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Auranofin, an antirheumatic drug, shows anticancer stem cell potential via suppression of the Stat3 signal

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Accumulating data have shown that targeting breast cancer stem cells (CSCs) is an auspicious way for anticancer therapies. This study demonstrated that the antirheumatic drug auranofin is a potent CSC inhibitor with anti-CSC action on breast cancer. This research focused on investigating the effect of auranofin on breast cancer and CSCs and its cellular mechanism. Mammosphere formation, colony formation, levels of $CD44^{high}/CD24^{low}$, and aldehyde dehydrogenase 1 expression in the cells were evaluated after auranofin treatment. The anti-CSC properties of auranofin were further examined by gel shift assay and cytokine detection. Auranofin suppressed cell growth, colony formation, migration, and mammosphere formation and triggered apoptosis in breast cancer. Auranofin decreased the $CD44^{high}/CD24^{low}$- and aldehyde dehydrogenase-expressed subpopulations, as well as the Stat3-DNA interaction and phosphorylated Stat3 level. Auranofin also decreased the extracellular levels of interleukin-8 (IL-8) in the mammosphere media. Auranofin suppressed the Stat3/IL-8 signal and killed CSCs; therefore, it may be a potential target for CSCs. [BMB Reports 2025; 58(7): 293-299]

INTRODUCTION

Breast cancer (BC) is characterized by the abnormal growth of cells within the breast, leading to uncontrolled cell division that eventually results in the formation of tumors. BC is the second most frequently diagnosed cancer and has become a prevalent cancer worldwide. It is the most aggressive cancer among women (1). BC cells develop within the milk ducts and/or milk-producing lobules in the breast tissue, representing heterogeneous diseases that originate from this tissue and exhibit diverse pathophysiological properties and clinical outcomes (2). BC stem cells (BCSCs) represent a small proportion of BC cells that contribute to the metastasis of BC cells to other tissues throughout the human body (3). Breast CSCs possess the ability to self-renew and differentiate into distinct tumor cells (4). These characteristics are believed to contribute to the malignancy of cancer or tumor (5).

Breast CSCs constitute a distinct population of tumor cells that differentiate into non-BCSCs and have self-renewal properties. BCSCs exhibit resistance to chemo- or radio-therapy and tumor-initiating capabilities. These properties are the major reason for the poor outcome of BC therapies (6). Targeting breast CSCs enhances drug sensitivity and efficiency, resulting in favorable outcomes for patients with BC (7). Understanding the resistance mechanism of breast CSCs in BC therapy can facilitate the development of targeted therapy aimed at these cells (8). $CD44^{high}/CD24^{low}$ and aldehyde dehydrogenase 1A levels are cellular markers of breast CSCs (9).

Metastatic BC demonstrates increased migratory and invasive potential, which was facilitated by the epithelial–mesenchymal transition (EMT). The EMT state has been related to CSC properties. EMT is an important step that initiates cell progression, invasion, and metastasis. The EMT pathway is controlled by several signaling pathways, such as TGF-β, Notch, Wnt, Hedgehog, and RTKs. Evidence indicates the association of EMT and cancer stem-like cells (10, 11). Oct4/Sox2/Nanog/KLF4/c-MYC regulates the stemness of cancer cells. Kynurenine, lactate, hypoxia, tumor-associated macrophages, cancer-associated fibroblasts, cancer-associated mesenchymal stem cells, extracellular matrix, and exosomes are crucial regulators for CSC survival (12). Therapies targeting breast CSCs have the potential to overcome drug resistance associated with cancer treatment and can be beneficial for patients with BC (13).

Auranofin has been an FDA-approved drug for the management of rheumatoid arthritis for 40 years; however, its biochemical reaction mechanism remains unclear (14). Auranofin suppressed the activation and mRNA expression of proinflammatory proteins induced by inflammation, including cyclooxygenase-2, inducible nitric oxide synthase, and nuclear

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factor kappa B (15). Auranofin, a pro-oxidant agent, works by disturbing the cellular reduction/oxidation system and targeting the thioredoxin (Trx) reductase system (Trx1 and TrxR2), inducing reactive oxygen species (ROS) production (16). The Trx system regulates the cellular redox state and, in particular, induces ROS levels through TrxR inhibition and apoptosis of cancer cells. TrxR overexpression is related to invasive tumor development and survival rates of patients diagnosed with breast, ovarian, and lung tumors (17). Thus, the TrxR-inhibitor auranofin may be a potent anticancer agent (18).

Although the antirheumatic drug auranofin has shown anticancer properties, the effect of anti-CSCs on BC remains unknown, and the mechanisms have not been explored. In this study, the suppression of BC-derived mammosphere formation by auranofin was investigated. In particular, the ability of auranofin to regulate mammosphere formation through Stat3 signaling was examined.

RESULTS

Auranofin suppresses cell growth, migration, and colony formation

The viability test was performed to assess the inhibitory effect of auranofin (Fig. 1A) on the growth of BC cells (MCF-7 and MDA-MB-231). BC cells were treated with auranofin at several concentrations (0, 0.2, 0.4, 0.6, 0.8, 1, and 2 μM) for 24 h. Auranofin reduced the proliferation of BC cell lines (Fig. 1B, C). Auranofin (1 μM) decreased the colony formation and migration of BC cells (Fig. 1D, E). Thus, auranofin suppresses the viability, colony formation, and migration of BC cells.

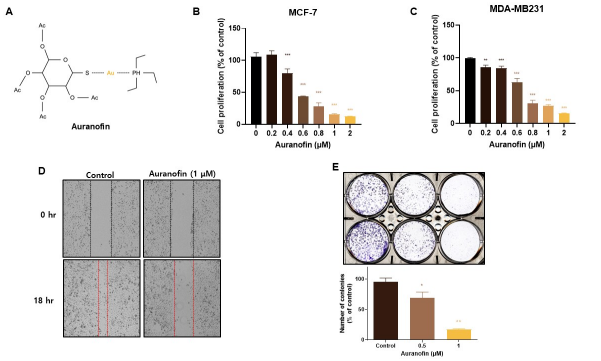

Fig. 1. Auranofin reduces the proliferation of breast cancer cell lines. (A) Molecular structure of auranofin. (B) Proliferation of MCF-7 cells. The cells were treated with increasing concentrations of auranofin for 24 h. (C) Proliferation of MDA-MB231 cells. The cells were treated with increasing concentrations of auranofin for 24 h. (D) Images of the wound-healing assay. MDA-MB231 cells were treated with 1 μM auranofin. Representative images were captured after 18 h of auranofin treatment (magnification, 10×). (E) Colony formation assay on MDA-MB 231 cells. The cells were treated with the indicated concentration of auranofin. The number of colonies was scanned and quantified after 10 days. Experimental values are represented as means ± SDs (n = 3, independent experiments). *P < 0.05; **P < 0.01 or ***P < 0.001 compared with control as determined by the one-way ANOVA followed by Dunnett post-hoc test.

Auranofin-induced apoptosis of BC

To examine the apoptosis effect of auranofin on BC cells, an apoptosis assay was performed using Annexin V-PI staining, caspase3/7 activity, and Hoechst 33258 dye. The apoptotic bodies of cancer cells were induced at 0.5 μM auranofin (Fig. 2A). The apoptotic cells were counted and enhanced by 0.5 μM auranofin (Fig. 2B). The proportion of early apoptosis increased from 8.9% to 37.0%. Caspase-3/7 activity testing revealed that auranofin increased caspase activity (Fig. 2C).

Auranofin suppresses mammosphere formation

To investigate whether auranofin can suppress mammosphere formation, auranofin was administered with the tumorsphere from human BC cells. In Fig. 3, auranofin decreased tumorsphere formation. The number of mammospheres decreased to 10%, as well as the size (Fig. 3A, B).

Auranofin decreased $CD44^{high}/CD24^{low}$ and ALDH-positive populations

The $CD44^{high}/CD24^{low}$ and ALDH1A-positive populations represent the breast CSC population (19). MDA-MB-231 cells were seeded in 6-well plates at 1×10^6 cells per plate for 24 h and administered with/without auranofin for 24 h. Auranofin decreased the cell proportion of the $CD44^{high}/CD24^{low}$ subpopulation from 39.8% to 16.0% (Fig. 4A). As a result, auranofin negatively influenced the traits of breast CSCs. Cancer cells were incubated with auranofin (0.5 μM) for 24 h, and an ALDEFLUOR

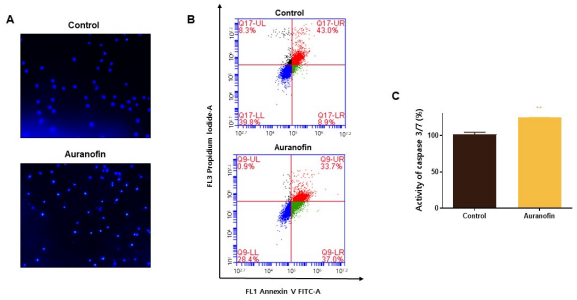

Fig. 2. Induction of apoptosis in breast cancer by auranofin. (A) Image of Hoechst 33342 staining for detecting apoptosis in MDA-MB231 cells. Images were captured after 24 h of 1 μM auranofin treatment (magnification, 10×). (B) Apoptosis assay using Annexin V/PI double staining in MDA-MB231 cells. The cells were treated with 1 μM auranofin for 24 h. The statistics show the percentages of the cells represented by alive (blue), early apoptosis (green), late apoptosis (red), and dead (black) cell populations. (C) Caspase 3/7 activity in MDA-MB231. The cells were treated with 1 μM auranofin for 24 h. The assay was performed using the Caspase-Glo® 3/7 Assay System. Experimental data are represented as means ± SDs (n = 3, independent experiments). **P < 0.01 compared with control as determined by the one-way ANOVA followed by Dunnett post-hoc test.

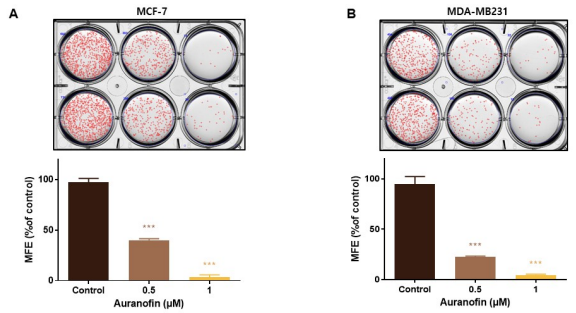

Fig. 3. Auranofin inhibits mammosphere formation. (A) Effect of auranofin on the mammosphere formation in MCF-7 cells. The cells were treated with the indicated concentrations of auranofin during mammosphere formation. Auranofin-treated mammosphere formation was reduced as shown in the images and statistics. (B) Effect of auranofin on the mammosphere formation in MDA-MB 231 cells. The cells were treated with the indicated concentrations of auranofin during mammosphere formation. The auranofin-treated mammosphere formation was reduced as shown in the images and graphs. Experiment data are represented as means ± SDs (n = 3, independent experiments). *** $P < 0.001$ compared with control as determined by the one-way ANOVA followed by Dunnett post-hoc test.

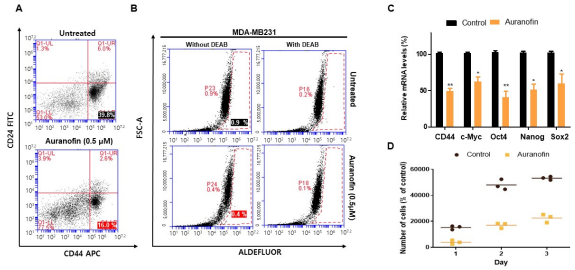

Fig. 4. Inhibitory effect of auranofin on the cancer stem cell marker of MDA-MB-231 cells. (A) Expression of the mammosphere marker $CD44^+/CD24^-$. Mammospheres derived from MDA-MB231 cells were treated with 1 μM auranofin for 24 h and detected using a flow cytometer. Data show the percentages of the cells represented by $CD44^+/CD24^-$ (black and red). (B) ALDH expression on auranofin-treated MDA-MB-231 cells. MDA-MB231 cells were treated with 1 μM auranofin for 24 h. The assay was detected using ALDEFLUOR™ Kit and flow cytometer. The data show the percentages of the cells represented by BAAA, a substrate for ALDH. (C) Expression of CSC-related genes on auranofin-treated mammospheres derived from MDA-MB231. The mammospheres were treated with 1 μM auranofin for 24 h. The mRNA levels of CD44, c-Myc, Oct4, Nanog, and SOX2 were assessed by RT-qPCR. β-actin was used as the loading control. (D) Inhibitory effect of auranofin on mammosphere formation. Cultured mammospheres were treated with 1 μM auranofin for 2 days. Auranofin-treated mammospheres dissociated into single cells and cultured in equal numbers. Experimental data are represented as means ± SDs (n = 3, independent experiments). *P < 0.05; **P < 0.01 compared with control as determined by the one-way ANOVA followed by Dunnett post-hoc test.

assay kit was used to investigate the effect of auranofin on ALDH-positive cells. Auranofin reduced the percentage of ALDH-positive cells from 0.9% to 0.4% (Fig. 4B). Auranofin suppressed the key CSC hallmarks, including $CD44^+/CD24^-$-expressing and ALDH1A-positive cells.

Auranofin inhibits the expression of CSC-related genes and reduces mammosphere growth

The results revealed that auranofin affected breast CSC formation. Moreover, auranofin suppressed the expression of CSC-related genes such as *c-myc*, *Oct4*, *CD44*, *Sox2*, and *Nanog* (Fig. 4C). To assess the effects of auranofin on mammosphere growth, mammospheres were treated with auranofin. An equal number of cancer cells derived from mammospheres with/without auranofin treatment were plated in 6-cm dishes everyday, and auranofin was found to inhibit mammosphere proliferation (Fig. 4D). These findings suggest that auranofin inhibits breast CSCs.

Auranofin suppresses the Stat3 and IL-8 signaling pathways

To explore the molecular mechanism of the effect of auranofin on breast CSCs, NF-κB and Stat3 signals and extracellular IL-6 and IL-8 levels in auranofin-treated mammospheres were analyzed. Initially, p65, Stat3, and phosphorylated Stat3 (pStat3) protein levels were examined under auranofin, and auranofin was found to reduce the levels of nuclear pStat3 proteins in breast CSCs. The pStat3 levels decreased in the nucleus fractions of CSCs (Fig. 5A). In addition, the DNA-binding activity in auranofin-treated nuclear extracts was assessed using a biotin-labeled Stat3 probe, and the results revealed that auranofin treatment reduced the Stat3-DNA-binding function (Fig. 5B, lane 3). The specificity of Stat3-binding was tested using a self-competitor (100×) (Fig. 5B, lane 4) and a mutated Stat3-oligo (100×) (Fig. 5B, lane 5). The band marked by the arrow (Fig. 5B) represents a Stat3-specific DNA complex. Extracellular IL-6 and IL-8 act as survival factors in CSCs (20). Stat3 controlled *IL-8* transcription by attaching to the *IL-8* promoter. To measure the IL-8 secretion, a cytokine assay was conducted with a mammosphere culture medium, revealing that auranofin treatment reduced the levels of IL-8 (Fig. 5C). Overall, auranofin inhibited mammosphere formation by inhibiting Stat3/IL-8 signals.

DISCUSSION

BC is the most common cancer in women (21). Although the therapeutic approaches for BC are well-known, they remain the primary reason for cancer-related mortality in this demographic (22). Stage IV metastatic BC can spread to other body parts, such as the bones, lungs, brain, or liver, and is the primary cause of BC-related deaths. The triple-negative BC showed significant heterogeneity, drug resistance, and tumor development, making it harder to treat and reducing the survival rate of patients (23).

The first experiment on the existence of CSCs started from the research of human acute myeloid leukemia (24); currently, CSCs have been recognized in many human tumors (25). Many researchers have demonstrated that CSCs are resistant to

standard chemotherapy and radiotherapy and that they may be the source of metastasis (26). Therefore, CSCs can be a prospective therapeutic target for BC. ALDH1A and CD44^{high}/CD24^{low} are recognized as biomarkers of breast CSCs (27), and targeting these CSCs in BC therapy presents an effective approach for treating patients with BC.

Several drugs were investigated as potential anti-cancer stem cell (CSC) agents, including metformin, salinomycin, and natural product, sulforaphane. Metformin, a diabetes medicine, has been found to selectively kill cancer stem cells in breast cancer models through AMPK activation. Salinomycin is an antibiotic that has shown potential for cancer treatment, particularly targeting cancer stem cells (CSCs) by interfering with ABC drug transporters. Sulforaphane (SFN) is one of the naturally occurring agents, and can target a specific cancer cell population displaying stem-like properties, known as cancer stem cells (CSCs).

Auranofin is a gold salt used as an antirheumatic drug and has exhibited antitumor effects on various cell and tumor models (28). Auranofin inhibited the activity of thioredoxin reductase (TrxR), which is a key enzyme involved in regulating the intracellular redox balance. TrxR inhibition in cancer increases cellular oxidative stress and triggers cell death (29). To our knowledge, the effects of auranofin and cellular mechanisms on anti-CSCs remain unclear, and limited information is currently available. This study showed the mechanisms that support the anticancer and anti-CSC action of auranofin.

In this study, the molecular mechanism of auranofin against breast CSCs was investigated. Auranofin inhibited cancer cell growth and tumorsphere formation (Fig. 1 and 3). Auranofin also increased the apoptotic BC cell population (Fig. 2). Auranofin reduced the levels of breast CSC biomarkers CD44^{high}/CD24^{low}, ALDH1A population levels, and expression levels of stem marker genes (*Oct4, Sox2, CD44, c-Myc,* and *Nanog*) (Fig. 4). In summary, the results suggest that auranofin effectively inhibits breast CSCs.

Stat3 has multiple biological roles and is constitutively activated in several human solid tumors such as head and neck cancer, BC, prostate cancer, and hepatocellular carcinoma (30, 31). Stat3 also controls the functions of the CSCs. Stat3 is crucial for initiating and progressing CSCs (32). CSCs are controlled by the tumor microenvironment components, growth factors, and cytokines. Cytokine networks such as the Stat3, IL-6, and IL-8 signals serve as key factors of breast CSCs (33). Our findings showed that auranofin inhibited breast CSC formation by suppressing Stat3 signaling (Fig. 5). Auranofin inhibits BCSCs through Stat3 signaling, although another mechanism may contribute to this effect. Extracellular IL-6 and IL-8 are recognized as crucial factors that support the survival and formation of breast CSCs (34, 35). Stat3 controlled *IL-8* expression by interacting with the *IL-8* promoter (36). To test the extracellular level of IL-8, a cytokine profiling experiment was performed using a mammosphere culture medium, which revealed that auranofin reduced the level of extracellular IL-8

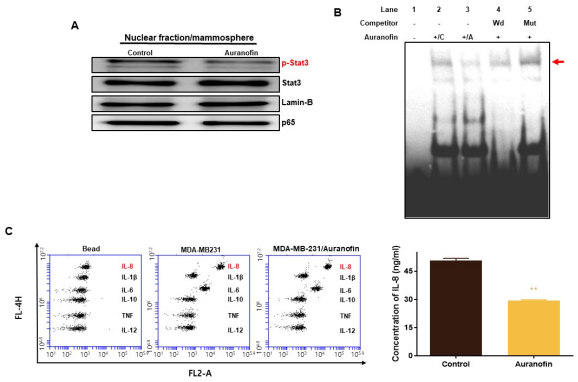

Fig. 5. Auranofin inhibits mammosphere formation through the Stat3/IL-8 axis. (A) Expression levels of Stat3, p-Stat3, and p65 protein in the nuclear fraction. The mammospheres derived from MDA-MB231 were treated with 1 μM auranofin for 1 day. (B) A gel shift assay was performed to analyze the interaction of the Stat3 protein with the Stat3 probe DNA. Mammospheres derived from MDA-MB231 cells were treated with 1 μM auranofin for 2 days. Lane 1, probe only; lane 2, nuclear proteins (with probe); lane 3, auranofin-treated nuclear proteins (with probe); lane 4, 10× self-competition. The arrow indicates the DNA/Stat3 interaction in the nuclear lysates. (C) Cytokine profiling in the auranofin-treated mammosphere. The amounts of cytokines were quantified using a flow cytometer, and the IL-8 level was represented in a graph. An assay was performed using an auranofin-treated mammosphere culture medium. Experimental data are represented as means ± SDs (n = 3, independent experiments). $^{**}P < 0.01$ compared with control as determined by the one-way ANOVA followed by Dunnett post-hoc test.

(Fig. 5). Eventually, auranofin inhibited mammosphere formation by disrupting the Stat3/IL-8 signaling pathway. Therefore, IL-8 secreted by breast CSCs promotes drug resistance and metastasis of the tumor cells (37). The inhibition of the IL-8 and Stat3 pathways as breast CSC-related targets for BC therapy requires additional research. The level of IL-8 is high in BC and is essential in regulating breast CSCs. A study showed that targeting IL-8 signaling suppressed breast CSC activity (38). Significantly, our findings reveal that auranofin inhibits IL-8 signals and CSC formation.

MATERIALS AND METHODS

Chemicals

Auranofin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Auranofin was made with dimethylsulfoxide with a stock concentration of 10 mM. Before treatment, auranofin was diluted to the required concentration in the medium.

Cell culture and media

MCF-7 and MDA-MB231 cancer cell lines were acquired from ATCC (Rockville, MD, USA) and cultured in Dulbecco's Modified Eagle Medium (Gibco, Carlsbad, CA, USA) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum

(Gibco). The medium was replaced every 2 days. All cells were incubated at 37°C under 5% CO_2.

Mammosphere formation assay

MCF-7 (4×10^4 cells per well) and MDA-MB-231 cells (2×10^4 cells per well) were seeded in ultralow attachment dishes, cultured with the mammosphere culture media (MammoCult™ Medium, STEMCELL Technologies, Vancouver, BC, CA), and then treated with two concentrations of auranofin (0.5 and 1 μM). After incubation for 7 days, the mammosphere (size > 60 μm) was observed. The number of mammospheres was counted using the NICE program following image scanning (39).

MTS assay

BC cells were plated in a 96-well plate. The cancer cells were administered with several concentrations (0, 0.2, 0.4, 0.6, 0.8, 1, and 2 μM) of auranofin for 24 h. The cell proliferation assay was performed through the MTS method, following the supplier's guideline of the Promega Cell Titer-Glo Cell viability kit (Promega, Madison, WI, USA).

Migration assay

MDA-MB231 cancer cells were seeded in a 6-well plate until the cells reached full confluency. A scratch wound was made in each well using a yellow tip and then washed with 1xDPBS. The cells were then treated with/without auranofin. Eighteen hours after scratching, the scratch wound area was examined under a microscope (Nikon, Tokyo, Japan).

Colony formation

Cells (1,000 cells per well) were plated in a 6-well plate and treated with auranofin for 10 days. The cells were rinsed with 1xDPBS, fixed with 4% formaldehyde, and stained with 0.05% crystal violet for 30 min. The NICE program following image scanning was employed to count the colonies.

Annexin V/PI staining

Cells (2×10^5 per well) were plated onto 60-mm plates for 24 h and treated with auranofin at 0.5 and 1 μM. The cells were rinsed with 1xDPBS, and the number of apoptotic cells was counted with the Annexin V Apoptosis Detection Kit I (BD, San Jose, CA, USA) for 15 min in the dark. A flow cytometer was used to detect apoptotic cells. The cancer cells were stained with Hoechst 33342 dye (Thermo Fisher Scientific Inc., Waltham, MA, USA) to visualize apoptosis, and images of apoptotic cells were captured using the Lionheart (Biotek, Agilent Technologies, Santa Clara, CA, USA).

Caspase 3/7 assay

Cells (2×10^5 per well) were seeded onto 60-mm plates for 1 day and cultured with auranofin. Caspase 3/7 activity was examined using the Caspase-Glo® 3/7 Assay kit (Promega, Madison, WI, USA) according to the protocol recommended by the manufacturer. Values were quantified using a plate reader (SpectraMax i3X, Molecular device, San Jose, CA, USA).

$CD44^{high}/CD24^{low}$ population analysis using a flow cytometer

Cells (2×10^5 per well) were plated onto 60-mm plates for 24 h and cultured with auranofin. The cells were trypsinized and then treated with FITC anti-CD44 and APC anti-CD24 antibodies for 30 min at 4°C. The labeled cells were examined through FACS after washing.

Aldehyde dehydrogenase (ALDH) expression analysis

Cells (2×10^5 per well) were plated onto 60-mm plates for 24 h and treated with auranofin. ALDH expression was analyzed using a flow cytometer and an ALDEFLUOR kit (STEMCELL Technologies). The cells were detached using trypsin and labeled according to the manufacturer's recommended protocol. Samples incubated with diethylamino benzaldehyde served as the negative control.

RNA extraction and quantitative reverse-transcription polymerase chain reaction (RT-qPCR)

Cells (2×10^5 per well) were seeded onto 60-mm plates for 24 h and cultured with auranofin. Total RNA was extracted using the Trizol RNA Extraction reagent. RT-qPCR was performed with TOPreal™ One-step RT-qPCR Kit (Enzynomics, Daejeon, South Korea). RT-qPCR conditions followed by the manufacturer's recommended protocol. *β-actin* was used as a control. The following primer pairs were used: Oct4, forward primer: AGCAAAACCCGGAGGAGT, reverse primer: CCACA TCGGCCTGTGTATATC; SOX2, forward primer: TTGCTGC CTCTTTAAGACTAGGA, reverse primer: CTGGGGCTCAAAC TTCTCTC; CD44, forward primer: AGAAGGTGTGGGCAGAA GAA, reverse primer: AAATGCACCATTTCCTGAGA; c-Myc, forward primer: AATGAAAAGGCCCCCAAGGTAGTTATCC, reverse primer: AGCAAAACCCGGAGGAGT; Nanog, forward primer: ATGCCTCACACGGAGACTGT, reverse primer: AAGTGGGT TGTTTGCCTTTG; and β-actin, forward primer: TGTTACCAAC TGGGACGACA, reverse primer: GGGGTGTTGAAGGTCTCAAA.

Western blot analysis

Protein solutions were isolated from the mammosphere using RIPA buffer supplemented with protease inhibitors and phosphatase inhibitors (Thermo Fisher Scientific Inc.). A method previously described for nuclear extraction was employed (40). The proteins were separated by gel electrophoresis (10% sodium dodecyl sulfate-polyacrylamide gel) and electrotransferred to polyvinylidene fluoride membranes (Millipore, Burlington, MA, USA). The membranes were blocked in 5% bovine serum albumin at room temperature for 1 h, and primary antibodies were then applied and incubated overnight at 4°C. Afterward, the membranes were rinsed with PBS-Tween and subsequently incubated with the secondary antibodies for 1 h. Anti-pStat3 (Cell Signaling, Danvers, MA, USA), anti-p65, anti-Stat3, and anti-β-actin (Santa Cruz Biotechnology, Dallas, TA, USA) were used.

Nuclear extract preparation

Nuclear extracts were prepared from mammospheres derived from MDA-MB-231 cells as described previously (40). In brief, mammospheres were cultured for 7 days, harvested, and washed with phosphate-buffered saline. All of the following steps were performed at 4°C. The cells were resuspended in sucrose buffer (0.32 M sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol [DTT], 0.5 mM PMSF, and 0.5% Nonidet P-40). The lysate was microcentrifuged at 500 g for 5 min to pellet the nuclei, which were washed with sucrose buffer Nonidet P-40. The nuclei were resuspended in low salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 0.02 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF), followed by addition of high salt buffer to extract the nuclei, with incubation for 20 min on a rotary platform. Diluent (2.5 vol. of 25 mM HEPES, pH 7.6, 25% glycerol, 0.1 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF) was added, and the sample was microcentrifuged at 13,000 g. Aliquots of supernatant (nuclear extracts) were stored at −80°C.

Gel shift assay

A previously documented protocol was performed (40). The gel shift assay was performed using a Lightshift Chemiluminescent EMSA kit (Thermo Fisher Scientific) according to the manufacturer's recommended protocol. Mammospheres were derived from BC cells treated with auranofin. The sense and antisense strands of the Stat3 probe labeled with biotin were annealed, and the double-strand Stat3-specific oligonucleotides were labeled at the ends of the biotin. The 5′-biotin-labeled upper and lower strands of the Stat3-specific probe were as follows: 5′-CTTCATTTCCCGGAAATCCCTA-Biotin-3′, 5′-TAG GGATTTCCGGGAAATGAAG-biotin-3′. The 5′-biotin-tagged DNA probes were combined with an auranofin-treated nuclear sample, resulting in a total volume of 20 µl of the gel shift assay buffer that contained 1 µg/µl poly (dI-dC). The reaction samples were applied to a 6% polyacrylamide non-denaturing gel and electrophoresed in 0.5X TBE. Super-shift bands were identified with the Lightshift Chemiluminescent EMSA kit (Thermo Fisher Scientific).

Inflammatory cytokine cytometric bead array

BC cell-derived mammospheres were incubated with auranofin for 2 days, and the culture media were then collected. Cytokine profiling of BC cells was conducted using a Human Inflammatory Cytokine Cytometric Bead Array kit (BD, San Jose, CA, USA) and analyzed with a flow cytometer. The procedures were performed according to the supplier's guidelines. Cytokine concentration was quantitated using BD FCAP array software (BD).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0. Values are presented as means ± standard deviations. Statistical tests included the one-way analysis of variance, followed by *Dunnett's* post hoc test. A P-value of 0.05 was regarded as significant.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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