ORIGINAL ARTICLE

Tocopherols inhibit estrogen-induced cancer stemness and OCT4 signaling in breast cancer

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Abstract

Estrogen plays an important role in breast cancer development. While the mechanism of the estrogen effects is not fully elucidated, one possible route is by increasing the stem cell-like properties in the tumors. Tocopherols are known to reduce breast cancer development and progression. The aim of the present study is to investigate the effects of tocopherols on the regulation of breast cancer stemness mediated by estrogen. To determine the effects of tocopherols on estrogen-influenced breast cancer stem cells, the MCF-7 tumorsphere culture system, which enriches for mammary progenitor cells and putative breast cancer stem cells, was utilized. Treatment with estrogen resulted in an increase in the CD44+/CD24− subpopulation and aldehyde dehydrogenase activity in tumorspheres as well as the number and size of tumorspheres. Tocopherols inhibited the estrogen-induced expansion of the breast cancer stem population. Tocopherols decreased the levels of stem cell markers, including octamer-binding transcription factor 4 (OCT4), CD44 and SOX-2, as well as estrogen-related markers, such as trefoil factor (TFF)/pS2, cathepsin D, progesterone receptor and SERPINA1, in estrogen-stimulated tumorspheres. Overexpression of OCT4 increased CD44 and sex-determining region Y-box-2 levels and significantly increased cell invasion and expression of the invasion markers, matrix metalloproteinases, tissue inhibitors of metalloproteinase and urokinase plasminogen activator, and tocopherols inhibited these OCT4-mediated effects. These results suggest a potential inhibitory mechanism of tocopherols in estrogen-induced stemness and cell invasion in breast cancer.

Introduction

Breast cancer stem cells, which represent a subset of tumor cells, are considered responsible for development, growth and progression of tumors (1). In addition, breast cancer stem cells are believed to be the main cause of metastasis and recurrence of cancer because of their strong tumor-initiating abilities and resistance to conventional therapies (1). Therefore, treatment that targets cancer stem cells may be of substantial benefit. Although the importance of estrogen in breast cancer is well established, the mechanism of its effects is not fully understood. Some studies have suggested that estrogen can promote cancer stem cell activity by inducing the secretion of paracrine growth factors from estrogen receptor (ER)-positive cells via fibroblast growth factor/Tbx3, epidermal growth factor and Notch signaling pathways (2,3). In contrast to these findings, estrogen was shown to reduce the self-renewal capacity of breast cancer stem cells by promoting differentiation through downregulation of stem cell genes (4).

Some dietary components and bioactive natural compounds potentially inhibit breast cancer development and progression in experimental systems. It appears that they work by inhibiting breast stem cells through regulation of their self-renewal pathways (5). Tocopherols, the major forms of vitamin E, are

Abbreviations

CTSD	cathepsin D
E2	estrogen
ER	estrogen receptor
GLI	glioma-associated oncogene homolog
MMPs	matrix metalloproteinases
OCT4	octamer-binding transcription factor 4
PGR	progesterone receptor
SFE	sphere-forming efficiency
SHH	sonic hedgehog
SOX2	sex-determining region Y-box-2
TFF	trefoil factor
TIMPs	tissue inhibitors of metalloproteinases
uPA	urokinase plasminogen activator.

particularly active in this regard. Tocopherols are a family of fat-soluble phenolic compounds consisting of a chromanol ring system and a 16-carbon side chain (6). Depending upon the number and position of methyl groups on the chromanol ring, they exist as α-, β-, γ- or δ-tocopherol (6). Many studies have shown that tocopherols inhibit cancer formation and development due to their strong antioxidant properties (7–9). Although α-tocopherol has been the most widely used form of tocopherols for cancer prevention studies, large-scale human trials with α-tocopherol did not find a cancer preventive effect (10,11). γ-Tocopherol is the most abundant tocopherol in the USA diet, mainly from vegetable oils and nuts (12). We have reported previously that treatment with γ- and δ-tocopherols and γ-TmT, a naturally occurring tocopherols mixture, inhibited mammary tumor growth in N-methyl-N-nitrosourea-induced Sprague Dawley rats (13). Recently, we demonstrated that γ- and δ-tocopherols were more active than α-tocopherol in inhibiting mammary tumorigenesis in ACI rats and breast xenograft tumor growth supplemented with estrogen as well as cell proliferation and oxidative stress in estrogen-treated MCF-7 cells (14,15). These studies suggest a potential inhibitory mechanism of tocopherols in estrogen-mediated events in breast cancer.

Recent studies have shown that signaling pathways involved in maintaining the stem cell population are activated in tumor-initiating cells (16). The main transcription factors such as octamer-binding transcription factor 4 (OCT4), sex-determining region Y-box-2 (SOX2) and nuclear factor-κB act as master regulators of pluripotency and maintain the undifferentiated state of cells (17). OCT4, one of the key transcription factors, is associated with the pluripotency and self-renewal properties in embryonic stem cells, germ cells and adult human stem cells (18). Hu *et al.* (19) reported that ablation of OCT4 expression leads to apoptosis of cancer stem cells through the OCT4/Tcl1/Akt1 pathway in MCF-7 breast cancer cells and inhibition of tumor growth. It is further reported that estrogen increases OCT4 expression and proliferation of tumorspheres as well as expands the breast cancer stem cell population in MCF-7 tumorspheres (20). In addition, SOX2 is expressed in derived spheres, those that have been generated from breast cancer tumors and cell lines (21). Evidence suggests that high levels of OCT4 and SOX2 lead to the activation of other pluripotency genes that aid in the activation of the pluripotency network (22). CD44 is one of the key cell surface markers for tumor-initiating cells in breast cancer (23). Recently, CD44 overexpression was shown to correlate with invasive, metastatic phenotype and nuclear localization of stemness factors in breast cancer (24). Since CD44 does not have intrinsic kinase activity, it modulates multiple intracellular signaling by interacting with other components of

signaling transduction (25). Therefore, identification of interacting molecules is important to understand the biological role of OCT4 and CD44 in human breast cancer stem cells.

In the present study, we investigated estrogen as an important positive modulator of cancer stem cell properties in ER-positive breast cancer and examined the effects of tocopherols on estrogen-mediated cancer stemness and OCT4 signaling in breast cancer.

Materials and methods

Cell culture and reagents

Tocopherols were prepared as described previously (14). Briefly, α- and δ-tocopherols were purified to ≥97% purity from the commercial grade α-tocopherol (T3634) and δ-tocopherol (T2028), respectively, from Sigma–Aldrich (St. Louis, MO). γ-Tocopherol was purified from γ-tocopherol-rich mixture of tocopherols (BASF Corporation, Kankakee, IL; Covi-ox T-90, Batch number 0008778732) to ≥97% purity with no other detectable forms of tocopherol. A CombiFlash Companion SL automated flash chromatographic system (Teledyne ISCO, Lincoln, NE) with a Redisep Rf Gold high-performance flash silica gel column (20–40 μm in particle size) was used for the purification. Each tocopherol was dissolved in dimethyl sulfoxide (Sigma–Aldrich). Estrogen was obtained from Sigma–Aldrich (E2758) and dissolved in dimethyl sulfoxide. The MCF-7 cell line was acquired from American Type Culture Collection (ATCC, Manassas, VA) and authenticated by short tandem repeat profiling at ATCC. MCF-7 cells were maintained in DMEM/F12 medium, 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C and 5% CO_2. When noted, MCF-7 cells were treated in 10% charcoal-stripped FBS/phenol red-free RPMI medium to examine effects of tocopherols on estrogen-mediated events.

Tumorsphere-forming assay

MCF-7 cells were harvested from monolayer culture using StemPro Accutase® (Life Technologies, Grand Island, NY). Cells were then plated at 10 000 cells/ml in six-well ultra-low attachment plates and maintained in a serum-free mammary epithelial basal medium without phenol red (MEBM, Lonza, Walkersville, MD), supplemented with B27 (GIBCO, Carlsbad, CA), 20 ng/ml epithelial growth factor (Sigma–Aldrich), 20 ng/ml basic fibroblast growth factor (GIBCO), 5 μg/ml bovine insulin (Sigma–Aldrich) and 0.5 μg/ml hydrocortisone (Sigma–Aldrich). Cells were incubated with vehicle control (DMSO), estrogen (1 nM) or different forms of tocopherol (1 μM) for 4 days without disturbing the plates and without replenishing the medium. The tumorsphere was gathered at the center of the well by slowly swirling plates, and the pictures were taken to measure number and size of tumorspheres for sphere-forming efficiency (SFE) using a TE200 microscope (Nikon Instrument, Melville, NY).

Flow cytometry

The detailed procedure was reported previously (26). MCF-7 cells were stained with antibodies against CD44-FITC (Cat. 555478) and CD24-PE (Cat. 561893) from BD Biosciences (San Jose, CA). The stained MCF-7 cells were analyzed by flow cytometry using an FC500 Analyzer (Beckman Coulter) to determine the percentage of different CD44⁻/CD24⁺, CD44⁺/CD24⁺, CD44⁻/CD24⁻ and CD44⁺/CD24⁻ subpopulations. For CD44/CD24/aldehyde dehydrogenase (ALDH) staining, tumorspheres were resuspended in 1 ml of Aldefluor buffer containing 5 μl of activated Aldefluor reagent (Stem Cell Technologies, Grenoble, France). One-half of the sample was transferred to a second tube containing 5 μl of the ALDH inhibitor, diethylaminobenzaldehyde. After 30 min of incubation with the Aldefluor reagent, cells were incubated with 15 μl of CD44-APC (Cat. 559945, BD Biosciences) and 15 μl of CD24-PE (Cat. 555428, BD Biosciences) for 30 min at RT in the dark. Then, cells were analyzed by flow cytometry using an FC500 Analyzer (Beckman Coulter).

ALDEFLUOR assay

To measure cells with ALDH activity, the ALDEFLOUR assay was carried out according to manufacturer's protocol (Stem Cell Technologies). Briefly, MCF-7 tumorspheres were resuspended in 1 ml of ALDEFLUOR assay

buffer containing 5 μl of activated Aldefluor reagent for 30 min at 37°C. As a negative control for all experiments, an aliquot of ALDEFLUOR stained cells was immediately quenched with 5 μl diethylaminobenzaldehyde. Cells were analyzed using the green fluorescence channel (FL1) on an FC500 Analyzer (Beckman Coulter).

Quantitative polymerase chain reaction analysis

The procedure was described previously ([27](#)); the labeled primers, including OCT4 (Hs00999634), CD44 (Hs01075861), NFKB1 (Hs00765730), SOX2 (Hs01053049), glioma-associated oncogene homolog (GLI; Hs00171790), sonic hedgehog (SHH; Hs00179843), TFF (Hs00907239), cathepsin D (CTSD; Hs00157205), progesterone receptor (PGR; Hs01556702), SERPINA1 (Hs00165475), MMP9 (Hs00957562), MMP13 (Hs00942584), MMP15 (Hs00233997), MMP16 (Hs00234676), TIMP1 (Hs00171558), TIMP2 (Hs00234278), TIMP3 (Hs00165949) and uPA (Hs00958880) were obtained from Applied Biosystems (Foster City, CA).

Nuclear and cytoplasmic fractionation

The nuclear and cytoplasmic fractionation was performed with NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Cat. 78833, Thermo Fisher Scientific, Waltham, MA). The nuclear and cytoplasmic fractions were isolated separately by following the manufacturer's instruction. The amount of a given protein in each fraction was determined by western blot analysis.

Western blot analysis

The detailed procedures have been described previously ([26](#)). The primary antibodies detecting OCT4 (1:1000, 2750S), SOX2 (1:1000, 3579S), p-p65 (1:1000, 3039S) and PGR (1:1000, 8757) were from Cell Signaling Technology (Danvers, MA); CD44 (1:500, sc-7297), ERα (1:500, sc-8002) and LaminA (1:1000, sc-20680) were from Santa Cruz Biotechnology (Santa Cruz, CA); β-actin antibody was from Sigma–Aldrich (1:2000, A1978). Secondary antibodies were from Cell Signaling Technology.

Fluorescence microscopy

The detailed procedure was described previously ([28](#)). Cells were incubated in PBS containing 10% goat serum to block non-specific binding and then incubated overnight at 4°C with a combination of primary antibodies against OCT4 (1:100, Cell Signaling Technology) and CD44 (1:100, Santa Cruz). Subsequently, the cells were incubated with fluorophore-conjugated secondary antibodies (Alexa Fluor 488 or 546; 1:200, Life Technologies) and TO-PRO-3 iodide nuclear antibody (1 μM, Life Technologies) at room temperature. The images were taken using confocal microscope (Eclipse Ti, Nikon Instrument) with laser at 488 nm (CD44), 546 nm (OCT4) and 633 nm (TO-PRO-3). The fluorescence was analyzed using ImageJ software (NIH, Bethesda, MD; http://rsbweb. nih. gov/ij).

OCT4 overexpression

The detailed procedures have been described previously ([29](#)). In brief, a lentiviral human OCT4 cDNA overexpression vector (pSin-EF2-OCT4-Pur) was obtained from Addgene (Addgene plasmid #16579). The control vector was obtained from Sigma–Aldrich. For the transient transfection of EF2-OCT4 vectors, DNAs were mixed with FuGene HD transfection reagent (Invitrogen, Carlsbad, CA), and this mixture was added to MCF-7 cells in Opti-MEM medium without serum for 24 h. Then, cells were treated with the estrogen (1 nM) or tocopherols (1 μM) for an additional 24 h in 10% charcoal-stripped FBS/phenol red-free RPMI medium.

Cell invasion assay

The inhibitory effect of tocopherols on cells invasion was assessed using Transwell chamber (8 μm pore size) according to the manufacturer's protocol (Cat. 354480, BD Biosciences). In brief, cell suspensions (8 × 10⁴ cells/ml) were added to the insert chambers in serum-free RPMI medium, and RPMI medium containing 10% FBS was placed in 24-well plates. Following incubation for 22 h in a humidified atmosphere containing 5% CO_2 at 37°C, non-invasive cells on the upper surface of the insert chamber were removed with a cotton swab. The invasive cells on the bottom surface of the chamber were fixed with 100% methanol and then stained with H&E stain. For each membrane, images of three different fields at random were captured at ×10 magnification. All experiments were performed in triplicate. Results were presented as images of invading cells and were quantified using ImageJ software (NIH; http://rsbweb. nih. gov/ij).

Statistical analysis

The significance of the difference between control or treatment groups and estrogen groups was evaluated by the Student's *t*-test or one-way analysis of variance followed by a Tukey test. The value of $P < 0.05$ was considered statistically significant.

Results

Effects of estrogen on the CD44/CD24 subpopulations and level of stem cell markers in MCF-7 monolayer culture and tumorspheres

A CD44⁺/CD24⁻/low subpopulation of breast cancer cells has been reported to have stem/progenitor cell properties ([30](#)). It has also shown that estrogen increases development and/or growth of CD44⁺/CD24⁻/low-derived breast tumors ([2](#)). We thus determined whether estrogen regulates the cancer stem cell population in MCF-7 monolayer and tumorspheres. Cells were treated with 1 nM estrogen for 4 days, and the expression profile of CD44/CD24 was evaluated by flow cytometry ([Figure 1A](#)). In monolayer culture, treatment with estrogen lowered levels of CD24 but had only a modest effect on CD44 levels. In contrast to MCF-7 monolayer cultures, estrogen substantially increased levels of CD44 and decreased levels of CD24 in tumorspheres, resulting in increased CD44⁺/CD24⁻/low stem cell-like populations.

We next evaluated the effects of estrogen on mRNA levels of pluripotency and stem cell-related markers including CD44, OCT4, NFKB, SOX2, GLI and SHH in MCF-7 monolayers and tumorspheres ([Figure 1B](#)). The levels of CD44 mRNA were increased with estrogen in MCF-7 tumorspheres but not in MCF-7 monolayer cells. The observation was consistent with results from flow cytometry, suggesting that CD44 may be an important target for a functional increase in the breast cancer stem cell population. We next determined the effect of estrogen on mRNA levels of several key transcription factors, such as OCT4, NFKB and SOX2, involved in the regulation of pluripotency and maintenance of self-renewal properties in stem cells ([17](#)). OCT4 mRNA was increased by estrogen in MCF-7 monolayer cells as well as tumorspheres. Interestingly, the levels of NFKB and SOX-2 mRNA were increased by estrogen in MCF-7 tumorspheres, not in MCF-7 monolayer cells. For the Hedgehog signaling pathway, the mRNA levels of GLI (a downstream effector) and SHH (a ligand) were determined. Hedgehog signaling is aberrantly activated in cancer stem cells ([31](#)). GLI and SHH were increased by estrogen in both MCF-7 monolayer and tumorspheres. Taken together, these results suggest that estrogen is a positive modulator on expression of cancer stem cell-related markers in MCF-7 cells.

Effects of tocopherols on formation of MCF-7 tumorspheres

To investigate the effects of tocopherols on formation of tumorspheres, MCF-7 cells were treated with estrogen and/or α-, γ- and δ-tocopherols for 4 days in ultra-low attachment plates. As shown in [Figure 2A](#), α-, γ- and δ-tocopherols without estrogen had little or no effect on the number or size of tumorspheres. However, estrogen markedly increased number and size of tumorspheres, and treatment with tocopherols decreased the number and size of tumorspheres in the estrogen-treated cultures: the SFE was reduced with α-, γ- and δ-tocopherols by 22.0, 31.7 and 27.7%, respectively ([Figure 2B](#)). The size of the tumorspheres was increased by estrogen, as shown by significantly increased number of large tumorspheres (>200 μm) to 42.5% in the estrogen treatment group
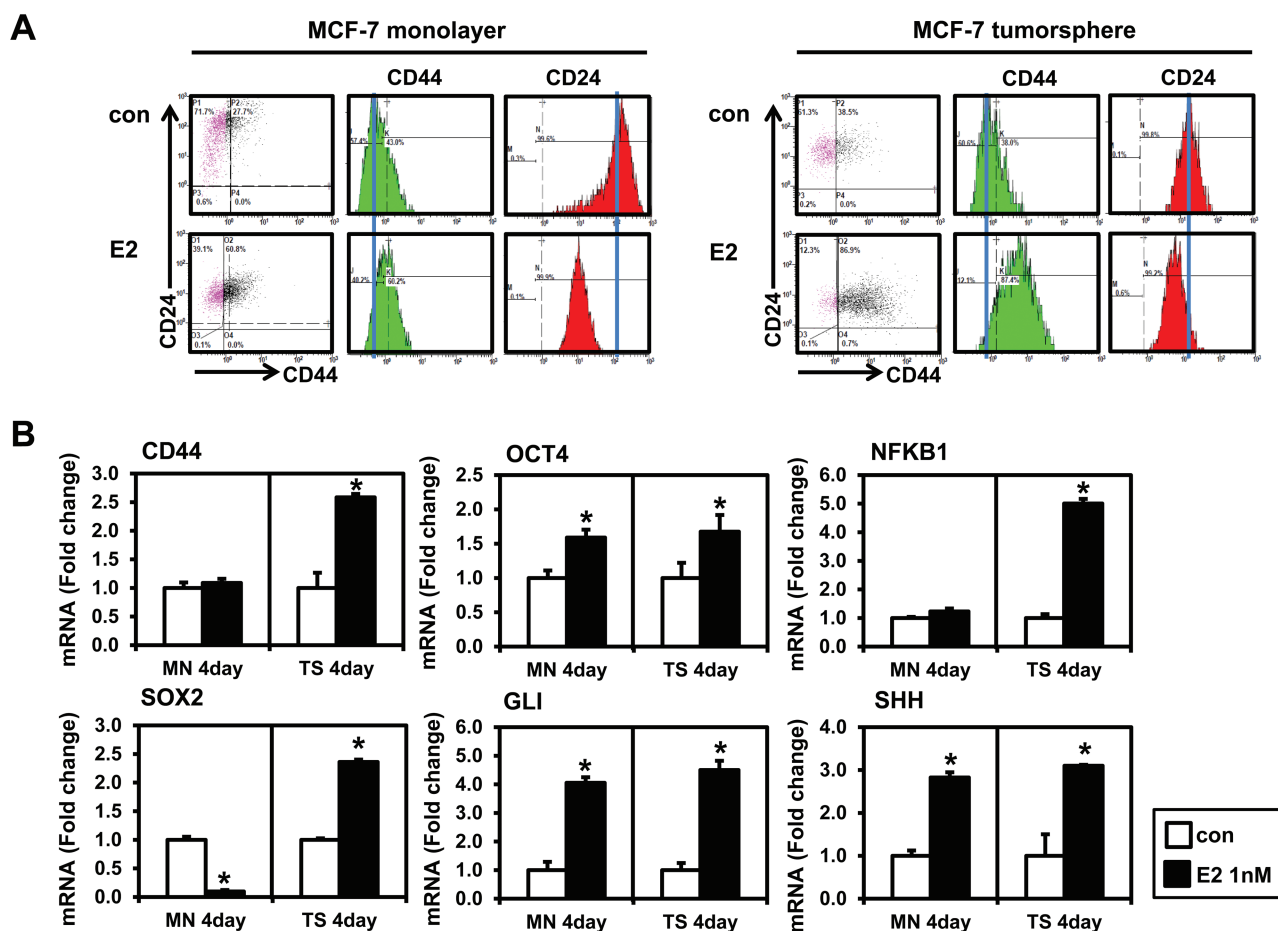
Figure 1. Effects of estrogen (E2) on the CD44/CD24 expression and the level of stem cell markers in MCF-7 monolayer cells and tumorspheres. (**A**) MCF-7 monolayer cells were plated at a density of 8×10^4 cells/ml in six-well plates and treated with the vehicle control (con) or E2 (1 nM) for 4 days. MCF-7 tumorspheres were formed by plating 10 000 cells/ml in ultra-low attachment six-well plates and treated with E2 (1 nM) for 4 days. Cells were stained with combinations of antibodies against CD24 and CD44, and then, flow cytometry was performed. Experiments were performed in triplicate, and representative histograms from flow cytometry are shown. Histograms show the mean fluorescence intensity of CD44 (PE-Green) and CD24 (FITC-Red). (**B**) qPCR analysis was performed on MCF-7 monolayer cells and tumorspheres treated with E2 and analyzed for markers of cancer stem cells. The data are represented as mean ± standard deviation (SD). Here, $n = 3$ represents independent experiments, *significantly different from the respective control ($P < 0.05$). Cycle numbers for genes related to CD44, OCT4, NFKB1, SOX2, GLI and SHH for control group in MCF-7 monolayer cells were 25, 25, 24, 31, 34 and 28 and for control group in MCF-7 tumorspheres cells were 25, 28, 26, 30, 35 and 30, respectively.

compared with 20.0% in no estrogen control group (Figure 2C). Treatment of MCF-7 tumorspheres with α-, γ- and δ-tocopherols decreased the size of large tumorspheres (>200 μm) by 23.6, 23.3 and 25.4%, respectively (Figure 2C). Since we found significant inhibitory effect of tocopherols on estrogen-induced formation of MCF-7 tumorspheres (Supplementary Figure S3), we tested the effect of tocopherols in another ER-positive human breast cancer line, T47D. Estrogen increased number and size of T47D tumorspheres. While α-, γ- and δ-tocopherols had little or no effect on the number and size of T47D tumorspheres in the absence of estrogen, treatment with α-, γ- and δ-tocopherols decreased the number and size of tumorspheres in estrogen-induced T47D tumorspheres (Supplementary Figure S1, available at *Carcinogenesis* Online). The results suggest that the effects of tocopherols on breast cancer stem cells were focused principally on the estrogen-mediated increase in the stem cell-like population.

Effects of tocopherols on the CD44/CD24 subpopulations and the ALDH activity in estrogen-treated MCF-7 tumorspheres

To further investigate the effects of tocopherols on the stem cell-like subpopulations in MCF-7 tumorspheres, we examined expression of CD44/CD24 in these cells by flow cytometry. As shown in Figure 3A, expression of CD44 increased and expression of CD24 decreased with estrogen treatment. Expression of these markers returned to near control (no estrogen) levels by treatment with α-, γ- and δ-tocopherols, with significant reduction of the CD44+/ CD24− subpopulation (Figure 3B). Since it has been shown that breast cancer stem cells present high levels of ALDH activity (32), we examined whether tocopherol decreases ALDH activity in estrogen-treated MCF-7 cells. We found that percentage of ALDH-positive cells was higher in MCF-7 tumorspheres ($P < 0.05$), compared with MCF-7 monolayer culture (Figure 3C). Estrogen increased the ALDH activity further in MCF-7 tumorspheres, and treatment with γ-tocopherol decreased the ALDH activity by 28.5% (Figure 3C). The representative dot plots of the experiments were shown in Supplementary Figure S4, available at *Carcinogenesis* Online. We next examined the effects of γ-tocopherol on expression of ALDH/CD24/CD44 in MCF-7 tumorspheres (Figure 3D). The percentage of cells with high ALDH and low CD24 was increased by estrogen (3.11%), and this induction was inhibited by γ-tocopherol (0.00%). In addition, the percentage of cells with high ALDH and high CD44 was increased by estrogen (6.86%). Expression of these markers returned to near
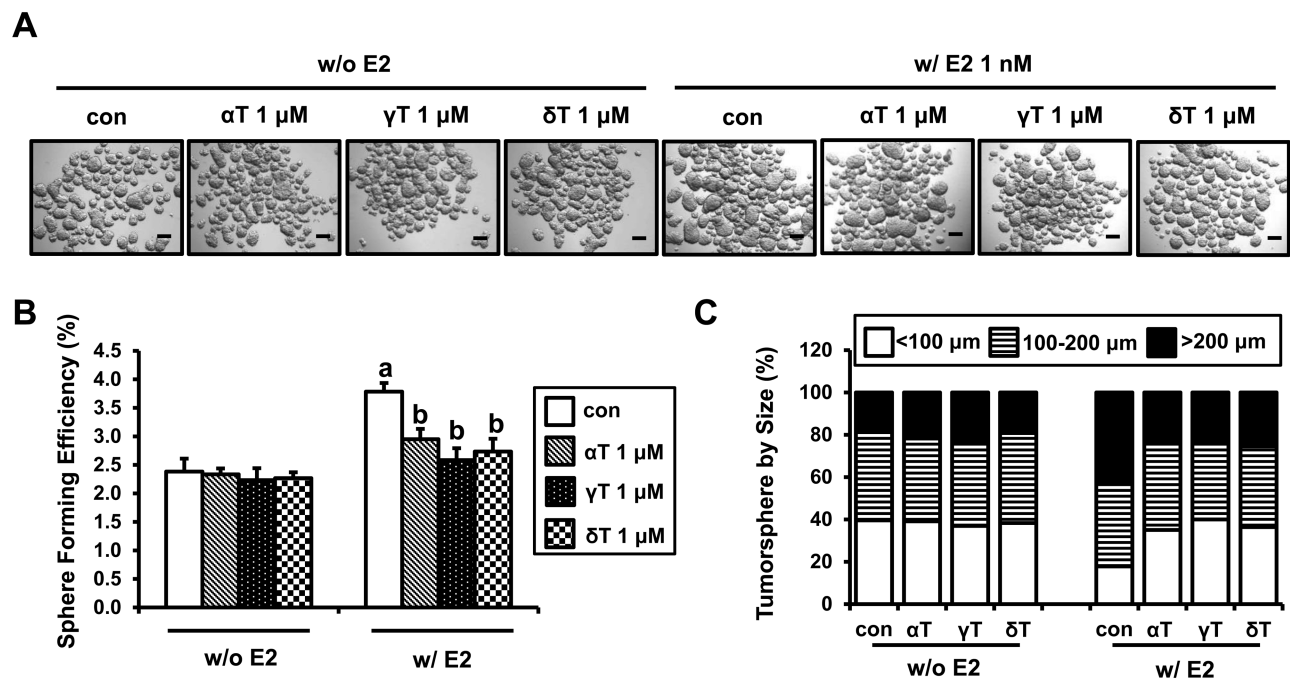
A

B

C

Figure 2. Effects of tocopherols on formation and size of MCF-7 tumorspheres. (**A**) MCF-7 cells were plated at a density of 10 000 cells/ml in ultra-low attachment six-well plates and grown for 4 days in the presence of E2 (1 nM) or/and α-, γ- and δ-tocopherols (1 μM). Representative pictures of MCF-7 tumorspheres are shown for phenotypic comparison, scale bar 100 μm. (**B**) SFE of MCF-7 tumorspheres is shown. SFE was calculated by dividing the number of tumorspheres (>100 μm) formed by the number of cells seeded presenting this as a percentage. The data are represented as mean ± SD. Here, $n = 3$ represents independent experiments in triplicate, [a,b]significantly different from the control and E2, respectively ($P < 0.05$). (**C**) The size of tumorspheres was divided into three ranges (<100, 100–200 and >200 μm). Average number of tumorspheres in each size range is shown in the graph.

control levels by treatment with γ-tocopherol (1.48%). MCF-7 tumorspheres treated with estrogen showed lower CD24 expression and higher CD44 expression, which is consistent with CD44/CD24 double staining. These findings suggest that ALDH-positive and the CD44^{+}/CD24^{−/low} subpopulation may be enriched in cancer stem/progenitor cells, and γ-tocopherol reduces estrogen-induced breast cancer stem cell subpopulations.

Effects of tocopherols on stem cell markers and estrogen target genes in MCF-7 tumorspheres

As shown above, estrogen increases sphere formation and the levels of stem cell-related markers in MCF-7 tumorspheres. We, therefore, tested the effects of α-, γ- and δ-tocopherols on the expression levels of stem cell-related markers as well as estrogen-related markers in MCF-7 tumorspheres. We first examined CD44 and OCT4 levels in MCF-7 tumorspheres by confocal microscopy. MCF-7 tumorspheres treated with estrogen showed stronger CD44 and OCT4 staining, which was decreased with α-, γ- and δ-tocopherols treatment in MCF-7 tumorsphere. The quantification of CD44 and OCT4 level has shown that γ-tocopherol had the most significant inhibitory effect (Figure 4A, Supplementary Figure S6). Next, we determined the protein levels of OCT4, CD44 as well as stem cell markers, p-p65 and SOX2, in estrogen-treated MCF-7 tumorspheres. As shown in Figure 4B, the protein levels of OCT4, CD44, p-p65 and SOX2 were increased by estrogen, whereas tocopherols reduced estrogen stimulation of these markers in MCF-7 tumorspheres. mRNA levels of OCT4, CD44, NFKB1 and SOX2 were not changed by the treatment with tocopherols in MCF-7 tumorspheres in the absence of estrogen. In contrast, mRNA levels of OCT4, CD44, NFKB1 and SOX2 were significantly increased by estrogen, and this induction was downregulated by α-, γ- and δ-tocopherols (Figure 4C). The mRNA level of Nanog, a transcription factor involved with self-renewal of stem cells, was too low for reliable

detection (Supplementary Figure S5, available at *Carcinogenesis* Online). Since inhibition of tumorsphere formation caused by tocopherols is dependent on estrogen, we investigated the effects of tocopherols on the modulation of the estrogen target genes, including TFF/pS2, CTSD, PGR and SERPINA1, in MCF-7 tumorspheres (Figure 4C). In the absence of estrogen, the mRNA levels of TFF/pS2, CTSD, PGR and SERPINA1 were unaffected by treatment with tocopherols in MCF-7 tumorspheres. However, levels of TFF/pS2, CTSD, PGR and SERPINA1 were significantly upregulated by estrogen treatment, and this stimulation was inhibited by α-, γ-, and δ-tocopherols.

Effects of estrogen and OCT4 overexpression on stem cell markers and the regulation by γ-tocopherol in MCF-7 cells

To further examine the role of OCT4, estrogen and tocopherols on breast cancer cell stemness, we used MCF-7 cells transiently transfected to overexpress OCT4. OCT4 is a well-known transcription factor that plays a crucial role in stem cell self-renewal and pluripotency (24). As γ-tocopherol inhibited the formation of tumorspheres most effectively among tocopherols, we chose γ-tocopherol to investigate further the roles of OCT4 in cancer stemness. MCF-7 cells were transduced with control vector (MCF-7) and lentiviral OCT4-expression vector (MCF-7-OCT4). We first investigated the levels of OCT4 and CD44 in MCF-7 and MCF-7-OCT4 cells treated with γ-tocopherol using confocal microscopy (Figure 5A). Expression of OCT4 was notably upregulated in the nuclei of MCF-7-OCT4 cells, compared with MCF-7 cells, as expected. MCF-7-OCT4 cells treated with estrogen showed stronger OCT4 staining in nuclei, which was decreased with γ-tocopherol treatment (Figure 5A). We found that the level of CD44 was increased by overexpressing OCT4, and the level was reduced by γ-tocopherol treatment (Figure 5A). These changes were further examined by western blot. As shown in Figure 5B,
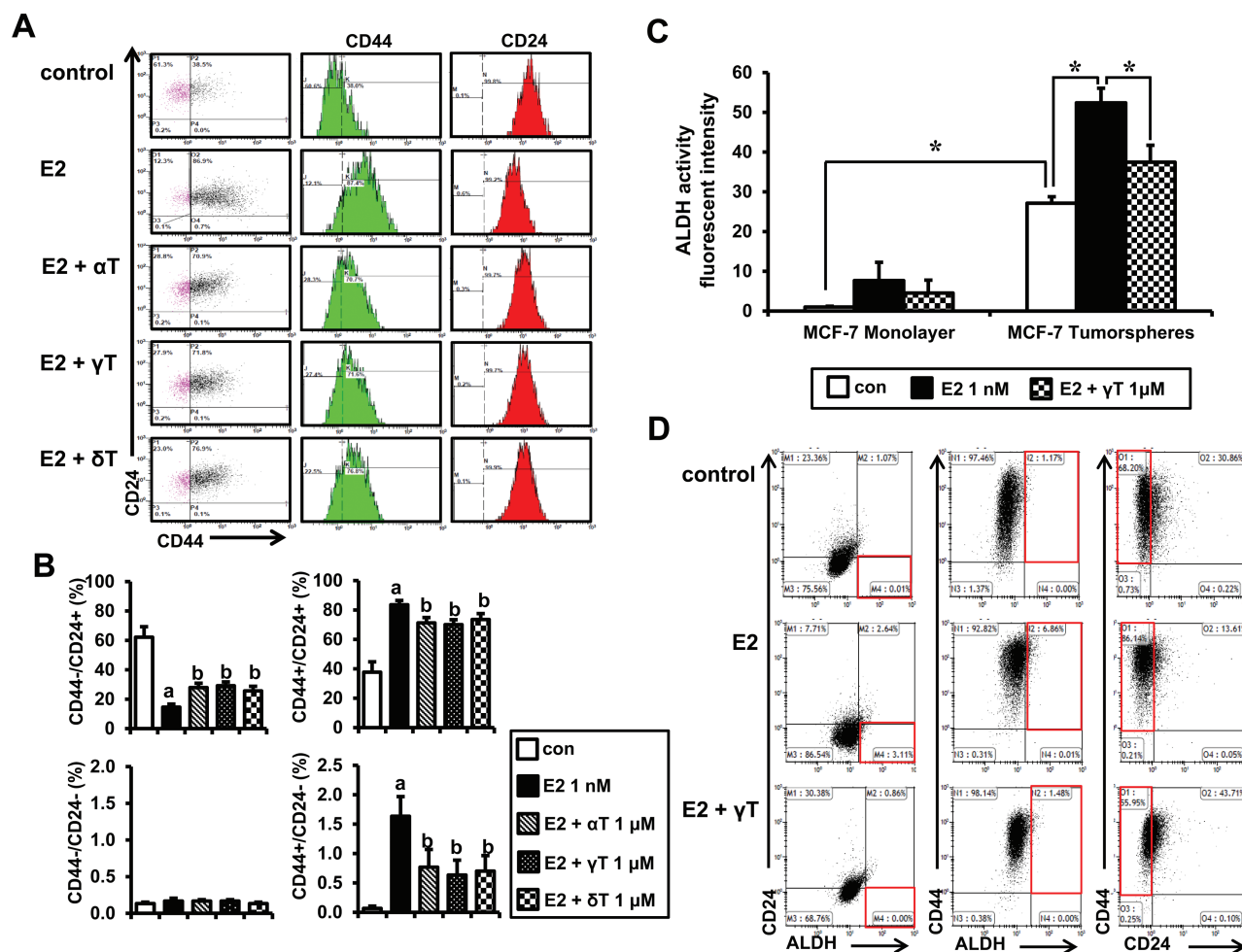
Figure 3. Effects of tocopherols on the CD44/CD24 subpopulations and ALDH activity in E2-treated MCF-7 tumorspheres. (**A**) MCF-7 cells were plated at a density of 10 000 cells/ml in ultra-low attachment six-well plates and grown for 4 days in the presence of E2 (1 nM) or/and tocopherols (1 μM). Cells were stained with combinations of antibodies against CD44 and CD24, and then, flow cytometry was performed. Histograms show the mean fluorescence intensity of CD44 (PE-Green) and CD24 (FITC-Red). (**B**) The average percentage of CD44⁻/CD24⁺, CD44⁺/CD24⁺, CD44⁻/CD24⁻ and CD44⁺/CD24⁻ subpopulations from three independent experiments are represented as a bar graph. The data are represented as mean ± SD. [a,b]Significantly different from the control and E2 control, respectively (P < 0.05). (**C**) The average fluorescent intensities of ALDH in MCF-7 cells treated with E2 (1 nM) and/or γ-tocopherol (1 μM). Data were calculated from immunofluorescence images from three independent experiments. *Significantly different from the respective control (P < 0.05). (**D**) Representative dot plots of flow cytometry show the expression of ALDH, CD24 and CD44 in MCF-7 tumorspheres treated with E2 (1 nM) and/or γ-tocopherol (1 μM) for 4 days. Cells were stained with ALDH, anti-CD44-APC and/or anti-CD24-PE.

MCF-7-OCT4 cells showed a significantly higher protein level of OCT4, CD44, SOX-2 and ERα than MCF-7 cells. Treatment with estrogen increased the levels of OCT4, CD44, SOX-2 and PGR, and this induction was decreased by γ-tocopherol in MCF-7-OCT4 cells (Figure 5B). We examined the cellular location of OCT4, CD44, SOX-2, PGR and ERα in the treated cells by immunoblotting of subcellular fractions (Figure 5C). We found that MCF-7-OCT4 cells showed a higher level of OCT4 in the nucleus than MCF-7 cells. Treatment with estrogen increased the level of OCT4 in the nucleus, and this stimulation was inhibited by γ-tocopherol in MCF-7-OCT4 cells. The levels of SOX-2 and PGR in the nucleus were further increased by estrogen, and γ-tocopherol down-regulated estrogen-stimulated levels of these markers in MCF-7-OCT4 cells (Figure 5C). Since CD44 is a cell surface marker, CD44 was not detected in the nucleus. However, MCF-7-OCT4 cells showed a higher level of CD44 in the cytosol than MCF-7 cells, which was decreased by treatment with γ-tocopherol. The ERα level in the nucleus was higher in MCF-7-OCT4 than MCF-7 cells. The level of ERα was not significantly changed by the treatment with estrogen or tocopherols in either the nucleus or cytosol fractions. Overall, these results suggest that OCT4 is a key

regulator of cancer stemness, that estrogen induces OCT4 and its downstream targets, and that γ-tocopherol reduces these effects in breast cancer cells.

Effects of estrogen and OCT4 overexpression on cell invasion and its regulation by γ-tocopherol

To investigate the possible biological effects of OCT4 expression, its stimulation by estrogen and inhibition by γ-tocopherol, we examined the invasive behavior of tumor cells through a basement membrane matrix barrier using the Matrigel transwell filter assay. As shown in Figure 6A, estrogen significantly increased cell invasion of MCF-7 cells (3.8-fold induction, P < 0.05), and this induction was decreased by γ-tocopherol (47.7% inhibition, P < 0.05). When compared with MCF-7 cells, MCF-7-OCT4 cells showed significantly increased invasive potential (3.2-fold induction, P < 0.05). Treatment with estrogen further increased the invasion potential of MCF-7-OCT4 cells (3.4-fold induction, P < 0.05), and γ-tocopherol effectively suppressed this estrogen-induced cell invasion in MCF-7-OCT4 cells (48.7% inhibition, P < 0.05) (Figure 6A). In addition, all the tocopherols tested, including α- and δ-tocopherols, blocked the cell invasion in
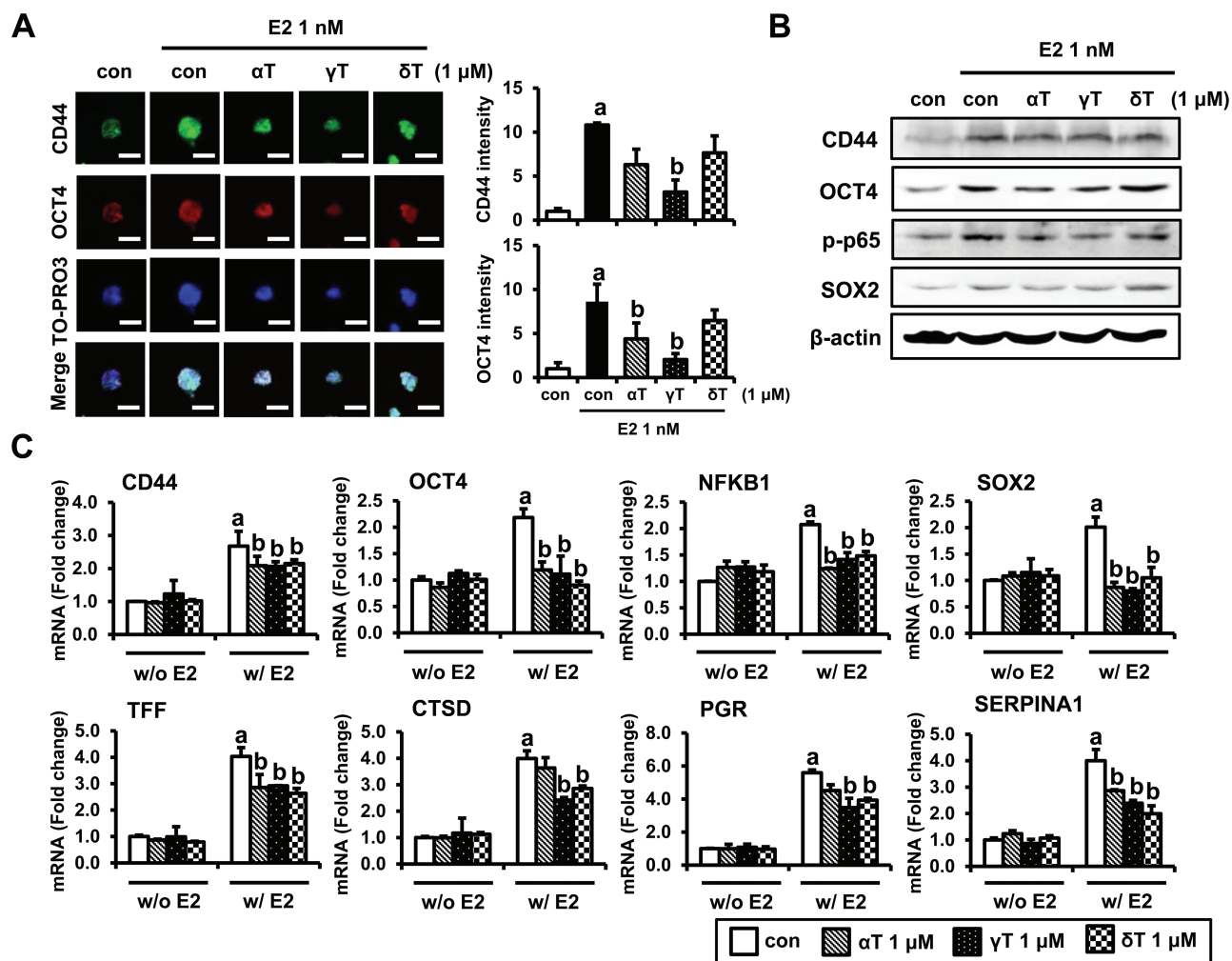
Figure 4. Effects of tocopherols on expression of E2-target genes and stem cell markers in E2-treated MCF-7 tumorspheres. (**A**) Immunofluorescence analysis of was performed on MCF-7 tumorspheres collected from 4 days of treatment with control, E2 (1 nM) or tocopherols (1 μM). MCF-7 tumorspheres were fixed using 4% para-formaldehyde and stained with antibodies against CD44 (green) and OCT4 (red). Nuclei were stained with TO-PRO3 (blue). Representative pictures are shown, scale bars: 200 μm. The quantification of CD44 and OCT4 level by ImageJ program from three independent experiments was shown. [a,b]Significantly different from the control and E2 control, respectively ($P < 0.05$). (**B**) Western blot analysis was performed on MCF-7 tumorspheres collected from 4 days of treatment with control, E2 (1 nM) or toco-pherols (1 μM) and analyzed for markers associated with stem cell signaling. β-Actin was used as a loading control. (**C**) qPCR analysis was performed on tumorspheres collected from 4 days of treatment with control, E2 (1 nM) or tocopherols (1 μM) and analyzed for markers associated with stem cell-related genes, including CD44, OCT4, NFKB1 and SOX-2, and E2-related genes, including TFF, CTSD, PGR and SERPINA1. The data are represented as mean ± SD. Here, $n = 3$ represents independent experiments, [a,b]significantly different from the control and E2 control, respectively ($P < 0.05$). Cycle numbers for genes related to CD44, OCT4, NFKB1, SOX-2, TFF, CTSD, PGR and SERPINA1 for control group were 25, 27, 26, 30, 18, 20, 30 and 32, respectively.

estrogen-treated MCF-7 and MCF-7-OCT4 cells (Supplementary Figure S2, available at *Carcinogenesis* Online). Next, to investigate the molecular changes underlying the changes in cell invasion, we analyzed the mRNA levels of cell invasion markers, including MMP9, MMP13, MMP15, MMP16, TIMP1, TIMP2, TIMP3 and uro-kinase plasminogen activator (uPA; Figure 6B). The mRNA levels of MMP9, MMP13, MMP15, TIMP1, TIMP2 and uPA were mark-edly increased by the overexpression of OCT4, while those of MMP16 and TIMP3 were not affected by the overexpression of OCT4. Treatment with estrogen increased the mRNA levels of MMP9, MMP13 and TIMP3 in MCF-7 and MCF-7-OCT4 cells, and this stimulation was inhibited by γ-tocopherol (Figure 6B). The level of uPA was increased by estrogen in MCF-7-OCT4 cells, but the level was not affected by γ-tocopherol. In contrast to these markers, treatment with estrogen decreased the mRNA levels of MMP15, MMP16 and TIMP1, and γ-tocopherol had little or no significant effect on mRNA levels of these genes in MCF-7 and

MCF-7-OCT4 cells (Figure 6B). The level of TIMP2 was not signifi-cantly changed by treatment with estrogen and tocopherols in MCF-7 and MCF-7-OCT4 cells.

Discussion

Accumulating evidence indicates that breast cancer stem cells are responsible for tumor initiation, recurrence, metastasis and progression of breast tumors (33). Hence, targeting breast can-cer stem cells with novel agents has become of great interest as a strategy to improve breast cancer treatment and preven-tion. Our present study demonstrates that tocopherols had significant inhibitory effects on estrogen-induced stemness and cell invasion by regulating OCT4. In these experiments, all forms of tocopherols tested had inhibitory effects, although γ-tocopherol inhibited tumorsphere formation better than α- or δ-tocopherol. In other systems, γ- and δ-tocopherols

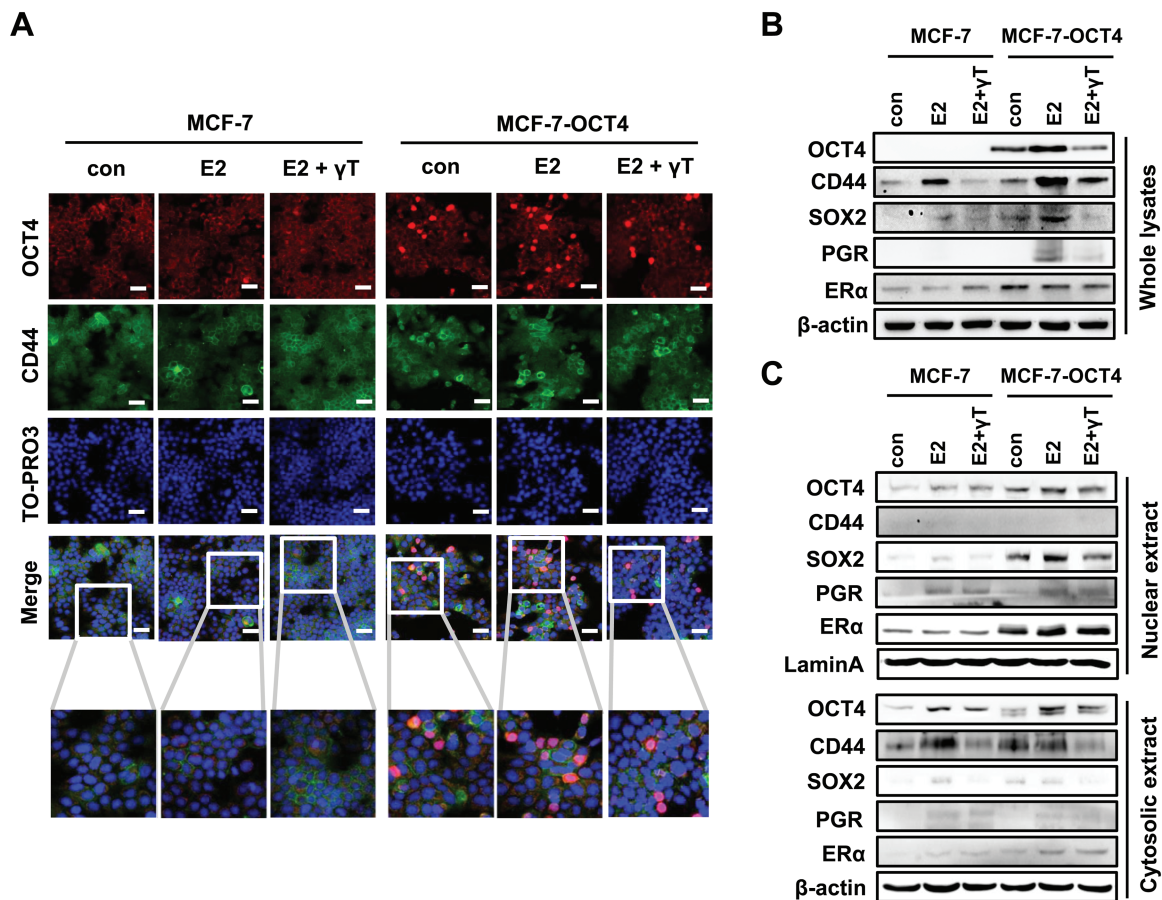**Figure 5.** Involvement of OCT4 in the regulation of E2-target genes and stem cell markers by γ-tocopherol. (**A**) MCF-7 cells were transfected with control vector (MCF-7) and pSin-EF2-OCT4-Pur vector (MCF-7-OCT4) for 24 h, and then, cells were treated with E2 (1 nM) and/or γ-tocopherol (1 μM) for an additional 24 h. MCF-7 and MCF-7-OCT4 cells were fixed using 4% paraformaldehyde and stained with antibodies against OCT4 (red) and CD44 (green). Nuclei were stained with TO-PRO3 (blue). Representative pictures are shown, scale bars: 100 μm. (**B**) The protein levels of OCT4, CD44, SOX2, PGR and ERα were examined by western blot analysis, with β-actin used as a loading control. (**C**) The protein levels of OCT4, CD44, SOX2, PGR and ERα in nucleus or in cytoplasm were examined by western blot analysis in MCF-7 and MCF-7-OCT4 cells. Lamin A and β-actin were used as loading controls for nucleus and cytoplasm fractions, respectively.

inhibited azoxymethane-induced colon carcinogenesis and PhIP-induced colon cancer (7,34); δ-tocopherol was more active than γ-tocopherol in inhibiting lung xenograft tumor growth (35); dietary administration of γ- and δ-tocopherols inhibited the *N*-methyl-*N*-nitrosourea -induced and estrogen-induced mammary tumorigenesis (13,15). Further studies are needed to understand the basis for the differences in the inhibition of cancer stem cells versus tumorigenesis by different tocopherols.

An earlier study identified the CD44$^+$/CD24$^{-/low}$ subpopulation of breast cancer cells from breast tumors as enriched in cancer stem cells (30). Some *in vivo* studies indicated that CD44 suppression not only inhibited tumor growth but also reduced the tumor-initiating ability of a human breast cancer cell xenograft (36). In addition, the overexpression of CD44 enhanced both tumorsphere formation and the expression of the stemness factors, Nanog, Sox2 and OCT4 (24). In this study, we found that estrogen increased the CD44$^+$/CD24$^-$ stem-like population and expression of CD44, and this was attenuated by treatment with tocopherols in MCF-7 tumorspheres. These findings suggest that CD44 is a useful cancer stem cell marker regulated by estrogen and tocopherols. Since the tumorsphere-forming assay enriches for stem and progenitor cells, the reduction of SFE with tocopherols could be at least in part due to the ability to suppress the putative CD44$^+$/CD24$^{-/low}$ breast cancer stem cell population. In addition, we found that ALDH activity was significantly higher in MCF-7 tumorspheres than MCF-7

monolayer, which was consistent with the previously reported findings by Charafe-Jauffret et al. (37). They demonstrated that the ALDH-positive population showed increased tumorsphere-forming capacity compared with ALDH-negative cells in SUM149 and SUM159 cells (37). Treatment with estrogen increased the ALDH activity, and this induction was decreased by γ-tocopherol, demonstrating that the suppression of ALDH-positive cells and the CD44$^+$/CD24$^-$ subpopulation by γ-tocopherol reduced breast cancer stemness. Interestingly, tocopherols without estrogen had little or no effect on the number and size of tumorspheres, suggesting that suppression of tumorspheres by tocopherols occurs at the level of estrogen stimulation of cell stemness. Alternatively, estrogen might selectively induce the replication of stem-like cells, and this might be suppressed by tocopherols.

Coordinated networks of stemness factors are the master regulatory mechanisms of pluripotency and differentiation in stem cells. OCT4, a transcription factor that forms a heterodimer with SOX2, plays a major role in the regulation of stem cell pluripotency and formation in breast cancer stem cells (38,39). OCT4 and SOX2 act synergistically to regulate their own transcription as well as the expression of other key stem cell genes (40). Jung *et al.* (20) reported that estrogen (1–20 nM) induced increase of OCT4 expression and proliferation of tumorspheres as well as the breast cancer stem cell population in MCF-7 tumorspheres. Our results also confirmed the findings of previous
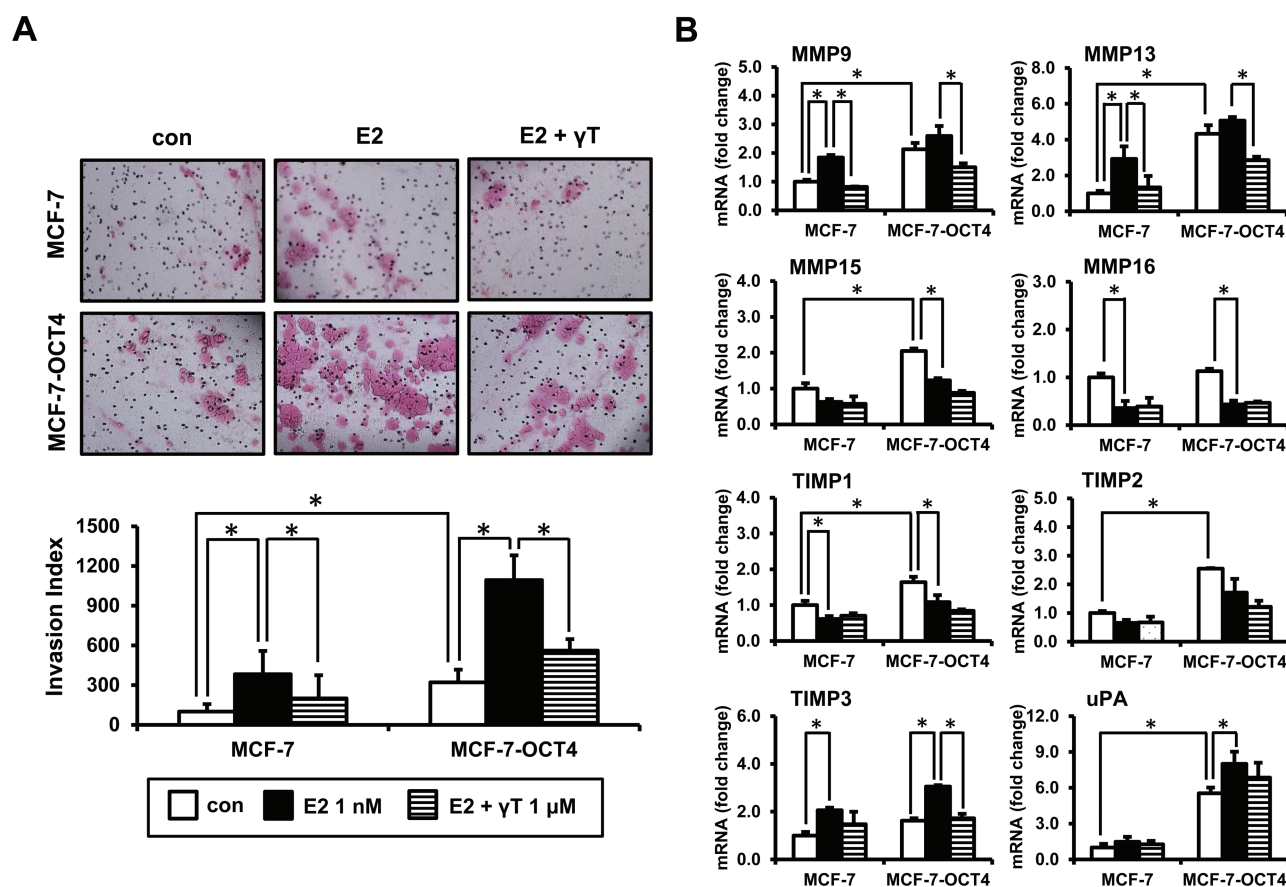
Figure 6. γ-Tocopherol inhibits cell invasion induced by E2 and OCT4 overexpression. Cells were transfected with control vector (MCF-7) and pSin-EF2-OCT4-Pur vector (MCF-7-OCT4) for 24 h, and then, cells were treated with E2 (1 nM) and/or γ-tocopherol (1 μM) for an additional 24 h. (**A**) Cells (8 × 10⁴ cells/ml) were loaded onto transwells coated with Matrigel. After 22-h incubation, the transwells were stained with H&E stain (top). Representative pictures are shown, ×10 magnification. The number of invading cells was counted in three representative fields per membrane, and the relative percentage of invasive cells was calculated from three independent experiments (bottom). (**B**) qPCR analysis was performed to detect genes involved in cell invasion, including MMP9, MMP13, MMP15, MMP16, TIMP1, TIMP2, TIMP3 and uPA. The data are represented as mean ± SD. n = 3 independent experiments. Cycle numbers for genes related to MMP9, MMP13, MMP15, MMP16, TIMP1, TIMP2, TIMP3 and uPA for MCF-7 control group were 27, 28, 25, 23, 22, 23, 24 and 27, respectively. *Significantly different from the respective control ($P < 0.05$).

studies indicating that the levels of pluripotency markers, such as OCT4 and SOX2, were increased by estrogen. Progesterone plays an important role in mammary gland pathophysiology, and PGR, TFF/pS2 as well as CTSD have been used as indicators of breast cancer progression and as a predictor for tamoxifen resistance of breast tumors (41,42). Recently, SERPINA1 has been reported to be a direct ER target gene (43). The expression of these genes is decreased by α-, γ- and δ-tocopherols in estrogen-stimulated MCF-7 tumorspheres, suggesting that downregulation of these estrogen-related genes could indicate selective inhibition of ER-regulated transcription by tocopherols. MCF-7 tumorspheres treated with estrogen increased the binding of ERα to the ERE site of POU5F1 (OCT4), suggesting that OCT4 acts through an ER-dependent mechanism in MCF-7 cells (20,44). Estrogen increased the ERE-luciferase activity, and this induction was decreased by γ-tocopherol in MCF-7 cells (Supplementary Figure S7, available at *Carcinogenesis* Online), suggesting that tocopherols may reduce estrogen-induced OCT4 expression by an ER-dependent mechanism in breast cancer cells.

We further examined the role of OCT4 on expression of stemness- and estrogen-related markers in MCF-7 ER-positive breast cancer cells. Overexpression of OCT4 increased the levels of CD44, SOX-2, PGR and ERα in the presence of estrogen, and this was attenuated by treatment with γ-tocopherol. Moreover, overexpression of OCT4 increased *in vitro* cell invasion through

regulation of matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs) and uPA gene expression in MCF-7 cells. MMPs are major players in tumor invasion and metastasis (45,46). MMPs may contribute to the development of breast cancer, while the increase in TIMPs expression may represent a feedback effect (47). In addition, uPA is a critical enzyme for cancer cell invasion converting plasminogen into plasmin, which degrades extracellular matrix and activates multiple MMPs (48). Hu *et al.* (49) reported that overexpression of OCT4 increases the migration and invasion properties by regulating miR-145 and downstream transcriptional factors, Snail, ZEB1 and ZEB2 in breast cancer cells. Knockdown of OCT4 suppresses the growth and invasion of pancreatic cancer cells through inhibition of Akt pathway-mediated PCNA and MMP2 expression (50) and inhibit colorectal cancer cell motility and invasion (51). Overexpression of OCT4 increases the invasion of breast cancer MCF-7 cells, and tocopherols suppress the estrogen-induced cell invasive properties by regulating these cell invasion markers, suggesting that OCT4 may be a potential target for prevention and/or treatment of breast cancer.

In conclusion, the inhibition of cancer stem-like cell properties and cell invasion by tocopherols in ER-positive breast cancer probably occurs via estrogen-dependent and OCT4-mediated mechanisms. Our findings suggest tocopherols as potentially useful agents for suppressing these estrogen effects on breast cancer development.

Supplementary material

Supplementary Figures S1–S7 can be found at *Carcinogenesis* online.

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