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Full paper

Estrogen receptor-α36 is involved in epigallocatechin-3-gallate induced growth inhibition of ER-negative breast cancer stem/progenitor cells

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ABSTRACT

Epigallocatechin-3-gallate (EGCG) is a type of catechin extracted from green tea, which is reported to have anticancer effects. EGCG is also reported to inhibit the cancer stem/progenitor cells in several estrogen receptor (ER)-negative breast cancer cell lines, such as SUM-149, SUM-190 and MDA-MB-231. And all these cancer cells are highly expressed a new variant of ER-α, ER-α36. The aim of our present study is to determine the role of ER-α36 in the growth inhibitory activity of EGCG towards ER-negative breast cancer MDA-MB-231 and MDA-MB-436 cells. We found that EGCG potently inhibited the growth of cancer stem/progenitor cells in MDA-MB-231 and MDA-MB-436 cells, and also reduced the expression of ER-α36 in these cells. However, in ER-α36 knocked-down MDA-MB-231 and MDA-MB-436 cells, no significant inhibitory effects of EGCG on cancer stem/progenitor cells were observed. We also found that down-regulation of ER-α36 expression was in accordance with down-regulation of EGFR, which further verified a loop between ER-α36 and EGFR. Thus, our study indicated ER-α36 is involved in EGCG's inhibitory effects on ER-negative breast cancer stem/progenitor cells, which supports future preclinical and clinical evaluation of EGCG as a therapeutic option for ER-α36 positive breast cancer.

1. Introduction

Breast cancer is one of the most commonly diagnosed cancers in women (1). Based on the presence or the absence of the specific estrogen receptor (ER), ER-α, human breast cancers can be divided into the ER-positive and -negative subtypes (2). While ER-positive breast cancers are often treated with ER antagonists, such as fulvestrant/tamoxifen and aromatase inhibitors, ER-negative tumors are unresponsive to endocrine-targeted therapy and are mostly treated with chemotherapy (3, 4). Considering the severe toxicity, side effects and poor response rate associated with chemotherapy, less toxic and more effective therapeutic agents are needed for the treatment of human ER-negative breast cancer.

Accumulating evidence indicated that a subpopulation of tumor cells, characterized with distinctive stem/progenitor properties, is responsible for tumor initiation, invasive growth, and metastasis (5, 6). These tumor-initiating or cancer stem/progenitor cells are capable of self-renewal and differentiation, resulting in the vast majority of the tumor bulk cells (7, 8). As cancer stem/progenitor cells are resistant to most conventional therapy, including chemotherapy and radiation therapy, novel and effective agents that specifically target these cells are urgently needed.

In 2006, Wang et al. identified and cloned a 36 kDa variant of ER-α, ER-α36 (9, 10). Unlike conventional ER-α and ER-β, ER-α36 is mainly expressed at the plasma membrane and in the cytoplasm, and mediates non-genomic estrogen signaling, including activation of the ERK1/2 and PI3K/AKT pathways (11, 12). Previous studies have revealed that ER-α36 expression was detected in breast cancer patients diagnosed as ER-negative (absence of ER-α66 expression) (11). ER-α36 is also over-expressed in ER-negative breast cancer cell lines, which is associated with the malignant growth of cancer cells (13). It has been verified that there exists an ER-36/EGFR cross-regulatory loop in which EGFR and ER-α36 positively regulate each other's expression (13). Recently, ER-α36 expression was reported

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to correlate with ALDH1 expression, a marker of breast cancer stem/progenitor cells, in clinical samples of breast cancer patients (14). Additionally, in SK-BR-3, an ER-negative breast cancer cell line, knockdown of ER-α36 resulted in a decrease of the ALDH1-positive cells (15). Thus, ER-α36 may play an important role in maintenance and expansion of cancer stem/progenitor cells, and also presents an attractive target for cancer stem cell-targeted therapy.

Epigallocatechin-3-gallate (EGCG), the most abundant and active constituent in green tea, has therapeutic benefits for a variety of pathological conditions, including cancer, neurodegenerative diseases, diabetes, and cardiovascular diseases (16). EGCG affects several signaling and metabolic pathways, leading to the inhibition of cancer cell growth, and tumor angiogenesis (17–19). Previous studies have shown that EGCG treatment inhibits ER-negative tumor growth (20, 21), and also inhibits growth of ER-negative breast cancer stem/progenitor cells (22). Recently, Chung et al. reported that the EGCG is more potent in ER-negative breast cancer MDA-MB-231 cells compared to ER-positive MCF7 cells (23). Thus, EGCG is potent growth inhibitor of malignant growth of ER-negative breast cancer cells. However, the mechanism by which EGCG mediates inhibition of the growth of ER-negative breast cancer stem/progenitor cells is not clear.

Previously, an ER-α36 specific down-regulator, Broussoflavonol B, a chemical purified from the bark of the Paper Mulberry tree, is reported to restrict the growth of ER-negative breast cancer stem/progenitor cells (24). Both EGCG and Broussoflavonol are phenolic compounds extracted from plants, and both have a flavonoid backbone (25). Since Broussoflavonol B shares a similar chemical structure with EGCG (Fig. 1), we decided to study whether EGCG functions through the ER-α36 signaling pathway.

2. Materials and methods

2.1. Chemicals and reagents

EGCG (≥95% pure) was obtained from Sigma–Aldrich (St Louis, MO, USA). EGFR antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). The monoclonal anti-ER-α36 antibody and ER-α36-specific shRNA expression vector were kindly provided by Dr. Zhao-Yi Wang (Department of Medical Microbiology & Immunology, Creighton University Medical School, Omaha, Nebraska, USA). β-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PerCP-Cy™ 5.5 mouse anti-human CD44 and PE mouse anti-human CD24 antibodies were obtained from BD Biosciences (San Jose, CA, USA). Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12), fetal bovine serum (FBS) and B27 were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Cell culture and treatment

MDA-MB-231 and MDA-MB-436 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.05 U/mL penicillin and 0.05 mg/mL streptomycin. Cells were maintained in phenol red-free media with 2.5% charcoal-stripped FBS (Thermo Scientific Hyclone, Logan, UT, USA) for 24 h prior to experimentation. All cell cultures were maintained at 37 °C with 5% CO_2 in a humidified incubator. Using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), the $CD44^+/CD24^-$ cell population was examined following incubation with PerCP mouse anti-human CD44 and PE mouse anti-human CD24 antibodies as per the manufacturer's instructions.

2.3. Tumorsphere formation assay and flow cytometry analysis

To evaluate the growth of cancer stem/progenitor cells, single cell suspensions of 5×10^4 MDA-MB-231 or MDA-MB-436 cells were seeded into low attachment 6-well dishes (Corning Incorporated, Corning, NY, USA) and cultured in the tumorsphere medium containing indicated concentrations of EGCG. The typical tumorsphere media are phenol-red free DMEM/F12 medium supplemented with 1 × B27, 20 ng/ml epidermal growth factor (Sigma–Aldrich, St. Louis, MO, USA), 20 ng/ml basic fibroblast growth factor (ProSpec, NJ, USA), and 0.5 μg/ml hydrocortisone (Sigma Aldrich Co., St. Louis, MO, USA). After seven days of culture with the indicated concentrations of EGCG, the number of tumorspheres and dissociated cells were counted, as previously described (13). Three dishes were used for each concentration tested, and all of the experiments were conducted in triplicate.

For analysis of the $CD44^+/CD24^-$ cell population, single cell suspensions were washed with cold PBS containing 1% BSA and then incubated for 30 min at 4 °C with PerCP-CyTM5.5 mouse anti-human CD44 and PE mouse anti-human CD24 in PBS containing 1% BSA. After incubation, cells were washed twice and resuspended in cold PBS containing 1% BSA for flow cytometry analysis. For the EGF-stimulated growth assay, EGF (10 ng/ml), EGCG (20 μM or 30 μM) or both EGF and EGCG were added to cell cultures and incubated for 72 h. Total cell counts were

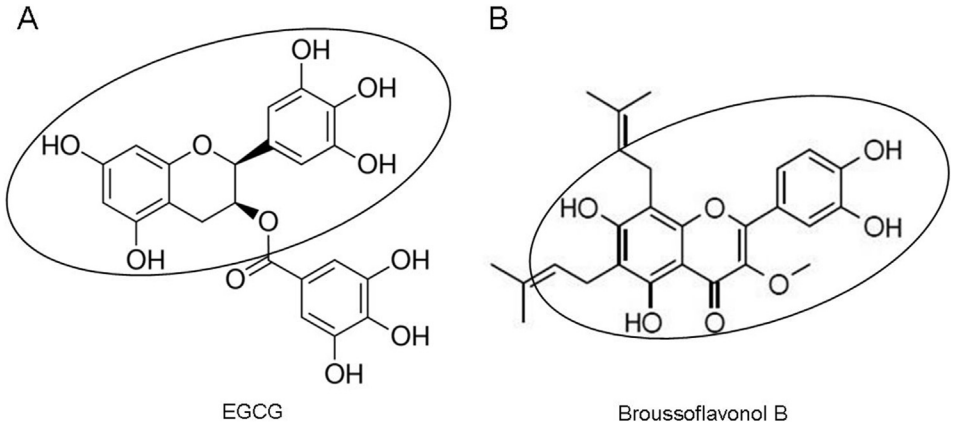

Fig. 1. Chemical structures of EGCG and Broussoflavonol B. (A). EGCG. (B). Broussoflavonol B. The core structure shared by EGCG and Broussoflavonol B was marked with an oval-shape ring, respectively.

determined following the experiment. Three dishes were used for each treatment, and all experiments were replicated more than three times.

2.4. Cell transfection and establishment of stable cell lines

To establish cell lines with ER-α36 expression knocked down by the shRNA method in breast cancer cells, stable cell lines were established as described previously (26). Briefly, cells transfected with the empty expression vector or the ER-α36-specific shRNA expression vector were selected in medium containing 300 μg/ml G418 for 3 weeks, and more than 20 individual clones of selected cells were pooled and were named as MB-231/shV or MB-436/shV and MB-231/sh36 or MB-436/sh36. The knocked-down level of ER-α36 expression was confirmed by Western blot analysis.

2.5. Western blot analysis

Western blot analysis was performed following the standard protocol. Briefly, cells were lysed, and the BCA assay was used to determine protein concentration of cell lysates. Protein samples were separated on a 10% gel using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then electro-transferred onto a PVDF membrane. The membranes were probed with appropriate primary antibodies followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. Bands were visualized using an enhanced chemiluminescence (ECL) system. Data within a linear range was quantified using ImageQuant software (GE Amersham, Piscataway, NJ, USA).

2.6. Statistical analysis

Data were summarized as the mean ± standard error (SE) using the GraphPad InStat software program. Tukey–Kramer Multiple Comparisons Test was also used, and the significance was accepted for $P < 0.05$.

3. Results

3.1. EGCG inhibits the growth of breast cancer stem/progenitor cells derived from ER-negative breast cancer cells

We tested the effects of EGCG on MDA-MB-231 and MDA-MB-436 tumorsphere cells. MDA-MB-231 and MDA-MB-436 cells were cultured in tumorsphere medium and in ultra-low attachment dishes. After seven days of treatment, total tumorsphere number and the cell number of dissociated tumorspheres were counted, respectively. We found that EGCG effectively inhibited the growth of tumorsphere cells from these two cell lines (Fig. 2A, B). The $CD44^+/CD24^-$ characteristic is often used to identify and characterize breast cancer stem cells. To test the effect of EGCG on breast cancer stem/progenitor cells, we tested the effect of EGCG treatment on the $CD44^+/CD24^-$ population in MDA-MB-231 and MDA-MB-436. We treated MDA-MB-231 and MDA-MB-436 cells with EGCG (20 μM) for seven days, and then we analyzed the $CD44^+/CD24^-$ population with flow cytometry. We found that EGCG treatment significantly reduced the $CD44^+/CD24^-$ cell population in these cells (Fig. 2C, D). These results indicated that EGCG effectively inhibited the growth of ER-negative breast cancer stem/progenitor cells.

3.2. EGCG down-regulates ER-α36 expression in ER-negative breast cancer cells

Previous studies indicated that ER-α36 is highly expressed in ER-negative breast cancer cells such as MDA-MB-231 and MDA-MB-436 cells (13) and is critical for the maintenance of stem/progenitor cells in both ER-positive and ER-negative breast cancer cells (14, 15). In the ER-negative breast cancer cell lines MDA-MB-231 and SK-BR-3, down-regulation of ER-α36 expression significantly reduced ALDH1-positive cell population (27). To probe the mechanism by which EGCG inhibited the growth of ER-negative cancer stem/progenitor cells, we decided to determine whether EGCG also down-regulated ER-α36 expression. Western blot analysis indicated that ER-α36 expression was down-regulated by EGCG treatment in a dose-dependent manner in MDA-MB-231 and MDA-MB-436 cells (Fig. 3A and B). Thus, our data indicated that down-regulation of ER-α36 expression is involved in growth inhibition of ER-negative breast cancer cells by EGCG.

3.3. EGCG also down-regulates EGFR expression and inhibits EGF-stimulated cell proliferation

Recently, Zhang et al. reported that there exists an ER-α36/EGFR positive feedback loop in ER-negative breast cancer cells; ER-α36 knockdown resulted in destabilization of EGFR protein (13). We decided to investigate whether EGCG-mediated down-regulation of ER-α36 expression also results in decreased EGFR expression. Accordingly, we examined EGFR expression in MDA-MB-231 and MDA-MB-436 following EGCG treatment. We found that EGCG treatment down-regulated EGFR expression in the two cell lines (Fig. 4A and B).

MDA-MB-231 and MDA-MB-436 breast cancer cell lines are characterized by the absence of estrogen receptor, progesterone receptor and HER2 expression and thus are named triple-negative breast cancer. As such, EGFR signaling is critical for the malignant growth of triple-negative breast cancer. As EGCG treatment down-regulated EGFR expression, we decided to examine the effect of EGCG treatment following EGF stimulation in MDA-MB-231 and MDA-MB-436 cells. As shown in Fig. 4C and D, under serum-starvation conditions, cell proliferation was stimulated by the addition of EGF. However, EGF-stimulated cell proliferation was inhibited by EGCG, indicating that EGCG attenuates EGF signaling possibly through the inhibition of EGFR expression. We then examined the time course of the inhibition of EGFR expression by ER-α36 knockdown. MDA-MB-231 and MDA-MB-436 cells were treated with 40 μM of EGCG and the expression levels of EGFR and ER-α36 were examined at 12, 24, 48, or 72 h. As shown in Fig. 4E, EGCG treatment was able to down-regulate ER-α36 expression at 24 h, which lasted for 72 h in MDA-MB-231 cells. EGFR expression began to be down-regulated within 48 h (Fig. 4E). We also observed similar change of EGFR expression in MDA-MD-436 cells, which lagged behind the down-regulation of ER-α36 expression (Fig. 4F). Thus, our results strongly suggested that ER-α36-mediated signaling is involved in the modulation of EGFR expression.

3.4. EGCG inhibited the MAPK/ERK and the PI3K/AKT signaling in breast cancer cells

Previous studies demonstrated that ER-α36 expression is closely related to the activation of the MAPK/ERK signaling in breast cancer (13). Several reports indicated that the MAPK/ERK and the PI3K/AKT signaling are the major non-genomic estrogen signaling pathways mediated by ER-α36, and both of the two signaling pathways are also involved in the positive feedback loop of ER-α36/EGFR (28–31). It has been reported EGCG treatment caused an

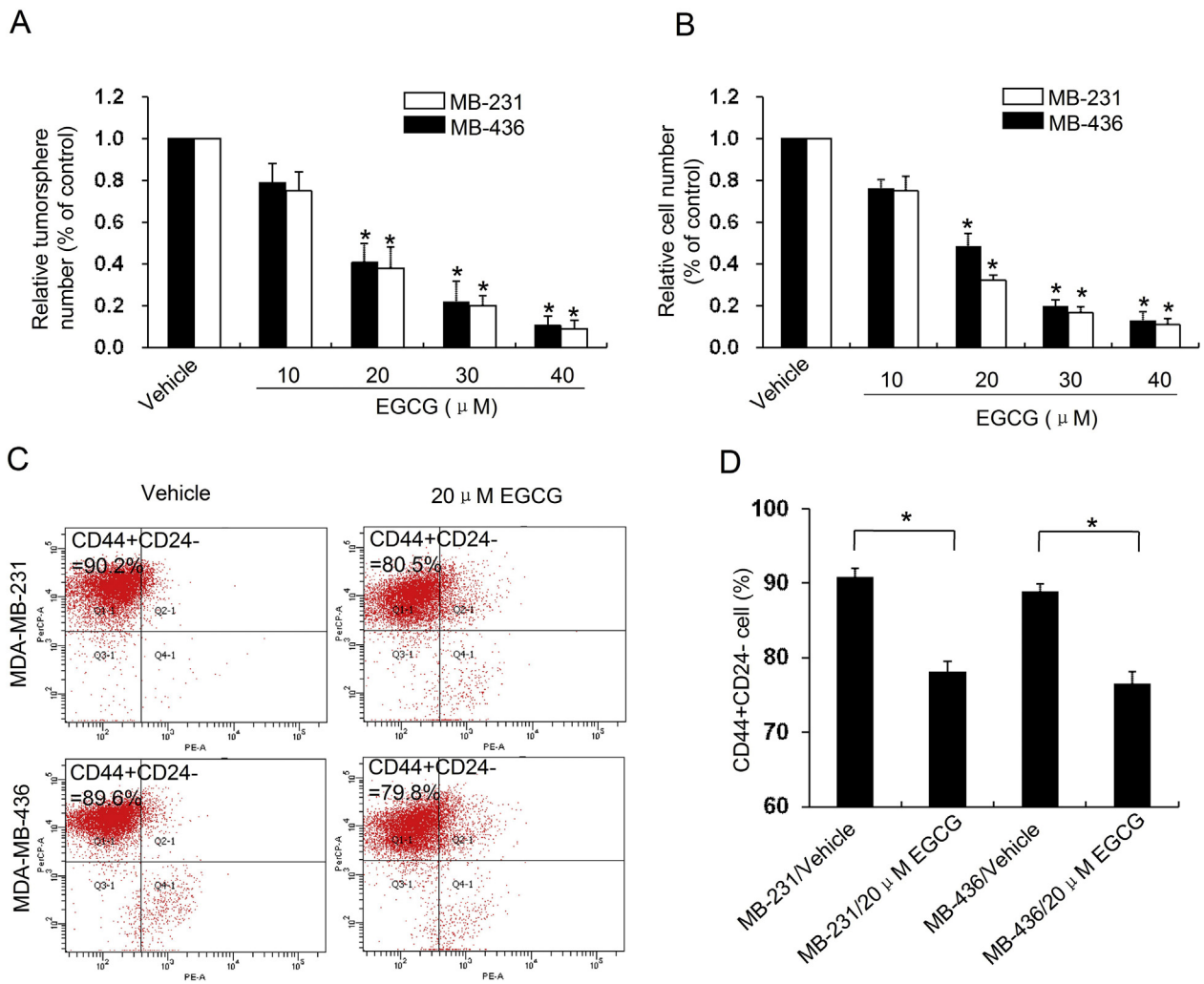

Fig. 2. EGCG reduces populations of the cancer stem/progenitor cells from ER-negative MDA-MB-231 and MDA-MB-436 cells. The tumorsphere formation assay and flow cytometry analysis of the $CD44^+/CD24^-$ cells were used to assess the population of breast cancer stem/progenitor cells. A and B, EGCG treatment decreases the number of tumorspheres and cells from dissociated tumorspheres derived from MDA-MB-231 cells (A) and MDA-MB-436 cells (B). C and D, EGCG treatment decreased the population of the $CD44^+/CD24^-$ cells in MDA-MB-231 and MDA-MB-436 cells. The MDA-MB-231 and MDA-MB-436 cells were treated with vehicle (DMSO) or indicated concentrations of EGCG for seven days. The $CD44^+/CD24^-$ population cells were analyzed after staining with fluorochrome-conjugated antibodies. C, The representative results are shown. D, The columns represent the means of three experiments; bars, SE. $^*P < 0.05$ for control cells treated with vehicle DMSO.

appreciable decrease in the phosphorylation levels of the ERK1/2 and AKT in MDA-MB-231 cells (32). To further test the possibility that EGCG may inhibit the MAPK/ERK and PI3K/AKT signaling, we examined the phosphorylated ERK and AKT in MDA-MB-436 cells treated with EGCG. The results showed that the phosphorylation level of the ERK1/2 was down-regulated by 20–40 μM EGCG in a dose-dependent manner (Fig. 5A). As showed in Fig. 5B, the PI3K/AKT signaling was also attenuated by EGCG in a dose-dependent manner. These results suggested that EGCG activity in suppression of the MAPK/ERK and the PI3K/AKT signaling pathways is involved in down-regulation of ER-α36 expression in breast cancer cells.

3.5. ER-α36 knockdown reduced the sensitivity of the breast cancer cells to EGCG

To further determine the role of ER-α36 in EGCG's effects on breast cancer stem/progenitor cells, we established two stable ER-α36 knockdown cell lines, MB-231/sh36 and MB-436/sh36. The ER-α36 expression in MB-231/sh36 and MB-436/sh36 with ER-α36 expression knocked down was dramatically decreased compared to the control cells transfected with the empty expression vector (Fig. 6A). We next examined the effect of ER-α36 knockdown on EGCG inhibitory activity in breast cancer stem/progenitor cells. As shown in Fig. 6B, MB-231/sh36 exhibited dramatically decreased sensitivity to EGCG compared to the control cells transfected with empty vectors. Similarly, MB-436/sh36 cells with ER-α36 knockdown were also less sensitive to EGCG than control MB-436/shV cells (Fig. 6C).

4. Discussion

In this study, we investigated the growth inhibitory potential of EGCG in ER-negative breast cancer stem/progenitor cells. We demonstrated that EGCG potently inhibited the growth of MDA-MB-231 and MDA-MB-436 stem/progenitor cells presumably through down-regulation of ER-α36 and EGFR expression.

A recent study indicated that the combination of curcumin and EGCG inhibited the growth of the cancer stem/progenitor cells from ER-negative breast cancer MDA-MB-231 cells (23) while EGCG

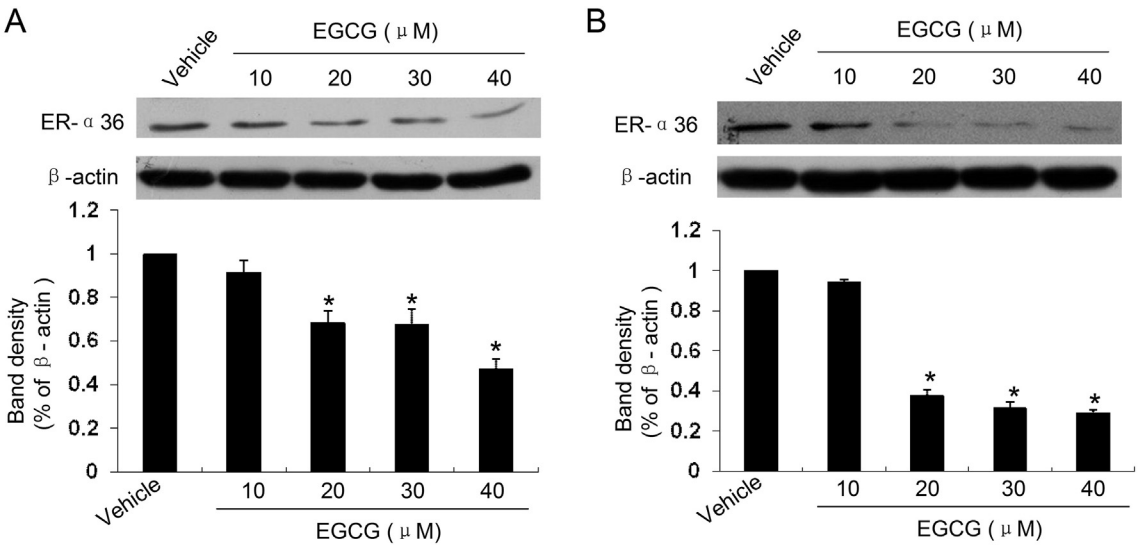

Fig. 3. EGCG treatment down-regulates ER-α36 expression in ER-negative MDA-MB-231 and MDA-MB-436 cells. Cells maintained in phenol red-free medium with 2.5% charcoal-stripped fetal calf serum were treated with vehicle DMSO and the indicated concentrations of EGCG for 24 h. Western blot analysis was performed to examine the expression of ER-α36 in MDA-MB-231 cells (A) and MDA-MB-436 cells (B). All membranes were stripped and re-probed with β-Actin antibody to ensure equal loading. The columns represent the means of three experiments; bars, SE. *$P < 0.05$ for control cells treated with vehicle DMSO.

alone was without any effect. Our results, however, indicated that EGCG alone effectively inhibited growth of ER-negative breast cancer stem/progenitor cells. The exact mechanism underlying this discrepancy is not clear. One possibility is that different concentrations of EGCG were used. They used 10 μM EGCG in their study, however, we failed to observe significant inhibition in cancer stem/progenitor cells with their dosage in our model.

As one of the most commonly consumed beverages worldwide, green tea has become a subject of interest for its potential anti-cancer properties. The most potent anti-cancer compound from green tea, EGCG, has been shown to affect cell growth, apoptosis, tumor angiogenesis and metastasis (33, 34). The molecular mechanisms underlying these anti-cancer properties are multiple facets, including catechins-based antioxidant/pro-oxidant activity, enzyme activity manipulation, and modulation of cancer-relevant molecular targets or signaling pathways (32, 35, 36). Accumulating evidence suggests that many cancers may originate from a small population of cancer stem/progenitor cells, which represent attractive new targets for cancer therapy. Zhang et al. reported that EGCG treatment inhibited cell viability and neurosphere formation as well as induced apoptosis of stem/progenitor cells in neuroblastoma U87 cells (37). Recently, a study has shown a dose-dependent reduction of cancer stem cell viability following EGCG treatment in human prostate cancer cell lines (38). EGCG treatment has also been shown to inhibit the proliferation of stem-like cells in inflammatory breast cancer (22). Additionally, EGCG treatment reduced growth and induced apoptosis of breast cancer stem/progenitor cells in culture (22) and decrease the growth of tumors derived from ALDH-positive stem/progenitor cells (22). Here, we reported that EGCG treatment effectively inhibits the growth of tumorsphere cells and $CD44^+/CD24^-$ cells from MDA-MB-231 and MDA-MB-436 cells, which further verified that EGCG is able to inhibit the growth of ER-negative breast cancer stem/progenitor cells.

Further investigation of the mechanism of tumor by which EGCG inhibits growth of breast cancer stem/progenitor cells revealed that EGCG treatment down-regulated ER-α36 expression in MDA-MB-231 and MDA-MB-436 cells. ER-α36, a variant of ER-α, is found to be highly expressed in ~40% of ER-negative breast cancer (11). ER-α36 has also been reported to mediate mitogenic activity of estrogen in ER-negative breast cancer MDA-MB-231 and MDA-MB-436 cells, which lack ER-α66 expression (13). Recently, Deng et al. reported that ER-positive breast cancer stem/progenitor cells express high levels of ER-α36 while shRNA-based knockdown of ER-α36 expression significantly reduced the percentage of breast cancer stem/progenitor cells (39). In addition, Kang et al. reported ER-α36 is critical in the maintenance of the ALDH1-positive cancer stem/progenitor cells (15), suggesting that ER-α36 expression is one characteristic of breast cancer stem/progenitor cells. Thus, down-regulation of ER-α36 expression may provide a novel therapeutic approach to treat ER-negative human breast cancer. Guo et al. reported that treatment with Broussoflavonol B, an ER-α36 specific down-regulator, restricted the growth of the ER-negative MDA-MB-231 and SK-BR-3 breast cancer cells (24). To further determine the role of ER-α36 in EGCG-induced inhibition of breast cancer stem/progenitor cells, two pairs of breast cancer cell lines, MDA-MB-231/shV and MDA-MB-231/sh36 cells as well as MDA-MB-436/shV and MDA-MB-436/sh36 cells, were used. ER-α36 knockdown was found to decrease the sensitivity of MDA-MB-231/sh36 and MDA-MB-436/sh36 cells to EGCG treatment, indicating that ER-α36 is involved in EGCG-induced inhibition of breast cancer stem/progenitor cells. Our study also indicated that the cells with ER-α36 knockdown formed much less tumorsphere compared to the control cells, suggesting that ER-α36 is involved in the maintenance of cancer stem/progenitor cells. In one of our previous studies, we demonstrated that the MCF7-HER2/18 cells that highly express ER-α36 formed more tumorspheres compared to the parent MCF7 cells while ER-α36 knocked-down in MCF-HER2/18 cells significantly decreased tumorsphere number (40). Consistent with our results, Zhang et al. reported that the MDA-MB-231 cells with ER-α36 knockdown showed significant reduction of the tumor growth in a xenograft mouse model, compared to the control MDA-MB-231 cells (31). Thus, both *in vitro* and *in vivo* studies indicated ER-α36 plays a critical role in maintenance of breast cancer stem/progenitor cells.

EGCG treatment has been suggested to exert different effects on several signal pathways in breast cancer (41, 42). EGCG, with the combination of raloxifene, has been revealed to inhibit the activation of the AKT, mammalian target of rapamycin (mTOR) and S-6-

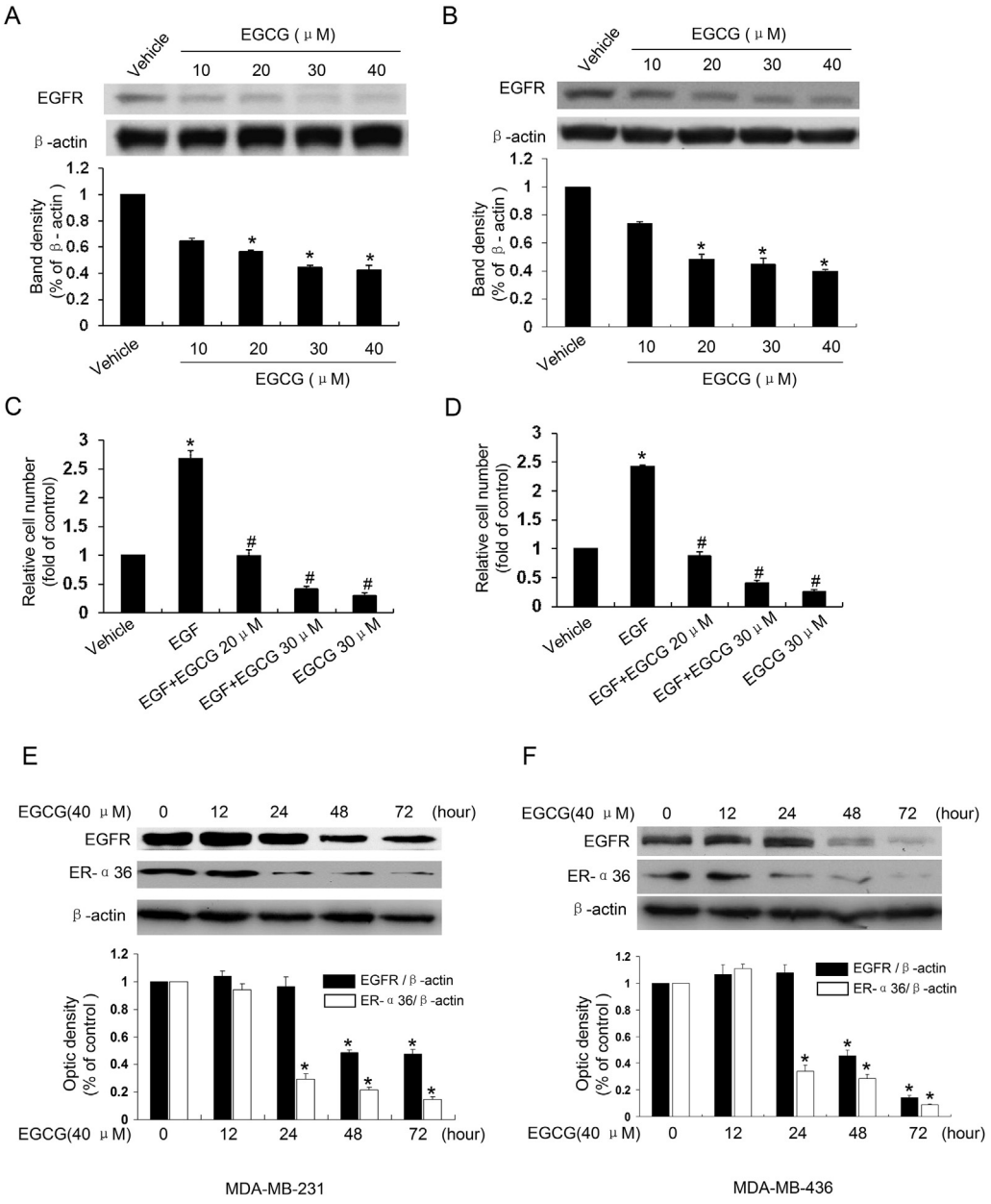

Fig. 4. EGCG down-regulates EGFR expression and attenuated mitogenic EGF signaling in MDA-MB-231 and MDA-MB-436 cells. A and B, Cells were treated with indicated concentrations of EGCG for 48 h. Cell lysates were subjected to Western blot analysis with an antibody for EGFR. The membrane was stripped and re-probed with a β-actin antibody to ensure equal loading. C and D, Cells maintained in phenol red-free medium with 2.5% charcoal-stripped FBS for 48 h. EGF (10 ng/ml) alone, together with indicated concentrations of EGCG or EGCG alone were added to cells and incubated for 72 h, and cell numbers were determined. Three dishes were used for each treatment and experiments were repeated more than three times. E and F, MDA-MB-231 (E) and MDA-MB-436 (F) cells were treated with 40 μM EGCG for different time periods (12, 24, 48 and 72 h) and Western blot analysis was performed to examine the levels of EGFR and ER-α36 expression. Columns: means ± SEM from three independent experiments. $^{*}P < 0.05$ versus control cells without treatment; $^{\#}P < 0.05$ versus cells treated with EGF (10 ng/ml).

kinase (S6K), which in turn represses the NF-κB signal in ER-negative breast cancer cells (35). Previous studies have also reported that MDA-MB-231 cells exposed to EGCG showed significant reduction in the NF-κB activation and the AKT phosphorylation (26, 36, 43). The MAPK/ERK pathway is one of the most important intracellular signal transductions in breast cancer. EGCG has been demonstrated to inhibit the MAPK/ERK activation and to reduce the expression and activity of matrix metalloproteinase 9 (MMP-9) in MDA-MB-231 cells (32). EGCG was also shown to repress the hepatocyte growth factor (HGF)-induced AKT and ERK phosphorylation in ER-negative breast cancer cells (37), further indicating the potent inhibitory activity of EGCG on the MAPK/ERK and PI3K/AKT pathways. Here, we found that EGCG attenuated the ERK and AKT signaling in ER-negative MD-MBA-436 cells. It has been reported that ER-α36 mediated the MAPK/ERK and the PI3K/AKT signal transduction. Thus, our results suggested EGCG inhibits the growth of ER-negative breast cancer cells through the repression of ER-α36-mediated MAPK/ERK and PI3K/AKT signaling.

In our study, we also found that EGCG treatment resulted in reduced EGFR expression, and cells treated with EGCG reacted poorly to EGF stimulation. Thus, EGCG treatment also attenuates EGF signaling in ER-negative breast cancer cells. Previous studies have reported that EGCG inhibits the EGFR signaling presumably through the inhibition of the ERK1/2 and AKT kinases (44). It has

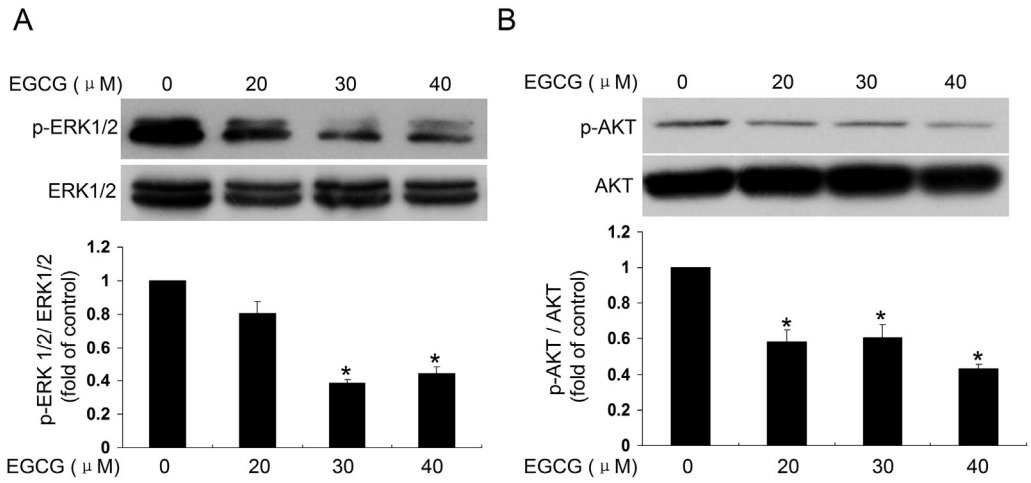

Fig. 5. EGCG inhibits the MAPK/ERK and PI3K/AKT signaling in breast cancer cells. A and B. MDA-MB-436 cells were treated with different concentrations of EGCG for 24 h. Western blot analysis was performed to examine the levels of the phospho-ERK1/2 (A) and phospho-AKT (B). The phosphorylation levels of these proteins were normalized with the expression levels of the total protein. Columns: means ± SEM from three independent experiments. *$P < 0.05$ versus control cells without treatment.

been confirmed that a positive feedback loop of EGFR and ER-α36 expression is critical for malignant growth of ER-negative breast cancer cells, in which ER-α36 mediates non-genomic estrogen signaling and stabilizes EGFR protein while EGF signaling up-regulates ER-α36 promoter activity through an Ap-1 binding site in the promoter region (13). In our study, we also noted that EGFR expression was inhibited by EGCG treatment, which lagged behind the down-regulation of ER-α36 expression. Thus, our results demonstrated that EGCG attenuates the EGF signaling presumably through down-regulation of the positive loop between ER-α36 and EGFR expression.

The function and underlying mechanism of ER-α36 in ER-negative breast cancer cells have been reported. The expression profiles and function of ER-α36 in ER-positive cells have not been well established. Wang et al. first reported that the expression of ER-α36 in ER-positive MCF7 cells and T47D cells (10). Chaudhri et al. has reported the high level of ER-α36 expression in ER-positive MCF7 cells (45), while Deng et al. found MCF7 cells expressed very low level of endogenous ER-α36 (39). Zhang et al. found the high-passage (>75 passages) MCF7 cells expressed high level of ER-α36 as well as EGFR and HER2. However, ER-α36 expression in low-passage (<35 passages) MCF7 cells was very low

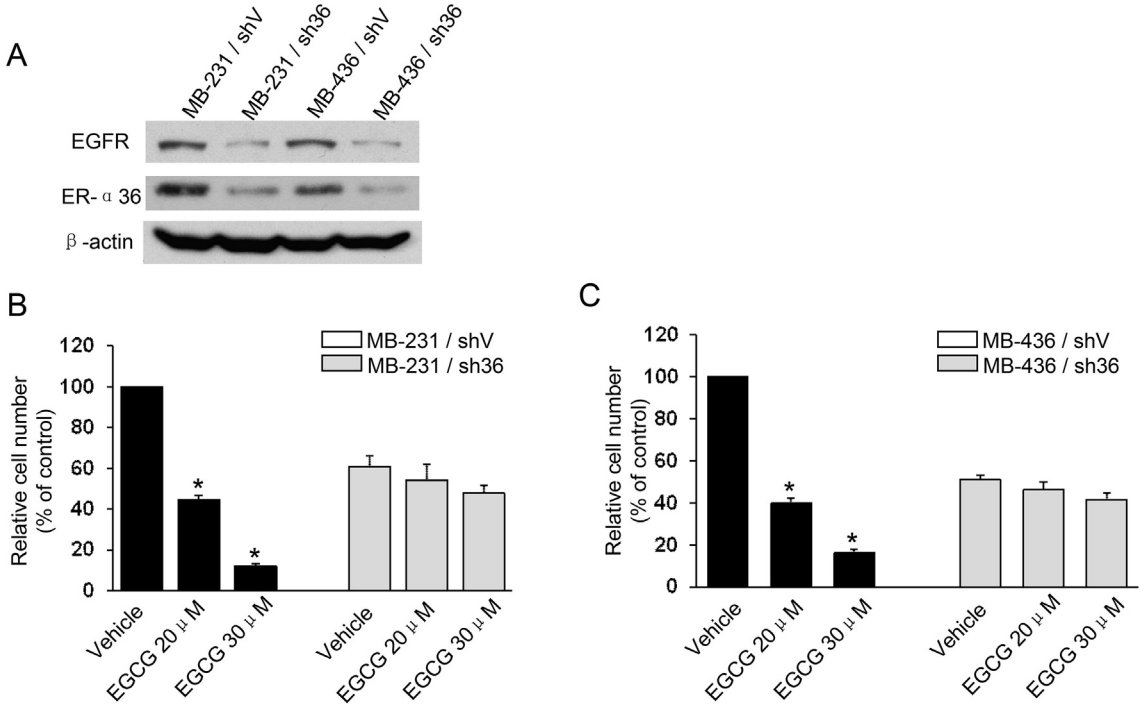

Fig. 6. ER-α36 knockdown desensitizes breast cancer cells to EGCG treatment. A. ER-α36 and EGFR expression levels were measured in MB-231/shV, MB-231/sh36, MB-436/shV and MB-436/sh36 cells using Western blot assays. B and C. The tumorspheres of MB-231/shV, MB-231/sh36, MB-436/shV and MB-436/sh36 cells were treated with vehicle (DMSO) or indicated concentrations of EGCG for seven days, and the cell numbers in tumorspheres were counted. The columns represent the means of three experiments; bars, SE. *$P < 0.05$ for control cells treated with vehicle DMSO. #$P < 0.05$ for cells treated with EGF (10 ng/ml) alone.

(28). They also observed enhanced ER-α36 expression in cells cultured in high density and fresh serum (28). Thus, different MCF-7 sub-lines as well as different culture conditions used in different lab may lead to variable results concerning ER-α36 expression. In our study, we found that consistent with the previous report (28), EGCG showed little growth inhibitory activity in ER-α36 knock-down cells, which suggested the growth inhibitory activity of EGCG depends on ER-α36 expression.

In summary, EGCG potently inhibits the growth of ER-negative stem/progenitor cells, presumably through down-regulation of the ER-α36 and EGFR positive regulatory loop. EGCG is an effective inhibitor of breast cancer proliferation and specific targets stem/progenitor cells in ER-negative breast cancer. Our results also provided evidence to support the view to develop ER-α36 as a therapeutic target for breast cancer.

Conflict of interests

There is no conflict of interests.

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