miR-28-5p inhibits the migration of breast cancer by regulating *WSB2*

LIANG MA[1], YUNFENG ZHANG[2] and FEN HU[1]

[1]College of Life Sciences, North China University of Science and Technology, Tangshan, Hebei 063210;
[2]Department of Life Sciences, Tangshan Normal University, Tangshan, Hebei 063000, P.R. China

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Abstract. MicroRNAs (miRNAs or miRs) play an important role in the tumorigenesis and progression of breast cancer. However, the function of miR-28-5p in breast cancer migration has yet to be determined. In the present study, Human MicroRNA Expression Database (HMED) analysis revealed that the expression level of miR-28-5p was significantly lower in breast cancer tissue than in normal breast tissue. Kaplan-Meier plotter (KMPLOT) analysis revealed that the low expression level of miR-28-5p was associated with a poor survival in breast cancer. In addition, reverse transcription-quantitative PCR (RT-qPCR) revealed that the expression of miR-28-5p was significantly lower in breast cancer cell lines compared with that in human mammary epithelial cells (HMECs). Moreover, transfection with miR-28-5p mimics suppressed the migration of MCF-7 cells, whereas an miR-28-5p inhibitor exerted the opposite effect. Gene chip assay identified 648 differentially expressed genes (DEGs) in cells overexpressing miR-28-5p. The DEGs are enriched in the 'focal adhesion' and 'pathway in cancer' pathways. The expression levels of Ras-related protein Rap-1b (*RAP1B*), WD repeat and SOCS box containing 2 (*WSB2*) and vascular endothelial growth factor A (*VEGFA*) were confirmed by RT-qPCR. Furthermore, transfection with miR-28-5p mimics decreased *WSB2* expression, whereas the miR-28-5p inhibitor increased the expression of *WSB2*, at both the transcriptional and translational levels. miR-28-5p targets the 3'UTR of *WSB2*, and the binding site is conserved in multiple species, with a consensus motif of 5'-AGCUCCUU-3'. Moreover, WSB2 overexpression promoted the migration of MCF-7 cells which had been inhibited by miR-28-5p. UALCAN analysis revealed that *WSB2* was significantly upregulated in primary breast tumor tissue, and a high expression level of *WSB2* was associated with a poor survival in breast cancer. Furthermore, immunohistochemistry revealed that the expression of WSB2 was markedly higher in breast cancer tissue compared with that in adjacent normal breast tissue. Taken together, the findings of the present study demonstrate that miR-28-5p inhibits the migration of breast cancer cells by regulating *WSB2* expression, and the miR-28-5p/WSB2 axis may be a novel therapeutic target in breast cancer.

Correspondence to: Dr Fen Hu, College of Life Sciences, North China University of Science and Technology, 21 Bohai Avenue, Caofeidian New Town, Tangshan, Hebei 063210, P.R. China
E-mail: ihufen@126.com

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Introduction

Breast cancer is one of the most common malignant types of tumor among women. In advanced stages, cancer cells metastasize to the liver, brain, lung and bone tissue, along the lymphatic and blood vessels. Metastasis is a complex process involving the dysregulation of multiple genes (1). Abnormally expressed microRNAs (miRNAs or miRs) are closely related to the development of breast cancer (2). Breast cancer-associated miRNAs (let-7, miR-155, miR-21, miR-510, miR-192, miR-200, etc.) have been shown to be involved in the regulation of cell proliferation, differentiation, apoptosis and the maintenance of breast cancer stem cells (3,4). Although a number of oncogenes and tumor suppressor genes have been implicated in the development and progression of breast cancer, the underlying molecular mechanisms remain poorly understood.

miRNAs are endogenous short-chain RNAs, of approximately 22 nucleotides in length (5). miRNAs are important post-transcriptional regulators that induce mRNA degradation and the translational repression of genes by binding to the 3' untranslated regions (UTRs) of their target mRNAs (6). The dysregulation of miRNA expression contributes to the development of multiple types of cancers. miRNA-28-5p is an intronic miRNA of the lipoma-preferred partner gene (*LPP*), which is located on chromosome 3q27-28. miR-28-5p directly binds to the *LPP* mRNA and suppresses its expression, which subsequently inhibits cell migration and adhesion (7). miR-28-5p has been found to function as a tumor suppressor, as it has been shown to be downregulated in various types of human malignancies, such as hepatocellular carcinoma (8), renal cancer (9), colorectal cancer (10) and in nasopharyngeal cancer cells (11). miR-28-5p is involved in the regulation of cell proliferation, apoptosis and metastasis. For example, it has been shown to inhibit the proliferation and migration of renal cancer cell lines by suppressing the expression of Ras-related

protein Rap-1b (*RAP1B*) (9); the expression of miR-28-5p is associated with the metastasis, recurrence and the poor prognosis of liver cancer (8). The overexpression of miR-28-5p in HCT116, RKO and SW480 cells has been shown to reduce cell migration and invasion (10). In addition, miR-28-5p overexpression has been shown to suppress nasopharyngeal cancer cell proliferation and induce cell cycle arrest and apoptosis (11). In the present study, bioinformatics analysis using the Human MicroRNA Expression Database (HMED) also revealed that miR-28-5p was expressed at a low level in breast cancer. However, the function of miR-28-5p in breast cancer cell metastasis, and the target genes of miR-28-5p in breast cancer are poorly understood.

The present study thus aimed to investigate the role of miR-28-5p in breast cancer migration, identify the target genes of miR-28-5p, and elucidate the molecular mechanisms through which miR-28-5p regulates its target genes. The results of the present study may enhance our understanding of the role of miR-218-5p in the development of breast cancer.

Materials and methods

Plasmid construction. The human WD repeat and SOCS box containing 2 (WSB2) coding sequence (CDS) (NM_018639.5) was amplified from MCF-7 cell cDNA using PCR with the following primers: Forward, 5'-TTCAAGCTTATGGAGGCC GGAGAGGAA-3' and reverse, 5'-TTAGGATCCTTAAAA AGTCCTGTATGTG-3'. The PCR fragments were recovered, digested with *Hin*dIII III (AAGCTT; indicated by underlined text above) and *Bam*HI (GGATCC; indicated by underlined text above), and then cloned into the pEGFP-C3 vector (BD Biosciences).

The 3'-UTR of *WSB2* was amplified from MCF-7 cell cDNA by PCR with the following primers: Forward, 5'-TCG CTCGAGATGACTATTCAGATGGCTAC-3' and reverse, 5'-AGAGCGGCCGCCTCCATAAAGCACCGATT-3'. The PCR fragments were recovered, digested with *Xho*I (CTCGAG; indicated by underlined text above) and *Not*I (GCGGCCGCC; indicated by underlined text above), and then cloned into the psiCHECK™-2 vector (Promega Corporation).

WSB2-3'-UTR target site-directed mutagenesis was performed using a Quik Change Site-Directed Mutagenesis kit (Stratagene; Agilent Technologies, Inc.) with the forward primer, 5'-TTCAACTCTACTGCGAAACAAAAATAACCC ATTAAAAGTACTGTTCTCCTTCAGTG-3' and the reverse primer, 5'-CACTGAAGGAGAACAGTACTTTTAATGGGT TATTTTTGTTTCGCAGTAGAGTTGAA-3'. Underlined base pairs indicate mutation sites.

Cells and cell culture. Human mammary epithelial cells (HMECs), and the T-47D, ZR-75-30, MDA-MB-231 and MCF-7 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM)-high glucose medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences), 100 U/ml penicillin and streptomycin, and incubated at 37˚C with 5% CO_2.

Cell transfection. The MCF-7 cells were transfected with the plasmids using Lipofectamine™ 2000 (Invitrogen, Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions.

The MCF-7 cells were seeded at a density of $1x10^4$ cells/well in 96-well plates, and transfected with 40 nM of miR-28-5p mimics, the miR-28-5p inhibitor, or controls (Shanghai GenePharma Co., Ltd.) for the migration assay. The cells were co-transfected with 40 nM of miR-28-5p mimics, or control, with 100 ng/well pEGFP-C3-WSB2 or pEGFP-C3 for the migration assay. The cells were co-transfected with 100 ng/well of WSB2 3'UTR-wt or WSB2 3'UTR-mut and 40 nM of miR-28-5p mimics, the miR-28-5p inhibitor, and controls for a dual-luciferase reporter (DLR) assay.

The MCF-7 cells were seeded at a density of $7x10^4$ cells/well in 24-well plates. The cells were transfected with 200 ng/well pEGFP-C3-WSB2, or pEGFP-C3 for subcellular localization and western blot analysis. The cells transfected with 40 nM of miR-28-5p mimics, the miR-28-5p inhibitor, or controls for reverse transcription-quantitative PCR (RT-qPCR) and western blotting. The cells were transfected with 20, 40 or 60 nM of miR-28-5p mimics and control for RT-qPCR. The cells were transfected with 40 nM of miR-28-5p mimics and control for gene chip assay.

RT-qPCR. Total RNA was extracted, purified and used for first-strand cDNA synthesis; RT-qPCR reagents and procedures were as previously described (12). Quantification was performed using the $2^{-\Delta\Delta Cq}$ method (13).

The specific product of human miR-28-5p was amplified by RT-qPCR using the following primers: miR-28-5p forward, 5'-GCGCATTGCACTTGTCTCG-3' and reverse, 5'-AGTGCA GGGTCCGAGGTATT-3'; and U6 forward, 5'-CTCGCTTCG GCAGCACATA-3' and reverse, 5'-CGAATTTGCGTGTCA TCCT-3'. U6 was used as an internal control.

MCF-7 cells were transfected as aforementioned and at 12, 24 and 48 h following transfection, total RNA was extracted. The specific products of human *RAP1B*, *WSB2* and *VEGFA* were amplified by RT-qPCR using the following primers: *RAP1B* forward, 5'-AAGAAAGTCCAAAG-3' and reverse, 5'-TTTCCTTCAACA-3'; *WSB2* forward, 5'-GTTAATTCG GAAGCTAGAGG-3' and reverse, 5'-CAAAGCCCATTG GTCATA-3'; *VEGFA* forward, 5'-ATTGGAGCCTTGCCT TGC-3' and reverse, 5'-TCCACCAGGGTCTCGATTG-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-TGACTTCAACAGCGACACCCA-3' and reverse, 5'-CACCCTGTTGCTGTAGCCAAA-3'. *GAPDH* was used as an internal control.

Cell migration assay. The MCF-7 cells were transfected as described above. After 24 h, a cell migration assay was performed as previously described using Transwell chambers (14). After 16 h, the cells passing the membrane were stained using 0.25% crystal violet (Sigma-Aldrich) at 37˚C for 10 min, and the crystal violet-stained cells were washed off using 33% acetic acid and measured on a spectrophotometer (Infinite M200; Tecan Group, Ltd.) at 570 nm.

DLR assay. MCF-7 cells were transfected as described above. After 24 h, DLR assay was performed using the DLR Assay system (Promega Corp.; Turner BioSystems, Inc.) according to the manufacturer's protocol. Luciferase activity was

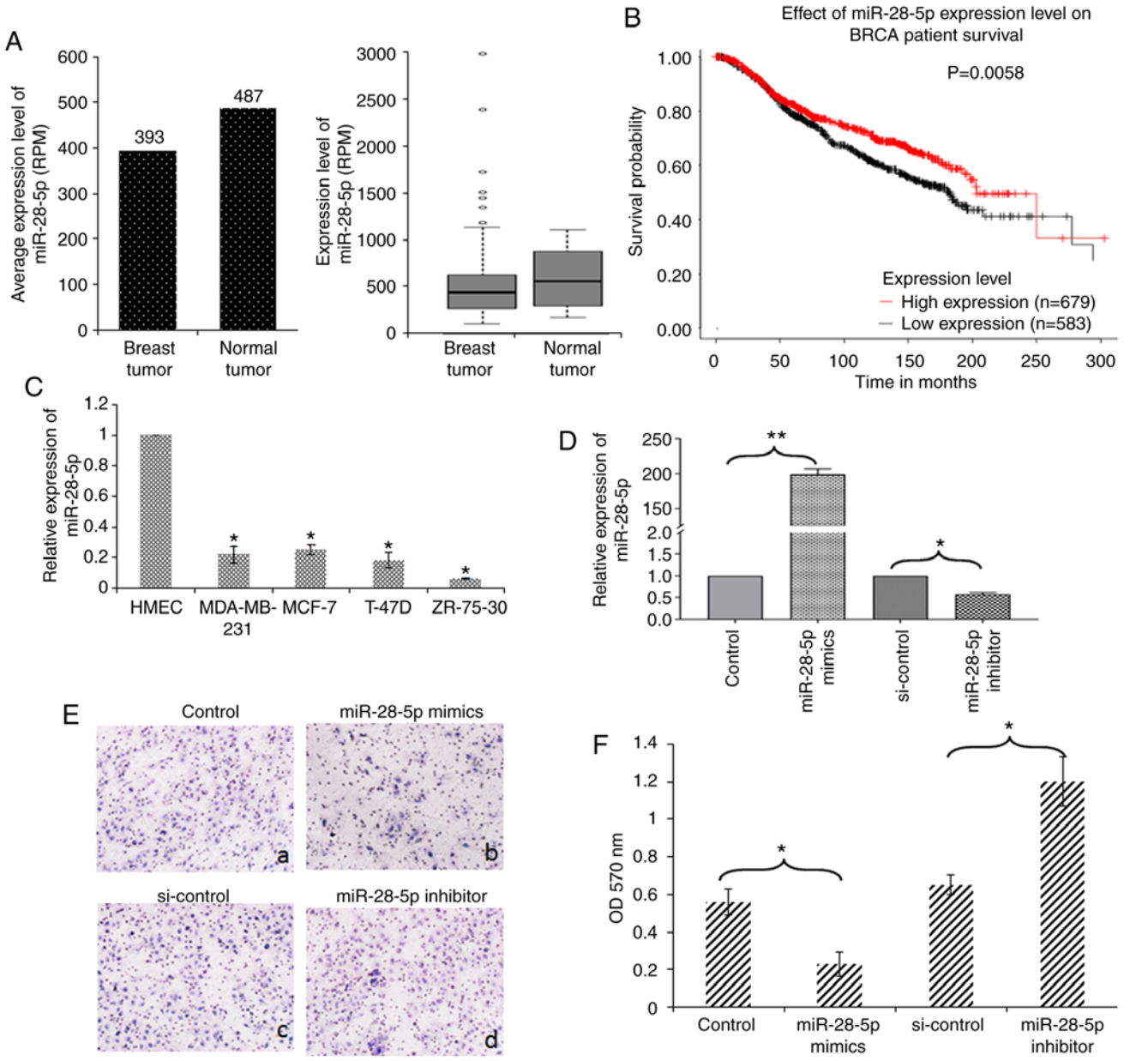

Figure 1. miR-28-5p inhibits the migration of MCF-7 cells. (A) miR-28-5p expression in breast tumor and normal breast tissue was analyzed using HMED. (B) Kaplan-Meier analysis of the effect of the miR-28-5p expression level on BRCA patient survival was analyzed using KMPLOT. (C) The expression levels of miR-28-5p in ZR-75-30, MDA-MB-231, T-47D, MCF-7 cells and HMECs were determined by RT-qPCR. *P<0.05 vs. HMECs. (D) MCF-7 cells were transfected with control, miR-28-5p mimics, si-control, or the miR-28-5p inhibitor, and the expression levels of miR-28-5p were determined using RT-qPCR. *P<0.05, **P<0.01 vs. the respective control. (E and F) MCF-7 cells were transfected with (a) control, (b) miR-28-5p mimics, (c) si-control, or (d) miR-28-5p inhibitor and subjected to Transwell assays. After 16 h, the cells passing the membrane were dyed using 0.25% crystal violet and assessed spectrophotometrically. *P<0.05 vs. the respective control.

normalized to *Renilla* luciferase activity. Experiments were performed in triplicate.

Subcellular localization. The MCF-7 cells were transfected as described above. At 24 h following transfection, the cell nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI) for 30 min at 37˚C and visualized under an inverted fluorescence microscope (TE 2000-U; Nikon Corporation), as previously described (15).

Western blot analysis. The MCF-7 cells were transfected as described above. At 24 h post-transfection, western blot analysis was performed as previously described (16). Anti-green fluorescent protein (GFP) antibody (SC8334; Santa Cruz Biotechnology, Inc.; 1:1,000 dilution), anti-WSB2 antibody (ab127176; Abcam; 1:1,000 dilution), anti-β-actin antibody (ab8227; Abcam; 1:10,000 dilution), and a secondary antibody (goat anti-rabbit IgG H&L) conjugated to horseradish peroxidase (HRP) (ab6721, Abcam, 1:10,000 dilution) were used. All antibodies were incubated with the membranes for 90 min at 37˚C.

Gene chip assay. The MCF-7 cells were transfected as described above. At 24 h post-transfection, RNA was extracted using TRIzol (15596-018; Thermo Fisher Scientific, Inc.), purified using an RNeasy mini kit (74106; Qiagen GmbH), amplified and labeled using the low input quick amp labeling

Table I. Genes regulated by miR-28-5p.

Entrez	Symbol	Description	Fold change miR-28 vs. control
7422	*VEGFA*	Vascular endothelial growth factor A	2.0758
5908	*RAP1B*	RAP1B, member of RAS oncogene family	-1.7277
55884	*WSB2*	WD repeat and SOCS box containing 2	-1.5569

kit (5190-2305; Agilent Technologies, Inc.); each slide was then hybridized, scanned; and data were extracted, and normalized as previously described (12,17). Two samples were used for gene chip assay. Differentially expressed mRNAs were identified as |log2(fold change)|≥1.5. The Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg, release number 91.0; July, 2019) pathway analysis was used to characterize the differentially expressed genes (DEGs).

Bioinformatics analysis. miR-28-5p expression in breast tumor and normal breast tissue was analyzed using HMED (http://bioinfo.life.hust.edu.cn/web/GEDS/) (18). The target gene of miR-28-5p was predicted using TargetScan7.1 (http://www.targetscan.org/), PicTar (http://pictar.mdc-berlin.de/) and miRDB (http://www.mirdb.org/) software. The association between miR-28-5p and WSB2 was analyzed using bc-GenExMiner v4.4 (http://bcgenex.centregauducheau.fr/BC-GEM/GEM-requete.php). The expression of *WSB2* in breast invasive carcinoma (BRCA) based on The Cancer Genome Atlas (TCGA) samples, was examined through UALCAN (http://ualcan.path.uab.edu/cgi-bin/TCGAExResultNew2.pl) (19). KMPLOT analysis of the association of WSB2 with BRCA patient survival was also examined through UALCAN (19). KMPLOT analysis of the association of miR-28-5p with BRCA patient survival was based on the Molecular Taxonomy Of Breast Cancer International Consortium (METABRIC) (http://kmplot.com/analysis/index.php?p=service&cancer=breast_mirna) (20).

Immunohistochemical analysis. A microarray containing samples from 40 cases of breast cancer and matching adjacent normal tissue was obtained (BR804a, Avilabio). Anti-WSB2 antibody (ab187987; Abcam, 1:50 dilution) was applied, and immunohistochemical analysis was performed as previously described (14). The results of staining were analyzed using ImageJ software version 1.46.

Statistical analysis. SPSS 9.0 software (SPSS, Inc.) was used for statistical analysis. Data were analyzed using the Student's t-test, and one-way analysis of variance followed by a Dunnett's test or Tukey's post hoc test. Pearson's correlation analysis was used to investigate the correlation between miR-28-5p and WSB2 expression. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

miR-28-5p inhibits the migration of MCF-7 cells. miR-28-5p expression in breast tumor and normal breast tissue was analyzed using HMED (18). miR-28-5p exhibited a lower expression in breast cancer tissue than in normal tissue (Fig. 1A). Kaplan-Meier analysis revealed that a low expression level of miR-28-5p was associated with a poor survival in breast cancer (Fig. 1B). Subsequently, RT-qPCR was performed to examine the expression levels of miR-28-5p in 4 human breast cancer cell lines, and HMECs. As shown in Fig. 1C, the expression of miR-28-5p was significantly lower in the 4 human breast cancer cell lines (ZR-75-30, MDA-MB-231, T-47D and MCF-7) compared with that in HMECs, suggesting that the decreased expression of miR-28-5p is associated with the development of breast cancer. The expression level of miR-28-5p in MCF-7 cells was higher than that in the other 3 breast cancer cell lines (Fig. 1C). Furthermore, the effect of miR-28-5p on the migration of MCF-7 cells was examined by a Transwell chamber assay. MCF-7 cells were transfected with miR-28-5p mimics, miR-28-5p inhibitor, or the respective controls. The expression level of miR-28-5p was confirmed by RT-qPCR (Fig. 1D). It was found that the MCF-7 cells transfected with miR-28-5p mimics exhibited a decrease in cell migration compared with that in the control, whereas transfection with miR-28-5p inhibitor resulted in an increased mobility of the MCF-7 cells compared with that in the control (Fig. 1E). The cells passing through the membrane were further quantified using crystal violet staining (Fig. 1F).

Target genes regulated by miR-28-5p. miRNAs regulate gene expression by binding to the 3'UTRs of their target mRNAs (6). In the present study, to determine the target genes of miR-28-5p, gene chip analysis we used to identify the DEGs between MCF-7 cells overexpressing miR-28-5p and the negative control miRNA. The overexpression of miR-28-5p was confirmed by RT-qPCR (Fig. 1D). The results of gene chip assay revealed that 648 genes were differentially expressed, including 283 upregulated genes and 365 downregulated genes in MCF-7 cells overexpressing miR-28-5p (Table SI). The DEGs were analyzed using KEGG pathway analysis. The top 10 significantly enriched pathways are shown in Fig. 2A. *RAP1B* and *VEGFA* are involved in the 'focal adhesion' and 'pathway in cancer' pathways, respectively. *WSB2* is an unannotated gene in KEGG, but was predicted as a target gene of miR-28-5p by TargetScan7.1, PicTar, and miRDB software. The alteration in the expression levels of *RAP1B*, *VEGFA* and *WSB2* was observed in breast cancer (Table I). RT-qPCR was performed to experimentally validate these findings (Fig. 2B-D). The results indicated that *RAP1B*, *VEGFA* and *WSB2* were regulated by miR-28-5p.

miR-28-5p targets the 3'UTR of WSB2 mRNA. To examine the effects of miR-28-5p on the expression of its target genes, MCF-7 cells were transfected with 20, 40 and 60 nM miR-28-5p mimics for 24 h. RT-qPCR analysis revealed that transfection

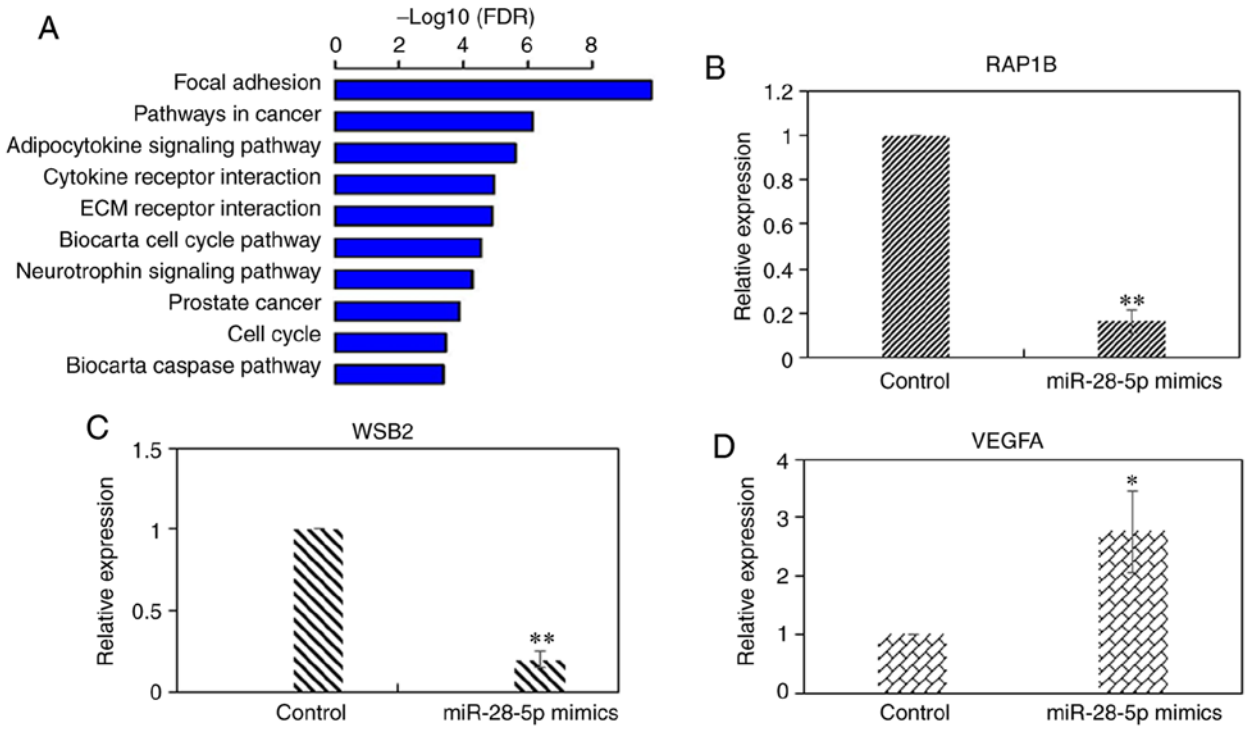

Figure 2. Target genes regulated by miR-28-5p. (A) Top 10 pathways from KEGG enrichment analysis of DEGs regulated by miR-28-5p mimics. MCF-7 cells were transfected with miR-28-5p mimics, and the expression levels of (B) *RAP1B*, (C) *WSB2*, and (D) *VEGFA* were examined by RT-qPCR. *P<0.05, **P<0.01 vs. the control.

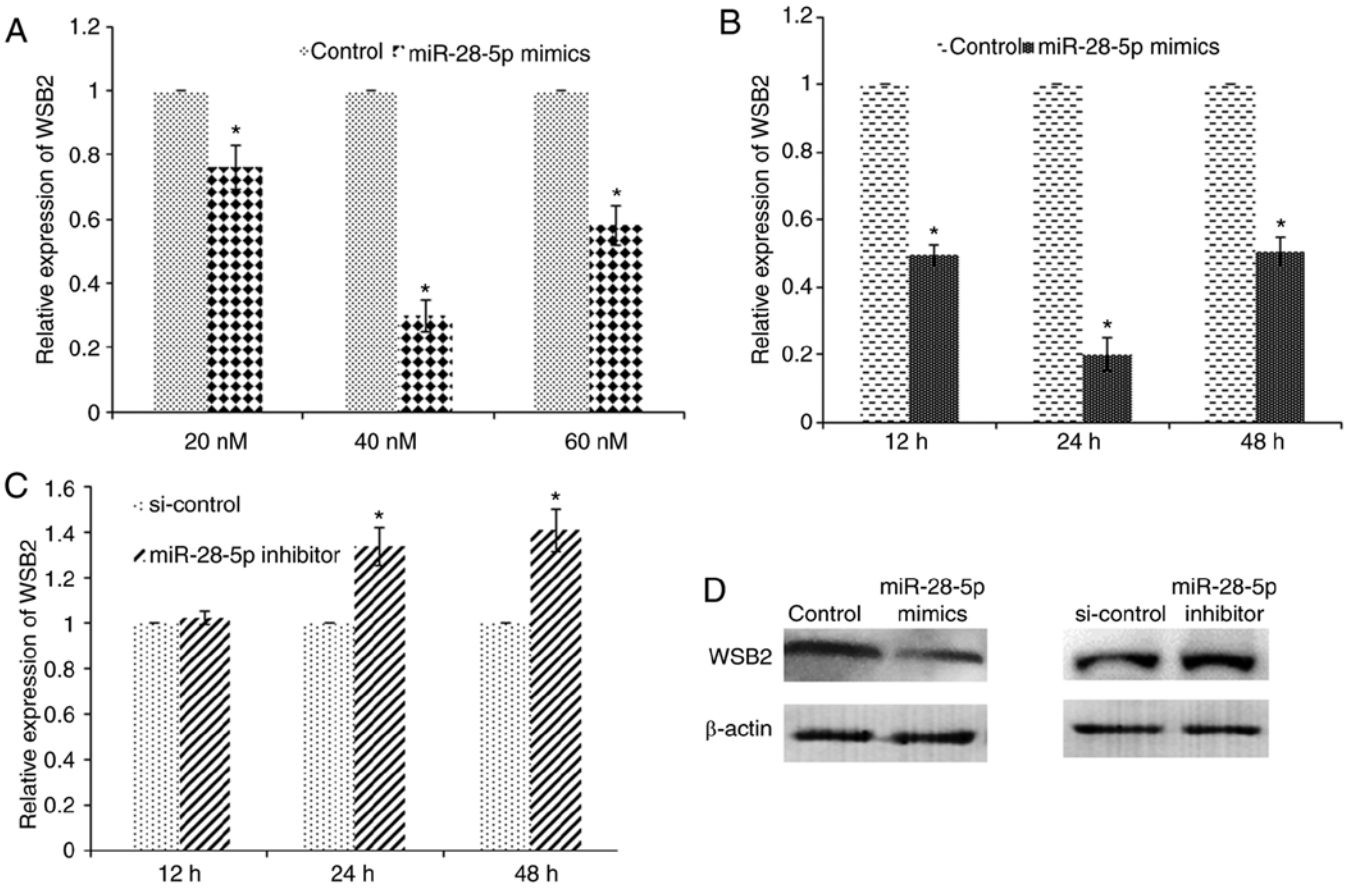

Figure 3. miR-28-5p inhibits *WSB2* expression. (A) MCF-7 cells were transiently transfected with miR-28-5p mimics for 24 h. *WSB2* mRNA downregulation was verified by RT-qPCR. *P<0.05 vs. the control. (B) MCF-7 cells were transfected with miR-28-5p mimics for 12, 24 and 48 h. *WSB2* mRNA downregulation was verified by RT-qPCR at the indicated time-points. *P<0.05 vs. the control. (C) MCF-7 cells were transfected with the miR-28-5p inhibitor for 24 and 48 h. *WSB2* mRNA expression was verified by RT-qPCR. *P<0.05 vs. the control. (D) WSB2 protein levels were determined by western blot analysis.

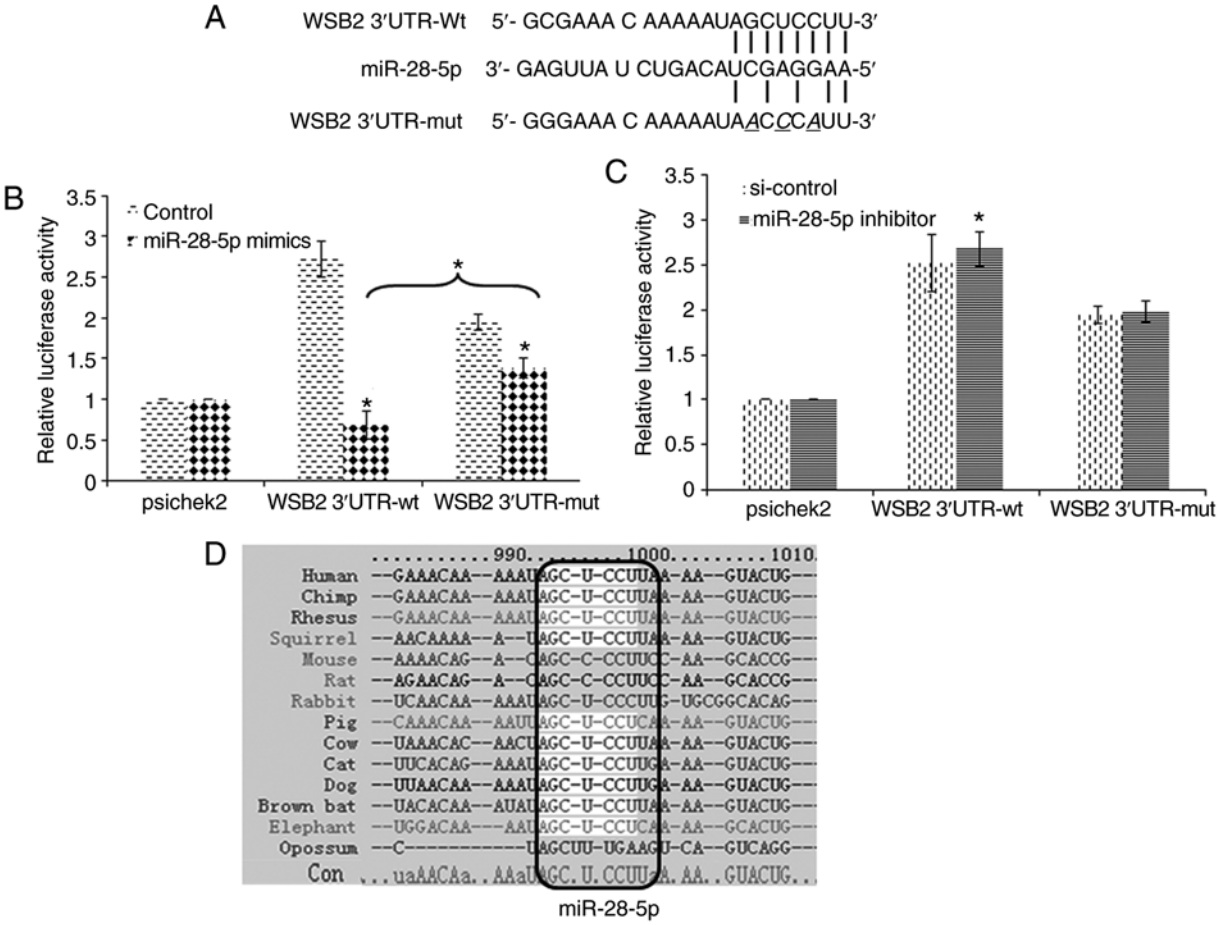

Figure 4. miR-28-5p targets the 3'-UTR of *WSB2* mRNA. (A) The miR-28-5p binding site in the predicted target sequences of the 3'-UTR of *WSB2* is indicated. (B and C) MCF-7 cells were co-transfected with WSB2-3'UTR-WT or WSB2-3'UTR-Mut and miR-28-5p mimics or the miR-28-5p inhibitor. The luciferase activity of the extracts was determined 24 h following transfection. Luciferase values were normalized to *Renilla* activity. *P<0.05. (D) The miR-28-5p binding site in the 3'-UTR of WSB2 is conserved in multiple species.

with miR-28-5p mimics led to the downregulation of *WSB2* expression in a dose-dependent manner (Fig. 3A) Furthermore, MCF-7 cells were transfected with 40 nM miR-28-5p mimics, and *WSB2* mRNA expression was measured at 12, 24 and 48 h post-transfection. RT-qPCR analysis revealed that transfection with miR-28-5p mimics led to the downregulation of *WSB2* expression in a time-dependent manner (Fig. 3B). miR-28-5p mimics led to a 2-fold decrease in *WSB2* mRNA expression at 12 h, and a 5-fold decrease at 24 h, compared with the control, respectively (Fig. 3B). By contrast, transfection with miR-28-5p inhibitor increased the expression of *WSB2* (Fig. 3C). At the protein level, the level of WSB2 decreased following transfection with miR-28-5p mimics, and increased following transfection with miR-28-5p inhibitor (Fig. 3D). The analysis of the correlation between miR-28-5p and WSB2 revealed that there was a weak negative correlation between miR-28-5p and WSB2 (R=-0.35) in triple-negative breast cancer (TNBC) (Fig. S1).

The binding sites for miR-28-5p were predicted in the 3'UTR of *WSB2*. We cloned 3'UTR of the *WSB2* gene into vector psiCheck-2 for a luciferase reporter assay (Fig. 4A). The luciferase reporter activity of the wild-type 3'UTR of *WSB2* decreased upon transfection with miR-28-5p mimics (Fig. 4B). By contrast, transfection with miR-28-5p inhibitor led to an increase in reporter activity (Fig. 4C). Furthermore, site-directed mutagenesis of the 3'UTR of *WSB2* was carried out, and the luciferase reporter activity of the mutant 3'UTR (WSB2-3'UTR-mut) was assessed (Fig. 4A). The regulatory effect of miR-28-5p mimics was significantly weakened in the mutant reporter, although the luciferase reporter activity of WSB2-3'UTR-mut decreased upon transfection with miR-28-5p mimics (Fig. 4B). The regulatory effect of miR-28-5p inhibitor was abolished in the mutant reporter (Fig. 4C). The 3'UTR of *WSB2* of human, chimp, rhesus, squirrel, mouse, rat, rabbit, pig, cat, dog, brown bat, elephant and opossum was analyzed using TargetScan7.1 to predict miR-28-5p binding sites. The binding site is conserved in multiple species, and the core consensus sequence is 5'-AGCUCCUU-3' (Fig. 4D). These results indicated that *WSB2* is the direct target gene of miR-28-5p, and this mechanism may be conserved in other species.

Overexpression of WSB2 attenuates the inhibitory effects on MCF-7 cell migration induced by miR-28-5p. The effect of WSB2 on the migration of MCF-7 cells was assessed using a migration assay. MCF-7 cells were transfected with empty pEGFP-C3 vector or pEGFP-C3-WSB2 that overexpresses *WSB2*. The overexpression of *WSB2* mRNA was confirmed by RT-qPCR (Fig. 5A). Western blot analysis revealed that pEGFP-C3-WSB2 expressed a 72-kDa WSB2-GFP fusion protein (Fig. 5B), which was primarily localized in the

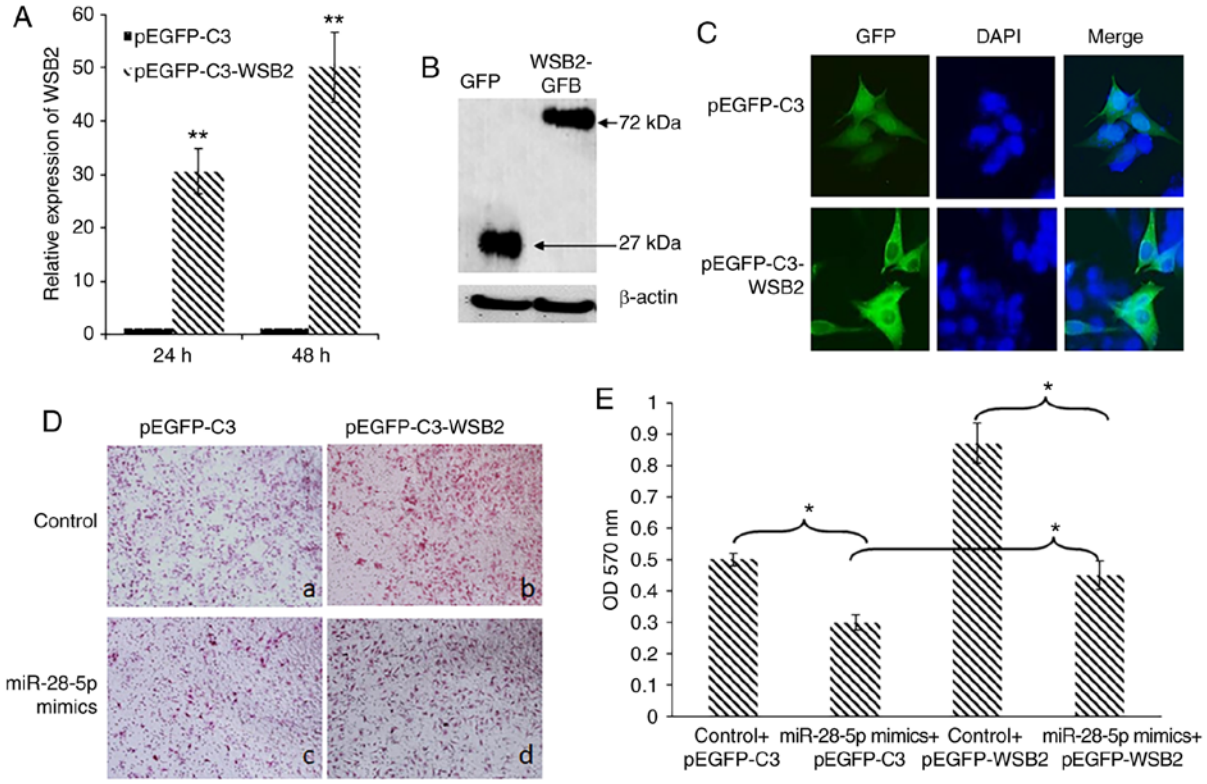

Figure 5. Overexpression of WSB2 attenuates the inhibitory effects of miR-28-5p on MCF-7 cell migration. MCF-7 cells were transfected with pEGFP-C3, or pEGFP-C3-WSB2. (A) mRNA and (B) protein levels of WSB2 were detected by RT-qPCR and western blot analysis, respectively. **P<0.01 vs. pEGFP-C3. (C) WSB2-GFP and DAPI staining were visualized by fluorescence microscopy (x10 magnification). (D) MCF-7 cells were co-transfected with miR-28-5p mimics or control, with pEGFP-C3-WSB2 or pEGFP-C3, and subjected to a Transwell assay. After 16 h, the cells passing the membrane were dyed using 0.25% crystal violet. (E) Crystal violet staining was assessed spectrophotometrically. *P<0.05.

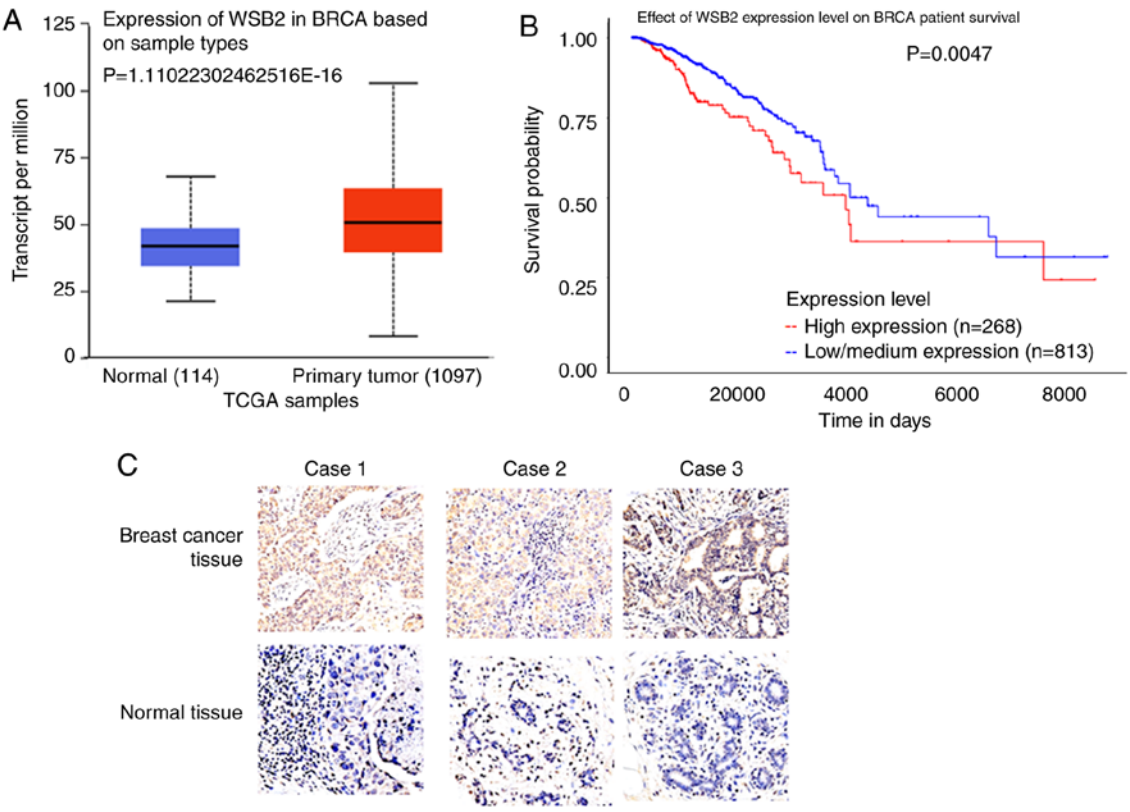

Figure 6. Expression of WSB2 in breast tissues. (A) WSB2 expression in BRCA was analyzed using UALCAN. (B) Kaplan-Meier analysis of the effect of the WSB2 expression level on BRCA patient survival was analyzed using UALCAN. (C) Representative images of immunohistochemistry of WSB2 in breast cancer and cancer-adjacent normal breast tissue from three cases. Magnification, x400.

Table II. Expression of WSB2 in breast cancer and normal breast tissues specimens.

Specimens	Number	Optical density	Integrated optical density
Breast cancer	40	0.018±0.001[a]	87±8.51[a]
Normal	40	0.01±0.001	49±5.02

Data are presented as the means ± SD; [a]P<0.05.

cytoplasm (Fig. 5C). Migration assays revealed that MCF-7 cells transfected with miR-28-5p mimics exhibited a decrease in migration compared with the control (Fig. 5D, panels a and c), which was attenuated by the overexpression of *WSB2* (Fig. 5D, panels b and d). Crystal violet staining further confirmed that *WSB2* overexpression attenuated the inhibitory effects of miR-28-5p on MCF-7 cell migration (Fig. 5E). These findings support the view that *WSB2* is a target of miR-28-5p, contributing to a better understanding of the mechanism through which miR-28-5p inhibits the migration of MCF-7 cells.

Expression level of WSB2 in breast cancer tissues. UALCAN analysis revealed that *WSB2* was significantly upregulated in primary breast tumor tissues, compared with that in normal tissues (Fig. 6A). Kaplan-Meier analysis revealed that a high expression level of WSB2 was associated with a poor survival in breast cancer (Fig. 6B). Furthermore, immunohistochemistry revealed that the integrated optical density of WSB2 was significantly higher in the malignant breast tumor tissue compared with that in the adjacent normal breast tissue (Fig. 6C and Table II). These results indicate that WSB2 promotes the malignancy of breast cancer cells.

Discussion

miRNAs regulate tumor development by modulating gene expression. The downregulation of miR-28-5p is involved in the development and progression of several types of human cancer (8-11,21). The present study demonstrated that miR-28-5p, which has a lower expression in breast cancer tissue and breast cancer cell lines, inhibited the migration of breast cancer cells. These data support the view that miR-28-5p functions as a tumor suppressor miRNA.

miRNAs regulate gene expression by binding to the 3'UTRs of their target mRNAs, and a single miRNA can regulate the expression of multiple genes. Studies have demonstrated that *LPP* (7), *RAP1B* (9), *NRF2* (22) and *ZEB1* (23) are direct target genes of miR-28-5p. The present study revealed that *RAP1B* was downregulated by miR-28-5p, which was consistent with the findings of a previous study (9). The present study demonstrated that *WSB2* was a previously unknown target gene of miR-28-5p, which targets the 3'UTR of *WSB2*. However, the mechanisms through which miR-28-5p inhibits *WSB2* require further investigation. Uncovering the miR-28-5p regulatory network is a challenging task that requires large-scale methods to identify miRNA targets.

WSB2 contains WD repeats and a SOCS box, which mediate intracellular signal transduction (24). Its homolog, WSB1, has been reported as an important regulator of aggressive metastasis in hormone receptor-negative breast cancer (25). It has been demonstrated that the expression of WSB2 is higher in human melanoma tissues; consistently, cell cycle progression and migration of A375 and G361 melanoma cells were shown to be significantly inhibited by *WSB2* knockdown (26). The present study demonstrated that WSB2 was upregulated in the breast cancer tissue, which was associated with a poor survival in breast cancer. Moreover, WSB2 promotes the migration of MCF-7 cells, which was consistent with previous findings; for example, WSB2 exerts a negative regulatory effect on IL-21R expression and signal transduction (27); the knockdown of *WSB2* decreased the levels of c-Myc, β-catenin, p-Rb, CDK4 and Cyclin D3 in G361 melanoma cells (26); *WSB1* knockdown has been shown to be associated with decreased matrix metalloproteinase (MMP) activity (25). However, the mechanisms through which WSB2 regulates breast cancer progression warrant further investigation.

In conclusion, the present study demonstrated that miR-28-5p exhibits a low expression in breast cancer tissues and breast cancer cell lines, and it inhibits the migration of breast cancer cells by regulating *WSB2* expression. *WSB2* mRNA is the direct target of miR-28-5p, containing an evolutionarily conserved binding site for miR-28-5p in its 3'UTR. WSB2 is highly expressed in breast cancer tissues, and its overexpression attenuates the inhibitory effects of miR-28-5p on MCF-7 cell migration, supporting the view that WSB2 functions downstream of miR-28-5p. In the future, this miR-28-5p/WSB2 axis may become a novel therapeutic target in breast cancer.

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Availability of data and materials

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

Authors' contributions

LM performed the RT-qPCR and western blot analyses. YZ and FH analyzed the gene chip data, and performed the immunohistochemical analysis. FH was a major contributor to the writing of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Patient data used in the present study were obtained from a microarray, as well as TCGA. Patient survival was analyzed with KMPLOT through UALCAN.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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