

MiRNA-92a Promote Angiogenesis in Colorectal Cancer by Inhibiting the Expression of PTEN

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Abstract

Background: The microRNA (miRNA) miRNA-92a has been implicated in colorectal cancer (CRC) pathology.

Methods: Here, we examined miRNA-92a involvement in CRC-associated angiogenesis, including examining a mechanism by which miRNA-92a may exert such effects. MiRNA-92a expression in 25 clinical CRC samples and in HT29, SW620, SW480 and HCT116 CRC cells was detected with quantitative real-time polymerase chain reaction (qRT-PCR). Blood microvessels were labeled by immunohistochemistry (IHC) with an anti-CD31 antibody. MiR-92a mimic or inhibitor were transfected into HCT116 and SW620 cells to up- and down-regulate miRNA-92a expression, respectively. The effects of altering miRNA-92a expression levels on HUVEC (human umbilical vein endothelial cell) tubule formation and PTEN protein expression were measured.

Results: MiRNA-92a was more highly expressed in CRC tissues and cell lines than in matched adjacent tissues ($p < 0.01$) and normal epithelial cells ($p < 0.05$), respectively. Microvessel density (MVD) was elevated in CRC tissues relative to adjacent tissues ($p < 0.01$), and miRNA-92a levels correlated with MVDs in CRC tissues (Pearson coefficient $r = 0.580$, $p = 0.01$). The cells culture supernatant of CRC cell with miRNA-92a overexpression (or suppression) can promoted (or reduced) the formation of HUVEC tubules ($P < 0.05$). Elevated miRNA-92a expression was associated with reduced PTEN expression in CRC cells ($p < 0.01$).

Conclusion: Modulation of miR-92a, which is highly expressed in CRC cells and tissues, is associated directly with differences in CRC angiogenesis. MiR-92a could promote tumor angiogenesis in CRC by inhibiting the expression of PTEN.

Background

Colorectal cancer (CRC) is a common human malignancy of the digestive system, and has the third highest incidence of all cancers worldwide (1). Unfortunately, CRC is insidious and has a poor prognosis.

Uncontrolled tumor cell proliferation and differentiation has been associated with altered expression microRNAs (miRNAs), which are non-coding small RNAs with distinctive abilities to act as oncogenes or tumor suppressor genes (2, 3). MiRNAs that are abnormally expressed in CRC interact with target genes that are involved in regulating important signaling pathways, such as cell proliferation, apoptosis, and invasion, and have been implicated in malignant transformation, CRC cell proliferation, and metastasis (10, 11). Notably, expression of the single-stranded miRNA known as miRNA-92a has been found to be abnormally elevated in CRC and other tumor tissues (4, 5), and miRNA-92a expression has been associated with lymph node metastasis and a poorer prognosis of colon cancer (6). MiRNA-92a has thus been classified as an oncogene and it has been suggested that it may play a key regulatory role in CRC occurrence and progression (7).

Uncontrolled angiogenesis is a well-established biological characteristic of malignant tumors, and angiogenesis of tumors is thought to enable their growth, invasion, and metastasis. Although miRNA-92a expression has been associated with vascular endothelial cell formation and tumor angiogenesis (8, 9), it has not yet been established whether it plays a role in CRC-associated angiogenesis and its regulatory mechanisms in CRC cells have yet to be clarified.

The aim of the present study was to explore miRNA-92a effects on CRC-associated angiogenesis and the mechanism by which miRNA-92a may exert such effects, with the long-term goal of contributing experimental evidence that may guide the development of molecularly targeted therapies for CRC. We thus analyzed the expression levels of miRNA-92a in CRC tissues, detected by quantitative real-time polymerase chain reaction (qRT-PCR), and whether they correlate with CRC tumor angiogenesis, detected by immunohistochemistry (IHC). Expression of miRNA-92a was manipulated by transfecting CRC cells with a miRNA-92a mimic or inhibitor. Subsequently, miRNA-92a effects on vascular endothelial cell angiogenesis *in vitro* and on the expression of a potential genetic target, namely the tumor suppressor phosphatase PTEN, were observed.

Methods

Samples. We obtained 25 CRC specimens (12 from males and 13 from females) with intact adjacent tissues (> 4-cm margins) from Shenzhen Nanshan Hospital (affiliated with Guangdong Medical College) between June of 2014 and December of 2015. The patients of origin had a mean ($\pm$ standard deviation) age of 59.2 ± 13.1 years (range, 32–84 years). All patients were informed of the sample donation and this study was approved by the ethical review committee of the hospital. None of the patients had received radiotherapy or chemotherapy before the tumor resection surgery. The specimens were preserved in liquid nitrogen or neutral formaldehyde.

Reagents. TRIzol reagent, Lipofectamine® 2000, RNA reverse transcription and amplification kits, and human endothelial SFM medium were purchased from Life Technologies (Carlsbad, CA). High-glucose DMEM was purchased from HyClone Laboratories (Logan, UT). Matrigel matrix glue was purchased from BD (Franklin Lakes, NJ). All primers were synthesized by Guangzhou Ruibo Biotechnology Co., Ltd (Guangzhou, China).

Monoclonal rabbit anti-human PTEN (ab154814, clone EPR7495; Abcam) and GAPDH (10494-1-AP; Proteintech Co.) primary antibodies and goat anti-rabbit secondary antibody (111-035-003; Jackson Laboratory, Bar Harbor, ME) were used for immunoblotting. Mouse anti-human CD31 (ZM-0044, clone 1A10; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China) direct antibody was used with a Vectastain Elite ABC system kit (PK-6102; Vector Laboratories, Burlingame, CA) for IHC.

Cell lines and culture. HCT116, SW620, HT29, and SW480 CRC cells (donated by the Chinese University of Hong Kong) were cultured in high-glucose DMEM complete medium. Human umbilical vein endothelial cells (HUVECs; donated by Sun Yatsen University) were cultured in complete medium at 37 °C under 5%

CO₂ and passaged until stable (2 ~ 3 generations). Cells were undergoing logarithmic growth when subjected to experiments.

qRT-PCR detection of miRNA-92a expression.

Total RNA was extracted from CRC tissues and cells with TRIzol reagent and 1-μg RNA samples were subjected to reverse transcription at 42 °C for 60 min in accordance with the RNA reverse transcription kit instructions. Subsequently, 1-μl cDNA aliquots were subjected to PCR amplification for 40 cycles under the following conditions: 50 °C for 2 min, 95 °C for 10 min, denaturation at 95 °C for 15 s, and extension at 60 °C for 1 min.

Expression of miRNA-92a in tissues was determined by the absolute quantitative method. A miRNA standard was subjected to 10-fold gradient dilution, and the standard and sample were detected by PCR. A standard curve and regression equation were established to calculate miRNA-92a concentrations in samples; miRNA quantification was standardized according to total RNA quantities in samples, and the results are shown as miRNA content (in fmol) per μg of total RNA. RNA extracted from cells was quantified relative to U6 as an internal reference, and the relative expression of miRNA-92a in each group was calculated by the $2^{-\Delta\Delta C_t}$ method. Each experiment was performed in triplicate and mean of the three values obtained was determined.

Detection of angiogenesis by IHC.

CRC tissue specimens were prepared by conventional dehydration and paraffin embedding. The paraffin-embedded specimens were sliced into 4-μm-thick sections with a microtome. High-pressure/heat epitope retrieval was performed with the sections in citrate buffer. Expression of CD31 in vascular endothelial cells was detected by IHC with mouse anti-human CD31 antibody (1:100 in phosphate buffered saline) performed according to the Vectastain Elite ABC system kit instructions (antibody omitted in negative control; known positive sample used as a positive control).

Angiogenesis was analyzed in terms of microvascular density (MVD) of the CD31-immunopositive signal (brown staining). High-vessel-density areas were selected under a low power microscope (100×), and then five fields of view were selected randomly for imaging under higher power microscopy (400×) and averaged.

Cell transfection.

HCT116 and SW620 cells in a logarithmic growth stage were subjected to transient transfection. The cells were seeded in 6-well plates, and then cultured in 1 × DMEM medium containing 10% fetal bovine serum with 5 μl of transfection reagent and an experimentally indicated miRNA molecule (50 nM miRNA-92a mimic or 100 nM miRNA-92a inhibitor) until ~ 60% fusion was observed. Serum-free 1 × Opti-MEM medium was added to each well to a final volume of 2 ml, and transfection was allowed to proceed for 6 h at 37 °C under 5% CO₂, and then the medium was exchanged for new medium. Cells were collected

48 h after transfection for RNA extraction, and cell proteins and serum-free culture supernatant (conditioned medium) were collected 72 h after transfection for use in subsequent experiments.

Tube formation assay. HUVECs (1×10^6 /well) were cultured for 24 h in Matrigel® matrix (50 µl/well) in 96-well plates. Conditioned medium (100 µl/well; from the above transfections) containing HCT116 or SW620 cells transfected with miRNA-92a mimic or inhibitor, respectively, was added to each well. HUVECs were allowed to grow into closed tubule-like structures for 6 h at 37 °C under 5% CO₂, and the number of tubules formed in each well was counted. Triplicate wells were set up for each group, and three views were imaged for each well. Averages of the three views were calculated for each well.

Immunoblot detection of PTEN.

Protein concentrations in the 72-h transfections of CRC cells described above were determined by Bradford assay (Sangon Biotech Co., Ltd). The proteins were extracted by cell lysate RIPA, and the total protein concentrations of the extracted protein samples were determined by the bicinchoninic acid assay method. Protein samples were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (30 µg/lane) and the resultant bands were electrotransferred to polyvinylidene difluoride membranes. Subsequently, the membranes were blocked in 5% skim milk for 1 h, incubated with rabbit anti-human PTEN antibody (1:2000) at 4 °C overnight, and then rinsed in Tris buffered saline with 0.1% Tween20 three times (10 min per rinse). The rinsed membranes were incubated with goat anti-rabbit antibody (1:4000) for 1 h at 37° C, and then ECL developer was applied to the membrane. The processed membranes were imaged with a Bio-Rad ChemiDoc XRS⁺ system for densitometry analysis of PTEN protein levels.

Statistical analysis.

Statistical analyses were conducted in SPSS19.0 software (IBM). Following confirmation of a normal distribution with Kolmogorov-Smirnov normality tests, *t* tests were used for inter-group comparisons. Correlations between miRNA-92a expression and angiogenesis MVD in CRC tissues were assessed by Pearson analysis. *P* values < .05 were considered significant.

Results

Expression of miRNA-92a in CRC tissues and cells.

As shown in Fig. 1A, we observed markedly higher levels of miRNA-92a in CRC tissues (1.102 ± 0.735 fmol/µg total RNA; *n* = 25) than in normal tissue margins (0.037 ± 0.031 fmol/µg total RNA; *t* = 7.405, *p* < .01). Likewise, as shown in Fig. 1B, miRNA-92a expression was confirmed to be highly elevated in all four CRC cell lines examined relative to levels in normal colorectal epithelial cells (*p* < .05 or *p* < .01). Among the cell lines, HCT116 cells had the lowest miRNA-92a expression, and SW620 cells had the highest.

Correlation between miRNA-92a expression and angiogenesis MVD in CRC cells.

As shown in Fig. 2, comparative analysis of 25 sample pairs revealed that the MVD of CD31-immunopositive CRC tissues (27.400 ± 7.560) was significantly higher than that of corresponding adjacent tissues (10.760 ± 3.140 ; $t = 9.829$, $p < .01$). Furthermore, miRNA-92a expression in CRC tissues correlated directly with CD31-immunopositive MVD (Pearson coefficient $r = 0.580$, $p = .01$).

Establishment of miR-92a expression modulated cell models.

Transient transfection with miRNA-92a mimic or miRNA-92a inhibitor upregulated and down-regulated miRNA-92a levels in the lowest miRNA-92 expressing cell line (HCT116) and in the highest miRNA-92a expressing cell line (SW620), respectively (Fig. 3). Compared with non-transfected controls, miRNA-92a levels were significantly increased in HCT116 cells transfected with miRNA-92a mimic for 48 h ($t = 7.439$, $p < 0.05$) and significantly decreased in SW620 cells transfected with miRNA-92a inhibitor for 48 h ($t = -45.916$, $p < 0.01$).

Effects of miRNA-92a expression modulation on HUVEC tubule formation.

Treatment with miRNA-92a–upregulating conditioned medium (from miRNA-92a upregulated HCT116 cells) for 6 h increased HUVEC tubule formation ($t = 5.563$, $p < 0.05$ vs. non-treated controls). In contrast, treatment with miRNA-92a–down-regulating conditioned medium (from miRNA-92a down-regulated SW620 cells) for 6 h decreased HUVEC tubule formation ($t = -6.107$, $p < 0.05$ vs. non-treated controls) (Fig. 4).

Effects of miRNA-92a on PTEN expression in CRC cells.

Targetscan software predicted that there should be a target binding site of miRNA-92a between the seed sequence of miRNA-92a and the 3' untranslated region of *PTEN* (Fig. 5A), suggesting that *PTEN* may be a regulatory target gene of miRNA-92a. As shown in Fig. 5B–D, transfection of miRNA-92a mimic that increased miRNA-92a levels in HCT116 cells resulted in a concomitant 26.40% reduction in PTEN protein band density ($t = -15.176$, $p < .01$ vs. non-transfected control). Meanwhile, transfection of miRNA-92a inhibitor that reduced miRNA-92a levels in SW620 cells resulted in a 21.98% reduction in PTEN protein band density ($t = 10.911$, $p < .01$; Fig. 5C–D).

Discussion

The present study confirmed that miRNA-92a levels are elevated in CRC tissues, relative to corresponding tumor-adjacent tissues, as well as in HCT116, SW620, HT29, and SW480 CRC cells, relative to normal intestinal epithelial cells. These results are consistent with previous reports implicating this miRNA in CRC (4, 6, 12) and further suggest that miRNA-92a may act as a tumor-promoting agent in CRC.

MiR-92a is a member of the miRNA-17-92 gene cluster family. Ng et al. found that miRNA-17-3p and miRNA-92 are elevated in CRC tissues and in CRC patient plasma (12). Moreover, abnormally high

expression of miRNA-92a has been associated with advanced clinical stage, lymph node metastasis, and poor outcomes in colon cancer patients (6, 13). Indeed, the abnormally high serum levels of miRNA-92a in patients with CRC could potentially be developed into a novel diagnostic marker for CRC (12, 14).

Angiogenesis is a necessary condition for solid tumor growth and metastasis, and angiogenesis plays an important role in tumorigenesis and development. Studies have shown that miRNAs can promote or inhibit tumor angiogenesis by targeting angiogenic factors and protein kinases (13, 15). Dews et al. (9) found that overexpression of the miRNA-17-92 gene cluster via K-Ras mediated transformation of intestinal cells can promote tumor vascular growth. In the present study, we found that the MVDs of CD31-immunopositive vessels were greater in CRC tissues with high miR-92a expression than in adjacent tissues with low miRNA-92 expression. Moreover, miRNA-92a levels correlated with MVD values in CRC tissues. Furthermore, miRNA-92a mimic-transfected HCT116 cell conditioned media and miRNA-92a inhibitor-transfected SW620 cell conditioned media augmented and reduced HUVEC tubule formation, respectively, suggesting that the high miR-92a expression in CRC may promote angiogenesis. It has been reported that CRC cells can act on vascular endothelial cells by secreting miRNA-92a containing microvesicles. These microvesicles have been reported to down-regulate expression of the miRNA-92a target gene *Dkk-3*, to promote endothelial cell proliferation and migration, and to help establish a suitable microenvironment for tumor angiogenesis that enables tumor growth (16).

PTEN is an important inhibitor on the PI3K/AKT signaling pathway, which can participate in the regulation of angiogenesis by mediating the expression of VEGF (17, 18). The deletion or down-regulation of PTEN can promote tumor angiogenesis (19, 20). Zhang et al. (21) showed that miRNA-92a can induce EMT transformation of CRC cells and promote invasion and metastasis of cancer cells by inhibiting PTEN expression. In this study, we established CRC cell models with enhanced expression and suppressed miRNA-92a expression and our experiments with these models confirmed that the expression level of miRNA-92a in CRC cells is inversely related to PTEN protein expression, suggesting that miRNA-92a may play a regulatory role in promoting angiogenesis of CRC by inhibiting the expression of PTEN.

In conclusion, miRNA-92a is highly expressed in CRC tissues, and we were able to relate its elevated presence closely to increased angiogenesis. Moreover, the present data suggest that miRNA-92a may promote angiogenesis in CRC neoplasms by inhibiting *PTEN* expression. MiRNA-92a should be further examined as a potential CRC molecular marker and therapeutic target. Because miRNAs can regulate multiple genes, the molecular mechanisms through which miRNA-92a influences tumor angiogenesis and its role in CRC pathogenesis and progression should be further explored.

Abbreviations

miRNA: microRNA; CRC: colorectal cancer; qRT-PCR: Real-time polymerase chain reaction; IHC: Immunohistochemistry; HUVEC: Human umbilical vein endothelial cell; MVD: Microvessel density.

Declarations

Ethics approval and consent to participate

This study was carried out in strict accordance with the International Council for Laboratory Animal Science. All experiments were conducted in accordance with protocols approved by the Experimental Ethical Committee of Shenzhen Nanshan People's Hospital and The 6th Affiliated Hospital of Shenzhen University Health Science Center.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed herein are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JHD conceived and designed the study. XW and HG analyzed the data. XW, YH, and LW performed the studies. XW and YH contributed reagents materials and analysis tools. XW, YSH, and JHD wrote the manuscript.

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Figures

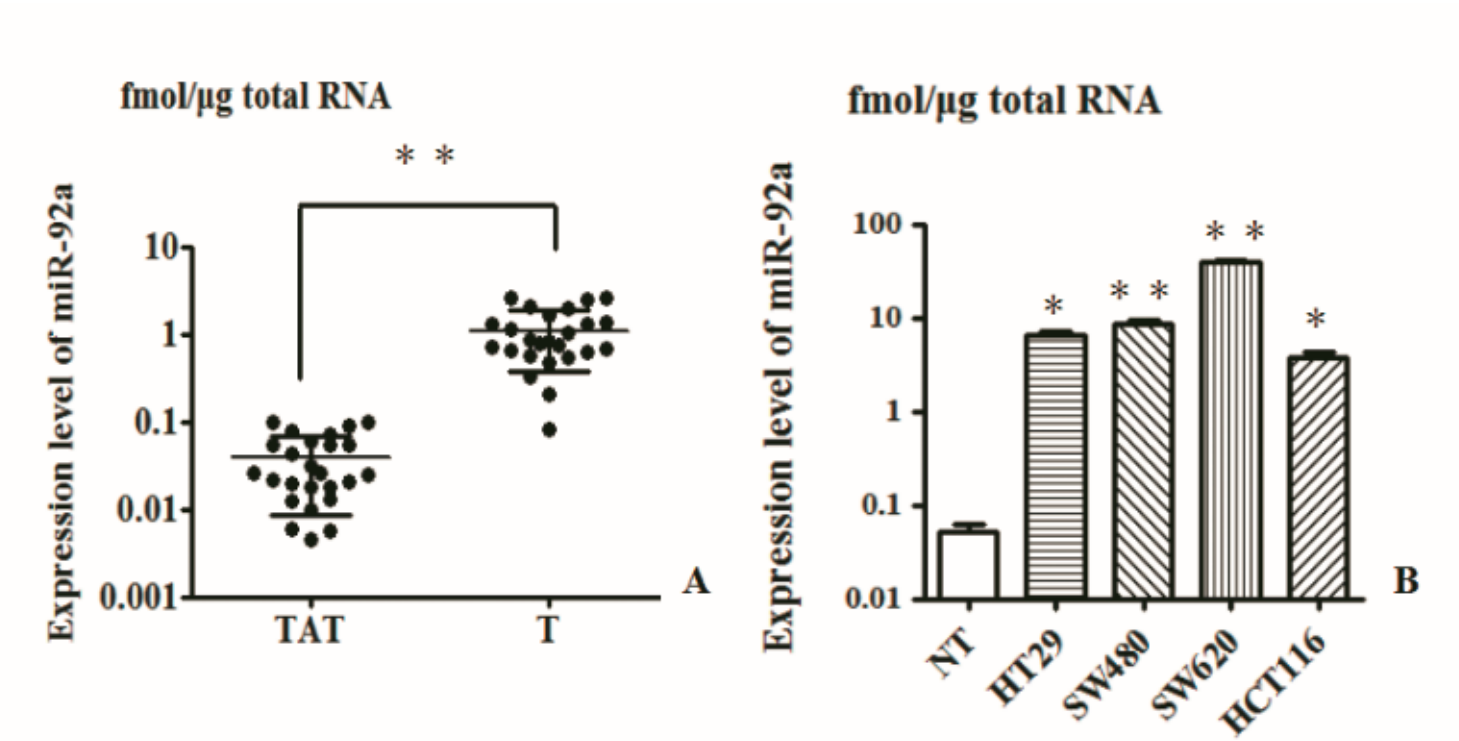


Figure 1

Expression of miRNA-92a in CRC tissues and cells. (A) Elevated miRNA-92a in CRC tumor tissue (T) compared to tissue adjacent to the cancer (TAT); **p < .01. (B) Elevated miRNA-92a levels in CRC cell lines compared to that in normal colorectal mucosa epithelial tissue (NT); *p < .05; **p < .01.

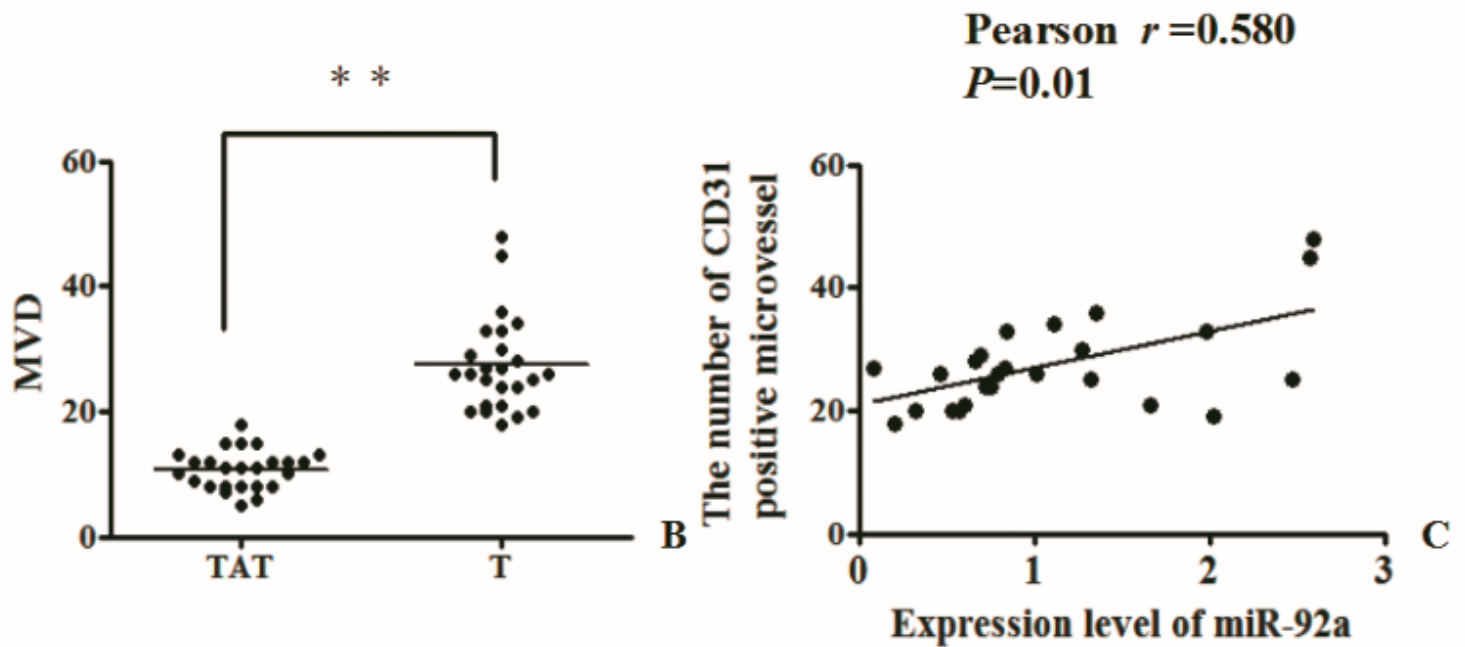
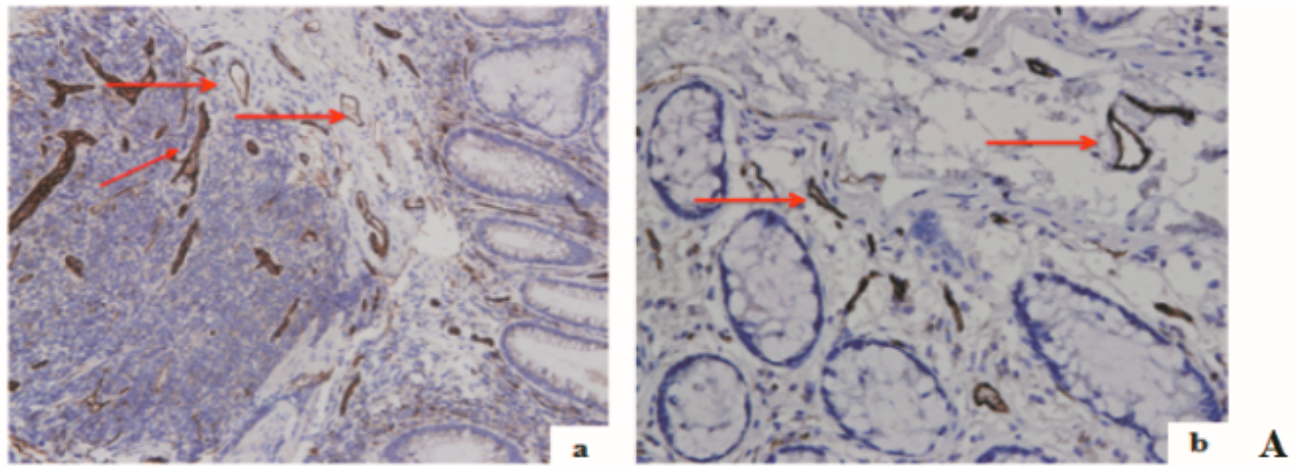


Figure 2

MiRNA-92a expression and tumor angiogenesis in CRC. (A) CD31-immunopositive microvessel (red arrows) in CRC (a) and adjacent (b) tissues (magnification, 400×). (B) Comparison of MVD (based on CD31 labeling) between CRC and adjacent tissues; ** $p < 0.01$. (C) Correlation between miRNA-92a expression and MVD in CRC tissues.

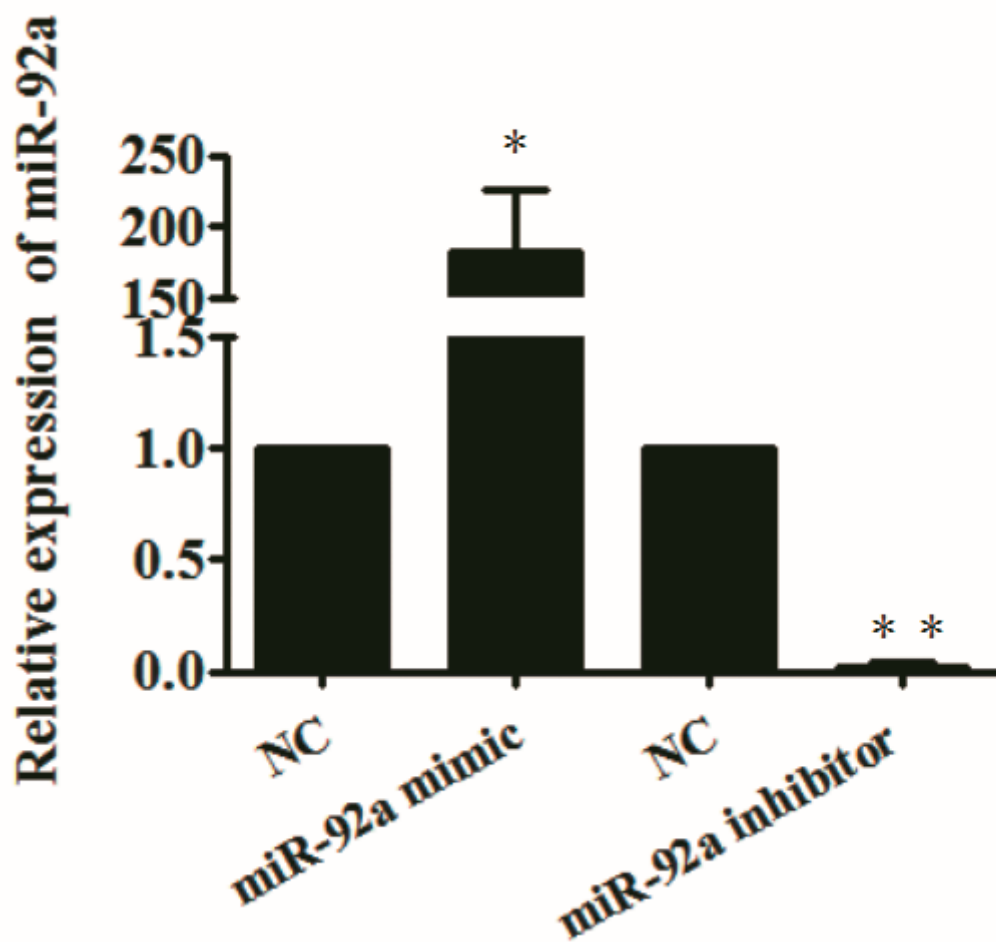


Figure 3

Expression of miRNA-92a in HCT116 cells and SW620 cells transfected with miRNA-92a mimic or inhibitor, respectively. NC, non-transfected control cells; * $p < .05$, ** $p < .01$.

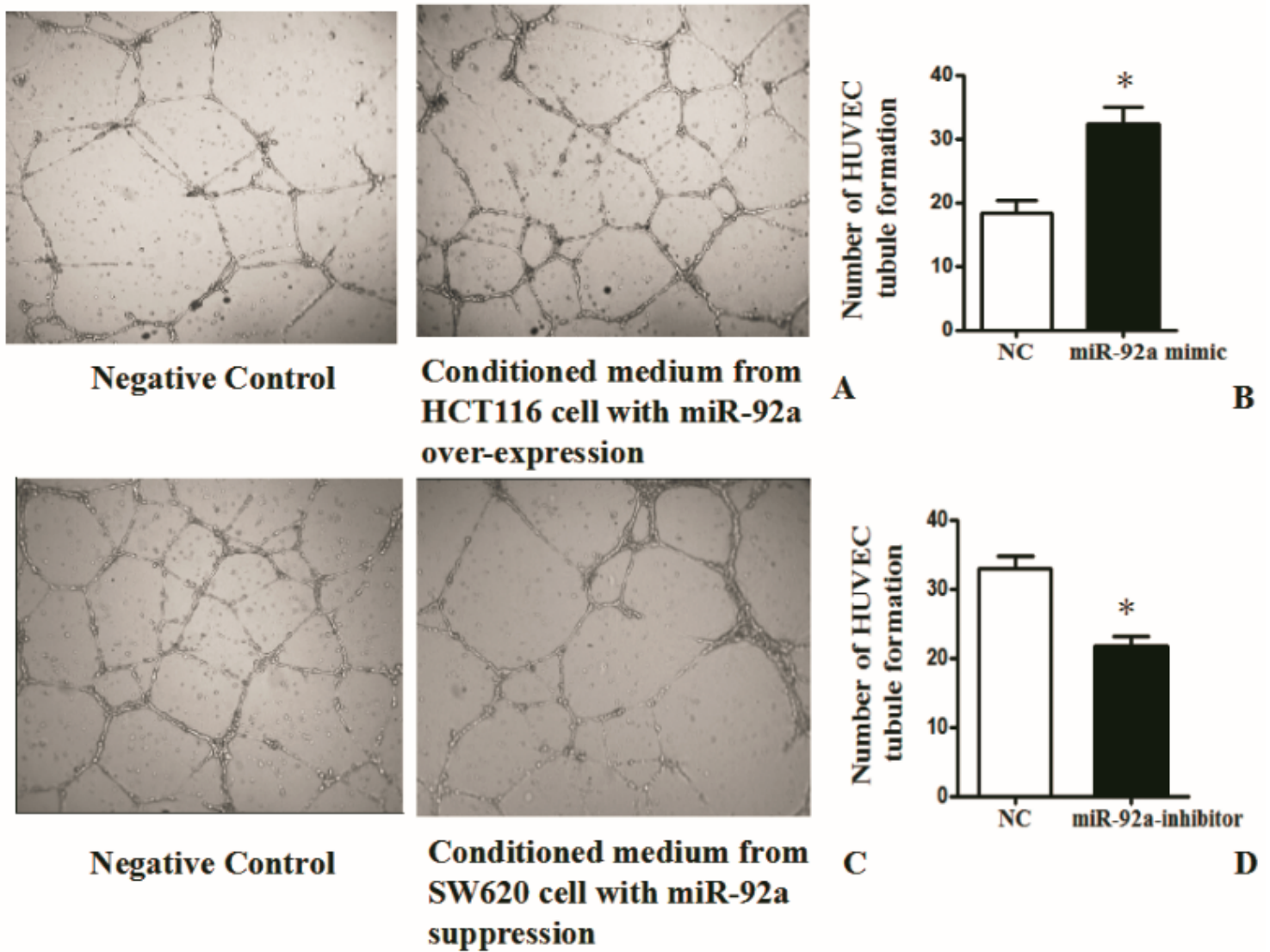


Figure 4

Effects of manipulating miRNA-92a levels on HUVEC tubule formation. (A) HUVEC tube formation in conditioned medium from miRNA-92a over-expressing HCT116 cells. (B) Quantitative analysis of the tube formation shown in panel A. (C) HUVEC tube formation in conditioned medium from miRNA-92a suppressing SW620 cells. (D) Quantitative analysis of the tube formation shown in panel A. In B and D, *p < .05 vs. non-treated controls (NC).

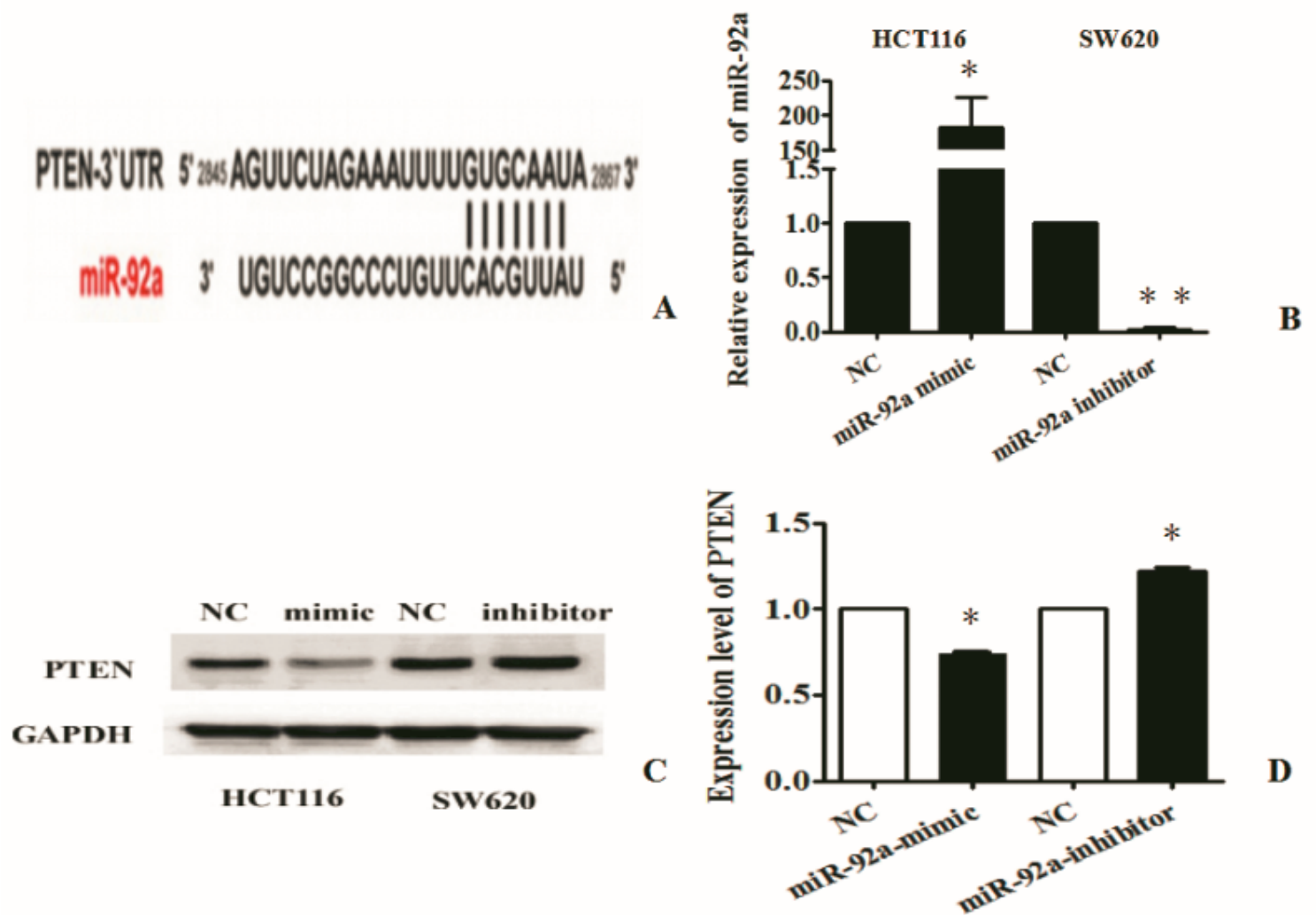


Figure 5

Effects of miRNA-92a manipulations on PTEN protein expression in CRC cells. (A) Targetscan-predicted binding site of miRNA-92a in the PTEN 3' untranslated region. (B) Relative miRNA-92a expression, determined by qRT-PCR, in CRC cells transfected with miRNA-92a mimic or inhibitor. (C) Immunoblot of PTEN protein with GAPDH loading control. (D) PTEN expression in CRC cells transfected with miRNA-92a mimic and inhibitor compared to non-transfected controls (NC); * $p < .05$; ** $p < .01$

Supplementary Files

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