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miR-144-3p Targets FosB Proto-oncogene, AP-1 Transcription Factor Subunit (FOSB) to Suppress Proliferation, Migration, and Invasion of PANC-1 Pancreatic Cancer Cells

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This study aimed to investigate the role of miR-144-3p in pancreatic cancer (PC) carcinogenesis and to explore the mechanism of its function in PC. miR-144-3p was downregulated in PC tissues and cells. miR-144-3p overexpression significantly inhibited PC cell proliferation, migration, and invasion. FosB proto-oncogene, AP-1 transcription factor subunit (FOSB) was a target gene of miR-144-3p. miR-144-3p could repress PC cell proliferation, migration, and invasion by inhibiting the expression of FOSB. In conclusion, miR-144-3p plays an important role in PC cell proliferation, migration, and invasion by targeting FOSB. miR-144-3p may provide a new target for the development of therapeutic agents against PC.

Key words: Pancreatic cancer (PC); miR-144-3p; FosB proto-oncogene, AP-1 transcription factor subunit (FOSB); Cell proliferation; Cell migration; Cell invasion

INTRODUCTION

Pancreatic cancer (PC) is an aggressive malignancy and is one of the most lethal among all cancers, leading to an estimated 227,000 deaths per year worldwide[1]. PC has poor prognosis, with a 5-year survival rate of only 5% for all stages combined[2]. The high mortality of PC is partly due to the invasive characteristics of PC cells during the early stages of carcinogenesis[3]. PC is usually diagnosed in an advanced state, and conventional chemotherapy is rarely curative. Therefore, understanding the carcinogenic mechanism of PC is indispensable for developing effective therapeutics.

MicroRNAs (miRNAs) are small, endogenous, noncoding RNAs that lead to mRNA degradation by binding with complementary sequences in the 3′-untranslated region (3′-UTR) of their target mRNAs[4]. It has been reported that miRNA regulation of gene expression plays an important role in cell proliferation and apoptosis and in tissue differentiation[5,6]. The aberrant regulation of specific miRNAs and their targets is associated with the development and progression of various human cancers[7]. More importantly, some miRNAs have been found to be aberrantly expressed in PC carcinogenesis, such as miR-96, miR-141, and miR-196a[8–11]. A recent study predicted that miR-144-3p was abnormally expressed in PC tissue[12], but the underlying molecular mechanism remained unclear.

The present study aimed to investigate the role of miR-144-3p in PC carcinogenesis and to explore the mechanism of its function in PC. With this purpose, we detected the expression of miR-144-3p on PC tissues and cell lines and then investigated the effects of its expression on proliferation, migration, and invasion. Furthermore, we investigated the target gene of miR-144-3p as well as studied the role of target in PC.

MATERIALS AND METHODS

Cell Line and Tissue Samples

Human PC cell lines PANC-1, SW-1990, and BxPC-3 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 mg/ml streptomycin in a 37°C incubator with 5% CO_2. Tissue samples were obtained from patients undergoing pancreatectomy or palliative surgery including implantation of ^{125}I seeds as well as choledochojejunostomy and gastroenterostomy, according to the National Comprehensive Cancer

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Network guideline for PC. The tissue samples were immediately snap frozen and stored at −80°C. All patients gave informed consent, and the study was approved by the local research ethics committee.

Cell Transfection

The synthetic, chemically modified short single- or double-stranded RNA oligonucleotides including anti-miR-144-3p and its appropriate negative control (anti-miR-NC), as well as miR-144-3p mimics and its appropriate negative control (miR-NC), were purchased from GenePharma (Shanghai, P.R. China). FosB proto-oncogene, AP-1 transcription factor subunit (FOSB)-siRNA (siFOSB) and its appropriate negative control (si-NC) were also purchased from GeneChem. For transfection, cells were seeded into a six-well plate and incubated overnight. Cell transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The expression levels were quantified after 24 h of transfection.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA in cells or tissues was extracted using TRIzol reagent (Invitrogen). Real-time (RT) reactions were performed using miR-144 special primer, and the specific stem-loop RT primers for miR-144 were designed according to a previous study[13]. Real-time PCR was performed based on the SYBR Green PCR Kit (Toyobo, Osaka, Japan). β-Actin and U6 were used as references for mRNAs and miRNAs, respectively. ΔCt values were normalized to U6 levels. The $2^{-\Delta\Delta}$Ct method was used to determine the relative gene expression levels. Primers used for target amplification were FOSB, 5′-CGGAATTCGAGCAGCGCACTTGGAGACG-3′ (forward) and 5′-CTGGATCCTCCCCTCTCCTCCTCATCTT-3′ (reverse); miR-144, 5′-GCTGGGATATCATCATATACTG-3′ (forward) and 5′-CGGACTAGTACATCATCTATACTG-3′ (reverse). Each sample was analyzed in triplicate.

Western Blot Analysis

Total protein was extracted from cells with lysis buffer after 72 h of transfection. Lysates were denatured with sodium dodecyl sulfonate (SDS) sample buffer at 100°C for 10 min and separated in 10% polyacrylamide gels, and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% nonfat milk powder in Tris-buffered saline containing 0.1% Tween 20 (TBST) and then probed with primary antibodies [FOSB, cyclooxygenase 2 (COX2), vascular endothelial growth factor (VEGF), matrix metallopeptidase 2 (MMP2), and MMP9; 1:1,000] overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase-labeled secondary antibodies (1:1,000). All antibodies were purchased from Invitrogen. Band signals were visualized using enhanced chemiluminescence (Pierce, Bradenton, FL, USA), and the band density was evaluated by Bio-Rad Quantity One software.

MTT Assay

Cells were collected, seeded into 96-well plates (3,000/well), and incubated at 37°C for 1 to 3 days after transfection. After incubation, 20 ml of MTT solution (5 mg/ml) was added to the medium of each well and incubated for another 4 h at 37°C. Afterward, the medium was replaced with 150 ml of dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA), and the absorbance was measured at 570 nm under an absorption spectrophotometer (Olympus, Tokyo, Japan). The MTT assay was repeated three times.

Colony Formation Assay

After 24 h of transfection, cells were seeded into a six-well plate (500/well) and incubated for 10 days. Medium was replaced with fresh medium on the fifth day. After incubation, cells were washed with PBS, fixed with 4% paraformaldehyde, and stained with 0.1% crystal violet. The colony formation assay was repeated three times.

Cell Migration and Invasion Assay

Cell invasion detection was evaluated using Transwell migration chambers (8-mm pore size; Millipore) precoated with a layer of Matrigel (excreta cellular matrix gel; Sigma-Aldrich). Cells were seeded in the upper chamber with 3×10^4 cells per well. The lower chamber contained 700 ml of medium with 30% FBS, which served as chemotaxin. After 48 h of incubation at 37°C, noninvaded cells on the upper membrane were removed with cotton swabs, and the invaded cells were washed with PBS, fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and then counted using light microscopy.

Construction of Reporter Plasmids and Luciferase Reporter Assay

FOSB 3′-UTR containing wild-type (wt) or mutated predictive miR-144 binding site was subcloned into pmirGlo Dual-luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) located 5′ to the firefly luciferase. The nucleotide sequences of the constructed plasmids were confirmed by DNA sequencing. For luciferase reporter assays, cells were seeded in a 96-well plate and cotransfected with the pmirGlo-FOSB 3′-UTR construct and miR-144-3p mimics or miR-NC. After 48 h of transfection, assays were performed using the Dual-Luciferase Reporter Assay System (Promega).

The firefly luciferase signals were normalized to the *Renilla* luciferase signals.

Statistical Analysis

All data are expressed as mean±standard deviation (SD) from at least three separate experiments, computed using the SPSS 16.0 software (SPSS, Chicago, IL, USA). The differences between groups were analyzed using Student's *t*-test and among groups were analyzed using a one-way ANOVA. Difference was deemed statistically significant at a value of $p<0.05$.

RESULTS

miR-144-3p Was Downregulated in PC Tissues and Cell Lines

The miR-144-3p expression in 10 pairs of PC tissues and their adjacent noncancerous tissues was examined by qRT-PCR. As shown in Figure 1A, the expression of miR-144-3p was significantly lower in PC tissues than in noncancerous tissues ($p<0.01$). In addition, the miR-144-3p expression in three PC cell lines was also detected by qRT-PCR. The result showed that miR-144-3p had the lowest expression in PANC-1 cells and had the highest expression in BxPC-3 (Fig. 1B). These results suggested that miR-144-3p may be associated with PC progression.

Effect of miR-144-3p on PC Cell Proliferation, Migration, and Invasion

To investigate the role of miR-144-3p in PC cells, we transfected PANC-1 cells with miR-144-3p mimic to upregulate the miR-144-3p expression. A significant increase in miR-144-3p expression was found after transfection ($p<0.01$) (Fig. 2A). MTT assay showed that the viability of PANC-1 cells was significantly repressed after 48 or 72 h of miR-144-3p mimic transfection ($p<0.05$) (Fig. 2B). Additionally, the ability of colony formation significantly decreased after overexpression of miR-144-3p ($p<0.01$) (Fig. 2C). Furthermore, the Transwell assay revealed that cell migration and invasion were weakened significantly after miR-144-3p overexpression ($p<0.01$) (Fig. 2D and E). The results indicated that miR-144-3p may have an inhibitory effect on PC cell proliferation, migration, and invasion.

FOSB Was a Target of miR-144-3p in PC

Online search for the target genes of miR-144-3p through miRanda, PicTar, and TargetScan databases revealed that the oncogene FOSB may be a potential target of miR-144-3p. The 3′-UTR of FOSB was found to have a binding site for miR-144-3p (Fig. 3A). qRT-PCR and Western blot analyses showed that FOSB expression was upregulated in PC tissues ($p<0.01$) (Fig. 3B) and was the highest in PANC-1 cells ($p<0.05$) (Fig. 3C). To ensure the direct miRNA–target interaction between miR-144-3p and FOSB, we set up a luciferase reporter assay. The luciferase activity in PANC-1 cells was decreased with wt construct by overexpression of miR-144-3p and was increased with mutant construct (Fig. 3D). These results suggested that the 3′-UTR of FOSB was a direct target of miR-144-3p. Subsequently, the effect of miR-144-3p overexpression or inhibition on FOSB was detected. As shown in Figure 3E and F, FOSB mRNA expression level was not affected, whereas FOSB protein expression level was significantly inhibited by anti-miR-144-3p ($p<0.01$). These results suggested that miR-144-3p could directly bind to the 3′-UTR of FOSB to decrease FOSB protein level.

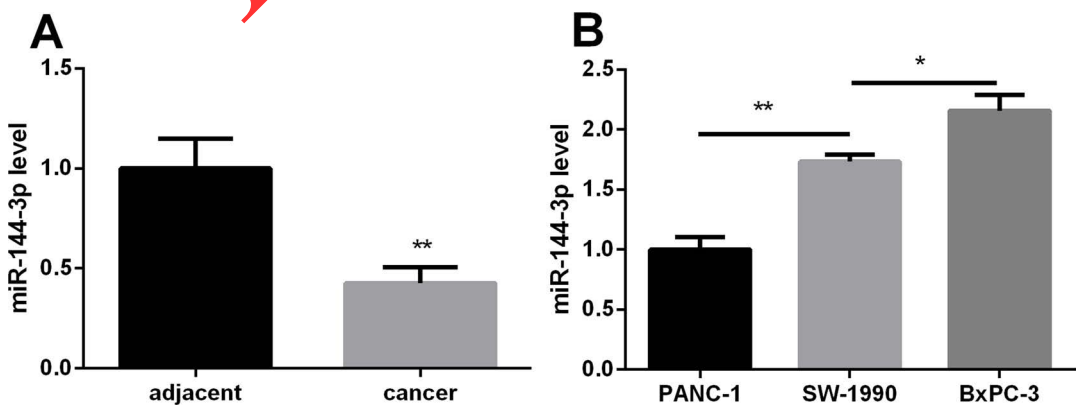


Figure 1. The relative expression levels of miR-144-3p in pancreatic cancer (PC) tissues and cells detected by real-time (RT)-PCR. (A) miR-144-3p was downregulated in PC tissues compared with the adjacent noncancerous pancreatic tissues. (B) Relative miR-144-3p expression in the PANC-1 PC cell line was significantly lower than that in the SW-1990 and BxPC-3 cell lines. *$p<0.05$, **$p<0.01$ compared with control.

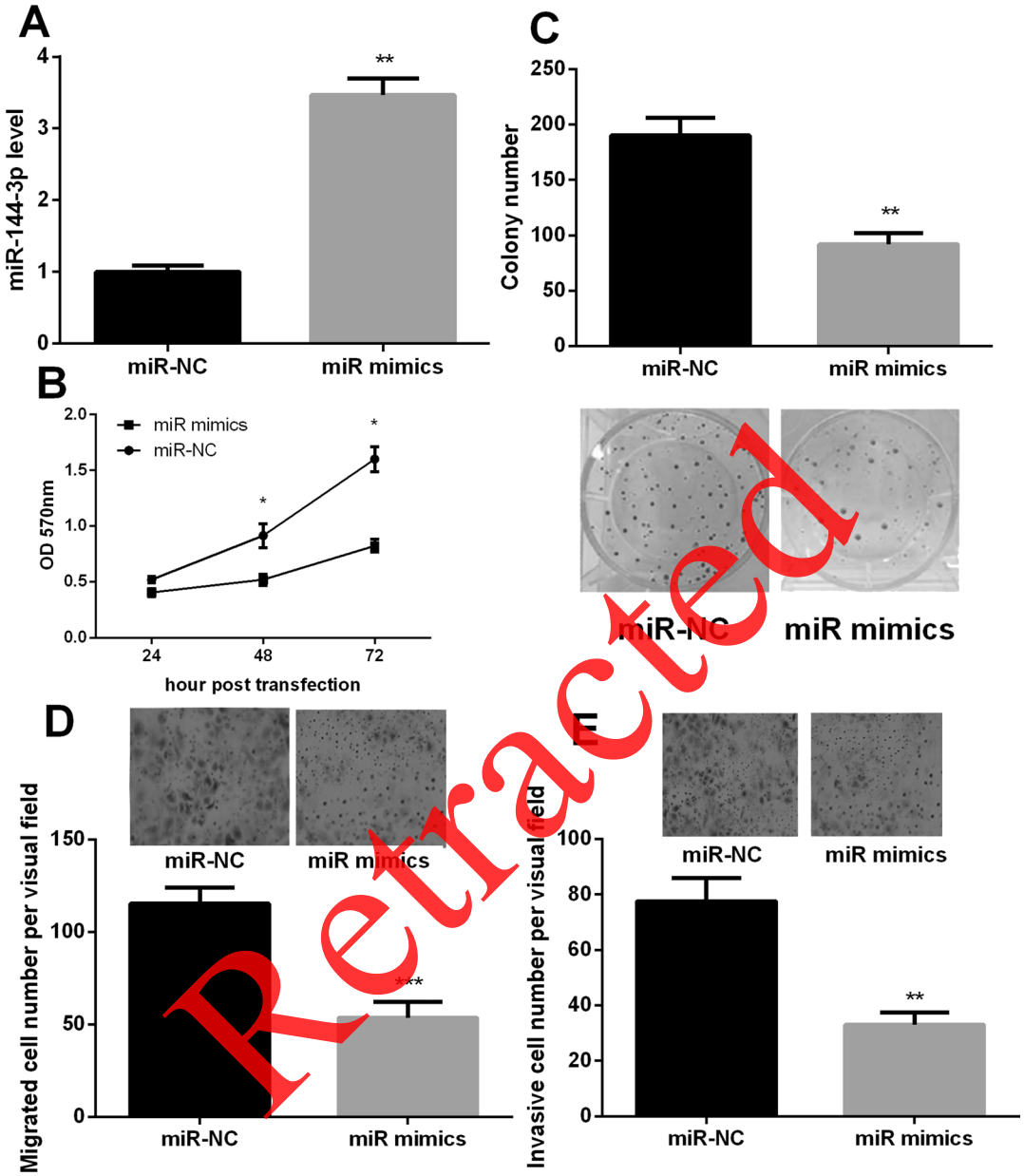


Figure 2. miR-144-3p inhibited proliferation, migration, and invasion of PANC-1 cells. (A) The relative expression levels of miR-144-3p after transfection of the miR-141 mimic with Lipofectamine 2000 detected by quantitative (q)RT-PCR. (B) The proliferation of PANC-1 cells was significantly inhibited by overexpressed miR-144-3p detected by MTT assay. (C) Overexpression of miR-144-3p reduced cell colony formation. (D) Overexpression of miR-144-3p decreased migration of PANC-1 cells detected by Transwell assay. (E) Overexpression of miR-144-3p decreased invasion of PANC-1 cells detected by Transwell assay. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with control.

FOSB Silencing Inhibited Proliferation, Migration, and Invasion of PC Cells

To investigate the biological role of FOSB in PC, we used siRNA to knock down FOSB expression and investigated the effect of FOSB silencing on proliferation, migration, and invasion of PANC-1 cells. Additionally, in order to abrogate the effect of miR-144-3p, we cotransfected siFOSB and anti-miR-144-3p into PANC-1 cells. The protein expression after transfection was confirmed by Western blot (Fig. 4A). As shown in Figure 4B, the viability of PANC-1 cells was significantly decreased after cells were transfected with siFOSB compared with its control group si-NC ($p<0.05$). After PANC-1 cells

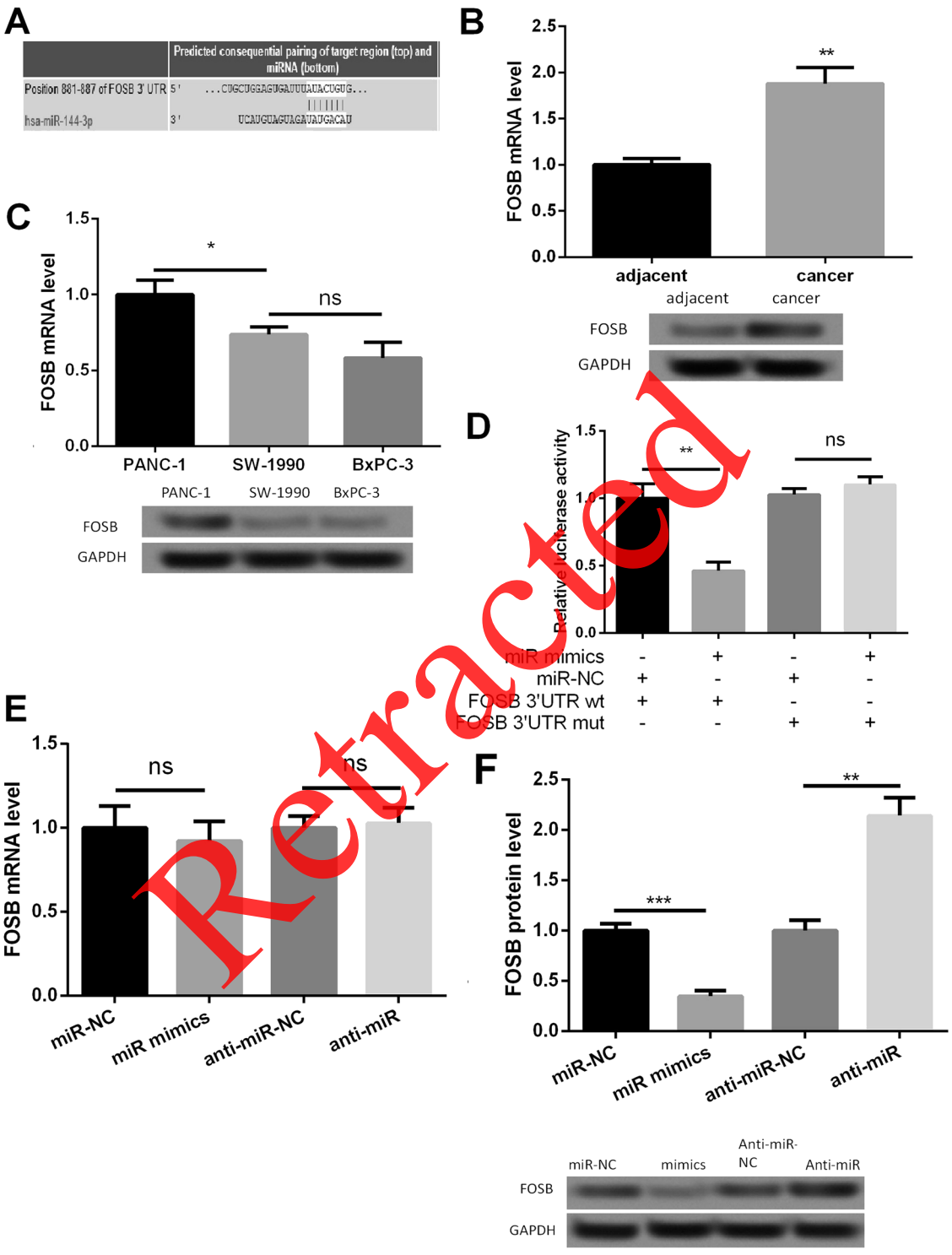
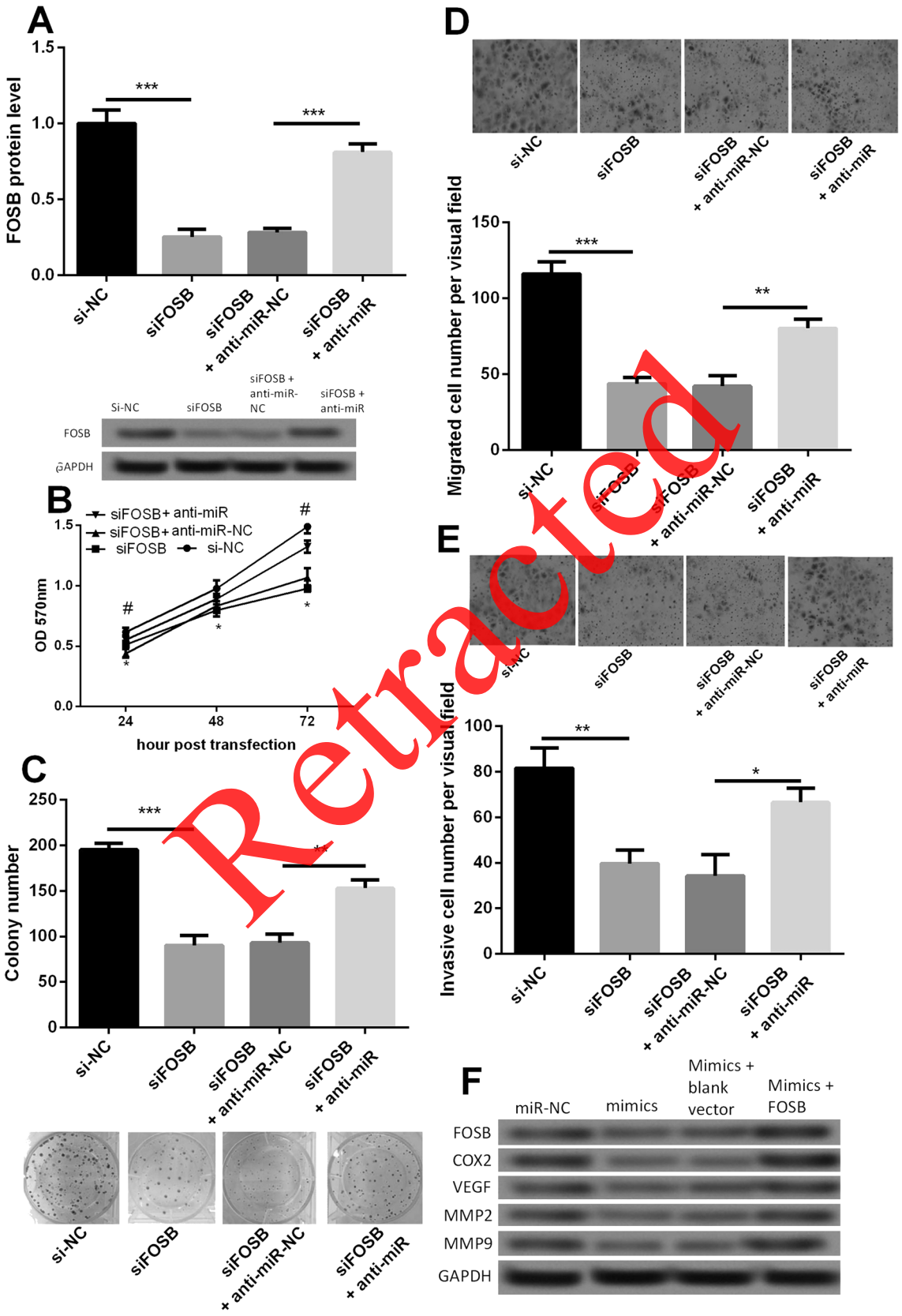


Figure 3. miR-144-3p directly targets the FOSB gene. (A) The gene sequences of FOSB regulated by miR-144-3p. (B) The expression level of FOSB was upregulated in PC tissues detected by RT-PCR and Western blot. (C) The relative FOSB expression in the PANC-1 PC cell line was significantly higher than that in the SW-1990 and BxPC-3 cell lines. (D) The relative luciferase activities in wild-type 3′-UTR of FOSB and mutant-type 3′-UTR of FOSB in transfected cells. (E) The relative mRNA expression level of FOSB after cell transfection detected by RT-PCR. (F) The relative protein expression level of FOSB after cell transfection detected by Western blot. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with control. ns, not significant.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MMP2
MMP9
GAPDH

were transfected with siFOSB and anti-miR-144-3p, the cell viability increased significantly compared with its control group siFOSB + anti-NC ($p < 0.05$). Similar results were also found in colony formation, cell migration, and invasion (Fig. 4C–E), suggesting that miR-144-3p regulated cell proliferation, migration, and invasion in PC cells by targeting FOSB.

We further investigated the protein expressions of COX2, VEGF, MMP2, and MMP9 in PANC-1 cells with miR-144-3p or FOSB overexpression. The result showed that decreased expressions of COX2, VEGF, MMP2, and MMP9 were found in the miR-144-3p mimic group, but FOSB overexpression increased their expressions (Fig. 4F). These data suggested that miR-144-3p may inhibit the expression of FOSB to repress the expressions of COX2, VEGF, MMP2, and MMP9 and then regulate PC cell proliferation, migration, and invasion.

DISCUSSION

Aberrant upregulation or downregulation of miRNAs is closely related to tumorigenesis. An increasing number of reports have indicated that miR-144 plays important roles in the development and progression of human cancers, such as nasopharyngeal carcinoma, colorectal cancer, and bladder cancer[14–16]. However, its function in PC is still not clarified. The present study focused on the role of miR-144-3p in PC cell proliferation, migration, and invasion. The results showed that miR-144-3p was downregulated in PC tissues and cell lines. Overexpression of miR-144-3p suppressed proliferation, migration, and invasion of PC cells by targeting FOSB.

Recently, abnormal miR-144 expression has been identified in nasopharyngeal carcinoma, colorectal cancer, and bladder cancer[14–16]. However, the definite role of miR-144 in regulating cancer progression is not very clear. In colorectal and bladder cancers, miR-144 is downregulated, but in nasopharyngeal carcinoma, miR-144 is upregulated with oncogenic properties. Here our data showed that the expression of miR-144-3p was markedly decreased in PC tissues and cell lines, suggesting its antitumor function.

Generally, miRNAs play important roles in diverse biological processes through degradation or inhibition of the translation of their target genes[17]. Therefore, miRNA target prediction is one of the key means for deciphering the role of miRNAs in disease[18]. Sureban et al.[19] proposed that Notch-1 is one of the downstream targets of miR-144 in a human PC cell line, contributing to key aspects of tumorigenesis. In this study, FOSB was revealed to be a target gene of miR-144-3p and was upregulated in PC tissues and cells. FOSB is a member of the Fos gene family, which can dimerize with proteins of the JUN family, forming the transcription factor complex activator protein 1 (AP-1)[20]. The AP-1 complexes are important regulators of cell proliferation, invasion, and metastasis[21–23]. Importantly, the FOSB gene has been reported to be a proto-oncogene. Milde-Langosch et al.[24] demonstrated that FOSB can promote breast cancer cell invasion. Recently, FOSB was reported to be associated with increased tumor growth and metastasis in ovarian cancer[20]. In agreement with the findings above, our result showed that FOSB silencing inhibited proliferation, migration, and invasion of PC cells. Moreover, FOSB increased the expressions of COX2, VEGF, MMP2, and MMP9.

COX2-initiated signaling pathways can control cell proliferation. Upregulation of COX2 is a frequent event in human malignancies[25]. VEGF is one of the most potent and specific angiogenic factors, which can induce proliferation and migration of vascular endothelial cells[26]. Angiogenesis is essential for continued tumor growth and metastasis[27]. MMP2 and MMP9 are members of the matrix metalloproteinase family that have been implicated in tumor cell invasion and migration[28]. Importantly, MMP2 and MMP9 have been reported to play major roles in PC cell growth, invasion, and migration[29]. In this study, miR-144-3p inhibited the expressions of COX2, VEGF, MMP2, and MMP9, whereas FOSB increased their expressions, which suggested that miR-144-3p may repress the expressions of COX2, VEGF, MMP2, and MMP9 to regulate PC cell proliferation, migration, and invasion by inhibiting the expression of FOSB.

In conclusion, our study revealed that miR-144-3p was downregulated in PC. miR-144-3p overexpression could repress PC cell proliferation, migration, and invasion by inhibiting the expression of FOSB. miR-144-3p may provide a new target for the development of therapeutic agents against PC.

ACKNOWLEDGMENT: The authors declare no conflicts of interest.

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Figure 4. Effect of FOSB on PANC-1 cell proliferation, migration, and invasion. (A) The relative protein expression level of FOSB after cell transfection with siFOSB or anti-miR-144-3p. (B) Growth curve among siFOSB, siFOSB + anti-miR-144-3p, and their appropriate controls. (C) Colony formation among siFOSB, siFOSB + anti-miR-144-3p, and their appropriate controls. (D) Transwell assay indicated that the migration ability of PANC-1 cells was markedly attenuated by downregulation of FOSB. (E) Transwell assay indicated that siFOSB significantly decreased the invasion ability of PANC-1 cells. (F) Effect of miR-144-3p mimic or FOSB on the expressions of COX2, VEGF, MMP2, and MMP9 detected by Western blot. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, #$p < 0.05$ compared with control.

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