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Original Article

WDR3 promotes malignant progression of triple-negative breast carcinoma

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Abstract: Background: Breast cancer is the second most frequently diagnosed cancer and the one with the highest mortality rate in females. Triple-negative breast cancer (TNBC) accounts for approximately 10-20% of all breast cancers. It generally has an aggressive biology, with higher proliferation and metastasis abilities, which promotes worse outcomes. Methods: WD repeat domain 3 (WDR3) expression in TNBC was analyzed using the Cancer Genome Atlas Program (TCGA) database and validated by immunohistochemistry and western blot. TNBC cells with WDR3 overexpression or downregulation were created to determine the effect of WDR3 expression on TNBC biological behavior. Results: Our study showed that elevated WDR3 expression boosted G2- and S-phase cell populations and accelerated the cell cycle, promoting tumor proliferation by reducing apoptosis. WDR3 also promoted tumor growth in vivo. WDR3 overexpression raised N-cadherin and Twist protein levels, and reduced E-cadherin protein levels. WDR3 overexpression boosted Matrix Metallopeptidase 2 (MMP2) protein levels. WDR3 overexpression increased extracellular matrix (ECM) breakdown and cell movement by activating the Wingless Integrated (WNT) signaling pathway. Conclusion: The level of WDR3 is high in TNBC. High expression of WDR3 accelerates the proliferation and cell cycle of TNBC cells. WDR3 promotes tumor growth in vivo. WDR3 also promotes migration and invasion by modulating epithelial-mesenchymal transition (EMT) and MMP2 by activating the WNT signaling pathway. Taken together, WDR3 could serve as a novel therapeutic target for human TNBC.

Keywords: TNBC, WDR3, proliferation, migration, invasion

Introduction

Breast cancer is the second most frequently diagnosed cancer and the one with the highest mortality rate in females. It has been reported that 12.4% of women are affected by breast cancer during their lifetime [1]. Breast cancer is classified into four subtypes, namely Human Epidermal Growth Factor Receptor 2 (HER2)-enriched, luminal A, luminal B, and triple-negative breast cancer (TNBC) [2]. TNBC is characterized by the absence of expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2-receptor (EGFR), and represents approximately 10-20% of all breast cancers. It generally has an aggressive biology, with higher proliferation and metastatic abilities, so it is associated with the worst outcomes [3, 4]. Standard chemotherapy and radiotherapy have been the only accepted treatment options for women with TNBC to increase the overall survival (OS) rate. However, these systemic treatments have adverse side effects and usually fail, resulting in high rates of recurrence and short OS [5-7]. Therefore, the clinical treatment of TNBC remains a major challenge, and understanding of the molecular mechanisms in the biology and pathogenesis of TNBC may help in identifying novel strategies for the prevention of cancer and the development of more effective treatment [5, 8].

WDR3 belongs to the WD-repeat family and is a component of the 80S complex of the small subunit processome, which participates in the 40S ribosome synthesis pathway [9]. Operation of ribosome biogenesis inducing the increased protein synthesis is required for maintaining cancer cell proliferation and growth [10]. In con-

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trast, perturbation of ribosome biogenesis results in ribosome stress and cell proliferation inhibition [11, 12]. Ectopic expression of WDR3 could induce genome stability, cell proliferation, signal transduction, and apoptosis [13, 14]. It has been reported that WDR3 is involved in malignant progression of thyroid cancer [15, 16]. However, it remains largely unknown whether and how WDR3 regulates the progression of TNBC.

In this study, we aimed to identify the role of WDR3 in TNBC. We found that the level of WDR3 was high in TNBC. High expression of WDR3 accelerated the proliferation and cell cycle of TNBC cells by inhibiting apoptosis. WDR3 promoted tumor growth in vivo. WDR3 also promoted migration and invasion by modulating epithelial-mesenchymal transition (EMT) and MMP2 via activating the WNT signaling pathway. These results suggested that WDR3 could serve as a novel therapeutic target for human TNBC.

Materials and methods

TNBC and nontumor tissues

Human TNBC samples and nontumor tissues were obtained from the Second Affiliated Hospital of Xuzhou Medical University. TNBC was histologically diagnosed based on the World Health Organization grading system. Written informed consent was obtained from the patients, and the study was approved by the ethics committee of the hospital.

Immunohistochemistry

Briefly, paraffin-embedded sections were heated at 60°C, deparaffinized in xylene, rehydrated in graded ethanol, and microwaved for antigen retrieval. The sections were treated with 3% hydrogen peroxide for 30 min, and then incubated with primary antibodies at 4°C overnight. The bound antibodies were detected by use of a streptavidin-peroxidase kit (Zhongshan Gold Bridge Bio, Beijing, China). The slides were counterstained with hematoxylin, dehydrated with ethanol and xylene, and covered with coverslips. The results are presented as the percentage of the cells with positive staining.

Cell culture and lentivirus infection

The human MDA-MB-231 cell line was purchased from Shanghai Cell Bank, Type Culture Collection Committee, Chinese Academy of Sciences. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (Gibco).

To obtain WDR3-overexpressing TNBC cells, the WDR3 cDNA was inserted into the plasmid cadherin (pCDH) backbone. TNBC cells were infected with lentivirus containing pCDH or pCDH-WDR3.

To obtain WDR3-downregulation TNBC cells, we generated lentivirus-based short hairpin RNA (shRNA) targeting human WDR3 with pLL3.7 as the backbone. Cells were infected with lentivirus containing WDR3 shRNA or a control scramble shRNA (Scramble).

Antibodies

The following antibodies were used: rabbit monoclonal anti-WDR3 (1:1000, Abcam), rabbit monoclonal anti-Cyclin-dependent kinases 4 (CDK4) (1:1000, Cell Signaling Technology), mouse monoclonal anti-Cyclin-dependent kinases 6 (CDK6) (1:1000, Cell Signaling Technology), rabbit monoclonal anti-N-cadherin (1:500, Millipore), rabbit monoclonal anti-E-cadherin (1:500, Millipore), rabbit monoclonal anti-Twist (1:500, Millipore), rabbit monoclonal anti-cleaved-caspase 3 (1:1000, Cell Signaling Technology), rabbit monoclonal anti-Glycogen Synthase Kinase 3 Beta (GSK3β) (1:1000, Cell Signaling Technology), rabbit monoclonal anti-p-GSK3β (1:1000, Cell Signaling Technology), mouse monoclonal anti-β-catenin (1:1000, Cell Signaling Technology), mouse monoclonal anti-p-β-catenin (1:500, Cell Signaling Technology), and mouse monoclonal anti-β-actin (1:4000, ABclonal).

Western blotting

Total proteins were evaluated using 10% Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred to Polyvinylidene fluoride (PVDF) membranes. The membranes were subsequently blocked with 5% BSA for 2 h at room temperature, incubated with the indicated primary antibodies, and detected by the Pierce enhanced chemiluminescence (ECL) Plus ImmunoBlotting Substrate (Thermo Fisher Scientific Inc.) and exposed by ChemiDoc Touch (BIO-RAD).

Cell growth assays

Cell viability was measured with a Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay every 24 h in accordance with the manufacturer's instruction.

Colony formation assay

Three days after lentivirus infection, the cells were seeded into six-well plates (300 cells per well). The medium was changed at 3-day intervals. After 14 days of culture at 37°C, the colonies were washed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. The colonies were then stained with 0.05% crystal violet for 10 min, washed with water, and air-dried. The total number of colonies with more than 50 cells was counted.

Flow cytometry

Cell cycle analysis was performed by flow cytometry with a commercial kit (propidium iodide/RNase Staining Buffer, BD) in accordance with the manufacturer's instructions.

EdU incorporation assay

Cells were seeded into 96-well plates at 5000 cells per well. After 24 h, the cells were exposed to 50 μM 5-ethynyl-20-deoxyuridine (EdU, Ribobio, China) for additional 2 h at 37°C. Then, the cells were fixed with 4% paraformaldehyde for 20 min and treated with 0.5% Triton-X-100 for another 20 min at room temperature. After being washed with PBS for 5 min, the cells were reacted with 100 μL 1* Apollo reaction cocktail for 30 min. Thereafter, the DNA contents of the cells were stained with 100 μL Hoechst (5 μg/mL) for 20 min and visualized under a fluorescence microscope (IX71, Olympus, Japan). Data were obtained from three independent assays performed in triplicate.

Wound healing assay

The cells were first incubated with the media containing mitomycin C (1 μg/mL) at 37°C for 2 h. Then, the media were removed, and the cells were detached and seeded in six-well plates following complete washing with phosphate buffered saline (PBS). Cell monolayers were disrupted with a plastic pipette tip, rinsed twice with PBS to remove dead cells, and incubated in serum-free media. At the designated time (0 and 24 h), three randomly selected fields at the lesion border were examined under Olympus IX-71 inverted microscope. Data were obtained from three independent assays performed in triplicate.

Transwell invasion assay

Cell invasion assay was performed using a transwell system that incorporated a polycarbonate filter membrane with a diameter of 6.5 mm and pore size of 8 μm (Corning). Briefly, transwell was coated with 50 μL Matrigel in cold serum-free medium at a final concentration of 1 mg/mL. The cells were added to the top chamber. In the lower chamber, DMEM medium containing 10% Fetal Bovine Serum (FBS) was added. After 24-h incubation, the cells were stained with 0.1% crystal violet for 5 min. The migratory cells were recorded with microscopy.

In vivo tumor xenograft model

All animal experiments were in accordance with the guidelines approved by the committee on the Ethics of Animal Experiments of Xuzhou Medical University. Female BALB/c nude mice (6-8 weeks old) were obtained from the Beijing Vital River Laboratory Animal Technology Co., Ltd., and raised at the specific pathogen-free (SPF) laboratory animal facility. Mice were maintained on a 12 h light/dark cycle at 25°C and provided free access to commercial rodent chow (sterilized by Cobalt-60) and tap water (high-temperature sterilization) before initiation of the experiments. Randomized grouping was used and the same group of mice were co-housed with less than 5 animals per cage. Tumor cells (5×10^6^ cells) were injected subcutaneously into the nude mice. Four weeks later, the mice were killed, and the tumors were weighed. Each mouse was anesthetized with 1.5% isoflurane to relieve the pain of the animals. For euthanasia, we calculated the required dose of anesthetic based on the animal's weight and drug concentration, then we prepared a 3% solution of pentobarbital sodium in sterile physiological saline at the usual dose of 30 mg/kg body weight and injected the prepared solution into the mouse's body.

Statistical analysis

The results are representative of experiments repeated at least three times and are present-

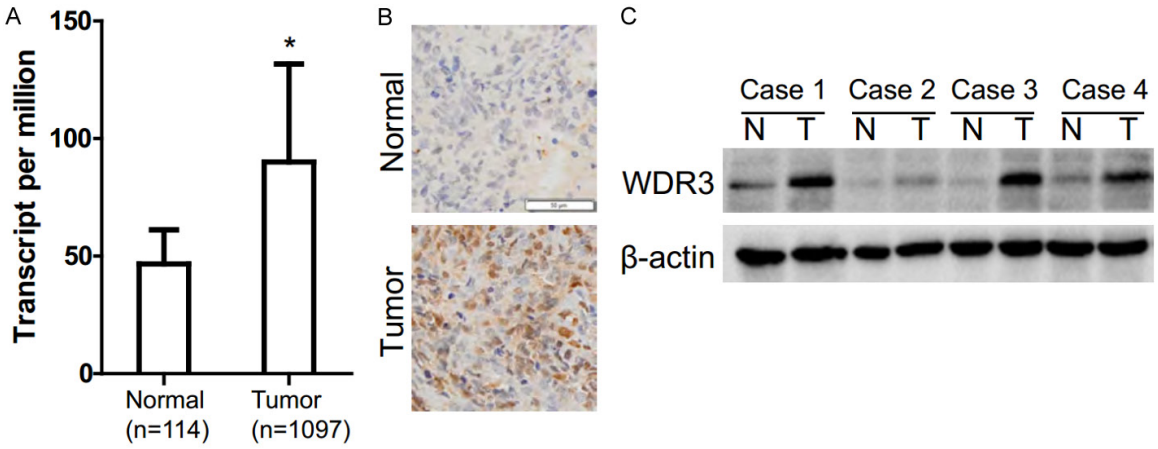


Figure 1. Clinical importance of WDR3 in TNBC patients. A. Expression of WDR3 in TNBC in TCGA database. B. The protein level of WDR3 in non-tumor and TNBC clinical specimens examined by immunohistochemistry (Scale bar = 50 μm). C. The protein level of WDR3 in non-tumor and TNBC clinical specimens examined by western blot.

ed as mean ± Standard Error of the Mean (SEM). Statistical comparisons of data from the experiments on cultured cells or mice were performed using the two-tailed Student's *t* test. *P* values lower than 0.05 were considered to indicate statistical significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Results

Clinical importance of WDR3 in TNBC patients

We first analyzed the WDR3 expression in TNBC patients in The Cancer Genome Atlas (TCGA). The data showed that the mRNA level of WDR3 was higher in cancer tissues than in normal tissues (**Figure 1A**). To determine whether WDR3-mRNA expression profiles are reflected at the protein levels, we examined WDR3-protein expression in four TNBC tissues and paired non-tumor tissues. There was a drastic increase of WDR3 expression in TNBC tissues compared with the non-tumor tissues (**Figure 1B** and **1C**).

Overexpression of WDR3 promotes proliferation of TNBC by accelerating cell cycle

Although WDR3 is highly expressed in TNBC tissues, the role and the molecular mechanism of WDR3 in TNBC progression have not yet been reported. CCK8 assays showed that the cell viability was much higher in WDR3-overexpressing cells (**Figure 2A**). Likewise, the number of colonies formed by WDR3-overexpressing cells was significantly increased (**Figure 2B** and **2C**). EdU assays showed that the WDR3 overexpression caused an obvious increase in the percentage of EdU-positive cells (**Figure 2D** and **2E**). To investigate whether the WDR3-induced increase in cell proliferation resulted from the acceleration of cell cycle progression, we evaluated the cell cycle distribution using flow cytometry assay (**Figure 2F**). As shown in **Figure 2G**, high expression of WDR3 increased the percentage of cells in G2 and S phases, whereas there was a significant decrease in the G1-phase population compared with the control group. Furthermore, overexpression of WDR3 significantly upregulated the level of CDK4 and CDK6, and inhibited the level of cleaved-caspase3 (**Figure 2H**).

Tumor cells were subcutaneously injected into mice. Four weeks after injection, the mice were killed, and the xenograft tumors were collected (Figure S1A). We found that overexpression of WDR3 resulted in larger tumor size (Figure S1B). These results suggest that WDR3 could promote tumor growth by accelerating cell cycle and inhibiting apoptosis.

Overexpression of WDR3 promotes the invasion and migration of TNBC by regulating EMT and MMP2

Next, we used the transwell assay in the presence of Matrigel to observe the effects of WRD3 overexpression on TNBC cell invasion. Upregulation of WDR3 significantly increased the number of TNBC cells that passed through Matrigel (**Figure 3A** and **3B**). We also found that WDR3-overexpressing TNBC cells healed the

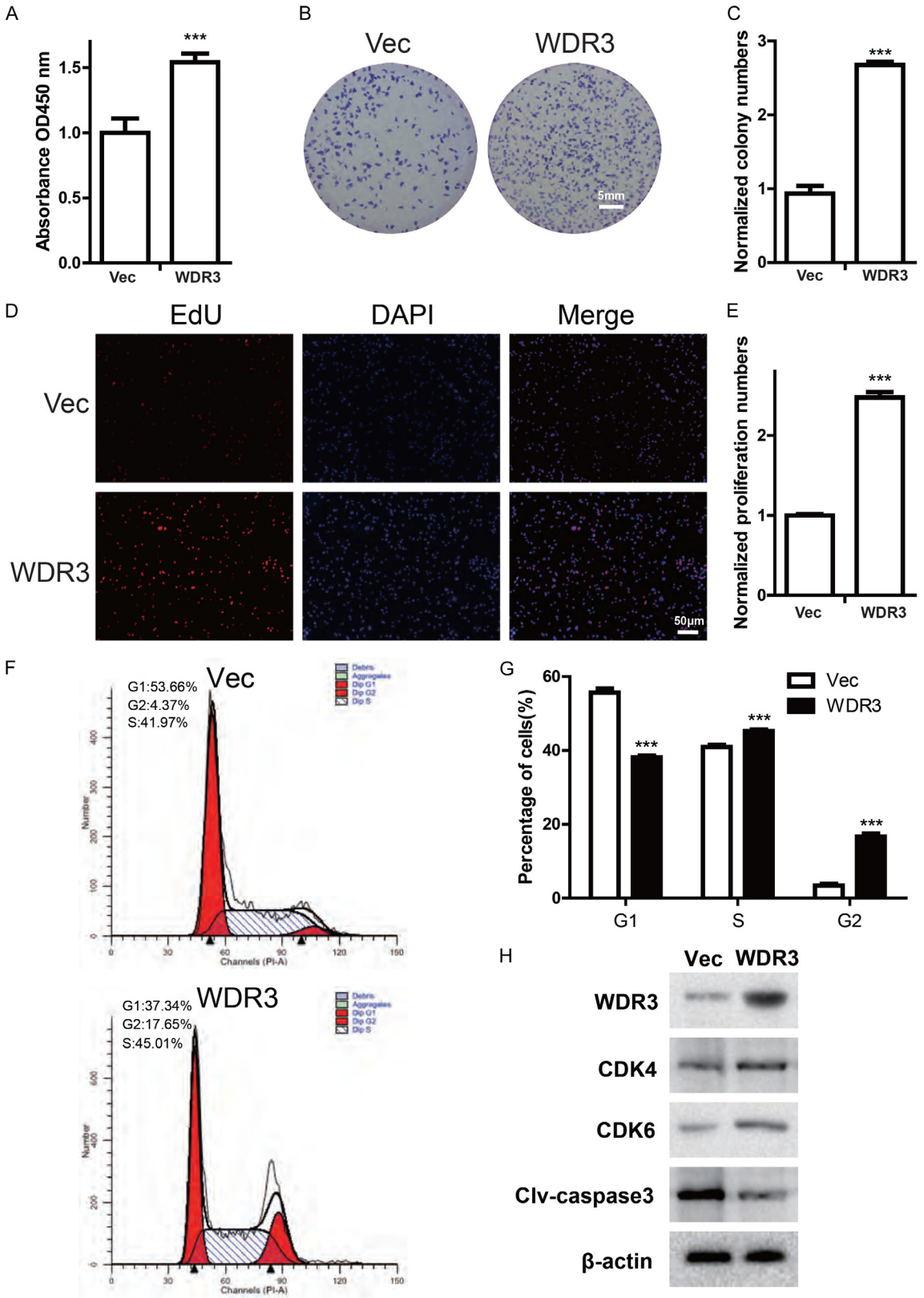


Figure 2. Overexpression of WDR3 promotes proliferation of TNBC by accelerating cell cycle. (A) Cell viability was detected by CCK8 assays. (B, C) Representative images (B) (Scale bar = 5 mm) and quantitative analysis (C) of TNBC cell colonies. (D) Typical pictures of EdU assay showing the cell proliferation rate of cells (Scale bar = 50 μm). (E)

Quantification results of (D). (F) Flow cytometry showing the cell cycle progression in TNBC cells. (G) Quantification results of (F). (H) Western blot was used to detect the protein level of cell cycle progression and apoptosis.

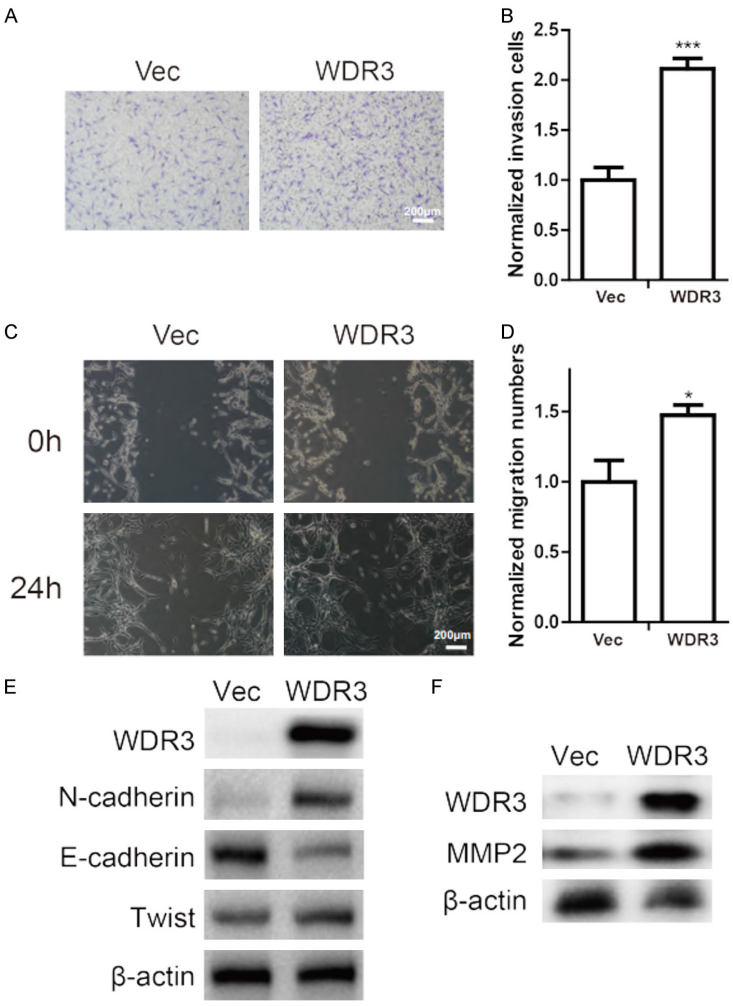
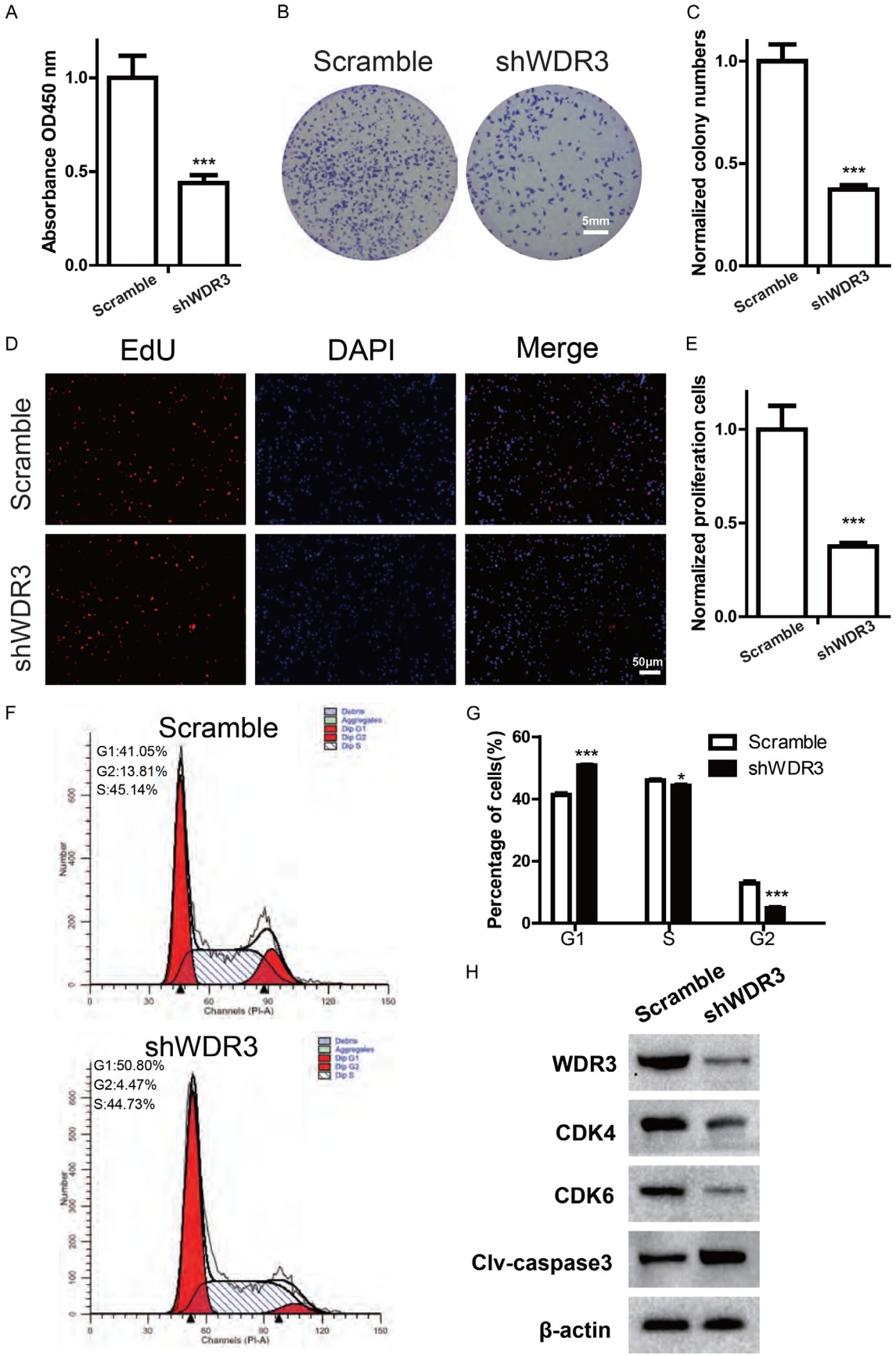


Figure 3. Overexpression of WDR3 promotes the invasion and migration of TNBC by regulating EMT and MMP2. (A) Invasion ability was examined by transwell assay (Scale bar = 200 μm). (B) Quantification results of (A). (C) Representative digital pictures of wound healing assay were taken at 0 and 24 h after scratching (Scale bar = 200 μm). (D) Quantification results of (C). (E, F) Western blot was used to detect the protein level of EMT and matrix metalloproteinase.

wound to a greater extent after 24 h of scratching (**Figure 3C** and **3D**). EMT is a process in which epithelial cells acquire mesenchymal features. In cancer, EMT is associated with tumor invasion and migration [17]. Therefore, we wondered whether WDR3 promoted TNBC cell invasion and migration through regulating EMT. As shown in **Figure 3E**, the protein levels of N-cadherin and Twist were significantly increased after WDR3 overexpression in TNBC cells. E-cadherin also showed a dramatic change; namely, the protein level of E-cadherin markedly decreased after WDR3 overexpression in TNBC cells (**Figure 3E**). The WNT signaling pathway plays an important role in EMT. Thus, we continued to investigate the impact of WDR3 on the WNT signaling pathway. We found that WDR3 upregulated the levels of act-β-catenin and p-GSK3β (Figure S1C). MMP2 is a critical enzyme for the degradation of the extracellular matrix (ECM) and thus contributes to tumor cell migration and invasion [18]. Overexpression of WDR3 significantly upregulated the MMP2 protein levels in TNBC cells (**Figure 3F**). These results indicate that WDR3 may contribute to TNBC invasion and migration by promoting EMT and MMP2.

Downregulation of WDR3 inhibits proliferation of TNBC by blocking cell cycle

To investigate whether there is a prospect of clinical value, we downregulated WDR3 and detected the proliferation ability of TNBC cells. CCK8 assays showed that the cell viability was reduced in WDR3-downregulated cells (**Figure 4A**). Similarly, the number of colonies formed by WDR3-downregulated cells significantly decreased (**Figure 4B** and **4C**). EdU assays showed that the WDR3 downregulation caused a significant decrease in the percentage of EdU-positive cells (**Figure 4D** and **4E**). Next, we detected the changes in the cell cycle after downregulation of WDR3 (**Figure 4F**). As shown in **Figure 4G**, low expression of WDR3 decreased the percentage of cells in G2 and S phases, whereas there was a significant increase in the G1-phase population compared with the control group. Furthermore, downregulation of WDR3 significantly inhibited the levels of CDK4 and CDK6,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Scramble
shWDR3
F
Scramble
G1:41.05%
G2:13.81%
S:45.14%
Debris
Aggregates
Dip G1
Dip G2
Dip S
Number
Channels (PI-A)
shWDR3
G1:50.80%
G2:4.47%
S:44.73%
G
Percentage of cells(%)
60
40
20
0
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G1
S
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Scramble
shWDR3
H
Scramble
shWDR3
WDR3
CDK4
CDK6
Clv-caspase3
β-actin

Figure 4. Downregulation of WDR3 inhibits proliferation of TNBC by blocking cell cycle. (A) Cell viability was detected by CCK8 assays. (B, C) Representatives images (B) (Scale bar = 5 mm) and quantitative analysis (C) of TNBC cell colonies. (D) Typical pictures of EdU assay showing the cell proliferation rate of cells (Scale bar = 50 μm). (E) Quantification results of (D). (F) Flow cytometry showing the cell cycle progression in TNBC cells. (G) Quantification results of (F). (H) Western blot was used to detect the protein level of cell cycle progression and apoptosis.

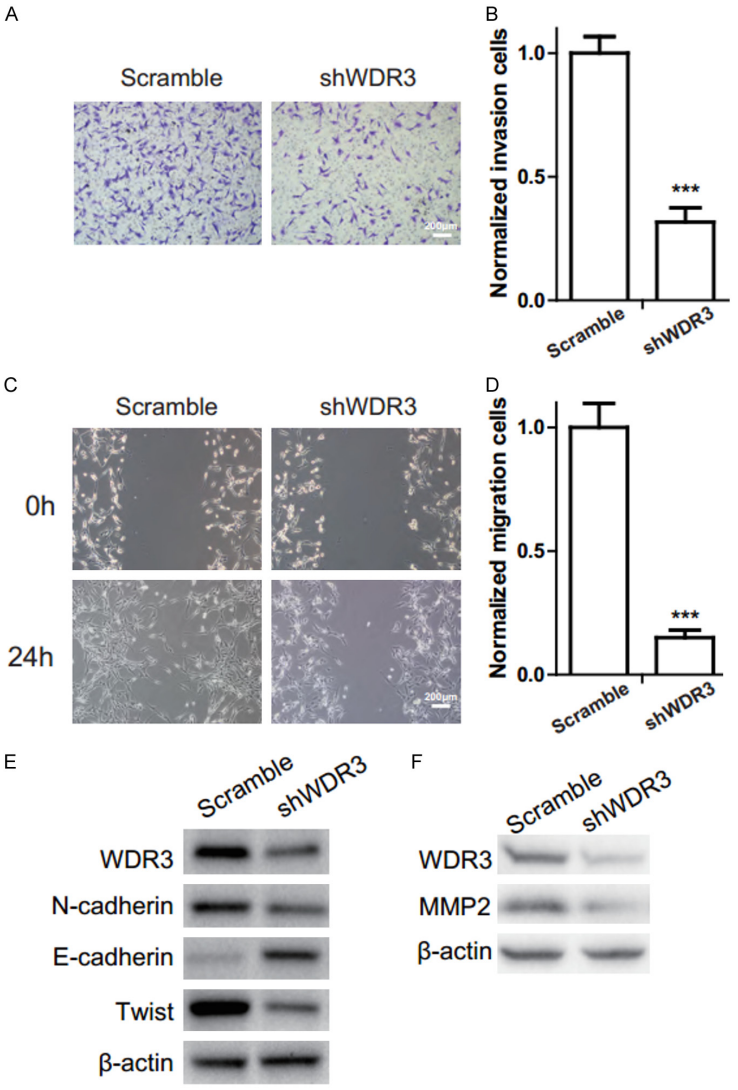

Figure 5. Downregulation of WDR3 prevents the invasion and migration of TNBC. (A) Invasion ability was examined by transwell assay (Scale bar = 200 μm). (B) Quantification results of (A). (C) Representative digital pictures of wound healing assay were taken at 0 and 24 h after scratching (Scale bar = 200 μm). (D) Quantification results of (C). (E, F) Western blot was used to detect the protein level of EMT and matrix metalloproteinase.

and upregulated the level of cleaved-caspase-3 (**Figure 4H**). Moreover, we found that downregulation of WDR3 resulted in a smaller tumor size in vivo (Figure S2A and S2B). These results suggest that downregulation of WDR3 inhibits proliferation of TNBC by blocking cell cycle and inhibiting apoptosis.

Downregulation of WDR3 prevents the invasion and migration of TNBC

Downregulation of WDR3 significantly decreased the number of TNBC cells that passed through Matrigel (**Figure 5A** and **5B**). We also found that WDR3-downregulation TNBC cells healed the scratch to a lower extent after 24 h (**Figure 5C** and **5D**). As shown in **Figure 5E**, the protein levels of N-cadherin and Twist were significantly decreased after WDR3 downregulation in TNBC cells. The protein level of E-cadherin markedly increased after WDR3 downregulation in TNBC cells (**Figure 5E**). We found that downregulation of WDR3 inhibited the levels of act-β-catenin and p-GSK3β (Figure S2C). Meanwhile, downregulation of WDR3 significantly inhibited the MMP2 protein levels in TNBC cells (**Figure 5F**). These results indicate that WDR3 can be used as a therapeutic target, and downregulation of WDR3 can inhibit the malignant progression of TNBC.

Discussion

TNBC is characterized by the absence of expression of ER, PR, and HER2. It represents approximately 20% of all breast cancers, and generally has a more aggressive biology, with earlier onset of metastatic disease, visceral metastases, rapidly progressive disease, short response duration to available therapies, and inferior survival outcomes [19]. Because TNBC lacks ER, PR, and HER2, it is not sensitive to endocrine therapy or HER2 treatment, and standardized TNBC treatment regimens are still lacking [20]. Therefore, development of new TNBC treat-

ment strategies has become an urgent clinical need.

It is well-documented that WD40 repeat proteins have important roles in a variety of cellular functions, such as cell proliferation and cell differentiation, cellular signaling, and gene transcription [21]. WDR3 is a member of the superfamily of WD40 repeat proteins that regulate many cellular functions, but its physiological functions have not been elucidated. Recently, it has been reported that WDR3 is overexpressed and serves as a risk factor in thyroid cancer [15]. Our studies have also shown that expression of WDR3 is increased in TNBC tissues compared with the non-tumor tissues. In pancreatic cancer, overexpressed WDR3 induces the activation of the Hippo pathway by interacting with GATA [22]. Many groups have also reported the biological function of WDR3 in modulating genome stability [16], promoting cell growth, and regulating cell cycle of cancer cells [23]. In this study, we showed that high expression of WDR3 increased the percentage of cells in G2 and S phases, but decreased G1-phase population compared with the control group. WDR3 also promoted tumor growth by accelerating cell cycle.

Cell migration and invasion include multiple processes, such as EMT, ECM degradation, cytoskeleton reorganization, de-adhesion and adhesion [24, 25]. The protein levels of N-cadherin and Twist were significantly increased after WDR3 overexpression in TNBC cells. In contrast, the protein level of E-cadherin markedly decreased after WDR3 overexpression in TNBC cells. MMP2 can degrade various protein components in ECM, destroy the histological barrier for tumor cell invasion, and play a key role in tumor invasion and metastasis [26]. We found that overexpression of WDR3 increased the protein level of MMP2. Our findings indicate that overexpression of WDR3 enhances ECM degradation to facilitate cell migration. It is generally considered that overexpression of WDR3 may reduce cell-cell adhesion, promote cell migration, and contribute to cancer cell dissemination and ultimately tumor progression.

We found that the level of WDR3 was high in TNBC. High expression of WDR3 accelerated the proliferation and cell cycle of TNBC cells. WDR3 also promoted tumor growth in vivo. WDR3 promoted migration and invasion by modulating EMT and MMP2 by activating the WNT signaling pathway. Our findings provide a theoretical and experimental basis for developing molecular targeted therapy for TNBC by targeting WDR3.

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Disclosure of conflict of interest

None.

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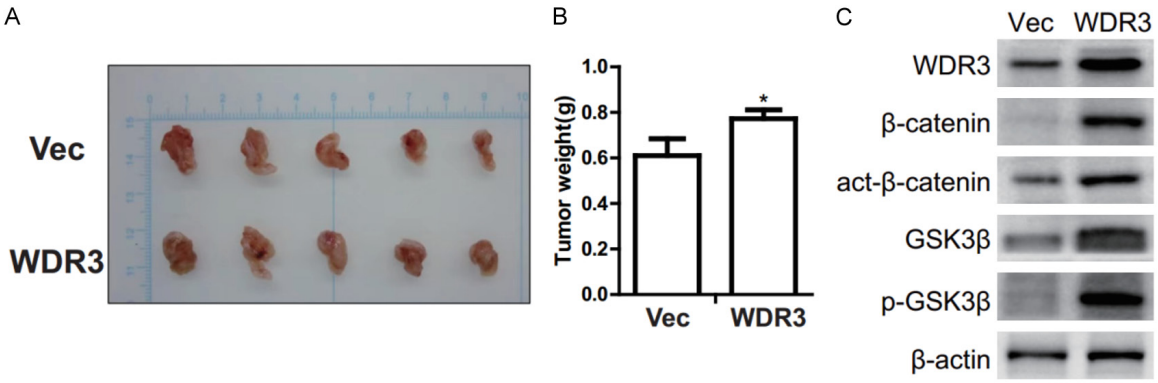

Figure S1. WDR3 promotes tumor growth in vivo and activates the WNT signaling pathway. A. A total of 5×10^6 tumor cells were subcutaneously injected into each mouse. *N* = 5 for each group. Four weeks after injection, the mice were killed, and the xenograft tumors were collected. B. The weight of the xenograft tumors was analyzed. C. Western blot was used to detect the protein level of the WNT signaling pathway.

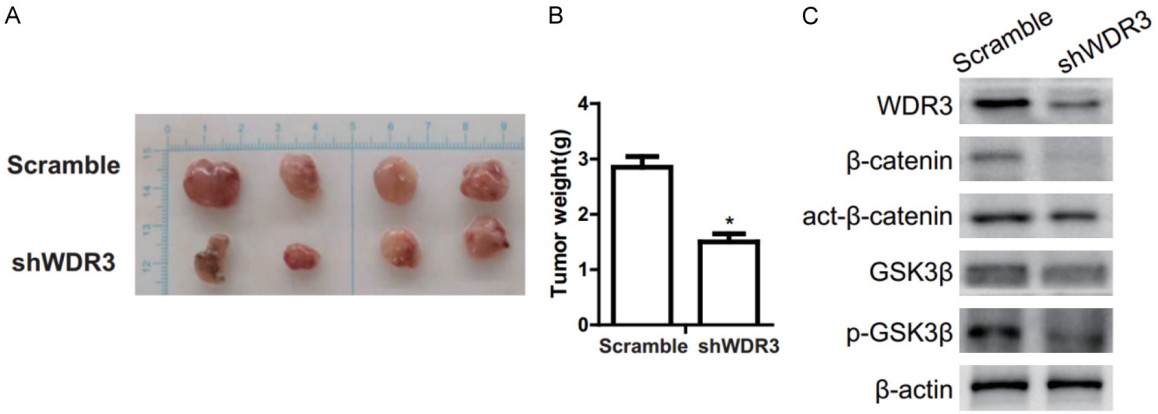

Figure S2. Downregulation of WDR3 prevents tumor growth in vivo and inhibits the WNT signaling pathway. A. A total of 5×10^6 tumor cells were subcutaneously injected into each mouse. *N* = 4 for each group. Four weeks after injection, the mice were killed, and the xenograft tumors were collected. B. The weight of the xenograft tumors was analyzed. C. Western blot was used to detect the protein level of the WNT signaling pathway.