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Original Article

Estrogen Induces Breast Cancer Cells Stemness by Activating PI3K /AKT / Mtor Signaling Pathway

Li Na[1,†], Fang Ma[1,2,†], Jing Chen[3], Li-bin Wang[1,4,5,*], Li-gang Wu[4,*]

[1]The Biobank of General Hospital of Ningxia Medical University, 750004 Yinchuan, Ningxia, China

[2]Clinical Laboratory of Yinchuan First People's Hospital, 750001 Yinchuan, Ningxia, China

[3]The drug preparation center of General Hospital of Ningxia Medical University, 750004 Yinchuan, Ningxia, China

[4]Surgical oncology of General Hospital of Ningxia Medical University, 750004 Yinchuan, Ningxia, China

[5]Shenzhen Nanshan People's Hospital; 518052 Shenzhen, Guangdong, China

[†]These Authors Contributed Equally.

[*]Corresponding Author: Li-bin Wang, Li-gang Wu

Abstract:
Background: Breast cancer (BC) is a malignant disease characterized by high morbidity and mortality. Estrogen plays a crucial role in the initiation and progression of BC, as well as in the development of BC stem cells (BCSCs). PI3K/AKT /mTOR Signaling Pathway holds significant importance in epithelial-mesenchymal transition and BCSCs generation. Therefore, thisthis study aims to explore the mechanisms by which estrogen influences the characteristics of BCSCs.
Methods:. MCF-7 BC cells were grouped as Control, Estradiol (E2), Fulvestrant and E2+Fulvestrant used for the study. CCK8, colony formation and wound healing assay were applied to identify the proliferation and migration of MCF-7 cells. study.CCK8, The Cell Counting Kit-8 (CCK-8), colony formation assay, and wound healing assay were employed to assess the proliferation and migration of MCF-7 cells. The expression of estrogen receptor alpha(ERα), BCSCs, Epithelial Mesenchymal transition (EMT), and the mTOR signaling pathway related genes and proteins were verified with qPCR and Western blot. The characteristics of BCSCs were identified using mammosphere formation assay and flow cytometry.
Results: Estrogen induced and promoted the expression of BCSCs-related genes, including ABCG2, Nanog, and Oct-4 in MCF-7 cells. Estrogen treatment also increased the proliferation, migration, and spheroid-forming ability of MCF-7 cells. Furthermore, estrogen enhanced EMT conversion via activation of the mTOR signaling pathway. **Conclusion**: Estrogen promotes BCSCs stemness by enhancing EMT and activating the mTOR signaling pathway.
Keywords: estrogen; breast cancer stem cells; epithelial-mesenchymal transition; mTOR signaling pathway

1. Introduction

Breast cancer (BC) is a malignant disease with high morbidity and mortality rates among women worldwide [1]. The occurrence of BC is associated with various factors, such as the environmental influences, hormonal changes, epigenetic modifications, gene mutations, and lifestyle differences [2]. Although surgical resection and neoadjuvant radiochemotherapy are standard treatments for BC, approximately 20% of patients still experience tumor metastasis and recurrence [3]. Therefore, understanding the mechanisms underlying BC progression and

identifying early diagnosis markers are important for improving BC treatment [4]. BC is classified into four subtypes based on the expression of three key receptor targets: Luminal A, Luminal B, HER2-positive, and Basal-like [5, 6]. Estrogen plays vital role in women's physiology and disease progression. Abnormal estrogen expression is a significant factor contributing to the increased incidence of BC.

The estrogen receptor (ER) is a group of proteins activated by the hormone estrogen. ER plays a key role in tumorigenesis by driving uncontrolled cancer cell division and is crucial in the initiation and progression of BC cells [7]. Estrogen can promote BC cell proliferation, migration, and chemoresistance, which are the basis of the occurrence and development of BC [8]. Endocrine therapy is often used to antagonize estrogen treatment of ER-positive BC. There are three common endocrine-targeted treatments for BC, including antagonizing ERs, downregulating estrogen expression, and inhibiting estrogen synthesis [9]. Tamoxifen and fulvestrant are two commonly used endocrine therapy medications that target ERα. Tamoxifen acts as an ER antagonist, while fulvestrant directly inhibits ERα and downregulates its expression. Although endocrine therapy is considered the most effective therapy, some ER-positive BC patients still exhibit resistance to this medical treatment [10].

Cancer stem cells (CSCs) are a distinct subset of cancer cells characterized by their high self-renewal ability and multi-lineage differentiation potential. CSCs drive cancer progression and contribute to resistance to clinical treatments [11]. Previous research has identified CSCs as key contributors to cancer cells metastasis and recurrence [12]. Breast cancer stem cells (BCSCs) are a specialized population of cancer cells that found within breast tumor tissues. BCSCs are characterized by the expression of $CD44^{+}/CD24^{-/low}$ and exhibit high activity of acetaldehyde dehydrogenase type 1 (ALDH1). This is often associated with drug resistance, recurrence, and poor prognosis in BC [13]. Abnormal genetic and epigenetic changes can induce somatic cells and normal stem cells to transform into BCSCs [14]. Epithelial-mesenchymal transition (EMT) is a crucial process for CSCs to maintain stemness. Through EMT, CSCs lose cell polarity and adhesion characteristics, which enhances tumorigenesis, facilitates distant metastasis, and increases their anti-apoptotic abilities [15].

It has been reported that EMT promotes the maintenance and differentiation of BCSCs in BC [16]. Therefore, elucidating the detailed molecular mechanisms between BCSCs and EMT is critical for developing effective BC treatment strategy [17]. The PI3K/AKT/mTOR signaling pathway is a key regulator involved in cancer cell proliferation, apoptosis, and survival [18]. In addition, this pathway helps maintain BCSCs' stemness by regulating the EMT of cancer cells[19]. The detailed mechanisms of this regulation are not yet fully understood and warrants further investigation.

In this study, we confirmed that estrogen can induce and promote the expression of BCSCs markers in MCF-7 cells. Estrogen can also influence the EMT process in MCF-7 cells and increase the proportion of BCSCs through mTOR signaling pathway. The results of this study clarify the molecular mechanism underlying the occurrence of BCSCs and provided a new potential treatment strategy for BC in the future.

2. Materials and Methods

2.1 Cell Culture

BC-related cell lines MCF-10A, MCF-7, BT474, and MDA-MB-231 were all purchased from the Chinese Academy of Sciences Cell Bank (CASCB). The catalog number of these cell lines are as follows: MCF-10A (Delf-10495), MCF-7 (SCSP-531), BT474 (TCHu143), and MDA-MB-231 (SCSP-5043). All cell lines were tested for mycoplasma contamination and have undergone Cell Line Authentication by STR profiling.The cells were cultured with Dulbecco's Modified Eagle Medium (DMEM) (11965118, Gibco, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS) (A5670701, Gibco, Grand Island, NY, USA) and 1% Penicillin-Streptomycin Solution (15140122, Thermo Fisher Scientific, Waltham, MA, USA).

To enrich BCSCs, the following supplements were added to DMEM/F12 medium: 1:50 B27 (17504044, Gibco, Grand Island, NY, USA), 1:50 Penicillin-Streptomycin Solution (Thermo Fisher Scientific, Waltham, MA,), 20 μg/L epidermal growth factor (EGF) (AF-100-15, PeproTech, Rocky Hill, NJ,),20 μg/L basic fibroblast growth

factor (bFGF) (AF-100-18B, PeproTech, Rocky Hill, NJ), and 5 μg/L insulin (I2643, Sigma-Aldrich, St. Louis, MO).

The cells were seeded in Ultra-Low Attachment 6-well plates (3471, Corning Incorporated, NY) at a density of 1000 cells/well, with 2 mL of prepared medium. The medium was refreshed every three days by adding an additional 1 mL of the aforementioned prepared medium. The cells were cultured under low oxygen conditions (volume fraction: 94% N_2, 5% CO_2, and 1% O_2) for nine days to facilitate the formation of BCSC spheres. These spheres were subsequently observed under a microscope for quantification and measurement of their diameter. Following the completion of the culture, cells were harvested for subsequent experimental procedures. BCSCs were cultured in DEME/F12 medium (A4192002, Thermo Fisher Scientific, Waltham, MA), supplemented with B27, bFGF, EGF, and insulin. All cells were maintained in a 37 °C, 5% CO_2 incubator.

2.2 Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded into each well of a 96-well plate at a density of 1×10^4 cells/well, with three replicate wells for each experimental group. After incubation in a cell culture incubator for 24 hours, the corresponding treatments were administered. CCK-8 reagent (KGA9305-1000, Keygen biotech, Nanjing, Jiangsu, China) was prepared in the culture medium and added to each well at a volume of 100 μL (containing 10 μL CCK-8 reagent per 100 μL of culture medium), followed by further incubation at 37 °C for 1 hour. Estrogen (802921, Sigma-Aldrich, St. Louis, MO, USA) was used at concentrations of 2 nM, 4 nM, 8 nM, 10 nM, and 15 nM, and added to the medium to culture the cells. The incubation times were 24 hours, 48 hours, and 72 hours respectively. The optical density (OD) value at wavelength 450 nm was measured with biotek synergy H1 system(Biotek Winooski, Vermont, USA) after incubation periods of 24 hours, 48 hours, and 72 hours. Based on the OD values, the cell proliferation rate was calculated, and the optimal estrogen concentration was determined. Fulvestrant (Sigma-Aldrich, St. Louis, MO, USA) was used at concentrations of 5 μM, 10 μM, 20 μM, 40 μM, 80 μM, 160 μM, 320 μM, and 640 μM. The cells incubation times with fulvestrant were 24 hours, 48 hours, and 72 hours. The OD value of each well was measured, and the cell inhibition rate was calculated. The half-maximal inhibitory concentration (IC50) value of the effect of fulvestrant was calculated according to the modified Kou's method. The IC50 of MK-2206 (S1078, Selleck Chemicals, Houston, TX, USA) was also calculated using the same methods.

2.3 qPCR

The cells were collected, and total RNA was extracted. Following reverse transcription of total RNA into cDNA, qPCR was performed to detect stemness-related genes, including Oct-4, ABCG-2, Nanog, Sox-2, ALDH-1, E-cadherin, N-cadherin, Vimentin, Slug, Snail, PI3K, AKT, and mTOR *et al*. The primer sequences are provided in **Supplementary Table 1**.

Supplementary Table 1. Primer sequences of quantitative real-time PCR.

Name		sequences (5'→3')
ABCG-2	Forward	GTCGTACTGGGACTGGTTATAG
	Reverse	TCTCTACCACAAAGAGTTCCAC
Nanog	Forward	GATGCAAGAACTCTCCAACATC
	Reverse	CTGGTGGTAGGAAGAGTAAAGG
Oct-4	Forward	GTGGTCCGAGTGTGGTTCTGTAAC
	Reverse	CCCAGCAGCCTCAAAATCCTCTC
ALDH1	Forward	GACAATGCTGTTGAATTTGCAC
	Reverse	AAGGATATACTTCTTAGCCCGC
Sox-2	Forward	GCCGAGTGGAAACTTTTGTCG
	Reverse	AAGGATATACTTCTTAGCCCGC
E-cadherin	Forward	ATTCTGATTCTGCTGCTCTTG
	Reverse	ATGTCCTGGTCCTCTTCTCC
N-cadherin	Forward	CATCATCCTGCTTATCCTTGTG
	Reverse	CATAGTCCTGGTCTTCTTCTCC

Slug	Forward	CGAACTGGACACACATACAGTG
	Reverse	CTGAGGATCTCTGGTTGTGGT
Snail	Forward	ACTGCAACAAGGAATACCTCAG
	Reverse	GCACTGGTACTTCTTGACATCTG
Vimentin	Forward	GCTGGAAGGCGAGGCGAGGAGAG
	Reverse	CAACCGTCTTAATCAGAAGTGTC
ZEB1	Forward	TTACACCTTTGCATACAGAACCC
	Reverse	TTTACGATTACACCCAGACTGC
PI3K	Forward	CTACAGGGCTTGCTGTCTCC
	Reverse	GTCTGGGTTCTCCCAATTCA
AKT	Forward	CACTGTCATCGAACGCACCT
	Reverse	TCCATCTCCTCCTCCTCCTG
mTOR	Forward	ACTGGAGGCTGATGGACACA
	Reverse	GGCTCTCCAAGTTCCACACC
β-actin	Forward	GGCAGCGTGTACTTATCCTTCT
	Reverse	GGATAGCACAGCCTGGATAGCAA

All primers were synthesized by Sangon Bioteck (Sangon Bioteck, Shanghai, China). The qPCR reaction conditions were set as follows: 95 ℃ for 10 min for pre-denaturation, 93 ℃ for 15 s for denaturation and 60 ℃ for 30 s for 40 cycles. β-actin was used as the internal reference gene, and $2^{-\Delta\Delta Ct}$ was used to calculate the gene expression.

2.4 Western Blotting

Proteins from each group of cells were isolated using the whole protein extraction kit (KGB5303-100) (Keygen Biotech, Nanjing, Jiangsu, China). The protein concentration was measured through BCA method. Protein were loaded at a concentration of 20 µg per sample, and electrophoresis was conducted at 80–120 V for 90 min. After electrophoresis, the proteins were transferred to a 0.45 µm PVDF membrane (IPVH00010, Merck Millipore Billerica, MA, USA) at 240 mA, 300 V. The PVDF membrane was placed into the blocking solution containing 5% skimmed milk powder and incubated with gentle shaking for 1 hour at room temperature The primary antibodies (Cell Signaling Technology, Danvers, MA, USA) were diluted according to the manufacturer's instructions. The PVDF membrane was incubated overnight with the primary antibody. The following day, the membrane was removed and washed with 1x TBST, then incubated with the rabbit IgG secondary antibody at room temperature for 1 hour. The membrane was exposed using a Bio-Rad Gel Doc XR+ Gel Documentation System(Bio-Rad Laboratories, California ,USA). The expression of each protein was calculated with internal reference GAPDH (Abcam Cambridge, MA, USA). The primary antibody was reconstituted in TBST at a 1:1000 dilution, and the secondary antibody in TBST at a 1:5000 dilution, following the provided instructions. The catalog number was: ERα (8644S), GAPDH (5174S), ABCG2 (42078S), Oct-4 (2750S), Nanog (4903S), E-Cadherin (3195S), N-Cadherin (13116S), Vimentin (5741S), Snail (3879S), SLUG (9585S), ZEB1 (3396S), PI3K-p85 (4292S), P-PI3K-p85 (Tyr458)/p55 (Tyr199), AKT (9272S), P-Akt (Ser473) (4060S), mTOR (2972S), P-mTOR (Ser2448) (2976S), P70S6k (34475S), p-P70S6k (Thr389) (9234S). Goat Anti-Rabbit IgG (H+L) Antibody (35401S).

2.5 Flow Cytometry

1×10^5 MCF-7 cells were cultured with 1 mL medium. After treatment with estrogen, the BCSCs were harvested and washed with 1x Phosphate-Buffered Saline (PBS)(G4202,Servicebio Biotechnology, Wuhan, Hubei, China). The cells were then resuspend in 5 µL of ALDEFLUOR detection buffer (01700, StemCell Technologies, Vancouver, BC, Canada). Incubated at room temperature for 15 min. The proportion of ALDH1+ stem cells was detected by flow cytometry (BD FASArial, USA) and the data was analyzed using FlowJo software(BD FASArial, USA).

2.6 Cell Colony -formation Assay

Cells in the logarithmic phase were digested and centrifuged, then inoculated into a 6-well plate at a density of 1000 cells/well. After the cells adhere

to the surface, estrogen or fulvestrant was added for treatment at 24 hours, 48 hours, and 72 hours. Colony formation was observed under a microscope daily. To fix the cell morphology, 4% paraformaldehyde (G1101, Servicebio Biotechnology, Wuhan, Hubei, China)were used. After fixation, crystal violet (GC307002, Servicebio Biotechnology, Wuhan, Hubei, China) was applied to stain the cells. After staining, the dye solution was rinsed off under running water. Photos were taken, and colonies counts were performed using ImageJ V1.8.0(NIH,USA).

2.7 Wound Healing Assay

The cells were seeded into 6-well plate at 1×10^{-6} cells/well. After the cells had adhere to the surface, a 200 µL tips was used to draw a straight line along the diameter of the wound. Cells were then washed twice with 1x PBS buffer, and take an initial photographwas taken and saved at 0 h. Estrogen or fulvestrant was added for treatment at 24 hours, 48 hours, and 72 hours. The healing of the cell scratches was observed daily using an Olympus inverted microscope system (Olympus, Tokyo, Japan), and images were captured. The scratch distance was measured using Image J V1.8.0 to calculate the wound healing rate(%).The wound healing rate = (initial scratch width - scratch width after 24 hours)/Initial scratch width x 100%.

2.8 Statistics

Data analysis was conducted using GraphPad Prism 8.0 software (Dotmatics, Boston, MA, USA) and Statistical Package for the Social Sciences (SPSS) version 25 (IBM Corp., Armonk, NY, USA). An independent samples *t*-test or one-way analysis of variance (ANOVA) was performed, as appropriate, for statistical analysis. The following post hoc analysis for the one-way ANOVA was performed using Bonferroni correction. All data were derived from three biological replicates. Difference were considered significant if $p < 0.05$, and highly significant if $p < 0.01$.

3. Results

3.1 ERα Expression in Different BC Cell Lines

Western blotting was used to detect the expression of ERα in the following cell lines: the breast epithelial cell line MCF-10A, the ER-positive BC cell lines MCF-7 and BT474, and triple-negative BC cell line MDA-MB-231. The results showed that ERα was barely expressed in MDA-MB-231 and MCF-10A cells (Fig. 1A), while ERα expression was significantly higher in MCF-7 and BT474 cells ($p < 0.01$). Based on these results, MCF-7 cells were selected for subsequent experiments. Mammosphere formation assays were used for identify BCSCs, while qPCR and Western blottin were used to detect BCSCs-related genes and proteins. The results demonstrated that the expression of stem-related genes and proteins ABCG-2, Nanog, Oct-4, and Sox-2 was significantly increased in BCSCs mammosphere ($p < 0.05$) (Fig. 1B,C).

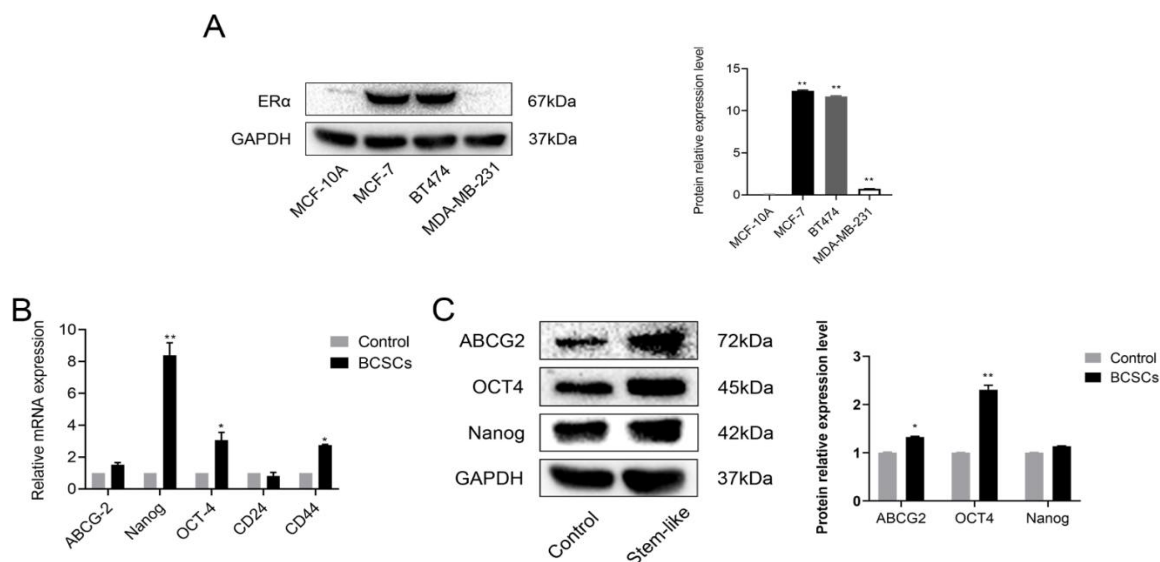
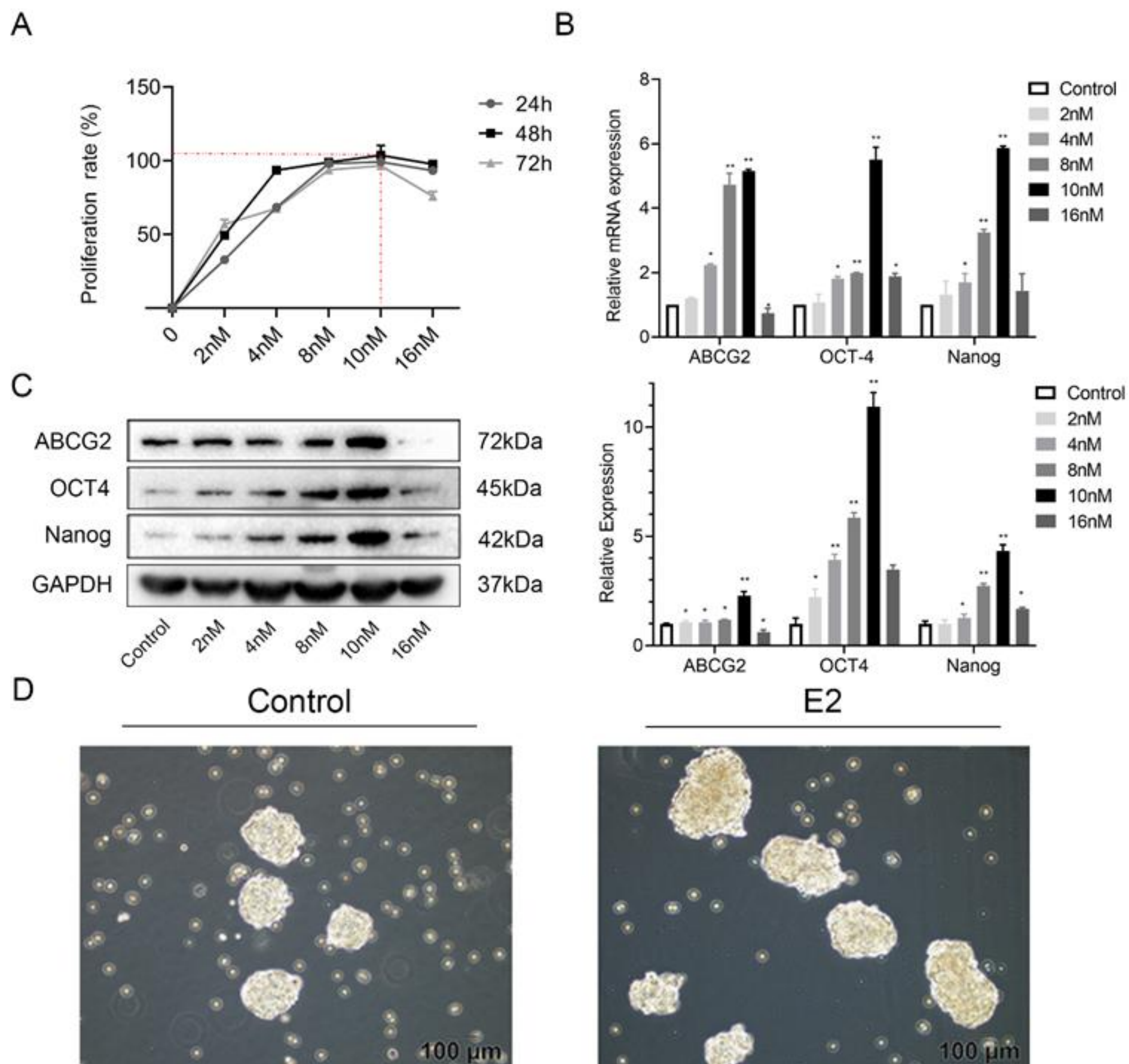


Fig. 1. Expression of ERα and stemness-related genes and proteins in BC cell lines and BCSCs. (A) Expression of **ERα in BC cell lines. (B,C) Expression of** stemness-related **genes and proteins in BCSCs**

(compare with MCF-10A, $p < 0.01$**,based on 3 replicates)). BC, breast cancer; BCSCs, breast cancer stem cells; CT, control; ERα, estrogen receptor alpha.**

3.2 Estrogen Effect of BCSCs Stemness

The effect of estrogen on **MCF-7** cell proliferation was measured using the CCK-8 assay. The results indicated that estrogen promotes MCF-7 cell proliferation, with the proliferation rate reaching its highest level at a concentration of 10 nM (Fig. 2A). The expression of BCSC stemness markers, both mRNA and protein, in MCF-7 cells was analyzed using qPCR and Western blotting. The results confirmed that ABCG-2, Nanog, Oct-4, were significantly increased at a concentration of 10 nM ($p < 0.01$) (Fig. 2B,C). The mammosphere formation assay results showed that both the volume and number of mammospheres were significantly increased following estrogen treatment ($p < 0.01$) (Fig. 2D). Additionally, the proportion of $ALDH1^+$ BCSCs was significantly increased in the estrogen treatment group ($p < 0.01$) (Fig. 2E). These results indicate that estrogen promotes BCSCs stemness in MCF-7 cells'.



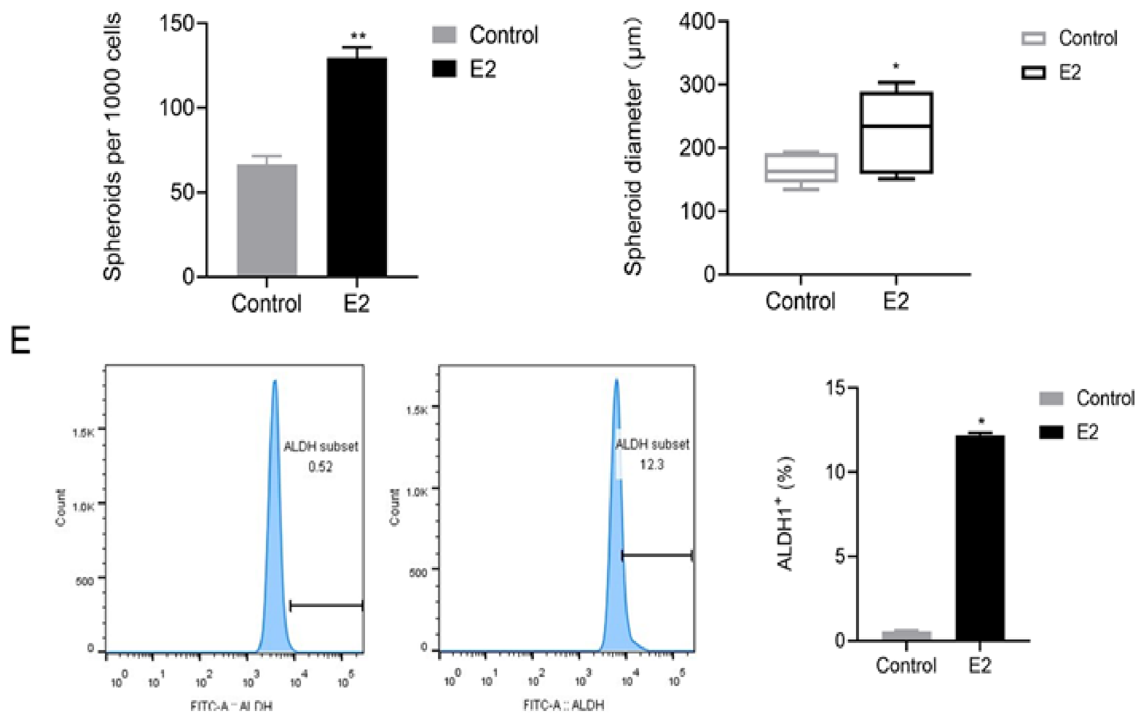


Figure 2. Effect of estrogen on BCSCs stemness. (A) Effect of estrogen on MCF-7 cell proliferation. (B,C) **mRNA and protein expression levels of BCSCs stemness markers in MCF-7 cells treated with estrogen**. (D) Effect of estrogen on **mammosphere formation in** BCSCs. (E) **Flow cytometry analysis of the proportion of ALDH1^{+} BCSCs in the estrogen treatment group (compare with control group, * $p < 0.01$, ** $p < 0.01$, based on 3 replicates).). ALDH1, acetaldehyde dehydrogenase type 1; BCSC, breast cancer stem cells; E2, Estradiol.**

3.3 Estrogen Effect on EMT and mTOR Signaling Pathway in MCF-7 Cells

The migration ability of MCF-7 cells affected by estrogen was tested using a wound healing assay. The results indicated that the healing rate and cell migration in the estrogen treatment group were significantly higher than in the control group (**p** < 0.05) (Fig. 3A). The clone formation assay was used to detect the effect of estrogen on the proliferation of MCF-7 cells. The number of cell clones formed in the estrogen treatment group was significantly increased (**p** < 0.01) (Fig. 3B). These results illustrate that estrogen can promote both proliferation and migration in MCF-7 cells.

The mRNA expression of EMT and mTOR signaling pathway-related genes was analyzed using qPCR. The results indicated that the expression of Vimentin, N-cadherin, Snail, Slug, ZEB1, PI3K, AKT, and mTOR was significantly increased in the estrogen treatment group (**p** < 0.01), whereas E-cadherin expression was significantly decreased (**p** < 0.01) (Fig. 3C,). Western blotting results demonstrated that E-cadherin protein expression was decreased in the estrogen treatment group, while the expression of Vimentin, N-cadherin, Snail, Slug, and ZEB1 was significantly increased). The expression of PI3K, AKT, mTOR, p70S6K proteins, was significantly increased (**p** < 0.01) (Fig. 3D). These results suggest that estrogen can influence EMT and the mTOR signaling pathway in MCF-7 cells.

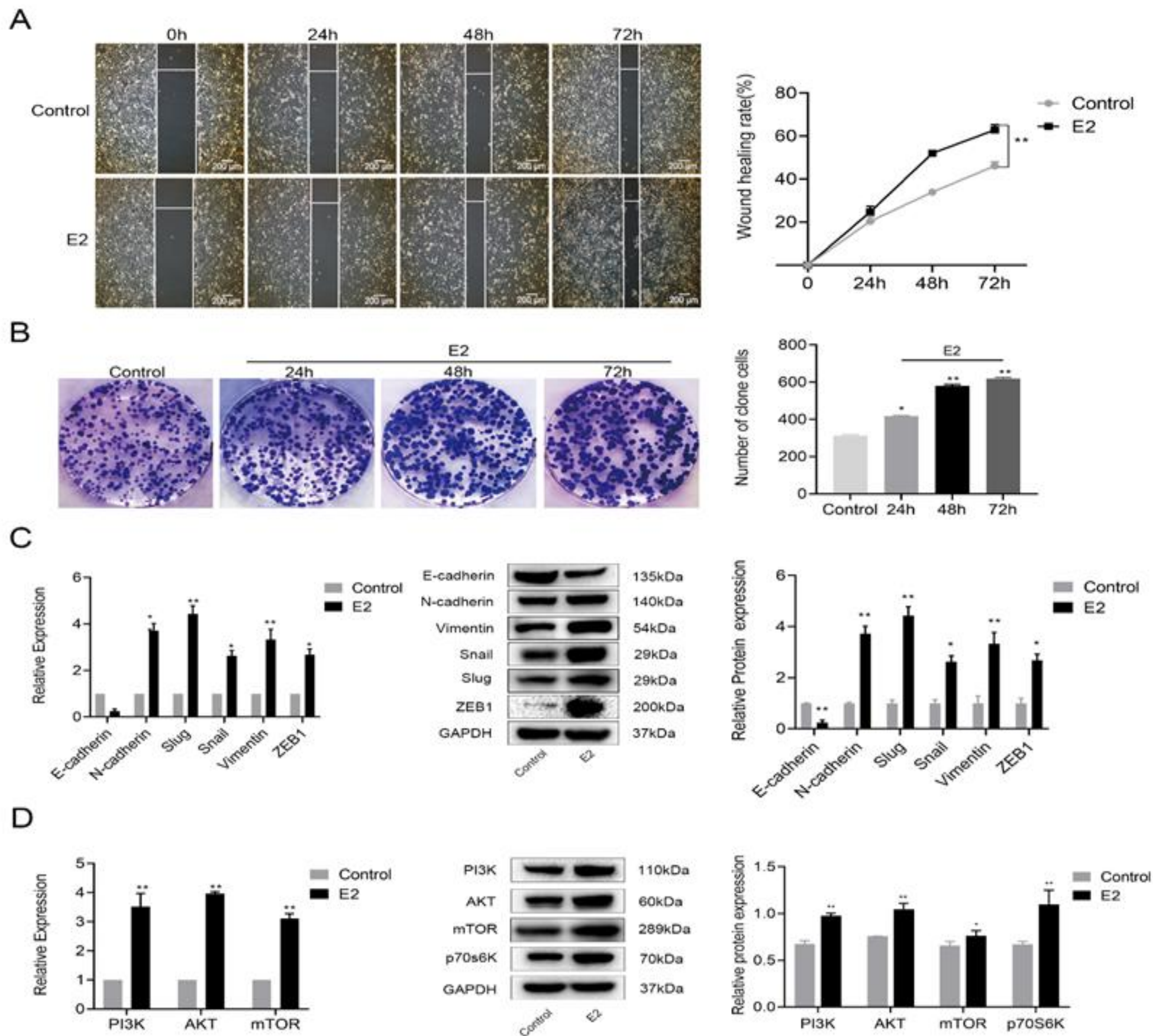


Figure 3. Effect of estrogen on EMT and PI3K/AKT/mTOR signaling pathway in MCF-7 cells. (A,B) Wound healing and colony formation assays demonstrated that estrogen accelerates MCF-7 cell proliferation. (C,) Impact of estrogen on the expression of EMT relatedpathway-related gene. (D) influenceEffect of estrogen on theexpression of PI3K signaling pathway-related protein expression (compare with control group,* p < 0.05, ** p < 0.01 based on 3 replicates).). E2, estradiol; EMT, epithelial-mesenchymal transition.

3.4 Influence of Fulvestrant on Estrogen Function through EMT and mTOR Signaling Pathway

Fulvestrant is an ER antagonist. It was used in this study to identify the relationship between estrogen and EMT in MCF-7 cells. The CCK-8 assay was used to determine the drug concentration and IC50 of fulvestrant. The results showed that fulvestrant inhibited MCF-7 cells proliferation, with the inhibition rate increasing with the drug concentration (Fig. 4A). The IC50 value was calculated using Kou's modified methods, resulting in a final cell concentration of 365 μM for a 48-hour exposure. Cell clone formation and wound healing assays were performed to assess the effect of fulvestrant i MCF-7 cells proliferation and migration. The results illustrated that both the number of cell clones and the migration rate were significantly reduced in the estrogen treatment group (**p** < 0.01) (Fig. 4B,C). These results confirm that fulvestrant inhibits MCF-7 cell proliferation and migration.

After fulvestrant treatment, proteins related to the EMT and mTOR signaling pathways were detected by Western blotting. The results proved that the expressions of Vimentin, N-cadherin, Slug, Snail, ERα, PI3K, and AKT were all significantly decreased in the estrogen+fulvestrant

treatment group. Meanwhile, E-cadherin expression was significantly increased (**p** < 0.01) (Fig. 4D,E). Consistent with these, fulvestrant can counteract the effects of estrogen on EMT through the PI3K/AKT/mTORsignaling pathway.

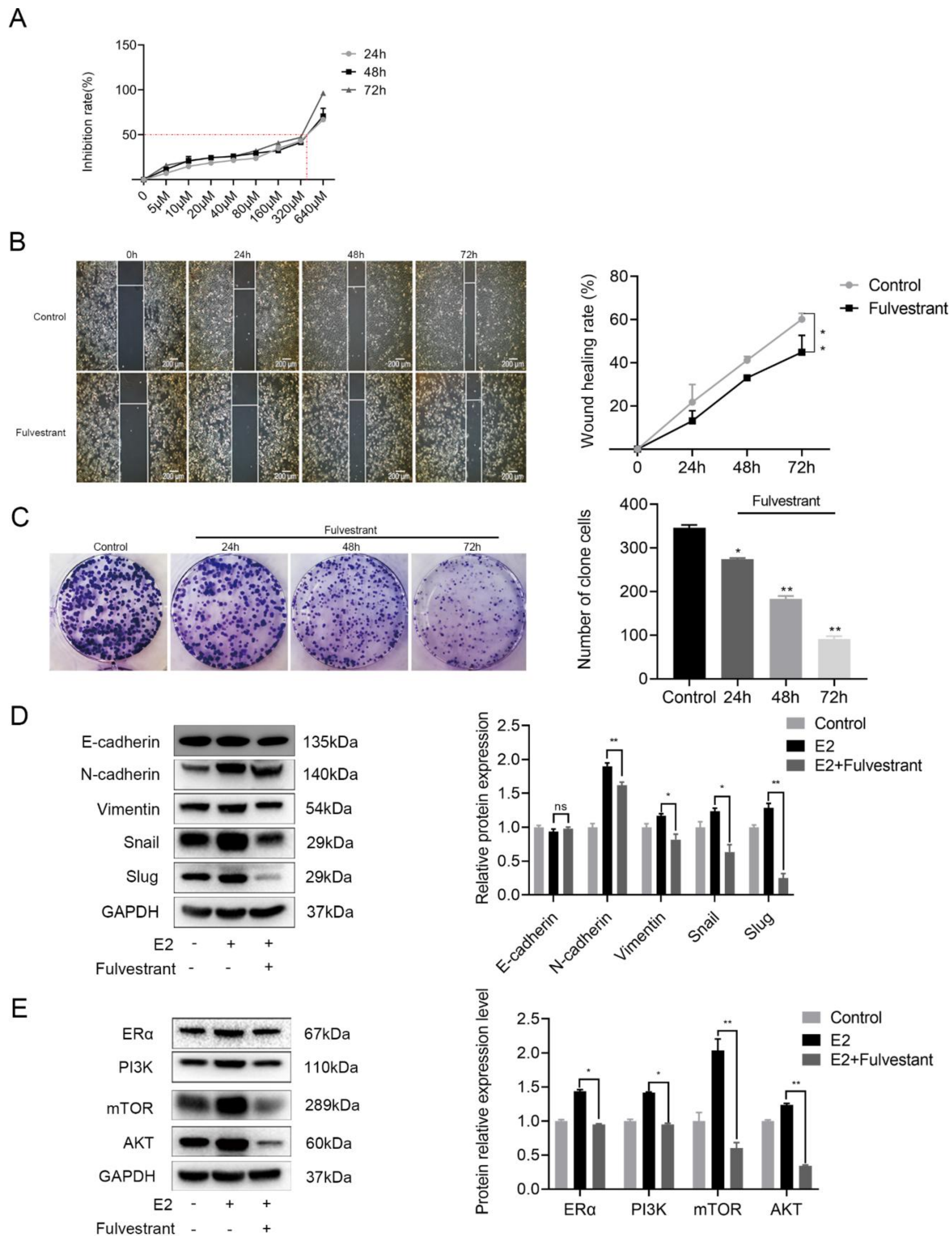
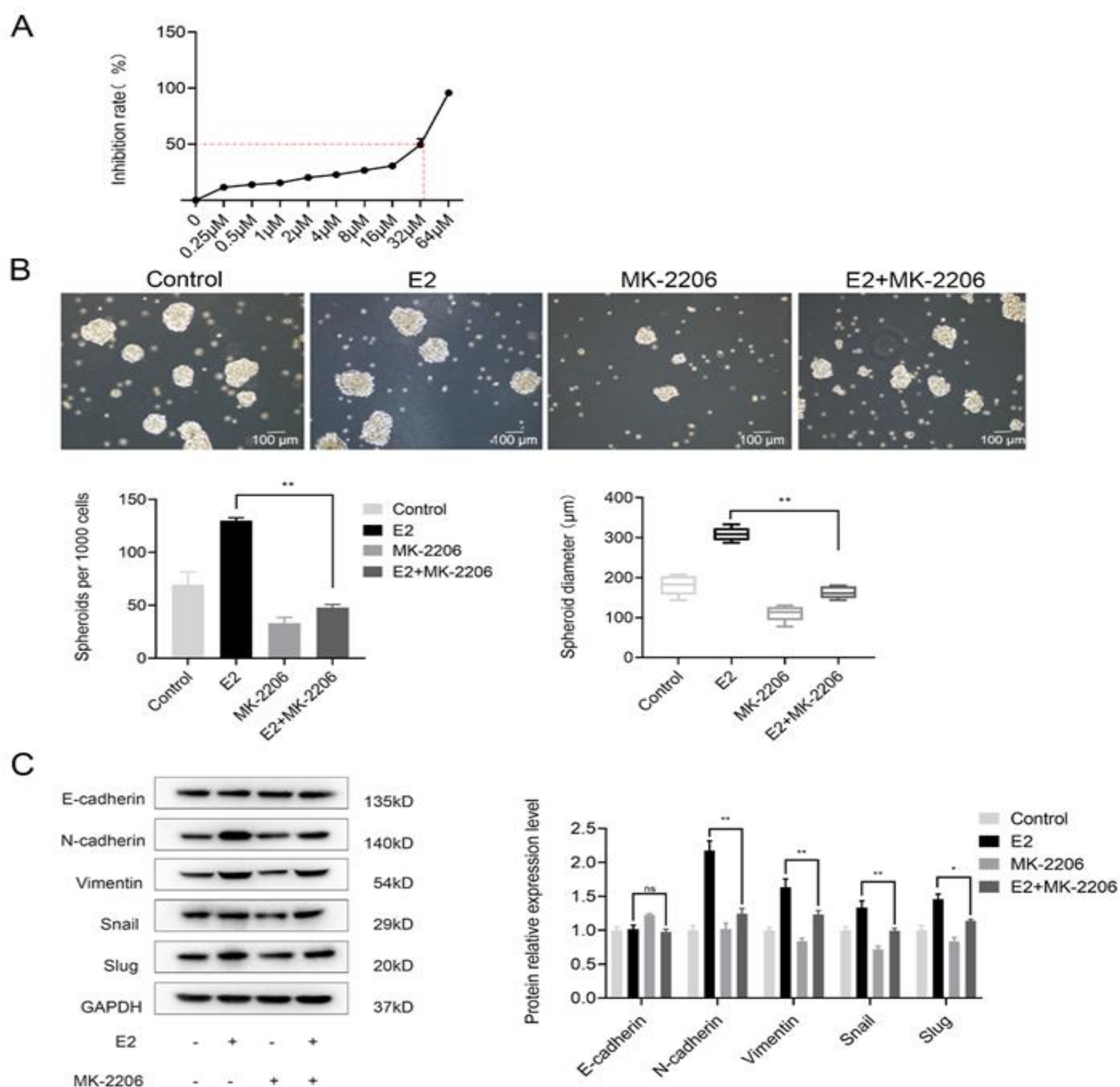


Fig. 4. Influence of fulvestrant on estrogen function through EMT and mTOR signaling pathway. (A) Treatment of MCF-7 cells with different concentrations of fulvestrant, with calculations of the IC50 value. (B,C) Effect of fulvestrant effect on MCF-7 cells proliferation and migration, assessed by colony formation and wound healing assays. (D,E) Effect of estrogen+fulvestrant on EMT and mTORPI3K/mTOR signaling pathway (compare to the E2 group,* p < 0.05, ** p < 0.01 based on 3 replicates).). E2: Estrodiol; EMT, epithelial-mesenchymal transition; IC50, half-maximal inhibitory concentration.

3.5 MK-2206 Inhibits BCSCs Stemness and mTOR Signaling Pathway

To further investigate the regulation of **mTOR signaling** pathway in BCSCs stemness, MCF-7 cells were treated with the AKT allosteric inhibitor MK-2206treat. The CCK-8 assay results demonstrated that MK-2206 had an inhibitory effect on MCF-7 cells in a concentration-dependent manner (Fig. 5A). The optimal conditions for MK-2206 treatment were 24 hours at 35.65 μM. By treating BCSCs using MK-2206, the mammosphere formation results showed that both the formation rate and diameter of BCSCsin the MK-2206 treatment group were significantly reduced (**p** < 0.01) (Fig. 5B). Western blotting analysis demonstrated that the protein expression EMT marker Vimentin, N-cadherin, Slug Snail and BCSCs stemness markers ABCG-2, Nanog, and Oct-4 was decreased in the MK-2206 treatment group (compare with E2 group , (Fig. 5C,D). These results indicate that MK-2206 inhibited E2 induced BCSCs self-renewal ability and suppressed stemness in MCF-7 cells.

Western blotting analysis was used to detect PI3K/AKT proteins related to the signaling pathway following MK-2206 treatment. AKT, mTOR, p70S6K, and the corresponding phosphorylated proteins P-mTOR, P-AKT, and P-p70S6K in the MK-2206 treatment group were all reduced, (**p** < 0.01) (Fig. 5E). Furthermore, the expression of mTOR-related protein (AKT, mTOR, p70S6K) were all significantly reduced in the MK-2206 treatment group (**p** < 0.01) (Fig. 5E). These results showed that MK-2206 can counteract the effects of estrogen on BCSCs. MK-2206 decreased the E2-inducedBCSCs stemness by inhibiting PI3K/AKT signaling pathway.



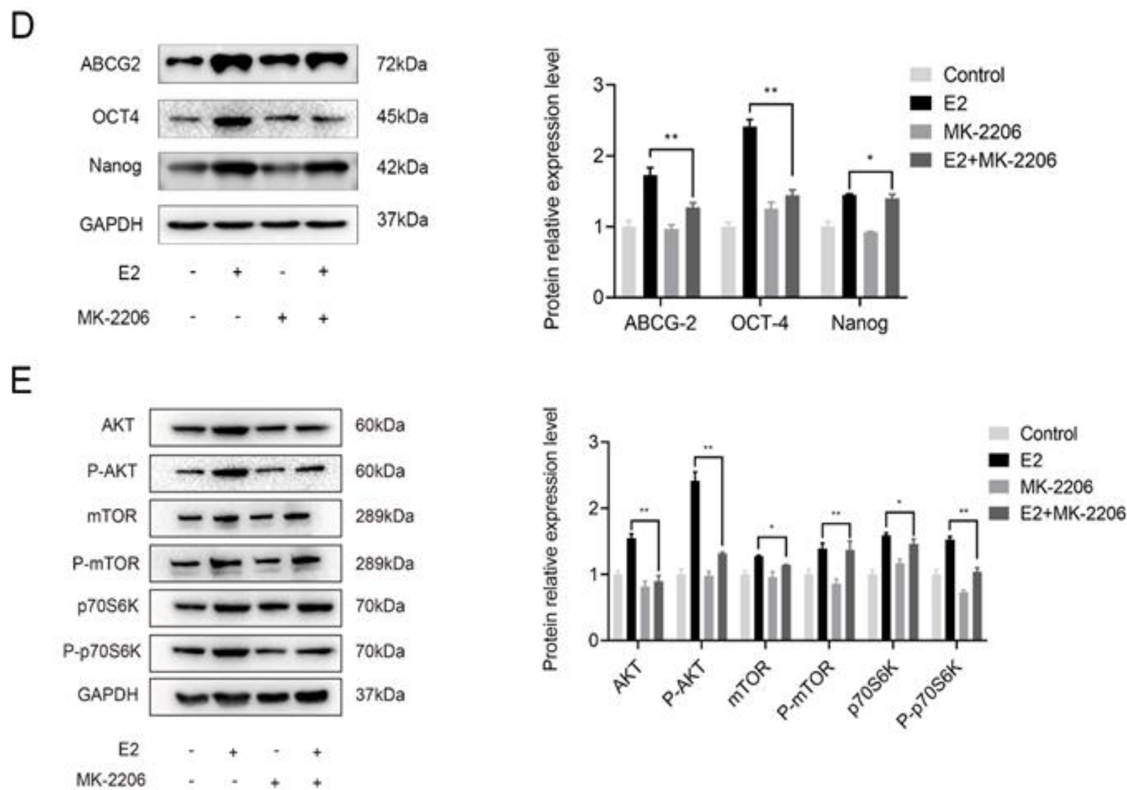


Fig. 5. MK-2206 inhibited E2 induced BCSCs stemness and the mTOR signaling pathway. (A) MCF-7 cells were treated with different concentration of MK-2206, and the IC50 value was calculated. (B) Mammosphere formation assays were conducted to determine the effect of MK-2206 on MCF-7 stemness. (C–E) Western blotting analysis was used to detect the expression of **stemness, EMT, and mTOR signaling pathway-related proteins, with or without MK-2206 (compare with E2 group,*treatment (* $p < 0.05$, ** $p < 0.01$, based on 3 replicates).). E2, Estrodiol; BCSC, breast cancer stem cell; EMT, epithelial-mesenchymal transition; IC50, half-maximal inhibitory concentration.**

4. Discussion

BC is a disease in which breast cells grow uncontrollably and is characterized by a range of molecular and genomic abnormalities [20]. According to recent statistics, approximately 75% of BC patients are diagnosed with ER-positive tumors [21]. The ER, including ERα and ERβ, is not only a typical sex hormone receptor but also functions as a transcription factor [22]. Expression of ER and estrogen secretion regulate the development of the reproductive system, including the ovary, endometrium, and breast. However, abnormal expression of ER and irregular estrogen secretion can lead to estrogen-dependent cancers or reproductive system hypoplasia [23]. Despite significant breakthroughs in the diagnosis and treatment of BC, the high recurrence rate remains a major challenge for cancer [24]. Commonly used targeted endocrine therapies can not only lead to tumor recurrence but also promote the development of more aggressive cells that enhance tumor metastasis [25].

To further understand the mechanism of tumor recurrence and metastasis, the concept of CSCs has been introduced. It is believed that tumors arise from CSCs with unlimited self-renewal and differentiation potential, which can generate different phenotypes and malignant cell populations [26]. The discovery of BCSCs provides new insights into the research and treatment of BC. Current methods for culturing BCSCs include suspension cell culture and 3D culture technology. These two methods are widely used in the research of BCSCs [27]. BCSCs are characterized by specific genes and cell surface markers, such as CD44, ALDH1, Nanog, Sox-2, and Oct-4, which can be used to identify them and reveal their unique functions [28]. Hu *et al.* demonstrated that reducing the expression of stemness markers can inhibit the number of BCSCs [29]. In the current study, we first identified that ERα was significantly expressed in

MCF-7 and BT474 cells (**p** < 0.01). We then cultured BCSCs using mammosphere formation assays and employed Western blotting and qPCR to detect BCSC-related genes and proteins. The results showed that stem-related genes and proteins ABCG-2, Nanog, Oct-4, and Sox-2, were all increased in BCSC mammosphere (**p** < 0.05). After treatment with estrogen, we confirmed that estrogen promotes BCSC stemness in MCF-7 cells.

EMT plays a key role in tumor cells invasion and metastasis. their also facilitates stem cell differentiation in different cancers [30,31]. Increased EMT and CSCs are considered to be the basis for BC metastasis, chemotherapy resistance, and tumor recurrence. E-cadherin, an epithelial cell marker, is an EMT-related gene that plays a crucial role in maintaining cell polarity and adhesion. In contrast, N-cadherin and Vimentin are markers of mesenchymal cells, often promoting cancer cells invasion and migration, which can lead to the growth of invasive cells [32]. Slug, Snail, ZEB1, and Twist are transcriptional regulatory genes that interact with the transcription of epithelial and mesenchymal cells, thereby regulating the EMT. Previous research has reported that during BC development, EMT promotes cancer cell self-renewal and accelerates the generation of BCSCs [33]. Estrogen can induce occurrence, invasion, and distant metastasis by enhancing EMT in ER-positive BC [34]. In this study, we demonstrated that estrogen promotes EMT in MCF-7 cells, while fulvestrant can inhibits this process.

The mTOR signaling pathway plays an important role in regulating cancer cell proliferation and apoptosis [35]. AKT, a a key factor in the mTOR signaling pathway, is widely involved and closely linked with the occurrence and development of almost all malignant cancers. AKT is considered a key component of the P13K/AKT/mTOR signaling pathway [36]. When activated by PI3K, AKT can further activate mTOR, p70S6K, 4E-BP1, and glycogen syntheses kinase 3 (GSK3) [37]. A series of physiological processes are closely linked to the abnormal activation of AKT substrate proteins in cancer cells. Activation of mTOR signaling pathways regulates cellular activities and promotes the occurrence of EMT in BC [38]. Our study identified that estrogen enhance EMT and increases the expression of the mTOR signaling pathway in MCF-7 cells. After treatment with the AKT inhibitor MK-2206, we found that the self-renewal ability and stemness of MCF-7 BCSC were inhibited. Additionally, we found that MK-2206 can counteract the effects of estrogen on BCSCs. The influence of MK-2206 on BCSC stemness is associated with the mTOR signaling pathway.

5. Conclusion

In this study, we identified that estrogen enhances the EMT conversion and promotes BCSCs stemness of in MCF-7 cells. As an ER antagonist, fulvestrant affects EMT conversion in BC cells by inhibiting the mTOR signaling pathway. Inhibiting AKT expression with MK-2206 can reverse the effects of estrogen on the mTOR signaling pathway and EMT conversion, and it can also restrain the BCSC stemness. The results of the study demonstrate that estrogen promotes BCSCs stemness by enhancing EMT and activating the mTOR signaling pathway. This study elucidates the molecular mechanism underlying BCSC development and provides a potential new treatment strategy for BC in the future.

Availability of Data and Materials

All the data of this study are available from the corresponding author upon reasonable request.

Author Contributions

LG W and LB W designed the research study. L N and F M performed the research. J C analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://....

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