Estradiol induces cell proliferation in MCF-7 mammospheres through HER2/COX-2

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Abstract. Cluster of differentiation (CD)44$^+$/CD24$^-$ breast cancer cells have stem cell-like characteristics and are potent initiators of tumorigenesis. Mammosphere cells can partially initiate breast tumorigenesis by inducing estradiol (E2)-dependent breast cancer cells. However, the mechanisms by which E2 mediates cancer formation in MCF-7 mammosphere (MS) cells have remained elusive. In the present study, MS cells were isolated by sphere culture. It was possible to maintain these MS cells in culture for long periods of time, while retaining the CD44$^+$/CD24$^-$ stem cell marker status. The CD44$^+$/CD24$^-$ status was confirmed by flow cytometry. Furthermore, the stem-cell markers Musashi-1, cytokeratin (CK)7 and CK19 were identified by immunofluorescence microscopy. It was revealed that treatment of MS cells with E2 increased the expression of CD44, whereas decreased the expression of CD24 on MS cells. In addition, treatment with E2 increased colony formation by MS cells. E2 also induced cyclooxygenase-2 (COX-2) expression in MS cells, which promoted their proliferation through the estrogen receptor/human epidermal growth factor receptor 2 (HER2)/mitogen-activated protein kinase/phosphoinositide-3 kinase signaling pathway. The results suggested a tumorigenic mechanism by which E2 promotes tumor cell proliferation via HER2/COX-2 signaling. The present study provided evidence for the molecular impact of E2 on breast tumorigenesis, and suggested possible strategies for preventing and treating human breast cancer.

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Introduction

Breast cancer is the most common cancer type among women worldwide, and it remains the second leading cause of cancer-associated mortality in women. In a previous study, it was identified that breast cancer consists of heterogeneous cell populations, and is derived from genetic aberrations and environmental factors (1). Further findings indicated that stem cells may be involved in carcinogenesis and that breast cancer is, at least in part, a stem cell-based disease (2). As a certain number of residual cancer cells can survive after chemotherapy, and have self-renewal and differentiation capacity, these cells may initiate a new tumor and cause relapse. These tumor-initiating cells are known as cancer stem cells (CSCs) (2-4). Therefore, determining the role of CSCs in primary tumorigenesis is critical for making appropriate treatment decisions for patients with breast cancer. In this regard, certain methods have been developed to collect breast CSCs, including cell sorting according to cell surface expression of cluster of differentiation (CD)44$^+$/CD24$^-$ (5).

The mammosphere assay was established based on the spheroid model (6), and mammospheres represent a pre-cancerous state and act as a surrogate indicator for the presence of CSCs (7). The mammosphere model partially resembles breast tumorigenesis (8,9). Notably, only epithelial cells survive in mammosphere suspension cultures, whereas other cells die via apoptosis, which is due to the higher self-renewal capacity of stem cells compared to other cells (10-12).

A previous study indicated that multicellular mammospheres have clone-initiating abilities and they reform following trypsin-mediated dissociation (8). Therefore, microsphere culture systems may be used to study the functions and tumorigenic aspects of CSCs, and assess the efficacy of therapeutic agents. In addition, some studies have indicated that human epidermal growth factor receptor 2 (HER2) mediates the self-renewal and proliferation of CSCs (13,14). It is estimated that 25% of patients with breast cancer present with aberrant expression of the gene encoding for HER2, which confers enhanced drug resistance (15) and increases the risk of mortality (16). HER2 activation may stimulate downstream signaling pathways, including the phosphoinositide-3 kinase (PI3K)/AKT serine/threonine kinase (17), mitogen-activated

protein kinase (MAPK)/extracellular signal-regulated kinase (18) and cyclooxygenase-2 (COX-2) (19) signaling pathways, thereby contributing to tumor and drug resistance.

However, HER2-positive tumors are usually heterogeneous and have a cellular phenotype that is consistent with $CD44^+/CD24^-$ CSCs. Overexpression of the intragenic HER2 enhancer in mammospheres and increased levels of the stem-cell marker aldehyde dehydrogenase increases the proportion of stem/progenitor cells compared with normal mammary epithelial cells (20). Previous findings indicated that cell surface HER2 expression was critical for mammosphere formation and maintenance of two-dimensional cell lines (21,22). Defects in HER2 function led to a pro-survival phenotype, and down-regulated signaling pathways that mediated cell proliferation and chemoresistance in breast cancer (23-25). A study also demonstrated that activation of the estrogen receptor (ER) on the cell membrane affects the HER2 signaling pathway and is a mechanism by which estrogen promotes proliferation (26).

To further understand the function of breast CSCs it is reasonable to assess mammospheres; however, the molecular mechanisms underlying mammosphere function remain elusive. In the present study, the effects by which estradiol (E2) increases the formation of mammospheres by MCF-7 breast cancer cells were examined and the underlying mechanisms were investigated. It was hypothesized that E2 mediates mammosphere formation through the HER2/COX-2 signaling pathway. The aim of the present study was to establish a therapeutic target that may contribute to the development of future clinical treatment strategies for eliminating breast CSCs.

Materials and methods

Cell culture and mammosphere generation. The MCF-7 human breast cancer cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in minimum essential medium (MEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere with 5% CO_2. For generation of mammosphere cultures, $1x10^5$ MCF-7 cells/ml were cultured in serum-free MEM supplemented with 10 ng/ml basic fibroblast growth factor (bFGF; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 20 ng/ml epidermal growth factor (EGF; Sigma-Aldrich; Merck KGaA) and 2% B-27 cell culture supplement (Invitrogen; Thermo Fisher Scientific, Inc.). The media was replaced during the 2 days of culture at 37°C and continue for 7 to 14 days, respectively, non-adherent spherical clusters were observed by light microscope (Nikon Eclipse TE 300; Nikon Corporation, Tokyo, Japan) and these were identified as MCF-7 mammosphere cells (hereafter referred to as MCF-7 MS cells) (27-29). In addition, $1x10^5$ cell were cultured in 6 well plate and when density reached ~80%, the cells were treated with $1x10^{-6}$ M E2 (Sigma-Aldrich; Merck KGaA) or $1x10^{-6}$ M Benzyl butyl phthalate (BBP; Sigma-Aldrich; Merck KGaA) and maintained in culture for experimental processes.

Flow cytometry. Flow cytometric analysis of CD44-, CD24- and 5-bromo-2'-deoxyuridine (BrdU)-stained cells, was used to identify MCF-7 and MCF-7 MS cells. For the analysis of cell surface expression of CD44 and CD24, $1x10^6$ cells/ml were incubated in the dark at 4°C for 30 min with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody against CD24 (1:400, cat. no. 555427; BD Biosciences, Franklin Lakes, NJ, USA) or phycoerythrin-conjugated antibody against CD44 (1:400; cat. no. 550989; BD Biosciences) in staining buffer (3% FBS + 0.01% Sodium azide). The staining buffer was removed and the cells washed twice with phosphate buffered saline (PBS; Sigma-Aldrich; Merck KGaA) to perform the blocking stain and the stained cells were analyzed on a BD FACSCalibur™ flow cytometer (BD Biosciences).

Cell proliferation was analyzed using the BrdU Cell Proliferation kit (EMD Millipore, Billerica, MA, USA), according to the manufacturer's protocol. The BrdU incorporation assay quantifies newly synthesized DNA in the S phase of the cell cycle. Briefly, cells were seeded in 6-well plates at a cell density of $1x10^4$ cells/well and treated with 5 ng BrdU for 24 h at 37°C. Subsequently, the cells were harvested and fixed in 70% ethanol for 10 min at 37°C. In addition, total DNA was stained with 2.5 μg/ml 7-aminoactinomycin D (7-ADD) for 15 min at 37°C. The DNA synthesized by replicating cells was detected by flow cytometric. The amount of BrdU and 7-ADD in the cells was detected using the BD FACSCalibur™ flow cytometer (BD Biosciences) and analyzed by WinMDI 2.9 software (Scripps Institute, La Jolla, CA, USA).

Immunofluorescence microscopy. A total of $1x10^4$ cells/well MCF-7 and MCF-7 MS cells were cultured on glass slides for 24 h, after which the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich; Merck KGaA) at 4°C for 10 min. The fixed cells were blocked with 0.5% (w/v) Triton X-100 and incubated overnight at 4°C with primary antibodies against Musashi-1 (1:500; cat. no. sc-135721; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), cytokeratin (CK)7 (1:500; cat. no. sc-53264; Santa Cruz Biotechnology, Inc.) and CK19 (1:500; cat. no. SC-6728; Santa Cruz Biotechnology, Inc.). Subsequently, cells were stained with secondary antibody FITC (1:1,000; cat. no. sc-65218; Santa Cruz Biotechnology, Inc.) for 60 min at 37°C. Cell nuclei were stained with DAPI (Sigma-Aldrich; Merck KGaA) for 1 min at 37°C, and three independent experiments images were acquired using an immunofluorescence microscope (magnification, x100).

Cell lysis and western blot analysis. MCF-7 MS cells were treated with E2 a dose ($1x10^{-11}$-$1x10^{-6}$ M) and time (5 min-48 h) dependent manner. After, MCF-7 and MCF-7 MS cells were washed with cold PBS (4°C) and resuspended in RIPA lysis buffer (EMD Millipore, Billerica, CA, USA) containing a protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA). The cells were incubated on ice for 30 min and the lysate was centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was collected and the protein concentration was determined by the Bradford method. Proteins were separated by 10% polyacrylamide SDS-PAGE and electrophoretically transferred onto a polyvinylidene difluoride membrane (EMD Millipore) for 90 min at 37°C. Non-specific binding protein was blocked by 5% non-fat dry milk with PBS for 1 h. The membrane was then stained with primary antibodies against HER2 (1:500; cat. no. sc-08; Santa Cruz Biotechnology, Inc.), COX-2 (1:500; cat. no. sc-19999; Santa Cruz Biotechnology, Inc.) and β-actin
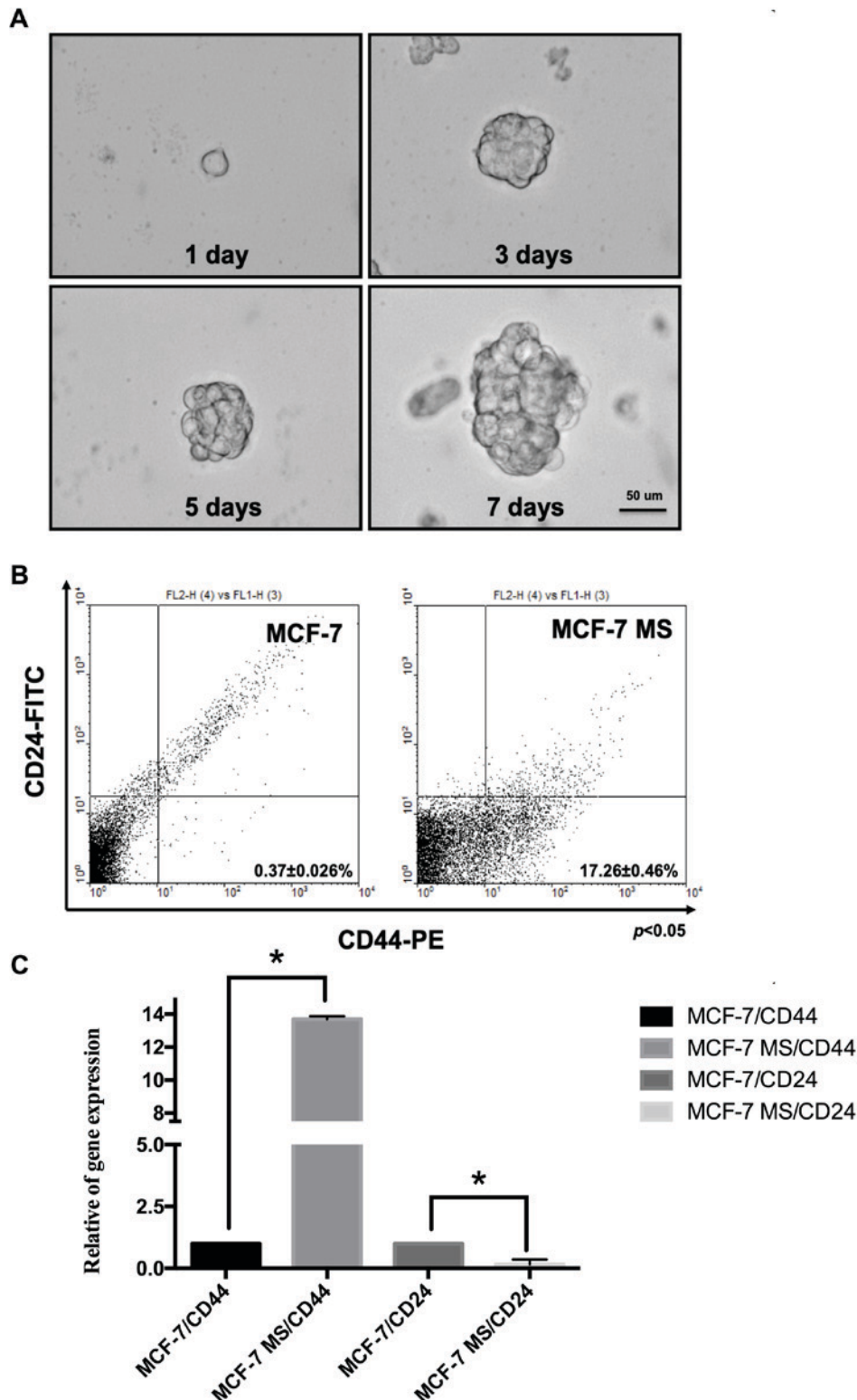
Figure 1. Morphology and proportion of CD44+/CD24− cells among MCF-7 MS cells. (A) MCF-7 cells were cultured in medium containing 10 ng/ml basic fibroblast proliferation factor, 20 ng/ml epidermal growth factor and 2% B-27 to induce the formation sphere-like MS for 7 days. Images were acquired with a phase-contrast microscope (scale bar, 50 μm). (B) The percentage of CD44+/CD24− cells in MCF-7 and MCF-7 MS cell cultures was determined by flow cytometry. (C) The gene expression levels of CD44 and CD24 were determined by reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean ± standard deviation from three independent experiments. Statistical significance was analyzed by Student's t-test. *P<0.05. CD, cluster of differentiation; FITC, fluorescein isothiocyanate; MS, mammosphere; PE, phycoerythrin.

(1;500; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.) overnight at 4˚C. The secondary antibodies goat anti-mouse IgG or anti-rabbit IgG (1:500; Santa Cruz Biotechnology, Inc.) were incubated at 37˚C for 1 h. The intensity of protein bands was assessed via enhanced chemiluminescence using the Western Lightning Plus-ECL kit (PerkinElmer, Inc.,

Waltham, MA, USA). In addition, ER antagonist Fulvestrant (ICI 182780, Sigma-Aldrich; Merck KGaA), MAPK inhibitors (SB203580 and PD98059, Sigma-Aldrich), a PI3K inhibitor (wortmannin; Sigma-Aldrich; Merck KGaA) and a HER2 inhibitor (Tyrphostin; AG-825, Sigma-Aldrich; Merck KGaA) were used to analyze the signalling pathway.

Colony formation assay. A total of $1x10^3$ MCF-7 cells were seeded in 6-well plates and $1x10^{-6}$ M E2 or $1x10^{-6}$ M BBP was maintained in the culture medium. The culture medium was replaced every 3 days and cells were maintained in culture for 14 days. The cells were fixed with 4% paraformaldehyde for 10 min at 4°C and then stained with 0.01% crystal violet (Sigma-Aldrich; Merck KGaA) for 30 min at 37°C to enable counting of colonies. The numbers of visible colonies in the control and MCF-7 MS cells were counted under a fluorescence microscope (Nikon Eclipse TE 300; Nikon Corporation, Tokyo, Japan; magnification, x100).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from MCF-7 and MCF-7 MS cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). Reverse transcription into complementary DNA was performed using the Deoxy+ HiSpec Reverse Transcriptase kit (Yeastern Biotech Co., Ltd., Taipei, Taiwan) according to the manufacturer's protocol. qPCR was performed using SYBR™ Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) on a 7900HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following primers were used: CD24, forward primer: 5'-TCA AGTAACTCCTCCCAGAGTA-3', reverse primer: 5'-AGA GAGTGAGACCACGAGA-3; CD44, forward primer: 5'-CGC TATGTCCAGAAAGGAGAAT-3', reverse primer: 5'-CTG CTCACGTCATCATCAGTAG-3 and GAPDH, forward primer: 5'-GGTGGCAGAGGCCTTTG-3'. Reverse primer: 5'-TGCCCATTTAGCATCTCCTT-3. The thermocycling conditions were: 94°C for 5 min; 35 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec. The relative gene expression data was analyzed using qPCR and the $2^{-\Delta\Delta Cq}$ method (30).

Statistical analysis. Data are expressed as the mean ± standard deviation from three independent experiments. Significant differences between two groups were determined using Student's t-test and comparisons between two groups were performed using Student's t-test and one-way analysis of variance using post hoc test to analyze differences among multiple groups. SPSS statistical software (version 13.0, SPSS. Inc., Chicago, IL, USA) was used for all analyses. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Mammosphere formation by MCF-7 cells. Previous studies have demonstrated that mammospheres cultured from breast cancer cell lines express the CD44+/CD24− biomarker signature (28) and possess side-population characteristics (31). For MCF-7 MS cell, the MCF-7 cells had attained the ability to grow as sphere-like mammospheres for 1, 3, 5 or 7 days, they were cultured in medium with proliferation factors (10 ng/ml bFGF, 20 ng/ml EGF and 2% B-27) and passaged every 2 days. The

Table I. Proportion of CD44+/CD24− cells among MCF-7 and MCF-7 MS cells.

Treatment groups	CD44−/CD24+ (%)	CD44+/CD24− (%)
MCF-7		
Control	5.04±0.46	0.37±0.46
E2	5.03±0.02	0.86±0.012
BBP	5.06±0.06	0.47±0.086
MCF-7 MS (7 day culture)		
Control	2.42±0.01	13.26±0.46[a]
E2	1.04±0.05	19.0±0.36[b]
BBP	1.03±0.09	16.57±0.42[b]
MCF-7 MS (14 day culture)		
Control	1.25±0.10	80.63±0.80[a]
E2	0.67±0.04	89.79±0.37[c]
BBP	0.78±0.04	86.04±0.67[c]

Data are presented as the mean ± standard deviation from three independent experiments. Statistical significance was analyzed by one-way analysis of variance followed by a one-way analysis of variance test. [a]$P<0.05$ vs. MCF Control. [b]$P<0.05$ vs. MCF-7 MS (7 day culture) Control. [c]$P<0.05$ vs. MS (14 day culture) Control. CD, cluster of differentiation; E2, estradiol; MS, mammosphere; BBP, benzyl butyl phthalate.

diameter of the mammospheres gradually increased over the 7-day period, reaching ~50 μm (Fig. 1A). Following mammosphere culture for 7 days, the proportion of CD44+/CD24− cells was detected by flow cytometry. As presented in Fig. 1B, the proportion of CD44+/CD24− cells reached 17.26±0.46% for MCF-7 MS cells, which was >40-fold higher compared with MCF-7 cells (0.37±0.026%). The gene expression of these biomarkers was also quantified by RT-qPCR. The results indicated that the expression levels of CD44 were increased whereas expression levels of CD24 were decreased in MCF-7 MS cells compared with MCF-7 cells (Fig. 1C). In addition, flow cytometry was used to investigate whether treatment with $1x10^{-6}$ M E2 or $1x10^{-6}$ M benzyl butyl phthalate (BBP) would affect the proportion of CD44+/CD24− cells among MCF-7 MS cells. The results revealed a significant increase in the proportion of CD44+/CD24− cells after MCF-7 MS cells were treated with E2 and BBP for 24 h (Table I). BBP is a plasticizer, which exhibits weak estrogenic activity (32). Previous studies by our group indicated that BBP could mediate cancer cell proliferation and angiogenesis in human breast cancer cell lines through the ER (26,32). In the present study, the results demonstrated that MCF-7 MS cells expressed CD44+/CD24− and that the proportion of CD44+/CD24− cells was increased upon treatment with estrogen.

Expression of stem cell markers in MCF-7 MS cell lines. Next, the expression of additional stem cell markers in MCF-7 MS cells was measured. Musashi-1, CK7 and CK19 are markers of progenitor stem cells (33-35). Musashi-1 mediates the self-renewal of stem cells and is overexpressed in a variety of tumor types (36). CK is a known CSC marker that indicates poor prognosis. CK7 and CK19 in particular are associated
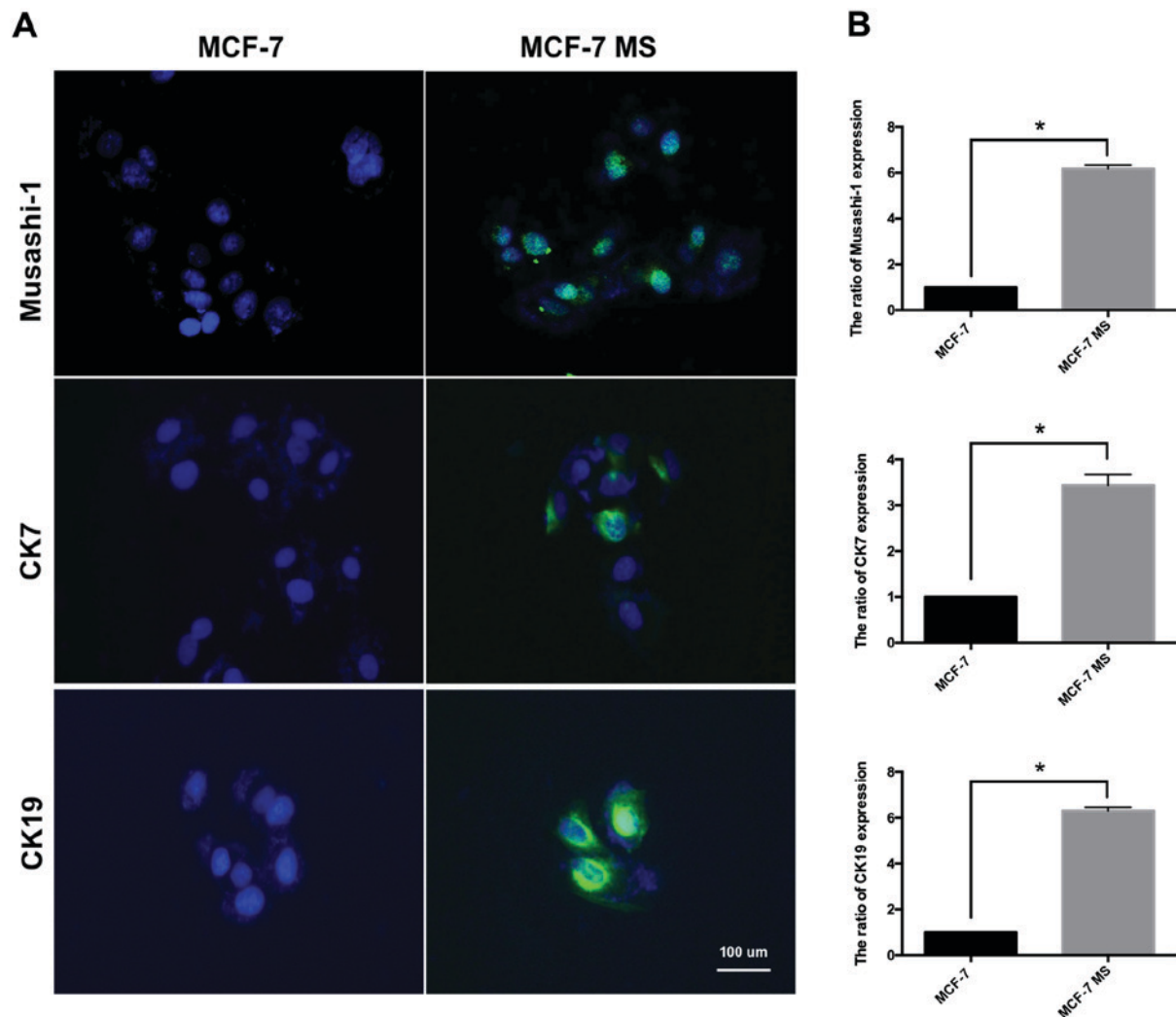
Figure 2. MCF-7 and MCF-7 MS cells express stem cell markers, including Musashi-1, CK7 and CK19. (A) MCF-7 and MCF-7 MS cells were immunofluorescently stained for Musashi-1, CK7 and CK19 using fluorescein isothiocyanate-conjugated antibodies (green). Nuclei were stained with DAPI (blue). Images were acquired with an immunofluorescence microscope (scale bar, 100 μm). (B) The gene expression levels of Musashi-1, CK7 and CK19 were determined by reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean ± standard deviation from 3 independent experiments. *P<0.05 vs. MCF-7. CK, cytokeratin; MS, mammosphere.

with signaling that regulates the epithelial-mesenchymal transition (37,38). Following mammosphere culture for 14 days, immunofluorescence and RT-qPCR were used to detect the expression of these stem cell markers in MCF-7 and MCF-7 MS cells. The immunofluorescence staining and RT-qPCR results indicated that Musashi-1, CK7 and CK19 were significantly increased in MCF-7 MS cells compared with in MCF-7 cells (Fig. 2A and B).

E2 and BBP induce colony formation by MCF-7 MS cells. A colony formation assay was used to measure the colony-forming ability of MCF-7 MS cells. Colony-forming ability is a sensitive *in vitro* indicator of undifferentiated CSCs (39). Following mammosphere culture for 14 days, the cells were treated with $1x10^{-6}$ M E2 and $1x10^{-6}$ M BBP, and maintained in culture for a further 14 days. The results revealed that treatment with E2 and BBP increased colony formation compared with control (Fig. 3A). The visible number of colonies in the control and treatment groups was counted under a microscope. The number of colonies was quantified and found that E2 and BBP

significantly induced colony formation in MCF-7 and MCF-7 MS cells (Fig. 3B).

E2 induces COX-2 expression through HER2 in MCF-7 MS cells. To date, it has remained elusive whether E2 stimulates the HER2 signaling pathway in MCF-7 MS cells. In the present study, western blotting was used to identify HER2 signaling in MCF-7 MS cells. The results revealed that the protein levels of HER2 and COX-2 were upregulated in MS cells compared with in MCF-7 cells (Fig. 4A). COX-2 is a downstream gene of HER2 (40) and regulates the proliferation of breast cancer cells (41). Following mammosphere culture for 14 days, MCF-7 MS cells were treated with E2 at different concentrations ($1x10^{-11}$-$1x10^{-6}$ M) and for different periods of time (5 min-48 h), and the expression levels of COX-2 were assessed. The results indicated that E2 increased the expression of COX-2 in a dose- and time-dependent manner (Fig. 4B and C). To confirm whether the cellular levels of COX-2 were dependent on ER, MCF-7 MS cells were treated with E2 and the ER antagonist Fulvestrant (ICI 182780).
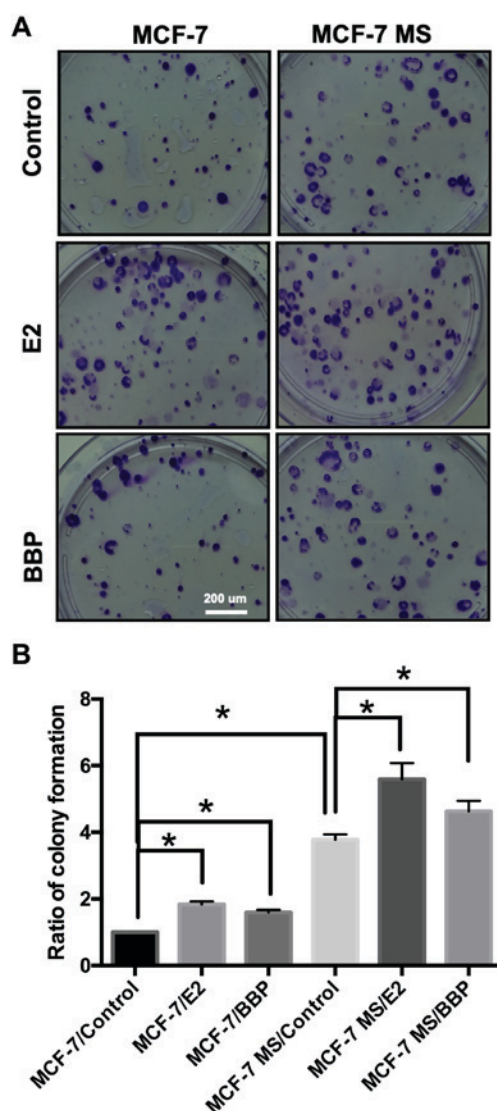
Figure 3. E2 induces colony formation of MCF-7 and MCF-7 MS cells. (A) MCF-7 and MCF-7 MS cells were treated with 1×10^{-6} M E2 or 1×10^{-6} M BBP for 21 days, before colony formation was assessed using a colony formation assay (scale bar, 100 μm). (B) The number of colonies was quantified. Data are presented as the mean ± standard deviation from three independent experiments. Statistical significance was analyzed by one-way analysis of variance followed by a one-way analysis of variance test. *P<0.05. E2, estradiol; BBP, benzyl butyl phthalate; MS, mammosphere.
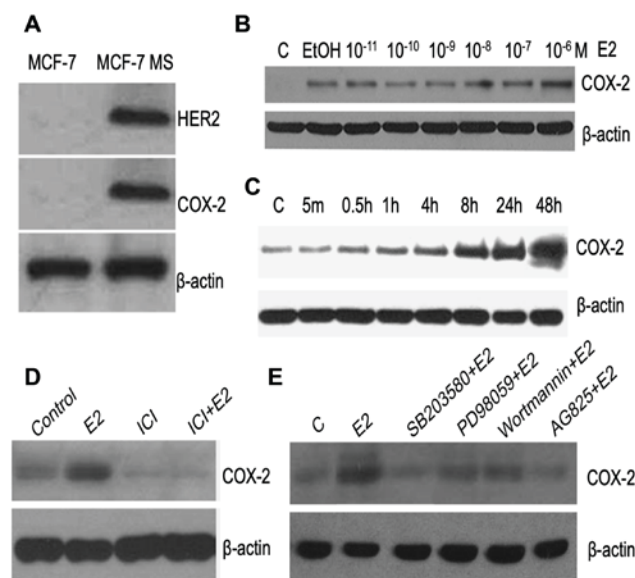

Figure 4. E2 induces COX-2 expression in MCF-7 MS cells through the ER/HER2/MAPK/PI3K signaling pathway. (A) Western blot analysis of HER2 and COX-2 in MCF-7 and MCF-7 MS cells. (B) COX-2 levels were assessed by western blotting after MCF-7 MS cells were treated with different concentrations of E2 (1×10^{-11}-1×10^{-6} M). (C) MCF-7 MS cells were treated 1×10^{-6} M for various durations (5 min-48 h) and COX-2 protein levels were measured. MCF-7 MS cells were treated with 1×10^{-6} M E2 alone or in combination with (D) 1×10^{-6} M ER inhibitor ICI182780, (E) 3×10^{-5} M MAPK inhibitor SB203580, 2×10^{-5} M MAPK inhibitor PD98059, 5×10^{-7} M PI3K inhibitor wortmannin or 5×10^{-6} M HER2 inhibitor AG-825, and COX-2 was detected by western blotting. β-actin was used as the loading control. The images are representative of 3 independent experiments. C, control; COX-2, cyclooxygenase-2; E2, estradiol; ER, estrogen receptor; EtOH, ethanol; HER2, human epidermal growth factor receptor 2; ICI, ICI182780; MAPK, mitogen-activated protein kinase; MS, mammosphere; PI3K, phosphoinositide-3 kinase.

Notably, treatment with ICI repressed the E2-mediated induction of COX-2 in MCF-7 MS cells (Fig. 4D). Furthermore, two MAPK inhibitors (SB203580 and PD98059), a PI3K inhibitor (wortmannin) and a HER2 inhibitor (Tyrphostin; AG-825) were used to analyze the signaling involved in E2-mediated induction of COX-2 in MCF-7 MS cells. All the inhibitors repressed E2-mediated induction of COX-2 (Fig. 4E), suggesting that E2 upregulated cellular COX-2 levels through ER/HER2/MAPK/PI3K signaling in MCF-7 MS cells.

E2 induces the proliferation of MCF-7 MS cells through COX-2. To determine whether E2 induces cell proliferation through COX-2 in MCF-7 MS cells, MCF-7 MS cells that had been in mammosphere culture for 14 days were treated with E2 plus HER2 antagonist AG-825 or COX-2 antagonist NS-398, and BrdU incorporation was measured by flow cytometry. Indeed,

both E2+AG-825 and E2+ NS-398 inhibited the proliferation of E2-treated MCF-7 MS cells (Fig. 5A and B), suggesting that E2 induced proliferation of MCF-7 MS cells through HER2/COX-2.

Discussion

The present study demonstrated that E2 increased the proportion of CD44^{+}/CD24^{-}MCF-7 MS cells and induced cell proliferation through ER/HER2/MAPK/PI3K/COX-2 signalling, which is consistent with a previous study, which indicated that E2 promotes cancer cell proliferation (42). CD44 is a hemagglutinin-binding glycoprotein surface marker, which is overexpressed in numerous solid malignancies and CSCs. There is evidence indicating that monoclonal antibodies against CD44 may be a favourable therapeutic strategy for the clinical treatment of cancer (43). The mammosphere culture model used in the present study was convenient and fast in obtaining CD44^{+} cells, which may aid in the development of clinical drugs in the future.

E2 is a steroid hormone that mediates various cell processes through the ER. The levels of E2 have an important role in breast cancer development and are associated with increased risk of breast cancer in women (44). Previous findings demonstrated that E2 significantly increases the percentage of cells in the S-phase during culture, and activates the phosphorylation
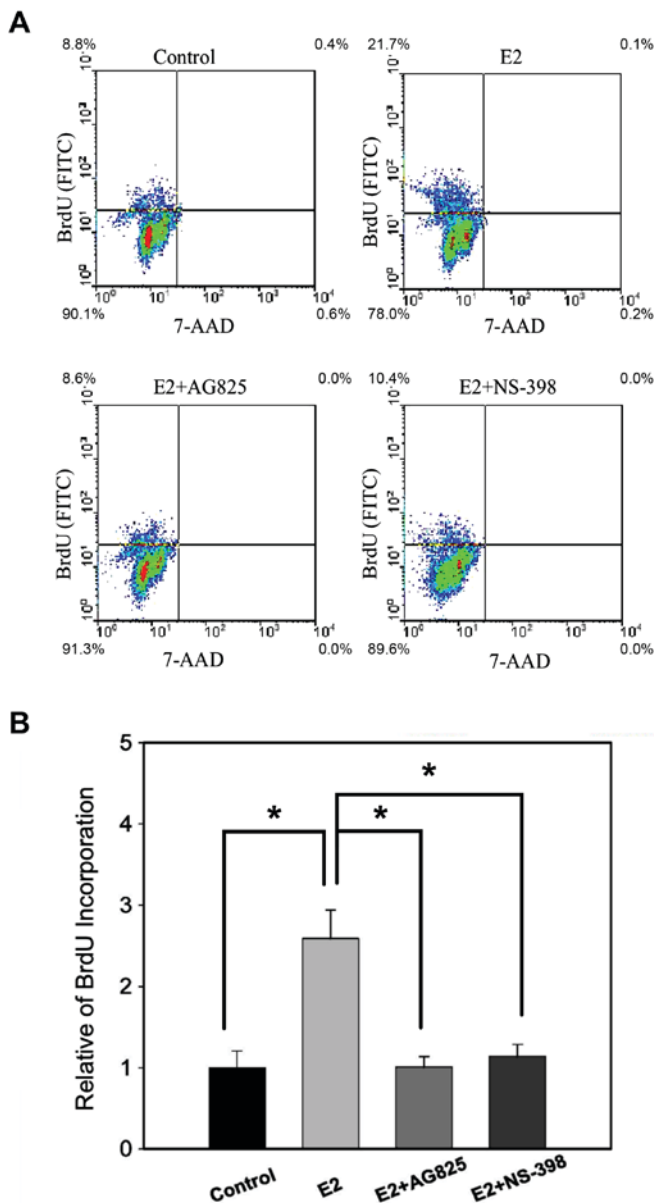
Figure 5. E2 meditates cell proliferation via human epidermal growth factor receptor 2/cyclooxygenase-2 in MCF-7 MS cells. (A) MCF-7 MS cells were treated with $1x10^{-6}$ M E2, $1x10^{-6}$ M E2 + $5x10^{-6}$ M AG-825 or $1x10^{-6}$ M E2 + $1x10^{-6}$ M NS-398 for 24 h, and cell proliferation was analyzed by examining BrdU incorporation using flow cytometry. (B) Quantified BrdU incorporation in the different groups. Data are presented as the mean ± standard deviation from three independent experiments. Statistical significance was analyzed by one-way analysis of variance followed by a one-way analysis of variance test. $^*P<0.05$. 7-AAD, 7-aminoactinomycin D; BrdU, bromodeoxyuridine; E2, estradiol; FITC, fluorescein isothiocyanate; MS, mammosphere.

of PI3K and MAPK in human mesenchymal stem cells (45). E2 also affects adipogenesis and osteogenesis through the ER (46). Therefore, regulation of stem cell differentiation by E2 is required for human tissue regeneration.

ER, HER2, MAPK, PI3K and COX-2 also have an important role in cell proliferation and stem cell differentiation/self-renewal. Side-population cells are stem cells that have a high drug efflux function and exhibit a 6-fold enriched expression of ER compared with non-side-population cells (47). In ER-positive breast cancer, HER2 is a CSC-selective marker for regulating self-renewal and proliferation (48). In addition, COX-2 is the rate-limiting enzyme for catalyzing the formation of prostaglandins and promoting cell proliferation in cancer. A previous study demonstrated that COX-2 is highly expressed in hematopoietic stem cells and mediates stem cell self-renewal and differentiation (49). Another study indicated that the COX-2-specific inhibitor celecoxib provided no clinical benefits for ER-positive patients with advanced disease, but had a greater effect in ER-negative patients (50). Therefore, the mechanisms of COX-2 and ER in breast cancer remains to be elucidated, and further clarification on their association is required to pave way for the development of novel drugs for the clinical treatment of breast cancer.

Furthermore, COX-2 is involved in osteogenesis by inducing the expression of core-binding factor $\alpha1$ and osterix, which mediate normal skeletal repair and stem cell differentiation (51). In addition, alterations of the MAPK/PI3K signaling pathway are involved in metastatic progression linked with CSC/progenitor cells (52). Therefore, it is indicated that ER, HER2, MAPK, PI3K and COX-2 have an important role in breast CSC self-renewal, differentiation and proliferation in mammospheres.

The mammosphere culture system based on human cancer cell lines can be relatively easily established and has been well characterized. Stem-like cells may be induced by growth factors and stem cell surface markers are identifiable by flow cytometry. However, the mammosphere culture system has certain limitations. It features a heterogeneous population with varying expression levels of CD44 and CD24 among cells (53). Furthermore, it has been reported that trastuzumab antibodies have poor penetration in the mammosphere model (22). In the future, cells from primary breast cancer may be isolated and induced to form mammospheres. These mammosphere-forming cells resembling a cancer stem-like cell population, which are resistant to clinical drugs may provide useful information for the development or selection of personalized therapies in the future.

In conclusion, the present study established an MCF-7 MS cell model, and revealed that E2 increased the proportion of CD44$^+$/CD24$^-$MCF-7 MS cells and mediated cell proliferation via ER/HER2/MAPK/PI3K/COX-2 signaling. This information may be used to examine the functions and tumorigenic aspects of stem cells, and assess the efficacy of novel therapeutic agents.

Availability of data and materials

All analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

THH, CHW, CLW and EMT conceived and designed the experiments. HYC, CYL and CYH performed the experiments. THH, CHW, CLW and EMT revised the manuscript and interpreting all data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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