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MiR-144 inhibits colorectal cancer cell migration and invasion by regulating PBX3

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Abstract. – OBJECTIVE: MicroRNAs (miRNA) are aberrantly expressed in various human cancers, including colorectal cancer (CRC). We aim to investigate the functional role and underlying mechanism of miR-144 in CRC.

PATIENTS AND METHODS: The expressional level of miR-144 and pre-leukemia transcription factor 3 (PBX3) in CRC tissues and cells was confirmed by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot. The migration and invasion of CRC cells were detected by transwell assay. Luciferase reporter assay was performed to determine the specific target of miR-144 in CRC cells.

RESULTS: The results displayed that miR-144 expression was significantly decreased in CRC tissues and cells compared to that in normal controls. Additionally, miR-144 mimic suppressed, while miR-144 inhibitor promoted the ability of CRC cell migration and invasion. More importantly, PBX3 was the direct target of miR-144 in regulating CRC development and PBX3 could reverse the inhibitory effect of miR-144 mimic on CRC cells. PBX3 expression was significantly increased in CRC and negatively correlated with miR-144 expression.

CONCLUSIONS: In conclusion, miR-144 suppressed CRC cell migration and invasion by targeting PBX3, suggesting its potential value in the diagnosis and treatment of CRC.

Key Words:
MiR-144, Migration, Invasion, CRC, PBX3.

Introduction

Colorectal cancer (CRC) is the most common malignant tumor and its main characteristics are high morbidity and high mortality. Despite considerable progress has been made in the treatment strategies of CRC, the prognosis is still poor[1]. Therefore, it is of vital significance to explore the molecular mechanisms that underlie the occurrence and progression of CRC

Recently, there has been many studies[2-5] showing that microRNAs participated in cell malignant progression of various tumors. MicroRNAs (miRNAs), evolutionary conservative non-coding RNAs, regulated protein expression by degrading the target mRNAs or hindering their translation[6]. MiRNAs acted as either oncogenes or tumor suppressors in regulating a widely variety of cancers[7]. For example, miR-30e, miR-503, miR-1, and miR-133b were all proved to function as tumor suppressors in CRC[8-11], while miR-18b, miR-4775, and miR-19a were served as oncogenes in regulating CRC progression[12-14]. MiR-144 expression was proved to remarkably decrease in CRC cells and associated with the progression of CRC[15]. However, the precise molecular mechanism through which miR-144 influences CRC progression remains unknown.

Pre-leukemia transcription factor 3 (PBX3), a member of the human PBX family, has been continuously reported to be associated with tumor growth and progression[16,17]. For instance, PBX3 was over-expressed in gastric cancer and promoted the progression of gastric cancer[18]. Moreover, PBX3 was upregulated in prostate cancer[19]. Ramberg et al[20] have shown that PBX3 expression was increased and its role in CRC cell migration and invasion was stimulative. Han et al[21] displayed that let-7c inhibited CRC metastasis by directly targeting PBX3. However, whether PBX3 played important role in miR-144-regulated CRC progression has not been fully explained until now.

In our study, we found that miR-144 showed an inhibition effect on CRC development. Over-expression of miR-144 inhibited, while silencing miR-144 promoted CRC cell migration and invasion. Moreover, we confirmed PBX3 as the specific target of miR-144 in CRC. We also found that the relevance between miR-144 and PBX3 mRNA expression in CRC tissues was negative and PBX3 reversed miR-144 inhibitory effect on CRC migration and invasion.

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Patients and Methods

Tissue Specimens

All CRC tissues were acquired from 30 paired patients who underwent surgery in the First Affiliated Hospital of the Zhengzhou University from 2012 to 2017. All patients signed written consent. The fresh specimens were snap-freezing quickly and stored at -80°C refrigerator. The Ethics Committee of the First Affiliated Hospital of Zhengzhou University approved all the studies of this experiment.

Cell Culture

CRC cell lines (SW480, HT-29, HCT8, HCT116) and normal colon epithelial cells (FHC) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) medium containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Solarbio, Beijing, China) and then maintained in an incubator at 37°C with 5% CO_2.

Cell Transfection

Control mimic/inhibitor, miR-144 mimic/inhibitor or PBX3 specific siRNA/vector were synthesized by Guangzhou RiboBio Co., Ltd (Guangzhou, China) and transfected into CRC cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). All these transfection procedures were conducted following the manufacturer's protocol.

RNA Isolation and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted from CRC cells or tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). QRT-PCR was performed using TaqMan PCR kit (TaKaRa, Otsu, Shiga, Japan). The primer sequences used were as follows: miR-144-F: ACACTCCAGCTGGGTACAGTATAGAT GATGTA, miR-144-R: CTCAACTGGTGTCGT-GGA; PBX3-F: GAGCTGGCCAAGAAATG-CAG, PBX3-R: GGGCGAATTGGTCTGGTTG; U6-F: ATTGGAACGATACAGAGAAGATT, U6-R: TC CAGTGCAGGGTCCGAG; glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-F: ACAACTT TGGTATCGTGGAAGG, GAPDH-R: GCCATCACGCCACAGTTTC. Measurements were normalized to U6 and GAPDH. The relative gene expression was calculated by $2^{-\Delta\Delta Ct}$ method.

Western Blot

Radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) containing protease inhibitor was used to lyse the cells. Bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) was used to determine the protein concentration. 50 μg total proteins were added to the hole of spacer gel and separated by the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and then, transferred onto NC membranes (Bio-Rad, Hercules, CA, USA). After blocking with the skim milk for 2 h, the membrane was incubated with the primary antibodies and secondary antibodies, respectively. The GAPDH values were used to normalize the amount of protein in the same lane.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) Assay

Cell viability was determined by MTT assay. The cells were seeded into 96-well plates and cultured in an incubator. Following incubation at 37˚C for 24 h, the culture medium was removed and MTT (20 μl) was added. After incubation for another 4 h at 37˚C, the culture medium was replaced with 150 μL dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). The cell viability was calculated by measuring the optical density (OD) at 490 nm.

Transwell Assay

The ability of cell migration and invasion was determined using transwell assay. For cell migration, the 8-μm pore size inserts were placed into the 24-well plates to separate the upper and the lower chambers. 1×10^5 cells were added into the upper chambers and RPMI-1640 medium containing 20% FBS was added to the lower chambers. After incubating at 37°C, 5% CO_2 atmosphere for 24 h, the cells were migrated into the lower chamber. The cells on the upper chambers were removed with a cotton swab, and the cells migrated to the lower chambers were fixed by 100% methanol and stained with the 0.1% crystal violet. Finally, the microscope (Olympus, Tokyo, Japan) was applied to quantify the cells number of migration. For cell invasion, all steps were the same as the migration procedure except for Matrigel (5 mg/mL) which was placed in the upper chamber.

Dual-Luciferase Reporter Assay

PBX3-3'-UTR-wild type and PBX3-3'-UTR-mutant type containing the miR-144-binding site were constructed. The Lipofectamine 2000 was applied

to co-transfect the miR-144 mimic and a pGL3-control vector containing the PBX3-3'-UTR-wild or mutant type into SW480 cells following the manufacturers' instruction. The measurement of the luciferase activity was performed using Dual-Luciferase Reporter Assay (Promega, Madison, WI, USA).

Statistical Analysis

All experiments were performed three times. Spearman's rank test was applied to analyze the correlation of miR-144 and PBX3 expression. Log-rank test was applied for analyzing the survival rate. The differences between the two groups were analyzed by the Student's *t*-test or more than two groups by Analysis of variance (ANOVA) followed by Tukey's post-hoc test. Multiple Array Viewer 4.9 software (MEV) was used to implement Pearson's correlation analysis. A statistically significant difference was considered as $p<0.05$.

Results

MiR-144 Expression is Lower and PBX3 is Higher in CRC Than Normal

QRT-PCR was applied to measure miR-144 expression in CRC, as Figure 1A shows; miR-144 expression was lower in all CRC cell lines than in normal cells. The average expression of miR-144 detected by qRT-PCR was lower in 30 pairs of CRC tissues than in normal tissues (Figure 1B). Moreover, we used qRT-PCR to detect PBX3 expression in CRC cell lines and tumor tissues too. As we saw in Figure 1C-1D, PBX3 expression was raised in both CRC cells and tumor tissues. Furthermore, the regression analysis results showed that miR-144 and PBX3 expression was negatively correlated (Figure 1E). In addition, Figure 1F displayed that high miR-144-3p expression predicted high survival of CRC patients. Taken together, miR-144 and PBX3 might play important role in CRC progression.

MiR-144 Inhibited CRC Cells Migration and Invasion

We transfected control mimic/inhibitor or the miR-144 mimic/inhibitor to the CRC cell lines (SW480, HT29) to examine the functional role of miR-144 in CRC. QRT-PCR analysis identified that miR-144 expression was remarkably increased in the miR-144 mimic group in both two CRC cell lines, while decreased in miR-144 inhibitor group (Figure 2A-2B). Before detecting cell migration and invasion, we first measured cell viability by MTT assay. As Figure 2C shows, miR-144 exhibited no effect on CRC cell viability.

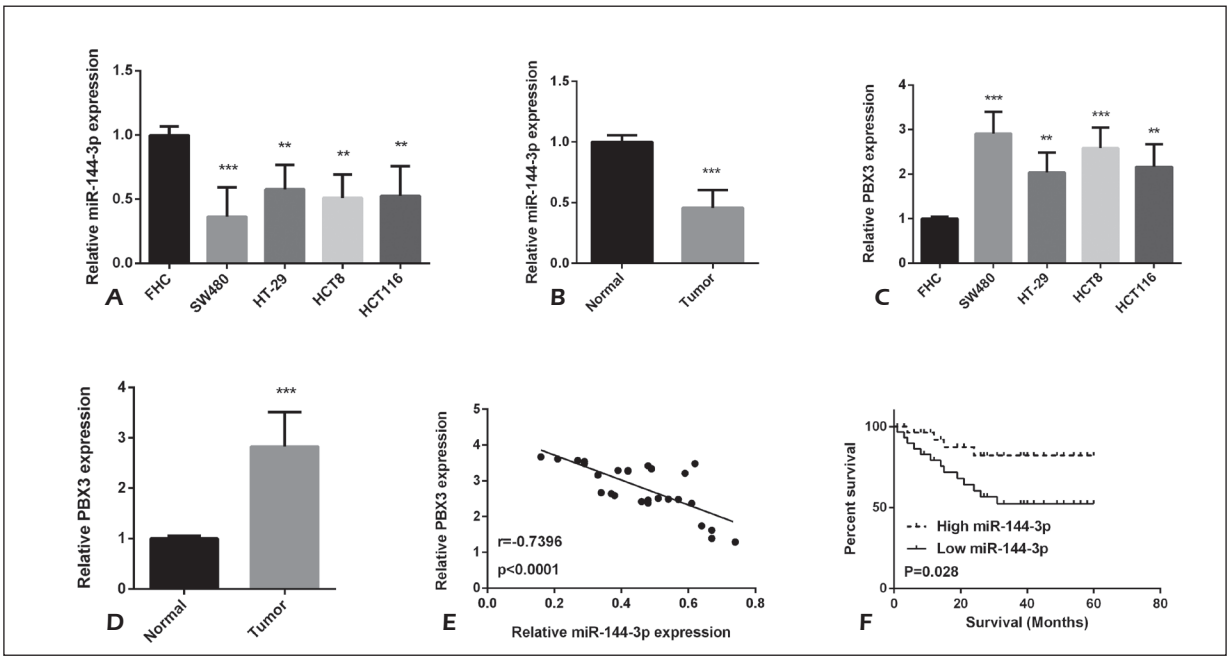

Figure 1. Decreased miR-144 expression and increased PBX3 expression in CRC. **A**, Detection of miR-144 expression in various CRC cell lines. **B**, Detection of average miR-144 expression in CRC tissues (n = 30). **C**, Detection of PBX3 expression in various CRC cell lines. **D**, Detection of average PBX3 expression in CRC tissues (n = 30). **E**, Relationship between miR-144 and PBX3 expression. **F**, Relationship between miR-144 and survival rate of CRC patients. **p<0.01, ***p<0.001.

Then, transwell assay was applied for measuring miR-144 effect on CRC cell migration and invasion. The results displayed that cell migration was significantly decreased by miR-144 mimic, while increased by miR-144 inhibitor (Figure 2D-2G). In addition, in miR-144 mimic group, the invasion ability of CRC cells was reduced distinctly and, in miR-144 inhibitor group, the invasion ability of CRC cells was significantly augmented (Figure 2E-2F). These data suggested that miR-144 restoration inhibited CRC cell migration and invasion.

PBX3 was the Direct Target of MiR-144

Next, the TargetScanHuman 7.2 was applied for predicting the potential targets of miR-144 to better understand the underlying mechanism of miR-144 in CRC cells. Figure 3A predicted the binding sites of miR-144 and PBX3. Then, we co-transfected miR-144 mimic and Luciferase report vectors into SW480 cells to detect the Luciferase activity. Re-expression of miR-144 showed a decreased Luciferase activity of PBX3-3'-UTR-wild type, but there were no evident change in PBX3-3'-UTR-mutant type as it was shown in Figure 3B.

We also detected PBX3 expression in SW480 cells after re-expression of miR-144 or silencing miR-144. In Figure 3C-3D, we saw that miR-144 mimic significantly reduced PBX3 expression, while miR-144 inhibitor increased PBX3 expression both in protein and mRNA level in SW480 cells. These results provided evidence that PBX3 was negatively regulated by miR-144 in CRC cells.

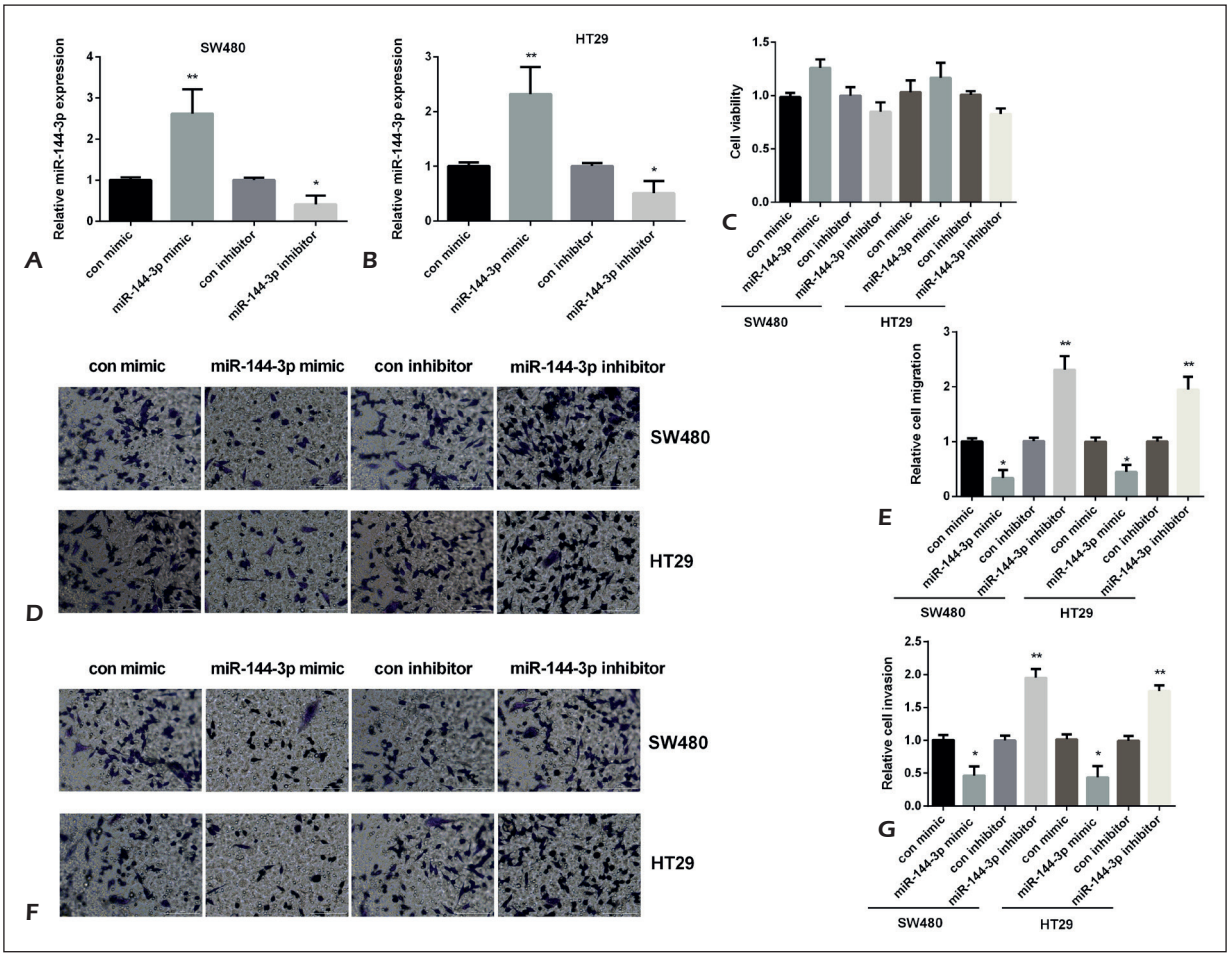

Figure 2. The suppression effect of miR-144 on CRC progression. **A**-**B**, Measurement of miR-144 expression in CRC cell lines (SW480, HT-29) after over-expression or knockdown of miR-144. **C**, Relative cell viability detected in SW480 and HT-29 cells respectively after increasing or decreasing miR-144 expression. **D**-**E**, Relative cell migration detected in SW480 and HT-29 cells respectively after increasing or decreasing miR-144 expression (×200 original magnification). **F**-**G**, Relative cell invasion detected in SW480 and HT-29 cells respectively after increasing or decreasing miR-144 expression (×200 original magnification). *p<0.05, **p<0.01.

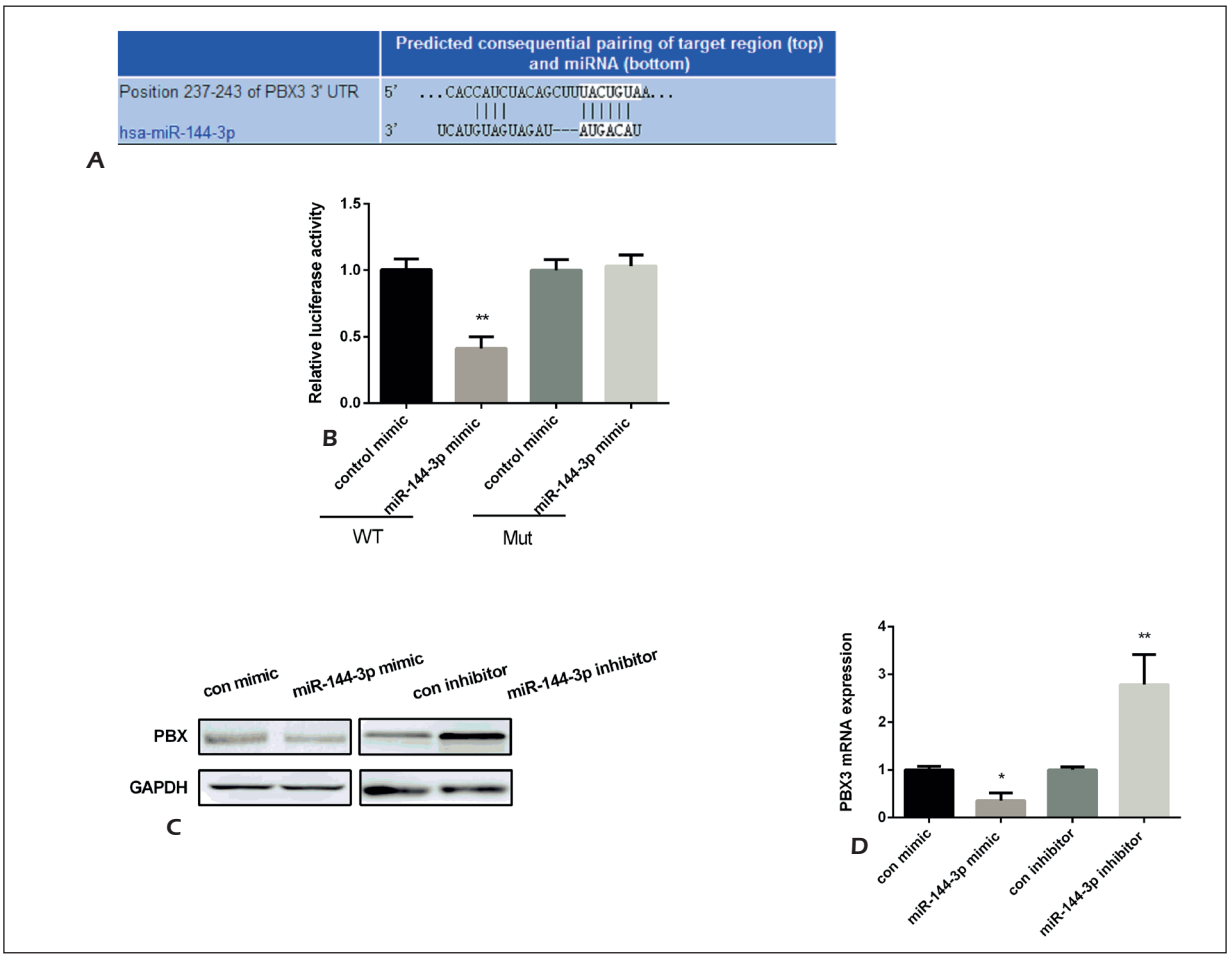

Figure 3. MiR-144 directly targeted PBX3 in CRC. **A**, Predicted consequential pairing of target region (top) and miR-144 (bottom). **B**, Measurement of relative luciferase activity in SW480 cells. **C**, Western blot analysis of the PBX3 protein level in SW480 cells after over-expression or knockdown of miR-144. **D**, PBX3 mRNA expression detected in SW480 cells after over-expression or knockdown of miR-144 by qRT-PCR. *$p<0.05$, **$p<0.01$.

Knockdown of PBX3 Inhibited CRC Cell Migratory and Invasive Ability

Then, PBX3 expression was altered to confirm the influence of PBX3 on CRC cell migration and invasion. The expression of PBX3 was significantly decreased after the knockdown of PBX3 (PBX3 siRNA) both in mRNA and protein level (Figure 4A-4B). Moreover, as Figure 4C-4D show, the silence of of PBX3 suppressed the migratory and invasive ability of CRC cells. These results stated that PBX3 siRNA showed inhibitory effect on CRC cell migration and invasion.

PBX3 Overturned MiR-144 Role in CRC Cell Migration and Invasion

Next, we investigated the function of PBX3 in CRC cells migration and invasion regulated by miR-144. Firstly, we detected the effect of miR-144 on PBX3. As we saw in Figure 5A-5B, PBX3 expression was lower in SW480 cells after over-expression of miR-144 than control group, whereas higher after over-expression of both miR-144 and PBX3 than miR-144 mimic group. We then detected CRC cell migration and invasion after over-expression of both miR-144 and PBX3 using transwell assay. As we saw in Figure 5C-5D, over-expression of PBX3 markedly reversed miR-144-medicated inhibition effect on CRC cell migration and invasion. Moreover, miR-144 mimic increased E-cadherin expression, while decreased N-cadherin and Vimentin expression. PBX3 could overturn miR-144 inhibitory effect on EMT of CRC (Figure 5E). Collectively, these data suggested that PBX3 might reverse partial function of miR-144 in CRC cell migration and invasion.

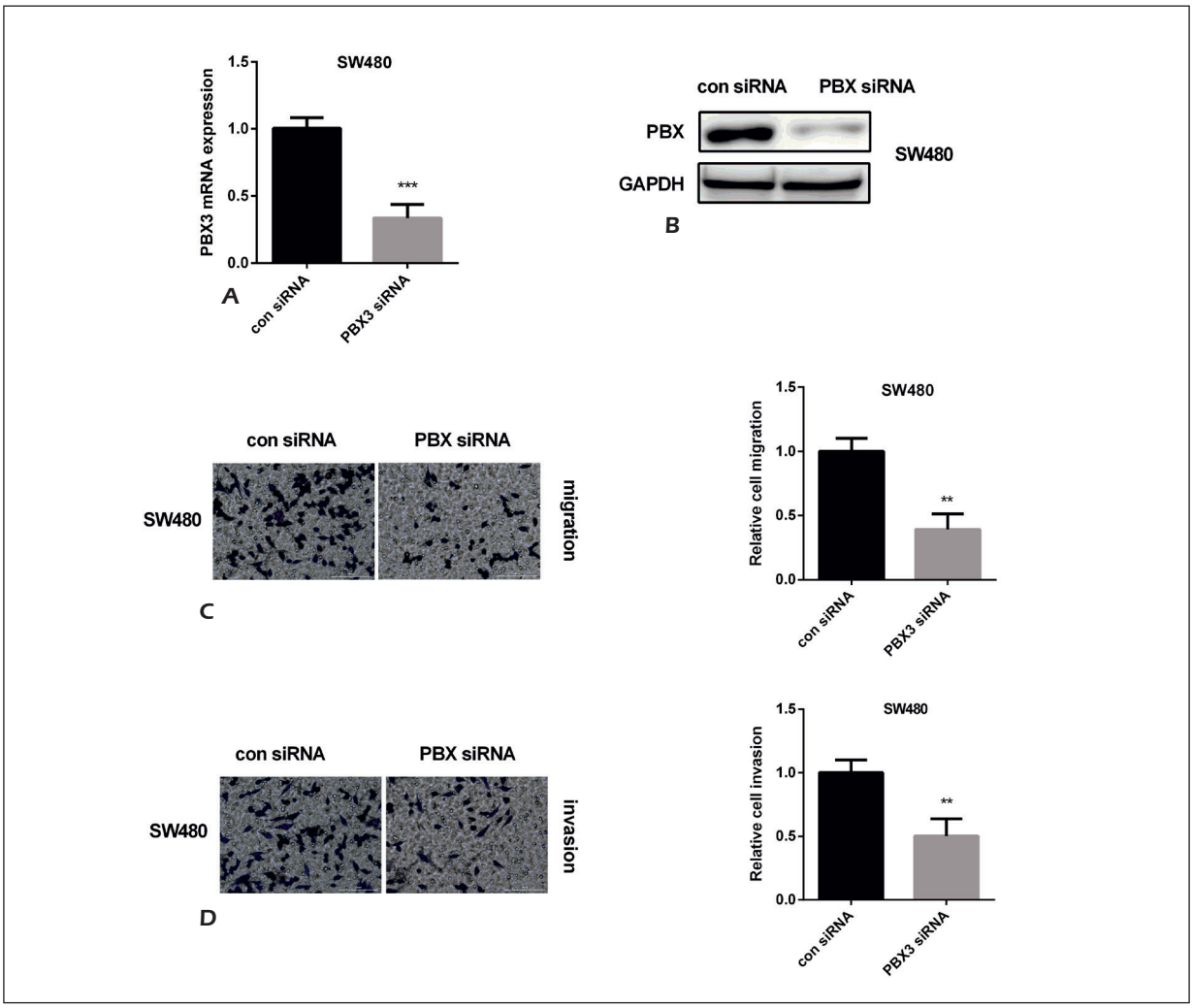

Figure 4. The inhibitory effect of siRNA PBX3 in SW480 cells. **A**, PBX3 mRNA expression detected in SW480 cells after transfected with siRNA PBX3 by qRT-PCR. **B**, PBX3 protein level detected in SW480 cells after transfected with siRNA PBX3 by Western blot. **C**, Relative migration of SW480 cells detected after knockdown of PBX3 by transwell assay (×200 original magnification). **D**, Relative invasion of SW480 cells detected after knockdown of PBX3 by transwell assay (×200 original magnification). $^{**}p<0.01$, $^{***}p<0.001$.

Discussion

In this present study, we observed that miR-144 expression was observably decreased in CRC and it participated in the development of CRC as a tumor suppressor. Restoration of miR-144 inhibited CRC cell migration and invasion. More importantly, we first identified PBX3 as the directly target of miR-144 using Luciferase assay and miR-144 repressed CRC migration and invasion by downregulating PBX3.

Han et al[22] revealed that the disorders of microRNA expression could lead to cellular alteration and oncogenesis. MiRNAs acted as either tumor promoters or suppressors in CRC development. Previous researches[23,24] have shown that miR-638, miR-548c, miR-6869, miR-5787, and miR-8075 expression were significantly increased, while miR-3180 and miR-486 expression was decreased in CRC patients. The researches about miR-144 were detected in many cancers. Jiang et al[25] showed that miR-144 over-expressed in insulinomas and promoted cell proliferation by targeting PTEN. MicroRNA-144 was also proved to promote the development and progression of nasopharyngeal carcinoma *via* inhibition of PTEN[25]. However, miR-144 inhibited gastric cancer cell growth by directly targeting RLIP76[26]. MiR-144-3p exerted anti-tumor role in pancreatic cancer cell migration and invasion by regulat-

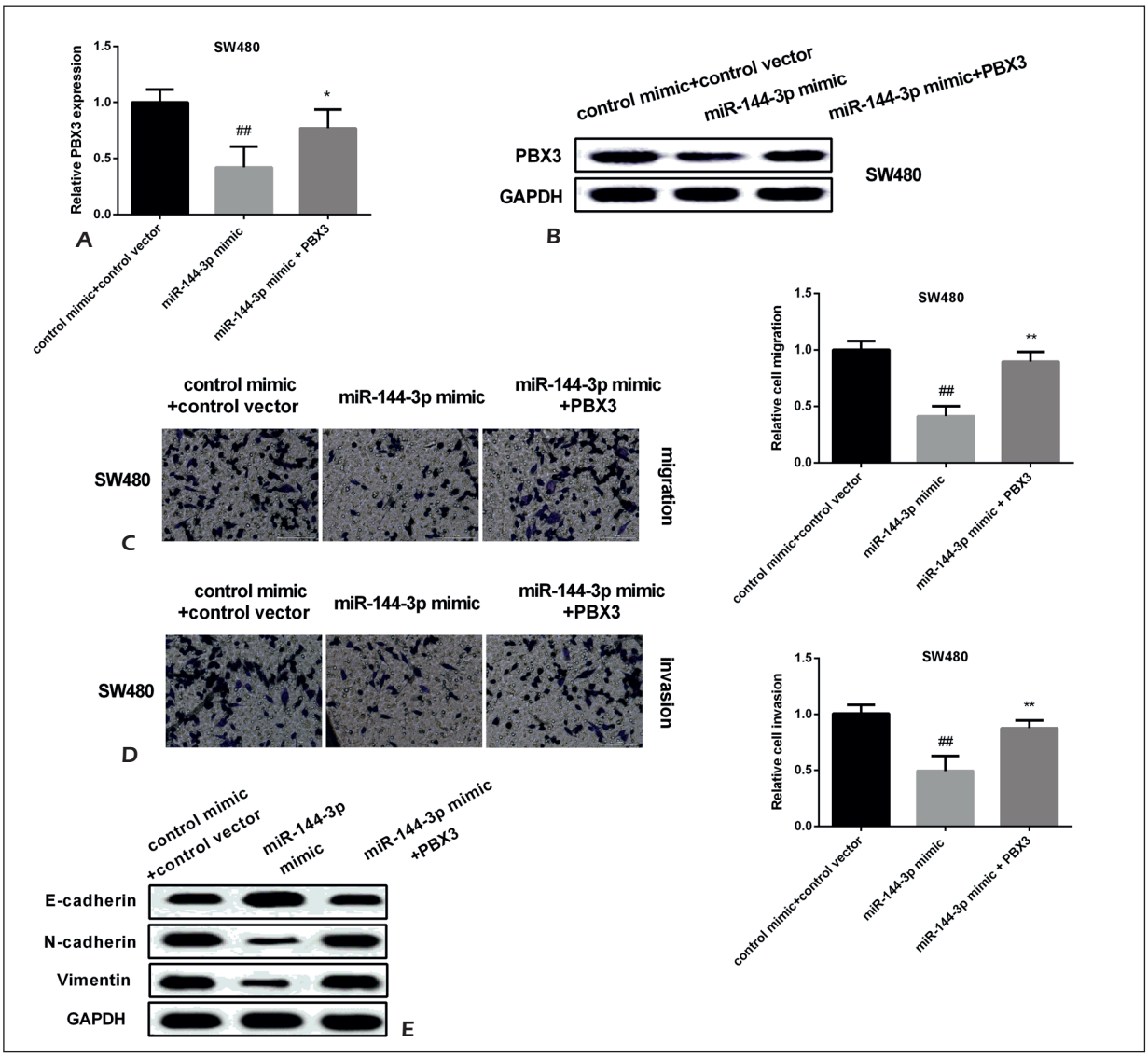

Figure 5. The function of PBX3 in CRC cell migration and invasion regulated by miR-144. **A**-**B**, Detection of relative PBX3 expression in SW480 cells after over-expression of miR-144 or combined with PBX3 by qRT-PCR and Western blot. **C**-**D**, Detection of relative migration and invasion of SW480 cells after over-expression of miR-144 or combined with PBX3 by transwell assay (×200 original magnification). **E**, Detection of E-cadherin, N-cadherin and Vimentin expression in SW480 cells after over-expression of miR-144 or combined with PBX3 by Western blot. *p<0.05, **p<0.01; $^{\#\#}p$<0.01.

ing FOSB[27]. Our present investigation stated that miR-144 was down-regulated in CRC, which was consistent with the previous study[15] showing that the lower expression of miR-144 was involved in the abnormal regulation of CRC progression.

Several reports suggested that PBX3 participated in many cancers' progression, including gastric cancer[17], prostate cancer[29], and glioma[30]. PBX3 was targeted by various miRNAs in regulating different cancers, including, miR-144 in lung cancer[31], miR-320a in hepatocellular carcinoma[32], and miR-144 in gastric cancer[33]. We suggested that PBX3 was over-expressed in CRC and PBX3 enhanced CRC cell migration and invasion, which was consistent with the results that PBX3 promoted CRC cell invasion and migration[34]. We also provided evidence[35] that miR-144 directly targeted PBX3 in regulating CRC progression. PBX3 expression was markedly reduced in miR-144 mimic group in SW480 cells. In addition, the suppression effect of miR-144 on SW480 cell invasion and migration could be reversed by the PBX3 vector.

Conclusions

We showed that miR-144 expression was lower in CRC and functioned as a tumor suppressor in CRC development, whereas PBX3 showed an opposite effect. It was the first time we found that PBX3 was the direct target gene of miR-144 in regulating CRC cell invasion and migration, which providing a novel idea of the treatment of CRC.

Conflict of Interests

TThe authors declare that they have no conflict of interests.

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