Supplementary materials and methods

Reagents

EGF was purchased from Sigma-Aldrich (St. Louis, MO). TGFα and HB-EGF were purchased from R&D Systems (Wiesbaden, Germany). The EGFR inhibitor AG1478, MEK inhibitor U0126, PI3K inhibitor LY294002, JNK inhibitor SP600125, and AKT inhibitor AI-IV were purchased from Calbiochem (Gibbstown, NJ). The EGFR inhibitors Lapatinib and Erlotinib were from purchased LC laboratories (Woburn, MA). Gefitinib was obtained from AstraZeneca. For control experiments, cells were incubated with the vehicle dimethylsulfoxide (DMSO) alone. Human FOXC1 siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human MMP-2 and MMP-9 Quantikine ELISA Kits were purchased from R&D system.

Real-time quantitative reverse transcription-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription was performed using the QuantiTect Reverse Transcription Kit. The qRT assay was done using an iCycler iQ Real-Time Thermocycler (Bio-Rad Laboratories, Hercules, CA). The following primers were used: *FOXC1*, Forward 5'-GGCAAAGAATTGATCCGGTA-3', Reverse 5'-TGGATGGCCATGGTGATGAGC-3'; *GAPDH*, Forward 5'-GATCGAATTAAACC TTATCGTCGT-3', Reverse 5'-AGCAGCAGAACTTCCACTCGGT-3', *GAPDH* was used as internal control.

Immunoblotting analysis

Immunoblotting analysis was performed using whole cell lysates prepared in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 10% glycerol) plus a protease inhibitor cocktail (Sigma, St Louis, MO). Nuclear protein was extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents from Pierce Biotechnology (Rockford, IL). Antibodies and their sources were as follows: phospho-EGFR (Y1068), EGFR, phospho–extracellular signal-regulated kinase (ERK; T202/Y204), ERK, phospho-Akt (S473), Akt, phospho–c-Jun NH2-terminal kinase (JNK; T183/Y185), JNK, E2F1, and phospho-Rb (S807/811) were from Cell Signaling Technology (Beverly, MA); β-actin, c-Myc, cyclin D1, cyclin A, cyclin E, p27, p21, MMP-2, and MMP-9 were from Santa Cruz Biotechnology (Santa Cruz, CA).

Transfection

MDA-MB-468 cells were transfected with the pGL4-FOXC1 promoter reporter construct and the β-galactosidase expression vector pSV-β-Gal (Promega Madison, WI) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the instruction manual. β-Galactosidase enzyme activity was detected using the β-Galactosidase enzyme assay system with reporter lysis buffer (Promega, Madison, WI). For co-transfection, 500 ng of a constitutively active p110α (PI3K 110 kDa catalytic subunit), Flag-ERK2, HA-Myr-Akt, pBABE-EGFR, or pBABE-EGFRvIII constructs were added along with 100 ng of the 2-kb (relative to the transcription start site) human FOXC1 promoter reporter construct pGL4-FOXC1. For the small interfering RNA (siRNA) experiment, MDA-MB-468 cells were transfected with 30 nM human FOXC1 siRNA for 48 h, and then treated with EGF for 24 h.

Immunohistochemistry (IHC)

We performed IHC analysis on formalin-fixed paraffin embedded (FFPE) tissue sections by using a highly sensitive streptavidin-biotin-peroxidase detection system. The sensitivity and specificity of the generated anti-FOXC1 monoclonal antibody against the FOXC1 N terminus were tested using western blotting and immunofluorescence of vector- and FOXC1-transfected breast cancer cells. This translational study was performed with institutional review board approval. Tissues were sectioned into serial 5-μm-thick tissue sections and subjected to IHC analysis for EGFR (Cell Signaling Technology) and FOXC1 expression. Semi-quantitative analysis was performed by one pathologist who scored the intensity of immunoreactivity on a scale of 0 (no staining) to 3 (strong staining). EGFR stains were considered positive if any membranous and/or cytoplasmic staining of invasive carcinoma cells was observed. FOXC1 protein expression status was considered positive only if any nuclear staining of tumor cells was observed and assigned a score of 1, 2, or 3 based on signal intensity. The Fisher's Exact Test was used to determine the correlation of EGFR and FOXC1 staining.

Cell migration and invasion assays

Cell migration was measured by transwell chamber assays. Cell invasion was measured by using BD BioCoat Matrigel Invasion Chambers (BD Biosciences, San Jose, CA). Briefly, 5×10^4 cells per well in serum-free DMEM medium were placed in the upper chamber. DMEM plus EGF was added in the lower chamber as a source of chemoattractants. Cells were allowed to migrate or invade through an 8-μM pore membrane for 36 h at 37˚C. Migratory or invasive cells on the lower surface were fixed

and stained with the HEMA3 Kit (Fisher, Middletown, VA), followed by counting under a light microscope.

Enzyme-linked immunosorbent assay (ELISA)

The human MMPs immunoassay was performed using the Quantikine ELISA Kit (R&D Systems) according to the manufacturer's instructions. Cell culture supernatant from control or FOXC1 siRNA knockdown cells treated with EGF was used for detection of the amount of secreted MMPs.

In vivo experiments

Animal studies were conducted with the approval of the Cedars-Sinai Medical Center and Shantou University Medical College Animal Care and Use Committees in accordance with the National Institutes of Health guidelines. An amount of 5×10^6 MDA-MB-468 cells in 200 µl PBS was inoculated subcutaneously into the flank of 6-week-old female nude mice. Tumors were measured every other day with calipers. Tumor volumes were calculated by the following formula: $a^2 \times b \times 0.5$, where a is the smallest diameter and b is the diameter perpendicular to a. Gefitinib was dissolved in 0.5% Tween-80 in sterile double-distilled water (vehicle) and kept at 4 ^{o}C. When the tumors grew to ~150 mm^3, the mice were randomly separated into 2 groups. Mice in each group were treated once daily by oral gavage with Gefitinib (100 mg/kg) or the same amount of vehicle for 20 days. Tumor xenografts were immediately removed after cessation of drug treatment for 8 days. The body weight, feeding behavior, and motor activity of each animal were monitored as indicators of general health.

Supplementary Figure Legends

Figure 1. EGF stimulation activates FOXC1 promoter in BLBC cell lines. (A) Human BLBC cells (MDA-MB-468 and BT-20), Luminal cancer cells (MCF-7 and T47D), and HER2 positive cells (BT474) were transiently transfected with the FOXC1 promoter reporter construct and treated with EGF for 24 h. Luciferase assays were performed, ****, $P<0.0001$. (B) MDA-MB-468 (left) and MCF-10A cells (right) were transiently transfected with the FOXC1 promoter-luc (see the supplement for details) or the vector pGL4-luc, and then treated with EGF for 24 h. Cell lysates were subjected to reporter luciferase assays, *, $P<0.05$; ***, $P<0.0001$. (C) MDA-MB-231 cells were transiently co-transfected with the FOXC1 promoter-luc and pBABE-EGFR construct for 24 h, and FOXC1 promoter activity was assessed by luciferase assays. ***, $P<0.0001$. (D) MDA-MB-468 cells were transiently transfected with the FOXC1 promoter-luc. After pretreatment with AG1478 (1 μM) for 1 h, cells were treated with EGF in the presence or absence of AG1478 for 24 h, followed by luciferase assays. **, $P<0.001$.

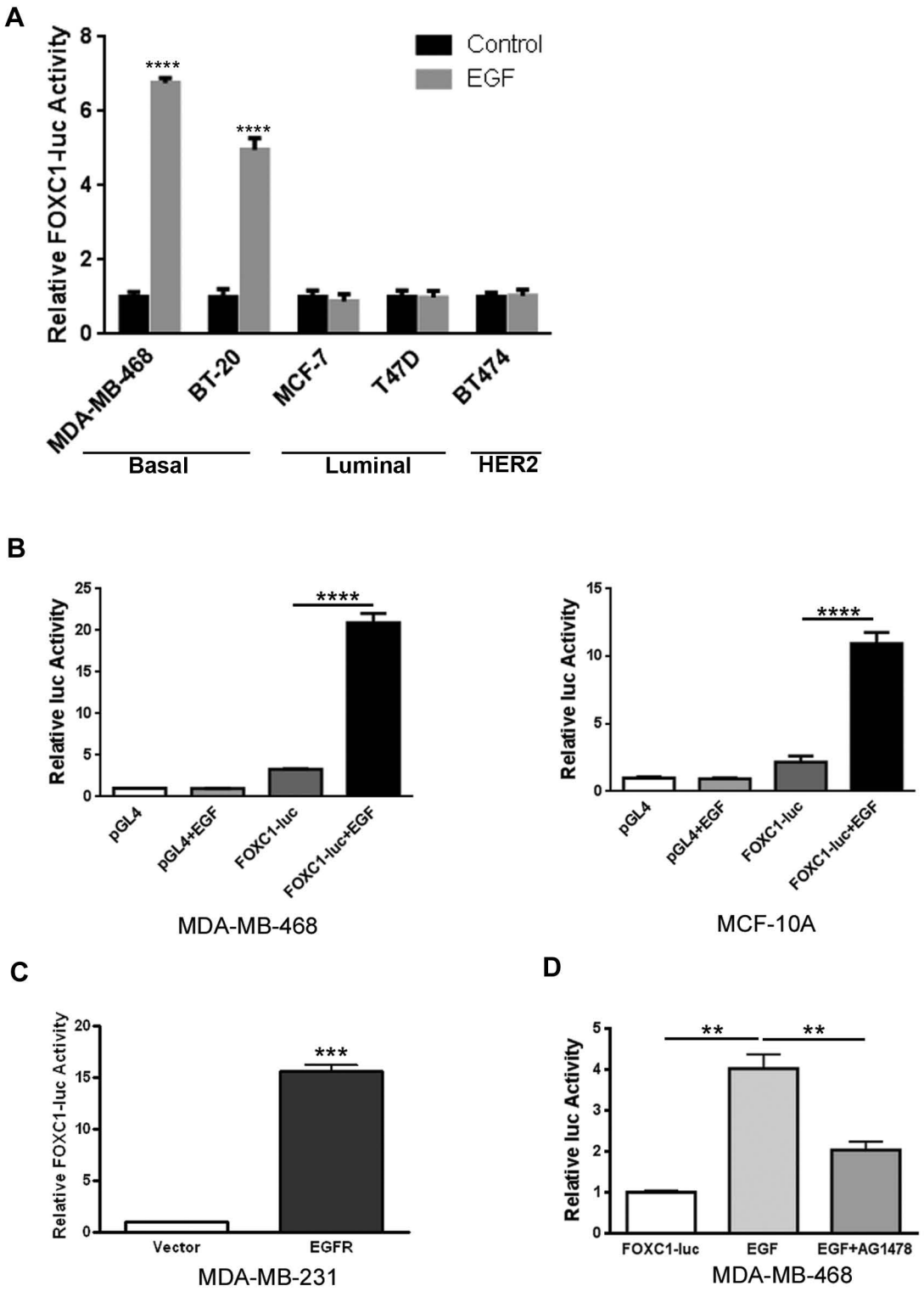
Figure 2. EGFR activation regulates FOXC1 levels through ERK and Akt pathways. (A) MDA-MB-468 cells were serum-starved overnight. After AG1478 treatment for 1 h, cells were stimulated with EGF for 24 h in the presence or absence of AG1478. (B) MDA-MB-231 cells were transiently co-transfected with the FOXC1 promoter reporter construct and the Flag-tagged ERK2 construct for 24 h, followed by luciferase assays. ****, $P<0.0001$. (C) MDA-MB-231 cells were transfected with the Flag-ERK2 construct for 24 h. FOXC1 mRNA expression levels were measured by qRT-PCR. **, $P<0.001$. (D) MDA-MB-231 cells were transfected with the Flag-ERK2 construct for 48 h. Levels of p-

ERK (T202/Y204), ERK, and FOXC1 were analyzed by immunoblotting. (E) MDA-MB-468 cells were transiently co-transfected with the FOXC1 promoter-luc and the H-Ras construct for 24 h, followed by luciferase assays. ****, $P<0.0001$. (F) MDA-MB-231 cells were transiently co-transfected with the FOXC1 promoter-luc and the constitutively active p110α construct (the PI3K catalytic subunit) for 24 h, followed by luciferase assays. **, $P<0.001$. (G) MDA-MB-231 cells were co-transfected with the FOXC1 promoter-luc with either the control or a constitutively active HA-Myr-Akt expression construct for 24 h, followed by luciferase assays. ****, $P<0.0001$. (H) MDA-MB-231 cells were transfected with a constitutively active HA-Myr-Akt construct for 48 h. FOXC1 mRNA expression was detected by qRT-PCR. **, $P<0.001$. Data represent mean ± SD from 3 independent experiments. (I) MDA-MB-231 cells were transfected with the constitutively active HA-Myr-Akt construct for 48 h. Levels of p-Akt (S473), Akt, and FOXC1 were analyzed by immunoblotting. Data represent mean ± SD from 3 independent experiments.

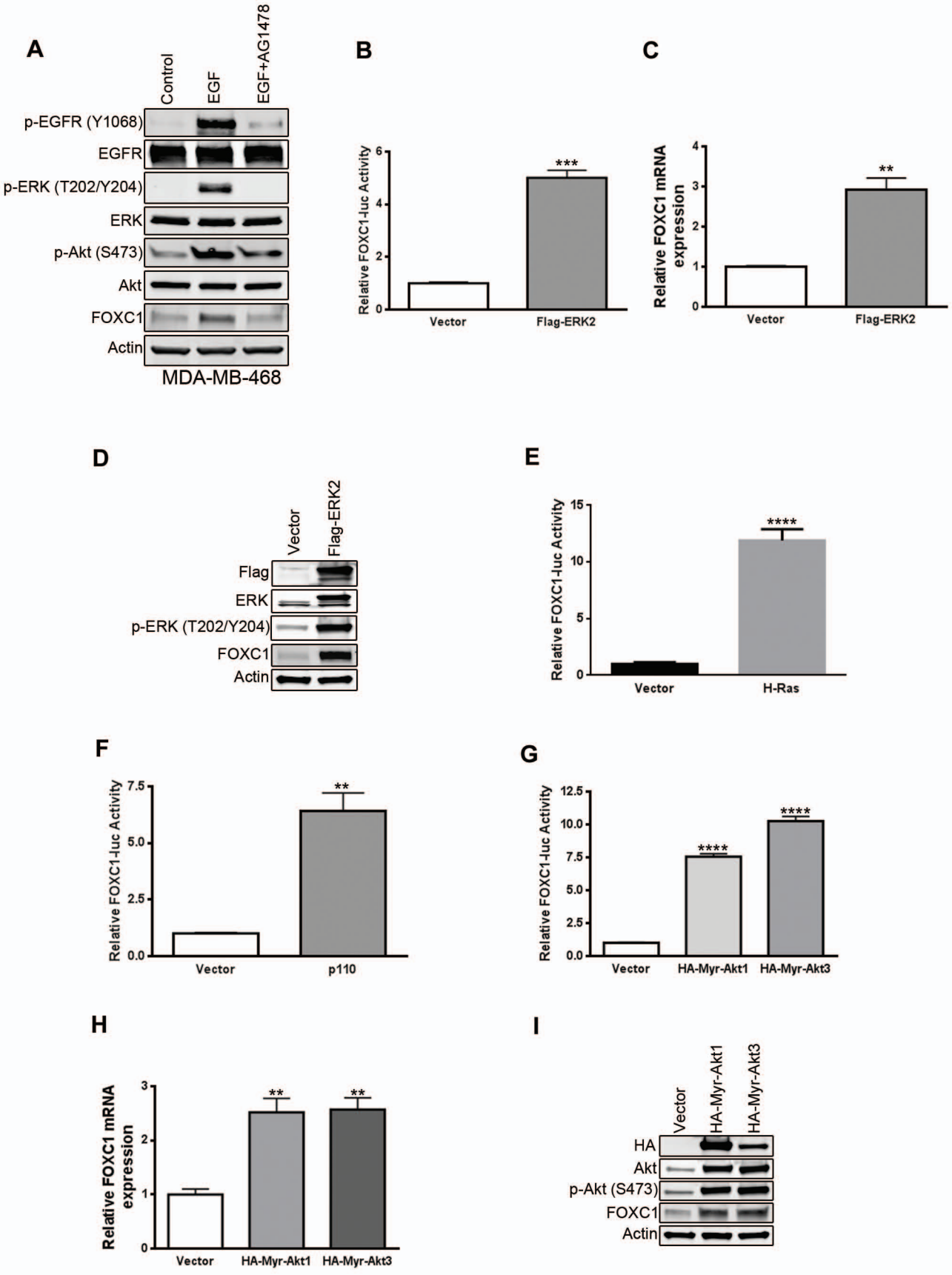
Figure 3. Overexpression of FOXC1 in human breast cancer cells increases expression of cell cycle-related proteins and MMPs. (A) c-Myc and cyclin D1 levels were measured by immunoblotting in control and FOXC1-overexpressing MDA-MB-157 and BT-20 cells. (B) MDA-MB-157 cells or BT-20 cells were transiently transfected with control or FOXC1 siRNA for 24 h and treated with EGF for another 24 h, cell cycle-related proteins were detected by immunoblotting. (C) MMP-2 and MMP-9 levels were determined by immunoblotting in control and FOXC1-overexpressing MDA-MB-468 and MDA-MB-231 cells. MDA-MB-468 cells were treated as in (Fig. 5C). MMP-2 and

MMP-9 protein levels and activities were detected by immunoblotting (D) and ELISA (E), respectively. **, P=0.0018 for MMP-2, **, P=0.0023 for MMP-9.

Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3