

# Exposure to EGF and 17 $\beta$ -estradiol irreversibly affects the proliferation and transformation of MCF7 cells but is not sufficient to promote tumor growth in a xenograft mouse model upon withdrawal of exposure

SARA I. CUNHA<sup>1</sup>, MIN JIA<sup>2</sup> and SERHIY SOUCHELNYTSKYI<sup>3</sup>

<sup>1</sup>Department of Immunology, Genetics and Pathology, Uppsala University, 75237 Uppsala;

<sup>2</sup>Department of Biosciences and Nutrition, Karolinska Institutet, 14157 Stockholm, Sweden;

<sup>3</sup>College of Medicine, Qatar University, Doha 2713, Qatar

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**Abstract.** Epidermal growth factor (EGF) and estrogen are potent regulators of breast tumorigenesis. Their short-term actions on human breast epithelial cells have been investigated extensively. However, the consequence of a long-term exposure to EGF and estrogen remains to be fully elucidated. The present study examined the effects of long-term exposure to EGF and 17 $\beta$ -estradiol on the proliferation, transformation, expression of markers of stemness, and tumorigenesis of MCF7 human breast adenocarcinoma cells. Exposure to EGF and/or 17 $\beta$ -estradiol irreversibly enhanced the proliferation rate of MCF7 cells, even following withdrawal. However, in a mouse xenograft experiment, no significant difference in tumor volume was observed between tumors derived from cells exposed to EGF, 17 $\beta$ -estradiol or EGF + 17 $\beta$ -estradiol. Immunohistochemistry performed on tumors derived from 17 $\beta$ -estradiol-exposed cells revealed reduced cell proliferation and vessel scores, according to the results obtained using Ki67 and von Willebrand factor staining, respectively. The EGF- and/or 17 $\beta$ -estradiol-treated cells exhibited an increased ratio of cluster of differentiation (CD)44<sup>+</sup>/CD24<sup>-</sup> cells and enhanced ability to form mammospheres. Furthermore, the long-term exposure of MCF7 cells to EGF and 17 $\beta$ -estradiol altered their responsiveness to short-term stimulatory or inhibitory

treatments with EGF, 17 $\beta$ -estradiol, transforming growth factor- $\beta$ 1 (TGF $\beta$ 1), Iressa and SB431542. Therefore, the findings indicated that sustained exposure of MCF7 cells to EGF and/or 17 $\beta$ -estradiol resulted in enhanced cell proliferation and mammosphere formation, an increased ratio of CD44<sup>+</sup>/CD24<sup>-</sup> cells, and altered responses to short-term treatments with EGF, 17 $\beta$ -estradiol, TGF $\beta$ 1, and drugs inhibiting these signaling pathways. However, this sustained exposure was not sufficient to affect tumor take or volume in a xenograft mouse model.

## Introduction

Human breast epithelial cells are constantly exposed to polypeptide growth factors and steroid hormones as part of their physiological control. The levels of epidermal growth factor (EGF) and estrogen have an impact on cell physiology, including cell proliferation rate, differentiation and migration (1-5). The exposure of cells to elevated levels of EGF and estradiol for a prolonged period of time may irreversibly affect their physiology, which may consequently impact on cell carcinogenic transformation.

Tumorigenesis is a complex process involving alterations of multiple genes, proteins and regulatory pathways (6,7). In breast cancer, the overexpression of epidermal growth factor receptor (EGFR) in the primary tumor correlates with increased metastatic dissemination and aggressive tumor progression (8). In total, >70% of breast cancer tumors express high levels of estrogen receptor- $\alpha$  (ER $\alpha$ ), and a large number of these tumors require estrogen to support cancer cell proliferation and tumor progression (9). As the EGFR and estrogen signaling pathways are closely associated with the development of breast cancer, they are targets for the treatment of breast cancer (10,11).

The EGF and estrogen signaling pathways share a number of intracellular signaling mechanisms due to cross-talk (10-12). The inverse correlation observed between the expression levels of EGFR and ER $\alpha$  has been explained by compensatory mechanisms, which are activated in malignant cells in order to maintain a high proliferative status.

*Correspondence to:* Professor Serhiy Souchelnytskyi, College of Medicine, Qatar University, Building H12, Al Jamiaa Street, Doha 2713, Qatar  
E-mail: serhiy@qu.edu.qa

**Abbreviations:** EGF, epidermal growth factor; vWF, von Willebrand factor; TGF $\beta$ , transforming growth factor- $\beta$ ; ER, estrogen receptor; DCIS, ductal carcinoma *in situ*; ECM, extracellular matrix

**Key words:** epidermal growth factor, 17 $\beta$ -estradiol, breast cancer, stemness, xenograft

When one of the above receptors is upregulated or down-regulated, the expression of the other receptor compensates for this alteration (12). It has been reported that EGF- and ER $\alpha$ -dependent transcription may operate in parallel, although with a marked overlap in the affected genes (13). Estrogen may also control the downregulation of EGFR (10). However, the exact molecular mechanisms of the crosstalk remain to be fully elucidated. In addition, how long-term exposure to EGF and estrogen may affect the carcinogenic properties of cells remains unclear.

Breast cancer stem cells are defined as self-renewing cells required to initiate a tumor and drive tumor growth when transplanted into mice (14,15). In human breast cancer, cancer stem cells show a cluster of differentiation (CD)44<sup>+</sup>/CD24<sup>-</sup> pattern of surface markers (16). This population of cells exhibits the ability to form three-dimensional mammospheres under low-adherence conditions and exhibit increased resistance to chemotherapeutic compounds (14-17). The EGFR signaling pathway has been implicated in the self-renewal of breast cancer stem cells (18). It has been reported that the EGFR tyrosine kinase inhibitor Iressa significantly decreases the formation of mammospheres by cells derived from a ductal carcinoma *in situ* (19). A number of studies have reported that estrogen treatment may expand the pool of breast cancer stem cells (20,21). The present study hypothesized that, if prolonged exposure to EGF and estradiol changes the physiology of breast cancer cells, then it may also modulate cell responsiveness to anticancer drugs. The results revealed that sustained exposure of conditionally tumorigenic MCF7 human breast adenocarcinoma cells to EGF and 17 $\beta$ -estradiol led to the generation of cells with increased proliferation rate, increased CD44<sup>+</sup>/CD24<sup>-</sup> cell fraction population and a different pattern of response to therapeutic drugs, including Iressa, tamoxifen and the transforming growth factor (TGF) $\beta$  type I receptor kinase inhibitor SB431542, compared with non-exposed control cells. The mouse xenograft experiments revealed that these changes in cell physiology were not sufficient to ensure additional tumor development in immunocompromised mice upon withdrawal of treatment.

## Materials and methods

**Cell culture.** The MCF7 human breast adenocarcinoma cell line was purchased from the American Type Culture Collection (Rockville, MD, USA; HTB-22<sup>TM</sup>) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 2 mM glutamine. The cells were maintained in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. To generate cell clones exposed to EGF, estrogen and EGF + estrogen, the MCF7 cells at an initial density of 1x10<sup>6</sup> cells/plate were grown on agarose-coated culture dishes with 5 ng/ml of EGF (Sigma-Aldrich; Merck KGaA), 5 nM of 17 $\beta$ -estradiol (Sigma-Aldrich; Merck KGaA) or 5 ng/ml of EGF + 5 nM 17 $\beta$ -estradiol. Agarose coating prevented the attachment of cells to the plate. After 4 weeks, the cells were transferred to 96-well plates coated with agarose. The growing clones of

cells were expanded, and 12 clones for each of the treatment conditions were randomly selected for evaluation of their proliferation status. For subsequent experiments, clones that represented the average proliferation status of the initial clones were selected, in addition to the 12 randomly selected clones (data not shown). The total time of cell exposure to EGF and/or 17 $\beta$ -estradiol was 40-42 days, prior to the random selection of 12 initial clones, from which other clones were selected for the mouse model and subsequent experiments. The selected clones were cultured and analyzed on regular culture dishes without agarose coating.

**MTT assay.** Cell proliferation was measured using the CellTiter 96<sup>®</sup> Non-Radioactive Cell Proliferation assay (Promega Biotech AB, Stockholm, Sweden). The MTT assay was performed according to the manufacturer's protocol. In brief, 1,000 cells were seeded per well in 96-well plates in triplicate, treated with 5 ng/ml EGF (Sigma-Aldrich; Merck KGaA), 5 nM 17 $\beta$ -estradiol (Sigma-Aldrich; Merck KGaA) or 5 ng/ml EGF + 5 nM 17 $\beta$ -estradiol, incubated in complete DMEM culture medium for 48 h, and then subjected to the MTT assay. The formazan crystals were dissolved in DMSO. The absorbance at 570 nm was recorded using a plate reader. Statistical significance of observed differences was evaluated using a one-way analysis of variance (ANOVA) with Tukey's honest significant difference (HSD) test.

**Colony formation assay.** Colony formation assays were performed in 6-well plates. Briefly, a bottom layer consisting of 0.5% agar in complete culture medium was poured and, once solidified, was covered by a layer containing 0.3% agar and 2,000 cells/well. Treatments were performed by the addition of 5 ng/ml EGF (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 5 nM 17 $\beta$ -estradiol (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) or 5 ng/ml EGF + 5 nM 17 $\beta$ -estradiol to the medium in the top layer on experimental day 1, considering the total volume of medium in the well. The plates were placed in the incubator, and the colonies were counted under a light microscope LeicaDHi1 (Leica Microsystems GmbH, Wetzlar, Germany) following 2 weeks of incubation. Treatments were introduced on day 1 of experiment, and the cells were under treatment for the 2 weeks of incubation. Colonies containing a minimum of 64 cells were counted. The statistical significance of observed differences was evaluated using a one-way ANOVA with Tukey's HSD test.

**Mouse xenograft tumorigenesis assay.** All experiments on mice were performed according to Swedish and International guidelines (ethical approval no. C123/6, granted by the Uppsala Animal Tests Committee of the Uppsala Court, Uppsala, Sweden). Five severe combined immunodeficiency (SCID) mice (females, 12 weeks of age, housed in pathogen-free conditions, at 25°C, 12-h day/night cycle, food and water *ad libitum* (free access); Charles Rivers Laboratory, Worcester, MA, USA) were injected subcutaneously in the flank with selected and tested cell clones per condition, and with parental MCF7 cells, and six mice were injected with wild-type cells. Each mouse received 5x10<sup>6</sup> cells/injection in a volume of 100  $\mu$ l suspended at a 1:1 ratio in PBS and Matrigel (BD Pharmingen, San Diego, CA, USA) in the mouse flanks.

The mice were monitored twice a week for overall health and tumor formation. The tumor diameters were measured with calipers, and the tumor volume in mm<sup>3</sup> was calculated using the following formula: Volume=width<sup>2</sup> x length x0.5. The mice were sacrificed at 29 weeks. All the tumors were excised, fixed in 4% paraformaldehyde for 24 h at 4°C and embedded in paraffin for analysis.

**Immunohistochemistry.** Paraffin-embedded sections of 5-μm were deparaffinized, rehydrated and subjected to antigen retrieval by incubation in citrate buffer (pH 6; Dako, Glostrup, Denmark) twice for 7 min at 95°C. Quenching of endogenous peroxidase was performed by incubation in 3% H<sub>2</sub>O<sub>2</sub> in PBS for 10 min at room temperature. Upon washing in PBS, the slides were incubated in 20% normal goat serum (cat. no. S-1000; Vector Laboratories, Inc., Burlingame, CA, USA) in PBST (PBS with 0.1% Tween-20) for blocking. The tumor sections were then incubated with anti-human Ki-67 clone MIB1 antibody (cat. no. M7240; Dako) diluted 1:250 and with anti-von Willebrand factor (vWF) antibody (cat. no. ab6994; Abcam, Cambridge, MA, USA) diluted 1:750 in blocking buffer overnight at 4°C. Following washing with PBST, a secondary biotinylated universal antibody (horse anti-mouse/rabbit IgG; cat. no. BA-1400; Vector Laboratories, Inc.) at dilution 1:50 was added and incubated for 45 min in room temperature. The slides were stained using a Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, USA) following the manufacturer's protocol, and then counterstained with Mayer's hematoxylin, dehydrated and mounted with Mountex (SouthernBiotech, Birmingham, AL, USA). Images of the stained tissues were captured using a Leica DFC camera and images were acquired with Leica QWin version 3 software (Leica Microsystems Imaging Solutions, Ltd., Cambridge, UK). The proliferative score was measured as the ratio of MIB1-stained cells to the total number of cells. The vessel score was measured as the number of vessels stained with anti-vWF antibody per tumor section.

**Flow cytometry.** A total of 1x10<sup>6</sup> cells were used per experimental condition. The cells were collected, washed twice in cold PBS + 1% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) and incubated at 4°C with either 1:25-diluted anti-CD44-fluorescein isothiocyanate antibody (1:25; cat. no. 555478; clone G44-26; BD Pharmingen) or anti-CD24-allophycocyanin antibody (1:25; cat. no. 561646; clone ML5; BD Pharmingen) in PBS + 1% BSA for 30 min in the dark. Subsequently, the cells were washed twice in cold PBS + 1% BSA and resuspended in 400 μl cold PBS + 1% BSA for flow cytometric analysis.

**Mammosphere assays.** The cells were trypsinized, mechanically separated and passed through 40-μm strainers to obtain a single cell suspension. Subsequently, the cells were plated at a density of 5,000 or 1,000 cells in 4 ml per well in super-low-attachment plates. Treatments were added on day 1 upon seeding of cells, and continued for 2 weeks as follows: EGF at 5 ng/ml, 17β-estradiol at 5 nM, TGFβ1 at 10 ng/ml, and Iressa, tamoxifen and SB431542 at 10 μM. The numbers of mammospheres formed were counted following 2 weeks of incubation with these drugs.

**Statistical analysis.** Significant differences were calculated using a one-way ANOVA with Tukey's HSD test. Data was analyzed using online software (www.icalcu.com online test) and with IBM SPSS software version 25 (IBM Corp., Armonk NY, USA). Results were presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Exposure to EGF and/or 17β-estradiol enhances the proliferation rate of MCF7 cells.** Following exposure of MCF7 cells to 17β-estradiol and/or EGF for 40-42 days, 12 clones per experimental condition were randomly collected. These clones were subjected to an MTT proliferation assay, and representative clones were used for subsequent experiments (data not shown). Prior to the proliferation experiments, EGF and 17β-estradiol were removed, and the cells were cultured in a standard culture medium without EGF or 17β-estradiol for two passages (~1 week). The proliferation rate of the cells was then measured. Control cells underwent the same manipulations as the EGF and 17β-estradiol-exposed cells. To evaluate whether the selection procedure by itself had an impact on the MCF7 cells, parental MCF7 cells were also included in the analysis. The parental cells were grown under standard culturing conditions as an adherent culture, and were not subjected to a substrate-independent selection.

The results indicated that cells exposed to EGF and/or 17β-estradiol exhibited a significantly enhanced rate of cell proliferation, whereas the control cells only exhibited a marginal increase in proliferation rate (Fig. 1A). These cell clones were used in all subsequent experiments. The prolonged exposure to EGF and/or 17β-estradiol resulted in enhanced proliferation rates *in vitro*.

Substrate-independent growth is one of the key features of transformed cells; these cells do not require attachment to a substrate for proliferation. A soft agar colony formation assay was used to analyze the ability of cells to grow unattached to a surface. It was observed that the control and EGF- and/or 17β-estradiol-exposed cells exhibited an enhanced ability to form colonies, compared with the parental cells (Fig. 1B). The difference between the parental and control cells may be due to the selection of control cells in the absence of adherence, whereas the parental cells were maintained as an adherent culture. Between the various selection conditions, the observed differences were not significant (P>0.05), the colonies did not differ significantly in their shapes, and no significant spreading of cells from the colonies observed (Fig. 1B). This higher level of colony formation in semi-solid medium may have resulted from the effect of the substrate-independent selection of cells during drug exposure (Fig. 1B). The proliferation-stimulating effect of the various treatments was observed when the cells were grown as a two-dimensional culture (Fig. 1A).

**EGF- and/or 17β-estrogen-exposed cells do not exhibit enhanced tumor formation ability in a xenograft mouse model.** To investigate whether long-term exposure to EGF and/or 17β-estradiol affects the ability of cells to form tumors *in vivo*, 5x10<sup>6</sup> exposed cells were inoculated in both flanks of



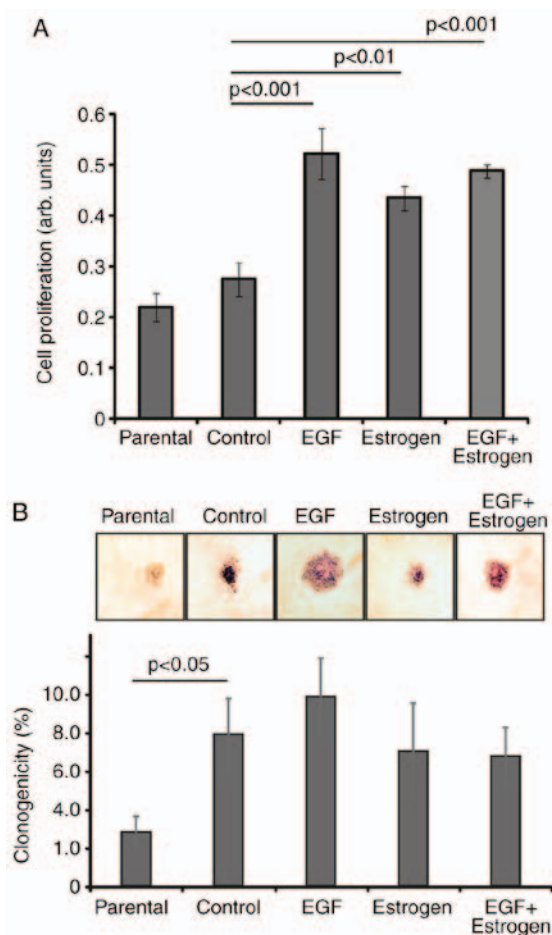


Figure 1. Prolonged exposure to EGF and 17 $\beta$ -estradiol increases the rate of proliferation of MCF7 cells. (A) MCF7 cell clones were generated following culture in anchorage independence on agarose and exposure to EGF and/or 17 $\beta$ -estradiol, alone or in combination. Cell proliferation was measured using an MTT assay. Statistical significance of differences was evaluated using a one-way ANOVA with Tukey's HSD. The experiment with representative clones is shown of five performed experiments. (B) Soft agar colony formation assay was performed with MCF7 cells exposed to treatments with EGF and/or 17 $\beta$ -estradiol, alone or in combination, as indicated. Statistical significance of differences was evaluated using a one-way ANOVA with Tukey's HSD. Images show examples of colonies formed by the cells, to illustrate shapes of colonies formed (magnification, x400). Representative results of three experiments are shown. EGF, epidermal growth factor.

each mouse (n=5 SCID mice per condition; n=6 mice with non-exposed control cells). The tumor take, growth and the overall state of the mice were monitored every second day for 29 weeks. It was observed that three of the five mice injected with parental MCF7 cells developed tumors, whereas all six mice injected with control cells, all five mice injected with cells exposed to EGF or 17 $\beta$ -estradiol, and four of the five mice injected with cells exposed to EGF + 17 $\beta$ -estradiol presented with tumors (Fig. 2A). The volume of the tumors collected from the injection sites was also measured (Fig. 2B). No significant difference in volume was observed among the tumors derived from cells subjected to different exposures.

To examine whether the molecular and functional features of the cells in the xenograft tumors had changed, the tumor sections were stained for proliferative status by Ki-67 staining, whereas the vessel density and apoptosis were evaluated with the vascular marker vWF and a terminal

deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, respectively.

The Ki-67 protein is a nuclear marker of proliferation (22). To investigate the cell proliferation rates, immunohistochemistry was performed by staining the collected mouse tumors with the anti-Ki-67 antibody MIB1 (Fig. 2C). Cell proliferation was scored based on the intensity and frequency of staining (Fig. 2D). The anti-Ki-67 immunohistochemistry revealed higher overall staining of cells in tumors derived from control and EGF-exposed cells, and lower staining of cells in tumors derived from 17 $\beta$ -estradiol-exposed cells, compared with the staining of tumors derived from parental MCF7 cells. The overall proliferation score revealed that tumors from the control and EGF-exposed groups exhibited the highest proliferation score, at 80 and 70%, respectively. Tumors from the EGF and 17 $\beta$ -estradiol-exposed cells exhibited marginally higher Ki-67 staining than the parental cells, at 50, vs. 40%, respectively. However, tumors from the 17 $\beta$ -estradiol-exposed cells exhibited only 20% positive staining for Ki-67 (Fig. 2D).

vWF is a glycoprotein that mediates platelet adhesion to the sub-endothelium at sites of vascular injury, and binds and stabilizes factor VIII in the blood (23,24). vWF appears to be expressed exclusively in endothelial cells, where it exhibits a granular pattern of reactivity. vWF is commonly used as an immunohistochemical marker of endothelial cells (23,24). To evaluate the angiogenic status of cells within the collected tumors, immunohistochemistry for vWF was performed and vessel density was analyzed in the present study (Fig. 2E). The tumors from the control, EGF-exposed and EGF + 17 $\beta$ -estradiol-exposed cells exhibited significantly enhanced angiogenesis compared with that of the parental group, with an increase in vessel density of >2.8-fold for the control and EGF-exposed cells, and a 3.5-fold increase for the EGF + 17 $\beta$ -estradiol exposed cells. The tumors derived from 17 $\beta$ -estradiol-exposed cells exhibited decreased angiogenesis by 33% compared with that of tumors derived from parental cells (Fig. 2F). No significant cell death was observed in tumors stained with TUNEL (data not shown). Therefore, the xenograft mouse study revealed that tumor volume and tumor take did not differ among tumors formed by cells exposed to EGF and/or 17 $\beta$ -estradiol. However, tumors formed by cells exposed to 17 $\beta$ -estradiol exhibited decreased expression of the proliferation marker Ki-67 and decreased vessel formation.

*EGF and 17 $\beta$ -estradiol exposure increases the breast cancer stem cell-like pool.* To examine whether exposure to EGF and/or 17 $\beta$ -estradiol affects the number of breast cancer stem cells, the proportion of stem-like cells was evaluated by flow cytometry and mammosphere formation assays. It was observed that, upon EGF, 17 $\beta$ -estradiol, and EGF + 17 $\beta$ -estradiol exposure, the proportion of CD44<sup>+</sup>/CD24<sup>-</sup> cancer stem-like cells was ~5-, 3- and 3-fold higher, respectively, than that of parental cells. No significant change in the proportion of CD44<sup>+</sup>/CD24<sup>-</sup> cells was detected in the control cells compared with the parental cells (Fig. 3A). This suggested that the change in expression of CD44<sup>+</sup>/CD24<sup>-</sup> markers was attributed to the long-term treatments.

Mammosphere formation is indicative of cell transformation and is associated with the degree of stemness exhibited

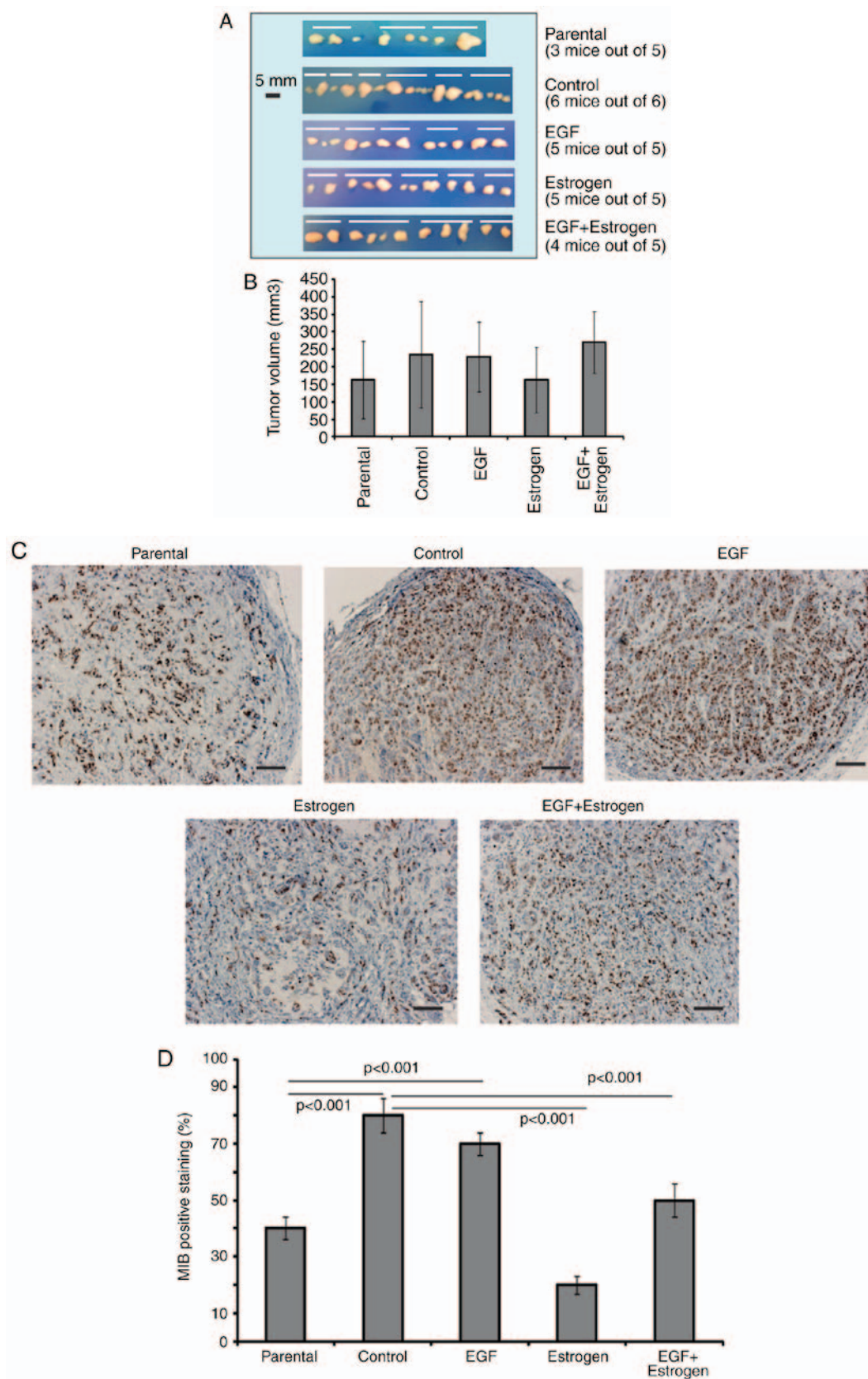


Figure 2. Tumorigenicity of MCF7 cell clones in the mice xenograft assay. (A) No significant difference in tumor take was found between different clones. The tumor take was calculated following retrieval of the tumors from the mice. The images show retrieved tumors from the mice, with white lines indicating tumors retrieved from the same mouse. In certain mice and in flanks, 2-3 tumors formed in the same injection site. Scale bar=5 mm. (B) Tumor volumes did not differ between experimental conditions. Tumor(s) formed in the same injection site were considered. (C) Immunohistochemical staining with MIB1 antibodies revealed that the tumors formed by the EGF- and 17 $\beta$ -estradiol-exposed cells showed higher proliferation rate. Representative regions of the sections are shown. Scale bar=200  $\mu$ m. (D) Quantification of MIB1 staining (Ki-67 expression) in retrieved tumors is shown as the percentage of positive cells. Statistical significance of differences was evaluated using a one-way ANOVA with Tukey's HSD (All P<0.001). Statistical significance of differences was evaluated using a one-way ANOVA with Tukey's HSD (all P<0.001). Annotations in the panels indicate parental and control cells and clones of cells exposed to EGF and/or 17 $\beta$ -estradiol. EGF, epidermal growth factor.

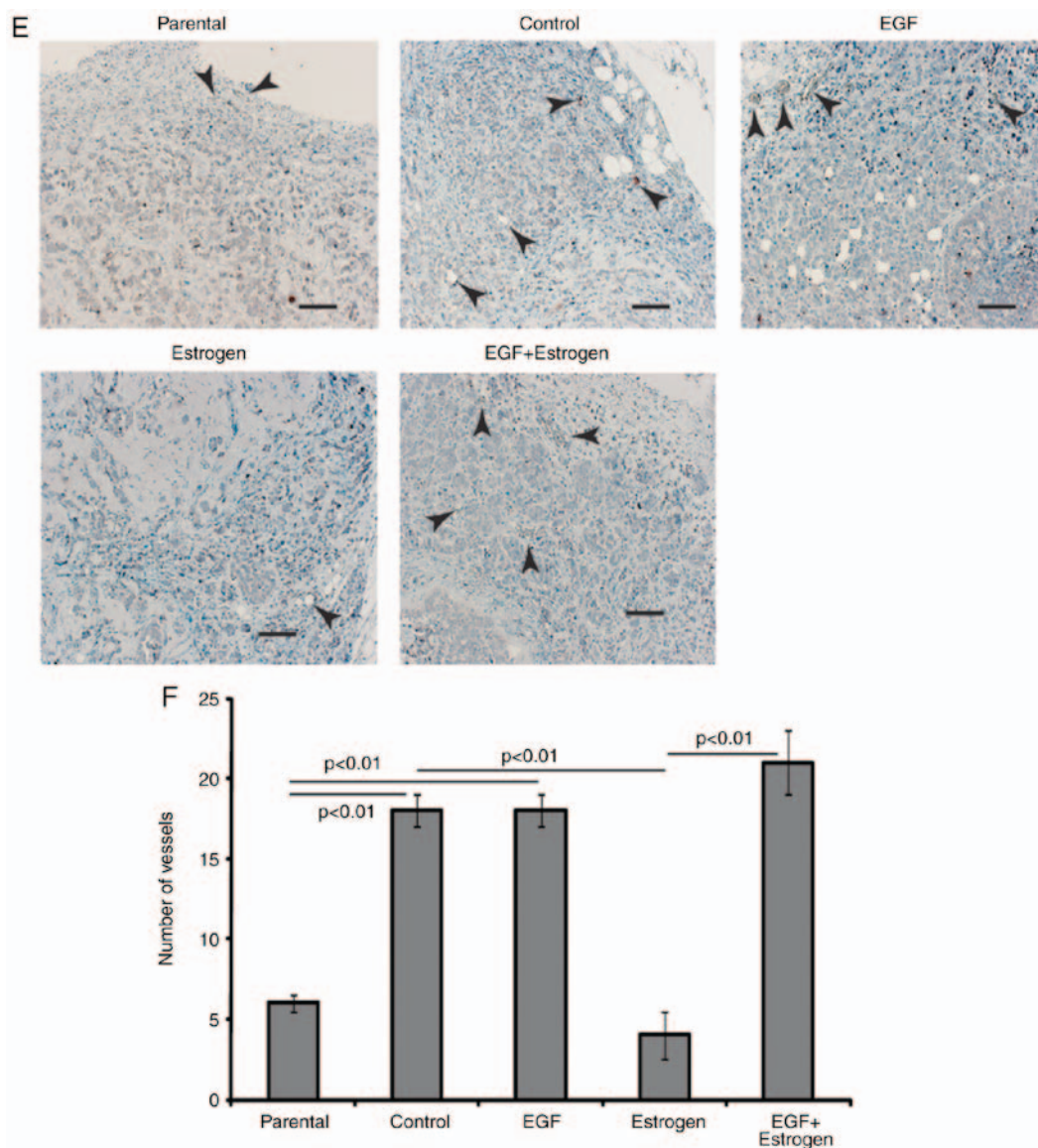


Figure 2. Continued. Tumorigenicity of MCF7 cell clones in the mice xenograft assay. (E) Immunohistochemical staining with vWF antibodies showed formation of vessels in the tumors formed by the EGF- and 17 $\beta$ -estradiol-exposed cells. Arrowheads indicates vessels. Representative regions of the sections are shown. Scale bar=200  $\mu$ m. (F) Vessel scores were calculated following evaluation of vWF staining in the sections. Statistical significance of differences was evaluated using a one-way ANOVA with Tukey's HSD (all  $P < 0.001$ ). Annotations in the panels indicate parental and control cells and clones of cells exposed to EGF and/or 17 $\beta$ -estradiol. EGF, epidermal growth factor; vWF, von Willebrand factor.

by the cells (25,26). The present study observed that the EGF, 17 $\beta$ -estradiol and EGF + 17 $\beta$ -estradiol-exposed cells formed 6-, 3- and 3.5-fold higher numbers of mammospheres, respectively, than the control cells (Fig. 3B). The control cells exhibited the same low level of mammosphere formation as the parental cells (Fig. 3B). The enhanced proportion of CD44<sup>+</sup>/CD24<sup>-</sup> cells and the increase in mammosphere formation suggested that exposure to EGF and/or 17 $\beta$ -estradiol promoted an increase of cells with cancer stem cell-like characteristics.

*EGF and 17 $\beta$ -estradiol exposure modulates mammosphere formation in response to treatments with Iressa, tamoxifen and SB431542.* As prolonged exposure to EGF and/or 17 $\beta$ -estradiol altered the physiology of MCF7 cells, the effects of Iressa, tamoxifen and SB431542 on the cells were further examined. These compounds are inhibitory agents targeting EGF, estrogen, and TGF $\beta$  signaling, respectively (27-30). The

mammosphere formation capacity of cells treated with EGF, 17 $\beta$ -estradiol, TGF $\beta$ 1, and the inhibitors of the corresponding signaling pathways, Iressa, tamoxifen and SB431542, was evaluated (Fig. 4A-C). It was observed that the parental and control cells had similar pattern of responses, suggesting that the selection in non-adherent conditions preserved the responsiveness mechanisms of the cells. By contrast, the cells exposed to 17 $\beta$ -estradiol had a lower amplitude of response to treatments compared with the other cells. Tamoxifen was the only drug that exhibited a consistent inhibitory effect in all the cells evaluated, which is consistent with the ER-positive status of MCF7 breast cancer cells (31). Iressa inhibited mammosphere formation in the EGF and EGF + 17 $\beta$ -estradiol-exposed cells. Exposure to EGF resulted in an overall increase of cell proliferation, which was inhibited upon treatment with 17 $\beta$ -estradiol, Iressa, tamoxifen and SB431542. Only TGF $\beta$ 1 stimulated mammosphere formation in the EGF-treated cells.



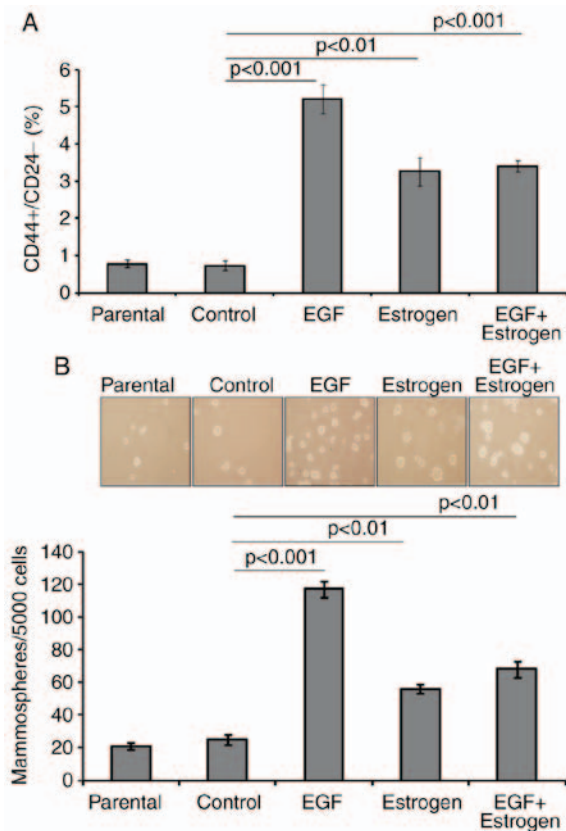


Figure 3. Prolonged EGF and/or 17 $\beta$ -estradiol exposure expanded cells with CD44<sup>+</sup>/CD24<sup>-</sup> phenotype. (A) Expression of CD44<sup>+</sup>/CD24<sup>-</sup> in EGF- and/or 17 $\beta$ -estradiol exposed cells was enhanced, compared with that in the control and parental cells. FACS sorting was performed with the same clones used for the xenograft mice experiments. Results are representative of two experiments performed. (B) Increased mammosphere formation was observed for the EGF- and/or 17 $\beta$ -estradiol-exposed cells. Images show examples of formed mammospheres (magnification, x100). Results are representative of three experiments performed. Statistical significance of differences in formation of mammospheres was evaluated using a one-way ANOVA with Tukey's HSD. EGF, epidermal growth factor; CD, cluster of differentiation.

A similar but less pronounced pattern of responsiveness was observed for cells exposed to EGF and 17 $\beta$ -estradiol (Fig. 4).

The above results suggested that prolonged exposure of human epithelial cells to EGF and/or 17 $\beta$ -estradiol altered the response pattern of the cells to short-term treatments with EGF, 17 $\beta$ -estradiol, TGF $\beta$ 1, and inhibitors of the corresponding signaling pathways (Fig. 4).

## Discussion

The period of time that is required for EGF and estrogen-exposed cells to acquire irreversible changes in tumorigenesis-relevant physiology remains to be elucidated. Short-term treatment, for hours or a few days, may not alter the cells irreversibly, as cancer cells are known to be robust (32,33). Therefore, the length of drug exposure required to induce a sustainable change in cellular physiology is of high relevance for understanding tumorigenesis. The present study demonstrated that drug exposure of MCF7 cells for 40-42 days affected their proliferation rate and transformation phenotype, but was not sufficient to affect tumor growth in mice (Fig. 5).

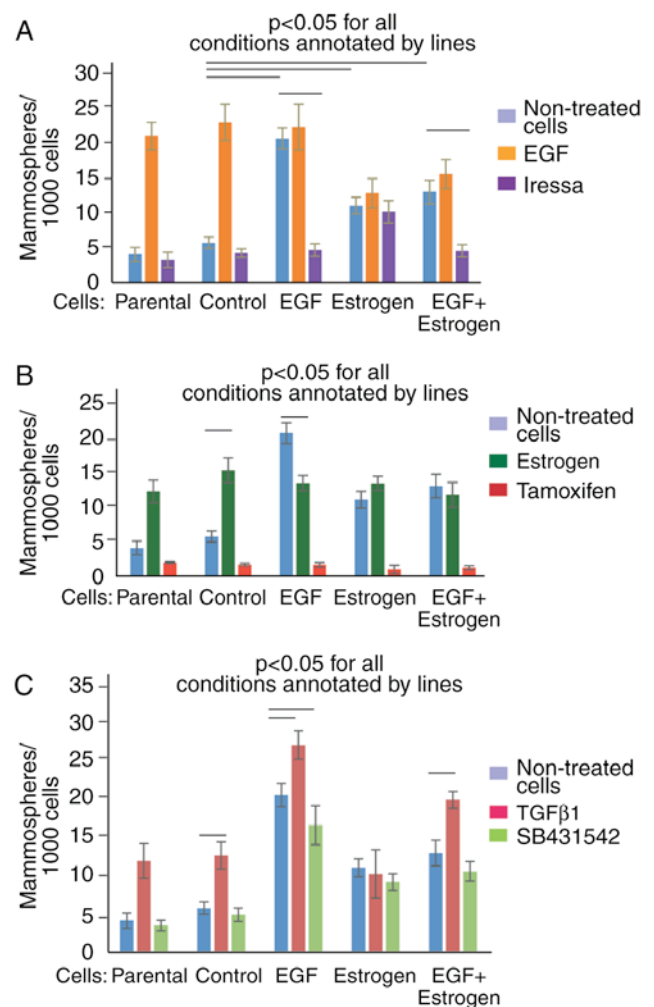


Figure 4. Exposure of cells to EGF and/or 17 $\beta$ -estradiol changes responsiveness of cells to treatments with TGF $\beta$ 1, Iressa, tamoxifen, and SB431542. Cells groups comprised Parental, Control, EGF, Estrogen (17 $\beta$ -estradiol), and EGF + Estrogen (EGF + 17 $\beta$ -estradiol), as indicated. Cells were treated with (A) EGF and Iressa, (B) estrogen and tamoxifen, and (C) TGF $\beta$ 1 and SB431542. The statistical significance of differences was evaluated using a one-way ANOVA with Tukey's HSD. Representative results of three experiments performed are shown. EGF, epidermal growth factor; TGF $\beta$ , transforming growth factor- $\beta$ .

EGF and 17 $\beta$ -estradiol are known stimulators of cell proliferation (3-9). The present study observed that prolonged exposure resulted in faster proliferation of MCF7 cells, and this higher rate of proliferation was maintained even upon removal of EGF and 17 $\beta$ -estradiol from the medium (Fig. 1). EGF and 17 $\beta$ -estradiol have been reported to promote tumor formation in mice (34,35). The present study observed that exposure to EGF and 17 $\beta$ -estradiol for 40-42 days did not affect the take or volume of the formed tumors when the treatments were withdrawn, and the cells injected in the mice were no longer under treatment (Fig. 2). However, immunohistochemistry revealed that exposure to 17 $\beta$ -estradiol resulted in lower proliferation rate and vascularization. This finding is in agreement with the reported roles of estrogen in the development of breast tissues and breast tumors (36,37). The data obtained in the present study suggested that long-term exposure to drugs did not alter the cellular physiology sufficiently to ensure more marked tumor growth upon withdrawal of treatment.

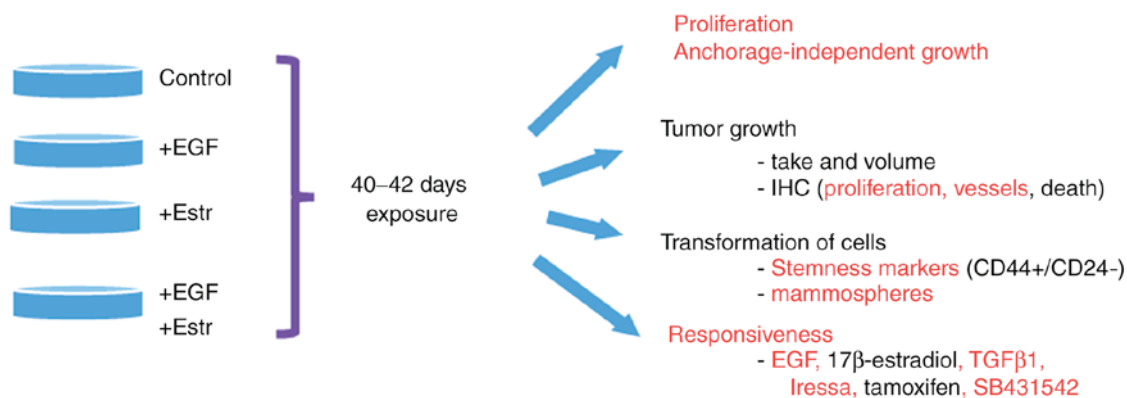


Figure 5. Long-term exposure of MCF7 cells to EGF and/or 17 $\beta$ -estradiol alters cell proliferation, mammosphere formation, expression of CD44<sup>+</sup>/CD24<sup>-</sup> markers, and responsiveness to treatments, but is not sufficient to affect tumor take or volume in a xenograft mice model. Cellular functions affected by the exposure of cells to EGF and/or 17 $\beta$ -estradiol, compared with parental or control cells, are annotated in red. EGF, epidermal growth factor; Estr, 17 $\beta$ -estradiol; TGF $\beta$ 1, transforming growth factor- $\beta$ 1; CD, cluster of differentiation; IHC, immunohistochemistry.

Cancer stem cells are considered to be responsible for the development of tumors (38). Human breast cancer stem cells can be identified by the expression profile of markers, including CD44 and CD24. The CD44<sup>+</sup>/CD24<sup>-</sup> phenotype is characteristic of breast cancer stem cells (38). The present study observed that the fractions of CD44<sup>+</sup>/CD24<sup>-</sup> cells followed the pattern of cell proliferation rate and formation of mammospheres (Figs. 1A, 3A and B). This suggested that cell proliferation, the expression of CD44<sup>+</sup>/CD24<sup>-</sup>, and mammosphere formation may be early sustainable features induced by carcinogenic exposure to EGF and 17 $\beta$ -estradiol.

Changes in cellular physiology may have an effect on cellular responsiveness to drugs. The present study evaluated whether the formation of mammospheres was affected upon treatment of the cells with EGF, 17 $\beta$ -estradiol, TGF $\beta$ 1, and drugs that inhibit the corresponding signaling pathways, Iressa, tamoxifen and SB431542 (Fig. 4). As expected, EGF and 17 $\beta$ -estradiol induced mammosphere formation in parental and control cells, however, this effect was negligible in the EGF- and/or 17 $\beta$ -estradiol-exposed cells, and even inhibitory in the EGF-exposed cells treated with 17 $\beta$ -estradiol. This response may indicate refractoriness of the EGF and 17 $\beta$ -estradiol signaling pathways. Cells exposed to 17 $\beta$ -estradiol were less susceptible to the effects of EGF, TGF $\beta$ 1, 17 $\beta$ -estradiol, Iressa, and SB431542. This result was expected, as treatment with the ligand 17 $\beta$ -estradiol has been shown to reduce the expression of human epidermal growth factor receptor 2 (HER2) in MCF7 wild-type cells (39). The potent inhibitory effect of tamoxifen on all cells suggested that the MCF7 cells preserved their ER $\alpha$ -positive status, thus tamoxifen remained effective independently of the exposure of cells to EGF and 17 $\beta$ -estradiol (Fig. 4). The cells exposed to EGF were particularly sensitive to TGF $\beta$ -induced proliferation, a clear indicator of crosstalk of these two pathways, for which there is accumulating evidence (40–42). In HER2-transformed cells, TGF $\beta$  further stimulated HER2 signaling to promote malignancy and induced resistance to anti-HER2 therapy. The observations in the present study showed changes in cellular responsiveness, and confirmed the previously reported promotion of transformation by EGF and TGF $\beta$ 1, the stimulatory effect of 17 $\beta$ -estradiol, and the

inhibitory effects of Iressa and tamoxifen (3–8,43,44). The observations also confirmed that long-term exposure may contribute to the development of resistance to Iressa in 17 $\beta$ -estradiol-exposed cells, and to SB431542 in EGF- and/or 17 $\beta$ -estradiol-exposed cells.

The response of tumor cells to external stimuli upon short-term treatment induces a number of regulatory processes. However, the majority of these induced responses reverse to the initial state in cells upon withdrawal of the stimulus. The present study hypothesized the existence of stimuli that are long-term effective and/or durable enough to irreversibly alter cellular responsiveness, as cells are robust regulatory systems (32,33). The roles of EGF and estrogen in breast cancer are well documented, and are associated with high levels of signaling as a response to constantly elevated levels of EGF and estrogen as ligands or due to mutations rendering their signaling levels elevated and independent from ligands (3,4,34–37,42–44). The results of the present study suggested that other stimuli may be required in addition to EGF and 17 $\beta$ -estradiol to promote tumor growth, or that EGF and 17 $\beta$ -estradiol stimulation may be constantly required during tumor growth. The results demonstrated that exposure to EGF and/or 17 $\beta$ -estradiol for 40 days was sufficient to alter cell proliferation rate; this transformation was evident in the ability of cells to form mammospheres and express stemness markers, but was not sufficient to affect the rate of tumor growth in mice.

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## Availability of data and materials.

Data and materials are available upon request. There is no data to be deposited in repositories.

## Authors' contributions

All authors contributed equally. SS designed and managed the project, SIC and MJ performed experiments. All authors were involved in writing the manuscript.

## Ethics approval and consent to participate

The animal experiments were performed according to the Swedish national and international guidelines (ethical permit no. C123/6, approved by the Uppsala Animal Tests Committee of the Uppsala Court).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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