Epigenetic silencing of miR-126 contributes to tumor invasion and angiogenesis in colorectal cancer

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Received June 7, 2013; Accepted July 5, 2013

DOI: 10.3892/or.2013.2633

Abstract. microRNAs (miRNAs) have been reported to play a crucial role in regulating a variety of genes pivotal for tumor metastasis. miR-126 is well known as one of the angiogenesis regulatory miRNAs. Recent studies have reported controversial roles of miR-126 in tumor progression. In this study, we sought to investigate the potential roles of miR-126 in colorectal cancer (CRC). By real-time PCR, miR-126 was shown to be downregulated in primary CRC tissues and cell lines. Restoration of miR-126 in CRC cells inhibited cell growth, migration and invasion. Using both *in silico* prediction and immunoblotting, we found that vascular endothelial growth factor (VEGF) was a target of miR-126. The interaction of miR-126 on the 3'UTR of VEGF mRNA was validated by luciferase reporter assay. Mechanistically, we found that the silencing of miR-126 was induced by promoter methylation of its host gene, EGFL7. Treatment with 5-aza-CdR restored miR-126 expression and thereby led to a decline in VEGF expression. Functionally, due to suppression of VEGF, enhanced miR-126 expression inhibited tumor neovasculature triggered by CRC cells. In conclusion, our findings suggest that DNA methylation-induced silencing of miR-126 contributes, at least in part, to tumor invasion and angiogenesis in CRC, through upregulation of VEGF expression. miR-126 may be a potential target for the therapeutic strategy against CRC.

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Abbreviations: VEGF, vascular endothelial growth factor; UTR, untranslated region; miRNA, microRNA; ELISA, enzyme-linked immunosorbent assay

Key words: miR-126, invasion, tumor angiogenesis, colorectal cancer, methylation

Introduction

Colorectal cancer (CRC) is the third most common cancer and the leading cause of cancer-related mortality worldwide (1), with a high potential for tumor invasion and metastasis. Similar to many other solid malignancies, metastasis of CRC involves proteolysis of the extracellular matrix, alterations in tumor cell adhesion and motility, and colonization in distant organs (2). In addition, formation of new blood vessels is also indispensable for the persistence of metastastic growth. Despite the obvious importance of metastasis, the molecular mechanism underlying these processes remains unclear.

Growing evidence has recently supported the cancer-related effects of microRNAs (miRNAs), a newly identified class of small non-coding RNA molecules which function through negatively regulating target gene expression. They can bind to specific complementary sites within the 3' untranslated regions (3'UTRs) of their target mRNA, to inhibit translation or to induce degradation, even to regulate mRNA transcription (3-5). Recent studies have revealed the critical role of miRNAs in regulating a variety of genes pivotal for invasion or metastasis (6,7).

miR-126, which locates within intron 7 of the epidermal growth factor-like domain 7 gene (EGFL7), is highly expressed in endothelial cells. Two studies have reported its crucial role in promoting embryonic angiogenesis by promoting vascular endothelial growth factor (VEGF) signaling in zebrafish (8) and in a mouse model (9). In contrast, miR-126 was identified to be one of the dysregulated miRNAs in multiple cancer types. Its downregulation was observed in breast, pancreatic and gastric cancer (10-12). Low expression of miR-126 in non-small cell lung cancer and renal cell carcinoma was significantly correlated with reduced patient survival (13,14). Through targeting oncogenic genes, such as SLC7A5, Crk and ADAM9, miR-126 plays a role as a tumor suppressor to inhibit tumor cell proliferation, invasion and the EMT process (11,15,16). Conversely, some recent studies have presented an oncogenic role of miR-126. They found that overexpression of miR-126 may contribute to gastric carcinogenesis by inhibiting SOX2 expression (17), and was highly associated with metastasis of prostate cancer (18). These contradictory results imply that miR-126 may function

by diverse mechanisms in different physiological and pathological contexts.

VEGF has commonly been acknowledged as the most prominent factor to promote tumor development (19). It operates to increase the permeabilization of blood vessels and induces formation of new blood vessels, to facilitate orthotopic and metastatic growth. In CRC, VEGF signaling involved neovascularity represents a key mediator of tumor initiation and dissemination (20).

Quite recently, studies have shown decreased expression of miR-126 in CRC (21), and that DNA methylation results in the silencing of miR-126 in bladder and lung cancer (22,23). Of specific note, *in silico* prediction indicates that miR-126 has a conserved binding site within the 3'UTR of VEGF mRNA. We thus hypothesized that overexpression of VEGF in CRC is, at least partly, due to silencing of miR-126 caused by DNA methylation.

Materials and methods

Tissue samples and cell lines. Twelve pairs of primary CRC and matched adjacent normal colonic epithelium, and 62 primary CRC tissues were collected. All samples were obtained from patients who underwent surgical resection at Nanfang Hospital (Guangzhou, China) and were snap-frozen in liquid nitrogen, and stored at -80˚C for further use.

HEK293 cell line and 6 human CRC cell lines, including LoVo, HT29, SW480, SW620, SW1116 and HCT116, were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained routinely in RPMI-1640 (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA) and cultured at 37˚C in a 5% CO_2 atmosphere.

TaqMan real-time-PCR analysis of miR-126 expression. TaqMan real-time-PCR was performed to detect mature miR-126 expression in tissue sample and cell lines. Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Expression of mature miR-126 was determined by the TaqMan miRNA assay (Applied Biosystems, Foster City, CA, USA). Data were processed using the $2^{-\Delta\Delta Ct}$ method. RNU6B (Ambion, Austin, TX, USA) was used as an endogenous control.

Ectopic miR-126 expression. Enforced expression of miR-126 in LoVo and SW620 cells was achieved by transfection with pre-miR-126 (Ambion). Cells were seeded in 6-well clusters or 96-well plates for 24 h and transfected with 30 nM pre-miR-126 using Lipofectamine 2000 (Invitrogen Life Technologies) for 24 or 48 h. Scramble precursor (Ambion) was used as a negative control. Transfected LoVo and SW620 cells were used in further functional assays or for RNA/protein extraction.

Cell proliferation assay. Alamar blue assay (Invitrogen Life Technologies) was conducted to measure cell proliferation. Cells were seeded in a 96-well plate at $0.5x10^4$/well for 24 h, then transfected with pre-miR-126 or scramble control. The transfected cells were incubated for 24, 48 and 72 h, respectively. Ten microliters of Alamar blue reagent was added to each well at 2 h before the end of the incubation. Following the incubation, the absorbance of each well at 570 nm (600 nm as reference wavelength) was determined using a microplate reader.

Cell invasion and migration assays. The invasive potential of LoVo and SW620 cells was evaluated using a cell invasion assay kit (Millipore, Billerica, MA, USA) following the manufacturer's instructions. Briefly, transfected cells were resuspended in serum-free RPMI-1640 medium at a density of $1.0x10^6$/ml. Cell suspension (300 μl) and 500 μl RPMI-1640 containing 10% FBS were respectively added to each insert and the matched lower chamber. After 48 h, non-invading cells were removed using a cotton swab, and then the underside of the insert was stained. Six random fields (at a magnification of x100) for each insert were counted. For the migration assay, the procedures were similar to those of the invasion assay, except that 200 μl cell suspension was cultured in each Transwell upper insert (Corning Incorporated, Corning, NY, USA) for 48 h. For human microvascular endothelial cell (HMVEC) migration, $1.0x10^5$ transfected LoVo cells were seeded in the lower chamber and incubated for 24 h. Then $5x10^4$ HMVECs were added in the matched upper insert, and co-cultured with LoVo cells for 4 h. The underside of the insert was stained. Six random fields (at a magnification of x100) for each insert were counted.

Western blot analysis. Immunoblotting was performed to detect the expression of VEGF in CRC cell lines after transfection. Protein (30 μg) was loaded onto a SDS-PAGE gel, transferred onto a PVDF membrane and subsequently probed with 1:1,000 diluted mouse monoclonal VEGF-A antibody (Cell Applications, San Diego, CA, USA) at 4˚C overnight, followed by incubation with HRP-conjugated secondary antibody. Signals were visualized using ECL substrates (Millipore). β-actin was used as an endogenous protein for normalization.

ELISA assay. An ELISA kit (Cusabio, China) was used to detect the VEGF concentration in the cell supernatant following the manufacturer's instructions.

Luciferase reporter assay. The full-length 3'UTR of VEGF (1923 nt) containing one miR-126 potential binding site was amplified by PCR using the following primers: VEGF-A forward, 5'-CCGctcgagGCCGGGCAGGAGGAAGGAG-3' and reverse, 5'ATAAGAATgcggccgcTGAGATCAGAATT AAATTCTTTAATAC-3'. The PCR product was subcloned into a psiCHECK-2 vector (Promega Corporation, Madison, WI, USA) immediately downstream to the luciferase gene sequence. A psiCHECK-2 construct containing 3'UTR of VEGF with a mutant seed sequence of miR-126 was also synthesized using the primers: mutVEGF-A forward, 5'-AAG AGAAAGTGTTTATATATCGATCTTATTTAATATCCC TTTTTA-3' and reverse, 5'-TAAAAAGGGATATTAAATAA GATCGATATATAAAACACTTTCTCTT-3'.

All constructs were verified by DNA sequencing. HEK293 cells were plated in 24-well clusters, then co-transfected with 500 ng constructs with or without miR-126 precursors. At 48 h after transfection, luciferase activity was detected using a dual-luciferase reporter assay system (Promega Corporation) and normalized to *Renilla* activity.

In vitro and in vivo angiogenesis assays. *In vitro* and *in vivo* angiogenesis assays were conducted to determine the potential of miR-126 to affect tumor vascularity. For the *in vitro* Matrigel tube formation study, HMVECs were seeded in a 96-well plate pre-coated with growth factor-reduced Matrigel (BD Biosciences). Conditioned medium (CM) obtained from miR-126 precursor or scramble control transfected cells was added, followed by incubated at 37˚C for 12 h. The capacity of tube formation was assessed by counting the tubes in 3 randomly chosen fields under an inverted microscope (at a magnification of x100). The tubes were defined as structures formed by 2 identifiable HMVECs connecting at both ends. *In vivo*, the chorioallantoic membrane (CAM) model was performed as previously described (24).

5-Aza-2'-deoxycytidine (5-aza-CdR) treatment. For the demethylation study, the 6 CRC cell lines were treated with 5-Aza-CdR (Sigma) at a concentration of 3 μM for 72 h, replacing the medium and the drug every 24 h.

DNA methylation analysis. To establish the methylation status of miR-126 CpG islands, we performed 2 types of PCR analysis of bisulfate-modified genomic DNA (Active Motif, Carlsbad, CA, USA). First, the methylation-specific PCR (MSP) of CRC cell lines was conducted using methylated and unmethylated primers: M forward, 5'-TTTAAGTTATTTTTTTTAGGTTCGG-3' and reverse, 5'-ATTATATAACCTCCTCCTAAAACGC-3'; U forward, 5'-TTTAAGTTATTTTTTTTAGGTTTGG-3' and reverse, 5'-TTATATAACCTCCTCCTAAAACACC-3'.

PCR products were subjected to 2.5% agarose gel electrophoresis and visualized by ethidium bromide staining and UV transillumination. Second, bidirectional bisulfite sequencing (BSP) was used to analyze the corresponding CpG islands. PCR products were subcloned into the pMD®18-T vector (Takara, China), 3 candidate clones were selected and sequenced. The primers used were as follows: forward, 5'-GTGTGGTTAGGGGTTGTGTT-3' and reverse, 5'-CACACCCAATACTCAAAAAATTTC-3'.

Statistical analysis. All data from 3 independent experiments are expressed as means ± SD and processed using SPSS 13.0. The expression of miR-126 in CRC tissues and matched adjacent colonic epithelium was compared by paired t-test. The difference between the experimental groups and control was estimated by one-way ANOVA. A P-value of <0.05 was considered to indicate a statistically significant result.

Results

miR-126 is commonly downregulated in CRC. We performed real-time PCR using TaqMan probe to detect the endogenous miR-126 level in primary CRC tissues and cell lines. miR-126 was significantly decreased in all of the 12 CRC tissues when compared to their matched adjacent normal colonic tissues (Fig. 1A). We extended the test to 6 human CRC cell lines: LoVo, SW620, SW480, SW1116, HT29 and HCT116. These 6 cell lines showed notable loss of miR-126, whereas the control normal colonic mucosa pooled from 3 individuals expressed a strong level of miR-126 (Fig. 1B).

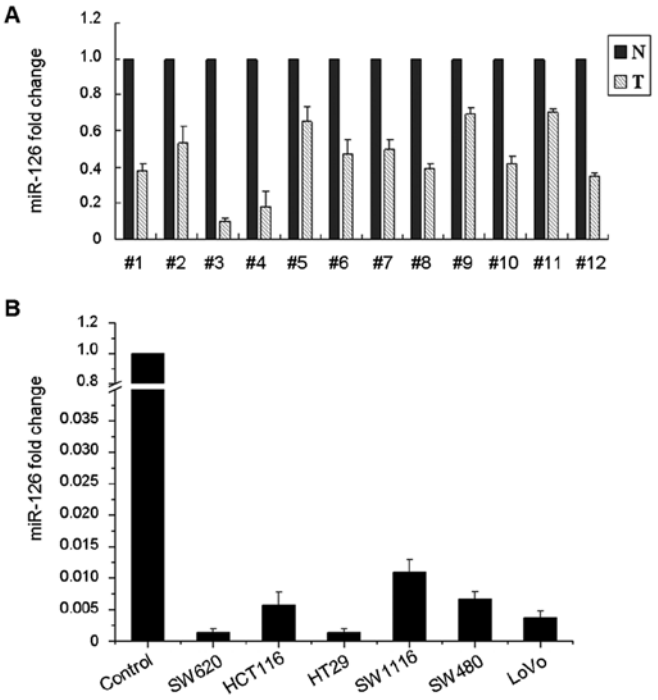

Figure 1. Decreased miR-126 expression in CRC tissues and cell lines. (A) TaqMan real-time PCR results for the fold change of miR-126 in 12 CRC tissues compared to their matched adjacent normal colon epithelium. (B) TaqMan real-time PCR results for the fold change of miR-126 in 6 CRC cell lines relative to the normal colon epithelium pooled from 3 healthy individuals. Results were normalized to RU6B. CRC, colorectal cancer.

miR-126 directly suppresses the expression of VEGF at the post-transcriptional level. Using *in silico* prediction database, we hypothesized that VEGF is a potential target gene of miR-126 (Fig. 2A). We initially tested the VEGF expression profile in 6 CRC cell lines. All of the cell lines exhibited notable expression of VEGF (Fig. 2B). LoVo and SW620 cells were selected to verify our hypothesis. Both LoVo and SW620 cells were transfected with pre-miR-126 to restore miR-126 expression. Restoration of miR-126 in the cell lines significantly suppressed VEGF protein expression (Fig. 2D). However, no alteration in VEGF mRNA was observed by qPCR (data not shown). This indicates that miR-126 may target VEGF at the translational level.

To further confirm whether the inhibition of VEGF is due to the interaction between miR-126 and the putative binding site in 3'UTR of VEGF mRNA, we constructed a luciferase reporter vector with the putative VEGF 3'UTR binding site for miR-126 and its mutant version by site direct mutagenesis (Fig. 2A). We transfected the luciferase reporter vector alone or together with pre-miR-126 or scramble control into HEK293 cells. A significant decrease in luciferase activity was noted when the VEGF 3'UTR vector was co-transfected with pre-miR-126, compared to the mutant vector, whereas the miR-126-mediated suppression of luciferase activity was abolished in the mutant VEGF 3'UTR vector (Fig. 2C). These results confirm a direct interaction of miR-126 on VEGF 3'UTR.

miR-126 inhibits CRC cell growth, migration and invasion. We performed gain of function assays to validate whether

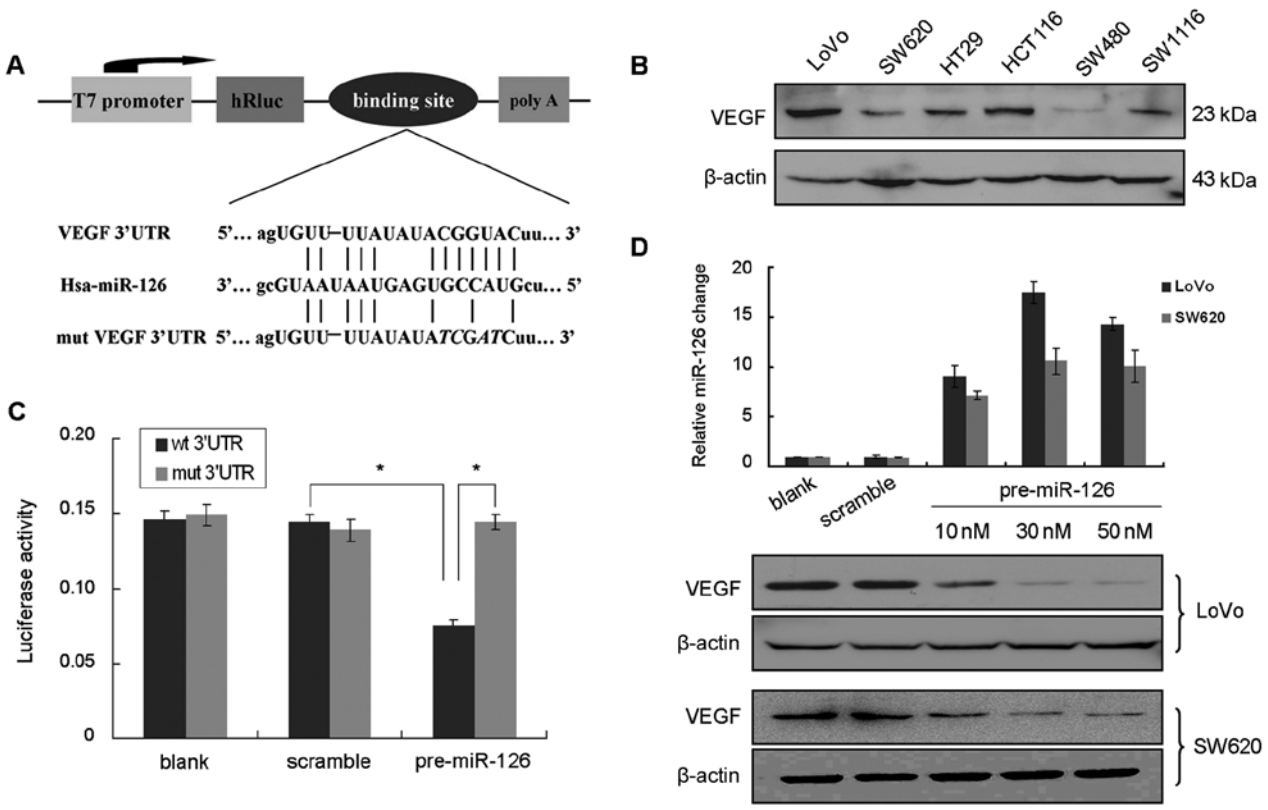

Figure 2. miR-126 suppresses VEGF expression by directly binding to its 3'UTR. (A) The wild-type (wt) VEGF 3'UTR and mutated (mut) VEGF 3'UTR were subcloned into psiCHECK-2 luciferase vector. The predicted miR-126 binding site within VEGF 3'UTR is shown. The mutated binding site is represented in italics. (B) Variable VEGF expression in 6 CRC cell lines as determined by western blotting. β-actin was used as the loading control. (C) The suppression of luciferase activity by VEGF 3'UTR was dependent on miR-126. Mutated VEGF 3'UTR abrogated miR-126-mediated suppression of luciferase activity (*P<0.05). (D) Restoration of miR-126 by pre-miR-126 transfection significantly suppressed VEGF protein expression in LoVo and SW620 cells. VEGF, vascular endothelial growth factor; 3'UTR, 3' untranslated region; CRC, colorectal cancer.

miR-126 regulates cellular processes, including cell growth, migration and invasion. Restoration of miR-126 in LoVo and SW620 cells significantly inhibited cell growth (Fig. 3A). In the Transwell migration assay, restoration of miR-126 in LoVo cells apparently impaired cell migration and invasion when compared to the blank and scramble control (Fig. 3B). Similar results were also observed in parallel assays using SW620 cells (Fig. 3C).

miR-126 exerts anti-angiogenenic effects on CRC. We investigated whether miR-126 regulates tumor vasculature via mediation of VEGF expression. First, ELISA assay was performed to detect VEGF secretion by CRC cell lines (Fig. 4A) and the concentration of VEGF in conditioned medium (CM) obtained from pre-miR-126-transfected LoVo cells (Fig. 4B). Restoration of miR-126 resulted in decreased VEGF secretion by LoVo cells to the culture medium (Fig. 4B). When human microvascular endothelial cells (HMVECs) were co-cultured with pre-miR-126-transfected LoVo cells in the Transwell system, the migration of HMVECs was significantly inhibited (Fig. 4C). Correspondingly, in the endothelial tube formation assay, we observed the reduced spontaneous ability of HMVECs to form capillary tubes in the presence of CM obtained from pre-miR-126-transfected LoVo cells, when compared with tube formation in the presence of CM from the blank and scramble controls (Fig. 4D). For further validation of the anti-angiogenic effect of miR-126 *in vivo*, we performed chick embryo chorioallantoic membrane assay (CAM) using gelatin sponge loaded with the different CM as mentioned. The neo-vessel formation was significantly inhibited in the presence of CM from the pre-miR-126-transfected LoVo cells (Fig. 4E).

miR-126 is epigenetically silenced in CRC. To validate whether miR-126 is silenced by DNA methylation, we examined the methylation status of EGFL7, the host gene of miR-126, in primary CRC tissues and cell lines. The CpG island status within the EGFL7 promoter region, along with the localization of PCR products, is presented in Fig. 5A. Clinicopathologic features of CRC patients are listed in Table I. Notably, there was no significant association observed between methylation status and TNM stage. Using methylation-specific PCR (MSP), all of the CRC cell lines showed extensively methylation of the EGFL7 promoter (Fig. 5B). Bisulfate sequencing (BSP) results exhibited extensive methylation throughout the promoter region of these cell lines (Fig. 5C). We treated the same panel of CRC cell lines with 5-aza-CdR. Compared to the untreated cells, enhanced expression of miR-126 and downregulation of VEGF were observed in the demethylated cells (Fig. 5D). These results suggest that promoter methylation resulted in the silencing of miR-126 in CRC, and may be partly responsible for the high VEGF expression in CRC.

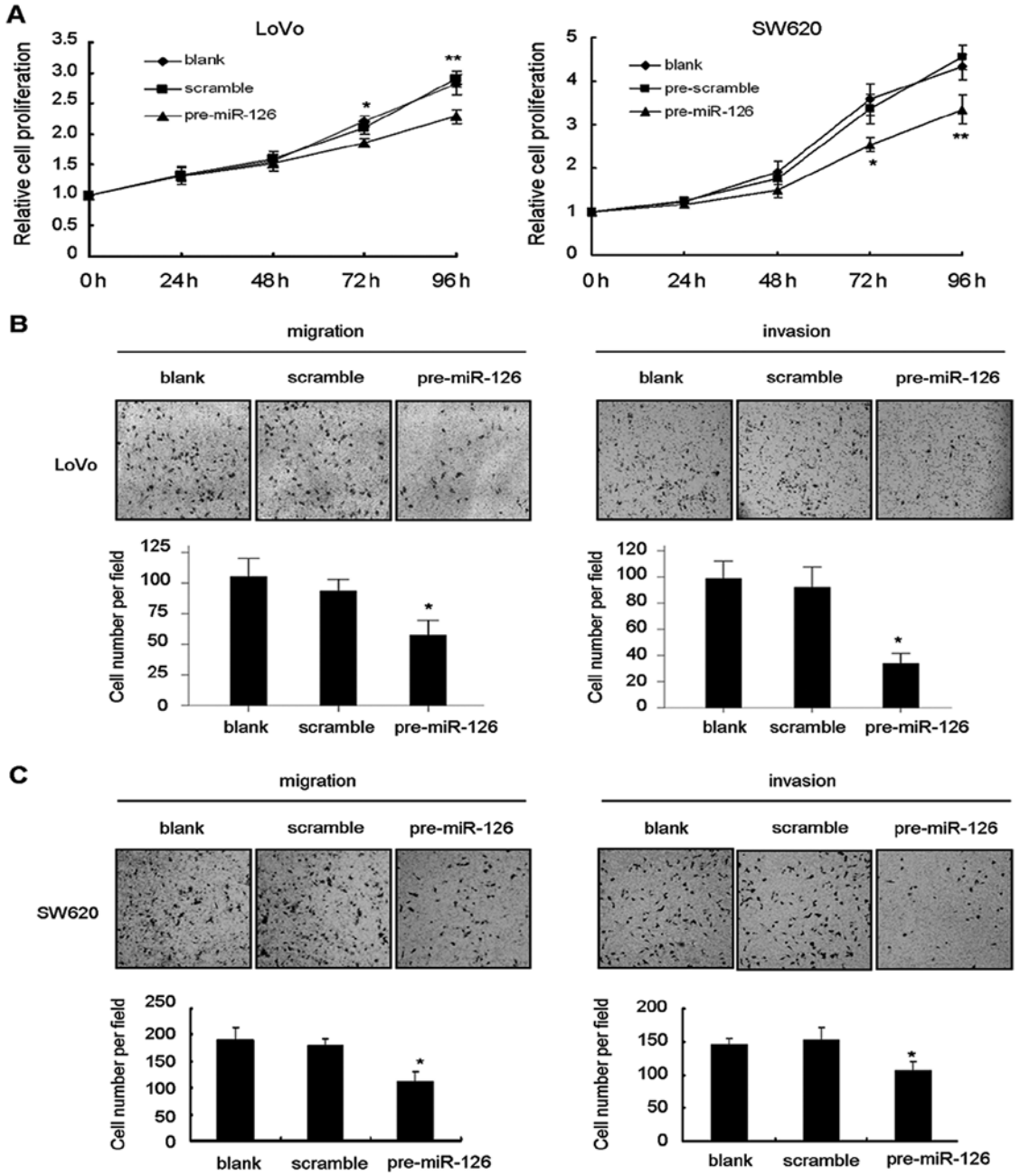

Figure 3. miR-126 suppresses cell growth, migration and invasion of CRC cells. (A) Enhanced expression of miR-126 inhibited cell growth of LoVo and SW620 cells (*P<0.05, compared with controls). (B and C) Transwell assay was performed to measure migration and invasion ability. (B) Reduced migration and invasion ability of LoVo cells at 48 h post-transfection with pre-miR-126 (*P<0.05, compared with controls). (C) Reduced migration and invasion ability of SW620 cells at 48 h post-transfection with pre-miR-126 (*P<0.05, compared with controls). CRC, colorectal cancer.

Discussion

The majority of deaths from tumors result from complications caused by metastasis. Therefore, targeting metastatic disease is a pivotal anticancer strategy. miRNAs have been implicated in the regulation of cellular processes which are deregulated in tumors, including proliferation, apoptosis, differentiation, cell migration and invasion (25), and even tumor angiogenesis (26). Recent studies have identified various miRNAs that may promote (27,28) or inhibit (29,30) tumor invasion and metastasis, providing potential therapeutic targets for anti-metastatic strategy. miR-126 is well known as one of the angiogenesis regulatory miRNAs that are termed angiomiRs (31). Emerging evidence indicates that miR-126 plays a regulatory role in tumor progression. Tavazoie *et al* (10) reported that miR-126 inhibits overall tumor growth and proliferation, and suppresses metastatic colonization and angiogenesis by blocking endothelial recruitment in breast cancer (32). In pancreatic cancer, restoration of miR-126 results in reduced cellular migration, invasion, and induction of the epithelial marker E-cadherin via suppression of ADAM9 (11). Enhanced expression of miR-126 was also reported to increase the sensitivity of lung cancer cells to anticancer agents (33). Based on these findings, we hypothesized that miR-126 may be involved in CRC metastatic processes. The most significant finding of our study was that restoration of miR-126 directly suppressed VEGF expression

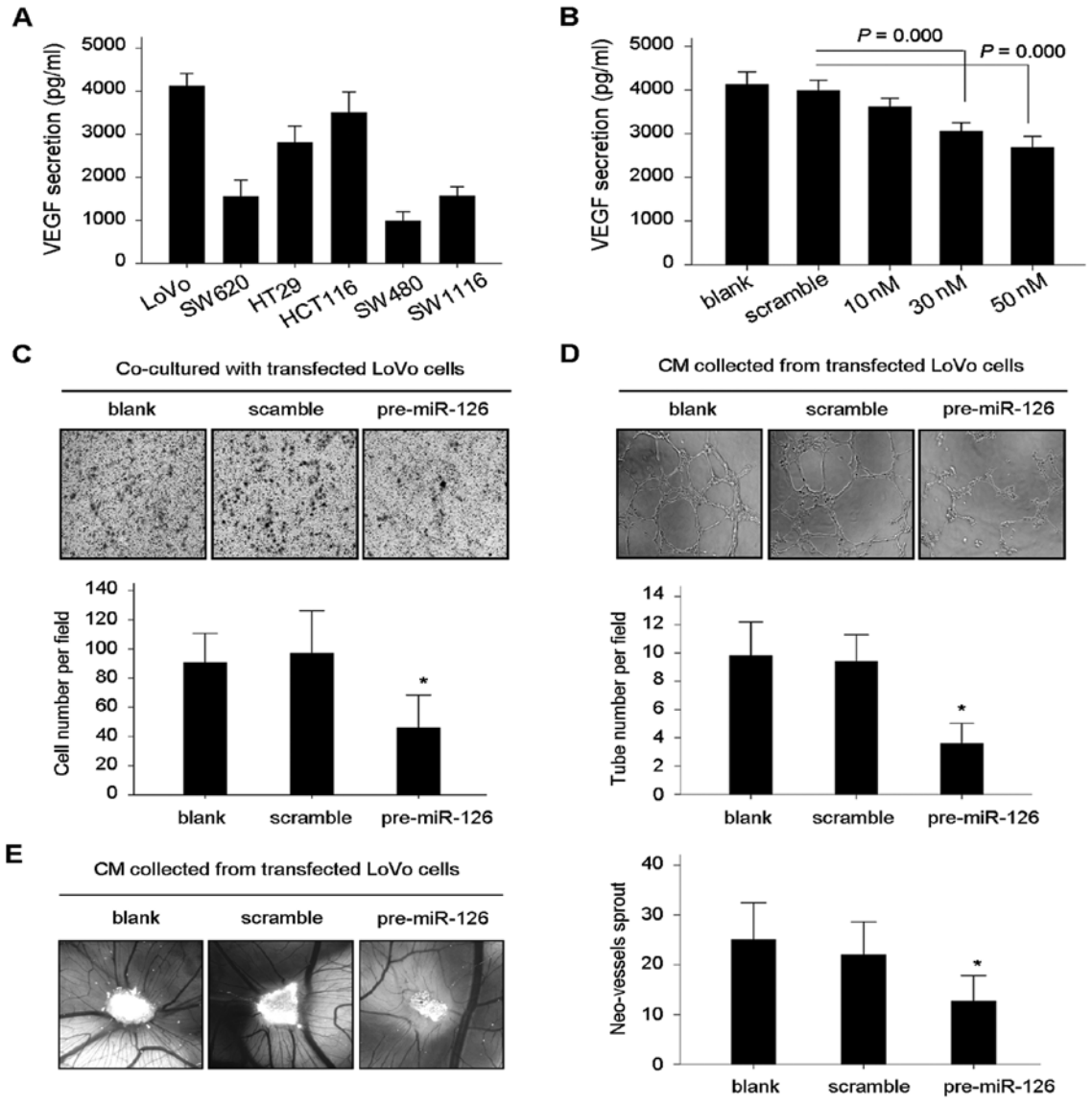

Figure 4. miR-126 inhibits CRC tumor angiogenesis. (A) Concentration of VEGF in CM obtained from 6 CRC cell lines was determined by ELISA. (B) Concentration of VEGF in CM obtained from pre-miR-126 or scramble transfected LoVo cells was determined by ELISA. Restoration of miR-126 suppressed VEGF secretion of LoVo cells. (C) HMVECs were co-cultured with LoVo cells in the Transwell system. The migratory ability of HMVECs was decreased in response to restoration of miR-126 in LoVo cells cultured in the lower chamber ($^*P<0.05$, compared with controls). (D) HMVECs were seeded on the top of ECM-matrix, in the presence of the CM as previously mentioned. The ability of HMVECs to form capillary tubes was inhibited, in response to restoration of miR-126 ($^*P<0.05$, compared with controls). (E) Angiogenic response induced by gelatin sponges loaded with CM was assessed using *in vivo* chick embryo chorioallantoic membrane (CAM) assay. Neo-vessels sprouted at the sponge-CAM boundary were counted under a stereomicroscope. Restoration of miR-126 impeded neo-vessel formation ($^*P<0.05$, compared with controls). CRC, colorectal cancer; VEGF, vascular endothelial growth factor; CM, conditioned medium; HMVECs, human microvascular endothelial cells.

consequently inhibiting cell invasion and tumor angiogenesis in CRC. In addition, DNA methylation was responsible, at least in part, for the silencing of miR-126 expression in CRC.

Our results showed that miR-126 was commonly down-regulated in 6 CRC cell lines and the 12 CRC patient tissues, which is consistent with a previous study (21). On the contrary, Otsubo *et al* (17) reported miR-126 was highly expressed in cultured and primary gastric cancer cells. This implies that the regulatory role of miR-126 may be specific to tumor context. Using a luciferase reporter, we revealed that miR-126 directly binds to a specific complementary site within the 3' untranslated region of VEGF mRNA. In the gain of function assays, we restored miR-126 expression in metastatic LoVo cells and found that re-expression of miR-126 suppressed VEGF expression post-transcriptionally. Moreover, restoration of miR-126 impaired cell growth, migration and invasion capability. It is essential for VEGF to regulate tumor progression through interaction with its tyrosine kinase receptors (VEGFRs) (34). Particularly, activation of VEGF/VEGFR1 signaling was found to lead to significant induction of cell motility and invasiveness of CRC cells (35). Therefore, our data indicated that miR-126 inhibited cell migration and invasion through inhibition of VEGF expression. Since tumor cell-released VEGF contributes to tumor vasculature via stimulation of the sprouting and proliferation of endothelial cells (26), we investigated the involvement of miR-126 in tumor angiogenesis of

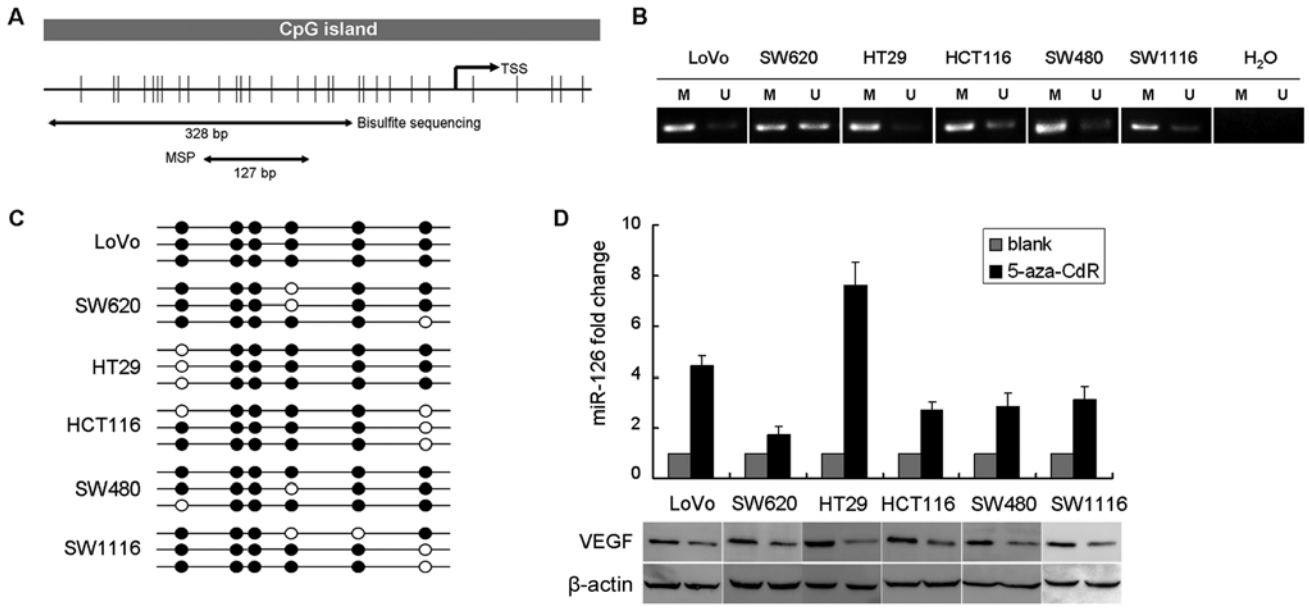

Figure 5. Analysis of the methylation status of miR-126 CpG islands. (A) miR-126 is an intronic miRNA located within intron 7 of the EGFL7 gene. Top, location of miR-126 within the EGFL7 gene. Bottom, map of the EGFL7 CpG island within the promoter region, with the positions of PCR products used for methylation analysis. Vertical tick marks, CpG sites; E, exon; TSS, putative transcribe start site. (B) Methylation status of the EGFL7 promoter region in 6 CRC cell lines by MSP. M, methylated; U, unmethylated. (C) Bisulfate sequencing of CpG islands within the EGFL7 promoter region in 6 CRC cell lines. Each circle indicates a CpG dinucleotide. Black circle, methylated CpG; open circle, unmethylated CpG. Three clones were sequenced for each cell line. (D) miR-126 fold change was determined by TaqMan real-time PCR and VEGF expression was determined by western blotting in 6 CRC cell lines following 5-aza-CdR treatment. miRNA, microRNA; EGFL7, epidermal growth factor-like domain 7; CRC, colorectal cancer; MSP, methylation-specific PCR; VEGF, vascular endothelial growth factor; 5-aza-CdR, 5-aza-2'-deoxycytidine.

Table I. Clinicopathological features of the CRC patients analyzed for miR-126 methylation.

Characteristics	miR-126 methylated n=52	miR-126 unmethylated n=10	Total n=62	P-value
Age (years), mean ± SD	57.83±13.76	57.1±11.61	57.71±13.35	0.156[a]
Age, ≥60 years	25 (48.1)	4 (40)	29 (46.8)	0.902[b]
Female, n (%)	27 (51.9)	5 (50)	32 (51.6)	0.911[b]
Proximal site, n (%)	8 (15.4)	2 (20)	10 (16.1)	1[b]
Poorly differentiation, n (%)	8 (15.4)	1 (10)	9 (14.5)	1[b]
Mucinous adenocarcinoma, n (%)	3 (5.8)	2 (20)	5 (8.1)	0.379[b]
TNM, n (%)				0.348[b]
I	9 (17.3)	4 (40)	13 (21.0)	
II	15 (28.8)	2 (20)	17 (27.4)	
III	23 (44.2)	4 (40)	27 (43.5)	
IV	5 (9.6)	0 (0)	5 (8.1)	

[a]Evaluated by Student's t-test; [b]evaluated by Chi-square test.

CRC. Expectedly, due to the decreased secretion of VEGF by CRC cells, the migration and sprouting of HMVECs were impaired *in vitro*, and neo-vessel formation decreased *in vivo*. Therefore, these findings suggest that miR-126 is involved in metastatic processes.

DNA methylation and associated silencing of tumor-suppressor genes is a molecular hallmark of human tumors (36). Recently, this phenomenon has been extended to miRNAs with tumor-suppressor features, which are downregulated in multiple tumors (37-39). Saito and Jones (40) reported that DNA methylation induced downregulation of EGFL7, and that demethylation treatment with 5-aza-CdR simultaneously resulted in restoration of miR-126 and its host gene, EGFL7, since as an intronic miRNA, miR-126 tends to co-express with EGFL7. In the present study, we observed extensive promoter methylation in 52 out of 62 primary CRC tissues and 6 cell lines. In addition, 5-aza-CdR treatment of the CRC cell lines restored miR-126 expression and thereby led to a decrease

in VEGF expression. Our results suggest that silencing of miR-126 by promoter methylation is an important mechanism underlying dysregulation of VEGF expression in CRC.

Tumor metastasis is defined as a consecutive process including local invasion, intravasation, cell survival in the circulatory system, extravasation, and colonization in a secondary site (41). Additionally, angiogenesis is also essential for tumor metastasis. Hurst *et al* (42) recently proposed a novel category of cancer-related miRNAs termed metastamiRs that are associated with metastatic processes. For instance, miR-21 is a mastermind of metastasis and promotes cell survival, migration, invasion, intravasation and metastasis (43,44), whereas the miR-200 family is delinquent and its absence contributes to the EMT phenotype (45). These metastamiRs are potential cancer prognostic markers and therapeutic targets for metastatic cancers. Our findings provide evidence for the role of miR-126 as a metastamiR through targeting VEGF.

In conclusion, we revealed the inhibitory effects of miR-126 on VEGF, and partly elucidated the potential mechanism by which miR-126 is implicated in CRC metastasis. miR-126 may be a potential target for the therapeutic strategy against CRC.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (30971520) and the Science and Technology Planning Project of Guangdong Province (2010B031600098).

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