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Estradiol, TGF-β1 and hypoxia promote breast cancer stemness and EMT-mediated breast cancer migration

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Abstract. Breast cancer is one of the most common cancer types among women, acting as a distinct cause of mortality, and has a high incidence of recurrence. External stimuli, including 17β-estradiol (E2), transforming growth factor (TGF)-β1 and hypoxia, may be important in breast cancer growth and metastasis. However, the effects of these stimuli on breast cancer stem cell (CSC) regulation have not been fully investigated. In the present study, the proportion of cluster of differentiation (CD)44^{+}/CD24$^{-/low}$ cells increased following treatment with E2, TGF-β1 and hypoxia in MCF-7 cells. The expression of CSC markers, including SOX2, KLF4 and ABCG2, was upregulated continually by E2, TGF-β1 and hypoxia. In addition, the expression levels of epithelial-mesenchymal transition-associated factors increased following treatment with E2, TGF-β1 and hypoxia. Therefore, the migration ability of E2-, TGF-β1- and hypoxia-treated MCF-7 cells was enhanced compared with control cells. In addition, the enhancement of apoptosis by 5-flurouracil or radiation was abolished following treatment with E2, TGF-β1 and hypoxia. These results indicate that E2, TGF-β1 and hypoxia are important for regulating breast CSCs, and that the modulation of the microenvironment in tumors may improve the efficiency of breast cancer therapy.

Introduction

Breast cancer is one of the most common cancer types among women, acting as a distinct cause of mortality, and has a high incidence of recurrence (1). Consequently, numerous studies have focused on the development of breast cancer therapies, including surgery, chemotherapy and radiotherapy (2-4). The cancer stem cell (CSC) hypothesis proposes that the preferential targets of oncogenic transformation are tissue stem cells or progenitor cells that are able to self-renew (5-8). Therefore, numerous studies have developed CSC phenotypic assays in order to identify CSCs (9-11). Breast CSCs have been characterized as cluster of differentiation (CD)44^{+}/CD24$^{-/low}$ cells that initiate carcinogenesis in NOD/SCID mice (12).

Hypoxia is involved in various tumors and often occurs when tumor growth surpasses blood supply (13). In particular, breast cancers are sensitive to hypoxia, as they outgrow nutrients or oxygen vascular supplies (14). In addition, hypoxia often causes chemo- and radiotherapy resistance, and contributes to tumor metastasis (15). Although the effect of hypoxia in patients with breast cancer has been widely reported, the regulatory mechanism of hypoxia in breast CSC and therapeutic resistance remain unknown (16-18).

17β-estradiol (E2) is the most effective female estrogen hormone and is pivotal in male and female physiology (19). Breast cancer is susceptible to estrogen hormones. Estrogen hormones promote the development and progression of breast cancer, and induce the invasion and metastasis of breast cancer cells that express estrogen receptors (ERs) to distant organs or lymph nodes (20). E2 also enhances the movement and invasion of breast cancer cells (21). However, due to the complexity of ER-triggered estrogen signaling, the effects of estrogen hormones on cancer are occasionally divergent.

The anti-inflammatory cytokine transforming growth factor (TGF)-β1 is associated with embryonic development and homeostasis in adult organisms (22,23). TGF-β1 is critical for angiogenesis, immunoregulation and cancer progression (24). In addition, TGF-β1 acts as a tumor suppressor in the early stages of breast carcinoma, and is involved in the progression of tumors by resisting inhibited cell growth during the later stages of disease (25). TGF-β1 also enhances breast cancer metastasis by inducing Smad family member 2 (Smad2) (26). However, the regulatory mechanisms of breast CSCs following treatment with E2 or TGF-β1 have not been investigated.

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In the present study, treatment with E2, TGF-β1 and hypoxia led to breast CSC ($CD44^+/CD24^{-/low}$) expansion. CSC markers and epithelial-mesenchymal transition (EMT)-associated factors were expressed in order to investigate the underlying mechanisms. Additionally, the effects of E2, TGF-β1 and hypoxia on cell migration and drug and radiation resistance were determined. The results indicate that E2, TGF-β1 and hypoxia are important for the regulation of breast CSCs, and that the modulation of the tumor microenvironment may improve the efficiency of breast cancer therapy.

Materials and methods

Cell culture. The human breast cancer MCF-7 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured at 37˚C in 20% O_2 and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Welgene, Daegu, South Korea) that contained 10% HyClone fetal bovine serum (FBS; GE Healthcare Life Sciences, Logan, UT, USA) and 1% Gibco antibiotic-antimycotic (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cells were incubated in a chamber containing a 5% CO_2 and 1% O_2 atmosphere for 48, 72 and 96 h and 1 week to create hypoxic conditions. The cells were treated with 10 nM E2 or 1 ng/ml TGF-β1 (R&D Systems Inc., Minneapolis, MN, USA).

Immunoblot analysis. The MCF-7 cells ($2x10^6$) were collected using 5 ml cold phosphate buffer solution (PBS; Welgene) and lysed in 100 µl lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2 ethylenediaminetetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin and 1 mM phenylmethanesulfonylfluoride (all Sigma-Aldrich, St. Louis, MO, USA). Following 1 hour of incubation on ice, samples were centrifuged at 13,000 x g for 20 min at 4˚C. Subsequently, the supernatant was removed and quantified using the Bio-Rad Protein Assay kit II (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturers's protocol. A total of 50 µg of total protein was loaded and electrophoresed on sodium dodecyl sulfate-polyacrylamide gels (Bio-Rad Laboratories, Inc.) and transferred to polyvinylidene difluoride membranes (GE Healthcare Life Sciences, Chalfont, UK). The transferred membranes were blocked using 5% milk (BD Biosciences, San Jose, CA, USA) dissolved in Tris-buffered saline (20 mM Tris; 137 mM NaCl; pH 7.6; Sigma-Aldrich) containing 0.02% Tween 20 (Sigma-Aldrich), and incubated overnight at 4˚C with specific primary antibodies. The membranes were subsequently incubated with specific horseradish peroxidase-conjugated secondary antibodies (detailed below). The blots were developed with the Super Signal Chemiluminescence reagent (Pierce Biotechnology, Inc., Rockford, IL, USA) by enhanced chemiluminescence (Thermo Fisher Scientific, Inc.).

Immunoblot antibodies. The following mouse monoclonal anti-human primary antibodies (1 µg; dilution, 1:1,000) were utilized for immunoblot analysis: Anti-CD44 (catalog no., ab78960), anti-CD24 (catalog no., ab76514), anti-ATP-binding cassette sub-family G member 2 (ABCG2; catalog no., ab130244), anti-JMJD1A (catalog no., ab107234) and anti-Kruppel-like factor 4 (KLF4; catalog no., ab75486) (all purchased from Abcam, Cambridge, MA, USA); epithelial cell adhesion molecule (EpCAM; catalog no., 2929) antibody was from Cell Signaling Technology, Inc. (Danvers, MA, USA); the cytokeratin 5/8 [mouse anti-human immunoglobulin G (IgG)]; catalog no., 550505), SOX2 (mouse anti-human IgG; catalog no., 561469) and β-catenin (mouse anti-human IgG; catalog no., 610154) antibodies were from BD Biosciences; the c-Myc (catalog no., sc-40) and E- and N-cadherin (catalog nos., sc-71008 and sc-271386, respectively) antibodies were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA); the hypoxia inducing factor (HIF)-1α (catalog no., NB100-105) antibody was from Novus Biologicals, LLC (Littleton, CO, USA); and the β-actin (catalog no., A-5441) antibody was from Sigma-Aldrich. The 3 µg of goat anti-mouse polyclonal secondary antibody (catalog no. 62-6520) was from Thermo Fisher Scientific, Inc. and was used at a dilution of 1:4,000.

Flow cytometry. MCF-7 cells ($2x10^6$) were washed twice using PBS and incubated with 2 µg/ml monoclonal mouse anti-human CD24-fluorescein isothiocyanate (FITC; catalog no., 555427) and monoclonal mouse anti-human CD44-allophycocyanin (APC; catalog no., 559250) antibodies (BD Biosciences; dilution, 1:100) in the dark on ice for 1 h, and washed twice using cold PBS. The labeled cells were analyzed using the fluorescence-activated cell sorting FACSAria cell sorter (BD Biosciences).

Migration assay. An *in vitro* wound-healing assay was used to assess two-dimensional cell motility. The MCF-7 cells ($2x10^6$) were treated for 96 h with E2 or TGF-β1 and hypoxia in 6-well plates. Then, a scratch was made on the cell layer with a micropipette tip, and the cultures were washed twice with serum-free medium to remove the floating cells. The cells were incubated in a chamber containing an atmosphere of 20% O_2 or in hypoxic conditions (5% CO_2 and 1% O_2) in DMEM medium at 37˚C. Wound healing was visualized by comparing photographs 48 h later using the Qimaging QI Click Camera system mounted on a phase-contrast Nikon microscope (TS100; Nikon, Inc., Tokyo, Japan). For the Transwell assay, MCF-7 cells ($2x10^4$) were seeded onto 8-µm Transwell-inserts (Costar brand; Corning Life Sciences, Tewksbury, MA, USA). The lower chambers were filled with DMEM containing 10% FBS. Migrated cells were stained with crystal violet and counted using the Image-Pro Plus 7.0 software (Media Cybernetics, Rockville, MD, USA) 24 h later.

Apoptosis assay. Cell apoptosis was assessed using the Annexin V/phycoerythrin (PE) Apoptosis Detection kit (BD Biosciences; catalog no. 559763). Briefly, the MCF-7 cells ($1x10^6$) were seeded in 100-mm dishes and incubated overnight. Then, the cells were treated with 5-fluorouracil (100 µg/ml) or radiation (10 Gy) for 48 or 72 h, respectively. Subsequently, the cells were washed twice with 5 ml PBS and stained with 2.5 µg/ml Annexin V/PE-conjugate and 5 µl 7-aminoactinomycin for 15 min on ice in the dark. Subsequent to staining, the cells were analyzed using the FACSAria cell sorter.

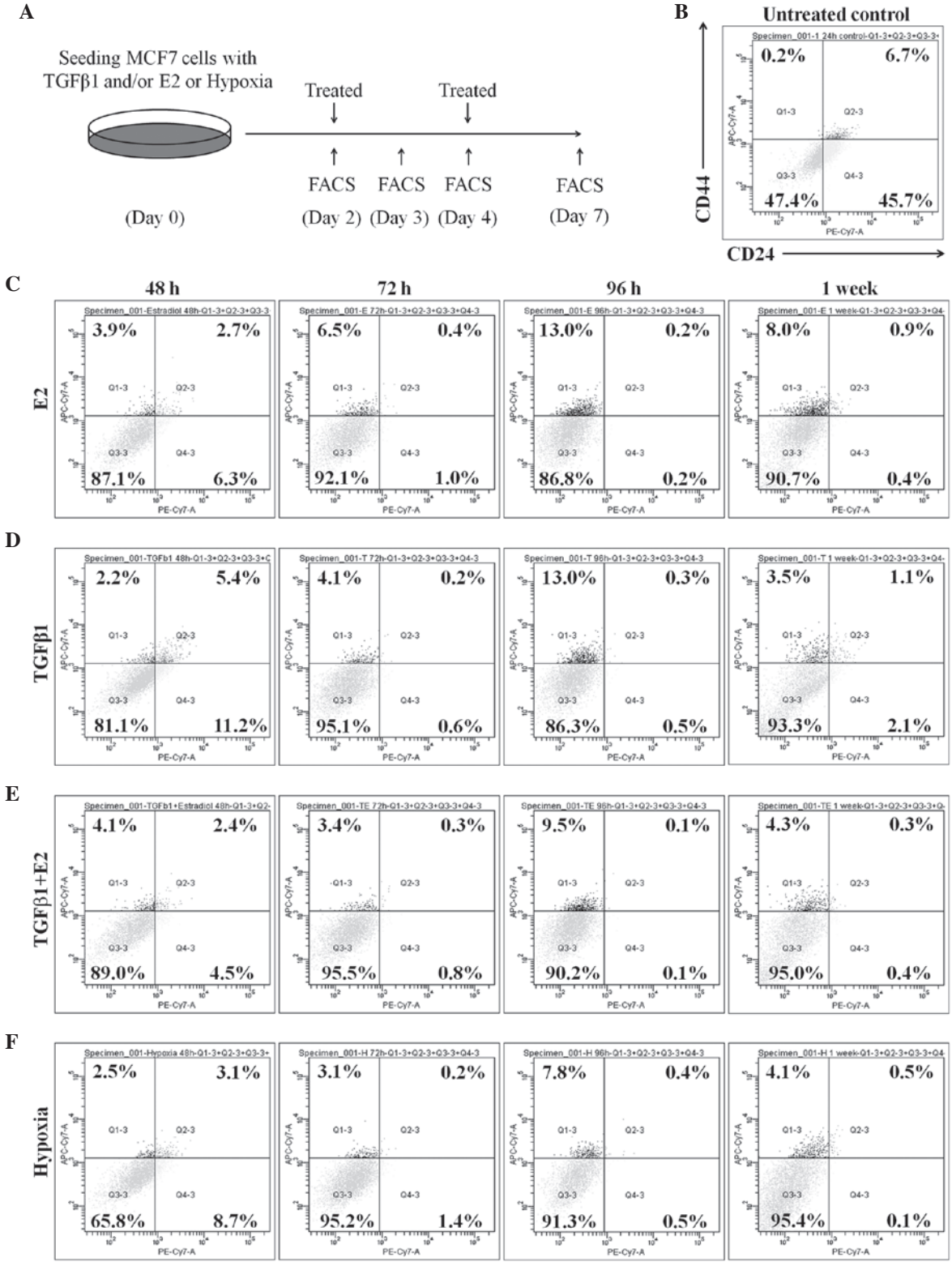

Figure 1. E2, TGF-β1 and hypoxia treatment increased the $CD44^{+}/CD24^{-/low}$ population of MCF-7 cells. (A) Schematic representation of the experimental procedure. (B-F) Representative FACS stained images. The FACS analysis was performed using specific cell-surface markers for basal (CD44-allophycocyanin) and luminal (CD24-phycoerythrin) epithelial cells, and the percentage of $CD44^{+}/CD24^{-/low}$ cells in each condition was assessed. The ratio of $CD44^{+}/CD24^{-/low}$ cells in E2 (10 nM), TGF-β1 (1 ng/ml) and hypoxia (1% O_2) treated MCF-7 cells is indicated. E2, estradiol; TGF-β1, transforming growth factor-β1; CD, cluster of differentiation; FACS, fluorescence-activated cell sorting.

Statistical analysis. Statistical analyses were performed using Excel (Microsoft Corporation, Redmond, WA, USA). A Student's *t*-test was used to make statistical comparisons and $P<0.05$ was considered to indicate a significant difference.

Results

Effect of E2, TGF-β1 and hypoxia on breast CSC expansion. The percentage of $CD44^{+}/CD24^{-/low}$ cells is considered to be

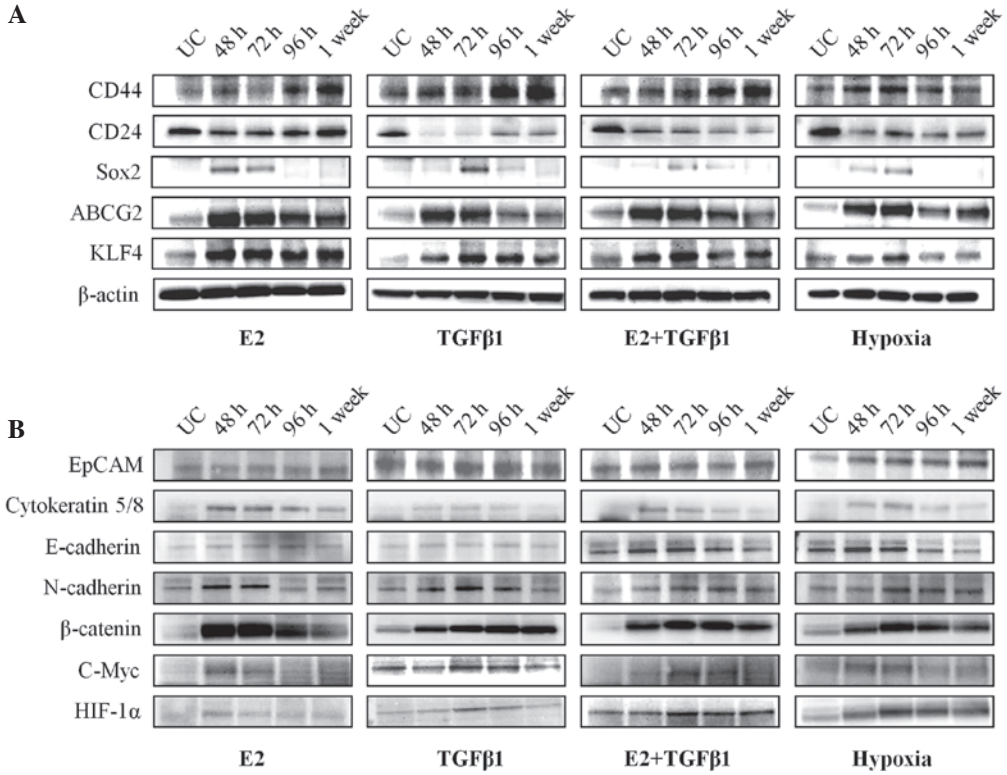

Figure 2. Patterns of cancer stem cell and epithelial-mesenchymal transition-associated marker expression in E2, TGF-β1 and hypoxia-treated MCF-7 cells. (A) The expression of CD44, CD24, Sox2, ABCG2 and KLF4 was determined in E2, TGF-β1, combined E2/TGF-β1 and hypoxia-treated MCF-7 cells by western blotting. β-actin was used as a loading control. Western blotting was performed at each time point (48, 72 and 96 h and 1 week). (B) EpCAM, cytokeratin 5/8, E-cadherin, N-cadherin, β-catenin, c-Myc and HIF-1α expression was determined by western blotting using same conditions that are indicated in Fig. 2A. E2, estradiol; TGF-β1, transforming growth factor-β1; CD, cluster of differentiation; Sox2, sex determining region Y-box 2; ABCG2, ATP-binding cassette sub-family G member 2; KLF4, Kruppel-like factor 4; EpCAM, epithelial cell adhesion molecule; HIF, hypoxia-inducible factor.

the breast CSC subpopulation. This percentage was assessed in MCF-7 cells, in the presence or absence of E2 and TGF-β1, in order to investigate whether E2 or TGF-β1 treatment affects the size of the CSC population. ER^+ MCF-7 cells were treated with 10 nM E2, 1 ng/ml TGF-β1 or a combination of the two for 48, 72 and 96 h, and 1 week. Following treatment, the proportion of stem-like cells was evaluated using flow cytometry (Fig. 1A). The results indicated that the proportion of $CD44^+/CD24^{-/low}$ cells was significantly expanded in E2-treated MCF-7 cells (48 h, 2.2%; 72 h, 4.1%; 96 h, 13%; 1 week, 1.1%) compared with untreated control cells (0.2%; Fig. 1B and C). TGF-β1-treated MCF-7 cells also demonstrated a notable increase in the percentage of $CD44^+/CD24^{-/low}$ cells at 48 (3.9%), 72 (6.5%) and 96 h (13.0%), and 1 week (8.0%) subsequent to treatment with TGF-β1 (Fig. 1D). However, no synergistic induction of the $CD44^+/CD24^{-/low}$ population by the TGF-β1/E2 combined treatment was observed (Fig. 1E). The effect of hypoxia on breast CSC expansion was also examined. The results show that the proportion of $CD44^+/CD24^{-/low}$ cells increased in hypoxia-treated MCF-7 cells (48 h, 2.5%; 72 h, 3.1%; 96 h, 7.8%; 1 week, 4.1%) compared with the proportion in normoxic conditions (Fig. 1B and F). These results indicate that treatment with E2, TGF-β1 and hypoxia in isolation expanded the CSC population of MCF-7 cells, but that the combined treatment had no synergistic effect.

Effect of E2, TGF-β1 and hypoxia on the expression of breast CSC markers and EMT-associated factors. CSC marker expression in E2-, TGF-β1- or hypoxia-treated MCF-7 cells was examined, as the E2, TGF-β1 and hypoxia treatment increased the CSC population in MCF-7 cells. MCF-7 cells were treated and incubated under the same conditions exhibited in Fig. 1A, and the CD44, CD24, SOX2, ABCG2 and KLF4 protein levels were measured using western blot analysis (Fig. 2A). Consistent with the flow cytometry results, CD44 expression increased in E2-, TGF-β1- or hypoxia-treated MCF-7 cells. However, CD24 expression decreased following E2, TGF-β1 and hypoxia treatment. Notably, the expression of the pluripotency-associated proteins SOX2 and KLF4 and the putative CSC marker ABCG2 increased when the cells were treated with E2, TGF-β1 and hypoxia. These results indicate that SOX2, KLF4 and ABCG2, which are associated with breast cancer stemness, were actively regulated by the E2, TGF-β1, and hypoxia treatments.

Several studies have demonstrated that CSCs and EMT-phenotypic cells have a tumor aggressiveness phenotype (27-29). Therefore, the present study investigated whether treatment with E2, TGF-β1 and hypoxia affects the expression of EMT-associated factors. The results demonstrate that EpCAM and E-cadherin expression was not affected by E2- or TGF-β1 stimulation (Fig. 2B). However, expression of the EMT markers cytokeratin 5/8 and N-cadherin, which increase cell motility, clearly increased following treatment with E2 or TGF-β1. In addition, the levels of β-catenin and the target gene c-Myc were upregulated in E2 and TGF-β1-treated MCF-7 cells compared with untreated control cells. EpCAM

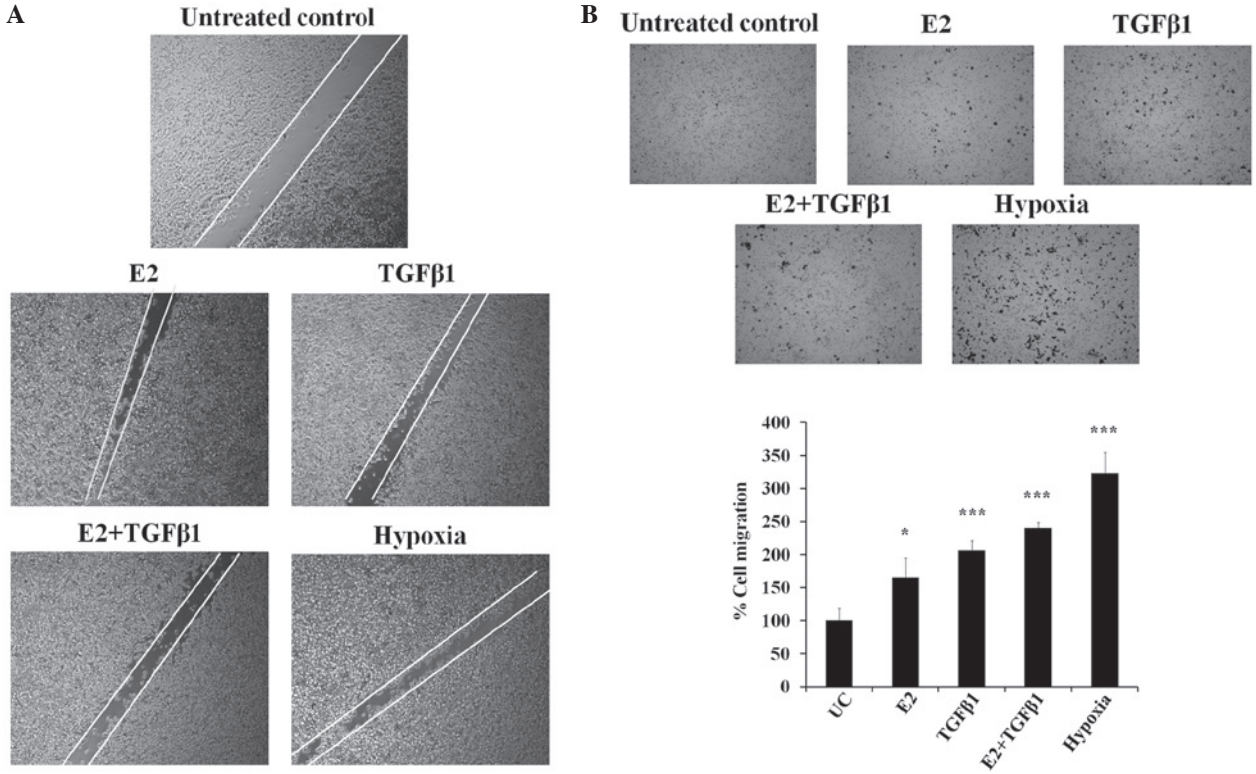

Figure 3. E2, TGF-β1 and hypoxia treatment enhances cell motility. (A) Representative images of the induction of MCF-7 cell migration by treatment with E2 (10 nM), TGF-β1 (1 ng/ml), combined E2 (10 nM)/TGF-β1 (1 ng/ml) and hypoxia (1% O_2). (B) Migration of MCF-7 cells treated with E2, TGF-β1, combined E2/TGF-β1 and hypoxia was evaluated using Transwell polystyrene membranes with 8-mm pores. The number of migrating cells was counted and scored in relative units. P-values were calculated using Student's *t*-test. *P<0.05, ***P<0.001. E2, estradiol; TGF-β1, transforming growth factor-β1.

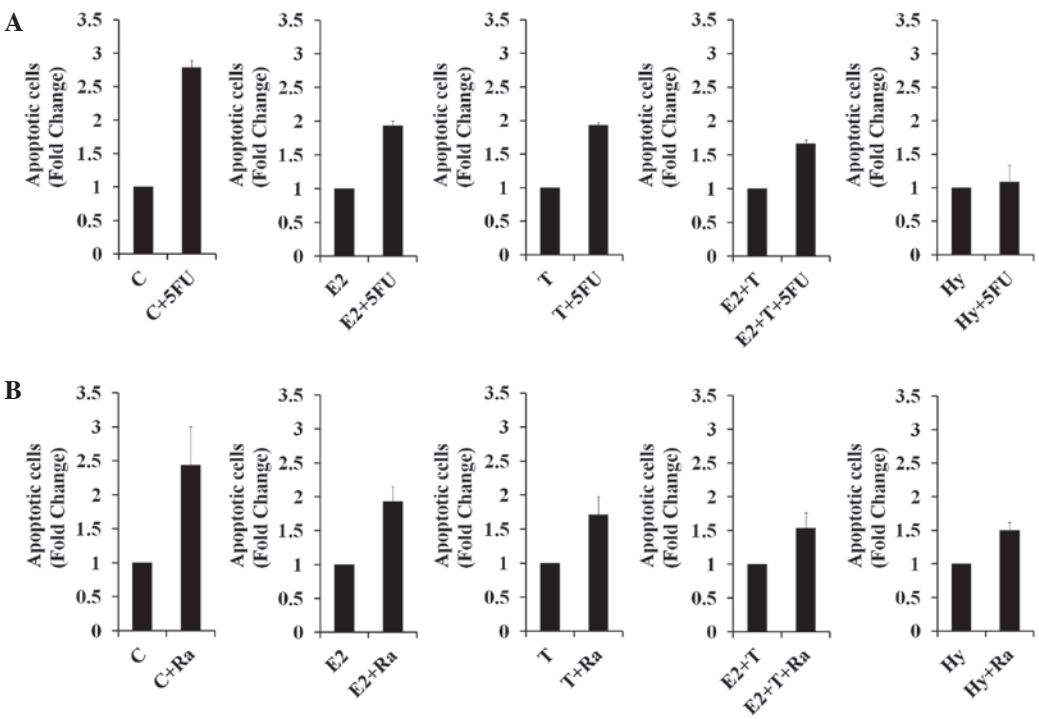

Figure 4. E2, TGF-β1, and hypoxia treatment increases chemo- and radioresistance. Each set of MCF-7 cells was treated with (A) 100 μg/ml 5-FU or (B) 10 Gy radiation to induce apoptosis. Then, apoptosis was analyzed with an Annexin V kit. E2, estradiol; TGF-β1, transforming growth factor-β1; 5-FU, fluorouracil.

expression increased, whereas E-cadherin expression decreased, in hypoxia-treated MCF-7 cells (Fig. 2B). Cytokeratin 5/8, N-cadherin, β-catenin and c-Myc levels increased under hypoxic conditions. These results indicate that the CSC expansion mediated by E2, TGF-β1 and hypoxia may promote EMT by regulating the expression of EMT-associated factors.

Enhancement of cell motility by E2, TGF-β1 and hypoxia. The increased number of CSCs and progression of the EMT are associated with cell motility and invasion. A wound-healing assay with the E2, TGF-β1 and hypoxia treatments was performed to visualize the effect on MCF-7 cell motility (Fig. 3A). E2 and TGF-β1-treated cells demonstrated increased cell migration compared with control cells. The hypoxia treatment also significantly enhanced cellular migration ability. Similarly, the Transwell assay results indicated that E2-, TGF-β1- and hypoxia-treated MCF-7 cells migrated more compared with control MCF-7 cells (Fig. 3B). The data indicate that treatment with TGF-β1, E2 and hypoxia induces migration ability in MCF-7 cells.

Effect of E2, TGF-β1 and hypoxia on drug and radioresistance. Fluorouracil (5-FU) is a chemotherapeutic agent used to treat various types of cancer, including breast cancer (30). In the present study, ABCG2 expression was induced by E2, TGF-β1 and hypoxia treatments (Fig. 2A). The effect of E2, TGF-β1 and hypoxia on 5-FU-induced-apoptosis was investigated as ABCG2 mediates multidrug resistance. The results showed that E2-, TGF-β- and hypoxia-treated MCF-7 cells were more resistant to 5-FU compared with untreated control MCF-7 cells. In particular, 5-FU-induced apoptosis was entirely abolished in hypoxia-treated cells (Fig. 4A). E2-, TGF-β1- and hypoxia-treated cells demonstrated inhibited radiation-induced apoptosis (Fig. 4B). These results indicate that the TGF-β1, E2 and hypoxia treatments enhance drug and radioresistance, which may be due to the increased expression of CSC-associated proteins, including ABCG2 and β-catenin.

Discussion

CSCs possess the stem cell properties of self-renewal and differentiation; therefore, tumors may be initiated, grow, invade and metastasize (31). Anticancer therapies at present emphasize killing CSCs or inhibiting the induction of the CSC population (32,33). However, CSC-targeted cancer therapies are problematic due to the characteristics of CSCs, including radio- and chemoresistance (34,35). The aim of the present study was to identify the regulatory mechanisms for breast CSC expansion. Treatment with E2 induces the proliferation of human ER^+ breast cancer cells (36,37). In addition, a previous study indicated that breast CSCs are stimulated by estrogens, through paracrine fibroblast growth factor/Tbx3 signaling (38). Numerous studies have demonstrated that estrogen hormones act as a negative regulator of TGF-β1-stimulated cellular responses (39,40). Therefore, the present study examined the effects of E2 and TGF-β1 treatment on the regulation of breast CSCs. The results of the present study indicated that treatment with E2 or TGF-β1 in isolation expanded the $CD44^+/CD24^{-/low}$ cell subpopulation, but that a combination of E2 and TGF-β1 treatment did not exhibit a synergistic effect (Fig. 1). These results demonstrate that E2 and TGF-β1 do not affect each other during regulation of the breast CSC population. Oxygen is a critical regulator of cellular metabolism and proliferation, and hypoxia regulates a variety of pro-angiogenic pathways and carcinogenesis (41,42). Although numerous studies have reported that hypoxia improves therapeutic efficacy by eliminating the CSC population (43-45), other studies indicate that hypoxia induces CSC characteristics by upregulating stemness-associated factors and a more aggressive phenotype (45,46). Therefore, in the present study, the $CD44^+/CD24^{-/low}$ cell population was monitored at each time point (48, 72 and 96 h and 1 week) following hypoxia treatment. As expected, the $CD44^+/CD24^{-/low}$ cell population expanded following hypoxia treatment (Fig. 1F). Although the peak percentage value varied for each treatment, the induced ratio of $CD44^+/CD24^{-/low}$ cells by E2, TGF-β1 and hypoxia decreased over time (Fig. 1). Therefore, breast CSCs with the $CD44^+/CD24^{-/low}$ phenotype appear to maintain a consistent CSC ratio from external stimuli.

EMT is an essential process in tumor metastasis and recurrence (47). Previous studies have demonstrated that the EMT is tightly linked to CSC biology (27-29). In addition, breast cancer is a distinct cause of mortality and has a high incidence of recurrence (48). Therefore, identifying the mechanisms or molecules used in the EMT is important for breast cancer therapy. In the present study, the E2, TGF-β1 and hypoxia treatments induced the expression of EMT-associated factors (Fig. 2B). Similarly, the migration ability of MCF-7 cells increased following treatment (Fig. 3). These results indicate that E2, TGF-β1 and hypoxia may be critical in EMT.

CSCs are hypothesized to demonstrate resistance to chemo- and radiotherapy (31), and ABCG2 is a key regulator of chemoresistance (49). The results of the present study indicated that ABCG2 expression increased significantly while screening target proteins that were induced by treatment with TGF-β1, E2 and hypoxia (Fig. 2A). The Annexin V analysis demonstrated that the E2, TGF-β1 and hypoxia treatments attenuated 5-FU-induced apoptosis (Fig. 4A). In addition, treatment with E2, TGF-β1 or hypoxia effectively blocked ionizing radiation-induced apoptosis (Fig. 4B). These results indicate that CSCs, which have been expanded by TGF-β1, E2 and hypoxia, may enhance chemo- and radioresistance. Components of the cell cycle machinery regulate asymmetric cell division, cell shape, and protein translation in stem cells (50). In addition, numerous studies have reported that the regulation of cell cycle-associated factors may contribute to inhibit the chemotherapeutic resistance of CSCs (50,51). The present study demonstrated that the expression of cell cycle-associated factors, including β-catenin and c-Myc, increased following treatment with E2, TGF-β1 and hypoxia (Fig. 2A). The present data suggest that these factors are involved in the regulation of the breast CSC population.

In conclusion, the results of the present study indicate that E2, TGF-β1 and hypoxia are important in breast CSC expansion and in the regulation of CSC-associated protein expression. In addition, the regulation of hormones, growth factors and the tumor microenvironment, which are potential breast cancer therapeutic targets, may improve the efficacy of breast cancer therapy by inhibiting chemo- and radioresistance.

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