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Sertaconazole, an Imidazole Antifungal Agent, Suppresses the Stemness of Breast Cancer Cells by Inhibiting Stat3 Signaling

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Abstract. *Background/Aim: Breast cancer stem cells (BCSCs) are a subpopulation of tumor cells that play a role in therapeutic resistance. In this study, we demonstrated that sertaconazole, an antifungal agent, displayed a potent inhibition on cancer stem cells (CSCs) and investigated the mechanism of action involved in its anti-BCSC effect. Materials and Methods: The effect of sertaconazole on BCSCs was investigated using a mammosphere formation assay, a colony formation assay, and a cell migration assay. In addition, $CD44^{high}/CD24^{low}$ and ALDEFLOR analyses, an apoptosis assay, quantitative real-time PCR, western blotting, an electrophoretic mobility shift assay, and a cytokine profiling assay were performed. Results: Sertaconazole inhibited cell proliferation, colony formation, cell migration, mammosphere formation, and mammosphere proliferation. It also induced apoptosis of breast cancer cells. It decreased the subpopulation of $CD44^{high}/CD24^{low}$ and aldehyde dehydrogenase-expressing cells. It also reduced the DNA binding of Stat3 and nuclear protein expression levels of phosphorylated Stat3. Furthermore, it reduced the IL-8 mRNA levels of the mammosphere. Conclusion: Sertaconazole can inhibit the Stat3 and IL-8 signaling pathways and induce CSC death. Thus, sertaconazole might be a potential inhibitor of BCSCs.*

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Breast cancer (BC) is the second most common cancer and the most invasive cancer in females (1). BC occurs in different tissues of the breast including lobules, ducts, and connective tissue. It is a heterogeneous disease that shows various physiological properties and different clinical outcomes (2). BC stem cells (BCSCs) are small subpopulations of BC cells that play a critical role in the metastasis of BC to other organs in the body (3). BCSCs are able to self-renew and differentiate into specialized breast tumor cells (4, 5). These properties are considered to contribute to the aggressiveness of metastatic cancer (3).

BCSCs are known to contribute to chemo-resistance, radio-resistance, and tumor initiation, making them major contributors to the failures of BC therapies (6). Targeting BCSCs could decrease drug resistance and increase drug efficacy, providing significant benefits to BC patients (7). Understanding the mechanisms involved in the resistance of BCSCs to cancer therapy can help us develop an effective BCSC-targeted therapy (8). Cellular characteristics of BCSCs are CD44(+)/CD24(–) tumorigenic cells and aldehyde dehydrogenase (ALDH)-positive cells (9). The Wnt, NF-κB, Notch, BMP2, STAT3, and Hedgehog (Hh) signaling pathways control the activation of epithelial-to-mesenchymal transition (EMT), tumor growth, and tumorigenesis of BCSCs (3, 10). The stemness of cancer cells is controlled by pluripotent transcription factors, such as OCT4,

Sox2, Nanog, KLF4, and c-MYC. Intracellular signaling pathways involving Wnt, NF-κB, Notch, Hedgehog, JAK-STAT3, PI3K/AKT/mTOR, TGF-β/SMAD, and extracellular factors containing vascular niches, hypoxia, tumor-associated macrophages, cancer-associated fibroblasts, cancer-associated mesenchymal stem cells, extracellular matrix, and exosomes, are known as important regulators of CSCs (11). BCSC-targeted therapies will overcome the drug resistance associated with cancer therapies applied to patients with BC (11).

Sertaconazole, an imidazole-type antifungal agent, possesses antifungal activities against pathogenic fungi, including yeast-like fungi (*Candida albicans*), dermatophytes, and opportunistic fungi, and other filamentous fungi (12, 13). Sertaconazole can block the synthesis of ergosterol by inhibiting the 14α-demethylase enzyme. Ergosterol is a critical component of the fungal cell membrane. Sertaconazole can disrupt fungal mycelial growth and replication by inhibiting ergosterol synthesis (14). It also shows anti-inflammatory and anti-itch effects by activating the p38-COX2-PGE2 pathway in keratinocytes and human peripheral blood mononuclear cells (PBMCs) (15, 16). Recently, it has been found that imidazole-based antifungal drugs are cytotoxic to diverse human cancers. For example, miconazole can induce apoptosis and inhibit the growth of colon cancer cells (17). Sertaconazole has also shown anticancer effects against non-small cell lung cancer (NSCLC) *via* promoting proapoptotic autophagy through stabilizing TRADD (18). Although recent studies have shown that sertaconazole, as antifungal agent, possesses anti-cancer effects, little is known about its impact on cancer stem cells in BC and the underlying mechanisms involved.

Thus, the objective of the present study was to determine whether sertaconazole could suppress BC-derived mammosphere formation. Specifically, the ability of sertaconazole to inhibit the formation of BCSCs *via* regulation of Stat3 signal was investigated.

Materials and Methods

Chemicals. Sertaconazole nitrate, with a purity ≥98% (HPLC), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sertaconazole nitrate was dissolved in DMSO to a concentration of 10 mM as a stock and stored at –20˚C. When treated, the stock was diluted to the required concentration using the medium.

Cell culture. BC cell lines (MCF-7 and MDA-MB231) were obtained from Korea Cell Line Bank (Seoul, Republic of Korea). MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) (Thermo Fisher Scientific Inc., Waltham, MA, USA). MDA-MB231 cells were cultured in RPMI 1640 medium supplemented with 1% penicillin/streptomycin and 10% FBS. These cells were cultured at 37˚C in a humidified atmosphere containing 5% CO_2. As needed, the cells were cultured on cell culture dishes and plates (SPL Life Sciences, Pocheon-si, Republic of Korea).

Mammosphere formation. MCF-7 cells (2×10^4 cells /ml) and MDA-MB-231 cells (1×10^4 cells/ml) were cultured with MammoCult™ medium (STEMCELL Technologies, Vancouver, Canada) supplemented with heparin and hydrocortisone on a cell floater plate. Mammospheres derived from cancer cells were formed at 37˚C in a humidified atmosphere containing 5% CO_2 for seven days. Cell floater plates, including 6-well ultra-low attachment plates, were purchased from SPL Life Sciences Co. and Corning (Corning, NY, USA). Mammosphere formation was quantified using the NICE program after image scanning (19).

Cell proliferation assay. MCF-7 and MDA-MB-231 cells were cultured in 96-well plates. The cells were cultured with various concentrations (0, 5, 10, 20, 40, and 80 μM) of sertaconazole for 24 h. Cell proliferation assay was performed using a CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) based on the MTS method following the manufacturer's protocol.

Wound-healing assay. MDA-MB-231 cells were cultured in a 6-well plate and allowed to reach confluency. A wound was made with a SPLScar™ Scratcher (SPL Life Sciences) on the cell layer, which was then treated with sertaconazole. The scratch wound area was imaged at 24 h post-scratching using a microscope.

Colony formation assay. MDA-MB-231 cells were cultured in a 6-well plate at a density of 2×10^3 cells per well and treated with increasing concentrations of sertaconazole. After seven days of incubation, the cells were fixed with methanol:acetic acid (3:1) solution and stained with 0.05% crystal violet. The number of colonies was quantified using the NICE program after image scanning.

Detection of apoptosis. MDA-MB-231 cells were cultured in a 6-well plate for 24 h and treated with sertaconazole. Live, dead, apoptotic, and necrotic cells were detected with a flow cytometer (BD Acuri C6, BD, San Jose, CA, USA) using FITC Annexin V Apoptosis Detection Kit I (BD) and following the vendor's recommendation. For apoptotic cell visualization, the cells were stained with Hoechst 33342 dye (Thermo Fisher Scientific Inc.). Photomicrographs of apoptotic cells were acquired using Lionheart LX (Biotek, Agilent Technologies, Santa Clara, CA, USA) (20).

Caspase 3/7 assay. MDA-MB-231 cells were cultured in a 6-well plate for 24 h and treated with sertaconazole. Expression of caspase 3/7 was analyzed with a Caspase-Glo® 3/7 Assay System (Promega) and following the vendor's recommendation. Values were quantified using a microplate reader, GloMax® Discover (Promega).

$CD44^{high}/CD24^{low}$ expression analysis. MDA-MB-231 cells were cultured in a 6-well plate for 24 h and treated with sertaconazole. Single cells were obtained by treating the cells with trypsin, followed by staining with FITC-conjugated anti-CD44 and APC-conjugated anti-CD24 antibodies for 20 min at 4˚C. After washing, stained cells were analyzed using Accuri™ C6 (BD, NJ, USA) in Bio-Health Materials Core-Facility, Jeju National University.

ALDH assay. MDA-MB-231 cells were cultured in a 6-well plate for 24 h and treated with sertaconazole. ALDH detection was performed using an ALDEFUOR kit (STEMCELL Technologies). These cells were trypsinized and stained following the vendor's recommendation. Stained cells were analyzed using a flow cytometer. Samples containing diethylamino benzaldehyde (DEAB) were used as negative controls.

Table I. *Primer sequences of target genes used in RT-qPCR.*

Gene name	Primer sequence	
	Forward	Reverse
OCT4	AGCAAAACCCGGAGGAGT	CCACATCGGCCTGTGTATATC
SOX2	TTGCTGCCTCTTTAAGACTAGGA	CTGGGGCTCAAACTTCTCTC
CD44	AGAAGGTGTGGGCAGAAGAA	AAATGCACCATTTCCTGAGA
c-Myc	AATGAAAAGGCCCCCAAGGTAGTTAT	AGCAAAACCCGGAGGAGT,
NANOG	ATGCCTCACACGGAGACTGT	AAGTGGGTTGTTTGCCTTTG
SNAIL	TGCCCTCAAGATGCACATCCGA	GGGACAGGAGAAGGGCTTCTC
β-actin	TGTTACCAACTGGGACGACA	GGGGTGTTGAAGGTCTCAAA

RNA isolation and RT-qPCR. The total RNAs were extracted from mammospheres derived from MDA-MB-231 cells using MiniBEST Universal RNA Extraction Kit (Takara, Tokyo, Japan). RT-qPCR was performed using the TOPreal™ One-step RT-qPCR Kit Enzynomics, Daejeon, Republic of Korea) following the manufacturer's recommendations. As an internal control, *β-actin* gene was used. Specific primer sequences used for RT-qPCR are listed in Table I.

Western blot analysis. Protein samples were extracted using RIPA buffer containing protease inhibitors and phosphatase inhibitors (Thermo Fisher Scientific) from mammospheres derived from MDA-MB-231 cells. Nuclear extraction was performed using a previously reported method (21). After electrophoresis on a 10% SDS-PAGE gel, proteins were electro-transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Burlington, MA, USA). Blots were incubated with 3% BSA at room temperature for 1 h and then reacted with primary antibodies at 4˚C overnight. After the membranes were washed at room temperature, they were incubated with secondary antibodies at room temperature for 1 h. The following antibodies were used: anti-phospho-Stat3 (Cell Signaling, Danvers, MA, USA), anti-p65, anti-Stat3, and anti-β-actin (Santa Cruz Biotechnology, Dallas, TX, USA).

Electrophoretic mobility shift assay (EMSA). EMSA was performed using a previously described method (21). EMSA was examined with a LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific) according to the manufacturer's recommendations. Biotin-labeled singles of the Stat3 probe (Table II) were annealed and double-strand oligonucleotides were end-labeled with biotin. These biotin-labeled DNA probes were incubated with sertaconazole-treated nuclear proteins in a final volume of 20 μl EMSA buffer containing 1 μg/μl poly (dI-dC). Reaction mixtures were electrophoresed on a 5% polyacrylamide nondenaturing gel in 0.5X TBE. Supershifts were detected using a chemiluminescent nucleic acid detection kit (Thermo Fisher Scientific Inc.).

Cytokine profiling. Mammospheres derived from MDA-MB-231 cells were treated with sertaconazole. The culture medium was then collected. Cytokine profiling of cells was performed using a Human Inflammatory Cytokine Cytometric Bead Array (CBA; BD) following the manufacturer's recommendations. All samples were measured by flow cytometry and data were quantitated using BD FCAP array software.

Statistical analysis. All statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). All data are represented as means±standard deviation (SD).

Table II. *Sequences of biotin-labeled Stat3 probe.*

	Sequences
Forward	CTTCATTTCCCGGAAATCCCTA-Biotin
Reverse	TAGGGATTTCCGGGAAATGAAG-Biotin

All data were analyzed by one-way ANOVA followed by Dunnett *post-hoc* test. A *p*-value less than or equal to 0.05 was considered statistically significant.

Results

Sertaconazole inhibits cell viability, cell migration, and colony formation. To examine the effect of sertaconazole (Figure 1A) on the viability of BC cells, an MTS assay was performed. BC cells were cultured with sertaconazole at various concentrations (0, 10, 20, 40, 60, and 80 μM) for 24 h. Results showed that sertaconazole inhibited the viability of BC cells (Figure 1B and C). Sertaconazole treatment at 10 μM reduced the colony formation and migration of BC cells (Figure 1D and E). Thus, sertaconazole suppressed the proliferation, colony formation, and migration of BC cells.

Sertaconazole induces apoptosis of BC. To evaluate the apoptosis effect of sertaconazole on BC cells, an apoptosis assay was performed using an Annexin V/PI staining kit, a caspase3/7 activity kit, and a Hoechst 33258 dye. Results showed that the number of apoptotic (Annexin V+) BC cells was increased by sertaconazole at 30 μM (Figure 2A). The subpopulation of early apoptosis was also increased from 3.7% to 16.4%. In addition, sertaconazole increased the caspase activity (Figure 2B). Apoptotic bodies of BC cells were also increased by sertaconazole at 30 μM (Figure 2C).

Sertaconazole inhibits mammosphere formation. To examine whether sertaconazole could inhibit mammosphere formation, we treated primary mammospheres derived from BC cells with sertaconazole. Results showed that sertaconazole decreased mammosphere formation. Sertaconazole not only

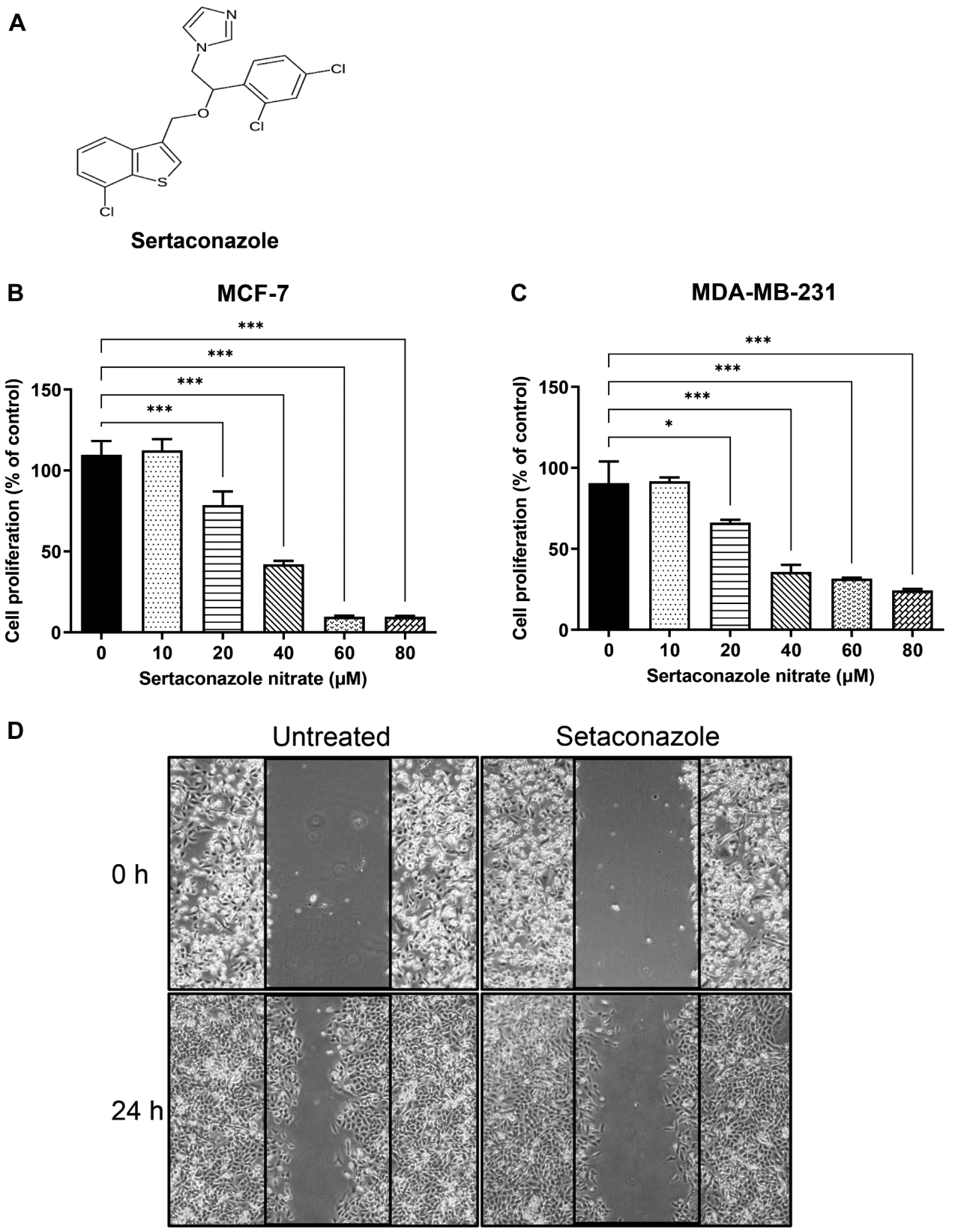

Figure 1. *Continued*

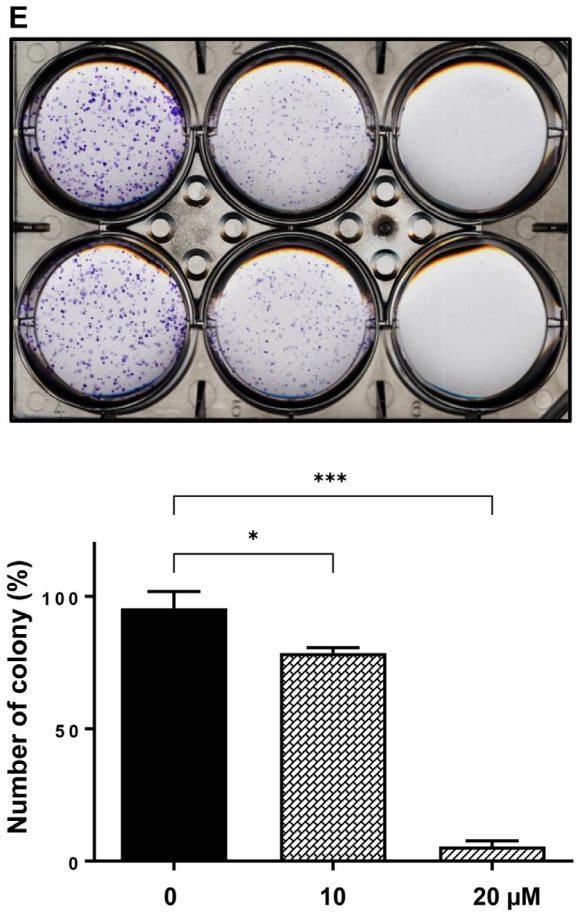

Figure 1. *Sertaconazole inhibits the proliferation of breast cancer cell lines. (A) Molecular structure of sertaconazole. (B) The proliferation of MCF-7 cells. MCF-7 cells were treated with various concentrations of sertaconazole for 24 h. (C) Proliferation of MDA-MB-231 cells. MDA-MB-231 cells were treated with various concentrations of sertaconazole for 24 h. (D) Representative images from the wound-healing assay of MDA-MB-231 cells treated with 30 μM sertaconazole. Migration images were captured after 24 h of sertaconazole treatment (magnification, 10×). (E) Colony formation assay using crystal violet staining in MDA-MB-231 cells. Cells were treated with 30 μM sertaconazole. The number of colonies was quantified after 10 days. Values of experiments are represented as the mean±SD of triplicates. *p<0.05; **p<0.01; ***p<0.001 using t-test or one-way ANOVA followed by Dunnett post-hoc test.*

decreased the number of mammospheres by 50% to 90%, but also decreased the size of mammospheres (Figure 3A and B).

Sertaconazole reduces $CD44^{high}/CD24^{low}$ subpopulations and ALDH-positive cells. Cancer cell subpopulations of $CD44^{high}/CD24^{low}$ represent the BCSC population (22). MDA-MB-231 BC cells were incubated in 6-well cell culture plates at 2×10^6 cells/plate for one day and treated or not treated with sertaconazole for one day. Sertaconazole reduced the cell subpopulation of $CD44^{high}/CD24^{low}$ from 45.6% to 35.9% (Figure 4A). Therefore, properties of BCSCs were negatively affected by sertaconazole. After cancer cells were incubated with sertaconazole (30 μM) for one day, an ALDEFLUOR assay kit was then used to examine the effect of sertaconazole on ALDH-expression cells. Sertaconazole decreased ALDH-positive cells from 1.2% to 0.6% (Figure 4B). Sertaconazole also suppresses CD44+/CD24–-expressing and ALDH-positive cells, which are known hallmarks of breast CSCs.

Sertaconazole suppresses the transcription of CSC-specific marker genes and mammosphere growth. We found that sertaconazole affected the formation of BCSCs. Additionally, sertaconazole inhibited CSC-specific gene expression of *c-myc*, *Oct4*, *CD44*, *Sox2*, *snail*, and *Nanog* (Figure 4C). To determine the effects of sertaconazole on mammosphere proliferation, MDA-MB-231 mammospheres were cultured with sertaconazole. The same number of cancer cells from mammospheres were treated with or without sertaconazole in 6 cm dishes every three days. Results showed that sertaconazole inhibited mammosphere proliferation (Figure 4D). Overall, these data indicate that sertaconazole can inhibit BCSCs.

Sertaconazole inhibits Stat3 and IL8 signaling. To examine the mechanism underlying the effects of sertaconazole on BCSCs, we investigated Stat3 signaling and secreted IL-6/IL-8 cytokine levels in mammospheres under sertaconazole treatment. First, we assayed Stat3 and phosphorylated Stat3 (pStat3) protein expression levels under sertaconazole treatment. We found that sertaconazole reduced nuclear pStat3 protein levels in BCSCs, with pStat3 protein levels significantly decreased in the nuclear fractions of MDA-MB-231 CSCs (Figure 5A). In addition, we examined the DNA binding affinity of sertaconazole-treated nuclear extracts using a Stat3 probe and found that sertaconazole treatment decreased Stat3-DNA binding (Figure 5B, lane 3). Stat3-binding specificities were examined using a self-competitor (100×) (Figure 5B, lane 4) and mutated Stat3-oligo (100×) (Figure 5B, lane 5). The band indicated by an arrow (Figure 5B) was a Stat3-specific DNA complex. Secreted IL-6 and IL-8 are known to be the survival factors of CSC formation (23). Stat3 regulated *IL-8* gene transcription by binding to the IL-8 promoter. Cytokine profiling was performed using a mammosphere culture medium to test the secretion level of IL-8. Treatment with sertaconazole decreased the IL-8 secretion level (Figure 5C). Overall, these results demonstrate that sertaconazole can inhibit the formation of mammospheres *via* Stat3/IL-8 signal dysregulation.

Discussion

BC is a malignant cancer type that accounts for approximately 30% of cancers in women (24). Although the novel therapeutic strategies of BC have been well-developed, it remains the leading factor of cancer-related death in women (25).

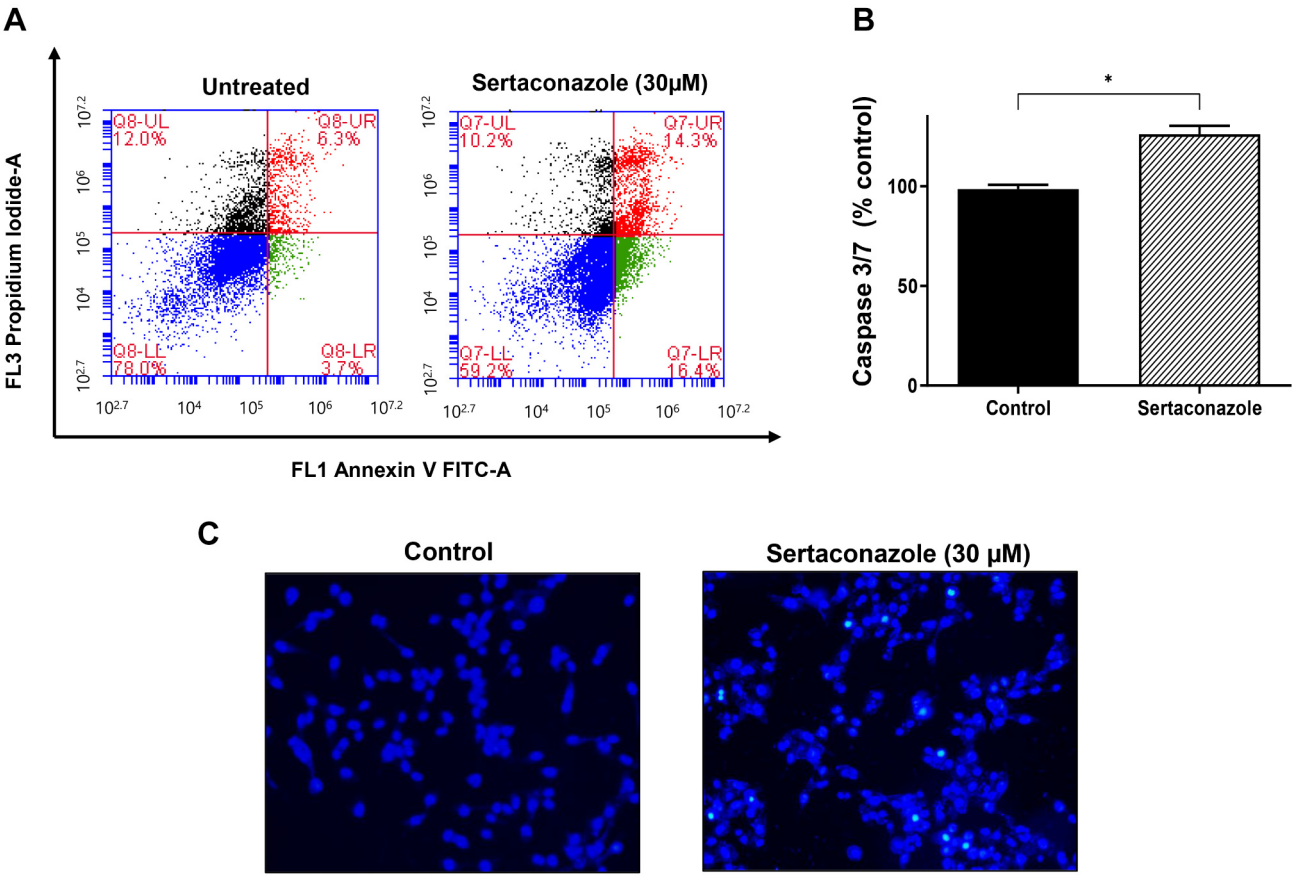

Figure 2. *Sertaconazole induces the apoptosis of breast cancer cells. (A) FACS assay by Annexin V/PI double staining for detection of apoptosis in MDA-MB231 cells. Cells were treated with 30 μM sertaconazole for 24 h. (B) Caspase 3/7 activity using Caspase-Glo® 3/7 Assay System and MDA-MB-231 cells. Cells were treated with 30 μM sertaconazole for 24 h. Values of experiments are represented as the mean±SD of triplicates. (C) Apoptosis detection using Hoechst 33342 staining of MDA-MB-231 cells. Images were captured after 24 h of treatment with 30 μM sertaconazole (magnification, 20×). Values of experiments are represented as the mean±SD of triplicates. *p<0.05; **p<0.01; ***p<0.001 using t-test or one-way ANOVA followed by Dunnett post-hoc test.*

Metastatic BC, in which the tumor has spread to other organs, such as the bones, liver, lungs, and brain, is the main cause of BC-related death. Metastatic triple-negative BC cells show high heterogeneity, drug resistance, and tumor progression, making to treatment more challenging and leading to reduced overall survival for patients (26).

The first evidence of CSCs in humans was found in acute myeloid leukemia (27). CSCs are found in most human tumors (28). Most studies have shown that CSCs are resistant to conventional chemotherapy and radiation treatment and that they are very likely to be the origin of cancer metastasis (29). Thus, CSCs have been used as the potential therapeutic target of BC cells. ALDH1 and $CD44^{high}/CD24^{low}$ have been known as biomarkers of BCSCs (30). Targeting BCSCs for cancer treatment is an effective strategy for treating patients with BC.

Sertaconazole is an imidazole derivative used to treat skin and mucosa mycoses (31). It has been widely used as an antifungal agent. Research studies on its anticancer effects are relatively insufficient. Until now, anti-cancer and anti-CSC effects of sertaconazole are poorly understood with minimal information currently available. In this research, we investigated the mechanisms involved in the anticancer and anti-CSC action of sertaconazole.

In the present study, the anti-CSC mechanism of sertaconazole in breast CSCs was investigated. Sertaconazole suppressed cancer cell proliferation and mammosphere formation (Figure 1 and Figure 3). Sertaconazole also increased the apoptotic cell subpopulation of BC cells (Figure 2). Furthermore, sertaconazole decreased $CD44^{high}/CD24^{low}$- and ALDH-subpopulation levels (biomarkers of BCSCs) and expression levels of CSC-related genes (*Oct4, Sox2, CD44, c-Myc, Snail*, and *Nanog*) (Figure 4). Collectively, our data show that sertaconazole has an inhibitory effect on BCSCs.

The signal transduction and transcription activator family (STAT) includes STAT1, STAT2, STAT3, STAT4, STAT5a,

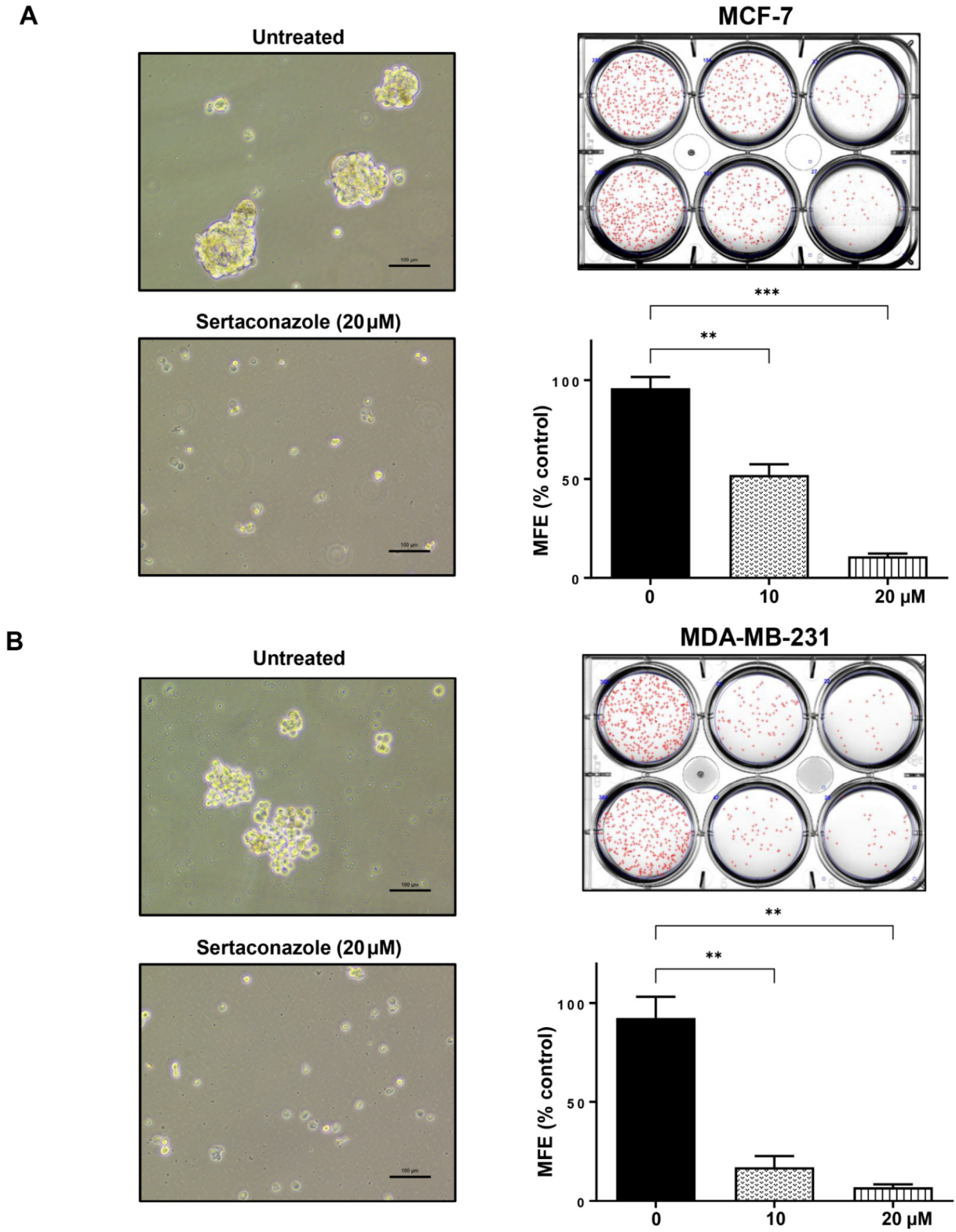

Figure 3. *Sertaconazole inhibits mammosphere formation. (A) Effect of sertaconazole on mammosphere formation of MCF-7 cells. As shown, sertaconazole-treated mammosphere formation was reduced. (B) Effect of sertaconazole on mammosphere formation of MDA-MB-231 cells. Sertaconazole-treated mammosphere formation was reduced. Values are presented as the mean±SD of triplicates. *p<0.05; **p<0.01; ***p<0.001 using t-test or one-way ANOVA followed by Dunnett post-hoc test.*

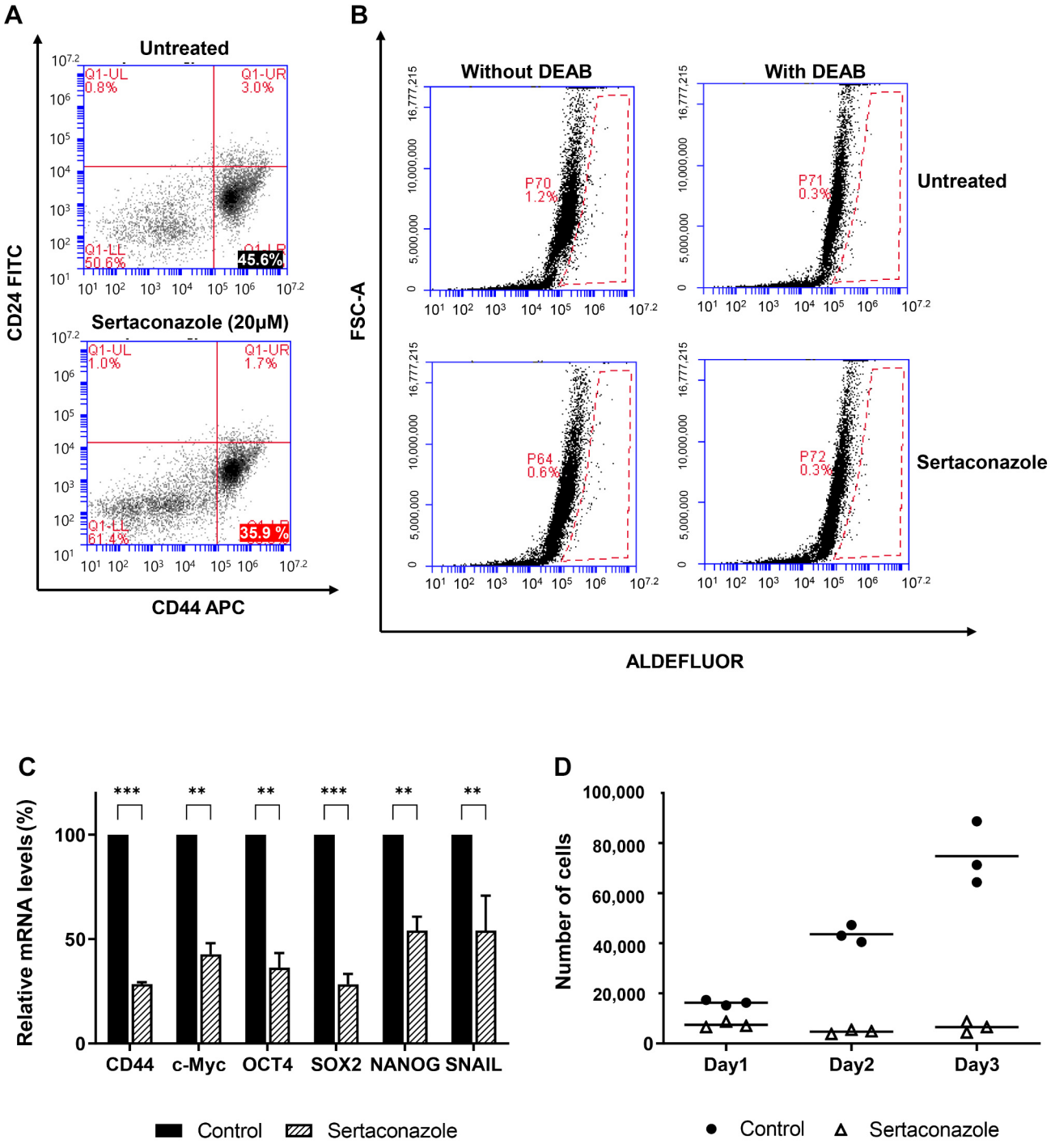

Figure 4. *The inhibitory effect of sertaconazole on mammospheres. (A) Expression of mammosphere markers CD44^{high} and CD24^{low} in MDA-MB-231 cells. Cells were treated with 10 μM sertaconazole for 24 h and double stained with CD44-FITC and CD24-APC. (B) ALDH expression assay using MDA-MB-231 cells. Cells were treated with 10 μM sertaconazole for 24 h. Expression of ALDH was detected by FACS and ALDEFLUOR™ Kit. (C) Expression of mammopshere-forming related genes in MDA-MB-231 cells. Cells were treated with 10 μM sertaconazole for 16 h. Then, mRNA levels of CD44, c-Myc, OCT4, SOX2, NANOG, and SNAIL were assessed by reverse-transcription quantitative polymerase chain reaction. β-actin was used as a loading control. Values are presented as the mean±SD of triplicates. (D) Inhibitory effect of sertaconazole on mammosphere formation. Cultured mammospheres were treated with 10 μM sertaconazole for two days. Sertaconazole-treated mammospheres were dissociated into single cells and cultured in equal numbers. The number of cells was counted daily for three days. Values are presented as the mean±SD of triplicates. *p<0.05; **p<0.01; ***p<0.001 using t-test or one-way ANOVA followed by Dunnett post-hoc test.*

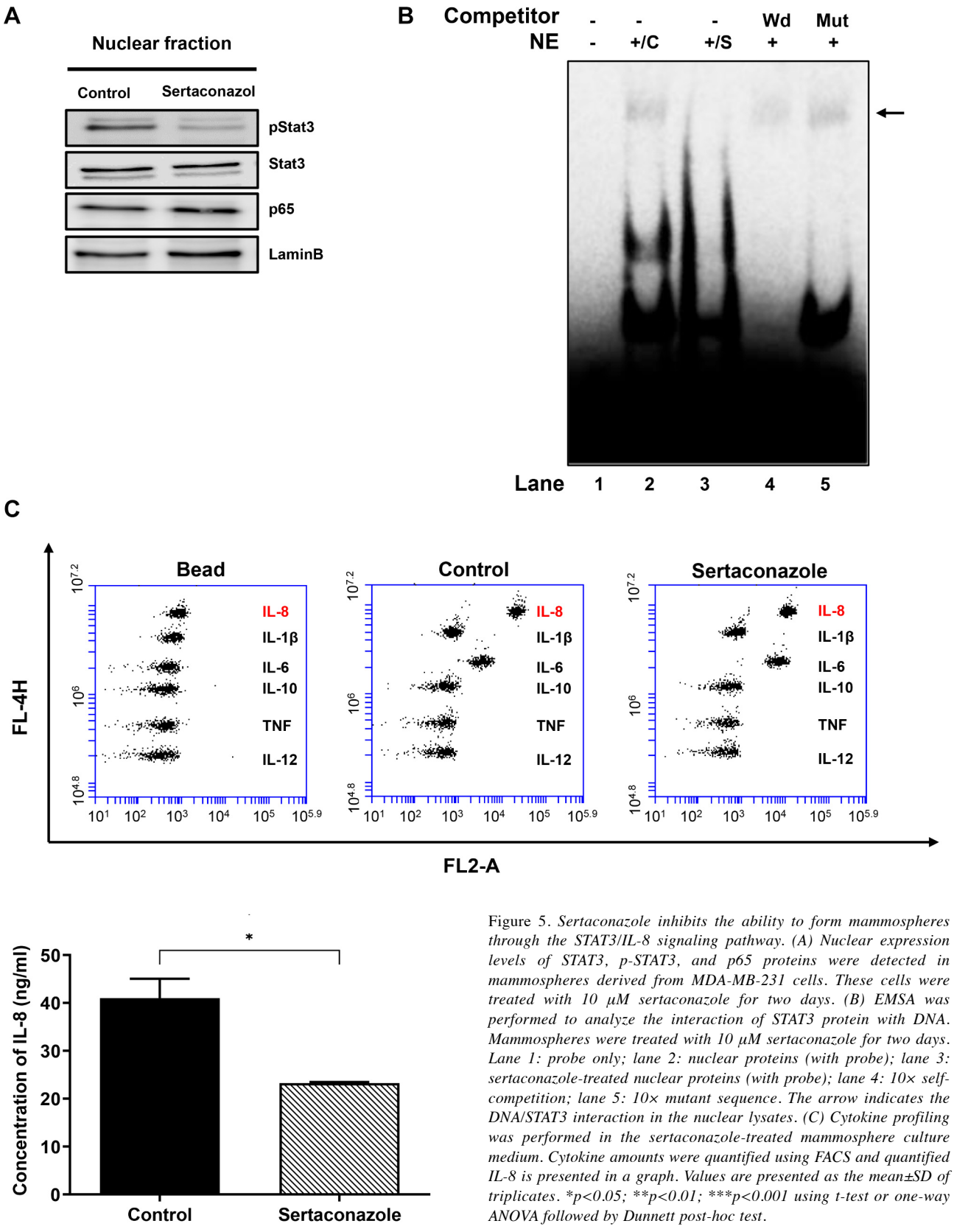

Figure 5. *Sertaconazole inhibits the ability to form mammospheres through the STAT3/IL-8 signaling pathway. (A) Nuclear expression levels of STAT3, p-STAT3, and p65 proteins were detected in mammospheres derived from MDA-MB-231 cells. These cells were treated with 10 μM sertaconazole for two days. (B) EMSA was performed to analyze the interaction of STAT3 protein with DNA. Mammospheres were treated with 10 μM sertaconazole for two days. Lane 1: probe only; lane 2: nuclear proteins (with probe); lane 3: sertaconazole-treated nuclear proteins (with probe); lane 4: 10× self-competition; lane 5: 10× mutant sequence. The arrow indicates the DNA/STAT3 interaction in the nuclear lysates. (C) Cytokine profiling was performed in the sertaconazole-treated mammosphere culture medium. Cytokine amounts were quantified using FACS and quantified IL-8 is presented in a graph. Values are presented as the mean±SD of triplicates. *p<0.05; **p<0.01; ***p<0.001 using t-test or one-way ANOVA followed by Dunnett post-hoc test.*

STAT5b, and STAT6. STAT3 is an important member. It is a transcription factor with several biological functions. It is constitutively activated in many types of human solid tumors (32-34). Stat3 also regulates the development and functions of CSCs. Stat3 signaling plays an important role in the initiation and progression of cancers and CSCs (35). CSCs are regulated by tumor microenvironment components through networks of growth factors and cytokines. Cytokine networks, including the Stat3/IL-6/IL-8 signal, are important regulators of BCSCs (36-38). Our data revealed that sertaconazole could inhibit BCSC formation by inhibiting Stat3 signaling (Figure 5). Although sertaconazole can inhibit BCSC through Stat3, there might be other pathways/mechanisms responsible for this inhibition. Secreted IL-6 and IL-8 are known to be the survival factors of CSC formation (23). Stat3 regulated *IL-8* gene transcription by binding to the *IL-8* promoter (39). Cytokine profiling was performed using a mammosphere culture medium to test the secretion level of IL-8. It was found that sertaconazole treatment decreased the IL-8 secretion level (Figure 5). Overall, these results indicate that sertaconazole can inhibit the formation of mammospheres *via* Stat3/IL-8 signal dysregulation.

Thus, IL-8 secreted by BC stem cells can promote therapeutic resistance and metastasis of bulk tumor cells (40). The blockade of the IL-8/Stat3 pathway requires further investigation as a potential BCSC-related target for BC therapy. As an inflammatory cytokine, IL-8 is up-regulated in BC. Such up-regulation is associated with a poor prognosis. IL-8 is also an important regulator of BCSCs. Indeed, targeting IL-8 signaling is known to inhibit BCSC activity (41). Importantly, our data revealed that sertaconazole can inhibit IL-8 signaling and CSC formation.

Conclusion

In summary, sertaconazole can suppress BCSCs by inhibiting mammosphere formation and growth, colony formation, and cell migration. Sertaconazole A can also suppress the Stat3/IL-8 signaling pathway. Collectively, our results indicate that BCSCs can be inhibited *via* inhibition of the Stat3/IL-8 signal pathway and that sertaconazole could be developed for use in BCSC therapy.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

Choi HS and Kim SL designed this study and participated in all the experiments. Choi HS and Kim SL wrote the manuscript. Hong HK did data clearance and identification. Lee DS supervised the study. All Authors have read and agreed to the published version of the manuscript.

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