

Regulation of the stem-like properties of estrogen receptor-positive breast cancer cells through NR2E3/NR2C2 signaling

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Abstract. Cancer stem cells (CSCs) are major drivers of metastasis, drug resistance and recurrence in numerous cancers. However, critical factors that can modulate CSC stemness have not been clearly identified. Nuclear receptor subfamily 2 group E member 3 (*nr2e3*) expression has been previously reported to be positively associated with drug sensitivity and favorable clinical outcomes in patients with estrogen receptor (ER)⁺ breast cancer. This suggests that *nr2e3* expression may be inversely associated with CSC stemness in this type of tumor cells. The present study aimed to investigate the regulatory roles of NR2E3 in the stem-like properties of ER⁺ breast cancer cells and to identify the underlying mechanisms. Bioinformatics analysis was performed using the data derived from the Cancer Genome Atlas database. *Nr2e3*-specific shRNA and nuclear receptor subfamily 2 group C member 2 (*nr2c2*) overexpressed plasmids were constructed to silence and enhance the expression of *nr2e3* and *nr2c2*, respectively. Transwell and wound healing experiments were conducted to evaluate the migration and invasion ability of MCF7 cells, while colony formation tests were used to evaluate the clonality. Flow cytometry was used to detect the percentage of CD44⁺CD24^{-low} cells. Reverse transcription-quantitative PCR and western blotting were performed to detect expression at the mRNA and protein levels. The results showed that compared with normal breast tissues and MCF10A cells, the

expression of *nr2e3* was increased in ER⁺ breast tumor tissues and cell lines. *Nr2e3* silencing promoted the migration, invasion and colony-forming ability of the ER⁺ MCF7 cells. It also increased the expression of epithelial-mesenchymal transition markers and stem cell-related transcription factors, in addition to the percentage of CD44⁺CD24^{-low} cells. The expression of *nr2e3* and *nr2c2* was found to be positively correlated. *Nr2e3* knockdown decreased the mRNA and protein expression levels of *nr2c2*, whereas *nr2c2* overexpression reversed the elevated CD44⁺CD24^{-low} cell ratio and the increased migratory activity caused by *nr2e3* silencing. The results of the present study suggest that NR2E3 may serve an important role in modulating the stem-like properties of ER⁺ breast cancer cells, where NR2E3/NR2C2 signaling may be a therapeutic target in ER⁺ breast cancer.

Introduction

Nuclear receptor subfamily 2, group E member 3 (NR2E3) serves an important function in retinal photoreceptor cell development and maintenance (1). Mutations in the human *nr2e3* gene have been reported to cause several retinal degenerative diseases, such as enhanced S-cone syndrome and retinitis pigmentosa (2). In recent years, research on the molecular function of NR2E3 in other tissues and its role in various diseases, such as liver injury and breast cancer, has been attracting attention (3,4). In estrogen receptor (ER)⁺ breast cancer, the level of *nr2e3* expression has been found to be positively associated with recurrence-free survival. In addition, patients with higher *nr2e3* expression tended to be more sensitive to tamoxifen treatment, which in turn confers more positive clinical outcomes compared with those with lower *nr2e3* expression (5). *Nr2e3* is typically expressed at low levels in ER⁺ breast cancer tissues and its overexpression induces cancer cell growth, invasion and metastasis (6). In addition, elevated levels of *nr2e3* expression have been associated with improved clinical prognosis in patients with hepatic carcinoma, and with the occurrence and progression of lung carcinoma and pancreatic cancer (7-9). These findings suggest that outside the retina, NR2E3 can serve biological functions in cancers.

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The cancer stem cell (CSC) theory hypothesizes that a subgroup of malignant cancer cells is endowed with stem-like properties that are undifferentiated, express self-renewing capacities and can replenish other differentiated bulk tumor cells (10). Clinically, conventional radiotherapy and drug treatments can terminate the majority of differentiated tumor cells, but they have limited efficacy against CSCs. CSCs also appear to form the basis of tumor heterogeneity and the pathological cause of tumor growth, drug resistance, metastasis and recurrence (11,12). In this regard, promoting the differentiation of CSCs into drug-sensitive cancer cells, known as differentiation therapy, has been proposed to be a promising treatment strategy for eradicating cancer cells (13-15). Therefore, identifying novel biomarkers that match the specific molecular signatures of cancer cells to broaden the target spectrum may prove beneficial.

The present study hypothesized that *nr2e3* is expressed at higher levels in differentiated tumor cells in ER⁺ breast cancer compared with breast cancer stem cells based on the following considerations: i) CSCs form a fraction of the tumor cell population that can differentiate into the majority of the bulk tumor cell types, which can then contribute to poor prognosis and drug resistance (16); ii) patients with breast cancer with higher *nr2e3* expression levels tend to have superior clinical outcomes and exhibit favorable responses to tamoxifen treatment (5); and iii) *Nr2e3* expression was previously found to be increased in ER⁺ breast tumor tissues compared with that in normal and ER⁻ breast tissues (17). In addition, it was hypothesized that NR2E3 may facilitate the differentiation of breast CSCs into bulk tumor cells, not too dissimilar to its activity in promoting the differentiation of rod photoreceptors from retinal pluripotent cells in the retina (18). Therefore, the present study aimed to investigate the relationship between *nr2e3* expression and the stem-like characteristics of ER⁺ breast cancer cells to evaluate the suitability of NR2E3 as a diagnostic and therapeutic biomarker for ER⁺ breast cancer.

Materials and methods

Cell culture. Human normal mammary epithelial cells MCF10A (cat. no. CL-0525) and the ER⁺ cell line MCF7 (cat. no. CL-0149) were purchased from Procell Life Science & Technology Co., Ltd. The two cell lines were cultured in their cell-specific complete medium (cat. nos. CM-0525 for MCF10A and CM-0149 for MCF7; Procell Life Science & Technology Co., Ltd.). When MCF10A cells were cultured, 5% horse serum, 20 ng/ml epidermal growth factor, 0.5 µg/ml hydrocortisone, 1% non-essential amino acid (NEAA), 10 µg/ml insulin and 1% penicillin-streptomycin solution (P/S) were added to DMEM/F12 medium. When MCF7 cells were cultured, 10% fetal bovine serum (FBS), 1% NEAA, 10 µg/ml insulin and 1% P/S were added to the MEM. According to a previous study (19), paclitaxel-resistant MCF7 cells were cultured with 10 µg/ml paclitaxel (cat. no. ab120143; Abcam) over 6 months. All cells were cultured at 37°C with 5% CO₂.

Plasmids and transfection. The negative control (NC) and *nr2e3* short-hairpin RNAs (shRNAs) were designed and purchased from RiboBio Co., Ltd. The target sequences (Table I) were cloned into the pRNAT-U6.1/Neo plasmid

Table I. shNC and NR2E3-specific shRNA sequences.

shRNA used	shRNA sequence (5'-3')
shNC	Sense: CAACAAGATGAAGAGCACCAA Antisense: TTGGTGCTCTTCATCTTGTG
shRNA1	Sense: GAAGGATCCTGAGCACGTA Antisense: TACGTGCTCAGGATCCTTC
shRNA2	Sense: GGGAAGCACTATGGCATCT Antisense: AGATGCCATAGTGCTTCCC
shRNA3	Sense: CATGGCCAGCCTTATAACA Antisense: TGTATAAGGCTGGCCATG

NC, negative control; *nr2e3*, nuclear receptor subfamily 2 group E member 3; sh, short hairpin RNA.

(RiboBio Co., Ltd.). The full-length cDNA sequence of human nuclear receptor subfamily 2 group C member 2 (*nr2c2*) was cloned into the pEGFP-N1 vector (Miaoling Biotechnology Co., Ltd.). LipofectamineTM 3000 reagents (cat. no. L300000; Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect the shRNA-carrying plasmids and/or the *nr2c2*-overexpression plasmids into MCF7 cells. Plasmids were incubated with LipofectamineTM 3000 reagents and Opti-MEMTM (cat. no. 11058021; Invitrogen; Thermo Fisher Scientific, Inc.) for 20 min at room temperature. Dishes with a diameter of 35 mm and 15 mm were transfected with 6 and 4 µg of plasmids, respectively. For the *nr2c2* overexpression experiment, MCF7 cells were co-transfected with *nr2e3* shRNA plasmids and the *nr2c2*-overexpression vector. 36 h after transfection, cells were harvested for subsequent experiments.

The Cancer Genome Atlas (TCGA) data analysis. According to our previous study (19), the expression data of *nr2e3* and *nr2c2* in 808 ER⁺ breast tumor and 113 normal tissues were downloaded from the TCGA database (<https://portal.gdc.cancer.gov/>; version 32.0) for bioinformatics analysis. Furthermore, the expression profile of *nr2e3* in 1,109 breast tumor samples that were not categorized according to ER content was also downloaded from the TCGA database. BRB-ArrayTools (<http://brb.nci.nih.gov/BRB-ArrayTools/download.html>, version 4.6.2) was used to analyze these data. Briefly, data collation and gene labeling modules were used, differentially expressed genes in the dataset were screened with $P < 0.05$ and $\log_{2}FC > 1$ as criteria, and hierarchical cluster analysis was performed.

MTT assay. The MTT cytotoxicity assay was conducted using an MTT Cell Proliferation and Cytotoxicity Detection kit (cat. no. C0009S; Beyotime Institute of Biotechnology). Briefly, $\sim 2 \times 10^3$ MCF7 cells transfected with either NR2E3 shRNAs or shNC were seeded into 96-well plates, before 10 µl MTT reagent was added. After incubation at 37°C for 4 h, 100 µl formazan solvent (DMSO) was added. After incubation for another 4 h (at 37°C), absorbance at 570 nm was measured.

Wound healing assay. MCF7 cells were cultured in complete medium. shRNA-transfected MCF7 cell monolayers were scratched when the confluence reached $\sim 80\%$. Scratched cells

Table II. Primers used for reverse transcription-quantitative PCR.

Gene (accession number)	Primer sequence (5' to 3')
<i>Nr2e3</i> (NM_016346.4)	F: GATCCTGAGCACGTAGAGGC R: GCAATTTCCCAAACCTCACGG
<i>Nr2c2</i> (NM_003298.5)	F: GGCGCCAAATCCTGAGGTAA R: GGTGAGGCTACAGCAGAGTC
<i>Nanog</i> (NM_024865.4)	F: TCCTCCTCTTCCTCTATACTAAC R: CCCACAAATCACAGGCATAG
<i>Klf4</i> (NM_004235.6)	F: ATCTCGGCCAATTTGGGGTT R: CCAGGTGGCTGCCTCATTA
<i>Oct4</i> (NM_002701.6)	F: ATCGAGAACCGAGTGAGA R: AACTCGGACCACATCCTT
<i>Sox2</i> (NM_003106.4)	F: GGGAAATGGGAGGGGTGCAAAAGAGG R: TTGCGTGAGTGTGGATGGGATTGGTGT
<i>E-cadherin</i> (NM_001792.5)	F: CCTCCAGAGTTTACTGCCATGAC R: GTAGGATCTCCGCCACTGATTC
<i>N-cadherin</i> (NM_004360.5)	F: GGCGCCACCTGGAGAGA R: TGTCGACCGGTGCAATCTT
<i>Vimentin</i> (NM_003380.5)	F: TACAGGAAGCTGCTGGAAGG R: ACCAGAGGGAGTGAATCCAG
<i>GAPDH</i> (NM_002046.7)	F: GGAGCGAGATCCCTCCAAAAT R: GGCTGTTGTCATACTTCTCATGG

Nr2e3, nuclear receptor subfamily 2 group E member 3; Nr2c2, nuclear receptor subfamily 2 group C member 2; Nanog, nanog homeobox; Klf4, Krüppel-like factor 4; Oct4, POU class 5 homeobox 1; Sox2, SRY-box transcription factor 2; E-cadherin, cadherin 1; N-cadherin, cadherin 2. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward primer sequence; R, Reverse primer sequence.

were serum starved, and incubated at 37°C with 5% CO₂. Images were captured at 0 and 24 h post-scratching using an inverted fluorescent microscope (cat. no. CKX53; Olympus Corporation) under the same magnification.

Two-dimensional colony formation assay. A total of 1,000 transfected MCF7 cells were seeded into 6-cm dishes, maintained in complete medium, and incubated for 2 weeks at 37°C with 5% CO₂. Colonies were fixed with 4% paraformaldehyde (cat. no. P0099; Beyotime Institute of Biotechnology) for 30 min and stained with crystal violet (cat. no. C0121; Beyotime Institute of Biotechnology) for 10 min, all at room temperature. Images were taken using an Ordinary camera (cat. no. VlogR7; Canon Corporation). Colonies that contain more than 50 cells were counted manually.

Transwell assays. Transwell chambers (cat. no. 3422; BD Biosciences) were used to investigate cell migration capacities. In every well, a total of ~2x10³ cells were resuspended in 200 µl serum-free DMEM and placed into the upper chamber, whereas 600 µl complete medium with 10% FBS was added to the lower chamber. After incubation for 24 h at 37°C, the cells were fixed in 4% formaldehyde for 30 min and stained with crystal violet for 10 min, both at room temperature. The number of cells on the underside of the membrane was counted under an Olympus CKX53 inverted microscope at x200 magnification, before cells in three randomly selected fields were counted.

Quantitative PCR (qPCR). MCF7 cells that were transfected with *nr2e3* shRNAs were harvested when the confluence reached ~90%. RNAiso Plus Reagent (cat. no. 9108; Takara Bio Inc.) was used to extract the total RNA. cDNA was synthesized through reverse transcription using the BeyoRT™ II kit (cat. no. D7168M; Beyotime Institute of Biotechnology). Briefly, total RNA, oligo(dT)18 primer, reaction buffer, RNase inhibitor, dNTP Mix, BeyoRT™ II M-MLV and DEPC-treated water formed a reaction system. Following the manufacturer's instruction, this system was incubated at 42°C for 60 min, and then for 10 min at 80°C. For the qPCR experiment, AceQ™ qPCR SYBR® Green Master Mix (cat. no. Q111-02; Vazyme Biotechnology Co., Ltd.) was used. According to the manufacturer's instructions, thermocycling conditions for PCR were 95°C for 10 sec and then 60°C for 30 sec. The relative mRNA expression was calculated using the 2^{-ΔΔC_q} method. The primer sequences used for qPCR are listed in Table II. GAPDH was used as the normalization control.

Western blotting. MCF7 cells that were transfected with *nr2e3* shRNAs were harvested when the confluence reached ~90%. Total proteins were extracted using cell lysis buffer for western blotting (cat. no. P0013; Beyotime Institute of Biotechnology). The protein concentration was determined with a bicinchoninic acid kit (cat. no. P0010; Beyotime Institute of Biotechnology). A 10% gel was used for electrophoresis and each lane was loaded with 20 µg protein. Proteins were then transferred to a polyvinylidene fluoride membrane (cat. no. FFP78; Beyotime

Table III. Antibodies used for WB and FCM.

Protein	Experiment	Company	Cat. no.
NR2E3	WB	Proteintech Group, Inc.	14246-1-AP
NR2E3	WB	Santa Cruz Biotechnology, Inc.	sc-374513
E-cadherin	WB	ProteinTech Group, Inc.	20874-1-AP
N-cadherin	WB	ProteinTech Group, Inc.	22018-1-AP
VIMENTIN	WB	Affinity Biosciences, Ltd.	BF8006
SLUG	WB	Affinity Biosciences, Ltd.	AF4002
NR2C2	WB	ABclonal Biotech Co., Ltd.	A6422
LSD1	WB	ABclonal Biotech Co., Ltd.	A1156
H3K4me2	WB	ABclonal Biotech Co., Ltd.	A2356
GAPDH	WB	ProteinTech Group, Inc.	60004-1-Ig
Goat anti-rabbit IgG	WB	Proteintech Group, Inc.	SA00001-2
Goat anti-mouse IgG	WB	Proteintech Group, Inc.	SA00001-1
CD44-FITC	FCM	Invitrogen; Thermo Fisher Scientific, Inc.	11-0441-82
CD24-PE	FCM	Invitrogen; Thermo Fisher Scientific, Inc.	12-0247-42

NR2E3, nuclear receptor subfamily 2 group E member 3; E-cadherin, cadherin 1; N-cadherin, cadherin 2; NR2C2, nuclear receptor subfamily 2 group C member 2; LSD1, Lysine-specific histone demethylase 1A; H3K4me2, Histone H3 lysine 4 dimethylation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; WB, western blotting; FCM, flow cytometry.

Institute of Biotechnology), and incubated in blocking buffer (cat. no. P0023B; Beyotime Institute of Biotechnology) for 2 h at room temperature. After washing with TBST buffer (containing 20% Tween), the membranes were then incubated with the primary and secondary antibodies (primary antibodies, 1:500; secondary antibodies, 1:10,000). A visualization reagent (cat. no. KF8005; Affinity Biosciences) was the applied. The primary and secondary antibodies used for western blotting are listed in Table III. GAPDH was used as the normalization control.

JASPAR prediction. The online prediction software JASPAR (<https://jaspar.genereg.net>) was used to predict the NR2E3 binding site on the *nr2c2* gene promoter. The human *nr2c2* gene promoter sequence (https://www.ncbi.nlm.nih.gov/nucleotide/NC_000003.12?from=14947583&to=15049273&report=fasta) with FASTA format was scanned at 2 kbp length.

Flow cytometry. A total of $\sim 1 \times 10^6$ *nr2e3*-silenced MCF7 cells and the negative control cells, as well as the *nr2c2*-overexpressed MCF7 cells were incubated with 5 μ l FC receptor blocker (cat. no. abs9476; Absin Bioscience, Inc.) at 4°C for 10 min, and then incubated on ice with 0.25 μ g FITC-conjugated CD44 and 0.25 μ g PE-conjugated CD24 antibodies for 30 min. The percentages of CD44⁺CD24^{-low} subgroup cells were detected using flow cytometry with the Beckman CytoFlex system (Beckman Coulter, Inc.) and analyzed using FlowJo software (version 10.8.1). The antibodies used for flow cytometry are listed in Table III.

Statistical analysis. All data were analyzed using GraphPad Prism (version 6; Dotmatics). One-way ANOVA followed by Tukey's post hoc test was used for all comparisons. Data are presented as the mean \pm SD. All data were obtained from

at least three independent experiments. $P < 0.05$ was used to indicate a statistically significant difference.

Results

Expression of *nr2e3* is increased in ER⁺ breast cancer tissues and cell lines. To investigate the biological roles of NR2E3 in ER⁺ breast cancer, its expression was first investigated in ER⁺ breast cancer tissues compared with that in normal breast tissue samples. Data from 808 ER⁺ breast tumors and 113 normal samples were downloaded from TCGA database, where the subsequent analysis revealed that the expression of *nr2e3* was significantly higher in the tumor samples (Fig. 1A). When the tumor types were not categorized according to the ER content, the expression of *nr2e3* remained significantly higher in breast cancer tissues (Fig. 1B). The protein expression level of NR2E3 was found to be elevated in the ER⁺ cell line MCF7 compared with that in the MCF10A normal human breast epithelial cell line (Fig. 1C). These results suggest that *nr2e3* expression is increased in ER⁺ breast cancer and tumor types not categorized according to the ER content.

Nr2e3 silencing promotes the migration, invasion and colony-formation by MCF7 cells. The association between *nr2e3* expression and the migration, invasion and colony-formation of MCF7 cells was next assessed as an indication of the stemness property of CSCs *in vitro*. In total, three shRNAs targeting *nr2e3* were designed and transfected into MCF7 cells. None of the three *nr2e3*-specific shRNAs, sh1, sh2 and sh3, nor the shNC, exhibited cytotoxicity towards MCF7 cells according to the MTT assay (Fig. S1A). In addition, mRNA and protein expression levels of *nr2e3* were found to be significantly decreased in shRNA-transfected cells compared with those in the shNC group (Fig. 2A and B). Among them, sh1

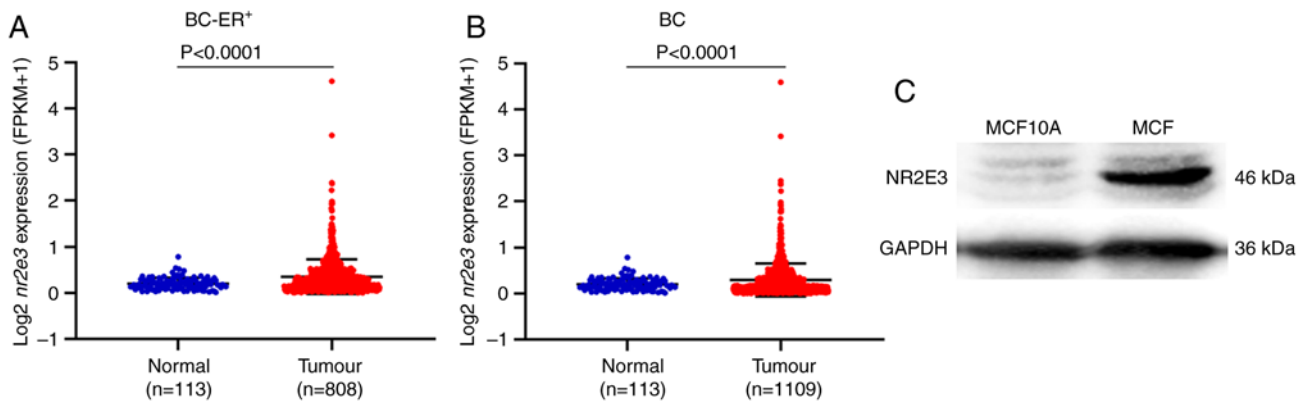


Figure 1. Expression of *nr2e3* is upregulated in ER⁺ breast adenoma tissue cells. *Nr2e3* expression was analyzed in (A) ER⁺ and (B) total breast adenoma tissues compared with normal tissue samples. Data were downloaded from The Cancer Genome Atlas database. (C) Western blotting showing the protein expression levels of NR2E3 in MCF10A and MCF7 cells, with GAPDH used as a loading control. BC, breast cancer; ER, estrogen receptor; NR2E3, nuclear receptor subfamily 2 group E member 3; FPKM, fragments per kilobase of exon model per million mapped fragments.

exerted the highest silencing effect and so was selected for use in further experiments. The results of the Transwell and wound healing assays showed that *nr2e3* knockdown markedly increased both their migratory and invasive capabilities (Figs. 2C-E and S1B). Two-dimensional colony formation tests showed that silencing *nr2e3* expression markedly promoted colony-formation (Figs. 2F and S2A). Considering that CSCs contribute to the migration and invasion ability of tumor cells (12), while *nr2e3* knockdown increases the migration and invasion ability of MCF7 cells and promotes the colony formation of MCF7 cells, it can be inferred that *nr2e3* knockdown enhances the stem cell-like properties of ER⁺ tumor cells.

In addition, paclitaxel treatment terminated most differentiated tumor cells, whereas the ratio of stem-like cells was elevated in paclitaxel-resistant (PR) breast tumor cells (20). In the present study, it was found that *nr2e3* was expressed at lower levels in MCF7-PR cells (Fig. S2B). This finding supported the notion that *nr2e3* is mainly expressed in differentiated tumor cells, whereas in stem-like breast tumor cells *nr2e3* expression is low.

nr2e3 silencing promotes epithelial-mesenchymal transition (EMT), enhances the expression of stem cell-related transcription factors and increases the proportion of CD44⁺CD24^{-low} cells. Tumor cells that undergo epithelial-mesenchymal transition (EMT) usually have enhanced migration, invasion and drug resistance. Additionally, EMT gives carcinoma cells the capacity to renew themselves and increases the ratio of tumor stem cells (21). Thus, the relationship between *nr2e3* expression and the expression of EMT-related marker genes was next evaluated. It was shown that *nr2e3* knockdown significantly reduced the mRNA expression levels of *E-cadherin*, but increased the expression of *N-cadherin* and *vimentin* mRNA (Fig. 3A). Similar directions of changes in the protein expression levels of E-cadherin, N-cadherin and vimentin were found using western blotting (Figs. 3B and S3). The protein expression of slug, a transcription factor that can promote EMT (22), was also shown to be increased when *nr2e3* expression was knocked down (Figs. 3B and S3). These results support the hypothesis that *nr2e3* knockdown can promote the EMT process.

Furthermore, *nr2e3* knockdown was found to significantly increase the mRNA expression levels of stem cell-associated transcription factors *sox2*, *oct4*, *nanog* and Kruppel-like factor 4 (Fig. 3A). The association between NR2E3 and the proportion of the CD44⁺CD24^{-low} population was investigated, where it was found that *nr2e3* knockdown significantly enhanced the proportion of CD44⁺CD24^{-low} in MCF7 tumor cells (Fig. 3C and D).

NR2C2 is a potential downstream target of NR2E3. Using genome-wide chromatin immunoprecipitation assays and publicly available database analysis (NCI-60 database), Park *et al* (5) previously showed that NR2E3 can directly regulate the expression of the ER α (*esr1*) gene. In addition to ESR1, NR2E3 also has the highest number of correlated genes encoding nuclear receptors, such as peroxisome proliferator activated receptor α (PPARA), thyroid hormone receptor α (THRA), estrogen related receptor α (ESRRA), hepatocyte nuclear factor 4 α (HNF4A) and NR2C2, suggesting that there may be further cross-talk between NR2E3 and these nuclear receptors (5). In the present study, using TCGA database, the correlation between mRNA expression of *nr2e3* and *ppara*, *thra*, *esrra*, *hnf4a* and *nr2c2* in ER⁺ breast tumor cells were evaluated, where it was found that there was little or no correlation between *nr2e3* expression and the expression of *ppara*, *thra*, *esrra* and *hnf4a* (Fig. S4). By contrast, a significant positive correlation between the mRNA expression of *nr2e3* and *nr2c2* was found (Fig. 4A). Compared with that in the normal breast samples, *nr2c2* expression was significantly lower in ER⁺ breast cancer tissues compared with that in normal tissues (Fig. 4B).

To determine if NR2E3 can directly regulate *nr2c2* expression, the mRNA and protein levels of *nr2c2* were detected in *nr2e3*-silenced MCF7 cells. It was shown that *nr2e3* silencing markedly reduced *nr2c2* expression (Fig. 4C and D). JASPAR (<https://jaspar.genereg.net>) was used to scan the 2 kb promoter sequence upstream from the transcriptional start site of the *nr2c2* gene to identify an NR2E3 binding site. In total, two predicted NR2E3 binding sites were identified on the proximal promoter of the *nr2c2* gene (-1748 to -1742 bp, and -1516 to -1510 bp), with the predicted binding sequence being xAAGCTT (x represents nucleotide A, T, C or G; Fig. S5). This suggested that NR2E3

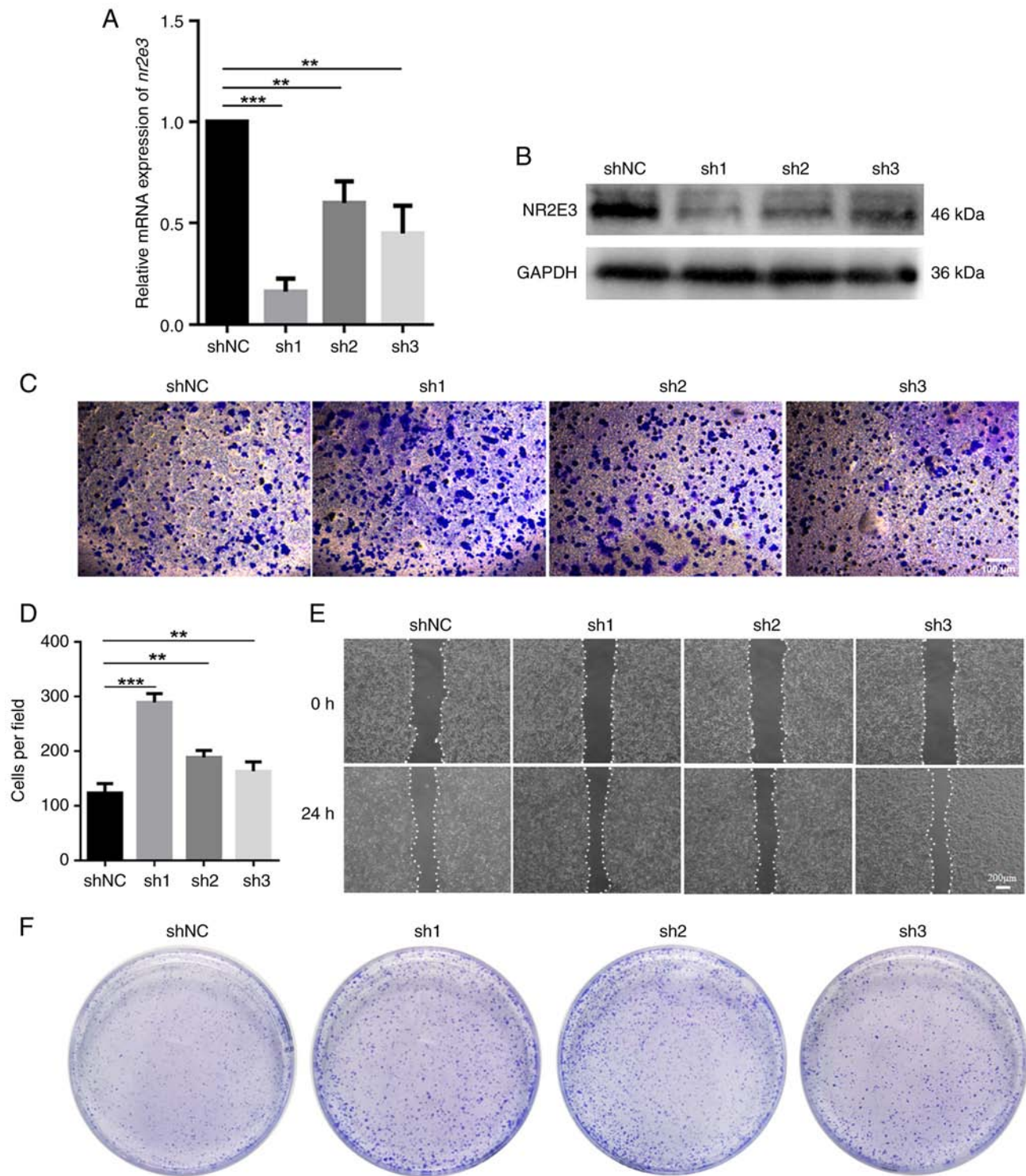


Figure 2. Efficiency of *nr2e3* shRNA transfection on the migration, invasion and colony-formation of MCF7 cells. Evaluation of the effectiveness of *nr2e3*-targeting shRNAs using (A) reverse transcription-quantitative PCR and (B) western blotting, with GAPDH used as a control. (C) Representative images and (D) evaluation of the migratory ability using Transwell assays. The migratory cells were observed using an Olympus CKX53 inverted microscope at x200 magnification. Scale bar, 100 μm. (E) Evaluation of the migratory ability was examined by wound healing experiments. (F) Evaluation of the colony-formation ability. ***P*<0.01 and ****P*<0.001. C, control; NR2E3, nuclear receptor subfamily 2 group E member 3; ns, no significance; shRNA or sh, short hairpin RNA.

can directly bind to the *nr2c2* promoter to regulate its transcription. Furthermore, *nr2e3* knockdown was found to decrease the expression levels of the active histone marker histone H3 lysine 4 dimethylation (H3K4me2), in addition to markedly increasing the protein expression level of lysine-specific histone demethylase 1A (Lsd1) (Fig. 4E), which usually retains the suppressive

histone status (9). These results suggest that NR2E3 may also serve a role as an epigenetic modification factor that can sustain *nr2c2* promoter chromatin accessibility. Taken together, these findings suggest that the orphan nuclear receptor NR2C2 may be implicated in the NR2E3 signaling pathway upstream of the regulation of ER⁺ breast cancer cell physiology.

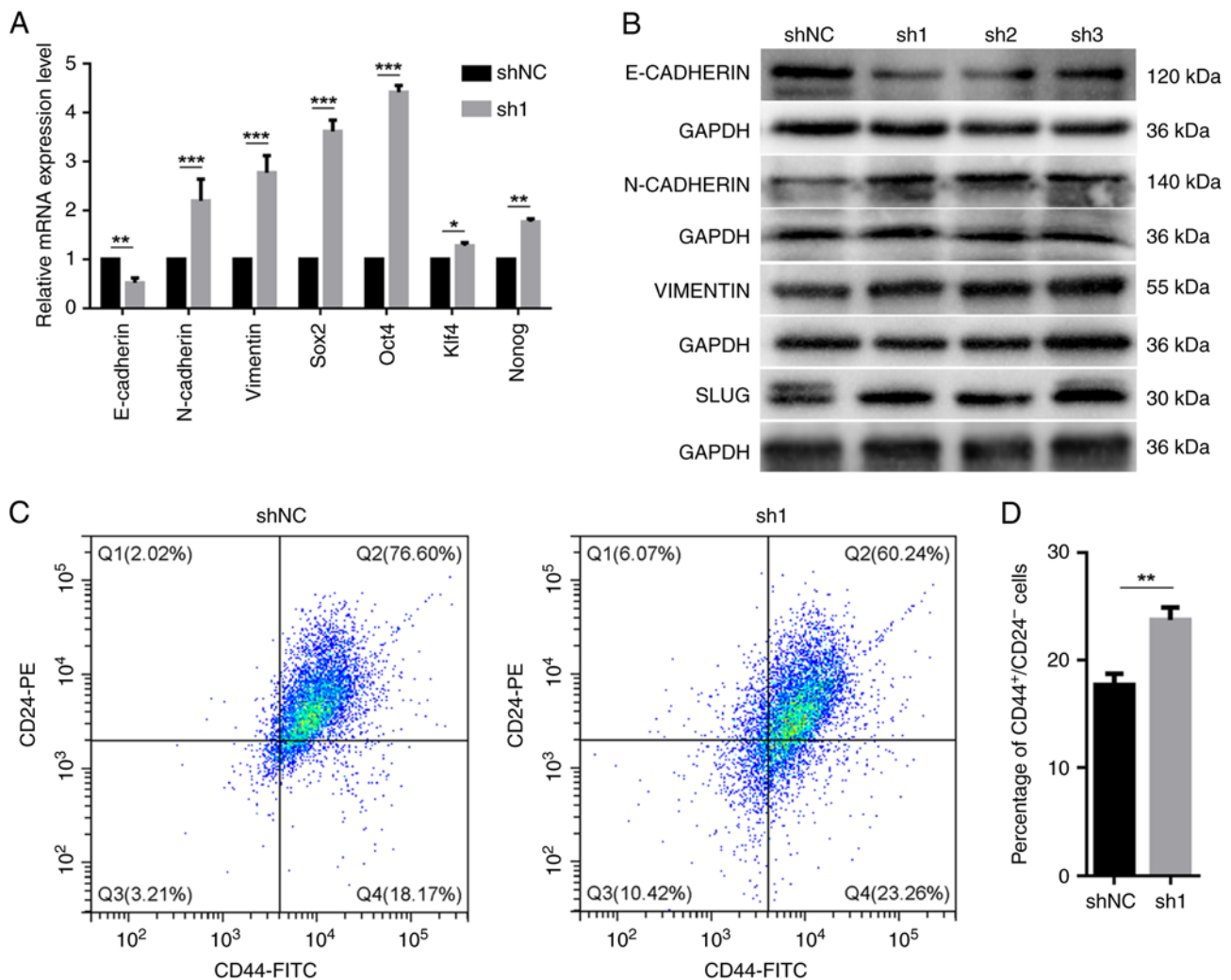


Figure 3. Effects of *nr2e3* silencing on the expression of EMT markers, stem cell-related transcription factors and the proportion of CD44⁺CD24^{-low} cells. Detection of the mRNA and protein expression levels of EMT markers and stem cell-related transcription factors using (A) reverse transcription-quantitative PCR and (B) western blotting, respectively, with GAPDH used as the endogenous control. (C) Proportion of CD44⁺CD24^{-low} subgroup cells was analyzed using flow cytometry. (D) Semi-quantification of the expression ratio from (C). *P<0.05, **P<0.01 and ***P<0.001. C, control; EMT, epithelial-mesenchymal transition; NR2E3, nuclear receptor subfamily 2 group E member 3; sh, short hairpin RNA; klf4, Krüppel-like factor 4.

Nr2c2 overexpression decreases the proportion of CD44⁺CD24^{-low} cells and suppresses migratory activity. To determine if NR2E3 can modulate the stem-like characteristics of ER⁺ breast carcinoma cells through NR2C2, vectors containing the full-length coding sequence of the human *nr2c2* cDNA were transfected into the *nr2e3*-silenced MCF7 cells. The protein expression levels of *nr2c2* were found to be increased following *nr2c2* overexpression (Fig. S6). It was then observed that *nr2c2* overexpression reversed the elevated ratio of both CD44⁺CD24^{-low} cells and the increased number of migratory cells caused by *nr2e3* silencing (Fig. 5A-C). These findings suggest that an NR2E3/NR2C2 network can modulate the stem-like activities of ER⁺ breast tumor cells.

Discussion

Nr2e3 was initially thought to be uniquely expressed in the retinal photoreceptor cells, it was therefore also called the photoreceptor-specific nuclear receptor (23). However, *nr2e3* has also been reported to be expressed in other tissues, such

as liver, mammary-glands, adrenal gland, thyroid gland, prostate, testis, uterus, trachea, digestive tract and salivary glands (4,24,25). In addition, its expression has been reported in several cancer cell lines, including the Y79, HepG2, MCF7, T47D, HeLa and HCT116. *Nr2e3* expression has been associated with the occurrence, progression and drug sensitivity depending on the cancer type (5-9,26,27).

Proteins specifically designed for the development of photoreceptors in the retina are employed to direct the proliferation of tumor cells. Neuroretinal leucine zipper protein and cone-rod homeobox transcription factor, two pivotal transcription factors that can form functional complexes with NR2E3 to regulate photoreceptor differentiation, are closely associated with the growth of the medulloblastoma (28). In patients with liver cancer and ER⁺ breast carcinoma, high levels of *nr2e3* expression are associated with favorable clinical outcomes and higher sensitivity to tamoxifen treatment (5,9). In ER⁻ breast cancer, *nr2e3* overexpression has been previously found to induce migration and metastasis (6), suggesting that NR2E3 serves

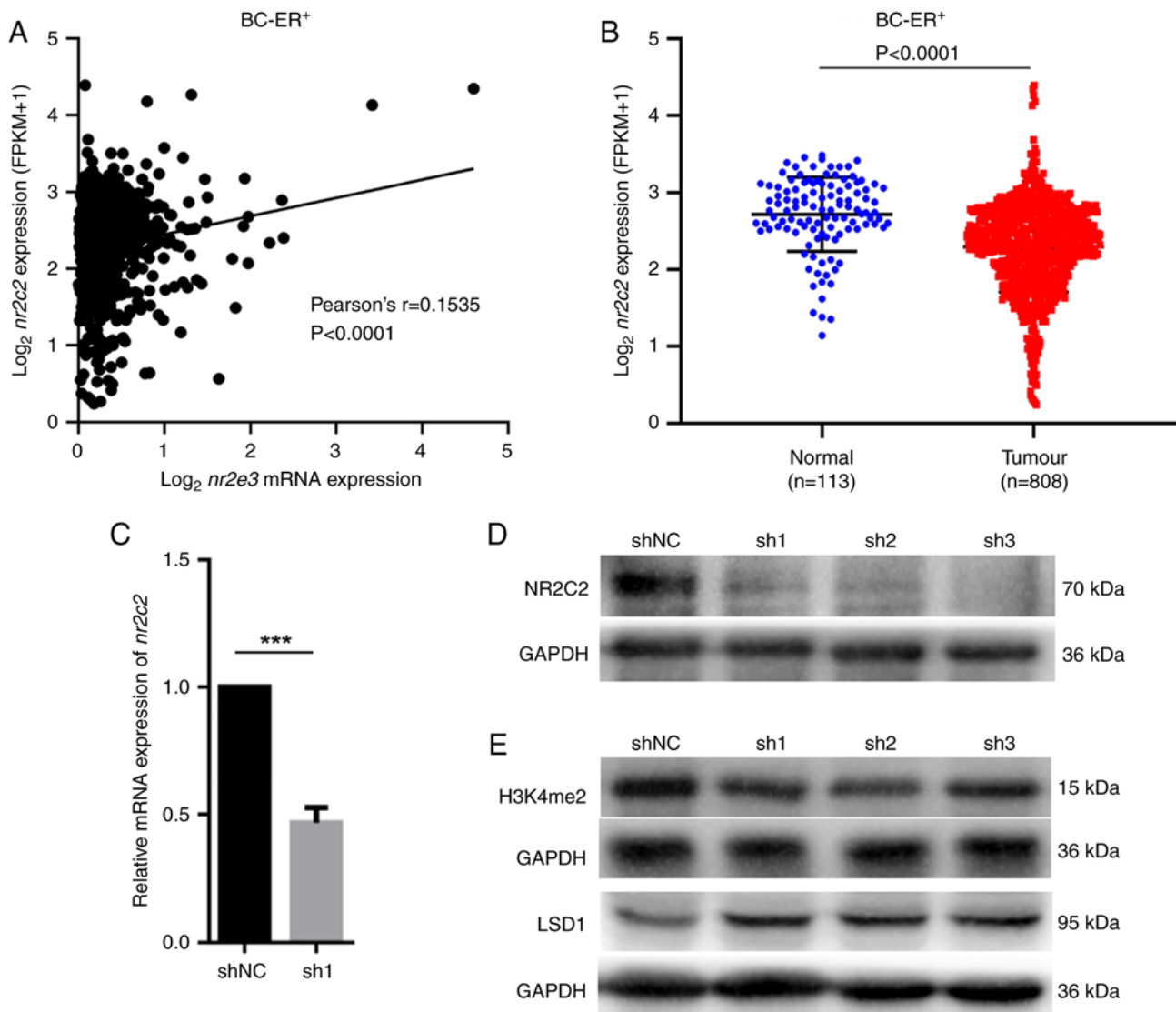


Figure 4. *Nr2e3* knockdown decreases the expression level of *nr2c2*. (A) Correlation analysis between the mRNA expression levels between *nr2e3* and *nr2c2* in ER⁺ breast cancer tissues. Data were downloaded from TCGA database. (B) *Nr2c2* expression was investigated in ER⁺ breast tumor samples compared with normal specimens from TCGA database. Expression levels of *nr2c2* (C) mRNA and (D) protein were detected in *nr2e3*-silenced MCF7 and shNC-transfected cells using reverse transcription-quantitative PCR and western blotting, respectively, with GAPDH used as the control. *** $P < 0.001$. (E) Protein expression levels of LSD1 and H3K4me2 were detected in *NR2E3*-depleted MCF7 and shNC cells using western blotting. GAPDH served as the endogenous control. BC, breast cancer; ER, estrogen receptor; LSD1, lysine-specific histone demethylase 1A; NR2C2, nuclear receptor subfamily 2 group C member 2; NR2E3, nuclear receptor subfamily 2 group E member 3; TCGA, The Cancer Genome Atlas.

a tumorigenic and antineoplastic function influenced by the molecular environment.

In the present study, *nr2e3* expression was found to be increased in ER⁺ breast cancer tissues and cell lines, which is consistent with previously reported data from TaqMan PCR assays and data re-elaboration (4,17). In the present study, *nr2e3* silencing promoted EMT progression, increased the ratio of CD44⁺CD24^{-low} cells and promoted the expression of stem cell-related transcription factors. By contrast, knocking down *nr2e3* expression enhanced the ability of migration, invasion and colony formation of ER⁺ MCF7 cells. Data in the present study also verified that *nr2e3* expression is inversely associated with the stem-like properties of ER⁺ breast tumor cells. In addition, changes to the stem-like properties of the MCF7 cells appeared to be in part mediated by the regulation of *nr2c2* expression. Therefore, the present study provided

a novel finding that the NR2E3/NR2C2 nuclear receptor network can modulate the physiological behaviors of breast cancer cells.

In retinal cells, NR2E3 mediates the expression of photoreceptor genes such as rhodopsin and *gnat1* on the transcriptional level (29). In tumor cells, NR2E3 can function as an epigenetic modulator to regulate the chromatin accessibility of target genes, such as *esr1*, aryl hydrocarbon receptor and long non-coding RNA damage-induced noncoding (3,9,30). NR2E3 can also regulate protein activity through post-translational modifications. NR2E3 has been reported to enhance the stability of p53 proteins by increasing acetylation, thereby strengthening p53 signaling (27). These results suggest that NR2E3 can modulate signal transduction on pre-transcriptional, transcriptional and post-translational levels.

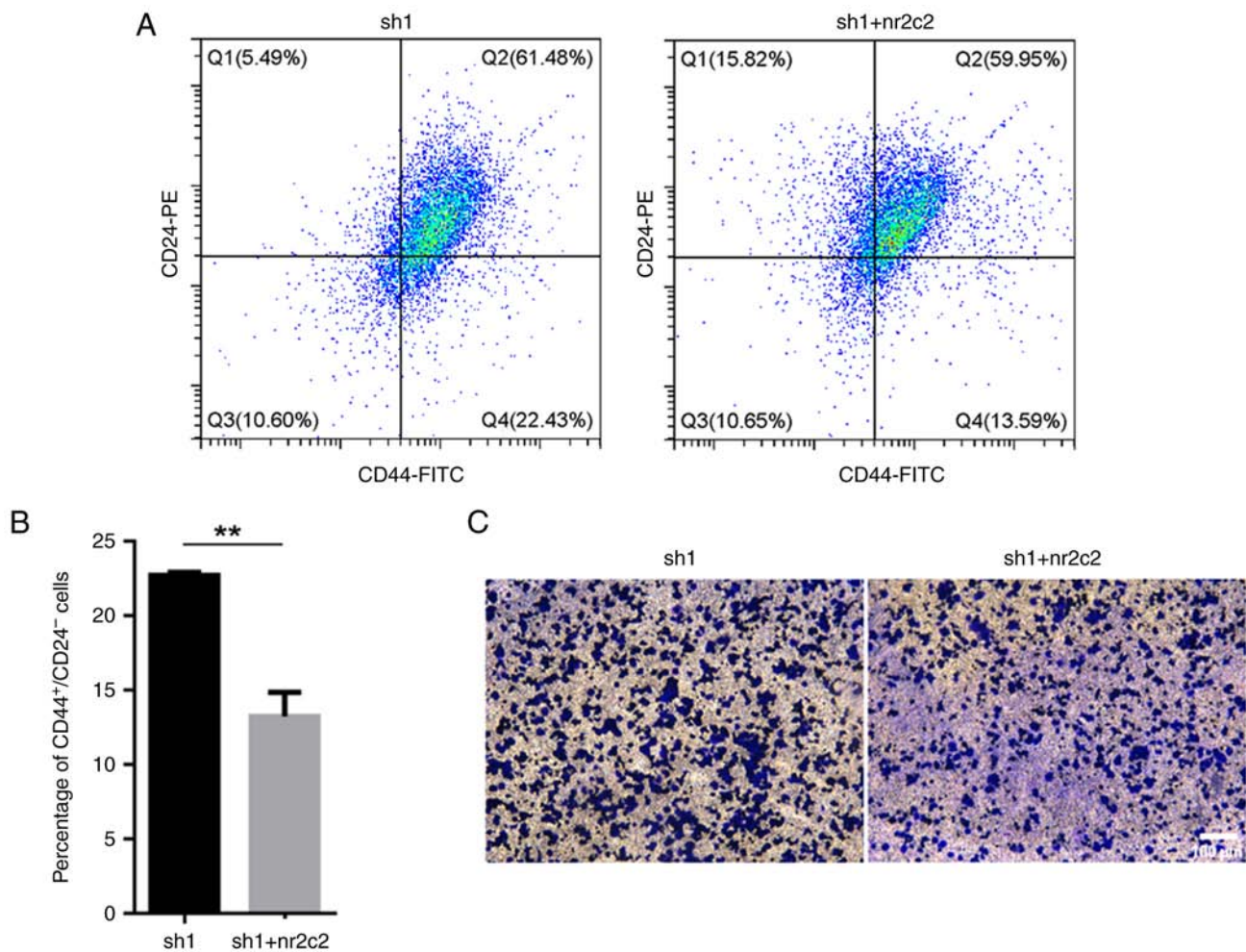


Figure 5. *Nr2c2* overexpression reverses the elevated proportion of CD44⁺CD24^{low} cells and the increased migratory ability originally induced by *nr2e3* silencing. (A) Flow cytometry was used to analyze the proportion of CD44⁺CD24^{low} subgroups in *nr2c2*-overexpressing MCF7 tumor cells with *nr2e3* expression knocked down. (B) Semi-quantification of the CD44⁺CD24^{low} ratio in (A). (C) Migratory activity of MCF7 cells following *nr2c2* overexpression and *nr2e3* silencing was analyzed using Transwell assay. The migratory cells were observed using an Olympus CKX53 inverted microscope at x200 magnification. Scale bar, 100 μ m. ***P*<0.01. sh, short hairpin RNA; NR2C2, nuclear receptor subfamily 2 group C member 2; NR2E3, nuclear receptor subfamily 2 group E member 3.

The expression of *nr2e3* and *nr2c2* mRNA was positively correlated in ER⁺ breast carcinoma, although not as high as the correlation between *nr2e3* and *esr1* (5). In the present study, *nr2e3* knockdown markedly downregulated the mRNA and protein expression of *nr2c2*. Mechanistically, several predicted binding sequences (for example, xAAGCTT) of NR2E3 were predicted at the proximal promoter of the *nr2c2* gene (-1748 to -1742 bp, and -1516 to -1510 bp), suggesting that NR2E3 may directly activate *nr2c2* transcription. *nr2c1*, a homologous gene that is associated with *nr2c2* and with high degrees of sequence homology (the overall structural identity is 65%, and the DNA binding domain is 82%), was previously identified as a direct target of NR2E3 (31,32). The consensus sequence AAGTCA recognized by NR2E3 proteins in retinal photoreceptors is also present on the *nr2c2* promoter (33). Further studies to determine the binding sequences of NR2E3 in ER⁺ breast cancer cells are warranted using specific antibodies in chromatin immunoprecipitation experiments. Another potential mechanism by which NR2E3 can regulate *nr2c2* expression could be by the modulation of chromatin accessibility, since *nr2e3* knockdown was found to increase the

expression of LSD1 whilst decreasing that of the active histone marker H3K4me2, consistent with previous findings (3,9,30).

Nr2c2 is ubiquitously expressed in the human brain, lung, kidney, skeletal muscle, prostate, ovary and testis, where they serve as a factor in neuronal development, glucose metabolism, hematogenesis and spermatogenesis (34). Since it is abundantly expressed in testicular tissues, it is also called testicular orphan nuclear receptor 4 (35). Depending on the tumor type, NR2C2 may function as a tumorigenic or tumor-suppressive factor. In prostatic carcinoma, non-small-cell lung carcinoma and malignant neuroglioma, NR2C2 was found to enhance the migratory and infiltrative capabilities of tumor cells (35-37). In hepatocellular carcinoma and bladder cancer, the opposite effect is observed (38,39). Consistent with a previously reported TaqMan array analysis (17), the present study showed that *nr2c2* was expressed at lower levels in ER⁺ breast tissues. In ER⁺ breast carcinoma, NR2C2 breaks the ER homodimers by binding to monomeric ESR1, thereby reducing cell proliferation (40). In addition, NR2C2 can alter the oxygen state of MCF7 cells by decreasing the expression of oncogenic microRNAs (miR)-526b and miR-655, which then suppresses

tumor migration and invasion (41). These data suggest that NR2C2 may inhibit the tumorigenicity of ER⁺ breast cancer cells.

The molecular mechanism underlying the NR2E3-mediated regulation of the characteristics of ER⁺ breast cancer cells can be complex. In addition to the aforementioned NR2C2, NR2E3 can enhance *esr1* transcription by interacting with protein inhibitor of activated STAT protein 3 (PIAS3), a representative inhibitor of STAT3 (5,42). Although ESR1 functions in cancer progression (43,44), its high expression has been associated with superior recurrence-free survival in ER⁺ breast cancer (5). Furthermore, patients with higher levels of expression of both *nr2e3* and *esr1* tended to show the optimal recurrence-free survival (5). *Esr1* expression was no longer associated with prognosis when patients were treated with tamoxifen. However, *nr2e3* expression was still relevant (5), suggesting that NR2E3 can modulate the characteristics of breast tumor cells through distinct pathways in patients who received hormonal therapy. PIAS3 acts as an essential protein that recruits NR2E3 to the *esr1* promoter (5). Although PIAS3 is an inhibitor of STAT3, a transcription factor that facilitates self-renewal and metastasis of breast cancer cells (13,14), ectopic expression of PIAS3 was shown to enhance the proliferation of MCF7 cells, attenuate the cytotoxicity of tamoxifen and decrease the survival time of patients with ER⁺ breast cancer (42). Therefore, according to the ER content, further studies are needed to investigate the molecular association of NR2E3 with these factors.

In conclusion, results from the present study suggest that *nr2e3* expression is inversely associated with the migratory and invasive capability of ER⁺ breast cancer cells. *Nr2e3* silencing reinforced the EMT process, enhanced the expression of stem cell-related transcription factors and elevated the proportion of CD44⁺CD24^{-low} cells. In addition, NR2E3 may perform its function by targeting NR2C2. Therefore, NR2E3/NR2C2 signaling may represent a target to eliminate stem-like cells in this type of breast cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SX was responsible for the study conceptualization, investigation, and writing and editing of the manuscript. YH was responsible for performing experiments, writing and reviewing of the manuscript. JJ was responsible for performing

experiments and methodology carried out. LF was responsible for performing experiments. CZ was responsible for the methodology and data analysis. QY was responsible for the project administration and data interpretation. YN was responsible for the methodology. ZS was responsible for the conceptualization, funding acquisition and reviewing of the manuscript. SX and YH confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Bengbu Medical College (approval no. 2022-138).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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