Knockdown of metadherin inhibits angiogenesis in breast cancer

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Abstract. Angiogenesis plays an important role in cancer growth, invasion and metastasis. It has been confirmed that metadherin (MTDH) is associated with angiogenesis. However, the detailed mechanism of MTDH on angiogenesis has not yet been reported. In this study, we demonstrate the anti-angiogenic function of MTDH in breast cancer. With RNA interference strategies, we found that knockdown of MTDH inhibits cellular angiogenesis both *in vitro* and *ex vivo*. Furthermore, we revealed that ERK1/2 pathway is involved in the anti-angiogenic function of MTDH, and the function can be partially reversed via upregulation of microRNA-21 (miR-21). In conclusion, knockdown of MTDH can inhibit angiogenesis in breast cancer. These results show that MTDH is a viable therapeutic target for anti-angiogenesis in breast cancer.

Introduction

Breast cancer is the most frequently diagnosed cancer and leading cause of cancer deaths among women in the world. Currently, breast cancer is the most common cancer among women in China; new cases account for 12.2% and the mortality rate is 9.6% of all breast cancer patients worldwide (1). In 2013, a total of 232,340 cases of invasive breast cancer and 39,620 breast cancer deaths were reported among US women (2). Although the mortality has dropped over the past decades, distant metastasis is still a main cause of death among breast cancer.

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By supplying nutrients and providing the vascular route for haematogenous metastasis, vascular-dependent diseases such as breast cancer can be affected by angiogenesis (3-5). Since angiogenesis plays a pivotal role in breast cancer development, and seriously effects cancer cell invasion and metastasis, inhibition of tumor angiogenesis is considered as an attractive and effective strategy for the therapy of breast cancer (6). Angiogenesis is a complex process, which is regulated by different molecular pathways (7).

Metadherin (MTDH), as a novel multifunctional oncogene, originally identified in 2002 (8). In our previous studies, we have found that MTDH improves the invasiveness of breast cancer cells by inducing epithelial to mesenchymal transition, is involved in inflammation-induced tumor progression, modulates TRAIL-resistance in breast cancer cells through caspase-8 downregulation and Bcl-2 upregulation, mediates estrogen-independent growth and tamoxifen resistance through PTEN downregulation and that >40% tumors overexpress MTDH, which correlates with metastasis and poor-prognosis of breast cancer (9-13). MTDH is frequently overexpressed in tumor tissues and its expression level is associated with the progression and worse prognosis of malignant tumor such as hepatocellular carcinoma, lung cancer, bladder cancer, laryngeal squamous cell carcinoma and breast cancer (14-19) Therefore, knockdown of MTDH can significantly inhibit prostate cancer progression, sensitize endometrial cancer cells to cell death induction by TRAIL and sensitize breast cancer cells to AZD6244 (20-22). In this study, we explored the inhibition of angiogenesis through knockdown of MTDH in breast cancer and the potential of MTDH as a therapeutic target for anti-angiogenesis.

Materials and methods

Reagents. Antibodies against ERK1/2, p-ERK, PTEN, MMP-2 and VEGF were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-MTDH rabbit antibody was obtained from Invitrogen (Carlsbad, CA, USA). Anti-CD31 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. Breast cancer cell line MDA-MB-231 and human umbilical vein endothelial cells (HUVECs) were purchased

from American Type Culture Collection. They were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) at 37˚C and under 5% CO_2 incubator.

Plasmid construction and transfection. The MTDH knockdown plasmids were constructed as previously described (23). In brief, the 19-nt sequence 5'-ATGAACCAGAATCAGTCAGC-3' was used to construct MTDH shRNA. The 60-nt oligonucleotides were annealed and inserted into the pSUPER.retro.pure (OligoEngine, Seattle, WA, USA). According to the manufacturer's protocol, the MDA-MB-231 cells were transfected with Lipofectamine 2000 (Invitrogen). The shRNA interference vector was applied to establish the MDA-MB-231-prpM cell line and the empty vector was used to establish the MDA-MB-231-prpn cell line. Cells were selected with 0.5 μg/ml puromycin to generate stable cell lines. miR-21-mimics and the corresponding negative control (NC) (Gene Pharma, Shanghai, China) were used for upregulation of miR-21. Transiently transfected cells were harvested at 48 h for mRNA and at 72 h for protein analysis.

Quantitative real-time PCR. Total RNA was isolated using TRIzol by the manufacturer's protocol (Takara, Dalian, China). Total RNA was reverse transcribed to cDNA by applying Prime Script RT reagent kit (Takara). Quantitative RT-PCR was performed with the SYBR green detection (Takara) in Applied Biosystems StepOne Plus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The level of GAPDH was used as the endogenous control for detection of mRNA expression analysis and U6 was used for microRNAs. The $2^{-\Delta\Delta Ct}$ method was employed for data analysis.

Western blot analysis. Cells were harvested and lysed in ice-cold RIPA buffer (1X PBS, 1% NP40, 0.1% SDS, 5 mM EDTA, 0.5% sodium deoxycholate and 1 mM sodium orthovanadate) with protease and phosphatase inhibitors. The proteins were quantified using the BCA Protein Assay kit (Merck, Darmstadt, Germany). Same amount of protein was separated by 10% SDS-PAGE gel and then transferred onto PVDF membrane (Millipore, Bedford, MA, USA). After blocking with 5% non-fat dry milk, the PVDF membrane was first incubated overnight at 4˚C with primary antibodies, rinsed and incubated with the secondary antibodies. The corresponding signals were detected with enhanced chemiluminescence (ECL).

Tube formation assay. Tumor cell conditioned medium (TCM) without FBS was obtained as described (24). The 96-well plates were covered with 50 μl Matrigel and placed at 37˚C for 30 min to polymerize. The HUVECs were suspended using different TCM and 100 μl of HUVECs were added to each well. Then HUVECs were incubated for 7 h. Tube-like structures were photographed with an Olympus digital camera and macroscopic quantities were recorded by counting at least 10 microscopic fields.

Mouse aortic ring assay. Mouse aortas were dissected from BALB/c mice and cut into ~1-mm long sections as previously described (25). A 48-well plate was first covered with 100 μl of Matrigel and incubated for 30 min at 37˚C. The aortic rings were put into the wells and then covered with an overlay of 100 μl Matrigel, followed by addition of 200 μl of TCM. The cultures were kept at 37˚C in a humidified environment for a week and the result of the fields covered by sprouting from the aortic rings was examined with an Olympus microscope at appropriate magnification.

Immunohistochemistry (IHC). Seventy-seven breast cancer tissue samples were obtained from the Department of Pathology of Qilu Hospital of Shandong University from 2011 to 2014. To quantify the microvessel density (MVD), the SP-9000 Histostain™-Plus kits (Zhongshan Goldenbridge Biotechnology Co.) were used to detect CD31 expression following standard steps as previously described (26,27).

MicroRNA array analysis. Total RNA was isolated using TRIzol by the manufacturer's protocol. A microarray with 873 miRNA probes was designed in accordance with Sanger miRbase release 12.0. RNA labeling and hybridization were performed as previously described (28). After hybridization, microarrays were investigated by the LuxScan 10K Microarray Scanner (CapitalBio, Beijing, China), and the images were analyzed by GenePix Pro 6.0 software (Axon Instruments, Foster City, CA, USA). The data are available in the Gene Expression Omnibus (GEO).

Statistical analysis. Statistical software SPSS 18.0 was used. The data are shown as mean ± SD. The difference in statistics was analyzed through the Student's t-test and regarded as statistically significant for P-values <0.05.

Results

Establishment of the MTDH knockdown cell line. Due to the high level expression of MTDH in MDA-MB-231 cells, we first designed the short hairpin RNA and then transfected the plasmid into MDA-MB-231 cells to establish the MTDH knockdown cell line. The stable cell line was selected by adding puromycin to DMEM. MTDH expression levels were detected by qRT-PCR and western blot analysis. As shown in Fig. 1, MTDH expression levels were obviously lower in MDA-MB-231-prpM cells than that in control MDA-MB-231-prpn cells.

Knockdown of MTDH inhibits angiogenesis in vitro. To confirm whether the knockdown of MTDH could inhibit angiogenesis of breast cancer cells, a tube formation assay was performed as an important indicator of endothelial function in angiogenesis. HUVECs cultured on Matrigel rapidly align and finally form tube-like structures. Since the tube formation can be affected by different medium components, TCM obtained from different breast cells was added to HUVECs cultured on Matrigel and the tube-like structures were quantitatively investigated. Compared to the control MDA-MB-231-prpn cells, HUVECs cultured with TCM from MDA-MB-231-prpM cells caused an obvious decrease in tube formation, as shown in Fig. 2A.

Knockdown of MTDH inhibits angiogenesis ex vivo. To investigate the inhibition of angiogenesis *ex vivo*, we detected

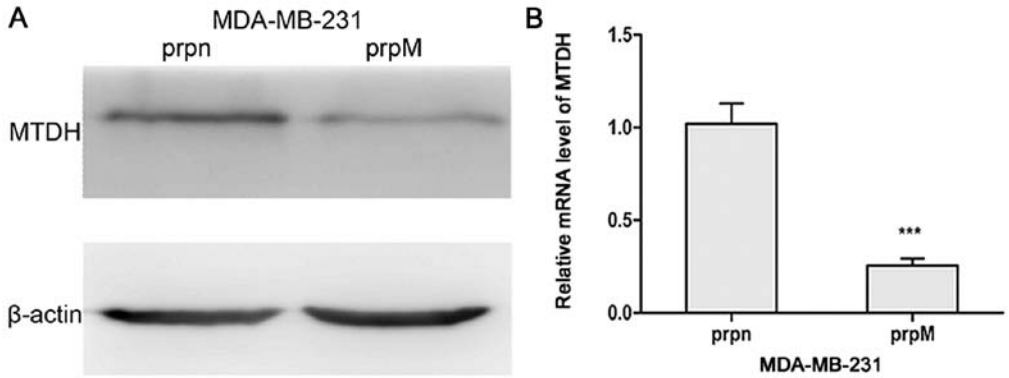

Figure 1. Knockdown of MTDH in the MDA-MB-231 cell line. (A) Protein levels of MTDH were validated through western blot analysis. (B) The mRNA levels of MTDH were validated by qRT-PCR. The panels show the data from three independent experiments. ***P<0.01.

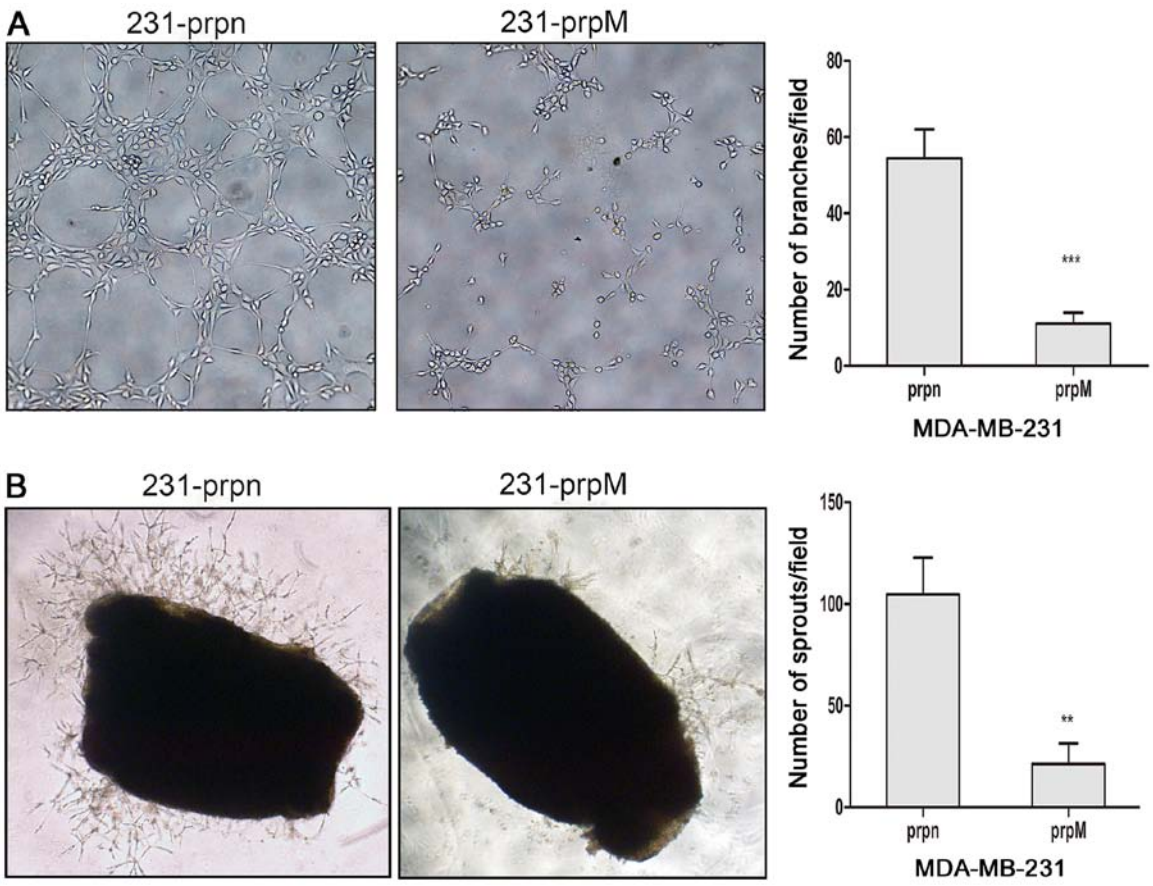

Figure 2. Knockdown of MTDH inhibited angiogenesis. (A) HUVECs were suspended using different TCM and incubated on Matrigel-covered 96-well plates. Tube-like structures were assessed after 7 h. Original magnification, x100. The bar graph shows the number of tube-like structures. (B) Mouse aortic rings were cultured in different TCM. The images of sprouting at day 7. Original magnification, x100. The bar graph shows the number of sprouts. Data are from three independent experiments. **P<0.05. ***P<0.01.

the sprouting of vessels from mouse aortic rings. Mouse aortas cultured in Matrigel were treated with TCM from MDA-MB-231-prpM cells and the control group. The effect on angiogenesis of MTDH was demonstrated through comparing the fields covered by sprouting from the aortic rings. As shown in Fig. 2B, the knockdown of MTDH significantly inhibited the formation of microvessel structures around the mouse aortic rings.

The knockdown of MTDH downregulates the expression of p-ERK1/2 and decreases the levels of VEGF and MMP2. ERK1/2 molecule was widely regarded as a signal pathway activator of angiogenesis (29,30). Therefore, the protein levels of ERK1/2 and p-ERK1/2 were monitored in our test to explore a potential mechanism of action. As shown in Fig. 3, the knockdown of MTDH downregulated the expression of p-ERK1/2 in western blot analysis. Because p-ERK1/2 could

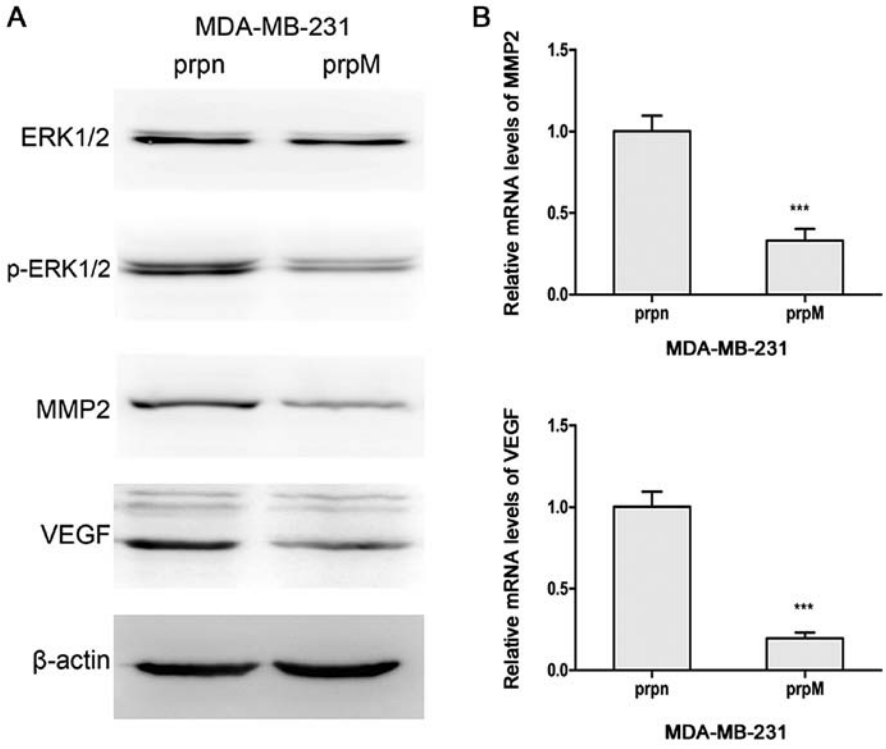

Figure 3. Knockdown of MTDH inhibits the ERK1/2 pathway in MDA-MB-231 cells. (A) The protein levels of ERK1/2 p-ERK1/2 MMP-2 and VEGF was analyzed using western blot analysis. β-actin was employed as the loading control. (B) The mRNA levels of MMP-2 and VEGF were assessed by qRT-PCR. ***P<0.01.

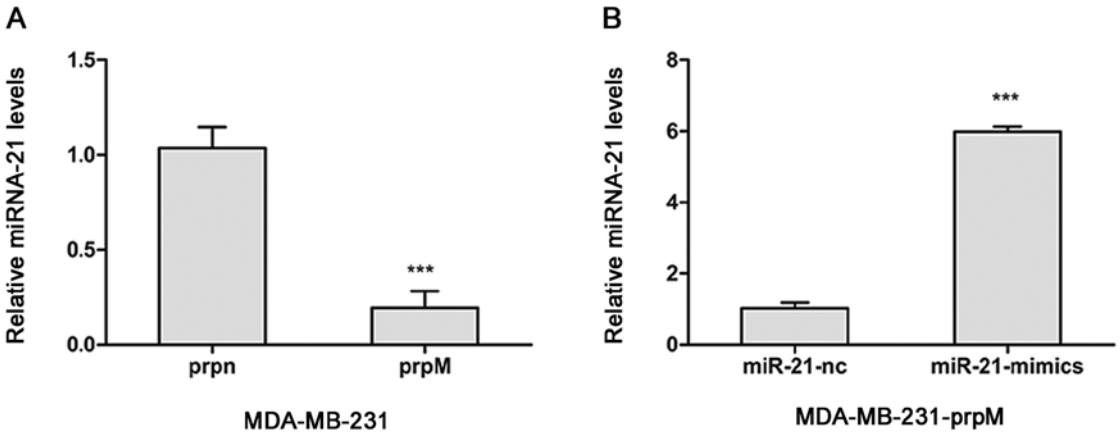

Figure 4. miR-21 levels were assessed using qRT-PCR. (A) The miR-21 levels were detected in MDA-MB-231-prpM cells and control. (B) The miR-21 levels were detected after transfection of miR-21-mimics in MDA-MB-231-prpM cells.

regulate angiogenesis through MMP-2 and VEGF (31), we measured the mRNA levels and protein levels of MMP-2 and VEGF in MTDH knockdown cells. The levels of the two markers were significantly reduced in MDA-MB-231-prpM cells.

miR-21-mimics increase miR-21-inducing ERK1/2, MMP2 and VEGF expression, and promote tumor angiogenesis. To investigate the influence of MTDH knockdown on the inhibition of angiogenesis, by regulating levels of miRNAs, the miRNA arrays was adopted to detect the changes of miRNAs after MTDH knockdown. From the miRNA array data, we found MTDH regulated miRNA expression in MDA-MB-231-prpM cell (data not shown). Among them, miR-21 level was significantly decreased. To investigate whether miR-21 was involved in the inhibition of angiogenesis, we transected miR-21-mimics with Lipofectamine 2000 into MDA-MB-231-prpM to upregulate the level of miR-21. As shown in Fig. 4, the level of miR-21 obviously increased. Then, we applied tube formation assay and mouse aortic ring assay to explore the role of miR-21 in angiogenesis. The results showed that upregulated expression of miR-21 could partially reverse the inhibition of angiogenesis in MDA-MB-231-prpM cells (Fig. 5). Previous studies indicated that miR-21 regulated expression of PTEN (32,33). Therefore, we detected PTEN and p-PTEN in miR-21 mimic-transfected MDA-MB-231-prpM cells. As shown in

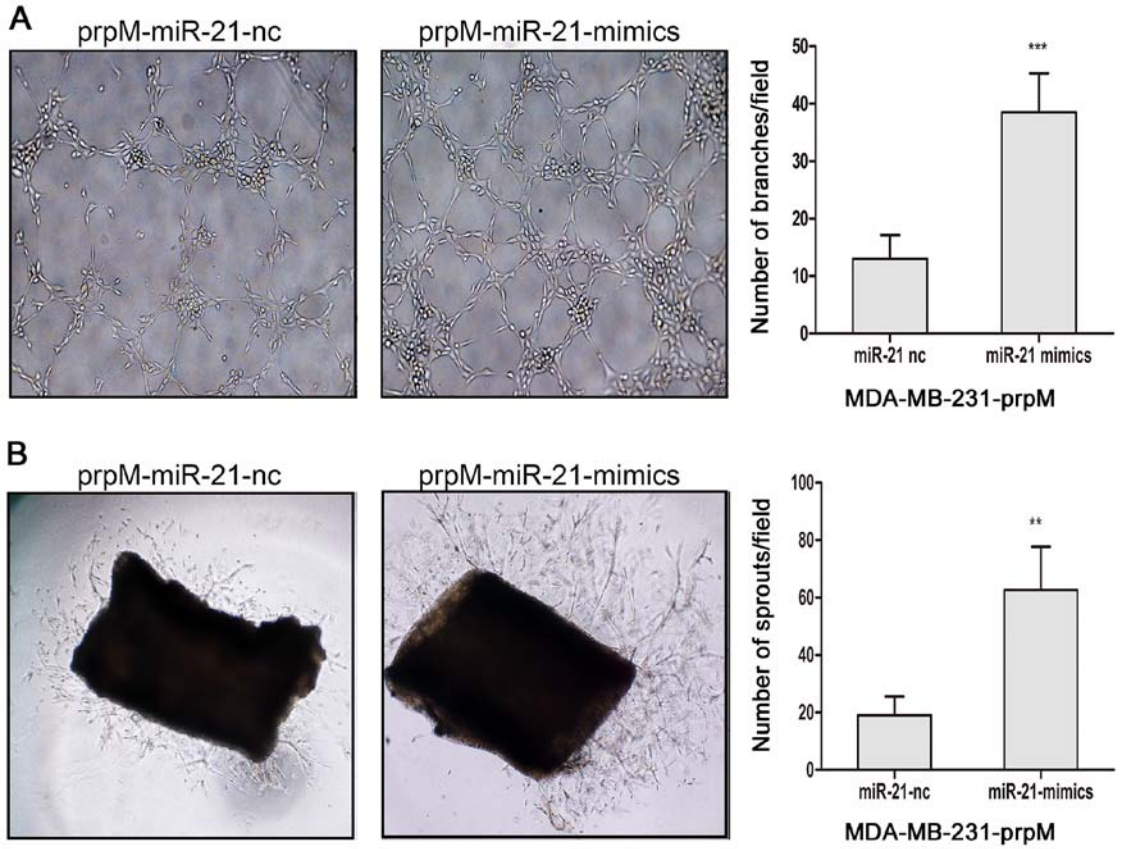

Figure 5. Transfection of miR-21-mimics partially reversed the inhibition of angiogenesis in MAD-MB-231-prpM cells. (A) Effect of the miR-21-mimics on the tube formation of HUVECs. Tube-like structures were assessed after 7 h. Original magnification, x100. The bar graph shows the number of tube-like structures. (B) Effect of the miR-21-mimics on the aortic ring assay. The bar graph shows the number of sprouts. Original magnification, x100. Data are from three independent experiments. **P<0.05. ***P<0.01.

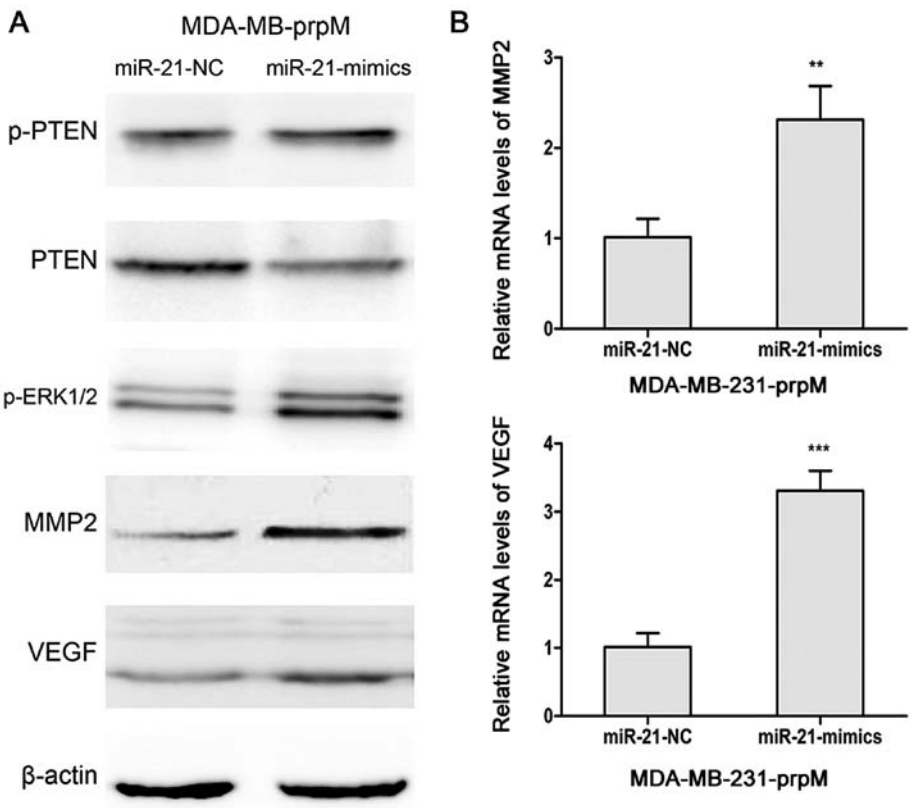

Figure 6. The transfection of miR-21-mimics promotes the ERK1/2 pathway in MDA-MB-231-prpM cells. (A) The protein levels of PTEN ERK1/2 p-ERK1/2 MMP-2 and VEGF were analyzed using western blot analysis. (B) The mRNA levels of MMP-2 and VEGF were measured using qRT-PCR. ***P<0.01.

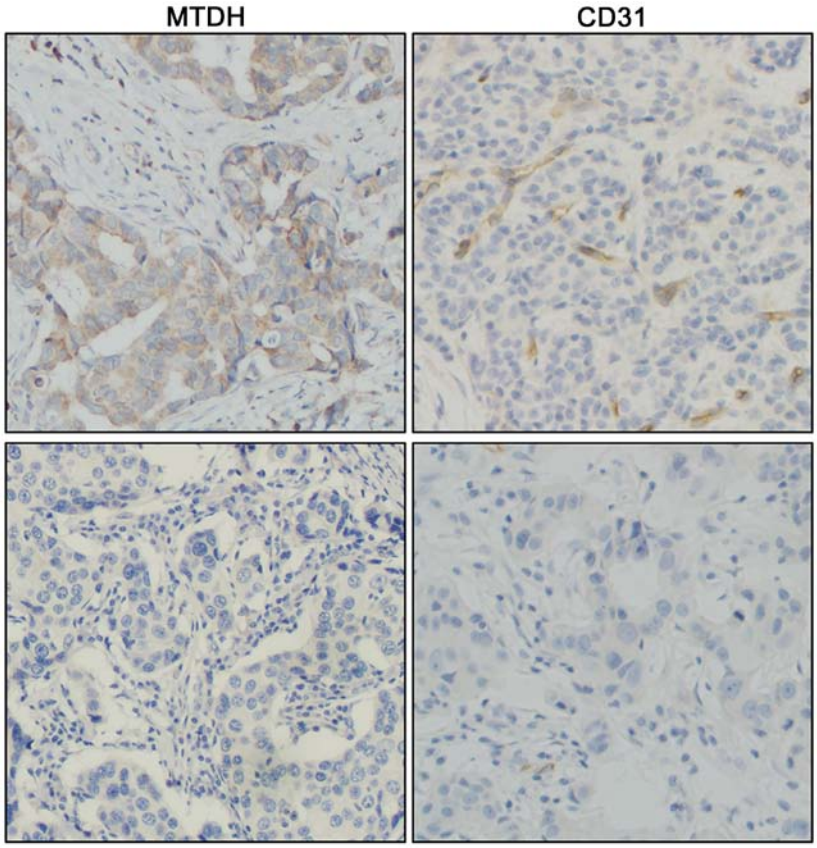

Figure 7. Sections of breast cancer samples were stained for MTDH and CD31. Representative images of sections with low expression of MTDH and CD31 (bottom), the sections with high expression of MTDH and CD31 (top).

Fig. 6, upregulated expression of miR-21 increased the protein level of p-ERK1/2 via suppressing PTEN, and then increased the levels of MMP2 and VEGF.

Lower MVD is linked with low expression of MTDH. To confirm the relationship between MTDH and angiogenesis in breast cancer tissue, we stained 77 breast cancer tissue samples with the MTDH and CD31 antibody. As shown in Fig. 7, 43 cases were CD31-positive in the 61 MTDH-positive cases in total. Of these, 6 cases were CD31-positive in the total 16 MTDH-negative staining cases. Therefore, compared to the MTDH overexpressing tissues, the result showed that low expression of MTDH was linked with lower MVD. The data were evaluated with the χ^2 test (P=0.032).

Discussion

Angiogenesis is mediated by multiple molecules including vascular endothelial growth factor (VEGF), epiderma1 growth factor (EGF), epidermal growth factor receptor (EGFR) and matrix metalloproteinases (MMPs).

Due to the increased secretion of pro-angiogenic factors, malignant cells become more angiogenic originating from the activation of the oncogene or inactivation of the tumor suppressor gene. For example, in breast cancer, P53 mutations promote angiogenesis by upregulation of EGFR (34); activated RhoA promotes VEGF expression and angiogenesis by decrease of P53 stability (35); 53BP1 inhibits angiogenesis by decrease of MMP2 and MMP9 (36). Previous studies demonstrated that MTDH as an oncogene promotes invasion and metastasis of malignant cells. In triple-negative breast cancer, it was reported that MTDH correlates with angiogenesis and worse clinical outcomes through immunohistochemical staining of 125 specimens. However, the mechanism was not clarified (37).

To explore the function of the knockdown of MTDH to modulate angiogenesis in breast cancer, the expression of MTDH was manipulated with RNA interference in MDA-MB-231 cells. Our results showed that the knockdown of MTDH was able to suppress tube formation of HUVECs and sprouting of the mouse aortic rings. To further investigate the potential molecular mechanism of the knockdown of MTDH in inhibition of angiogenesis, we focused on the ERK1/2 signaling pathway, which is essential in cell proliferation, differentiation, apoptosis and angiogenesis (29,38,39). The knockdown of MTDH downregulated the level of p-ERK1/2 in MDA-MB-231-prpM cells, and then decreased the levels of MMP2 and VEGF. These results demonstrated that the knockdown of MTDH is an effective method of anti-angiogenesis in breast cancer and p-ERK1/2 signaling is an essential event in this process.

MicroRNAs (miRNAs), 20-25 nucleotides non-protein-coding RNAs, have been proven to be involve in regulation of gene expression (40). The function of miRNAs have been confirmed not only in many biological processes but also in various pathological situations including cancer (41).

Angiogenesis is a key process in cancer development, and the process can also be regulated by many miRNAs such as miR-29b in hepatocellular carcinoma, miR-18a in gastric cancer, miR-497 in ovarian cancer, miR-1246 in colorectal cancer cells, and miR-21 in prostate cancer through different molecular pathways (24,33,42-44). As one of the best-evaluated miRNA, miR-21 has been reported as an oncogene (45). Knockdown of miR-21 can inhibit angiogenesis in VEGFR2-luc mouse breast tumor model and reverse EMT by targeting PTEN in breast cancer cells (46,47). The significant decrease of miR-21 expression was confirmed by microRNA array and quantitative PCR method in MAD-MB-231-prpM cells. Our data showed that the upregulated expression of miR-21 could partially reverse the inhibition of angiogenesis through the ERK1/2 pathway activation in MAD-MB-231-prpM cells, MTDH knockdown inhibited angiogenesis via decreasing the expression level of miR-21.

To further validate the relation of MTDH and angiogenesis in breast cancer samples, we evaluated the angiogenic markers CD31 by using immunohistochemistry. The result showed that MTDH was related with CD31 and the CD31 decreased in samples with the lower expression of MTDH, thus, demonstrating that low expression of MTDH suppressed angiogenesis in breast cancer.

Anti-angiogenesis is considered to be a prospective novel therapeutic strategy for malignant tumors. However, to find an efficient target gene or molecule is still an unsolved problem. As an important oncogene, MTDH plays an important role in tumor progression and prognosis. In this study, we provided evidence that the knockdown of MTDH significantly inhibited angiogenesis. Our findings demonstrate that MTDH is a potential therapeutic target for anti-angiogenesis in breast cancer.

Acknowledgements

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