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ORIGINAL ARTICLE

Disruption of ZNF334 promotes triple-negative breast carcinoma malignancy through the SFRP1/ Wnt/β-catenin signaling axis

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Abstract

Zinc-finger proteins (ZNFs) constitute the largest transcription factor family in the human genome. The family functions in many important biological processes involved in tumorigenesis. In our research, we identified ZNF334 as a novel tumor suppressor of triple-negative breast cancer (TNBC). ZNF334 expression was usually reduced in breast cancerv (BrCa) tissues and TNBC cell lines MDA-MB-231 (MB231) and YCCB1. We observed that promoter hypermethylation of ZNF334 was common in BrCa cell lines and tissues, which was likely responsible for its reduced expression. Ectopic expression of ZNF334 in TNBC cell lines MB231 and YCCB1 could suppress their growth and metastatic capacity both in vitro and in vivo, and as well induce cell cycle arrest at S phase and cell apoptosis. Moreover, re-expression of ZNF334 in TNBC cell lines could rescue Epithelial-Mesenchymal Transition (EMT) process and restrain stemness, due to up-regulation of SFRP1, which is an antagonist of Wnt/β-catenin signaling. In conclusion, we verified that ZNF334 had a suppressive function of TNBC cell lines by targeting the SFRP1/Wnt/β-catenin signaling axis, which might have the potentials to become a new biomarker for diagnosis and treatment of TNBC patients.

Keywords Breast cancer · ZNF334 · Wnt · β-catenin · SFRP1 · Transcription factor

Abbreviations

ZNFs	Zinc-finger proteins
TNBC	Triple-negative breast cancer
BrCa	Breast cancer
MB231	MDA-MB-231
EMT	Epithelial-Mesenchymal Transition (EMT)
PR	Progesterone receptor
ER	Estrogen receptor
HER2	Human epidermal growth factor receptor 2
KRAB-ZFPs	Zinc-finger proteins containing a Kruppel associated box
FZD	Frizzled
Aza	5-Aza-2'-deoxycytidine
TSA	Trichostatin A
MethylTarget	Multiple targeted bisulfite enrichment sequencing
MSP	Methylation-specific PCR
IHC	Immunohistochemistry

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Introduction

According to Global Cancer Statistics 2020, BrCa has become the most prevalent cancer and the primary reason of cancer deaths in women worldwide [1]. According to molecular characteristics, BrCa is usually divided into five types: luminal A, luminal B, HER2-enriched, basal-like and claudin-low BrCa [2]. TNBC, characterized by losing progesterone receptor (PR), estrogen receptor (ER), and human epidermal growth factor receptor 2 (HER2), accounts for 10 – 20% of total BrCa diagnoses [3]. TNBC is usually

poorly differentiated and easier to metastasize and relapse than other kinds of breast cancers, making it the most difficult subtype of BrCa to cure [4]. Therefore, finding more effective strategies for TNBC treatment is an urgent need.

ZNFs family is the largest family of transcription factor within the human genome, participating in a number of important biological processes such as differentiation, and development [5]. ZNFs containing a Kruppel associated box (KRAB-ZFPs) account for one-third of the approximate 800 different ZNFs, functioning in transcriptional repression and regulation of cell differentiation, proliferation, and apoptosis [6]. In previous research, many KRAB-ZNFs have been found regulating various genes involved in the promotion of tumorigenesis as well as the suppression of tumor development [7–10]. ZNF334, a newly described member of the KRAB-ZNFs family, was previously found to be associated with rheumatoid arthritis [11, 12], although no study has been carried out to evaluate its association with cancers.

DNA methylation is an epigenetic alteration by which gene activity can be regulated. Methylation of promoter CpG islands can silence gene expression, resulting in tumor initiation and development [13]. Epigenetic changes are thought to appear early in the tumor progression prior to genetic alterations. As such, identification of such changes may be a useful means by which to detect early forms of cancer, allowing for improved cancer treatment [14]. Silence of anti-tumor ZNFs by promoter methylation is a universal phenomenon involved in tumorigenesis [15]. For instance, ZNF582 was found to be commonly methylated in cervical intraepithelial neoplasia grade 3 or more severe lesions, and its reduced expression was associated with a poorer prognosis [16]. Our previous study revealed that promoter methylation contributed to decreased expression of the transcription inhibitor ZNF382 in diverse tumors [17, 18]. Promoter hypermethylation of ZNF471 in esophageal squamous cell carcinoma and breast cancer contributes to its decreased expression, leading to loss of its tumor-suppressive function [19, 20].

Wnt/β-catenin signaling has been reported taking part in various biological processes including migration, invasion, proliferation, apoptosis [21, 22]. Unusual activation of this signaling has a close relationship with the tumorigenesis of various kinds of cancers [23–27]. Many reports have illustrated the association between it and the proliferation, metastasis, stemness maintenance, and drug resistance of breast cancer [28–31]. There are a number of core components responsible for Wnt/β-catenin signaling activation. Combining Wnt ligands to Frizzled (FZD) receptors are vital for β-catenin translocating from cytoplasm to nucleus, wherein binding to TCF/LEF to induce transcription of various downstream target genes [32]. Up to date, some antagonists have been identified working on this signaling [33]. SFRP1 belongs to one of its antagonists, which is homologous to the extracellular cysteine-rich domain of the Wnt receptor FZD but lacks transmembrane and intracellular domains. SFRP1 plays its suppressive role in Wnt/β-catenin signaling through hetero-dimerization with FZD, forming a receptor complex with no function to isolate Wnt protein [34–36]. SFRP1 has been found playing a tumor-suppressive role in various cancer types [37–42]. In breast cancer, the expression of SFRP1 was found commonly lost and inversely correlated with tumor stage [43]. Ectopic expression of SFRP1 can impair breast cancer cells growth by suppressing Wnt signaling pathways according to previous reports [44, 45].

In the present study, we investigated the correlation between ZNF334 and BrCa, including the expression status of ZNF334 as well as its biological function and molecular effects on TNBC cell lines.

Materials and methods

Tumor tissues

Tumor or normal human tissues of the breast were acquired from the First Affiliated Hospital of Chongqing Medical University. This research was authorized by the Institutional Ethics Committees of the First Affiliated Hospital of Chongqing Medical University (Approval notice: #2016–75) abode by the Declaration of Helsinki. All patients have received informed consent.

Tumor cell lines

YCCB1, MB231 and MB468 cell lines belonging to TNBC cell lines were chosen for analysis. YCCB1 cell lines were presented by our collaborators, MB231 and MB468 cell lines were acquired from American Type Culture Collection (ATCC).

Bioinformatics analysis

Analyses of ZNF334 expression status in BrCa and breast normal tissues were performed online as well as the correlations between breast cancer clinical-pathological parameters and the expression status of ZNF334 using UALCAN (http://ualcan.path.uab.edu/analysis.html) with the Cancer Genome Atlas (TCGA) database. The correlation between ZNF334 and SFRP1 in breast tissues was analyzed online using Gepia2 (http://gepia2.cancer-pku.cn/#correlation) with TCGA database.

Establishments of lentiviral plasmids and ZNF334 stably expressing cell lines

An effective coding region of ZNF334 was inserted into the lentiviral vector pEZ-Lv242 (GeneCopoeia, Rockville, MD,

USA) to establish the ZNF334 lentiviral plasmid. Next, pEZ-Lv242-ZNF334-Flag or vector plasmids were transfected into 293 T cells using LipoD293™ (SignaGen Laboratories, Rockville, MD, USA) together with packing plasmids. Supernatants embodying ZNF334 lentivirus were gathered and filtered to infect MB231 and YCCB1 cells. Puromycin (ab141453, Abcam, 1ug/ml) was applied to screening ZNF334 stably expressing MB231 and YCCB1 cell lines for further analysis. Semi-quantitative PCR and western blotting were performed for ZNF334 expression identification.

Quantitative real-time PCR (qRT-PCR) and semi-quantitative PCR

RNA was isolated by TRIzol (Invitrogen) reagent. Reverse transcription (RT) of RNA samples was performed before polymerase chain reaction (PCR) with a system for RT from Promega (Madison, WI) to obtain cDNA. A 10 µL reaction mixture containing Go-Taq DNA polymerase (Promega) together with 2 µL cDNA was applied to Semi-quantitative PCR [46] and gel electrophoresis was performed to determine the amplified PCR products. SYBR green (Invitrogen) and 2 µL cDNA were added to the reaction mixture applied to qRT-PCR analysis, and the mixture was then detected with the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA). Primers for PCR were lined up in Supplementary Table 1.

5-aza-2′-deoxycytidine (Aza) and trichostatin A (TSA) treatment assay

10 µmol/L Aza (Sigma-Aldrich, Steinheim, Germany) was applied to playing demethylation function in cells for the first 3 days, followed by adding the inhibitor of histone deacetylases (HDAC) TSA or not (100 ng/mL) for another 1 day.

Multiple targeted bisulfite enrichment sequencing (MethylTarget) analysis and Methylation-specific PCR (MSP) assay

Methylation status of ZNF334 was evaluated by MSP assay and MethylTarget analysis as previously described [46]. QIAamp DNA mini kit (Qiagen, Hilden, Germany) was applied to isolating genomic DNA. AmpliTaq-Gold DNA Polymerase (Applied Biosystems) was applied to conducting MSP. Electrophoreses on 2% agarose gels of PCR products were then performed. Gel imaging system (Bio-RAD Gel Doc XR + , USA) was used to visualize all the MSP results. Some tissues were processed and high-throughput sequenced at Genesky Biotechnologies Inc. Shanghai.

Small interfering RNA (siRNA)

ZNF334 siRNA (siZNF334) and SFRP1 siRNA (si-SFRP1) kits were bought from OriGene (OriGene Technologies, Rockville, MD). Lipofectamine 2000 (Invitrogen) was applied to siRNA or siNC (control) transfection. Cells were collected for the next experiments 48–72 h post-transfection.

Colony formation assays

Cells were uniformly spread in a six-well plate, and each well was added with 800 cells incubated in 1 ml RPMI 1640 with 10% FBS and 1 ug puromycin. After incubating about 2 weeks, colonies were fixed (4% paraformaldehyde, 30 min) and stained (crystal violet, 20 min). Microscope (Leica DMI4000B, Milton Keynes, Buckinghamshire, UK) was used to photograph the colonies. Photoshop software was applied to counting colonies number (only colonies containing cells more 50 were counted).

Cell viability assay

Cells which need analyses of their proliferation ability were plated in 96-well plates and grew for the next 72 h. Cell Counting Kit-8 (CCK-8, Beyotime) was applied to evaluate cell proliferation ability. OD450 value was measured at 0, 24, 48 and 72 h, respectively [47].

Transwell® assay

Transwell® assay was applied to evaluate cell migration and invasion ability according to previously mentioned [48]. Transwell® chambers (8 µm, Corning, Tewsburry, USA) were applied to evaluate migration capacity. Chambers with Matrigel (BD Biosciences, San Jose, CA) were applied to evaluate invasion capacity. 100 µL serum-free medium with cells (2×10^4 cells for MB231; 5×10^4 cells for YCCB1) was added to the upward side of each chamber. 500 µL medium containing 10–20% FBS was put below each chamber. After incubation (24 h for MB231 cells; 48 h for YCCB1 cells), cells were fixed and stained as previously mentioned (see part 2.9 for details). Cells that had not migrated or invaded through the pores remained at the upward side were finally erased by cotton swabs. Microscope (Leica DMI4000B) was used to photograph the stained migrated or invaded cells. For cells counting, five fields of each chamber with evenly distributed cells were selected and averaged.

Flow cytometry analysis

Flow cytometry was conducted according to previously mentioned for cell cycle and apoptosis analysis [47]. Propidium iodide (PI) stain of cells was applied to evaluate cell

cycle distribution. Annexin V-fluorescein isothiocyanate together with PI double stain were applied to evaluate cell apoptosis. CELL Quest kit (BD Biosciences) was used for data analysis.

Immunohistochemistry (IHC)

Immunohistochemistry (IHC) was conducted as previously mentioned [17]. In short, all tumor slices from nude mice and human tissues were treated as the following steps: dewax, rinse, rehydrate, and then antigen repaired. Afterwards, the slices were incubated with anti-Ki67 (ab15580; Abcam, Cambridge, UK), anti-Flag (#G188; Abm, Richmond, BC) or anti-ZNF334 (HPA050022; Sigma-Aldrich) antibody at 4 °C through the night. Then, they were incubated with a secondary antibody the next day (37 °C, 30 min). Last, slices were treated with diaminobenzidine (DAB) and hematoxylin for color development and counterstaining. Images were photographed by a microscope (Leica DMI4000B).

Tumor xenograft model in nude mice

Four to six weeks of female nude mice were chosen for the evaluation of TNBC cells proliferation ability in vivo. Mice were purchased from the Vital River Laboratory Animal Technology (Beijing, China). They were treated with subcutaneous injection into the left or right side of their backs respectively with 100 μL PBS containing 2×10^6 MB231 cells stably expressing ZNF334 or vector ($n = 3$). Then, we measured tumor diameter every 3d after injection and tumor volume was determined by $0.5 \times \text{length} \times \text{width} \times \text{width}$. IHC assay was performed as previously described (see part IHC for details) to assess the characteristics of xenografts. Primary antibodies for IHC analysis were anti-Flag (#G188; Abm) and anti-Ki-67 (ab15580; Abcam) antibodies. This study was approved by the Institutional Ethics Committees of the First Affiliated Hospital of Chongqing Medical University.

Western blotting

This experiment was conducted according to the previously mentioned [46]. In short, western blotting was performed in the following steps: lysis of cells, proteins separation, proteins transfer, blocking, primary antibody incubation, secondary antibody incubation, and detection. Primary antibodies used in western blotting were listed as follows: ZNF334 (HPA050022; Sigma-Aldrich), β-actin (sc-8432; Santa Cruz), active β-catenin (#19,807; Cell Signaling Technology), total β-catenin (sc-7963; Santa Cruz), c-Myc (sc-40; Santa Cruz), MMP1 (sc-21731; Santa Cruz), SFRP1 (#3534S; Cell Signaling Technology), Cyclin D1 (sc-8396; Santa Cruz), E-cadherin (sc-8426; Santa Cruz), Flag (#G188; Abm), N-cadherin (sc-8424; Santa Cruz), SNAI2 (sc-166476; Santa Cruz). The next day, after incubating with secondary antibodies (37 °C, 1 h), proteins were detected with an Enhance Chemiluminescence kit (ThermoFisher scientific). Goat Anti-Mouse IgG, Peroxidase-Conjugated (BL001A; Biosharp, Hefei, China) and Goat Anti-Rabbit IgG, Peroxidase-Conjugated (BL003A; Biosharp, Hefei, China) were used as secondary antibodies.

Dual-luciferase reporter assays

Plasmid pGL3/Basic was connected with the target gene and the reporter to establish the target gene reporter plasmid. Plasmid pRL-TK with Renilla luciferase reporter (Promega) was co-transfected with the target gene plasmid into cells as an internal control. 48 h after transfection, a detecting kit from Promega was applied to assess the luciferase activity according to the instructions.

Chromatin immunoprecipitation (ChIP) assay

This assay was conducted according to the previously mentioned [49]. In brief, DNA and proteins had been cross-linked before cells were lysed. Then the ultrasonic treatment was performed to shear chromatin and obtain DNA fragments. Then, samples were incubated with the following antibodies: anti-Flag (#14793S, Cell Signaling Technology), anti-IgG (#2729, Cell Signaling Technology), anti-histone H3 antibody (#4620, Cell Signaling Technology). After incubation, protein A/G magnetic beads (#9006, Cell Signaling Technology) were applied to capture the samples for 2 h. Last, after purifying, DNA was used for semi-quantitative PCR analysis. Primers for ChIP analysis were listed in Supplementary Table 1.

Sphere-forming assay

Cells were incubated 2 weeks in non-adherent dishes (Costar, Corning, NY, USA) with serum-free DMEM-F12 (Gibco, Carlsbad, CA, USA) medium comprised of basic fibroblast growth factor (15 ng/mL, #10,014, Sino Biological;), epidermal growth factor (30 ng/mL, #10,605, Sino Biological), 0.4% BSA (Sigma-Aldrich, St. Louis, MO, USA), 2% B27 (Gibco, Carlsbad, CA, USA), insulin (10 mg/mL, #11,038, Sino Biological). Microscope (Leica DMI4000) was used to photograph and assess the number of spheres.

In vivo tumor imaging

MB231 cells with luciferase activity stably expressing ZNF334 or vector were injected through the tail vein into nude mice ($n = 3$, 2×10^6cells in 0.2 mL PBS). In vivo

fluorescence imaging was performed with an in vivo imaging system (PerkinElmer, Waltham, MA, USA). The fluorescence intensity of the lung in nude mice was monitored at 30 days post-injection. Lung tissues were excised right after fluorescence detection. IHC assay was performed as previously described (see part IHC for details) to evaluate Flag-ZNF334 expression status in lung tissues. Primary antibody used was anti-Flag (#G188; Abm) antibody.

EdU (5-ethynyl-2′-deoxyuridine) incorporation assay

This assay was conducted according to the previously mentioned [49]. Briefly, cells were treated using EdU (37 °C, 2 h) firstly followed by being fixed, permeabilized, and treated with Click Additive Solution together with Hoechst (for nucleus staining). Pictures were then photographed using a microscope (Leica DMI4000B). The percentage of EdU stained cells to total cells was considered as representative of the DNA replication status.

Immunofluorescent (IF) staining

Cells were planted on coverslips and fixed (4% paraformaldehyde, 30 min). Then cells were treated with 0.5% Triton X-100 (10 min) for cell permeabilization. Next, cells were treated with Immunol Staining Blocking Buffer (Beyotime) 1 h. Then cells were incubated with primary antibodies SFRP1(#3534S; Cell Signaling Technology) and Flag (F1804;Sigma) at 4 °C overnight. The next day, cells were treated with DyLight-conjugated anti-rabbit or anti-mouse secondary antibodies at room temperature for 1 h. And then DAPI was used for nucleus staining. Finally, a fluorescence microscope was used for taking images.

Statistical analysis

All data were statistically analyzed with SPSS25.0 (IBM, Almonck, USA). χ^2 test, Fisher's exact test and Two-tailed Student's t test were performed based on the specific situations. Difference was regarded as statistically significant when $p < 0.05$. All experiments were repeated three times independently.

Results

ZNF334 expression is decreased in BrCa tissues and is related to clinical-pathological parameters

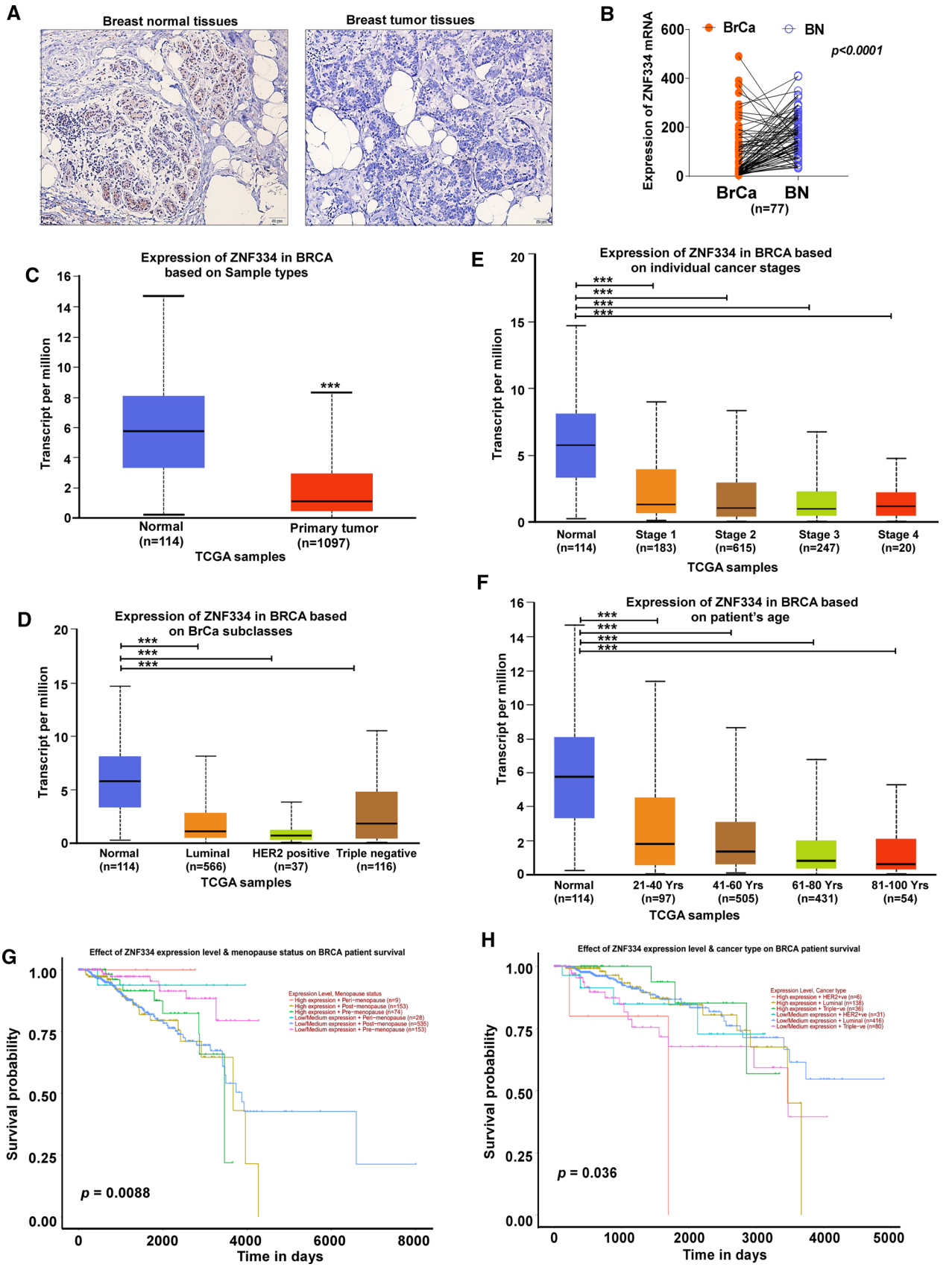
To investigate the characteristics of ZNF334 in BrCa, we firstly performed an online analysis using UALCAN with the TCGA database. Analyses showed that ZNF334 expression is decreased in BrCa tissues compared to normal breast tissues (Fig. 1B and 1C). IHC staining for ZNF334 using tissues gained from our hospital also demonstrated a decreased ZNF334 expression in human BrCa tissues compared to paired normal tissues (Fig. 1A). Furthermore, using online analysis, we observed that the expression of ZNF334 was decreased in all four stages and every kind of BrCa. However, it did not show an obvious correlation with the progression of tumor stage and molecular subtypes (Fig. 1E + 1D). Besides, the online analysis also showed that the expression of ZNF334 in BrCa patients may negatively correlate with their ages. With the age growing, the expression of ZNF334 was decreasing (Fig. 1F). Moreover, survival probability analysis showed that BrCa patients in pre-menopause status with high expression of ZNF334 held a worse prognosis compared with those who have a lower expression of ZNF334. But this correlation between the expression of ZNF334 and menopause status was reversed in peri-menopasue BrCa patients, indicating that the status of the hormone may influence the function of ZNF334 in BrCa patients (Fig. 1G). Besides, survival probability analysis indicated that TNBC patients with greater expression of ZNF334 held better outcomes than those with lower expression (Fig. 1H).

ZNF334 was down-regulated by promoter methylation in BrCa tissues and cells

Next, using MethylTarget® analysis, we found much higher methylation levels of ZNF334 CpG sites in BrCa tissues (Fig. 2A). MSP assay illustrated that promoter methylation was detected in 46/64 (71.88%) primary breast tumors, suggesting promoter methylation of ZNF334 is a universal event in breast cancer (Fig. 2B). To further identify the correlation between promoter methylation and ZNF334 expression, we treated MB231 and YCCB1 cell lines with Aza for demethylation, with or without HDAC inhibitor TSA. We observed that ZNF334 expression was restored by treating with Aza (sFig.1A) accompanied by a decreased methylation status and an increased unmethylated status of a promoter in TNBC cells (sFig.1B and 1C). Together, those results demonstrated that promoter methylation might be the reason for decreased ZNF334 expression in BrCa tissues and cells.

ZNF334 suppressed proliferation ability of TNBC cells in vitro and in vivo

Because TNBC patients with higher expression of ZNF334 appear to have a better prognosis, we focused on exploring the function of ZNF334 in TNBC cells. The lentivirus containing Vector (pEZ-Lv242) and pEZ-Lv242-ZNF334-Flag were infected into the TNBC cell lines MB231 and YCCB1 to establish ZNF334 stably expressing cell lines.


A
Breast normal tissues
Breast tumor tissues
B
Expression of ZNF334 mRNA
BrCa
BN
p<0.0001
BrCa BN
(n=77)
C
Expression of ZNF334 in BRCA based on Sample types
Transcript per million
Normal (n=114)
Primary tumor (n=1097)
TCGA samples
D
Expression of ZNF334 in BRCA based on BrCa subclasses
Transcript per million
Normal (n=114)
Luminal (n=566)
HER2 positive (n=37)
Triple negative (n=116)
TCGA samples
E
Expression of ZNF334 in BRCA based on individual cancer stages
Transcript per million
Normal (n=114)
Stage 1 (n=183)
Stage 2 (n=615)
Stage 3 (n=247)
Stage 4 (n=20)
TCGA samples
F
Expression of ZNF334 in BRCA based on patient's age
Transcript per million
Normal (n=114)
21-40 Yrs (n=97)
41-60 Yrs (n=505)
61-80 Yrs (n=431)
81-100 Yrs (n=54)
TCGA samples
G
Effect of ZNF334 expression level & menopause status on BRCA patient survival
Survival probability
p = 0.0088
Time in days
H
Effect of ZNF334 expression level & cancer type on BRCA patient survival
Survival probability
p = 0.036
Time in days

◂**Fig. 1** Expression of ZNF334 is lower in BrCa tissues and correlated with clinical-pathological parameters. **A** IHC staining of ZNF334 in human normal breast tissues (left) and breast tumor tissues (right). **B**–**H** Online analysis: **B**, **C** The expression of ZNF334 in BrCa and normal breast tissues. **D** Correlation between the expression of ZNF334 and BrCa subclasses. **E** Correlation between the expression of ZNF334 and individual cancer stages of BrCa. **F** Correlation between the expression of ZNF334 and patient's age. **G** Survival analysis of correlation between ZNF334 expression and menopause status. **H** Survival analysis of the correlation between ZNF334 expression and BrCa subtype. Data are presented as the mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

Semi-quantitative RT-PCR and western blotting identified ZNF334 expression status in them (Fig. 3A, B). Next, cell viability and colony formation assays illustrated that ectopic expression of ZNF334 suppressed cell proliferation and colony formation ability of TNBC cells (Fig. 3C, D). Further, we injected nude mice subcutaneously with vector or ZNF334 stably expressing MB231 cells. Growth curves of the tumors demonstrated reduced tumor progression in mice injected with cells stably expressing ZNF334 compared to mice that received vector-infected cells. Likewise, the average tumor weight of xenografts was lighter in ZNF334 over-expressing group (Fig. 3E). IHC and hematoxylin and eosin (HE) staining were used to assess ZNF334 expression and tumor characteristics of xenografts. Decreased Ki-67 expression was found in MB231 cells stably expressing ZNF334 (sFig. 2A + 2B). These results illustrated that ectopic expression of ZNF334 suppressed TNBC cells proliferation both in vitro and in vivo.

ZNF334 induced cell apoptosis and cell cycle arrest at S phase

Using flow cytometry, we explored the influence of ZNF334 on apoptosis and cell cycle distribution of MB231 and YCCB1 cells. We observed that ectopic expression of ZNF334 increased the number of apoptotic and S phase cells in both MB231 and YCCB1 cells (Fig. 4A,C). At S phase, it was difficult to distinguish between cell cycle arrest and increased DNA replication. We, therefore, evaluated DNA replication activity by supplemented Edu assay. Results demonstrated that DNA replication activity was decreased in ZNF334-expressing tumor cells compared to control cells (fewer cells were stained by Edu in ZNF334-expressing cells) (Fig. 4B). As such, we considered that DNA replication was not responsible for the increase of S phase cells which may result from cell cycle arrest. In conclusion, we observed that re-expression of ZNF334 induced apoptosis and cell cycle arrest at S phase of TNBC cells.

ZNF334 reversed epithelial-mesenchymal transition (EMT) and restrained stemness of TNBC cells

To evaluate the functional effects of ZNF334 on TNBC cells, we performed Transwell® and sphere-forming assays. Results showed that cellular migration and invasion ability were reduced in MB231 and YCCB1 cells stably expressing ZNF334 together with a decrease in the number of formed spheres, suggesting it had functional effects on metastatic capacity and stemness of TNBC cells (Fig. 5A, B and E). Therefore, we detected alterations in EMT-related proteins which were believed closely related to metastasis and stemness-related genes. Results of qRT-PCR revealed a decrease in several stemness-related genes in MB231 and YCCB1 cells stably expressing ZNF334 (Fig. 5C). Moreover, western blotting demonstrated a decreased expression of N-cadherin (N-cad) and SNAI2 expression in ZNF334 stably expressing MB231 and YCCB1 cells together with an increased expression of E-cadherin (E-cad) in YCCB1 cells stably expressing ZNF334, suggesting a partial reverse of EMT process (Fig. 5D). But we failed to detect the expression of E-cad in MB231 cell line. In a previous study, MB231 was reported as an E-cad deficient cell line[50] and the expression of E-cad in MB231 was always barely to be detected [51–53]. Therefore, we thought this is the reason for the loss of the expression of E-cad in MB231 cells in our western blotting result. In conclusion, ectopic expression of ZNF334 appeared to inhibit the metastasis and stemness of TNBC cells by reversal of the EMT process and by reducing the expression of stemness-related genes.

ZNF334 suppressed TNBC cells metastasis in vivo

Because overexpression of ZNF334 had an inhibitory function of migration and invasion in MB231 and YCCB1cells in vitro, we explored its effects on metastasis in vivo. The lentivirus containing Vector (pEZ-Lv242) and pEZ-Lv242-ZNF334-Flag were infected into the MB231 cells with luciferase activity (MB231-LUC). Next, vector and ZNF334 stably expressing MB231-LUC cells were injected into nude mice through the tail vein to evaluate their lung metastatic potential. Thirty days after injection, using in vivo imaging system, we observed that two of three mice injected with vector stably expressing MB231-LUC cells had obvious luciferase signals within their lungs while only one of three mice injected with ZNF334 stably expressing MB231-LUC cells having less obvious luciferase signals within their lungs (sFig. 3A), suggesting a reduced metastatic capacity for MB231-LUC cells transfected with ZNF334. HE staining and IHC staining for Flag of lung tissues were then performed (sFig. 3B). In conclusion, ectopic expression of ZNF334 could inhibit TNBC cells metastasis in vivo.

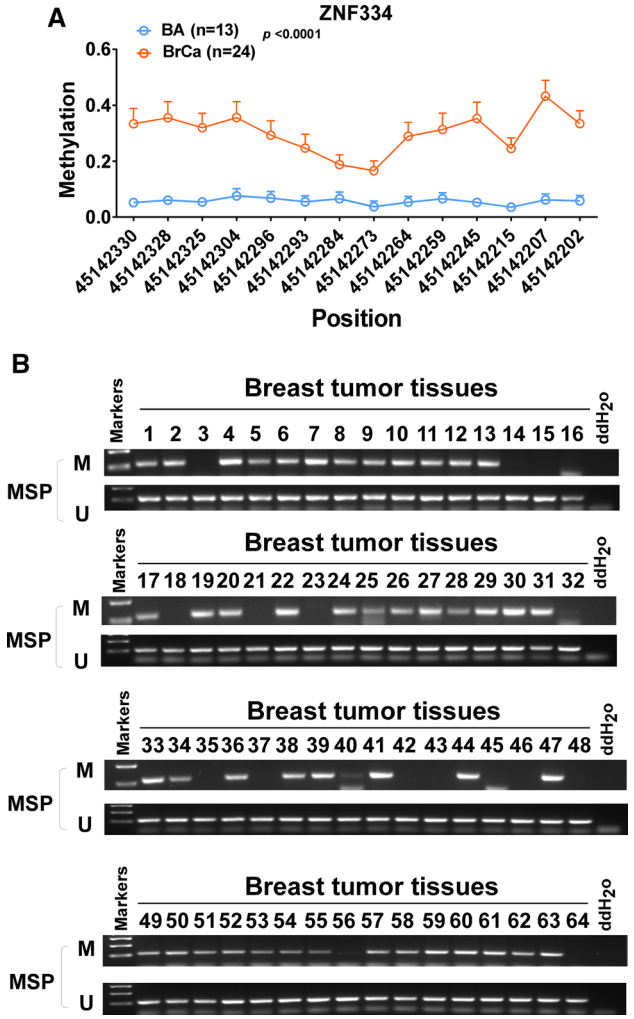


Fig. 2 Promoter of ZNF334 was always hypermethylated in BrCa tissues. **A** MethylTarget analysis of 24 breast cancer cases (BrCa) and 13 normal breast tissues (BA). **B** ZNF334 promoter methylation status was detected in 64 BrCa tissues using MSP assay. Data are presented as the mean ± SD

ZNF334 suppressed Wnt/β-catenin signaling by up-regulating SFRP1

Wnt/β-catenin signaling is believed to have a close relationship with the EMT process as well as the maintenance of stemness. So we then focused on the correlation between ZNF334 and it. Using TOP/FOP flash dual-luciferase reporter assay, we observed reduced luciferase reporter activity in 293T and YCCB1 cells with ZNF334 overexpression (Fig. 6C). Western blotting demonstrated that ZNF334 reduced the expression of active β-catenin together with some downstream targets such as c-Myc and cyclin D1 in MB231 and YCCB1 cells while total β-catenin had not been affected (Fig. 6A). In another study (have not been published), we performed high-throughput sequencing to analyze differently expressed genes between vector and ZNF334 transfected colon cancer cells. The results of the differently expressed genes in TNBC cells were partly similar to those in colon cancer cells, with confirmation by qRT-PCR (data not shown). Among those differentially expressed genes related to Wnt/β-catenin signaling, we focused on SFRP1, which had been reported as an antagonist of it and suppressing breast cancer initiation and development [45]. Western blotting showed that SFRP1 expression was up-regulated in MB231 and YCCB1 cells with ZNF334 overexpression (Fig. 6A). Moreover, the dual-luciferase reporter and ChIP assays demonstrated that ZNF334 could directly bind to the promoter of SFRP1, increasing its luciferase reporter activity (Fig. 6B and 6D). Moreover, we detected the expression of SFRP1 in BrCa tissues and xenografts of nude mice. Results showed that the expression of SFRP1 was also higher in the normal breast tissues with a high expression of ZNF334 compared to BrCa tissues (sFig.4B) and its expression was increased in the ZNF334 over-expressed tumor of mice (sFig.2A). Besides, we performed an online analysis using GEPIA2 with TCGA database to explore the relationship between the expression of ZNF334 and SFRP1 in normal breast tissues and BrCa tissues. Results showed that the expression of SFRP1 is both positively correlated with the expression of ZNF334 in normal breast tissues and BrCa tissues, especially in normal breast tissues (sFig.4A). We thought it may be because, in BrCa tissues, the expression of SFRP1 and ZNF334 was at a low level and can not show an obvious correlation. Furthermore, we performed IF assay to explore the expreesion of SFRP1 in ZNF334 infected MB231 and YCCB1 cells. Results showed that ectopic expression of ZNF334 could increase the expression of SFRP1 in those cells (sFig.4C). Based on these results, we hypothesized that ZNF334 inhibited the activity of the Wnt/β-catenin signaling by up-regulating of SFRP1.

Knockdown of ZNF334 promoted migration and invasion of TNBC cells as well as activated Wnt/β-catenin signaling

Further to evaluate the function of ZNF334, another TNBC cell line, MB468, was chosen for the knockdown experiment. MB468 cells were transfected with siNC (control) or ZNF334 siRNA (siZNF334) and then assessed for functional effects by cell viability and Transwell® assays.

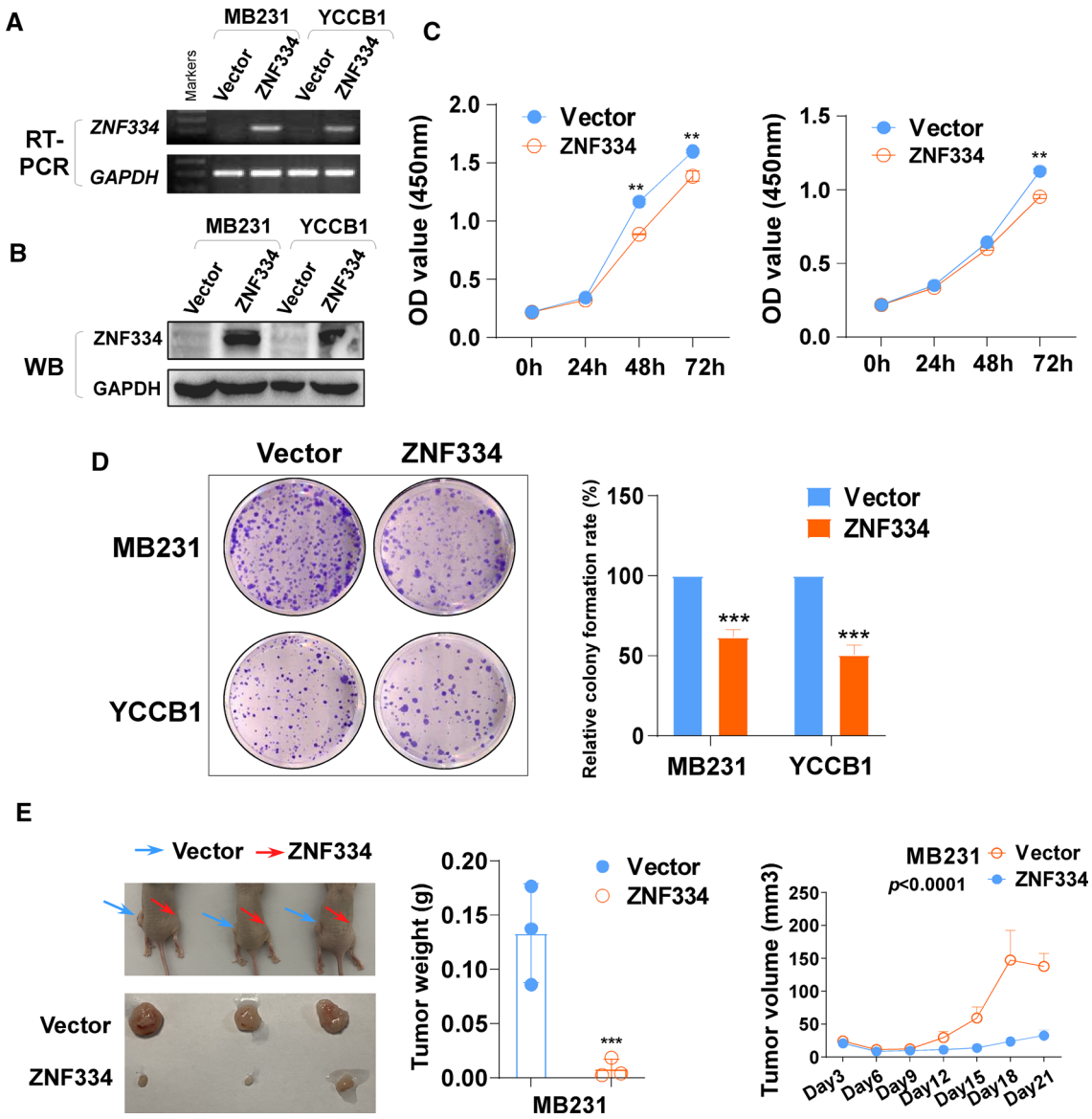


Fig. 3 ZNF334 suppressed TNBC cells proliferation. **A**, **B** Expression of ZNF334 in TNBC cells MB231 and YCCB1 were verified by Semi-quantitative RT-PCR and western blotting assay. **C** Cell viabilities measured at 0, 24, 48 and 72 h in vector and ZNF334 stably expressing MB231 and YCCB1 cells. **D** Colony formation ability of ZNF334 stably expressing MB231 and YCCB1 cells (left). Histograms of relative colony formation rates from three independent experiments (right). **E** Images of human TNBC tumor xenografts (left). Comparative histogram of tumor weights in the two groups (vector group vs. stably expressing ZNF334 group) of nude mice (mid). Comparative analyses of tumor growth curve of vector and ZNF334 stably expressing MB231 cells in nude mice xenografts (right). Student's test was used. Data are presented as the mean ± SD. $*p<0.05$, $**p<0.01$, $***p<0.001$

Results showed that knockdown of ZNF334 promoted the migration and invasion ability of MB468 cells in vitro (Fig. 7B, C), while no obvious effect on proliferation was observed (Fig. 7A). TOP/FOP flash dual-luciferase reporter assay indicated that knocking down ZNF334 could increase the reporter activity which demonstrated an increased activity of Wnt/β-catenin signaling in MB468 cells transfected with siZNF334 (Fig. 7D). Western blotting showed that Wnt/β-catenin signaling was more active in MB468 cells transfected with siZNF334, with increased

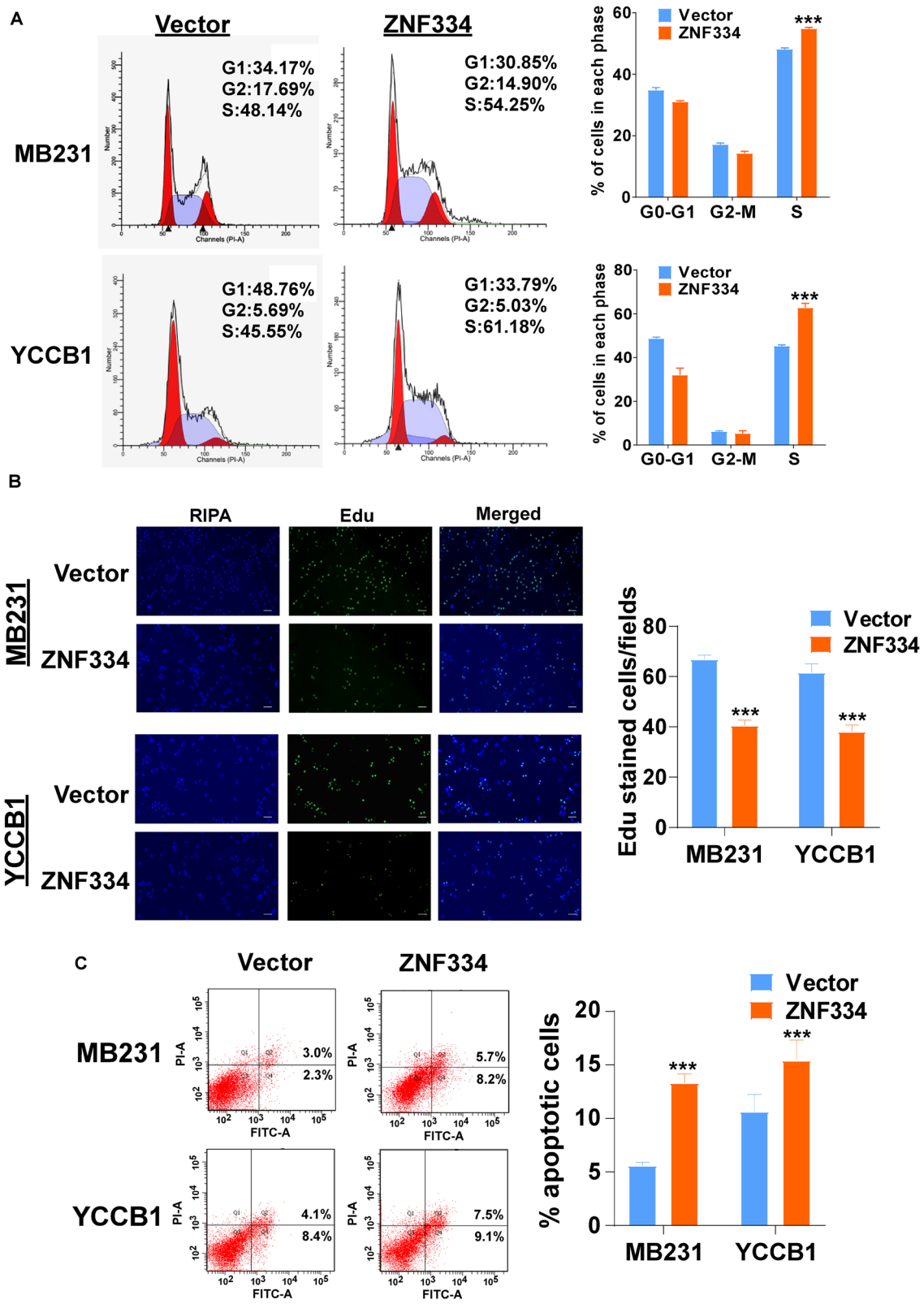


Fig. 4 ZNF334 induced TNBC cells apoptosis and cell cycle arrest at S phase. **A** Cell cycle distribution measured in vector and ZNF334 stably expressing MB231 and YCCB1 cells by flow cytometry. **B** DNA replication activity was measured by Edu assay. Images of Edu stained cells (left) and histograms of a number of Edu stained cells per fields (right) in vector and ZNF334 infected MB231 and YCCB1 cells are shown. **C** Percentages of apoptotic cells in MB231 and YCCB1 cells after expressing with ZNF334. Histograms of percentages of apoptotic cells (right). Student's test was used. Data are presented as the mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

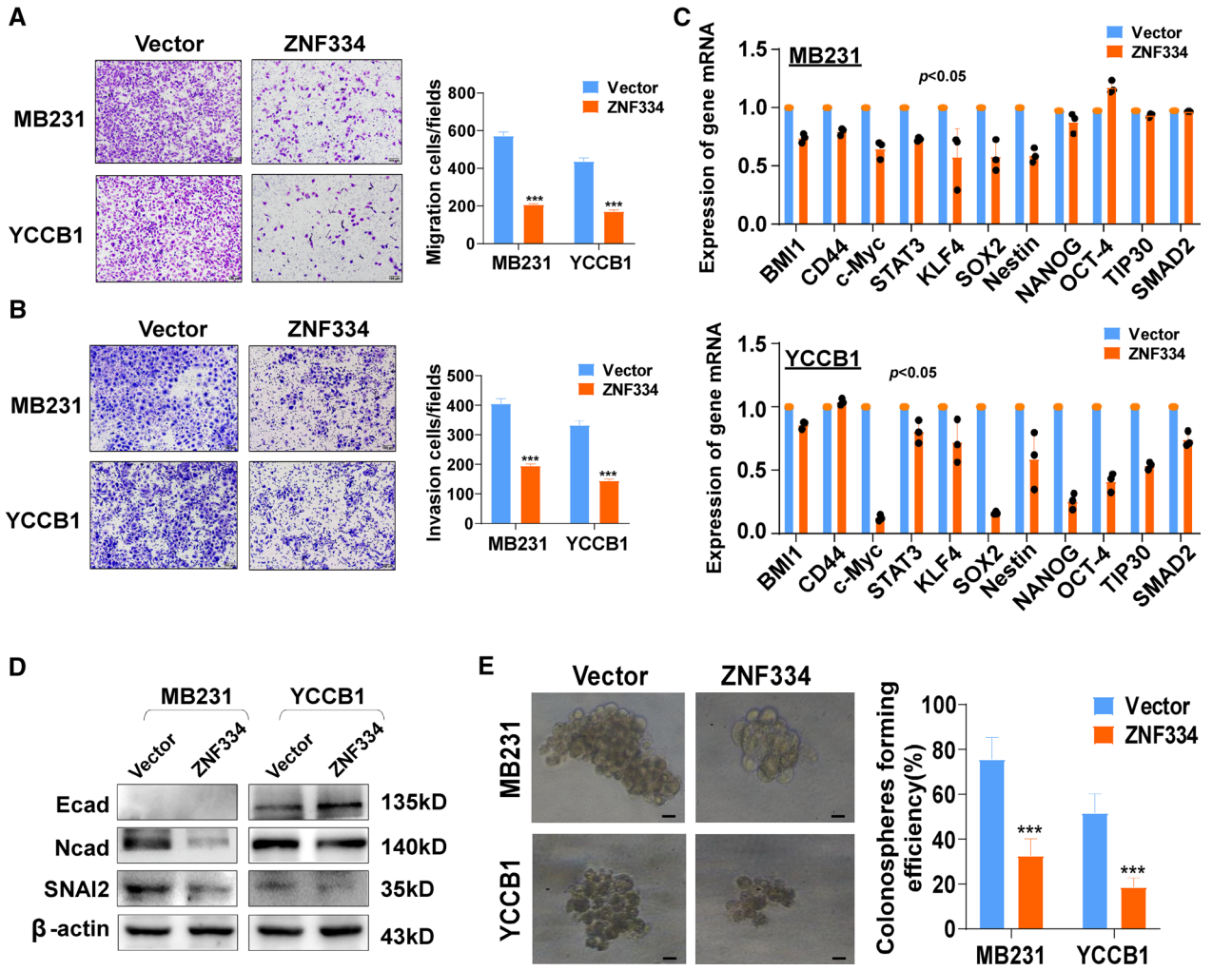


Fig. 5 ZNF334 suppressed the metastasis capacity and restrained stemness of TNBC cells. **A**, **B** Images of migrated **A** and invaded **B** TNBC cells with or without ectopic expression of ZNF334 detected by Transwell® assays. Scale bars: 100 μm. **C** qRT-PCR analysis of expression status of stemness related genes of vector and ZNF334 stably expressing MB231 and YCCB1 cells. **D** Western blotting analysis of EMT-related proteins. **E** Images of sphere-forming assay (left) and histograms of clon-spheres forming efficiency (right) in vector and ZNF334 stably expressing MB231 and YCCB1 cells. Student's test was used. Data are presented as the mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

expression of active β-catenin and downstream target genes c-Myc, MMP1. Furthermore, the expression of E-cad was reduced while N-cad and SANI2 were increased (Fig. 7E). These results suggested that the knockdown of ZNF334 promoted the metastasis ability of TNBC cells and activated Wnt/β-catenin signaling.

SFRP1 may be the key molecule by which ZNF334 inhibited the Wnt/β-catenin signaling

To further verify the correlation between ZNF334 and SFRP1, we knocked down the expression of SFRP1 in ZNF334 stably expressing MB231 and YCCB1 cells with siRNA. Knockdown of SFRP1 in ZNF334 over-expressing TNBC cells restored their migration and invasion capacity (Fig. 8A and sFig. 5). Moreover, knockdown of SFRP1 in ZNF334 over-expressing TNBC cells reversed the restraint of Wnt/β-catenin signaling (Fig. 8B). These results suggested that SFRP1 may be the key molecule by which ZNF334 inhibited the Wnt/β-catenin signaling, resulting in a tumor-suppressive function in TNBC cells.

Discussion

The ZNFs transcription factor family consists of many members with a zinc-finger domain for binding with the promoter of various genes, regulating their expression. Members of this family carry out many important cellular biological processes in cells, influencing the initiation and development of

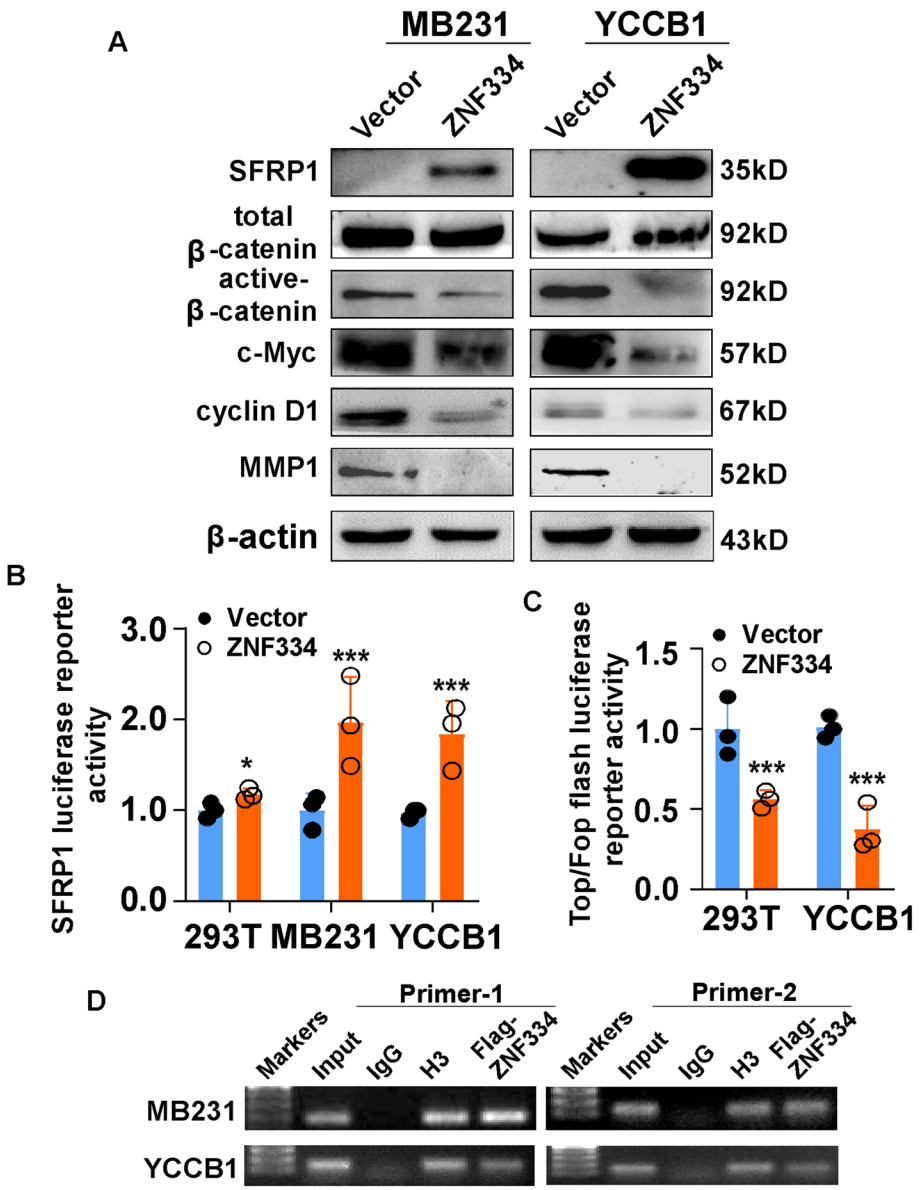


Fig. 6 ZNF334 suppressed Wnt/β-catenin signaling pathway by up-regulating SFRP1. **A** Western blotting illustrated alterations of Wnt/β-catenin signaling-related proteins. **B**, **C** Dual-luciferase reporter assay showed increased SFRP1 luciferase reporter activity and decreased TOP/FOP flash reporter activity in ZNF334 transfected 293 T and TNBC cells. **D** ChIP assay demonstrated a direct combination between ZNF334 and the promoter of SFRP1. Student's test was used. Data are presented as the mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

various tumors [5]. ZNF334 is a newly described member belonging to KRAB-ZNFs family, which has been shown related to rheumatoid arthritis in previous researches [11, 12]. Up to date, no research has been performed to study the relationship between ZNF334 and cancers. In this research, we first explored the effects of ZNF334 on BrCa, especially on TNBC cells. Using online analysis with the TCGA database, we observed a decreased ZNF334 expression in BrCa tissues and TNBC patients with greater expression of ZNF334 had a better prognosis. Further, ZNF334 stably expressing TNBC cell lines MB231 and YCCB1 were established to explore its specific function. We found ectopic expression of ZNF334 in TNBC cell lines could suppress their proliferation and metastatic capacity both in vitro and in vivo, induce apoptosis and cell cycle arrest at S phase, reverse EMT process, and restrain their stemness. Those results illustrated us that ZNF334 may be a tumor suppress gene functioning in TNBC cells.

Interestingly, in the present research, we observed that ZNF334 had a greater influence on metastatic capacity and stemness than proliferation. Hence, we focused on the underlying basis for those observed effects. It firstly reminded us of the well-studied Wnt/β-catenin signaling related to metastasis, stemness and promotion of the EMT process of tumor cells [54]. To explore whether the Wnt/β-catenin signaling was affected by ZNF334, we then performed TOP/FOP flash

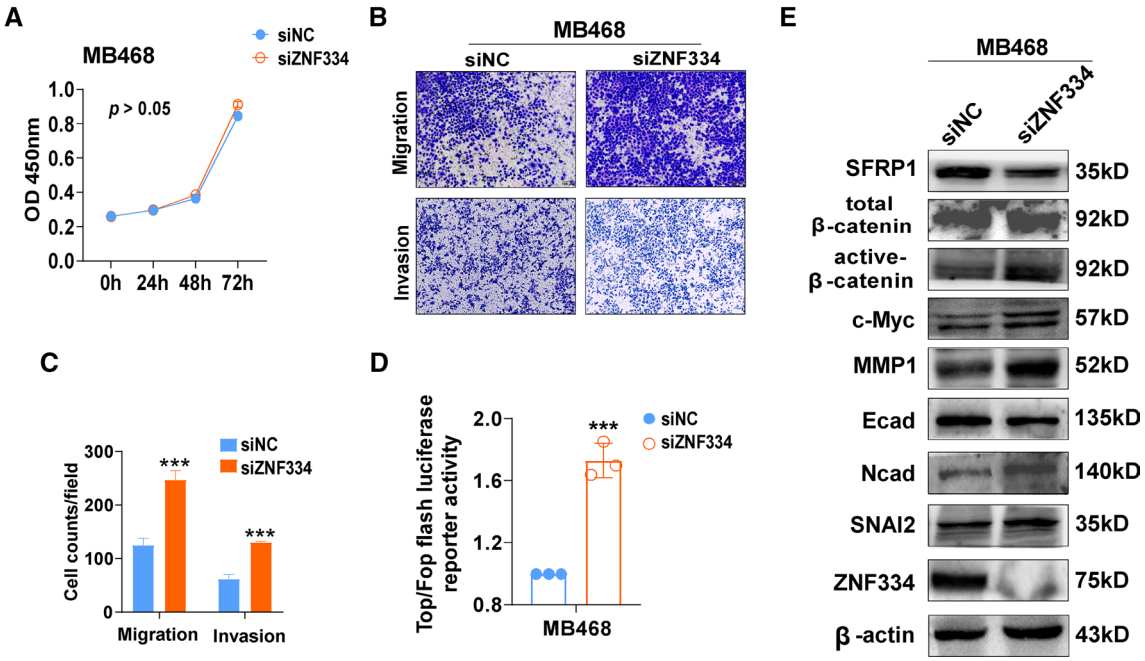


Fig. 7 Knockdown of ZNF334 promoted metastasis capacity and activated Wnt/β-catenin signaling pathway in TNBC cells. **A** The effect of knock-down ZNF334 on cell viability measured by cell viability assays. **B** Images of migrated and invaded siNC or siZNF334 transfected MB468 cells detected by Transwell® assay. Scale bars: 100 μm. **C** Histograms of number of migrated and invaded cells in si-NC and siZNF334 transfected MB468 cells. **D** TOP/FOP flash luciferase reporter activity after knocking down ZNF334 in MB468 cells. **E** Expression of Wnt/β-catenin related proteins in siNC and siZNF334 transfected MB468 cells. Each experiment was repeated three times. Student's test was used. Data are presented as the mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

dual-luciferase reporter assay and western blotting. Results showed that re-expression of ZNF334 could reduce the luciferase reporter activity and the expression of active β-catenin as well as some downstream targets of Wnt/β-catenin signaling pathway. Therefore, we speculated that ZNF334 played an inhibitory role in metastasis, stemness and proliferation of TNBC cells by suppression of Wnt/β-catenin signaling.

Wondering the detailed mechanism of how ZNF334 influenced Wnt/β-catenin signaling, we referenced our RNA-sequencing results in colon cancer cell lines. In HCT116 and HT29 cells, the expression of several genes closely correlated with Wnt/β-catenin signaling were altered by ectopic expression of ZNF334 (data not shown). In the present study, we observed some similar changes caused by ZNF334 in MB231 and YCCB1 cells. Among them, we finally focused on SFRP1, a well-known antagonist of this signaling [37, 38]. To identify the relationship between ZNF334 and SFRP1, we then performed dual-luciferase reporter, ChIP-PCR and western blotting assays which showed that ZNF334 could bind to the promoter of SFRP1, increasing its expression. Moreover, we found that knockdown of SFRP1 could restore the suppressed metastasis ability as well as the expression of EMT and Wnt/β-catenin signaling-related proteins in TNBC cells. Taking all results into consideration, we hypothesized that ZNF334 played its tumor suppressor role in TNBC cells through up-regulating SFRP1 which is an antagonist of Wnt/β-catenin signaling.

Wnt/β-catenin signaling is crucial for tumor initiation and development in various kinds of cancers, but no drugs targeting this signaling pathway have been proven for clinical treatment by now. In previous reports, Wnt/β-catenin signaling has been observed influencing TNBC resistance to chemotherapy. Inhibition of the pathway may increase the sensitivity of TNBC to chemotherapeutic treatment. A combination of chemotherapeutic drugs with Wnt/β-catenin signaling inhibition may be a potential therapeutic method for TNBC treatment [31, 55]. As such, we believed that ZNF334 could become a new target by which to influence Wnt/β-catenin signaling, with benefit for TNBC patients.

Furthermore, we explored the potential mechanism responsible for the decreased ZNF334 expression in BrCa tissues and cells. Using MethylTarget® analysis and MSP assay, we verified that ZNF334 promoter methylation was more common in BrCa tissues. Furthermore, Aza and TSA treatment assay of TNBC cells demonstrated that promoter methylation may contribute to the decreased ZNF334

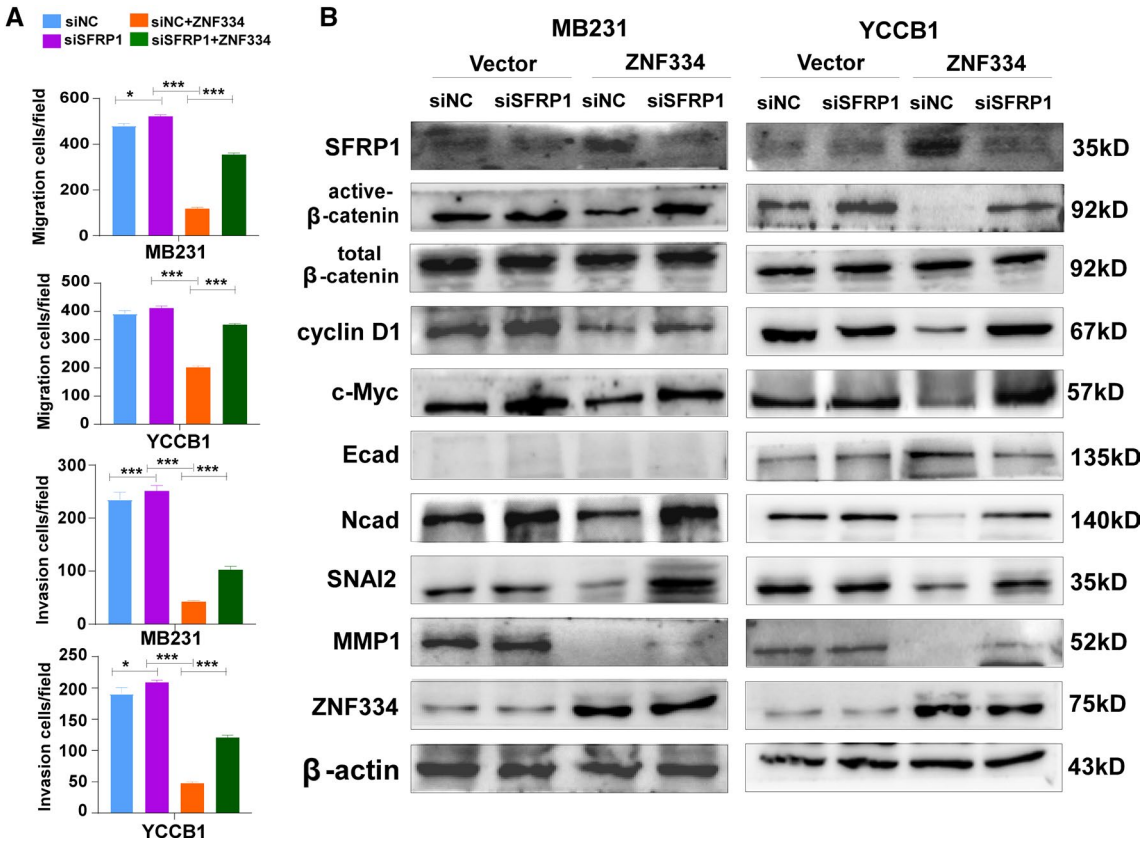


Fig. 8 Knockdown of SFRP1 could reverse the suppress function of ZNF334 in TNBC cells. **A** Histograms of number of migrated and invaded cells in siNC and siSFRP1 transfected MB231 and YCCB1 cells with or without ectopic expression of ZNF334. **B** Western blotting assay illustrated knockdown of SFRP1 restored the expression of Wnt/β-catenin related proteins affected by ectopic expression of ZNF334. Each experiment was repeated three times. Student's test was used. Data are presented as the mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

expression in BrCa. As DNA methylation is a vital epigenetic modification affecting gene activity which was regarded as an early diagnostic marker for various kinds of cancers [13–15], we believe that the promoter methylation status of ZNF334 could become a new molecular biomarker for early TNBC patient diagnosis.

In conclusion, we verified a new tumor suppress gene ZNF334 of TNBC cells, which functioned by targeting SFRP1/Wnt/β-catenin signaling axis. Whether ZNF334 influences other biological processes is still unclear. Further work is needed to comprehensively analyze the function of ZNF334 in the breast or other kinds of cancer.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00018-022-04295-1.

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Author contributions TX: conception and design. ZC, RY: performed majority of experiments. TX, LL, FW, JM: performed experiments and analyzed data. YL, XZ: collected samples. TX, ZC, RS: drafted the manuscript. TX, RS, XZ, YW: reviewed data and manuscript. TX, RS: reviewed data and finalized the manuscript. All authors reviewed and approved the final version.

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Availability of data and material Online analysis was performed using UALCAN (http://ualcan.path.uab.edu/ analysis.html) and Gepia2 (http://gepia2.cancer-pku.cn/#correlation) with the Cancer Genome Atlas (TCGA) database. Other data used in this study are available on reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval This research was authorized by the Institutional Ethics Committees of the First Affiliated Hospital of Chongqing Medical University (Approval notice: #2016–75) abode by the Declaration of Helsinki.

Consent to participate All patients have received informed consent.

Consent for publication Consents had been received from patients for reporting individual data.

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