

GRWD1 affects the proliferation, apoptosis, invasion and migration of triple negative breast cancer through the Notch signaling pathway

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Received March 4, 2022; Accepted April 8, 2022

DOI: 10.3892/etm.2022.11400

Abstract. Breast cancer is a highly heterogeneous tumor, among which triple negative breast cancer (TNBC) is the most invasive and prone to recurrence and metastasis. The present study aimed to investigate the regulatory mechanisms of glutamate-rich WD-repeat-containing protein 1 (GRWD1) in TNBC cells. The expression of GRWD1 in the normal human breast epithelial cells and human breast cancer cells was detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis. The transfection effects of small interfering RNA (siRNA)-GRWD1 and overexpression (Ov)-Notch1 were also confirmed by RT-qPCR and western blotting. The proliferation, apoptosis, invasion and migration of transfected cells were in turn analyzed by Cell Counting Kit-8, 5-Ethynyl-2'-deoxyuridine, Matrigel and wound healing assays. The expression of proteins related to proliferation, apoptosis, metastasis, epithelial-mesenchymal transition and the Notch signaling pathway was detected by western blotting. As a result, GRWD1 expression was upregulated in breast cancer cells and was revealed to be highest in MDA-MB-231 and HCC1937 cells. GRWD1 knockdown suppressed TNBC cell proliferation, invasion and migration and promoted TNBC cell apoptosis. Furthermore, the expression of Notch1 and Notch4 was inhibited by GRWD1 knockdown. The expression of downstream genes of the Notch signaling pathway Hes1, Hes5, Hey1, Hey2, p21, c-Myc, cyclin D1, human epidermal growth factor 2 receptor and NF- κ B were all suppressed after siRNA-GRWD1 transfection. However, Notch1 overexpression reversed the effect of GRWD1 knockdown on biological

behaviors of TNBC cells. In conclusion, GRWD1 knockdown could suppress the proliferation, invasion and migration and promoted apoptosis of TNBC cells through inhibiting the Notch signaling pathway.

Introduction

Breast cancer is one of the most common malignant tumors in women. According to the latest global cancer data in 2020, there were 2.26 million new cases of breast cancer worldwide in 2020, accounting for 11.7% of the total cancer cases, and ~860,000 deaths, accounting for 6.9% of the total cancer-related deaths (1). There are five different types of breast cancer: Luminal A breast cancer, Luminal B breast cancer, triple negative breast cancer (TNBC), human epidermal growth factor 2 receptor (HER2) negative breast cancer and HER2 positive breast cancer. TNBC accounts for ~15% of breast cancers and is characterized by loss of expression of estrogen receptor, progesterone receptor and HER2 (2,3). Local invasion and distant spread are the main causes of tumor progression (4). Although surgery and chemotherapy are effective, they still fail to meet expectations (5). Therefore, the development of effective and safe treatment strategies for TNBC should not be underestimated.

Previous studies have found that p53 mutations are diagnostic and prognostic indicators of breast cancer, and breast cancer with p53 mutations is more aggressive, such as TNBC (6,7). Glutamate-rich WD-repeat-containing protein 1 (GRWD1) is a multifunctional protein rich in glutamic acid and is involved in numerous cellular regulatory pathways, particularly ribosome metabolism and cell growth (8). Recently, GRWD1 was found to bind to the tumor suppressor gene p53 and negatively regulate the expression of p53 (9), which leads to the attention of GRWD1 as a potential oncogene in an increasing number of tumors. For example, GRWD1 promotes tumor cell growth and drug resistance by regulating p53 in colorectal cancer (10). Concurrently, highly expressed GRWD1 can promote the growth and metastasis of non-small cell lung cancer (NSCLC) through the Notch pathway (11). However, the role of GRWD1 in TNBC has not been studied.

The present study aimed to detect the expression levels of GRWD1 in breast cancer cells and to explore the regulatory

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Key words: glutamate-rich WD-repeat-containing protein 1, proliferation, invasion and migration, triple negative breast cancer, the Notch signaling pathway

effect of GRWD1 on the proliferation, apoptosis, invasion and migration of breast cancer cells through the Notch pathway.

Materials and methods

Cell culture. Normal human breast epithelial cells (MCF-10A) and human breast cancer cells (MDA-MB-231, HCC1937, MCF-7, SK-BR-3 and BT474) were provided from Procell Life Science & Technology Co., Ltd. The MCF-10A cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Procell Life Science & Technology Co., Ltd.) containing 5% horse serum (Procell Life Science & Technology Co., Ltd.), 20 ng/ml EGF, 0.5 μ g/ml hydrocortisone, 10 μ g/ml insulin, 1% non-essential amino acids and 1% penicillin/streptomycin. The breast cancer cells were cultured in DMEM (Hyclone; Cytiva) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin. All cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Cell transfection. Small interfering RNA (siRNA)-negative control (NC) (siB06525141922-1-5), siRNA-GRWD1 (siG000083743A-1-5), overexpression (Ov)-NC and Ov-Notch1 were synthesized and purchased from Guangzhou RiboBio Co., Ltd. A total of 50 nM siRNA-NC, siRNA-GRWD1, Ov-NC and Ov-Notch1 were transfected into MDA-MB-231 and HCC1937 cells when their confluence reached 80% using Lipofectamine® 3000 kit (cat. no. L3000015; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Then, cells were cultured at 37°C for 48 h before subsequent experiments.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from MDA-MB-231 and HCC1937 cells (1 \times 10⁶ cells) using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Then, the PrimeScript™ First Strand cDNA Synthesis kit (Takara Bio, Inc.) was used to reverse transcribe the RNA to first-stranded cDNA at 30°C for 10 min. qPCR was performed with SYBR Premix Ex Taq (Takara Bio, Inc.) on a CFX96 System (Bio-Rad Laboratories, Inc.). The thermocycling conditions were as follows: 10 min initial denaturation at 94°C, 15 sec denaturation at 94°C and 30 sec of annealing at 55°C (40 cycles) and final extension for 1 min at 72°C. GAPDH were used as internal control. The relative expression of genes was calculated using the 2^{- $\Delta\Delta$ C_q} method (12). The primer sequences used for qPCR (designed by Thermo Fisher Scientific, Inc.) were as follows: GRWD1 forward, 5'-ATCACACAGTGGGACCTGGCA-3' and reverse, 5'-TCAGACGCTGATGGTGC GGAA-3'; Notch1 forward, 5'-CTGGTCAGGGAAATCGTG-3' and reverse, 5'-TGGGCA GTGGCAGATGTAG-3'; Notch4 forward, 5'-ACACACACA TGAGGATCTCTGGCA-3' and reverse, 5'-AGTTGGCCT TGTCTTTCTGGTCTCT-3'; and GAPDH forward, 5'-GAC AGTCAGCCGCATCTTCT-3' and reverse, 5'-TTAAAGCA GCCCTGGTGAC-3'.

Western blot analysis. Proteins were extracted from MDA-MB-231 and HCC1937 cells using RIPA lysis buffer (Thermo Fisher Scientific, Inc.). The protein concentration

was detected by the BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Proteins (15 μ g) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk for 2 h at room temperature and subsequently incubated with primary antibodies against GRWD1 (cat. no. ab176815; 1:2,000), Ki67 (cat. no. ab92742; 1:5,000), proliferating cell nuclear antigen (PCNA; cat. no. ab92552; 1:1,000), Bcl-2 (cat. no. ab32124; 1:1,000), cleaved caspase-3 (cat. no. ab32042; 1:500), Bax (cat. no. ab32503; 1:1,000), caspase-3 (cat. no. ab32351; 1:5,000), MMP9 (cat. no. ab76003; 1:1,000), MMP2 (cat. no. ab92536; 1:1,000), E-cadherin (cat. no. ab40772; 1:10,000), N-cadherin (cat. no. ab76011; 1:5,000), Vimentin (cat. no. ab92547; 1:1,000), Notch1 (cat. no. ab52627; 1:1,000), Notch4 (cat. no. ab184742; 1:1,000), Hes1 (cat. no. ab108937; 1:1,000), Hes5 (cat. no. ab194111; 1:1,000), Hey1 (cat. no. ab154077; 1:1,000), Hey2 (cat. no. ab167280; 1:1,000), p21 (cat. no. ab109520; 1:1,000), c-Myc (cat. no. ab32072; 1:1,000), cyclin D1 (CCND1; cat. no. ab16663; 1:200), HER2 (cat. no. ab134182; 1:1,000), NF- κ B (cat. no. ab32536; 1:1,000) and GAPDH (cat. no. ab9485; 1:2,500; all from Abcam) overnight at 4°C. Following 0.1% Tween TBST washing, membranes were incubated with the HRP-conjugated goat anti-rabbit IgG secondary antibody (ab6721; 1:2,000; Abcam) for 1 h at room temperature. The blots were developed with enhanced chemiluminescence Western Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc.), visualized using the Gel Imager System (Bio-Rad Laboratories, Inc.) and quantified using ImageJ version 1.8.0 (National Institutes of Health).

Cell Counting Kit-8 (CCK-8) assay. The MDA-MB-231 and HCC1937 cells were inoculated in 96-well plates with each well including 2,000 cells. Following transfection, 10 μ l of CCK-8 solution (Dojindo Molecular Technologies, Inc.) was added to each well; then, MDA-MB-231 and HCC1937 cells were incubated for 24, 48 and 72 h at 37°C. Subsequently, the cells were incubated with CCK-8 solution for another 2 h and the absorbance at 450 nm was detected by a SpectraMax Absorbance Reader (Molecular Devices, LLC).

5-Ethynyl-2'-deoxyuridine (EdU) staining assay. The MDA-MB-231 and HCC1937 cells were seeded into 96-well plates with each well including 1 \times 10⁴ cells for overnight culture at 37°C. Each well was supplemented with EdU (10 μ M) and plates were incubated for 2 h at 37°C. After that, cells were fixed with 4% polyoxymethylene at room temperature for 20 min and decolorized with glycine (2 mg/ml) at room temperature for 10 min; Hoechst 33342 was added to stain the nucleus at room temperature for 25 min. The fluorescence of cells was observed by a fluorescent microscope (magnification, \times 200; BX41; Olympus Corporation). A total of 3 fields per group were counted. The cell proliferation was quantified using ImageJ version 1.8.0 (National Institutes of Health).

TUNEL assay. The MDA-MB-231 and HCC1937 cells (1 \times 10⁵ cells) were seeded in 24-well plate and transfected for 48 h. Then, cells were fixed with 4% paraformaldehyde for 0.5 h at room temperature and incubated with 0.3% Triton X-100 for 5 min at 4°C. Subsequently, 50 μ l TUNEL reaction

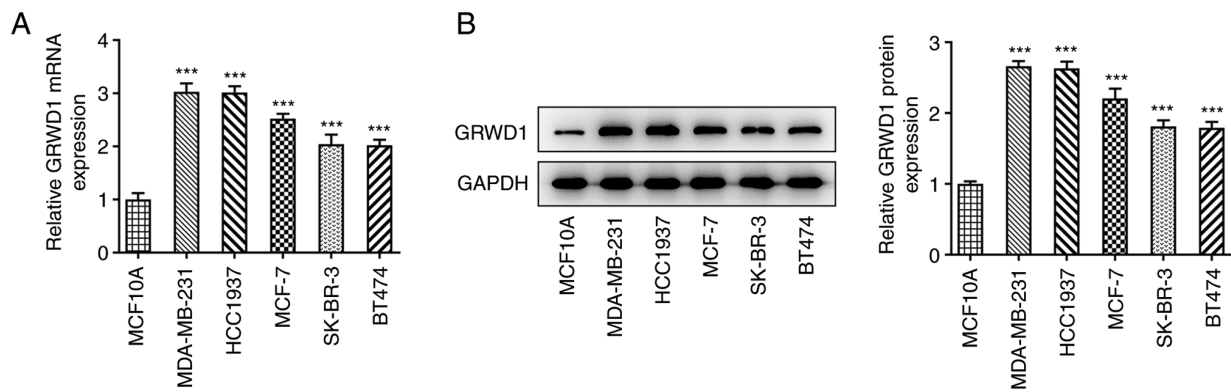


Figure 1. GRWD1 is highly expressed in breast cancer cells. (A) The mRNA and (B) protein expression of GRWD1 in breast cancer cells and normal human breast epithelial cells was detected by reverse transcription-quantitative PCR and western blot analysis, respectively. *** $P < 0.001$ vs. MCF-10A group. GRWD1, glutamate-rich WD-repeat-containing protein 1.

buffer was added and the plate was incubated for 1 h at 37°C; 1 mg/ml DAPI was added to counterstain the nucleus for 1 min at room temperature in dark; the slides were mounted with neutral balsam mounting media (Sangon Biotech Co., Ltd.). Finally, the stained apoptotic cells were visualized under a fluorescent microscope (BX41; Olympus Corporation) and quantified using ImageJ version 1.8.0 (National Institutes of Health). A total of three randomly selected fields per group were counted.

Transwell invasion assay. MDA-MB-231 and HCC1937 cells were seeded at a density of 5×10^4 cells/well in the upper chamber with 8- μ m pore filters (MilliporeSigma) of Transwell plates precoated with Matrigel at 37°C for 30 min. The upper chamber contained the serum-free medium and the lower chamber contained DMEM supplemented with 10% FBS. After 48 h of incubation at 37°C, cells that passed through the membrane were fixed with 4% paraformaldehyde for 15 min at room temperature and stained with 0.1% crystal violet at room temperature for 20 min. Invasive cells were then observed under a light microscope (magnification, $\times 100$) and quantified using ImageJ version 1.8.0 (National Institutes of Health). A total of 3 fields per group were counted.

Wound healing assay. MDA-MB-231 and HCC1937 cells were seeded in a six-well plate with each well containing 5×10^5 cells and incubated overnight at 37°C. The next day, a sterile 200 μ l pipette tip was used to create a 0.5~1 cm horizontal line, followed by PBS washing. Then, cells were incubated with the serum-free medium at 37°C with 5% CO_2 for 24 h. Cells migrating into the scratch were observed under a light microscope (magnification, $\times 100$) at 0 and 24 h. The relative mobility of the cells was then calculated using ImageJ version 1.8.0 (National Institutes of Health).

Statistical analysis. Data analysis was conducted by GraphPad Prism version 7 (GraphPad Software, Inc.) and data were expressed as the means \pm standard deviation (SD) of at least three independent experiments. ANOVA followed by Tukey's post hoc test was used to compare differences between ≥ 3 groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

GRWD1 is highly expressed in breast cancer cells. GRWD1 mRNA expression in breast cancer cells was significantly higher than that in MCF10A cells (Fig. 1A). In addition, the GRWD1 protein expression was higher in breast cancer cells compared with MCF10A cells (Fig. 1B). The aforementioned results indicated that the expression of GRWD1 in MDA-MB-231 and HCC1937 cells was higher than that in other breast cancer cells. Therefore, MDA-MB-231 and HCC1937 cells were chosen for the subsequent experiments.

GRWD1 knockdown suppresses TNBC cell proliferation. When MDA-MB-231 and HCC1937 cells were transfected with siRNA-GRWD1, the GRWD1 mRNA expression in both cell lines was decreased compared with the siRNA-NC group (Fig. 2A). Downregulation of GRWD1 resulted in the decreased proliferation of MDA-MB-231 and HCC1937 cells (Fig. 2B), accompanied with the decreased expression of Ki67 and PCNA (Fig. 2C). The Edu staining also indicated that the proliferation of MDA-MB-231 and HCC1937 cells was inhibited in the siRNA-GRWD1 group (Fig. 2D).

GRWD1 knockdown promotes TNBC cell apoptosis. There were no obvious changes in apoptosis of MDA-MB-231 and HCC1937 cells between the control group and the siRNA-NC group. The apoptosis of MDA-MB-231 and HCC1937 cells was significantly increased after siRNA-GRWD1 transfection (Fig. 3A and B). GRWD1 knockdown suppressed the expression of Bcl-2 and promoted the expression of Bax and cleaved caspase-3 in both cell lines (Fig. 3C and D).

GRWD1 knockdown suppresses TNBC cell invasion and migration. The invasion and migration of MDA-MB-231 and HCC1937 cells was not changed in the siRNA-NC group compared with the control group and was inhibited in siRNA-GRWD1 group (Fig. 4A and B). The expression of MMP9 and MMP2 was also decreased in both MDA-MB-231 and HCC1937 cells transfected with siRNA-GRWD1 (Fig. 4C). siRNA-GRWD1 also enhanced the expression of E-cadherin and suppressed the expression of N-cadherin and Vimentin in both MDA-MB-231 and HCC1937 cells (Fig. 4D).

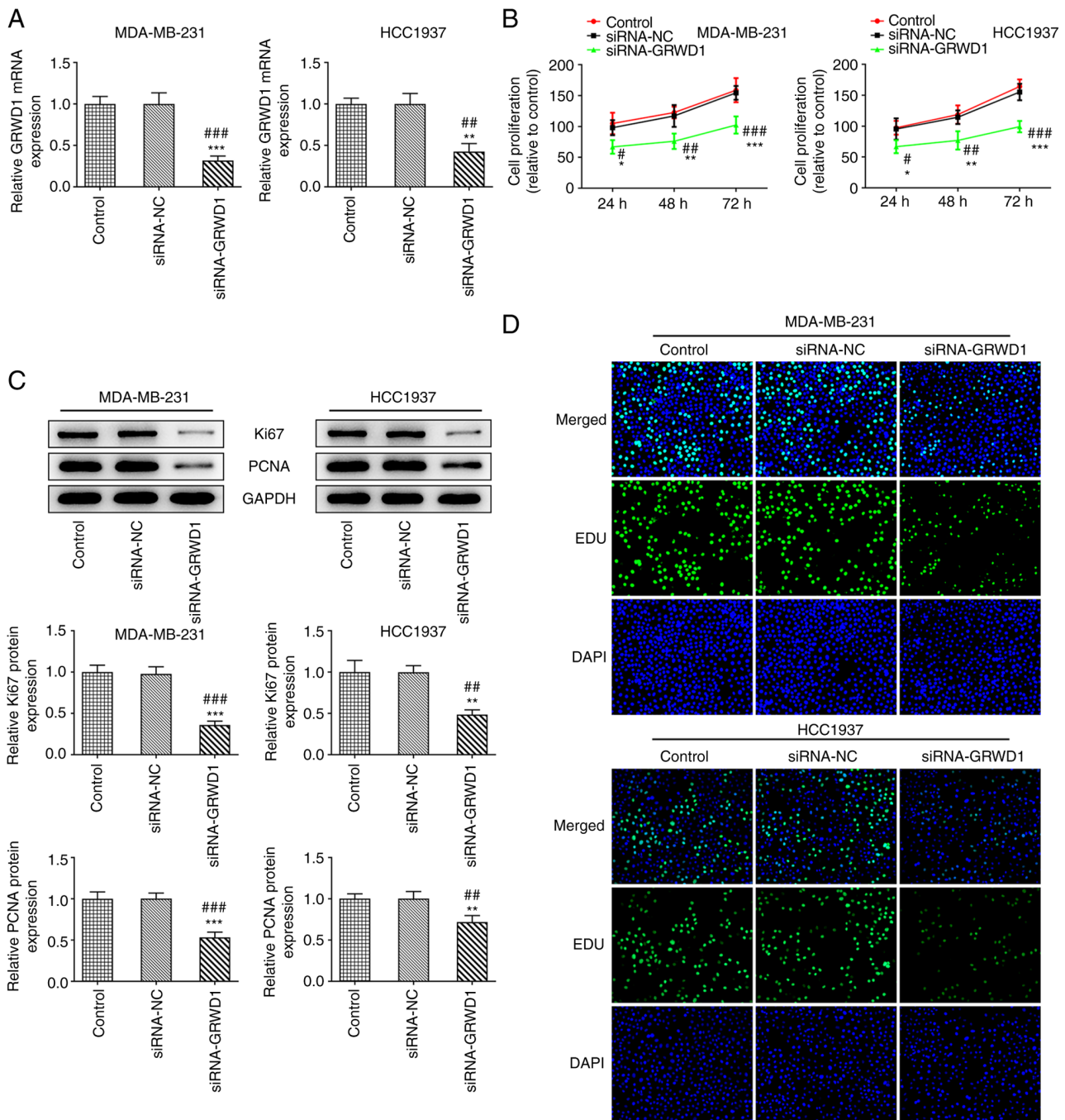


Figure 2. GRWD1 knockdown suppresses triple negative breast cancer cell proliferation. (A) The mRNA expression of GRWD1 in MDA-MB-231 and HCC1937 cells transfected with siRNA-GRWD1 was detected by reverse transcription-quantitative PCR. (B) The proliferation of MDA-MB-231 and HCC1937 cells transfected with siRNA-GRWD1 was determined by Cell Counting Kit-8 assay. (C) The protein expression of proliferation-related proteins in MDA-MB-231 and HCC1937 cells transfected with siRNA-GRWD1 was detected by western blot analysis. (D) The proliferation of MDA-MB-231 and HCC1937 cells transfected with siRNA-GRWD1 was also determined by 5-Ethynyl-2'-deoxyuridine staining (magnification, x200). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. Control group. # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ vs. siRNA-NC group. GRWD1, glutamate-rich WD-repeat-containing protein 1; siRNA, small interfering RNA; NC, negative control; PCNA, proliferating cell nuclear antigen.

GRWD1 knockdown inactivates the Notch signaling pathway. The mRNA and protein expression levels of Notch1 and Notch4 in MDA-MB-231 and HCC1937 cells were increased compared with MCF10A cells (Fig. 5A and B). Following transfection of both cell lines with siRNA-GRWD1, the protein expression of Notch1 and Notch4 was decreased (Fig. 5C). GRWD1 knockdown reduced the expression of Hes1, Hes5, Hey1, Hey2, p21, c-Myc, CCND1, HER2 and NF- κ B (Fig. 5D and E) in both cell lines.

Notch1 overexpression reverses the effect of GRWD1 knockdown on TNBC cell proliferation and apoptosis. The mRNA and protein expression of Notch1 was increased in MDA-MB-231 and HCC1937 cells transfected with Ov-Notch1 (Fig. 6A and B). Notch1 overexpression improved the decreased proliferation of MDA-MB-231 and HCC1937 cells induced by GRWD1 knockdown, as well as increased the expression of Ki67 and PCNA (Fig. 6C and D). Notch1 overexpression reduced the apoptosis of MDA-MB-231 and

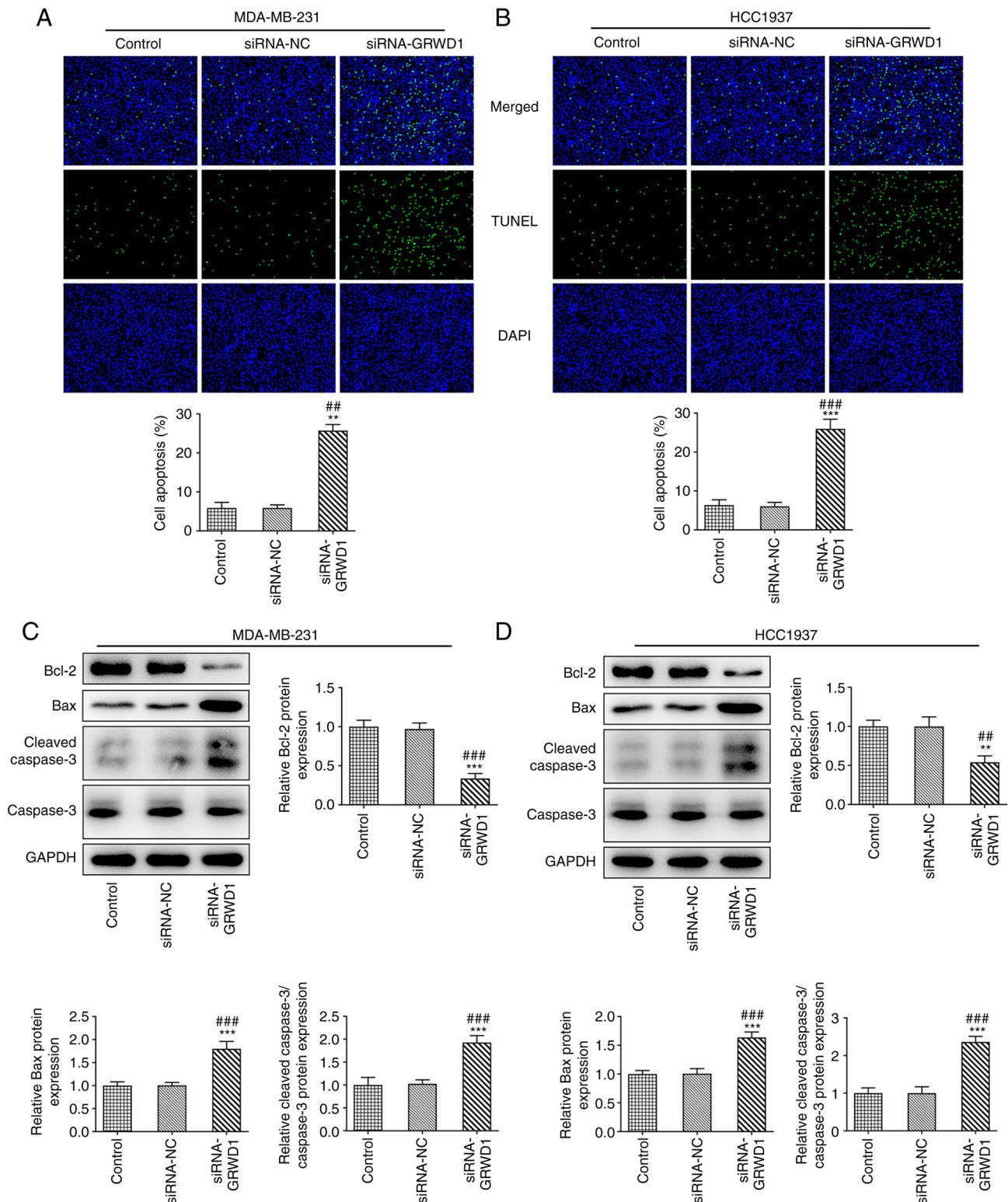


Figure 3. GRWD1 knockdown promotes triple negative breast cancer cell apoptosis. (A and B) The apoptosis of (A) MDA-MB-231 and (B) HCC1937 cells transfected with siRNA-GRWD1 was analyzed by TUNEL assay. (C and D) The protein expression of apoptosis-related proteins in (C) MDA-MB-231 and (D) HCC1937 cells transfected with siRNA-GRWD1 was detected by western blotting. ** $P < 0.01$ and *** $P < 0.001$ vs. Control group. ## $P < 0.01$ and ### $P < 0.001$ vs. siRNA-NC group. GRWD1, glutamate-rich WD-repeat-containing protein 1; siRNA, small interfering RNA; NC, negative control.

HCC1937 cells which were transfected with siRNA-GRWD1 (Fig. 6E). Notch1 overexpression upregulated the expression of Bcl-2 and downregulated the expression of Bax and cleaved caspase-3 in siRNA-GRWD1- transfected MDA-MB-231 and HCC1937 cells (Fig. 6F).

Notch1 overexpression reverses the effect of GRWD1 knockdown on TNBC cell invasion and migration. The invasion and migration of MDA-MB-231 and HCC1937 cells were suppressed by GRWD1 knockdown, which was reversed by Notch1 overexpression (Fig. 7A and B). Notch1 overexpression

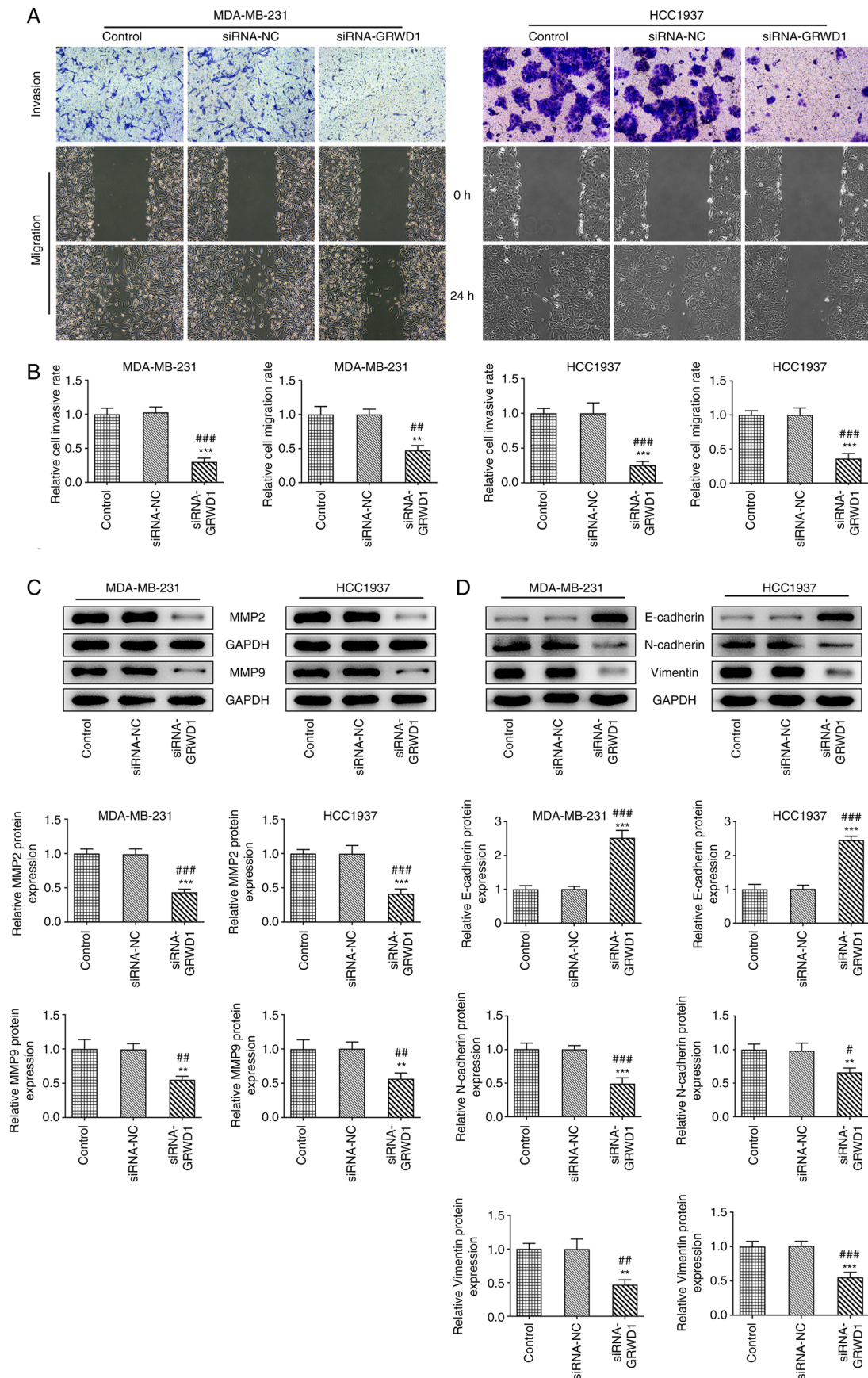


Figure 4. GRWD1 knockdown suppresses triple negative breast cancer cell invasion and migration. (A and B) The invasion and migration of (A) MDA-MB-231 and (B) HCC1937 cells transfected with siRNA-GRWD1 was detected by Matrigel and wound healing assays, respectively. (C) The expression of metastasis-related proteins in MDA-MB-231 and HCC1937 cells transfected with siRNA-GRWD1 was detected by western blotting. (D) The expression of epithelial-mesenchymal transition-related proteins in MDA-MB-231 and HCC1937 cells transfected with siRNA-GRWD1 was detected by western blotting. ** $P < 0.01$ and *** $P < 0.001$ vs. Control group. # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ vs. siRNA-NC group. GRWD1, glutamate-rich WD-repeat-containing protein 1; siRNA, small interfering RNA; NC, negative control.

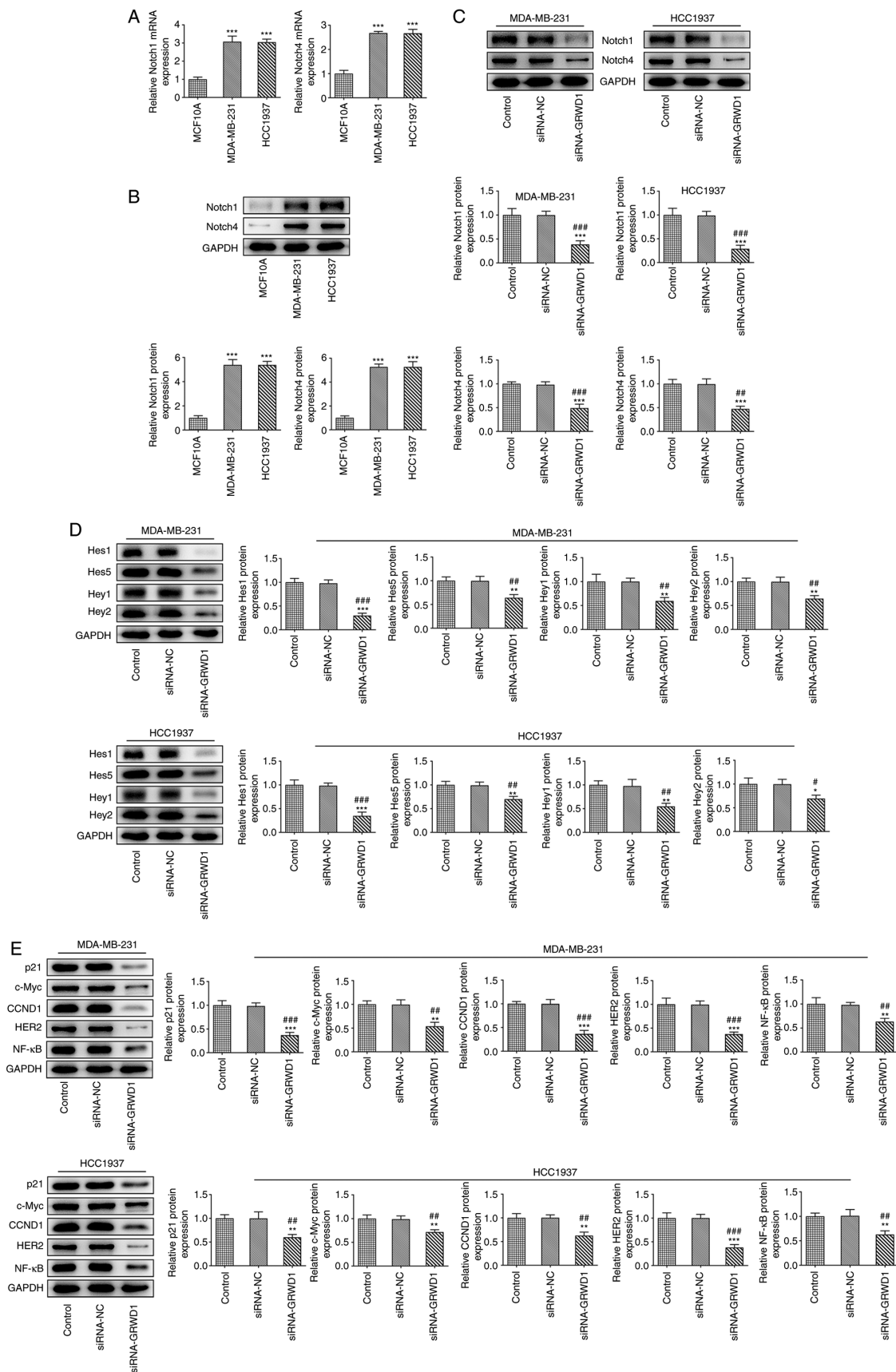


Figure 5. GRWD1 knockdown inactivates the Notch signaling pathway. (A) The mRNA and (B) protein expression of Notch1 and Notch4 in breast cancer cells and normal human breast epithelial cells was detected by reverse transcription-quantitative PCR and western blotting, respectively. **** $P < 0.001$ vs. MCF-10A group. (C) The expression of Notch1 and Notch4 in MDA-MB-231 and HCC1937 cells transfected with siRNA-GRWD1 was detected by western blot analysis. (D) The expression of Hes1, Hes5, Hey1 and Hey2 in MDA-MB-231 and HCC1937 cells transfected with siRNA-GRWD1 was detected by western blot analysis. (E) The expression of p21, c-Myc, CCND1, HER2 and NF- κ B in MDA-MB-231 and HCC1937 cells transfected with siRNA-GRWD1 was detected by western blotting. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. Control group. # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ vs. siRNA-NC group. GRWD1, glutamate-rich WD-repeat-containing protein 1; siRNA, small interfering RNA; NC, negative control.

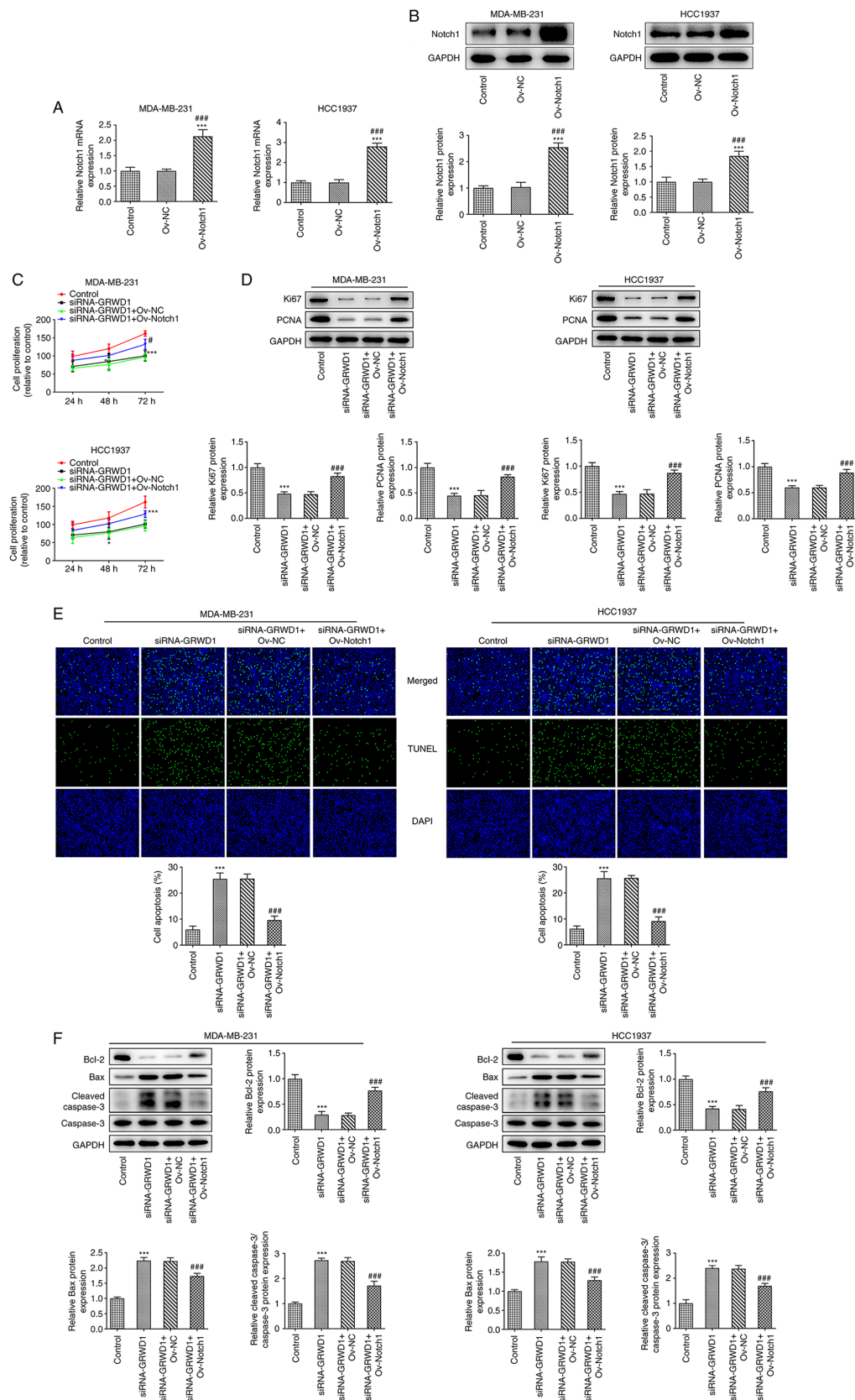


Figure 6. Notch1 overexpression reverses the effect of GRWD1 knockdown on triple negative breast cancer cell proliferation and apoptosis. (A) The mRNA and (B) protein expression of Notch1 in MDA-MB-231 and HCC1937 cells co-transfected with siRNA-GRWD1 and Ov-Notch1 was detected by reverse transcription-quantitative PCR and western blotting, respectively. *** $P < 0.001$ vs. Control group. ### $P < 0.001$ vs. Ov-NC group. (C) The proliferation of MDA-MB-231 and HCC1937 cells co-transfected with siRNA-GRWD1 and Ov-Notch1 was determined by Cell Counting Kit-8 assay. (D) The protein expression of proliferation-related proteins in MDA-MB-231 and HCC1937 cells co-transfected with siRNA-GRWD1 and Ov-Notch1 was detected by western blotting. (E) The proliferation of MDA-MB-231 and HCC1937 cells co-transfected with siRNA-GRWD1 and Ov-Notch1 was also determined by Edu staining (magnification, x200). (F) The protein expression of apoptosis-related proteins in MDA-MB-231 and HCC1937 cells co-transfected with siRNA-GRWD1 and Ov-Notch1 was detected by western blot analysis. * $P < 0.05$ and *** $P < 0.001$ vs. Control group. * $P < 0.05$ and *** $P < 0.001$ vs. siRNA-GRWD1 + Ov-NC group. GRWD1, glutamate-rich WD-repeat-containing protein 1; siRNA, small interfering RNA; NC, negative control; Ov, overexpression.

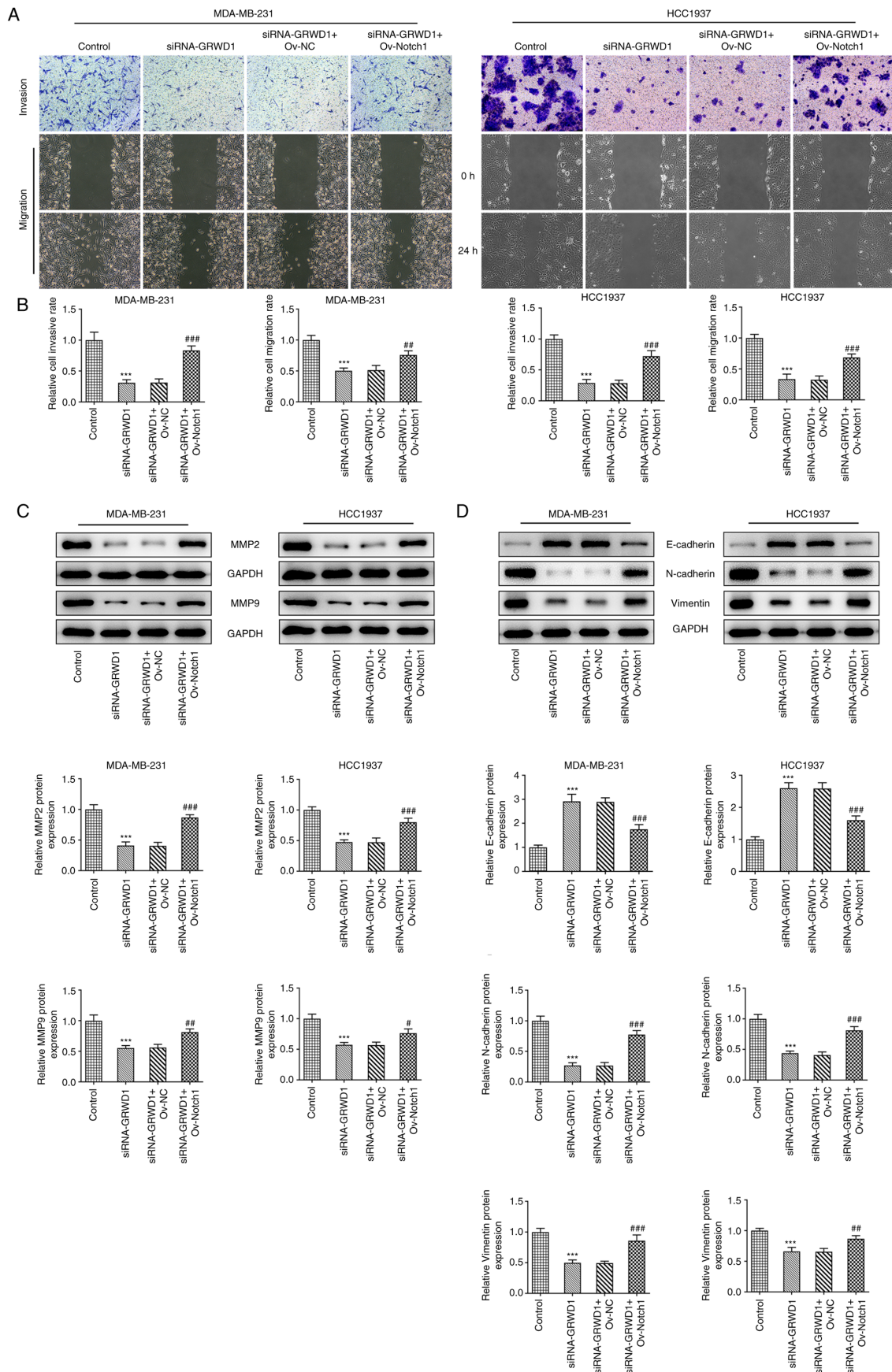


Figure 7. Notch1 overexpression reverses the effect of GRWD1 knockdown on triple negative breast cancer cell invasion and migration. (A and B) The invasion and migration of MDA-MB-231 and HCC1937 cells co-transfected with siRNA-GRWD1 and Ov-Notch1 was detected by Matrigel and wound healing assays. (C) The expression of metastasis-related proteins in MDA-MB-231 and HCC1937 cells co-transfected with siRNA-GRWD1 and Ov-Notch1 was detected by western blotting. (D) The expression of epithelial-mesenchymal transition-related proteins in MDA-MB-231 and HCC1937 cells co-transfected with siRNA-GRWD1 and Ov-Notch1 was detected by western blotting. *** $P < 0.001$ vs. Control group. # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ vs. siRNA-GRWD1+ Ov-NC group. GRWD1, glutamate-rich WD-repeat-containing protein 1; siRNA, small interfering RNA; NC, negative control; Ov, overexpression.

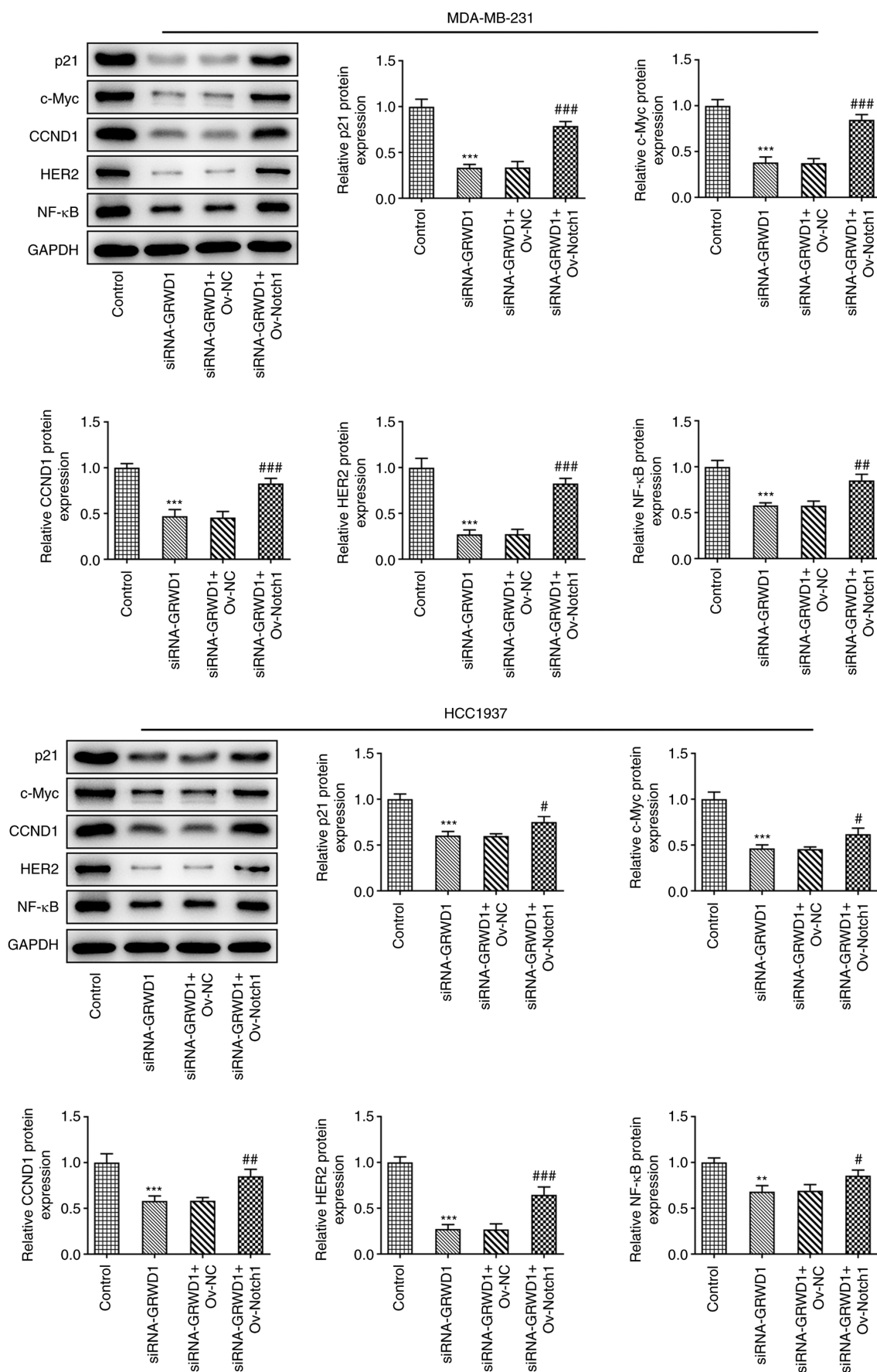


Figure 8. Notch1 overexpression reverses the effect of GRWD1 knockdown on the Notch signaling pathway in triple negative breast cancer cells. The expression of p21, c-Myc, CCND1, HER2 and NF-κB in MDA-MB-231 and HCC1937 cells co-transfected with siRNA-GRWD1 and Ov-Notch1 was detected by western blotting. **P<0.01 and ***P<0.001 vs. Control group. #P<0.05, ##P<0.01 and ###P<0.001 vs. siRNA-GRWD1 + Ov-NC group. GRWD1, glutamate-rich WD-repeat-containing protein 1; CCND1, cyclin D1; HER2, human epidermal growth factor 2 receptor; siRNA, small interfering RNA; NC, negative control; Ov, overexpression.

upregulated the expression of MMP2 and MMP9 which were inhibited by GRWD1 knockdown in MDA-MB-231 and HCC1937 cells (Fig. 7C). Both cell lines co-transfected with siRNA-GRWD1 and Ov-Notch1 presented decreased expression of E-cadherin and increased expression of N-cadherin and Vimentin compared with the siRNA-GRWD1 group (Fig. 7D).

Notch1 overexpression reverses the effect of GRWD1 knockdown on the Notch signaling pathway in TNBC cells. The expression of p21, c-Myc, CCND1, HER2 and NF- κ B in MDA-MB-231 and HCC1937 cells was reduced in siRNA-GRWD1 group, which was reversed by Ov-Notch1 transfection (Fig. 8).

Discussion

Breast cancer is a malignant tumor occurring in the glandular epithelial tissue of the breast, and the cancer cells are easy to fall off and dissociate, leading to distal metastasis. The metastasis of vital organs such as lung, bone and brain can directly threaten the lives of patients, adding great difficulties to the clinical treatment of breast cancer (13). Therefore, effectively inhibiting the malignant biology of breast cancer cells has become an important measure for the treatment of breast cancer (14).

GRWD1 was confirmed to be a novel negative regulator of p53 induced by nucleolar stress and a latent oncogene (15). GRWD1 can competitively bind ribosomal protein L11, thus promoting the recovery of MDM2 ubiquitin activity and negatively regulating p53 (16). GRWD1 overexpression could promote tumor cell growth by inhibiting p53 (9,16). In a previous study by Pan *et al* (17), 156 patients with TNBC were included and it was found that the mutation rate of p53 reached 71.3%, and the mutation of p53 was closely related to TNBC histological grading. In a recent study by Xu *et al* (18), the expression and prognosis of p53 in TNBC tissues was investigated and it was found that p53 was highly expressed in TNBC tissues and was associated with tumor node metastasis staging, histological grade, lymph node metastasis and poor prognosis in patients with TNBC. Considering that GRWD1 overexpression could inhibit the highly expressed p53 in TNBC tissues, it was found in the present study that GRWD1 expression was also upregulated in MDA-MB-231 and HCC1937 cells, and downregulation of GRWD1 could suppress the proliferation and promoted the apoptosis of these two cell lines.

In tumor cells, in addition to the downregulation of E-cadherin expression, the expression of N-cadherin protein is also upregulated, and the downregulation of E-cadherin expression is a marker for the occurrence of epithelial-mesenchymal transition (EMT) (19). In the present study, it was observed that downregulation of GRWD1 increased the expression of E-cadherin while decreased the expression of N-cadherin and Vimentin, suggesting that GRWD1 could promote the EMT process of TNBC cells. MMP2 and MMP9 belong to the MMP family, whose main function is to maintain the dynamic balance of remodeling and degradation of extracellular matrix, and participate in the process of tumor cell migration and invasion (20,21). In the present study, the expression of MMP2 and

MMP9 was increased in TNBC cells, which was decreased by GRWD1 knockdown.

The Notch signaling pathway is a common regulated signaling pathway mainly involved in organ development, differentiation, cell proliferation and apoptosis. Previous studies have shown that it can be involved in the regulation of malignant tumor diseases such as NSCLC, breast cancer and T-cell leukemia (22-24). Activated Notch protein is over-expressed in alveolar epithelial cells and can interact with Myc Pathway co-promoting the development of NSCLC (25). Notch1 inhibits p53-mediated apoptosis by regulating the stability of p53, and is necessary for the growth of lung adenocarcinoma (26). In addition, the Notch pathway plays an important role in the proliferation, metastasis and treatment of breast cancer (23,27). Notch1 was demonstrated to be related to the invasion and migration steps which characterize the EMT process in TNBC (28). Notch1 was highly expressed in Cisplatin-resistant MDA-MB-231 TNBC cells, and this helped to induce chemoresistance via activating the AKT pathway and promoting EMT (29). It was indicated in the present study that Notch1 expression was also higher in MDA-MB-231 and HCC1937 cells than MCF10A cells. Furthermore, Notch1 overexpression could enhance the proliferation, invasion and migration and suppress the apoptosis of MDA-MB-231 and HCC1937 cells which were transfected with siRNA-GRWD1. Despite being epithelial and lymphoblast cells, respectively, MDA-MB-231 and HCC1937 cells are both TNBC cells and yielded similar results, which is consistent with previous studies (30-32).

The KEGG PATHWAY database (<https://www.genome.jp/kegg/pathway.html>) indicates that Notch1/4 affects downstream expression of p21, c-Myc, CCND1, HER2 and NF- κ B through Hes1/5 and Hey in TNBC. The most well-known Notch target genes are transcription factors of the Hes and Hey families (33). Hes and Hey members are helix-loop-helix proteins, forming homo- or heterodimers that regulate transcription of genes relating to cell fate determination (33-35). Other Notch pathway targets include cell cycle regulators CCND1 and p21, NF- κ B family members, c-Myc and Deltex (36-39). Hes1 protein is downstream of the Notch1 signaling pathway and affects cell proliferation and differentiation (40-42). Salidroside inhibited the hepatocellular carcinoma cell metastasis by suppressing the Notch1 signaling pathway, which was manifested by the downregulation of Hey1, Hes1 and Hes5 (43). In the present study, it was revealed that GRWD1 knockdown suppressed the expression of Notch1/4, Hes1/5, Hey1/2, p21, c-Myc, CCND1, HER2 and NF- κ B and Notch1 overexpression promoted the expression of p21, c-Myc, CCND1, HER2 and NF- κ B in both TNBC cell lines used. In addition, regulation of the Notch signaling pathway could indeed affect the proliferation, invasion and migration and apoptosis of TNBC cells.

In conclusion, GRWD1 knockdown suppressed the proliferation, invasion and migration and promoted the apoptosis of TNBC cells through the activation of the Notch signaling pathway. The present study may provide a potential biomarker for the diagnosis and treatment of TNBC.

Acknowledgements

Not applicable.

Funding

No funding was received.

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

LY wrote the manuscript and analyzed the data. FT carried out the experiments, supervised the present study, searched the literature and revised the manuscript. LY and FT confirm the authenticity of all the raw data. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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