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Targeting estrogen mediated CYP4F2/CYP4F11-20-HETE metabolic disorder decelerates tumorigenesis in ER+ breast cancer

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ABSTRACT

Purpose: As the most common subset of breast cancer (BC), estrogen receptor positive (ER+) BC accounting for 80% of cases, has become a global public health concern. The female hormone estrogen (E2) unequivocally drives ER + breast malignancies. The reasons that estrogen affects BC development has long been considered, yet further study remains to be conducted of the molecular events in the E2-estrogen receptor α (ERα) signaling pathway in ER + BC progression, especially lipid metabolism, so providing more options for tailored and individualized therapy. Our aim is to find out new targets and clinical biomarkers for ER + breast cancer treatment from the perspective of lipid metabolism.

Methods: Lipid metabolomics profiling was used to examine the membrane phospholipid stimulated by E2. Clinical BC samples were used to assess the association of CYP4F2, CYP4F11 expression with clinicopathological characteristics and patient outcomes. Some inhibitors of main enzymes in AA metabolism were used combined with E2 to assess roles of CYP4F2/CYP4F11 in the progression of ER + BC. CYP4F2, CYP4F11 overexpression and knockdown BC cell lines were employed to examine the effects of CYP4F2, CYP4F11 on cellular proliferation, apoptosis and tumor growth. Western blotting, qPCR, Immunohistochemical staining and flow cytometry were also conducted to determine the underlying mechanisms related to CYP4F2, CYP4F11 function.

Results: The activation of the CYP450 signaling pathway in arachidonic acid metabolism contributed to ER + BC tumorigenesis. In ER + BC, CYP4F2 and CYP4F11 overexpression induced by E2 could promote cancer cell proliferation and resistance to apoptosis by producing the metabolite 20-HETE and activating the antiapoptotic protein Bcl-2. CYP4F2 and CYP4F11 elevation correlates with poorer overall survival and disease-free survival in ER + BC patients.

Conclusion: CYP4F2, CYP4F11 and their metabolite 20-HETE could serve as effective prognostic markers and attractive therapeutic targets for novel anticancer drug development about ER + BC.

1. Introduction

According to Wilcock and Webster [1], breast cancer has the highest incidence and mortality rate among all female malignancies [1]. In 2020, there were 2.26 million new cases of BC worldwide and 680,000 deaths. Based on hormone receptor status and histological markers, BC can be divided into four subtypes: luminal A, luminal B, HER2 enriched, and triple-negative breast cancer (TNBC) [2,3]. Both luminal types account for a large proportion of BCs and generally have high ER expression. Estrogen is required for mammary gland development and

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unequivocally drives the majority of breast malignancies (~80%) [4]. It was proven that higher serum estradiol increases BC risk up to three fold [5]. Hence, the E2-ER pathway has long been regarded as an important oncogenic pathway in BC development, and several endocrine therapies, such as aromatase inhibitors (AIs) and selective estrogen receptor (ER) degraders, were invented based on the rationale for *anti*-ER treatment [6]. Although endocrine therapy alone or combined with CDK4/6 inhibitors, mTOR inhibitors [7,8], PI3K inhibitors, reduce the recurrence and mortality rate of ER + BC patients, acquired drug resistance is still an obstacle [9,10], and up to 24% of patients still experience recurrence at 10 years [11,12]. This suggests that an in-depth understanding of the E2-ER pathway mechanism and synergistic effects with other molecular pathways is of great importance for developing novel therapeutic strategies.

Recently, with the development of lipid metabolomics, the study of lipid metabolism in tumors has become more in-depth [13]. Arachidonic acid (AA) is a kind of ω-6 eicosatetraenoic acid that is produced by membrane phospholipids catalyzed by phospholipase. AA metabolism is excessively and abnormally activated in various tumors [14–16], and it mainly includes three branches, namely, the COX [17], LOX [18,19] and CYP450 [20,21] pathways. Among these, CYP4F enzymes can biosynthesize 20-hydroxyeicosatetraenoic acid (20-HETE) [22,23], an important eicosanoid that regulates angiogenesis and metastasis in cancer development [24,25]. CYP450 enzymes and their metabolites could promote tumor development and enhance the antiapoptotic ability of tumor cells [26,27]. But there has been little research on the relationship between estrogen and AA metabolism, especially CYP450 metabolism in ER + BC.

In this study, our work is aimed at discovering new targets belong to CYP450 metabolism regulated by E2-ER signaling, and determining new prognostic biomarkers for ER + breast cancer treatment.

2. Materials and methods

2.1. Cell lines and cell culture

Human BC cell lines (MCF-7, T47D, BT474, MDA-MB-231, MDA-MB-468 and MDA-MB-453) were purchased from American Type Culture Collection (ATCC) and cultured in DMEM or RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin and streptomycin. All cells were maintained at 37 °C with 5% CO2.

2.2. Antibodies and reagents

The antibodies we used were as follows: *anti*-CYP4F2 (Abcam # ab230709), *anti*-CYP4F11 (Proteintech # 20012-1-AP), *anti*-ERα (H184) (Santa Cruz# sc-7207), *anti*-BCL2 (CST), anti-Cl-caspase 3 (CST), *anti*-β-Actin (CST), anti-Cl-PARP (CST), *anti*-P-Erk (CST), 17-β-estradiol (Sigma–Aldrich), NS398 (Cayman Chemical), indomethacin (Sigma–Aldrich), estradiol cypionate (HY–B1100), HET0016 (ApexBio, C5344), and 20-HETE (Cayman, 90030).

2.3. Construction of small hairpin RNAs and lentiviral infection

Short hairpin RNA (shRNA) sequences targeting endogenous CYP4F2 or CYP4F11 were purchased from TsingKe. To generate lentiviruses, HEK293T cells were transfected with CYP4F2 or CYP4F11 shRNA or the nontargeting shRNA vector Plko.1-EGFP-PURO in combination with Fugene transfection reagent (Promega) and the PLP1/PLP2/VSVG system. Scrambled shRNA used as negative control. The target cells infected with recombinant lentiviruses were subjected to 1 μg/mL puromycin selection.

2.4. Stable overexpressing upregulation

To generate stable overexpression cell lines, pcDNA specifically expressing human CYP4F2 or CYP4F11 was cloned into the pCDH-CMV-MCS-EF1-GFP + Puro vector and used to overexpress CYP4F2 and CYP4F11. Empty plasmid used as negative control. The target cells infected with recombinant lentiviruses were subjected to 1 μg/mL puromycin selection.

2.5. siRNA and plasmid transfection

siRNAs and plasmids were synthesized by Gene Pharma (Suzhou, China). For overexpression, the full-length target genes were inserted into pcDNA3.1. Plasmid or siRNA was transiently transfected into BC cells using Lipofectamine 2000 (Invitrogen) and Opti-MEM medium. The siRNA sequences targeting endogenous human ERα, CYP4F2, and CYP4F11 are described in Supplementary Table 2.

2.6. RT–qPCR

Total RNA was isolated from tissue samples or cultured cells with an RNA extraction kit (TianGen, DP430, and DP431). Two micrograms of RNA were reverse transcribed to cDNA using All-in-One cDNA Synthesis SuperMix (Bimake, B24408) and then subjected to RT–qPCR using Super Real Premix Plus (SYBR Green) (TianGen, FP205). β-Actin was used as an internal control. The $2^{-\Delta\Delta Ct}$ method was applied to quantify gene expression. The primers used for RT−qPCR was listed in Supplementary Table 1.

2.7. Cell viability assay

Cell viability was determined by MTT assay (Sigma–Aldrich). Cells were cultured in 96-well plates. After incubation for 12 h, the cells were treated with E2 or inhibitors. At the end of the treatment, MTT reagent was added to each well, and the cells were incubated for 4 h at 37 °C in the dark. After the supernatants were aspirated, DMSO was added to each well. The absorbance at a wavelength of 570 nm was finally measured using a Tecan InfiniteF50 microplate reader.

2.8. Cell death assay

Cell death was measured using a PI exclusion assay and crystal violet staining. Cells were plated in the 96-well black plates and incubation with 100 μL propidium iodide (5 μM) in PBS for 45 min at 37 °C. Fluorescence read at 530 nm/620 nm. After that, cells were fixed with 100 μL of formalin for 5 min then aspirate formalin and add 100 μL crystal violet to each well for cell number normalization. Data represented as percentage of dead cells relative to the total number of cells as detected by crystal violet staining.

2.9. Animal studies

All animal studies were approved by the Institutional Animal Care and Use Committee at Nankai University. Female BALB/c nude athymic mice (Vital River Beijing, China) aged 6–8 weeks were used in all studies. Starting one week before MCF-7 cancer cell line injection, the mice were treated with estradiol cypionate (2 mg/kg in corn oil, MCE, Shanghai, China, Cat: HY-B1100) subcutaneously at the nape of the neck every week[28]. In tumorigenesis assays, 5×10^6 MCF-7-vec/MCF-7--CYP4F2/MCF-7-CYP4F11 cells were suspended in 100 μL of PBS/Matrigel (1:1) and injected subcutaneously. In lung colonization assay, MCF-7, MCF-7-shCYP4F2, and MCF7-shCYP4F11-luciferase–expressing cells were intravenously inoculated into mice pretreated with E2 and examined by bioluminescent imaging in the chest regions. Animal studies were performed according to the Guidelines on Laboratory Animals of Nankai University and were approved by the Institute Research Ethics Committee at Nankai University (No: 2021-SYDWLL-000464).

2.10. Bioluminescent imaging

Prior to imaging, mice were given D-luciferin (120 mg/kg, i.p.; PerkinElmer Inc.; #122799). Bioluminescence signals were recorded using the Xenogen IVIS Spectrum System. The total photon flux of chest regions was analyzed [29].

2.11. Immunoblotting

Cells were lysed using cold RIPA buffer supplemented with protease inhibitors (Sigma #P6730) and phosphatase inhibitors (Sigma #P1260), and lysate protein concentrations were measured by a Pierce BCA protein assay kit (Thermo #23227). 20 μg of total protein was electrophoresed using SDS–PAGE and blotted onto PVDF membranes. Membranes were blocked with 5% BSA solution for 1 h. Samples were probed with primary antibodies overnight at 4 °C. HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG secondary antibodies were diluted at 1:5000.

2.12. Cell apoptosis assay

To detect the antiapoptotic ability of CYP4F2- and CYP4F11-overexpressing MCF-7 cells, H_2O_2 was used to induce apoptosis. Then, the cells were collected and stained with an Annexin V-APC/7-AAD apoptosis kit (MultiSciences Biotech, China) and analyzed using flow cytometric measurement.

2.13. Lipid metabolomics profiling

There were three groups: the control, E2-treated 24 h group, and siERα plus E2-treated group. The cells were centrifuged at low speed (5 min, 800×*g*, RT). The culture medium was aspirated and discarded, and 1 mL of ice-cold (−48 °C) 80% (v/v) methanol: water per 2×10^6 cells (calculated from cell count) was immediately added to simultaneously resuspend and quench the cells. Then the samples were sent to Dalian ChemDataSolution Information Technology Co. Ltd to test.

2.14. Immunohistochemistry (IHC)

Staining of the tissue sections of xenograft tumors, breast tumor and adjacent tissue was performed (5-μm-thick) according to protocols. The tissue sections were immunostained with *anti*-CYP4F2 (1:200), *anti*-Ki67 (1:500), *anti*-CYP4F11 (1:200). The slides were scanned at 20 × using the Aperio Scan Scope XT pathology system (Leica Microsystems, Germany).

2.15. Clinical samples

Breast tumors, matched uninvolved lymph nodes and serum samples acquired from 38 consenting BC patients were provided by Peking Union Medical College Hospital. Tumors were histologically classified as luminal A/B breast cancer according to World Health Organization (WHO) criteria.Table S3 shows the detailed information.

2.16. Quantification of 20-HETE levels

Levels of 20-HETE in human serum (Peking Union Medical College Hospital), cell lysis solution or animal tumor tissues were measured using ELISA kits (BlueGene Biotech) following the manufacturer's procedure.

2.17. Statistical analysis

The data are presented as the mean ± SEM. Statistical analysis and data plotting were performed using GraphPad Prism 8.0. All in vitro experiments were repeated at least three times unless stated otherwise. Statistical analysis was performed using Student's *t*-test when comparing two groups for in vitro and in vivo studies. Mann–Whitney tests were used for 20-HETE quantification in clinical data. $P < 0.05$ was used to define statistical significance.

3. Results

3.1. E2 increases ER + BC cell metabolic activity and most probably proliferation through ERK signaling and regulates AA metabolism

To identify an E2-stimulated cell metabolic activity and most probably proliferation signature in ER + BC cells, we performed an MTT assay in MCF-7, T47D, BT474, SKBR3, MDA-MB-231 and SUM159 cells (Fig. 1A and S3A). We confirmed that the pro-proliferation effect of E2 only occurred in ER + BC cells. In addition, 10 nM E2 was the optimal concentration to induce proliferative effect [30]. ER triggers E2-sensitive gene transcription by binding to specific E2 response elements, and it can increase p44/42–MAPK T202/Y204 phosphorylation in a short time [31]. To examine and verify the cell response to E2, we performed an immunoblot assay and found that E2 increases p44/42–MAPK T202/Y204 phosphorylation at 15 min, 6 h and 24 h in MCF-7 cells; at 2 h and 6 h in T47D cells; and at 6 h, 24 h and 72 h in BT474 cells (Fig. 1B).

We also found that lipid metabolism was activated by E2, and cell membrane phospholipids and certain fatty acid generation were elevated (Fig. S1). The expression of several genes in AA metabolism was compared among different BC subtypes. Genes belonging to CYP family 4 were expressed at higher levels in ER + BC than in the ER-subtype (Fig. 1D and S2). The expression profile analysis revealed that CYP450 enzymes were upregulated in AA metabolism in ER + BC (http://ualcan.path.uab.edu/analysis.html) (Figs. S3C and S3D).

To determine the genes regulated by E2 in ER + BC, the transcript levels of genes in AA metabolism were measured by RT–qPCR assay in MCF-7 cells treated with E2 for 15 min, 6 h, and 24 h (Fig. 1C). We also examined *CYP4F2 and CYP4F11* in BT474 and T47D cells for 24 h (Fig. S3B). We confirmed *CYP4F2* and *CYP4F11,* which are key ω-hydroxylases in the CYP450 pathway that convert AA into 20-HETE [23], were upregulated after E2 treatment in the ER + BC.

3.2. CYP4F2/CYP4F11 play key roles in the progression of ER + BC induced by E2 through ERα

HETEs are products of CYP450 enzymes, especially CYP family 4 and subfamily F (CYP4F). We found only CYP4F2 and CYP4F11 upregulated by E2 induction in MCF-7 and BT474 cells (Fig. 2A and S4A). 20-HETE is the principal proinflammatory metabolite and was also increased in ER + BC cells after E2 treatment (Fig. 2B and S4B).

To define the molecular mechanisms responsible for E2-induced CYP4F2 and CYP4F11 expression and 20-HETE production, we overexpressed and knocked down ERα in BC cells, and the efficiency was confirmed by immunoblot and RT–qPCR analyses (Fig. 2C, D, 2F and 2G). Several genes in AA metabolism were influenced (Fig. 2E–I). ER-cell MDA-MB-468 overexpressed ERα and treated with E2 also resulted in an increase of CYP4F2 and CYP4F11 expression (Fig. S4C). To confirm that CYP450 pathway was the most affected among the three AA metabolism pathways, cell viability was tested. MCF-7 cells were treated with the EP2 antagonist PF-04418948 (30 μM), the EP3 inhibitor L-798106 (10 μM), the EP4 inhibitor CJ-42794 (60 μM), the COX-2 inhibitor NS398 (50 μM), the MAPK inhibitor PD98059 (50 μM), the cPLA2 and iPLA2 inhibitor MAFP (5 μM), the sPLA2 inhibitor LY315926 (50 μM), and the CYP4F inhibitor HET0016 (100 μM) alone or combined with E2 (10 nM) for 72 h and then measured by MTT assay. The results showed that only HET0016 effectively inhibit E2 stimulated MCF-7 cell proliferation (Fig. S4D). Meanwhile, E2 had little effect on the COX2 pathway with no significant change in the COX2 metabolite PGE2 in MCF-7 cells (Fig. S4E). While Inhibition of CYP4F2 and CYP4F11 with

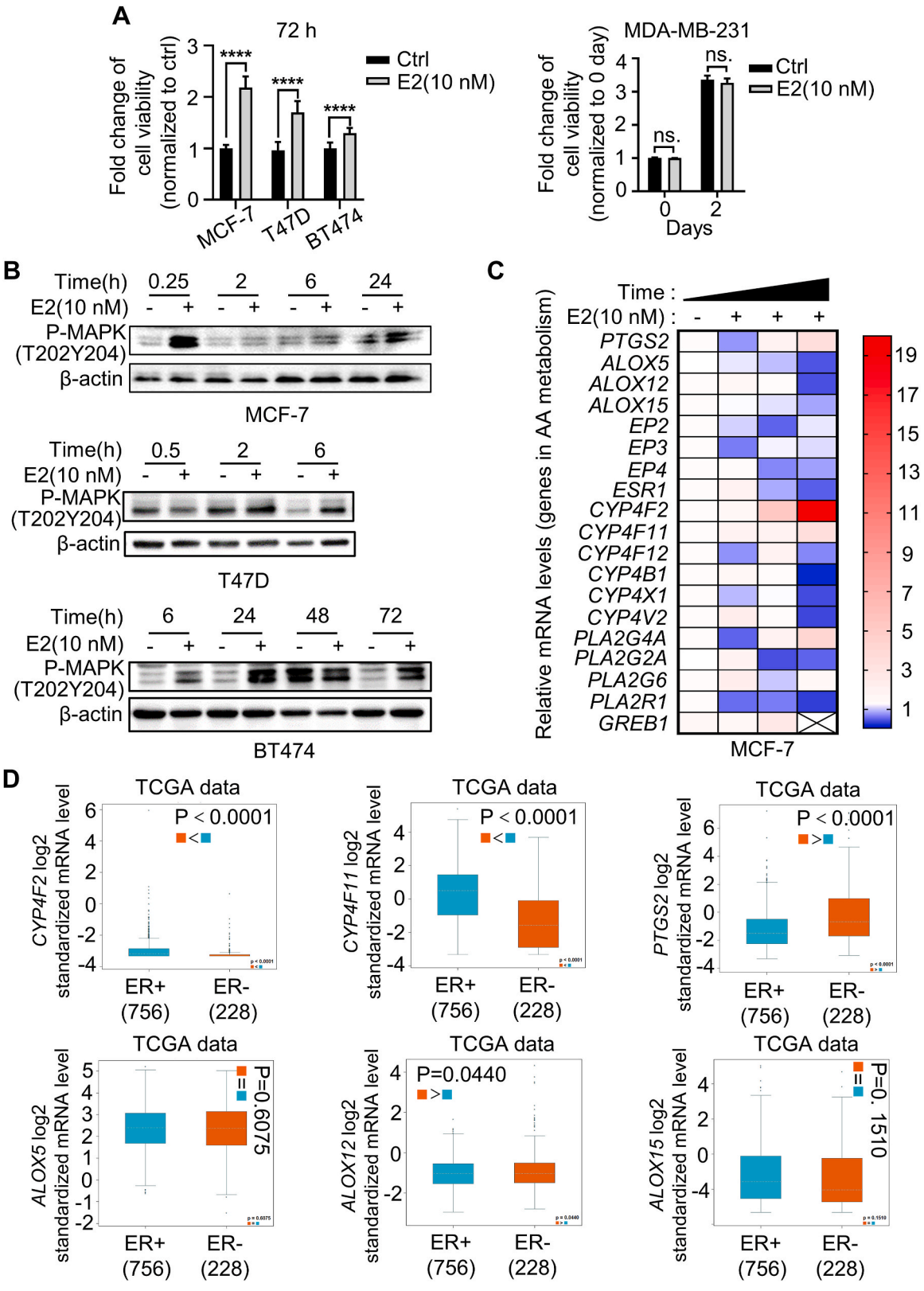

Fig. 1. Identification of an E2-stimulated cell metabolic activity and most probably proliferation signature by inducing arachidonic acid (AA) metabolism in ER + BC cells. (A) MCF-7, T47D, BT474 cell viability was tested under 10 nM E2 treatment for 72 h; the viability of MDA-MB-231 cells was measured by MTT assay at the indicated times for 2 days. (B) Immunoblot analysis of MCF-7, T47D, and BT474 cells treated with E2 at the indicated times. β-Actin was used as a loading control. (C) Transcript levels of AA metabolism-related genes were measured using RT−qPCR on RNA prepared from MCF-7 cells treated with E2 for 15 min, 6 h, and 24 h (n = 3 each). (D) The Cancer Genome Atlas analysis showed the expression levels of AA metabolism-related genes according to the ER status of BC. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; ns, not significant. Student's *t*-test.

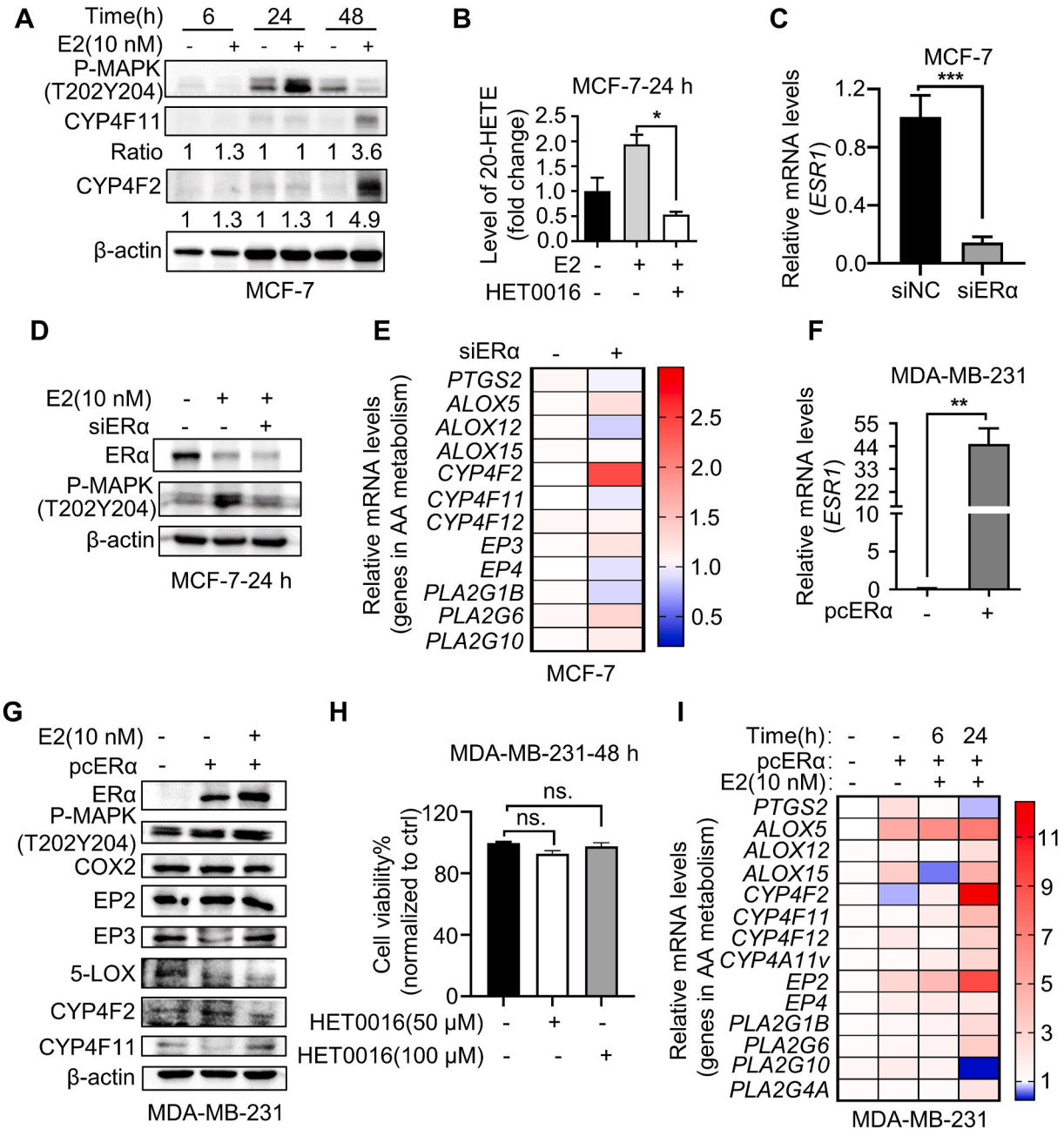

Fig. 2. CYP4F2/CYP4F11 play key roles in the progression of ER + BC induced by E2 through ERα. (A) Immunoblot analysis of CYP4F2 and CYP4F11-induced expression in MCF-7 cells treated with E2 for 6, 24, and 48 h through MAPK signaling. (B) 20-HETE levels were quantified in cell lysates collected from cells treated with HET0016, E2 or control. (C) ERα in MCF-7 cells was knocked down with siRNA. RT−qPCR analysis of the levels of ERα was performed. (D) Immunoblot analysis of ERα, P-Erk expression in MCF-7 cells knocked down with siERα and treated with E2. (E) Transcript levels of AA metabolism-related genes were measured using RT−qPCR on RNA prepared from MCF-7-vec and MCF-7-siERα cells. (F) ERα was overexpressed in MDA-MB-231 cells. RT−qPCR analysis of the levels of ERα was performed. For the transfection assay, we used empty plasmid as negative control. (G) Immunoblot analysis of ERα, *p*-Erk, 5-LOX, COX2, CYP4F2, CYP4F11, EP2, and EP3 expression by overexpression of ERα in MDA-MB-231 cells in the presence or absence of E2. (H) MDA-MB-231 cell viability after treatment with HET0016 was tested by MTT assay. (I) Transcript levels of AA metabolism-related genes were measured using RT−qPCR on RNA prepared from MDA-MB-231 and 231-pcERα cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. Student's *t*-test.

HET0016 had no effect on ER- BC MDA-MB-231 cell viability (Fig. 2H). These data demonstrate that E2-ER is associated with induces CYP4F2 and CYP4F11 expression and stimulates 20-HETE biosynthesis. The inhibitory effect on CYP450 enzymes highly depends on positive ER status.

3.3. The prognostic value of CYP4F2 and CYP4F11 in luminal BC patients

We performed a meta-analysis via an online Kaplan–Meier plotter BC survival analysis to further assess the roles of CYP4F2 and CYP4F11 in survival prediction (http://bcgenex.ico.unicancer.fr/BC-GEM/GEM-Accueil.php?js=1). We took advantage of publicly available gene expression datasets of primary BC with associated clinical data, including disease recurrence and survival. The results revealed that

tumors with higher CYP4F2 and CYP4F11 expression had worse overall survival; in addition, high CYP4F2 and CYP4F11 expression decreased DFS and DMFS in luminal BC (Fig. 3A). To further validate the prognostic value of CYP4F2 and CYP4F11 in ER + BC patients, we performed immunohistochemistry (IHC) with ER + BC tissue samples and found that CYP4F2 and CYP4F11 were highly expressed in tumor tissues than in adjacent normal regions (Fig. 3B). To determine the effect of E2 on CYP450 metabolites in BC patients, we examined the levels of 20-HETE using ELISA. The level of 20-HETE in ER + BC patient serum was increased compared with that in healthy people (Fig. 3C). Gene expression analysis in ER + BC tissues also revealed that *CYP4F2* and *CYP4F11* were upregulated (Fig. 3D). These data demonstrate that E2-ER is associated with induces CYP4F2 and CYP4F11 expression and stimulates 20-HETE biosynthesis through ERα in vivo. Thus, it could be a reasonable explanation for the negative correlation between CYP4F2/CYP4F11 expression and BC patient survival.

3.4. Overexpression of CYP4F2 or CYP4F11 enhances ER + BC cell viability and antiapoptotic ability

We further investigated the impact of CYP4F2 and CYP4F11 on cell functions. Hence, we stably expressed CYP4F2 or CYP4F11 in BC cells and evaluated the cellular outcomes. The efficiency was confirmed by immunoblot and RT−qPCR analysis (Fig. 4A–C). Overexpression of CYP4F2 or CYP4F11 in BC cells enabled the cells to produce more 20-HETE than control cells (Fig. 4B and S5A-S5C) and reduced cell death in serum-free medium (Fig. 4D–S5D and S5E). The metabolite 20-HETE also promoted MCF-7 and T47D cell proliferation under prolonged stimulation (Fig. 4E and S5F).

One of the cancer hallmark capabilities is self-sufficiency in growth signals to sustain persistent proliferation. Since CYP4F2 or CYP4F11 is highly expressed in ER + breast tumors and promotes cell proliferation, we hypothesized that CYP4F2 or CYP4F11 might enhance tumorigenesis. To prove this hypothesis, we constructed CYP4F2 stably overexpressed cell line MCF-7-CYP4F2 and CYP4F11 stably overexpressed cell line MCF-7-CYP4F11, empty plasmid stably transfected cell line MCF-7-NC as control. A total of 30 mice were divided into 3 groups and 10 mice per group. We injected 5×10^6 of MCF-7-CYP4F2, MCF-7-CYP4F11 or MCF-7-NC cells subcutaneously into athymic nude mice without E2 supplementation, and tumor growth was monitored. Normally, the growth of MCF-7 cells depended on E2 in vivo and MCF-7 xenograft tumor is hard to development without E2 supplement. The purpose of this experiment is to compare the number of tumor formation. In this experiment, we found that even without exogenous E2 supplementation, MCF-7-CYP4F2 and MCF-7-CYP4F11 groups showed higher tumor formation rates than MCF-7-NC group after 5 weeks (Fig. 4F). Furthermore, we observed that tumors originating from MCF-7-CYP4F2 and MCF-7-CYP4F11 cells had a better proliferation ability by immunostaining of a proliferative marker, Ki67 (Fig. 4G). Overexpression of CYP4F2 or CYP4F11 also made MCF-7 cells more resistant to H_2O_2 induced apoptosis (Fig. 4H–I).

3.5. Inhibition of CYP4F2 or CYP4F11 attenuates E2 treated ER + BC cell viability

Knocking down of CYP4F2 or CYP4F11 decreased cell growth and attenuated the proliferative effect induced by E2 (Fig. 5G). The knock down efficiency was confirmed by immunoblot and RT-qPCR analysis (Fig. 5A–D). Depletion of CYP4F2/CYP4F11 from MCF-7-CYP4F2 and MCF-7-CYP4F11 cells by siRNA or HET0016 treatment undermined 20-HETE production (Fig. 5E and F). To further determine the biological function of CYP4F2/CYP4F11 in cell survival, we treated BC cells with selective CYP450 inhibitor and selective 20-HETE synthase inhibitor ([32] October; [33]) HET0016 for 72 h in serum-free medium and then analyzed cell apoptosis. Treatment with 100 μM HET0016 combined with 10 nM E2 significantly promoted apoptosis up to 5-fold in MCF-7 cells compared to E2 alone (Annexin V+ cells 35% vs. 6.88%) (Fig. S5G). HET0016 increased the proportion of apoptotic cells in BC cells, indicating cancer cell cytotoxicity. We also performed immunoblotting and found that HET0016 promotes cell apoptosis by altering the Bcl-2 family balance, inhibited Erk phosphorylation and ERα expression. Bcl-2 is an antiapoptotic protein, and the Bcl-2 interacting mediator Bad is a critical activator of apoptosis. E2 can increase Bcl-2 levels [34], HET0016 combined with E2 treatment decreased Bcl-2 protein levels by elevating of the cell apoptosis markers Cl-Caspase3 and Cl-PARP (Fig. 5H). Overall, these data indicate that Bcl-2 overexpression stimulated by E2 enhances the resistance of ER + BC cells to apoptosis. Inhibition of E2-induced CYP4F2 and CYP4F11 may alter tumor homeostasis, and increase cancer cell apoptosis, which demonstrated the effect of HET0016 in attenuating E2-mediated apoptosis protection.

To further investigate CYP4F2 and CYP4F11 function in vivo, a lung colonization experiment has been arranged. The bioluminescent photon flux reflects the cell number colonized in the lung. We established CYP4F2 stably knock down cell line MCF-7-shCYP4F2, CYP4F11 stably knock down cell line MCF-7-shCYP4F11 and MCF-7-shNC as control, all of the 3 cell lines stably expressed luciferase. We divided 15 mice into 3 groups and 5 mice per group. All the mice were given 40 mg/mL estradiol cypionate subcutaneously a week earlier and then 6×10^5 MCF-7-shNC, MCF-7-shCYP4F2, or MCF7-shCYP4F11-luciferase-expressing cells treated with estradiol for 48 h were intravenously inoculated into mice. The mice examined by bioluminescent imaging (Fig. 5I). At cell injection timepoint (baseline), 120 mg/kg of D-luciferin was intraperitoneally injected to comparable levels of bioluminescence in the lung of all mice. At 2 h after cell injection, the bioluminescence in the chest regions of mice inoculated with MCF-7-shCYP4F2 and MCF-7-shCYP4F11 cells was decreased by 58% and 44% compared with MCF-7-shNC (Fig. 5J). MCF-7-NC cells highly expressed CYP4F2 and CYP4F11 after estradiol treatment but MCF-7-shCYP4F2 or MCF-7-shCYP4F11 cells not, which suggesting CYP4F2 and CYP4F11 sustains the lung colonization of ER + BC cells in vivo.

4. Discussion

The role of estrogen in breast cancer development began with the early observation that bilateral oophorectomy significantly reduced the risk of ER + breast cancer. It is well accepted that ER + BC progression is strongly driven by the E2-ER signaling pathway. Estrogen exerts its physiological effects by binding to specific steroid receptors named ER. ER is the primary regulators of the estrogen signaling pathway because it can regulate gene expression through interacting with promoters, thereby mediating a range of effects in the cells, such as PKCs, Ras/Raf/MAPK, PI3K/AKT, cAMP/PKA and so on [35]. Endocrine therapy is the backbone treatment for patients with ER + BC. However, traditional endocrine therapy based on an *anti*-ER regimen was less effective in advanced ER + BC due to the activation of other cell signaling pathways [11]. Hence, the identification of new therapeutic targets or more specific biomarkers remains important. Here, through TCGA database analysis, we found that the CYP450 pathway plays an important role in ER + BC progression. We identified an E2-stimulated cell proliferation signature in ER + BC cells and found that abnormal CYP4F expression was hyperactivated and regulated by E2 in ER + BC cells.

We characterized the critical roles of E2-induced CYP4F2 and CYP4F11 overexpression in ER + BC. First, E2 induces the expression of CYP4F2 and CYP4F11 and 20-HETE production in ER + BC cells. This result was also observed in TNBC cells transfected with the ERα plasmid. 20-HETE, synthesized via CYP4F, stimulated ER + BC cell proliferation. Moreover, even with serum-free medium, overexpression of CYP4F2 or CYP4F11 improved cell resistance to death.

Second, shRNA knockdown of CYP4F2/CYP4F11 inhibited cell viability and induced cell apoptosis, resulting in decreased lung colonization ability in vivo. We also demonstrated that CYP4F2 and CYP4F11 are abundant in ER + BC tissue and that the serum level of 20-

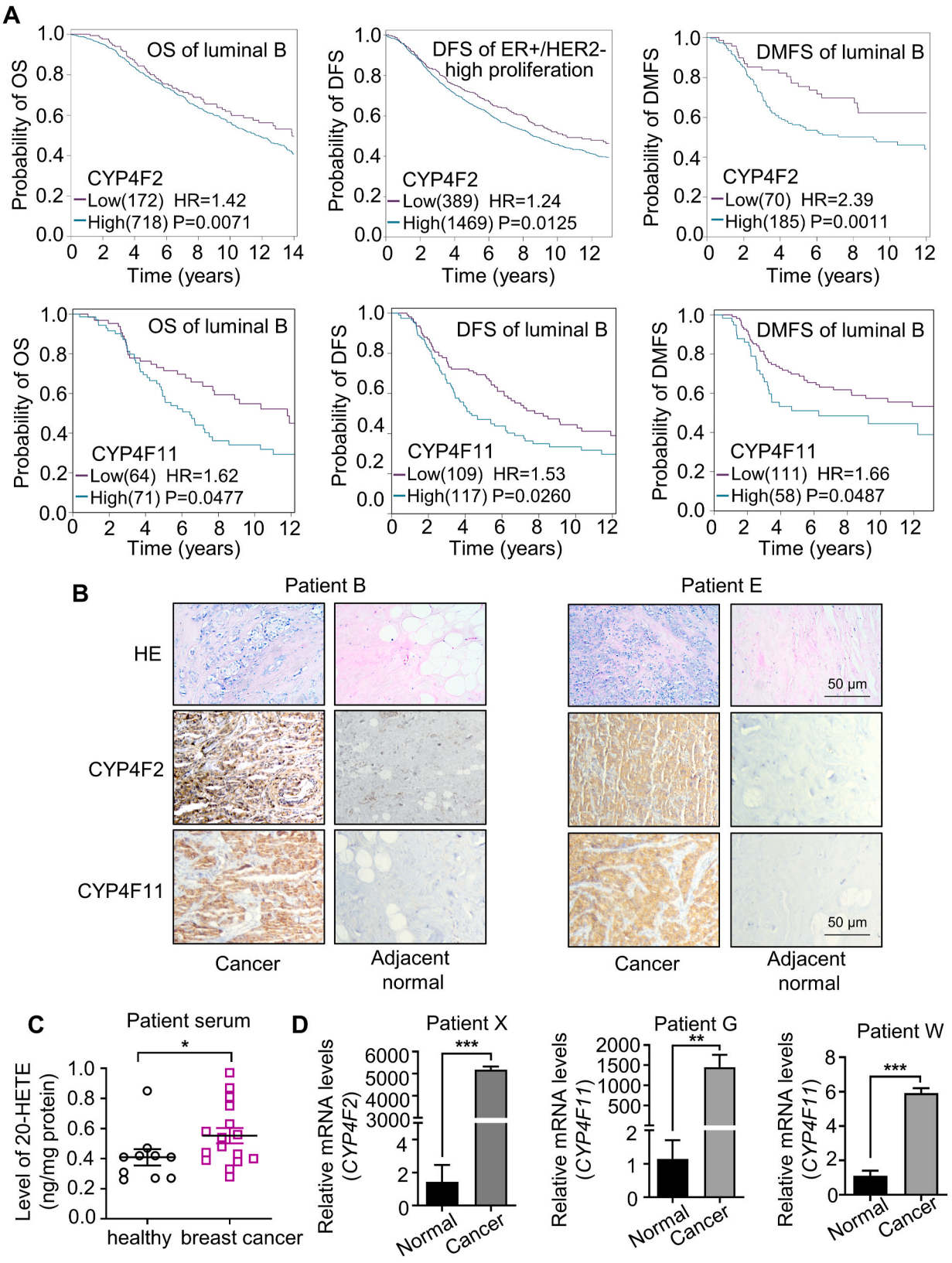

Fig. 3. CYP4F2/CYP4F11 accumulated in ER + BC. (A) Kaplan–Meier analysis of CYP4F2 and CYP4F11 expression for different molecular subtypes of ER + BC. (B) Immunohistochemical and HE analysis of CYP4F2 and CYP4F11 protein expression in ER + BC tissues. Representative IHC images in matched normal tissues and tumors from three patients are shown. (C) Serum levels of 20-HETE in ER + BC patients (n = 16) and healthy women (n = 10) were assessed (ELISA). (D) RT–qPCR analysis of the transcript levels of CYP4F2 and CYP4F11 in ER + BC patients. *, P < 0.05; ***, P < 0.001; ns, not significant. Student's *t*-test for transcript levels; Mann–Whitney test for 20-HETE quantification in clinical data.

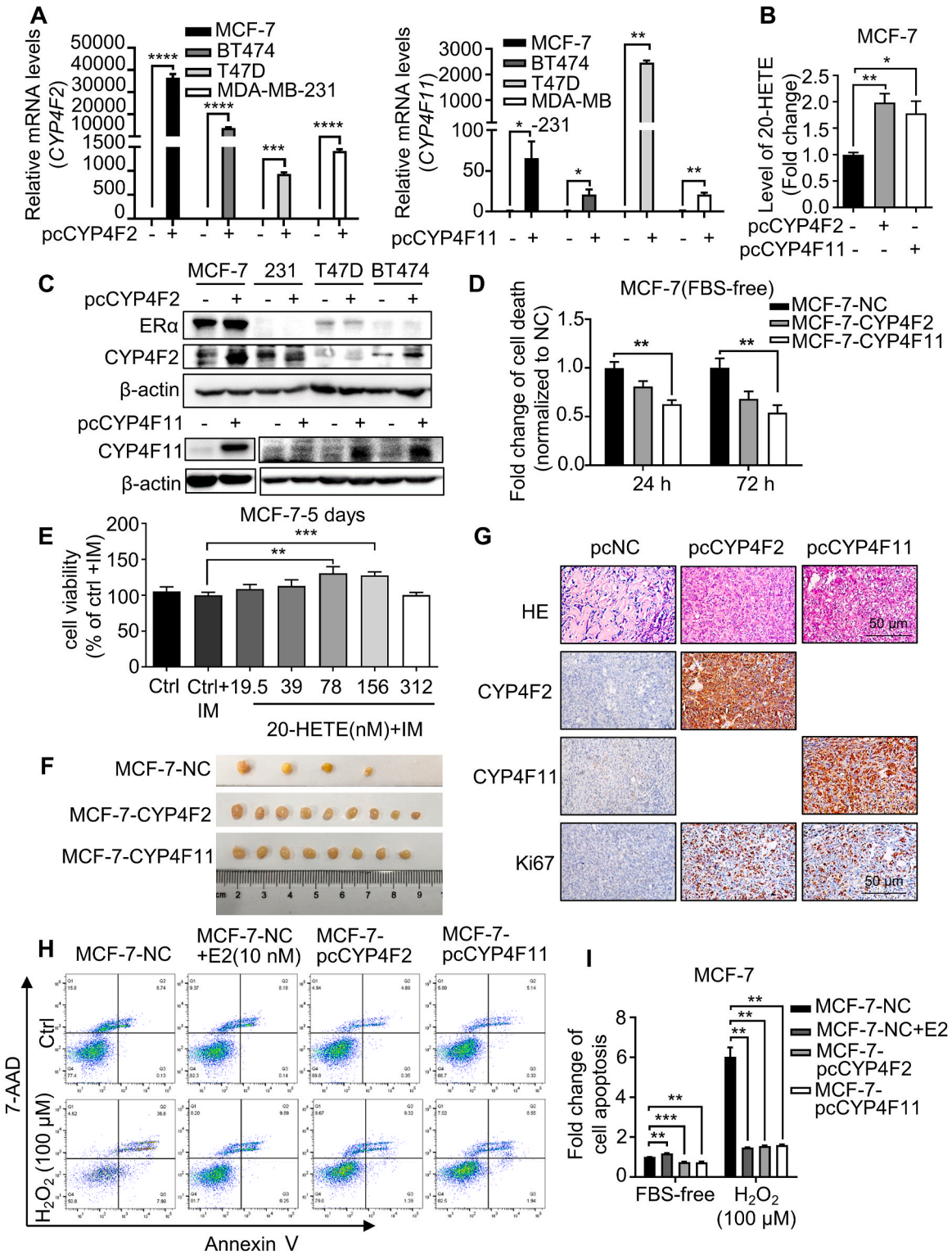

Fig. 4. CYP4F2/CYP4F11 overexpression enhances ER + BC cell viability and antiapoptotic ability. (A and C) Western blotting and RT−qPCR assays were performed to verify the transfection efficiency. (B) The metabolite 20-HETE levels were quantified in cell lysates collected from MCF-7 cells overexpressing CYP4F2 and CYP4F11 (ELISA). (D) MCF-7, MCF-7-CYP4F2, and MCF-7-CYP4F11 cells in the absence of FBS. Cell death was measured using a PI exclusion assay. For the stably overexpressed cell line construct, we used empty plasmid as negative control. (E) Proliferation of MCF-7 cells treated with 20-HETE and 5 μM indomethacin was measured by MTT assay. (F) Tumor growth of MCF7-vec, MCF-7-CYP4F2, and MCF-7-CYP4F11 cells implanted subcutaneously in athymic mice in the presence of growth factor-reduced Matrigel. The tumor formation rate was 80–90% after MCF-7 cells were transfected with the CYP4F2 or CYP4F11 plasmid. (G) H&E staining and IHC analysis of the expression of CYP4F2, CYP4F11, and Ki67 in tumors. (H and I) MCF-7, MCF-7-CYP4F2 and MCF-7-CYP4F11 cells were cultured in FBS-free medium and treated with 10 nM E2 and 100 μM H_2O_2 for 60 h. Cells were stained with Annexin V-APC and 7-AAD, and apoptosis was determined (n = 3). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Student's *t*-test.

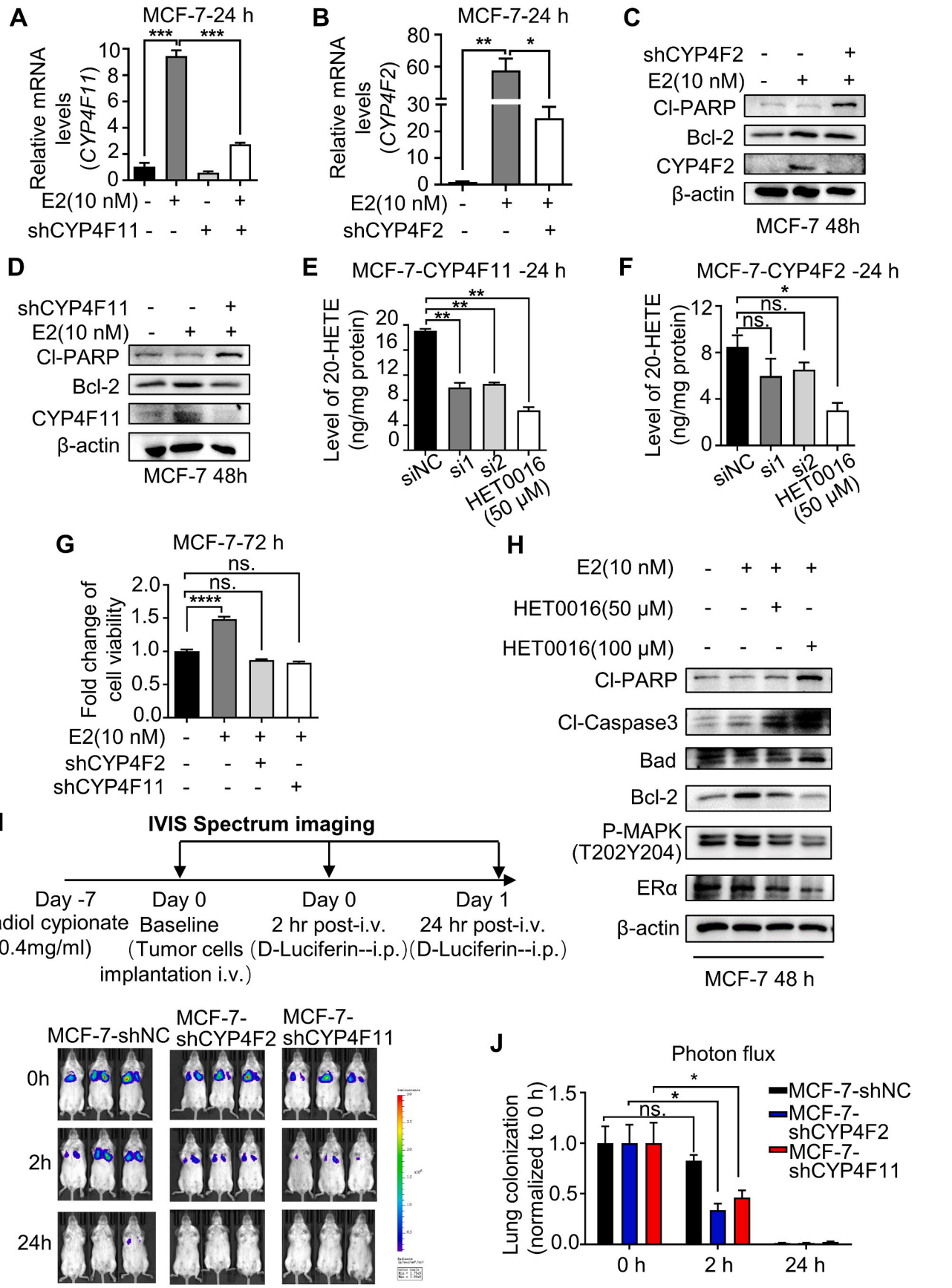

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Fig. 5. Inhibition of CYP4F2 or CYP4F11 attenuates E2 treated ER + BC cell viability. (A and B) RT−qPCR was performed to measure *CYP4F2* or *CYP4F11* expression in MCF-7-shCYP4F2 and MCF-7-shCYP4F11 cells treated with or without E2. (C and D) Immunoblots of lysates from MCF-7 cells treated with E2 for 48 h and then *CYP4F2* and *CYP4F11* were knocked down with Bcl2, Cl-PARP, CYP4F2, and CYP4F11 antibodies. For the stably knock down cell line construct, we used scrambled shRNA as negative control. (E and F) 20-HETE levels were quantified in cell lysates collected from MCF-7-CYP4F2 and MCF-7-CYP4F11 cells transfected with CYP4F2/4F11 siRNA and control siRNA or treated with 50 μM HET0016. (G) MCF-7, MCF-7-shCYP4F2, and MCF-7-shCYP4F11 cell proliferation treated with or without E2 was measured by MTT assay for 72 h. (H) Immunoblot analysis of Cl-Caspase3, Cl-PARP, Bcl-2, Bad and ERα in MCF-7 cells treated with 50 μM and 100 μM HET0016 combined with or without E2 for 48 h. (I) MCF-7, MCF-7-shCYP4F2, and MCF-7-shCYP4F11 cells (7×10^5) were intravenously injected into female SCID mice. Baseline bioluminescent images were captured immediately after cell injection. Mice received imaging and final imaging at 2 and 24 h after cell injection, and bioluminescent images are shown. (J) Relative photon flux was calculated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant. Student's *t*-test.

HETE was elevated in ER + BC patients. Collectively, these data suggested that CYP4F2 and CYP4F11 may promote ER + BC pathogenesis.

HET0016 is a selective CYP450 inhibitor and selective 20-HETE synthase inhibitor. As expected, we found that ER + BC cells treated with HET0016 combined with E2 exhibited reduced 20-HETE levels and reduced cell viability.

In cancer, apoptosis evasion through the dysregulation of specific Bcl-2 family genes is a recurring event. Bcl-2 is an antiapoptotic protein, and the Bcl-2–interacting mediator of cell death Bad is a critical activator of apoptosis [26,27]. We found that E2 treatment can promote the expression of Bcl-2 proteins in ER + BC cells and increase MCF-7 cell apoptosis resistance [36,37]. HET0016 induced BC cell apoptosis by decreasing the level of Bcl-2 protein and promoting Bad protein expression. Knocking down of CYP4F2 and CYP4F11 in E2 treated MCF-7 cells also induced downregulation of Bcl-2 and cell apoptosis. Our finding indicates that CYP4F2/CYP4F11 inhibition could interfere with CYP450 metabolism, which ultimately inhibits ER + BC progression.

5. Conclusion

In summary, our findings suggest that CYP4F2/CYP4F11 play an important role in the E2-induced cell signal cascade and that its product, 20-HETE, facilitates tumor progression and suppresses apoptosis via interaction with Bcl-2 family proteins. This implies that CYP4F2/CYP4F11 inhibition could serve as a promising strategy for ER + BC treatment and may exert synergistic effects with the combination of *anti*-ER therapy. Future preclinical and clinical investigations are warranted to further investigate the efficacy and safety issues of this novel modality.

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Data availability

All data generated that are relevant to the results presented in this article are included in this article. Other data that were not relevant for the results presented here are available from the corresponding author (C L) upon reasonable request.

Abbreviations

BC	breast cancer
E2	17-β- estradiol
ER	estrogen receptor
PR	Progesterone receptor

Ethics statement

Animal studies were performed according to the Guidelines on Laboratory Animals of Nankai University and were approved by the Institute Research Ethics Committee at Nankai University (No: 2021-SYDWLL-000464).

Samples from patients with BC were collected by the Department of Breast Surgery, Peking Union Medical College Hospital. Informed consent was received from each patient before surgery. The procedures related to human subjects were approved by the Ethics Committee of Peking Union Medical College Hospital and Nankai University (No: NKUIRB2021105) and performed according to established ethical guidelines for experimentation with human samples via the Helsinki declaration.

CRediT authorship contribution statement

Juan Yang: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Yin Li:** Validation, Software, Investigation, Data curation. **Xiao Han:** Validation, Data curation. **Tianjiao Li:** Validation, Data curation. **Ding Li:** Resources, Funding acquisition. **Qiao Liu:** Validation, Investigation. **Lizhong Yan:** Validation, Data curation. **Fei Li:** Data curation. **Xiaolin Pei:** Software, Data curation. **Ya Feng:** Data curation. **Zhoujun Lin:** Software. **Zhenkun Fu:** Resources, Formal analysis. **Changjun Wang:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Qiang Sun:** Software, Resources. **Chenggang Li:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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We would like to thank Miaomiao Sheng (Kunming University of Science and Technology) for providing breast cancer cell lines, Gang Zhao (Department of Pathology, Tianjin Cancer Hospital) for supplying breast cancer sections, and Xinying Wu (Animal Center of Nankai University) for technical support of in vivo imaging.

AA	Arachidonic acid
COX	Cyclooxygenase
LOX	Lipoxygenases
CYP4F2	Cytochrome P450 family 4 subfamily F member 2
HETE	hydroxyeicosatetraenoic acid
PGE2	Prostaglandin E2
FBS	Fetal bovine serum
Indometacin	IM
Bcl-2	B cell lymphoma 2
BAD	Bcl-2-associated agonist of cell death
TCGA	The Cancer Genome Atlas
OS	Overall survival
DFS	Disease-free survival
DMFS	Distant metastasis-free survival

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101706.

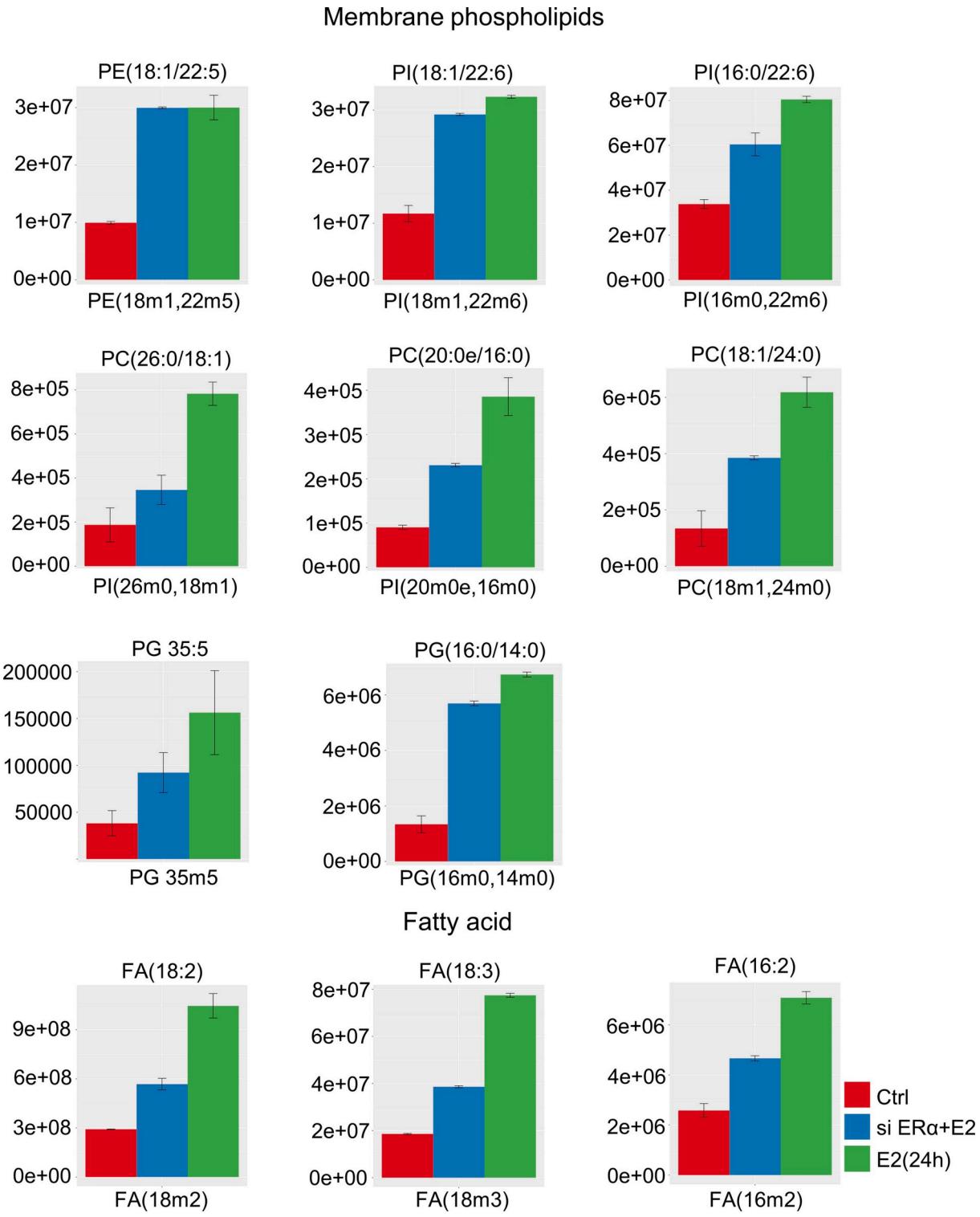

Figure S1**Lipid metabolism was activated by E2.** MCF-7 and MCF-7-siERα cells were treated with 10 nM E2 for 24 h, and cellular lipid metabolites were profiled by mass spectrometry (n = 2; Metabolon LC–MS/MS).

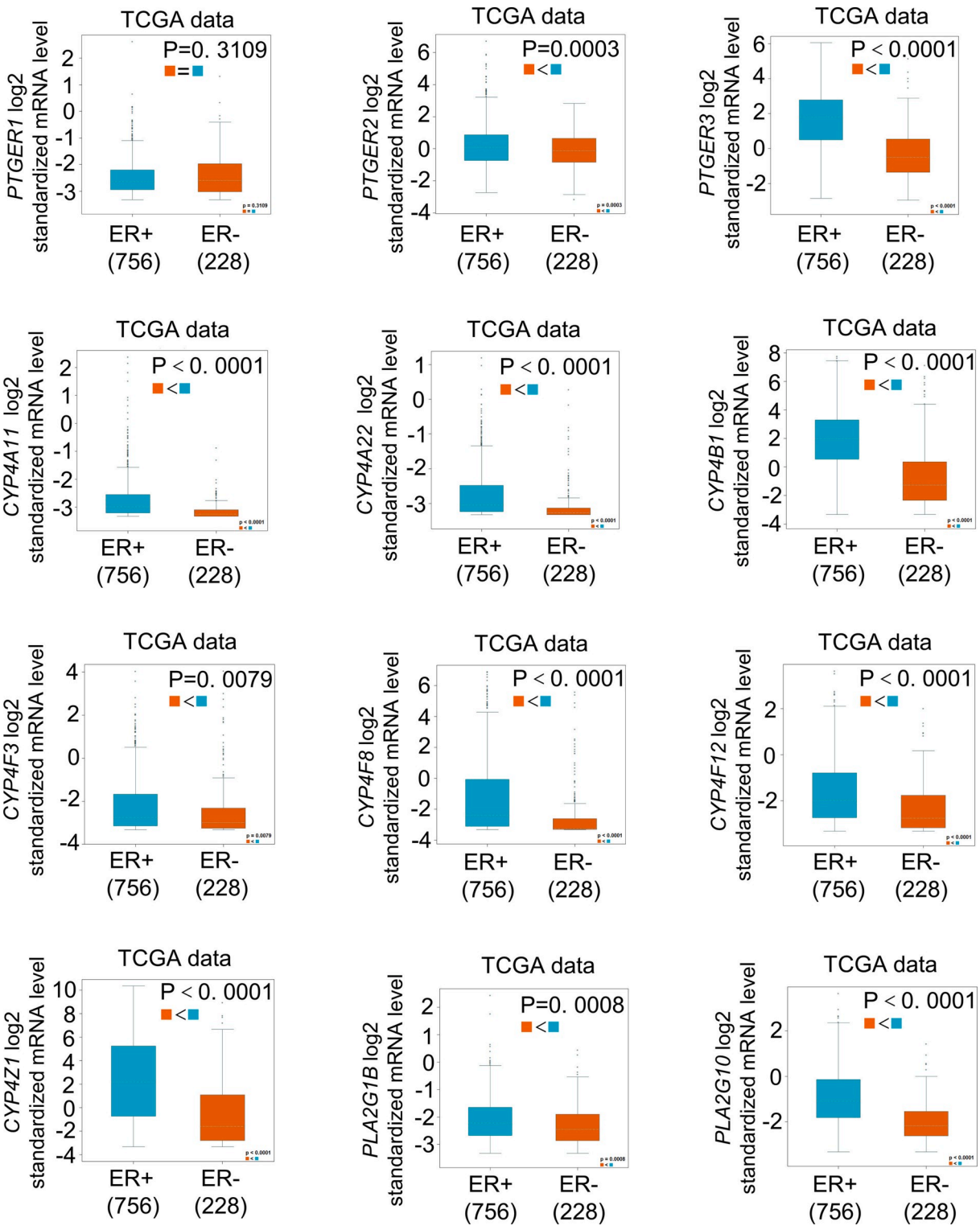

Figure S2The Cancer Genome Atlas analysis showed that the expression levels of CYP450 metabolism-related genes are higher in ER + than in ER- BCs. We compared *PTGER1*, *PTGER2*, *PTGER3* in COX pathway, *CYP4A11, CYP4A22, CYP4B1, CYP4F3, CYP4F8, CYP4F12, CYP4Z1* in CYP450 pathway, *and PLA2G1B, PLA2G10* expression levels between 756 ER + breast cancer patients and 228 ER-breast cancer patients using Breast Cancer Gene-Expression Miner v5.0 database (http://bcgenex.ico.unicancer.fr/BC-GEM/GEM-Citation.php) and found *CYP4F* related genes in CYP450 pathway are hyperactivated in ER + BCs. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Student's *t*-test.

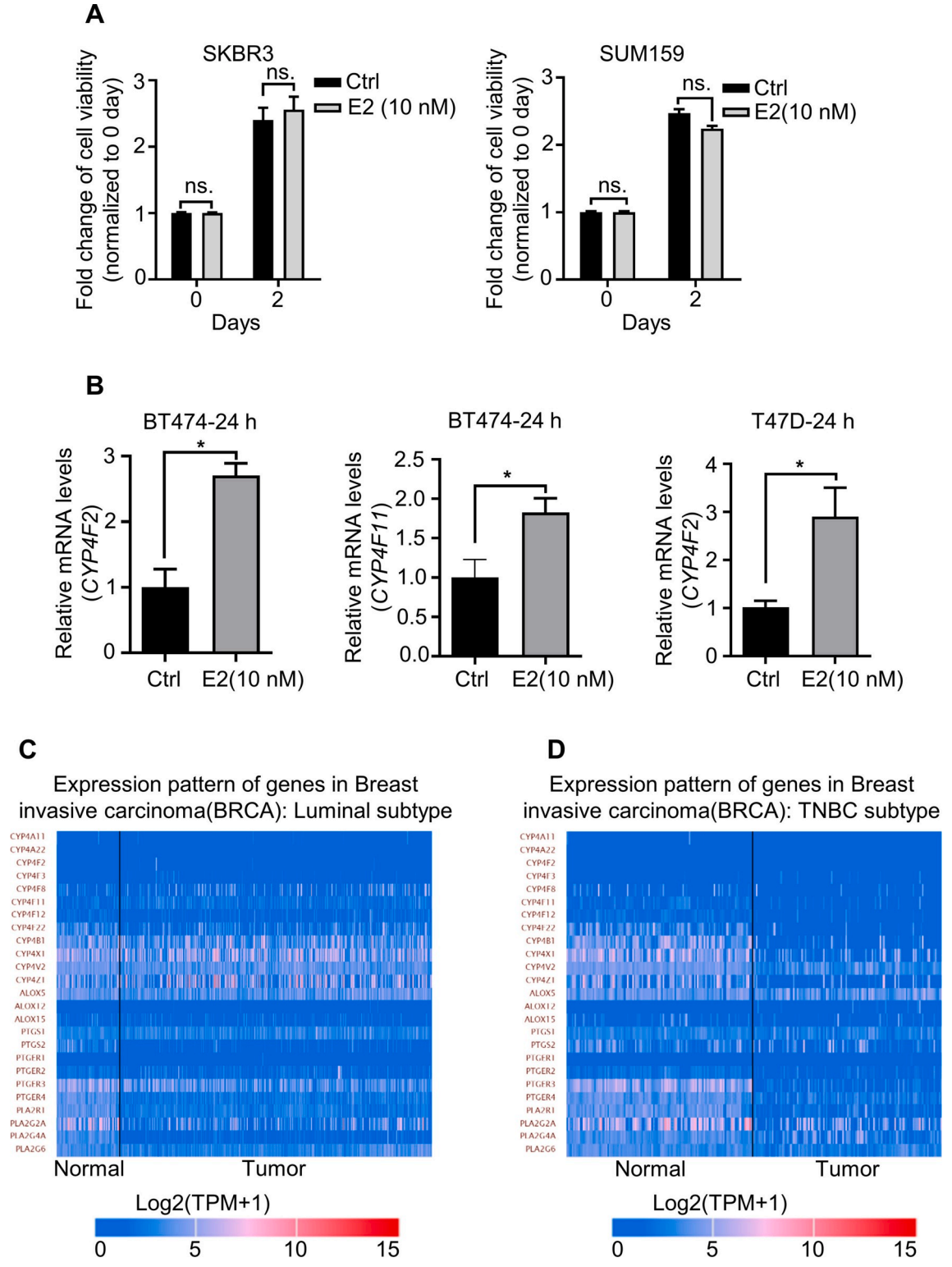

Figure S3**E2 stimulates AA metabolism in ER + BC progression.** (A) The viability of SUM159 and SKBR3 cells was measured by MTT assay at the indicated times for 2 days. (B) Transcript levels of CYP4F2 and CYP4F11 in BT474 and T47D cells treated with or without E2. (C and D) The expression pattern of AA metabolism-related genes in luminal subtype and TNBC subtype compared with normal tissues. *, $P < 0.05$; ns, not significant. Student's *t*-test.

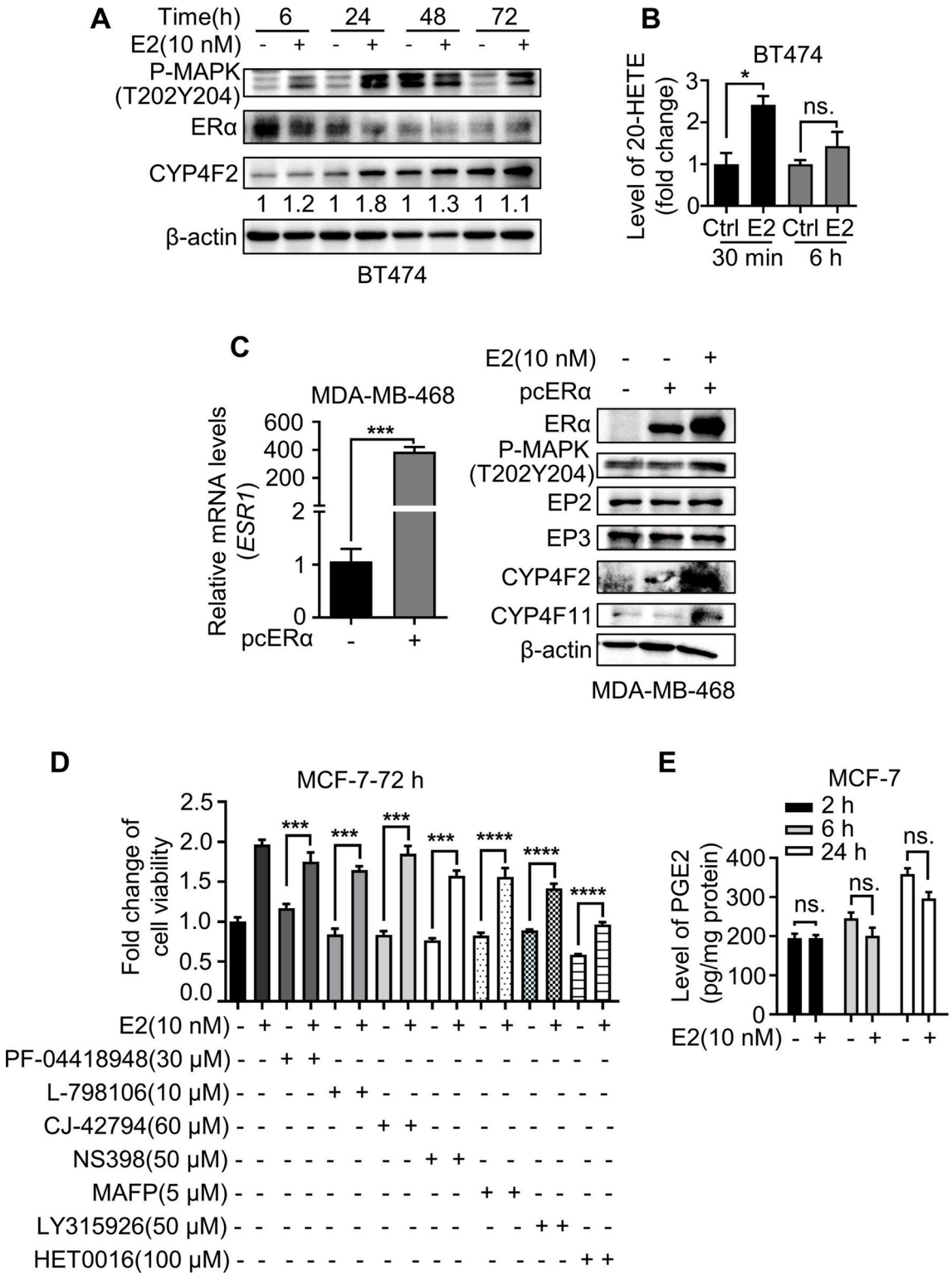

Figure S4E2 induces CYP4F2 and CYP4F11 expression through ERα. (A) Immunoblot analysis of CYP4F2-induced expression in BT474 cells treated with E2 for 6, 24, 48, and 72 h through MAPK signaling. (B) 20-HETE levels were quantified in cell lysates collected from BT474 cells treated with E2 or control. (C) MDA-MB-468 cells were transfected with plasmid ERα, empty plasmid pc-DNA3.1 as negative control, and the gene levels of ERα were measured by RT−qPCR. Immunoblot analysis of ERα, *p*-Erk, CYP4F2, CYP4F11, EP2, and EP3 expression was performed by overexpression of ERα in MDA-MB-468 BC cells in the presence or absence of E2. (D) MCF-7 cells were treated with the EP2 antagonist PF-04418948 (30 μM), the EP3 inhibitor L-798106 (10 μM), the EP4 inhibitor CJ-42794 (60 μM), the COX-2 inhibitor NS398 (50 μM), the cPLA2 and iPLA2 inhibitor MAFP (5 μM), the sPLA2 inhibitor LY315926 (50 μM), and the CYP4F inhibitor HET0016 (100 μM) alone or combined with E2 (10 nM) for 72 h and then tested by MTT assay. (E) Secreted prostaglandin levels were quantified in conditioned media collected from cells treated with E2 or control, and data were normalized to the data of the control group (ELISA). *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant. Student's *t*-test.

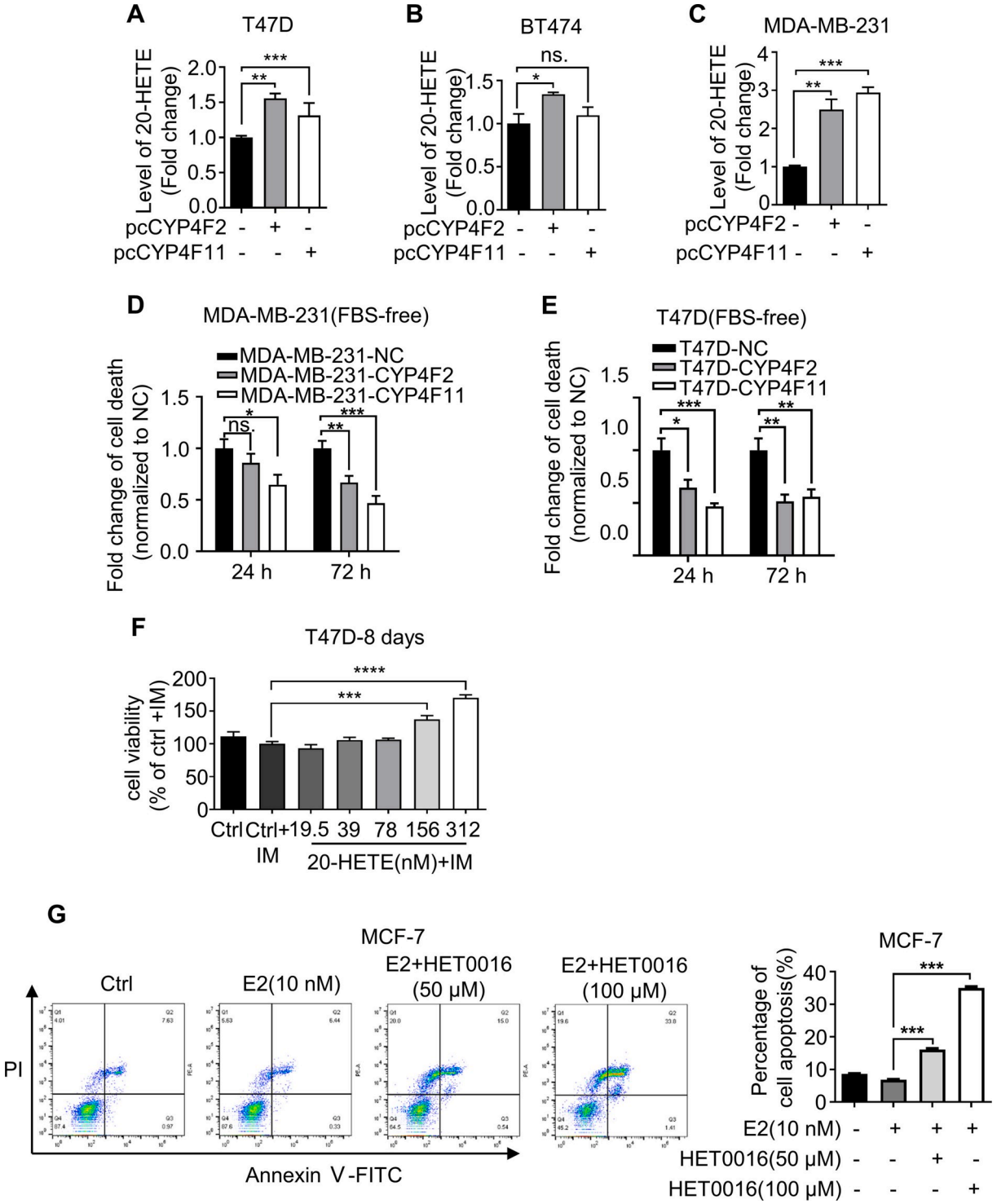

Figure S5**CYP4F2 and CYP4F11 overexpression promotes MCF7 cell survival.** (A-C) The metabolite 20-HETE levels were quantified in cell lysates collected from ER+ and ER- BC cells overexpressing CYP4F2 and CYP4F11 (ELISA). (D and E) MDA-MB-231 and T47D cells overexpressing CYP4F2 or CYP4F11 in the absence of FBS for 24 h and 72 h. Cell death was measured using a PI exclusion assay. (F) Proliferation of T47D cells treated with 20-HETE and 5 μM indomethacin was measured by MTT assay. (G) Cell apoptosis was measured in MCF-7 cells using the Annexin V-FITC/PI Apoptosis Detection Kit (n = 3). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant. Student's *t*-test.

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