 expression was assessed using immunoblotting. (D) Migration and invasion of control and FOXC1-knockdown cells were analyzed using transwell chamber assays.

Migrating or invasive cells were counted in 3 randomly selected fields. Data represent mean \pm SD. **, $P < 0.001$.