NRBP1 negatively regulates SALL4 to reduce the invasion and migration, promote apoptosis and increase the sensitivity to chemotherapy drugs of breast cancer cells

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Abstract. The incidence of breast cancer (BC) ranks first among all kinds of female malignancies. Its invasion, migration, apoptosis and resistance to chemotherapeutic drugs are the focus of current research. Nuclear receptor binding protein 1 (NRBP1) and spalt-like transcription factor 4 (SALL4), which are observed to be abnormally expressed in BC, are investigated herein to identify their involvement in invasion, migration, apoptosis and chemotherapeutic drug sensitivity of BC and to elucidate the underlying mechanism. After NRBP1 was overexpressed by cell transfection, wound healing and Transwell experiments were used to detect the abilities of cell invasion and migration, and western blotting was used to detect the expression of MMP2 and MMP9. Cell viability and apoptosis were detected by Cell Counting Kit-8 assay, TUNEL staining and western blotting, in which Doxorubicin (DOX) and cis-platinum (Cis) were administrated after overexpression of NRBP1. Finally, after overexpression of NRBP1 and SALL4, the cell invasion, migration and apoptosis, and the sensitivity to DOX and Cis, were detected to explore the underlying mechanism. Overexpression of NRBP1 inhibited the invasion and migration, promoted the apoptosis, and enhanced the chemotherapeutic effect of chemotherapy drugs in BC cells. Overexpression of SALL4 in cells blocked the effects of NRBP1 overexpression on invasion, migration, apoptosis and DOX and Cis drug sensitivity of BC cells. In conclusion, NRBP1 negatively regulated SALL4 to reduce the invasion and migration capacities, promote apoptosis and increase the sensitivity to chemotherapeutic drugs of BC cells.

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Introduction

According to statistics in 2012, the incidence of breast cancer (BC) ranks first among all kinds of female malignancies in the world, with ~1.671 million new cases of BC diagnosed globally every year and ~522,000 patients succumbing to the disease (1). However, the pathogenesis of this disease has not been fully elucidated. In addition, as a consequence of the lack of effective therapeutic targets, the treatment options for BC are relatively poor, which has contributed to a 5-year survival rate of <15% (2). The current mainstream treatment, which is chemotherapy, confers low therapeutic efficacy due to the development of drug resistance (3). Anthracyclines are one of the most effective drugs in the treatment of BC, but they have strong side effects and notable cardiotoxicity (4). Doxorubicin (DOX) is one of the basic drugs commonly used in the chemotherapeutic regimen of BC. However, its clinical application is hindered by drug resistance, a narrow therapeutic window and severe cardiorenal toxicity (5). Therefore, exploring the mechanism of drug resistance in BC will allow more specific probing methods to prevent drug resistance in tumor cells.

Nuclear receptor binding protein 1 (NRBP1), a newly discovered adaptor protein, is present in most normal human tissues and serves a crucial role in the regulation of intracellular signal transduction pathways (6). It can specifically regulate the binding between signaling protein molecules, thus regulating the occurrence and development of numerous diseases (7). In bladder cancer, high expression of NRBP1 promotes cell proliferation and is associated with a less favorable prognosis (8). Similarly, high expression of NRBP1 in prostate cancer is linked to poor clinical outcomes and increased cancer cell growth (9). A previous study reported that NRBP1 is downregulated in patients with BC, and overexpression of NRBP1 can inhibit the proliferation of cancer cells through the Wnt/ β-catenin signaling pathway (10). However, whether NRBP1 can affect the invasion, migration and apoptosis of BC cells as well as the its role in drug sensitivity still need to be studied in detail.

Spalt-like transcription factor 4 (SALL4) gene was first found to be highly expressed in embryonic stem cells, and it serves an important role in maintaining the pluripotency and self-renewal of these. Moreover, its expression level is significantly related to the process of cell cycle (11). A number of

studies have revealed that SALL4 is closely associated with the development of BC (12-14). Other studies have reported that NRBP1 can negatively regulate the expression of stem cell marker SALL4, and at the same time, SALL4 interference can inhibit the proliferation, invasion and migration capacities, and the apoptosis of BC, as well as reducing the drug resistance to DOX (15,16). Therefore, it was speculated that NRBP1 may serve a role in the invasion, migration and apoptosis, as well as the drug sensitivity, of BC cells by regulating SALL4.

In the present study, the role of NRBP1 in cell invasion, migration and apoptosis, as well as chemotherapy drug sensitivity of BC, were investigated, in addition to the underlying mechanisms. The current study provides a theoretical basis for targeted therapy and chemotherapy-sensitive therapy for BC.

Materials and methods

Cell culture. Breast cancer MDA-MB-231 cells were purchased from Shanghai Institute of Biological Sciences, Chinese Academy of Sciences. The cells were maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin solution (Hyclone, Thermo Fisher Scientific) at 37˚C at 5% CO_2. After transfection, the cells were treated with 0.1 μg/ml Doxorubicin and 2 μmol/l Cis at 37˚C for 24 h, and then related experiments were conducted.

Cell transfection. The NRBP1 and SALL4 overexpression plasmids (ov-NRBP1 and ov-SALL4, respectively) and their corresponding negative control transfection group (Ov-NC) were obtained from FitGene BioTechnology Co., Ltd. using the pcDNA3.1 vector. The ov-NRBP1 plasmid and ov-SALL4 plasmid or the control empty vector pcDNA3.1 (OV-NC; 1 μg each) were transfected into cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h at 37˚C, according to the manufacturer's instructions. Following incubation for 48 h, cells were used for subsequent experiments. The control group did not undergo any transfection treatment. The overexpression vector sequence is listed in Data S1.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from cells with RNAiso Plus (Takara Biotechnology Co., Ltd.) according to the manufacturer's specifications. Reverse transcription into cDNA was performed using the PrimeScript RT Reagent kit (cat. no. RR037A; Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol followed by qPCR using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Inc.) on a StepOne™ Real-Time PCR system (Applied Biosystems. The following thermocycling conditions were as follows: 95˚C For 10 min; followed by 40 cycles of denaturation at 95˚C for 10 sec and annealing/extension at 60˚C for 60 sec. The primer sequences were as follows: NRBP1 forward, 5'-GGA CTCATCAAGATTGGCTCTG-3' and reverse, 5'-TCTTCT GCTCTTCTCGACAAGT-3'; SALL4 forward, 5'-TCGATG GCCAACTTCCTTC-3' and reverse, 5'-GAGCGGACTCAC ACTGGAGA-3'; and GAPDH forward, 5'-GAGCCCGCA GCCTCCCGCTT-3' and reverse, 5'-CCCGCGGCCATCACG CCACAG-3'. Relative mRNA levels were calculated using the $2^{-\Delta\Delta Cq}$ method (17) and normalized to GAPDH.

Western blotting. The cultured cells were treated with RIPA lysis buffer (Beyotime Institute of Biotechnology) at 4˚C for 30 min. The lysates were cleared by centrifugation (22,000 x g) at 4˚C for 20 min, and supernatants were collected as protein samples. Protein concentration was determined using a BCA kit (Beyotime Institute of Biotechnology). Next, 30 μg proteins were separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to PVDF membranes (MilliporeSigma). Then, 5% non-fat milk was used to block PVDF membranes for 60 min at room temperature. The membranes were incubated with primary antibodies overnight at 4˚C. The antibodies are as follows: NRBP1 (cat. no. GTX84007), MMP2 (cat. no. GTX104577), MMP9 (cat. no. GTX100458), Bcl2 (cat. no. GTX100064), survivin (cat. no. GTX100065), P53 (cat. no. GTX70214), Bax (cat. no. GTX109683), cleaved PARP (cat. no. GTX009483) and GAPDH (cat. no. GTX100118) (all 1:1,000 dilution; GeneTex, Inc.). GAPDH was used for the loading control. On the next day, the membranes were then incubated with HRP-conjugated goat anti-rabbit IgG (cat. no. GTX213110-01) or HRP-conjugated goat anti-mouse secondary antibody (cat. no. GTX213111-01) (both 1:10,000 dilution; GeneTex, Inc.) for 60 min at room temperature. The membranes were developed using an enhanced chemiluminescence system (Beyotime Institute of Biotechnology). Images were captured with SynGene G: Box Chemi XRQ (AlphaMetrix Biotech GmbH), and intensity of blot bands was analyzed using ImageJ 1.8.0 (National Institutes of Health).

Wound healing. Cells were plated in a 6-well plate at a density of $5x10^5$ cells/well. After being transfected with ov-NRBP1 plasmid and ov-SALL4 plasmid at 37˚C for 48 h, the wounds were scratched using a sterile pipette tip. The remaining cells were washed twice in serum-free culture media, and wound closure was then observed. Images at 0 and 24 h were captured using a phase-contrast microscope (magnification, x100) and the percent of wound closure was calculated: [(Ai-At)/Ai] x 100, where Ai represents the initial area of the wound at 0 h and At represents the area of the wound after 24 h.

Transwell assay. After transfection of ov-NRBP1 plasmid and ov-SALL4 plasmid at 37˚C for 48 h, the cells were collected, seeded into the upper chamber (8-μm pores) pre-coated with Matrigel overnight at 37˚C at a density of $1x10^5$ cells/well (Corning, Inc.) and incubated with DMEM. The lower chamber was filled with 500 μl DMEM with 10% FBS. After 12 h of incubation at 37˚C, the cells on the upper surface of the membrane were removed. The cells in the lower chamber were fixed with formaldehyde and stained with crystal violet for 30 min at room temperature. The number of migrated cells was counted under an inverted microscope (Olympus Corporation; magnification, x100) in five random fields using ImageJ 1.8.0 (National Institutes of Health).

TUNEL. TUNEL Apoptosis Assay kit (cat. no. C1086; Beyotime Institute of Technology) was used for detection of apoptosis. A total of $1x10^6$ cells were seeded on chamber slides (Nunc Lab-Tek; Sigma-Aldrich, Merck KGaA) were treated accordingly. TUNEL staining was conducted according to the manufacturer's specifications. A light microscope (Leica DM6000B; magnification, x200) coupled with a camera

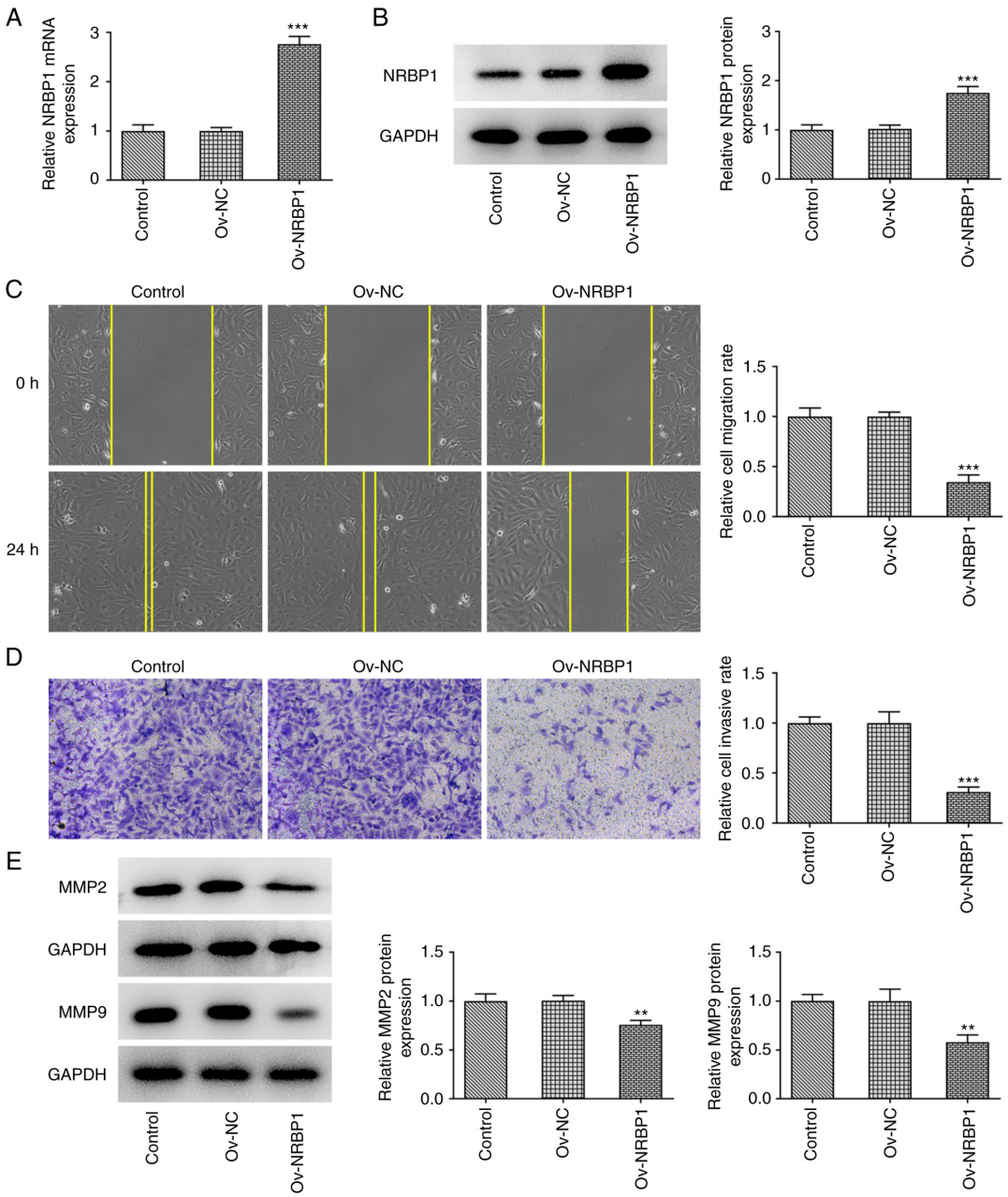

Figure 1. Overexpression of NRBP1 inhibits the invasion and migration of BC cells. (A) Reverse transcription-quantitative PCR was used to detect the expression of NRBP1. (B) Western blot analysis detected the expression of NRBP1. (C) Wound healing detected the migration of cells (magnification, x100). (D) Transwell assays detected the invasion of cells (magnification, x100). (E) Western blot analysis detected the expression of MMP2 and MMP9. **P<0.01, ***P<0.001 vs. Ov-NC. NRBP1, nuclear receptor binding protein 1; Ov-NC, overexpression-negative control.

(Leica DC490) was used for light microscopic evaluation of TUNEL-positive cells and Image J software (version 1.48v; National Institutes of Health) was utilized for analysis.

Cell Counting Kit-8 (CCK-8). Cell viability was measured via the CCK-8 (Dojindo Molecular Technologies, Inc.) according to the manufacturer's specification. After treatment of 0.1 μg/ml Doxorubicin and 2 μmol/l Cis at 37˚C for 48 h, the cells were stained with 10 μl CCK-8 reagent and incubated at 37˚C for 2 h. Finally, the number of viable cells was assessed by measuring the absorbance at 450 nm in each well.

Statistical analysis. All data are presented as the mean ± SD. All analyses were carried out with the SPSS 22.0 statistical software package (IBM Corp.). One-way ANOVA followed by Tukey's post hoc test was used to compare the differences between more than two groups. P<0.05 was considered to indicate a statistically significant difference. Three or more independent experiments were repeated.

Results

Overexpression of NRBP1 inhibits invasion and migration, and promotes apoptosis of BC cells. The overexpression plasmid of NRBP1 was constructed and the overexpression level of NRBP1 was detected via RT-qPCR and western blotting. The NRBP1 level in the OV-NRBP1 group was significantly higher than that in the OV-NC group (Fig. 1A and B).

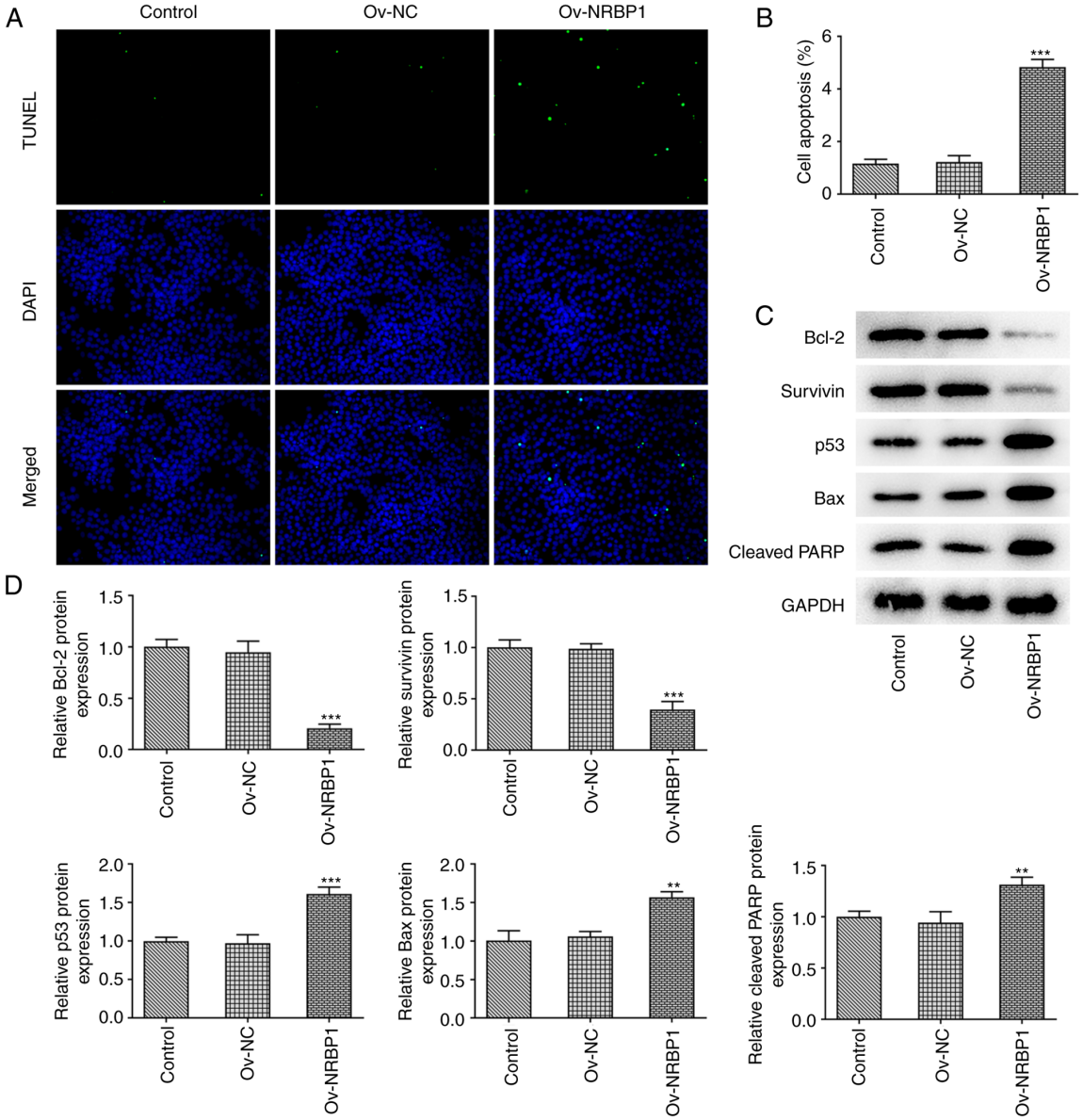

Figure 2. Overexpression of NRBP1 promotes the apoptosis of breast cancer cells. (A) TUNEL assay detected cell apoptosis (magnification, x200). (B) Quantification of apoptosis. (C) Western blot analysis detected the expression of apoptosis-related proteins. (D) Semi-quantification of apoptosis-related proteins. **P<0.01, ***P<0.001 vs. Ov-NC. NRBP1, nuclear receptor binding protein 1; Ov-NC, overexpression-negative control.

Then, wound healing and transwell assays were used to detect cell invasion and migration capabilities. It was revealed that the invasion and migration abilities of OV-NRBP1 group were significantly decreased compared with OV-NC group (Fig. 1C and D). In addition, the expression levels of MMP2 and MMP9 proteins in cells were detected by western blot analysis, and it was revealed that compared with OV-NC, the expression of MMP2 and MMP9 in the OV-NRBP1 group was significantly reduced (Fig. 1E). Cell apoptosis was detected by TUNEL staining and western blot analysis. Compared with the OV-NC group, apoptosis was significantly increased in the OV-NRBP1 group (Fig. 2A and B), accompanied by decreases in Bcl2 and survivin proteins and increases in P53, Bax and cleaved PARP proteins (Fig. 2C and D).

Overexpression of NRBP1 enhances the efficacy of chemotherapy drugs in BC cells. CCK-8 was used to detect the cell activity after DOX and Cis treatment. The results indicated that the cell activity of DOX 0.1 μg/ml group decreased significantly compared with that of the group without DOX. Cell viability was further decreased after overexpression of NRBP1 (Fig. 3A). Western blotting was used to detect cell apoptosis. Compared with the group without DOX, the expression of Bcl-2, an anti-apoptotic protein, was decreased in the 0.1 μg/ml DOX group. The expression levels of pro-apoptotic proteins Bax, cleaved caspase3, cleaved PARP and Bad were significantly increased. After the overexpression of NRBP1, the expression of Bcl-2 in cells was further decreased and the expression of pro-apoptotic proteins was further increased (Fig. 3B). The trend of cell viability and apoptosis after Cis administration was consistent with that of DOX administration (Fig. 3C). These data suggested that the overexpression of NRBP1 enhanced the efficacy of chemotherapy drugs in BC cells.

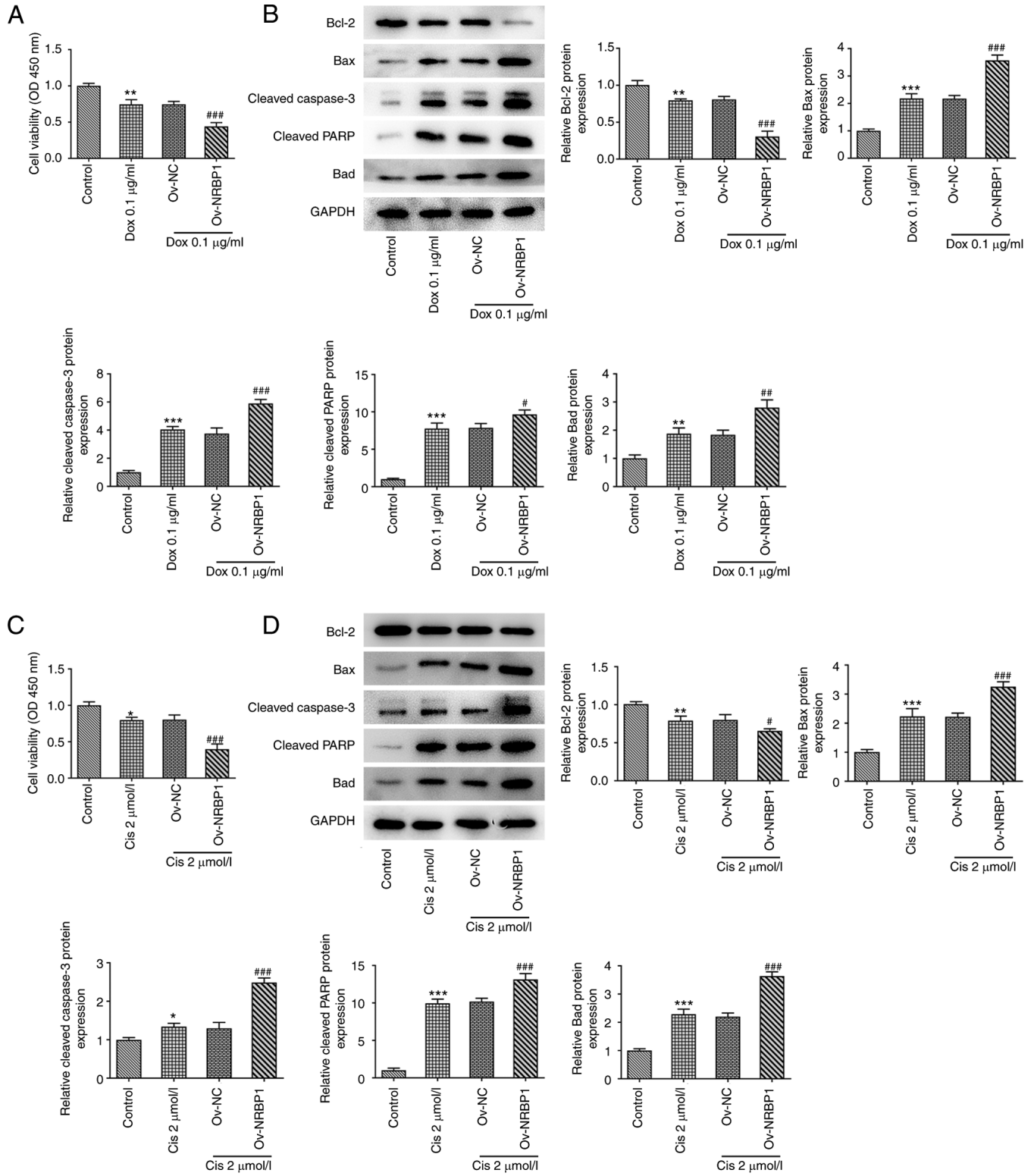

Figure 3. Overexpression of NRBP1 enhances the efficacy of chemotherapy drugs in BC cells. (A) CCK-8 detected the cell viability after Dox administration. (B) Western blot detected the expression of apoptosis-related proteins after treatment with Dox. (C) CCK-8 detected the cell viability after Cis treatment. (D) Western blot detected the expression of apoptosis-related proteins after given Cis treatment. *P<0.05, **P<0.01, ***P<0.001 vs. Control; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. Ov-NC. CCK-8, Cell Counting Kit-8; NRBP1, nuclear receptor binding protein 1; Ov-NC, overexpression-negative control.

Overexpression of SALL4 abrogates the inhibitory effects of NRBP1 on invasion, migration and apoptosis of BC cells. The expression level of SALL4 after cell transfection was detected by RT-qPCR and western blot analysis. Notably, the cells were successfully transfected (Fig. 4A and B). The cells were divided into control, Ov-NRBP1, Ov-NRBP1 + Ov-NC and Ov-NRBP1 + Ov-SALL4 groups. Wound healing and Transwell assays demonstrated that, compared with Ov-NRBP1 + OV-NC group, the cell invasion and migration abilities of the Ov-NRBP1 + Ov-SALL4 group were

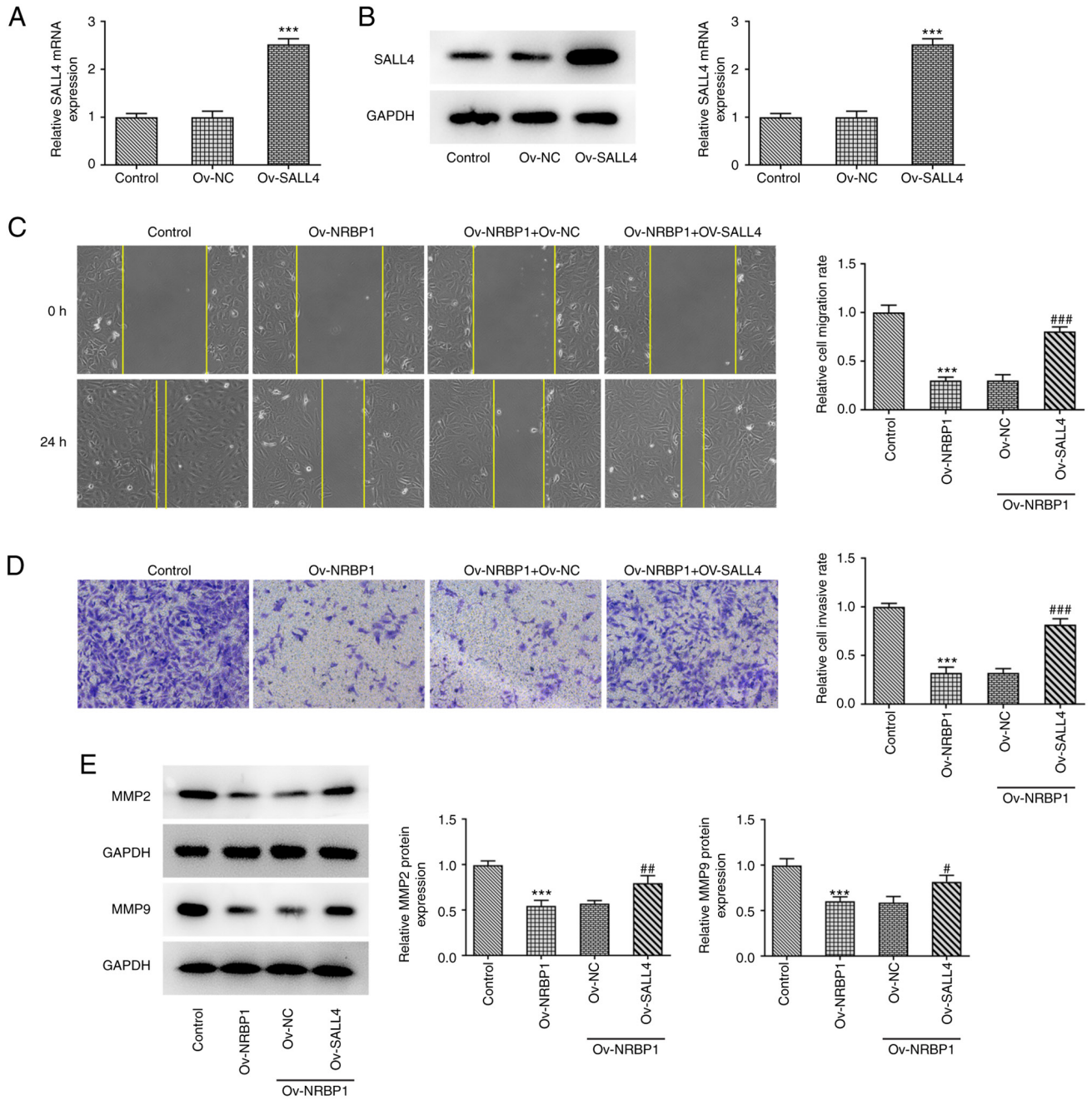

Figure 4. Overexpression of SALL4 abrogates the inhibitory effects of NRBP1 on invasion and migration of breast cancer cells. (A) RT-qPCR detected the expression of SALL4. (B) Western blot detected the expression of SALL4. ***P<0.001 vs. Ov-NC. (C) Wound healing detected the migration of cells (magnification, x100). (D) Transwell detected the invasion of cells (magnification, x100). (E) Western blot detected the expression of MMP2 and MMP9. ***P<0.001 vs. Control; #P<0.05, ##P<0.01, ###P<0.001 vs. Ov-NC. NRBP1, nuclear receptor binding protein 1; Ov-NC, overexpression-negative control; SALL4, spalt-like transcription factor 4.

significantly increased (Fig. 4C and D). The expression of MMP2 and MMP9 was significantly increased in comparison with the Ov-NRBP1 + Ov-NC group (Fig. 4E). As for cell apoptosis, the TUNEL assay demonstrated that, compared with the Ov-NRBP1 + Ov-NC group, apoptosis in the Ov-NRBP1 + Ov-SALL4 group was significantly decreased (Fig. 5A and B), accompanied by the increased expression of Bcl-2 and the decreased expression of pro-apoptotic proteins detected via western blotting (Fig. 5C and D). These results indicated that overexpression of SALL4 abrogates the inhibitory effects of NRBP1 on invasion, migration and apoptosis of BC cells.

Overexpression of NRBP1 enhances the effect of chemotherapy drugs on BC cells through SALL4. After overexpression of NRBP1 and SALL4 in cells, DOX and Cis were administered, respectively. CCK-8 and western blotting results revealed that, compared with the DOX 0.1 μg/ml + Ov-NRBP1 + Ov-NC group, the cell viability of DOX 0.1 μg/ml + Ov-NRBP1 + Ov-SALL4 group was significantly increased (Fig. 6A), and the expression of Bcl-2 in cells was also increased. Simultaneously, the expression of Bax, cleaved caspase 3, cleaved PARP and Bad was decreased (Fig. 6B). The expression trend of Cis was consistent with that of DOX

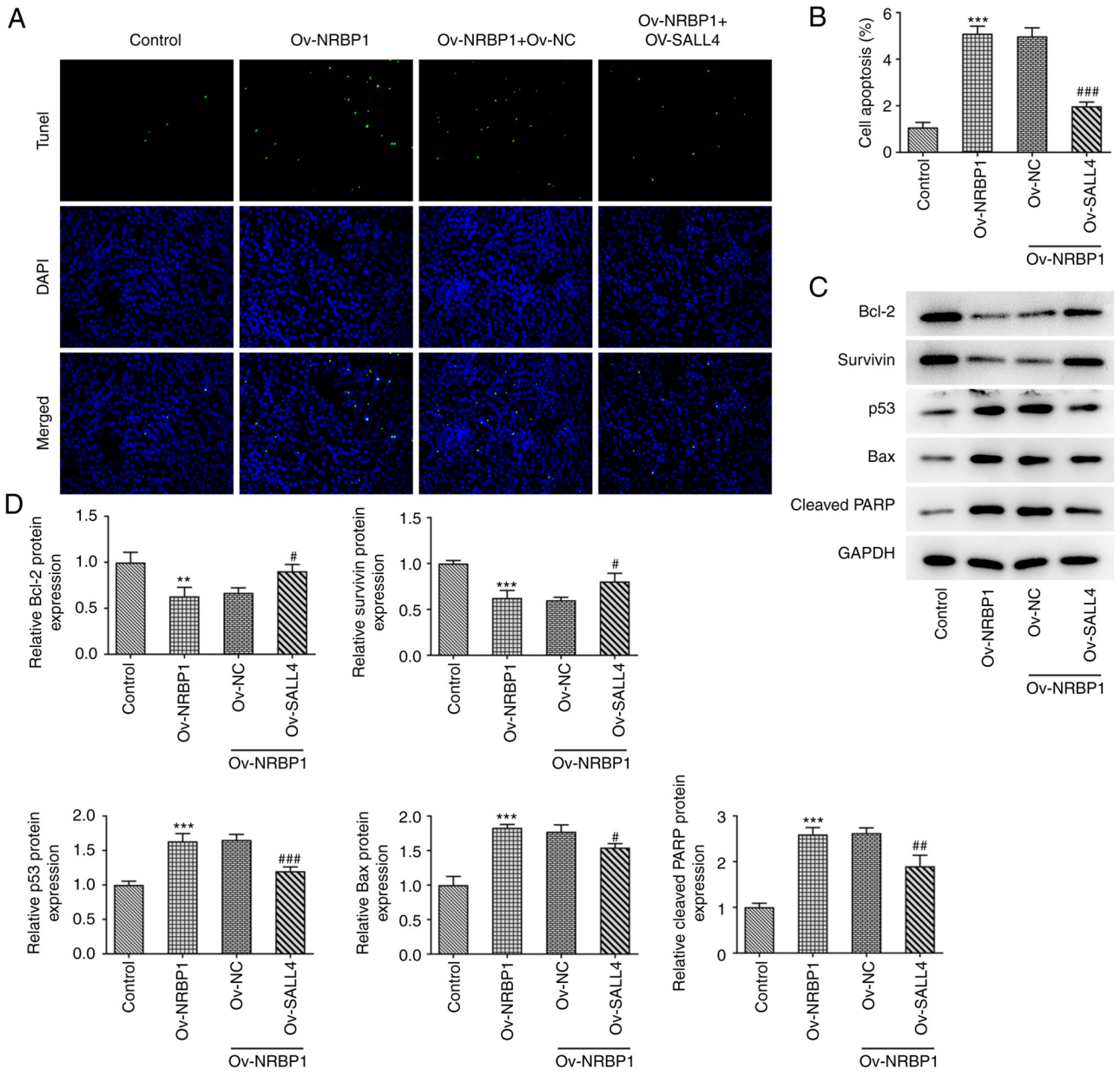

Figure 5. Overexpression of SALL4 abrogates the inhibitory effects of NRBP1 on apoptosis of breast cancer cells. (A) TUNEL assay detected cell apoptosis (magnification, x200). (B) Statistical map of apoptosis. (C) Western blot detected the expression of apoptosis-related proteins. (D) Statistical map of apoptosis-related proteins. **P<0.01, ***P<0.001 vs. Control; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. Ov-NC. NRBP1, nuclear receptor binding protein 1; Ov-NC, overexpression-negative control; SALL4, spalt-like transcription factor 4.

(Fig. 6C and D). These results indicated that the overexpression of NRBP1 enhanced the effect of chemotherapy drugs on BC cells via regulation of SALL4.

Discussion

Estrogen receptor-positive cell line MCF-7 was selected in the present experiments and multiple reports have investigated the mechanism underlying the malignant progression of BC by detecting proliferation, migration and invasion of the MCF-7 cell line (18-20).

Somatic deletion of NRBP1 in mice results in a progenitor cell phenotype that disrupts the normal program of cell differentiation and proliferation along the villus axis of the intestinal crypt (21). In addition, NRBP1 is downregulated in a range of human tumor types, and that its low expression is associated with the poor prognosis of these diseases (6). Based on the aforementioned information, NRBP1 is regarded as a conserved regulator of cell fate that serves an important role in tumor suppression. Consistent with the findings by Wei *et al* (10), the current study demonstrated that following NRBP1 overexpression, the invasion and migration abilities of BC cells were significantly decreased while apoptosis was increased, which may enhance the chemotherapeutic effect of chemotherapy drugs on BC.

In a study by Wilson *et al* (22), NRBP1 downregulated the expression of SALL4. Moreover, knockdown of NRBP1 increased levels of SALL4 and TSC22D2 in the intestinal tract, and SALL4 served an important role in invasion, migration and apoptosis in BC (12). In addition, in chronic myeloid leukemia,

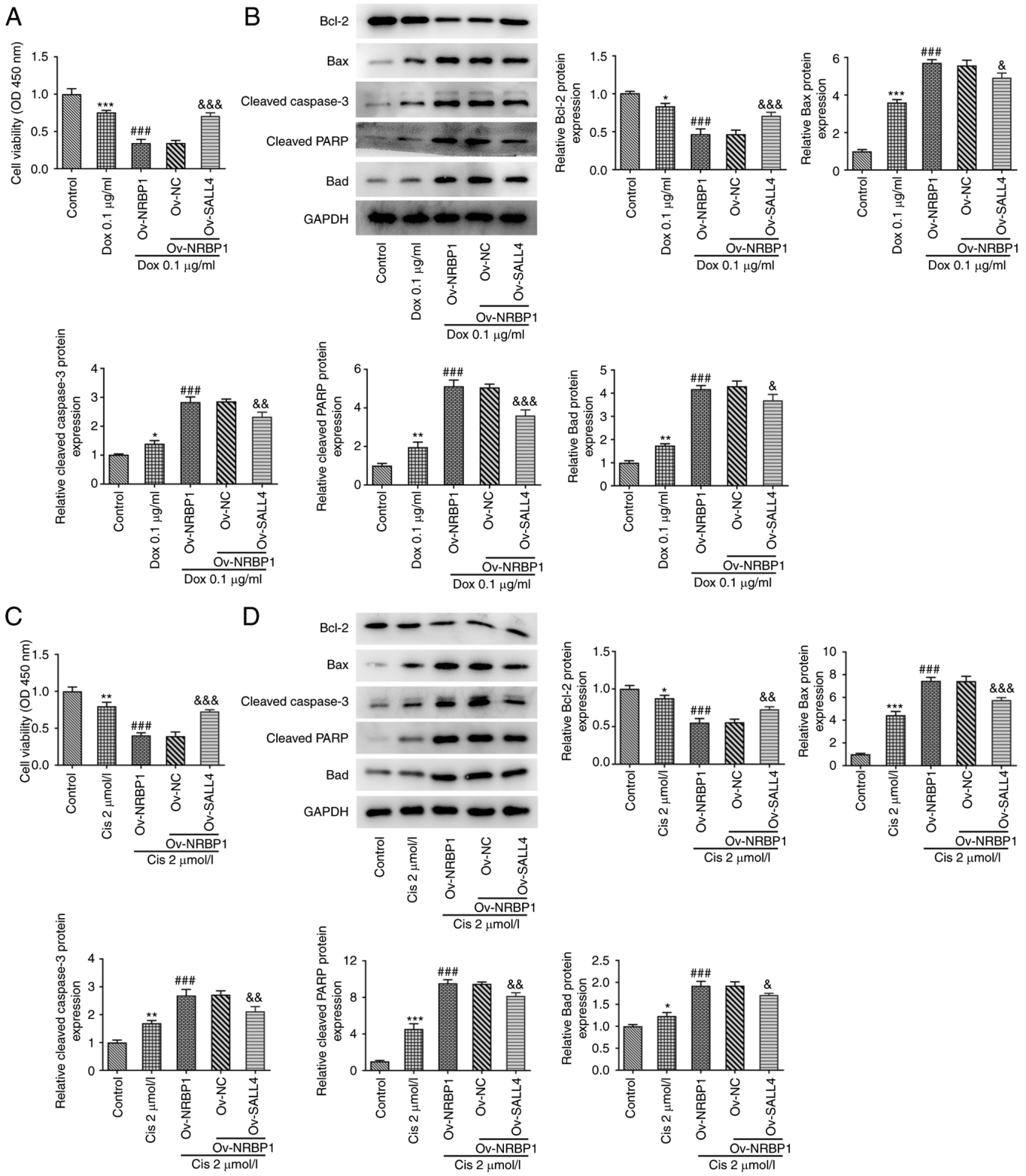

Figure 6. Overexpression of NRBP1 enhances the effect of chemotherapy drugs on breast cancer cells through SALL4. (A) CCK-8 detected the cell viability after given Dox. (B) Western blot detected the expression of apoptosis-related proteins after given Dox. (C) CCK-8 detected the cell viability after given Cis. (D) Western blot detected the expression of apoptosis-related proteins after given Cis. *P<0.05, **P<0.01, ***P<0.001 vs. Control; $^{\#\#\#}$P<0.001 vs. Dox 0.1 μg/ml; $^{\&}$P<0.05, $^{\&\&}$P<0.01, $^{\&\&\&}$P<0.001 vs. Ov-NC. NRBP1, nuclear receptor binding protein 1; Ov-NC, overexpression-negative control; SALL4, spalt-like transcription factor 4; Dox, doxorubicin; Cis, cis-platinum.

inhibition of SALL4 expression enhanced the sensitivity of leukemia cells to imatinib, dasatinib and nilotinib (23). In the present experiment, SALL4 was overexpressed in MCF-7 cells, and its transfection efficiency was detected by RT-qPCR and western blot analysis. Subsequently, in order to further explore the regulatory mechanisms of NRBP1 and SALL4 in the proliferation, invasion, migration and apoptosis of BC cells, SALL4 was overexpressed in cells under the condition

of overexpression of NRBP1. It was revealed that overexpression of SALL4 blocked the inhibitory effects of NRBP1 on invasion, migration and apoptosis of BC cells, which had significance for our conclusion.

Drug resistance of cancer cells to anti-cancer drugs can weaken the therapeutic effects of chemotherapy drugs. Succumbing to frequent recurrence and metastasis of tumor cells has demonstrated the low five-year survival rate of patients (24). Therefore, research on the resistance mechanism of antibody drugs and how to reverse the resistance of cancer cells remains a current focus for anti-cancer methods. In the current paper, it was revealed that NRBP1 increased the sensitivity of BC cells to DOX and Cis chemotherapy drugs by negatively regulating SALL4, thus providing a solid theoretical basis for the mechanism of BC cell sensitivity to DOX and Cis. Fig. S1 illustrates the specific mechanism of the experiment. In subsequent experiments, the effects on cell proliferation, invasion, migration and apoptosis caused by adding DOX or Cis into NRBP1-overexpressing cells will be investigated and compared with NRBP1 overexpressing cells alone.

There were several limitations in the present study. Multiple reports have investigated the mechanism of malignant progression of breast cancer by detecting proliferation, migration and invasion of the MCF-7 cell line (18-20). Thus, the focus was primarily on the phenotype of ER-positive cell line MCF-7, which is also one of the limitations of the paper. The findings should be validated further in other highly metastatic BC cell lines such as ER-positive, HER2 and triple negative cell lines in the following experiments. In addition, the roles and mechanisms of NRBP1 and SALL4 in the occurrence, development and drug sensitivity of BC should be examined using animal experiments. Moreover, the association between NRBP1 and SALL4 in BC stem cells should be explored, and mammosphere formation assays should be used to explore the role of SALL4.

In conclusion, the present study confirmed the role of NRBP1 in inhibiting the invasion and migration of BC cells and promoting their apoptosis and increasing the sensitivity to chemotherapeutic drugs by negatively regulating SALL4. The current results provide a theoretical basis for the discussion of the mechanism of BC and the mechanism of DOX and Cis resistance in BC.

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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

CY and XZ designed the experimental study. CG and KD carried out the experiments. YL analyzed and interpreted the experiment data. CG wrote the manuscript. CY helped to correct the manuscript. CY and KD confirm the authenticity of all the raw data. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patients consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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