17β-estradiol regulates the malignancy of cancer stem-like cells derived from the MCF7 cell line partially through Sox2

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Abstract. As a major common malignant tumor in women, the malignant behavior of breast cancer, which includes tumorigenesis and metastasis, is associated with estrogen, particularly 17β-estradiol (E2). With accumulating evidence demonstrating that cancer stem-like cells (CSCs) serve a function in the malignant behavior of breast cancer, including metastasis, recurrence and chemoresistance, the effects of E2 on the physiological processes of CSCs have been attracting more attention. In the present study, in order to investigate the effects of E2 on CSCs, CSCs from the MCF7 breast cancer cell line were isolated and treated with 1, 10 and 50 nM E2. Detection of cell proliferation following E2 treatment revealed that 10 nM E2 treatment inhibited cell proliferation, whereas 50 nM E2 treatment resulted in the induction of apoptosis on CSCs. In order to further investigate the effects of E2 treatment on migration, colony formation and the self-renewal capacity of CSCs *in vitro*, cells were treated with 1 and 10 nM E2. As expected, compared with mock group, the self-renewal capacity of the CSCs was slightly increased by 10 nM E2 treatment, while 1 nM exhibited no observable effect. E2 treatment demonstrated different effects on the proliferation, migration, colony formation and self-renewal capacity of CSCs in a dose-dependent manner.

Introduction

In women, breast cancer is a major cause of cancer-associated mortality globally. Each year, ~1.4 million women are diagnosed with breast cancer, and >0.45 million women succumb to the disease (1). According to data from the World Health Organization, since 2008, there has been an ~20% increase in the number of diagnosed patients with breast cancer per year. Of the multitude of factors associated with the tumorigenesis of breast cancer, age is the strongest risk factor. Unlike numerous cancers that demonstrate an increase in incidence rate during the fifth decade of life, the incidence rate for breast cancer increases in the third decade of life, which is believed to be due to the effects of ovarian hormones on breast tissue (2-4).

The association between certain hormone levels and the increased risk of breast cancer indicates the critical function of hormones in the processes of breast cancer. Estrogens, particularly 17β-estradiol (E2), have been demonstrated to drive the tumorigenic processes of breast cancer (5). It has been reported that E2 drives the tumorigenesis of breast cancer by binding to estrogen receptor α (ERα) and regulating the expression of the downstream genes (6-9). Yager and Davidson (10) described several potential pathways that may explain how E2 treatment promotes breast cancer proliferation, migration and invasion. However, it has also been reported that E2 serves a contradictory effect on breast cancer cells in a concentration-dependent manner. Zhao *et al* (11) demonstrated that a high concentration of E2 induces apoptosis independent of the presence of ERα, whereas a low concentration of E2 promotes the proliferation of breast cancer cells through ERα. A high dose of E2 treatment caused a change in the levels of metastasis-associated lung adenocarcinoma transcript-1 (non-protein-coding) in MCF7 cells, which consequently caused the inhibition of the proliferation of breast cancer cells, as well as inhibiting the migratory, invasive and colony-formation abilities. Further studies are required to confirm these potential mechanisms.

Stem cells or cells that possess stem-like cell properties are considered to be fundamental in breast cancer initiation and progression (12). The small subpopulation of stem cells that exist within solid tumors, cancer stem-like cells (CSCs), are heterogeneous and have been demonstrated to be responsible for the regeneration of breast tumors (13). In this previous study, the different mechanisms of CSCs were assessed, including cellular markers cluster of differentiation $44^{+}/24^{-/low}$, aldehyde dehydrogenase 1 expression, and mammosphere formation and self-renewal capacity. The differential gene expression patterns of breast cancer cells and the CSCs derived from breast cancer raise the following question: How does E2 treatment of these two types of cell affect their physiological processes?

In order to answer this question, in the present study, the effects of different concentrations of E2 treatment on breast

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cancer cells and CSCs were examined. To elucidate the potential molecular mechanisms underlying the effect of E2 on CSCs, the levels of the transcription factors associated with self-renewal capacity were determined. The results of the present study demonstrated the effects of E2 on CSCs derived from breast cancer, and the partial underlying molecular mechanism.

Materials and methods

Cell culture. The human breast adenocarcinoma cell line MCF7 was obtained from the American Type Culture Collection (Manassas, VA, USA) and frozen in liquid nitrogen (-196˚C) in the laboratory. Cells were kept in 100 cm^2 dishes that contained 10 ml RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.) in a humidified atmosphere containing 5% CO_2 at 37˚C. The medium was replaced every 3 days.

Culture of CSCs from MCF7 cells. The suspended MCF7 cells were diluted to a density of 10^6 cells/ml in sphere-forming medium (SFM; Gibco; Thermo Fisher Scientific, Inc.) which was supplemented with 10 ng/ml basic fibroblast growth factor (bFGF; PeproTech, Inc., Rocky Hill, NJ, USA), 20 ng/ml epidermal growth factor (EGF; PeproTech, Inc.) and 2% B27 (Thermo Fisher Scientific, Inc.). The medium was half-replaced every 3 days and the cells were passaged every 10-15 days.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In order to detect the expression levels of ERα, octamer-binding transcription factor 4 (Oct4), sex-determining region Y-box 2 (Sox2), Krüppel-like factor 4 (Klf4) and MYC proto-oncogene (c-Myc), total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Total RNA (0.5 μg) was added to the RT reaction mixture in a final volume of 25 μl using the RevertAid RT Reverse Transcription kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was used for qPCR using SYBRGreen SuperMix (Thermo Fisher Scientific, Inc.) on a ABI7500 device (Applied Biosystems; Thermo Fisher Scientific, Inc.). For each cycle: 10 sec at 95˚C for denaturation, 45 sec at 60˚C for annealing and extension, repeat 35 cycles. The primer pairs used for amplification were as follows: ERα forward, 5'-CCCACTCAACAGCGTGTCTC-3' and reverse, 5'-CGTCGATTATCTGAATTTGGCCT-3'; Oct4 forward, 5'-CTGGGTTGATCCTCGGACCT-3' and reverse, 5'-CCATCGGAGTTGCTCTCCA-3'; Sox2 forward, 5'-GCC GAGTGGAAACTTTTGTCG-3' and reverse, 5'-GGCAGC GTGTACTTATCCTTCT-3'; Klf4 forward, 5'-CCCACATGA AGCGACTTCCC-3' and reverse, 5'-CAGGTCCAGGAGATC GTTGAA-3'; c-Myc forward, 5'-GGCTCCTGGCAAAAG GTCA-3' and reverse, 5'-CTGCGTAGTTGTGCTGATGT-3'. For data analysis, the DDCq method was used (14). All experiments were performed three times.

Cell counting Kit-8 (CCK-8) assay. The proliferation of CSCs was measured using CCK-8 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocol. CSC spheres were signalized using TrypLE™ Express (Life Technologies, Grand Island, NY, USA) and a total amount of $5x10^3$ CSCs were seeded and incubated in 96-well plates for 24 h. Subsequently, the 0, 1, 10 or 50 nM of E2 (Sigma-Aldrich; Merck KGaA) was added and co-incubated with the CSCs for 1-5 days at 37˚C in 5% CO_2 incubator. Each day, 10 μl CCK-8 reagent was added to each well and incubated for 4 h at 37˚C. Absorbance was then measured at 450 nm. All experiments were performed in triplicate and repeated at least twice.

Caspase-3/7 activity assay. Target cells were seeded in 96-well plates at a concentration of $5x10^3$ cells/ml. Following exposure to 0, 1, 10 or 50 nM E2 for 24 h at 37˚C, caspase-3/7 activity was analyzed using Caspase-Glu™3/7 assay kit (Promega Corporation, Madison, WI, USA) by following the manufacturer's protocol. Briefly, Caspase-Glu™ reagents were added and incubated with cells for 1 h at 37˚C and the absorbance at a wavelength at 520-530 nm was determined using a microplate reader (Synergy 2 Multi-Mode Microplate Reader; BioTek Instruments, Inc., Winooski, VT, USA).

Immunofluorescence analysis. Cells were fixed in 4% paraformaldehyde for 15 min at room temperature, and incubated with PBS supplemented with 0.1% Triton X-100 for 10 min. Permeabilized cells were blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) and incubated with antibody against ERα (cat. no. ab32063; Abcam, Cambridge, UK) at a dilution of 1:2,000 overnight at 4˚C. Cells were washed and further stained with Alex Fluor® 594-conjugated goat anti-mouse secondary antibodies (cat. no. R37121; Life Technologies, Grand Island, NY, USA) at a dilution of 1:1,000 for 2 h in darkness. Following washing with PBS, cells were imaged under a X71 (U-RFL-T) fluorescence microscope (Olympus Corporation, Tokyo, Japan) at a magnification of x400.

Western blot. Cells were pelleted and washed three times with PBS and resuspended with lysis buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P40 (NP-40), and 0.25% sodium deoxycholate). Lysate was centrifuged for 5 min at 12,000 x g, 4˚C to remove cell debris. The supernatant was removed into a fresh tube before sample buffer was added (Guangzhou RiboBio Co., Ltd., Guangzhou, China). Following incubation at 100˚C for 10 min, samples were separated SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA), which were pre-treated with PBS containing 5% BSA and 0.3% Tween 20. Membranes were probed with antibodies against human ERα (Cat. No.: ab32063), activated caspase-3 (Cat. No.: ab2302), β-actin (Cat. No.: ab8226), GAPDH (Cat. No.: ab8245) which were bought from Abcam (Cambridge, UK) at dilution of 1:1,000. The signals were visualized using a enhanced chemiluminescence substrate (Supersignal West Femto Luminal/Enhancer Solution; Thermo Fisher Scientific, Inc.) and blotted on X-ray films in a dark room. To quantify the western blots, ImageJ software (Version. 1.48a; National Institutes of Health, Bethesda, MD, USA) was used to quantitatively measure the bands and normalized using β-actin.

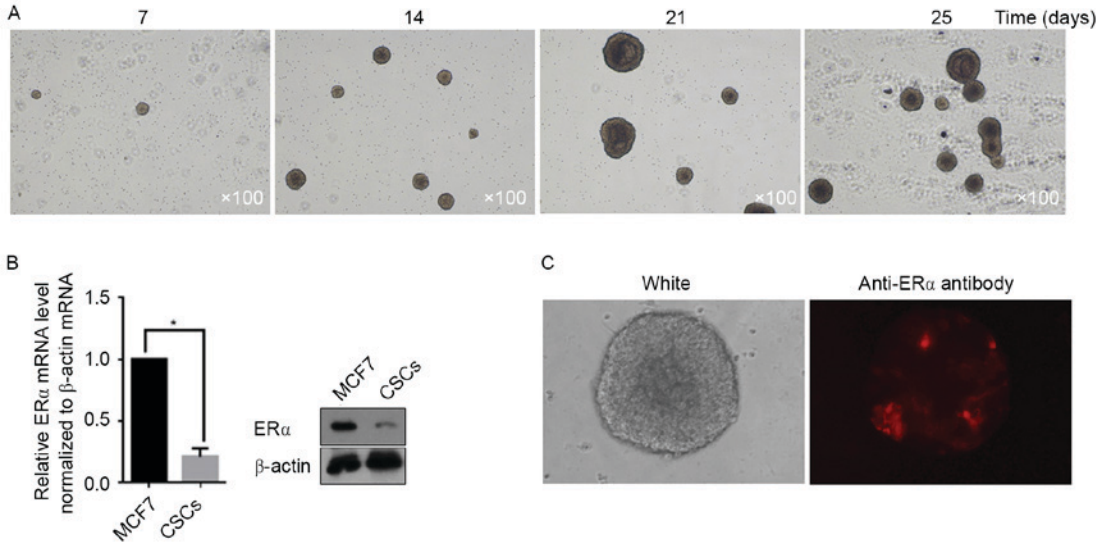

Figure 1. Isolation of CSCs from MCF7 cells and the detection of ERα expression on the sphere. (A) Following culture in sphere-forming medium, images of sphere formation were captured at days 7, 14, 21 and 25. (B) The reverse transcription-quantitative polymerase chain reaction was performed to detect the mRNA level of ERα (left panel) and a semi-quantitative western blot was performed to detect the protein level of ERα (right panel). (C) Immunofluorescence staining of ERα was used to identify the ERα-positive subpopulation in CSCs. *P<0.05, with comparisons indicated by lines. CSCs, cancer stem-like cells; ERα, estrogen receptor α.

Transwell migration assay. The migration of CSCs was quantified using a Transwell assay (EMD Millipore, Billerica, MA, USA). Cells ($1x10^4$) were suspended with RPMI-1640 medium containing 0, 1 or 10 nM E2 and seeded onto the surface of the upper chamber. RPMI-1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA) was added to the lower well. The plates were incubated for 24 h at 37˚C and, subsequently, migrated cells were stained with 0.5% crystal violet at room temperature for 30 min followed by three washes with PBS and imaged under a X71 (U-RFL-T) fluorescence microscope (Olympus Corporation) at a magnification of x200.

Self-renewal capacity assay. A total of $2x10^3$ signalized CSCs were plated into 24-well plates. The cells were cultured in the SFM in the presence of 0, 1 or 10 nM E2 for 7-15 days. The spheres >40 μm in diameter were counted under an X71 (U-RFL-T) fluorescence microscope (Olympus Corporation) at a magnification of x40.

Statistical analysis. Data are presented as the mean ± standard error of the mean. The data were evaluated statistically using one-way analysis of variance followed by the Tukey test for paired observations. The two-tailed Student's t-test was used to compare two groups. P<0.05 was considered to indicate a statistically significant difference. All experiments were performed at least three times independently.

Results

CSCs derived from MCF7 express a lower ERα level compared with MCF7 cells. To isolate the CSC subpopulation from MCF7 cells, $1x10^6$ MCF7 cells were incubated in Dulbecco's modified Eagle's medium/Ham's F12 supplemented with B27, bFGF and EGF for 25 days. Images were taken at days 7, 14, 21 and 25. As presented in Fig. 1A, the CSC spheres rapidly increased in size. Owing to the presence of ERα on the surface of MCF7 cells, the potential for CSCs derived from MCF7 to express ERα was examined. According to the RT-qPCR and semi-quantitative western blot assays, the total amount of mRNA and protein from ERα in CSCs decreased markedly when compared with that in MCF7 cells (Fig. 1B). In order to determine whether the decrease in ERα mRNA and protein levels occurred in each CSC, immunofluorescent staining was utilized to demonstrate the ERα-positive cells in spheres. As presented in Fig. 1C, a small section of ERα-positive CSCs in the sphere was detectable, whereas further cells exhibited an ERα-negative status.

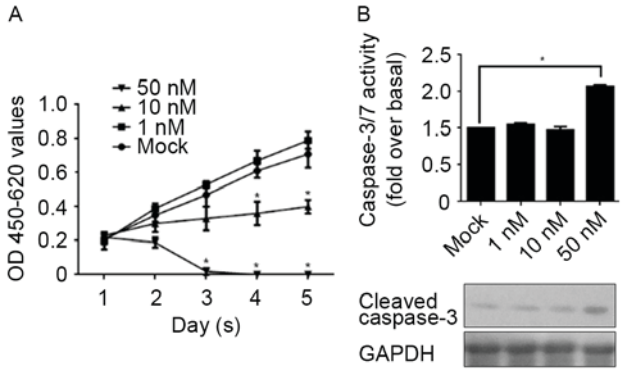

Figure 2. Identifying the effects of E2 treatment on cell proliferation and apoptosis. (A) A Cell Counting Kit-8 assay was performed to demonstrate the effect of 1, 10 and 50 nM E2 treatment on the proliferation of CSCs. (B) Caspase-3/7 activity was assayed (upper panel) and the cleaved form of caspase-3 was detected using a western blot. *P<0.05, with comparisons indicated by lines. E2, 17β-estradiol.

E2 treatment regulates the proliferation and apoptosis of CSCs in a dose-dependent manner. It has been reported that the effect of E2 treatment varies depending on the concentration. Low

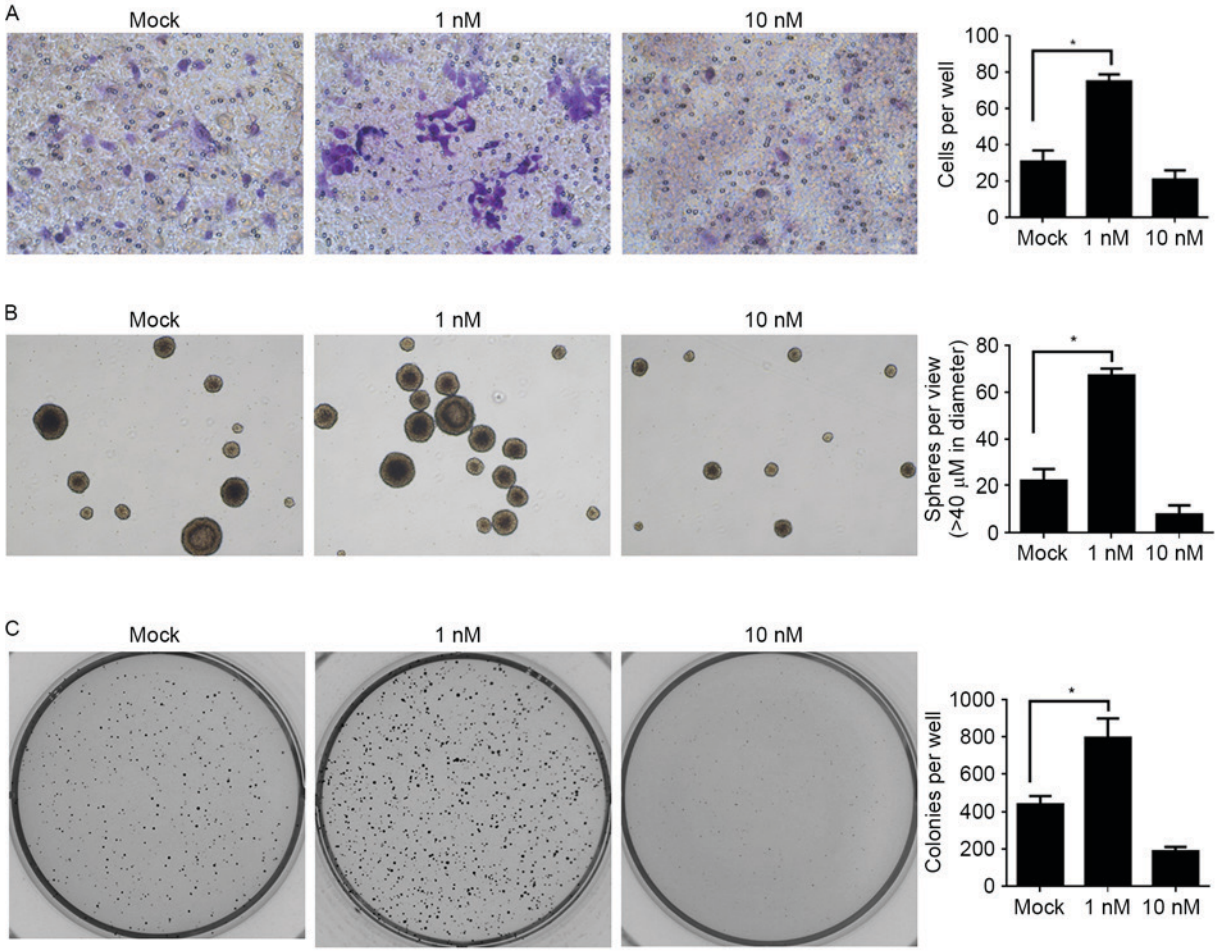

Figure 3. The effects of E2 treatment on CSC migration, self-renewal capacity and colony formation. (A) A Transwell assay without Matrigel was used for migration analysis. (B) Sphere formation was performed in sphere-forming medium for 14-20 days. (C) Colony formation in soft agar was performed. *P<0.05, with comparisons indicated by lines. E2, 17β-estradiol; CSCs, cancer stem-like cells.

doses or high doses of E2 treatment have opposing effects on cell proliferation. This indicates that, considering the decrease in ERα in CSCs, E2 treatment results in differential effects on CSCs according to the concentration. CSCs were treated with 1, 10 and 50 nM E2 for 1-5 days and assessed using a CCK-8 assay for cell proliferation. In Fig. 2A, 1 nM E2 treatment was demonstrated to present no detectable effect on cell proliferation, whereas 10 nM E2 treatment markedly decreased cell proliferation. Notably, 50 nM E2 treatment directly eliminated all cells, meaning that this concentration of E2 treatment is fatal to CSCs. In order to identify whether the elimination of CSCs following 50 nM E2 treatment was due to the induction of apoptosis, the activity of caspase-3/7 and the cleaved form of caspase-3 were detected separately. As expected, the results demonstrated that 50 nM E2 treatment increased the activity of caspase-3/7, accompanied by the increase in the levels of the cleaved form of caspase-3 (Fig. 2B).

E2 treatment affects migration, self-renewal capacity and colony formation, potentially due to the regulation of Sox2. In order to further investigate the effects of E2 treatment on the physiological processes of CSCs, the effects on migration, self-renewal capacity and colony formation were assessed. Taking into consideration the fatal effect of the 50 nM E2 treatment, Mock, 1 and 10 nM E2 treatments were employed

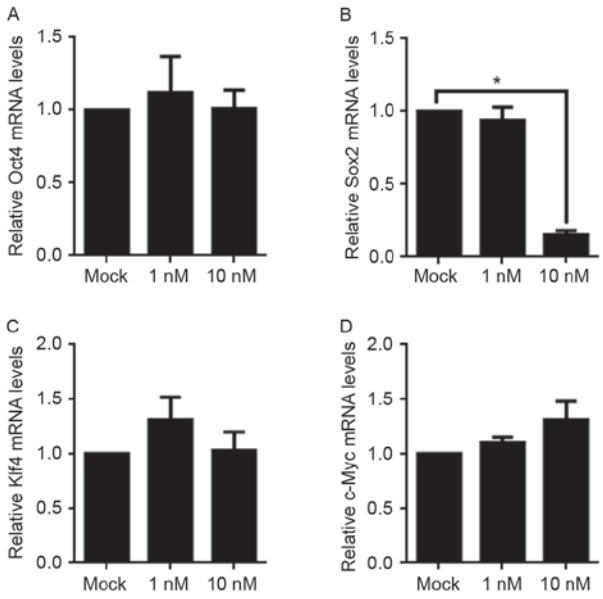

Figure 4. Reverse transcription-quantitative polymerase chain reaction analysis for the stem-maintaining factors following E2 treatment. Following the response to E2 treatment, the mRNA levels of Oct4 (A), Sox2 (B), Klf4 (C) and c-Myc (D) were detected. *P<0.05 vs. the Mock group, as indicated by lines. E2, 17β-estradiol; Oct4, octamer-binding transcription factor 4; Sox2, sex-determining region Y-box 2; Klf4, Krüppel-like factor 4; c-Myc, MYC proto-oncogene.

for the following assays: For the migration assay, a Transwell assay without Matrigel coating was used. Compared with the Mock group, the 1 nM E2-treated group promoted the migration of CSCs and, in contrast, the 10 nM E2 treatment inhibited the migration of CSCs, but the difference was not significant (Fig. 3A). The assays for self-renewal capacity and colony formation revealed similar tendencies: When compared with the Mock group, the lower dose of E2 treatment (1 nM) significantly promoted these processes and the higher dose of E2 treatment (10 nM) inhibited these processes (Fig. 3B and C).

Owing to the fact that Oct4, Sox2, Klf4 and c-Myc serve critical functions in maintaining cell stemness, the aforementioned result which revealed the decreased stemness following 10 nM E2 treatment prompted an interest in detecting the changes of the mRNA levels of these four factors. Using RT-qPCR, although Oct4, Klf4, and c-Myc levels were not altered, the mRNA level of Sox2 was significantly decreased. This indicated that a decrease in Sox2 mRNA expression may be the potential underlying molecular mechanism for the loss of stemness following E2 treatment (Fig. 4).

Discussion

E2 is believed to regulate the physiological processes of normal breast cells or breast cancer cells, depending on the presence of ER (15,16). This is supported by the fact that ERα is frequently highly expressed in ER-positive breast cancer cells, and thus regulates the cell cycle in these cells, indicating that E2-ERα signaling serves a critical function in cell proliferation (17,18). Notably, Zhao *et al* (11) reported that E2 also performs a regulatory function on breast cancer cells independent of ERα. Consistently, in their results, a low level of E2 (1 nM) was demonstrated to affect the proliferation of ER-positive MCF7 breast cancer cells, but not that of ER-negative MB231 breast cancer cells. However, a high dose of E2 (50-100 nM) markedly blocked proliferation and induced apoptosis in these two types of breast cancer cell.

In the present study, the difference in ERα expression between the CSCs derived from MCF7 cells and original MCF7 cells, and the effects of E2 treatment at a range of concentrations, were investigated. Initially, CSCs were obtained using a serum-free maintenance system and a confirmatory assay for their self-renewal capacity was performed (Fig. 1A). RT-qPCR and semi-quantitative western blot assays demonstrated that the mRNA and protein levels of ERα in MCF7 cells were significantly increased compared with that in CSCs derived from MCF7 cells (Fig. 1B). Notably, the fluorescent staining of ERα in the CSC sphere demonstrated that a small proportion of the CSCs presented an ERα-positive signal, indicating the existence of two subpopulations of CSCs: An ERα-positive subpopulation and an ERα-negative subpopulation (Fig. 1C). These two subpopulations of CSCs may be derived from two separate subpopulations of MCF7, or may be derived from the same population and subsequently differentiated into two subpopulations.

In the present study, the effects of different concentrations of E2 on a mixture of ERα-positive and ERα-negative CSCs were tested due to the failure to separate these two subpopulations (data not shown). Owing to the unknown ratio of ERα-positive and ERα-negative CSCs, no detectable promotion of proliferation was observed following 1 nM E2 treatment and, as expected, 10 nM E2 treatment resulted in inhibition of proliferation, and 50 nM E2 treatment directly eliminated cell viability in 48 h by inducing apoptosis (Fig. 2A and B). Despite the lack of clarity regarding whether low doses of E2 treatment promote the proliferation of CSC in these two subpopulations, it was confirmed that a high dose of E2 treatment is fatal for them, independent of the existence of ERα.

Because of the fatal effects of 50 nM E2 treatment, 10 nM E2 treatment was employed for further investigation of migration, self-renewal capacity and colony formation. It was observed that 10 nM E2 treatment universally inhibited these processes, whereas 1 nM E2 treatment had an opposing effect on them (Fig. 3).

The decreased maintenance of stemness of CSCs prompted an interest in detecting the expressional changes of Oct4, Sox2, Klf4 and c-Myc, which are critical for stemness maintenance (19), and it was revealed that the Sox2 mRNA level was significantly decreased by 50 nM E2 treatment (Fig. 4).

In summary, the results of the present study confirmed the regulatory effects of different concentration of E2 treatment on CSCs derived from MCF7. It was identified that there were two subpopulation of CSCs derived from MCF7 and this may have resulted in the differential effects following E2 treatment. In addition, a high dose of E2 treatment may inhibit the malignancy of CSCs by decreasing their stemness through the downregulation of the Sox2 expression level.

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