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ORIGINAL ARTICLE

Targeting of RhoE inhibits epithelial-mesenchymal transition during colorectal cancer cell migration

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Abstract

Objective Despite microRNA (miR-200b) being proved to promote the proliferation of colorectal cancer (CRC) cells, the relationship between miR-200b and epithelial-mesenchymal transition (EMT) of CRC cells remains poorly understood. The aim of the study was to investigate the relationship between miR-200b and EMT during CRC cell migration.

Methods The effect of miR-200b on EMT-associated markers E-cadherin and vimentin was evaluated by western blot in CRC cells (SW620 and HT-29) by treatment with miR-200b mimics and inhibitors. A luciferase reporter assay was employed to detect downstream targets of miR-200b. Transwell migration assays were used to detect CRC cell migration.

Results Western blots revealed that treatment with miR-200b mimics led to up-regulation of E-cadherin and down-regulation of vimentin, metalloproteinase (MMP)-9, and MMP-2, whereas treatment with miR-200b inhibitor exhibited opposite effects on expression of E-cadherin and vimentin. Luciferase reporter assays demonstrated that RhoE (RND3) was targeted by miR-200b. Two predicted target sites of miR-200b were present in the 3'-UTR of RhoE. Predicted target site 1 was from nucleotides 1584 to 1591, and site 2 was from nucleotides 1729 to 1735. RhoE knockdown cell lines were also established to investigate the impact of RhoE and miR-200b on EMT and cell migration. RhoE knockdown enhanced the effect of miR-200b mimics, up-regulating E-cadherin and down-regulating vimentin. RhoE knockdown also inhibited cell migration. Furthermore, miR-200b mimic treatment further promoted the inhibitory effect of RhoE knockdown on cell migration.

Conclusion miR-200b inhibited EMT and CRC cell migration partly via inhibiting RhoE expression in CRC. RhoE and miR-200b might therefore be promising target genes in the management of CRC.

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Key words: miR-200b; colorectal cancer (CRC); metalloproteinase (MMP); epithelial-mesenchymal transition (EMT); cell migration

Colorectal cancer (CRC) is one of most common cancers and the fourth leading cause of cancer-related death worldwide [1]. Metastasis is the most common reason for the death of CRC patients [2]. Despite application of screening strategies, such as fecal occult-blood test, sigmoidoscopy and colonoscopy, approximately 500 000 patients with CRC die due to uncontrolled cancer metastasis each year [3]. Therefore, the molecular mechanisms of CRC metastasis and therapies focusing on specific molecular targets of CRC metastasis attract wide attention.

Epithelial-mesenchymal transition (EMT) is essential for initiation of cancer metastasis. In breast cancer, ovarian cancer, esophageal cancer, and colon cancer models, EMT has been observed. It has been established that EMT is aberrantly reactivated in tumor progression and contributes to cancer invasion and metastasis *in vivo* and *in vitro* [4]. Key features of EMT include deficiency of epithelial cell markers such as E-cadherin, and elevated expression of mesenchymal proteins such as vimentin [5–6]. Recent studies have demonstrated that E-cadherin also can be regulated by micro-RNAs [4]. It is established that microRNAs (miRNA) are a class of small noncoding gene-regulatory RNAs, which are involved in regulation of expression of cancer-related genes, and play roles in cancer invasion and metastasis [7–8]. Among miRNAs, miR-200b is a member of the miR-200 family. A large amount of

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evidence supports the notion that miR-200b is associated with EMT of cancer cells. For instance, miR-200b is reported to suppress EMT and promote proliferation of intestinal epithelial cells [9]. It has been reported that miR-200b targets E-box-binding homeobox (ZEB) 1, which is involved in regulation of E-cadherin expression in gastric carcinoma [10]. Although it has been demonstrated that miR-200b promotes CRC cell proliferation through suppressing reversion-inducing cysteine-rich protein with Kazal motifs (RECK) [11], the relationship between miR-200b and EMT of CRC cells remains undefined. Here, we sought to reveal the role of miR-200b in EMT and CRC cell migration, and the potential underlying molecular mechanisms.

Rho proteins are important signaling molecules as a member of Rnd subfamily. Although RhoE lacks GTPase activity, it can bind GTP. These features give RhoE many unique functions that are different from other members of Rho family [12]. In recent years, it has been confirmed that Rho proteins show abnormal expression in many malignancies, such as colorectal cancer, breast cancer, stomach cancer, HCC, and pancreatic cancer. It has also been found that abnormal expression of Rho proteins was closely related to the tumor occurrence，invasion and metastasis [13]. Bioinformatic predictions have suggested that RhoE may be a target gene of miR-200b. However, there are few reports about the relationship between RhoE and miR-200b.

In this study, we studied the impact of miR-200b on the EMT-associated markers E-cadherin and vimentin in the colorectal cancer cell lines SW620 and HT-29, by treating the cells with miR-200b mimics and inhibitors. We also identified that miR-200b can inhibit EMT by regulating RhoE expression. Luciferase reporter assays were employed to detect downstream targets of miR-200b. We also investigated the impact of RhoE and miR-200b on cell migration. This study provides more clues regarding the molecular mechanism of miR-200b in CRC, and identifies novel targets of colon cancer treatment.

Materials and methods

Cell culture

Human CRC cell lines SW620 and HT-29 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, CA, USA), supplemented with penicillin (100 U/mL, Gibco, CA, USA), streptomycin (100 μg/mL, Gibco, CA, USA) and 10% fetal bovine serum (FBS) in an humidified incubator (37 ℃, 5% CO_2).

Cell transfection

For cell transfection, miR-200b mimics, miR-200b inhibitors, and a negative control were designed by and purchased from RiBoBio Company (Guangzhou, China). Transfections of miRNAs were performed using Lipofectamine2000 (Invitrogen, CA, USA) according to the manufacturer's protocol. After 6 hours, the culture medium was replaced with fresh DMEM. Total RNA and protein were prepared 48 hours after cell transfection for analysis by qRT-PCR or western blot, respectively.

Western blot

Western blotting was performed in accordance with standard procedures. Briefly, whole proteins were obtained from cell pellets lysed in RIPA buffer (Santa Cruz, USA) after required treatments, and were separated on 10% SDS-PAGE gels. The proteins were transferred onto PVDF membranes (Millipore, USA) which were blocked 1 h with 5% skim milk at room temperature (25 ℃) and incubated with primary antibodies overnight at 4 ℃. E-cadherin antibody was purchased from BD Bioscience (USA). Vimentin antibody, MMP-2 antibody, MMP-9, and GAPDH antibodies were purchased from Cell Signaling Technology (USA). RhoE antibody was purchased from R&D System. After washing with TBST (TBS + 0.1% TWEEN-20) three times, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, USA) for 60 minutes at room temperature. Signals were visualized using the ECL system (Millipore, USA). GAPDH was detected as an internal control. Protein expression levels were quantified using ImageJ software (National Institute of Health, MA, USA) [14].

Quantitative real-time PCR

Total RNA, including miRNAs, was isolated from cells using TRIzol reagent (Invitrogen, USA) after required treatments according to the manufacturer's instructions. From 2.0 μg of total RNA, complementary DNA (cDNA) was randomly primed in a final volume of 20 μL using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Canada). Human GAPDH was amplified as an internal control. Quantitative real time-PCR reaction was conducted using ABI SYBR Green Master Mix (Applied Biosystems). Quantitative PCR (95 ℃ for 10 min, 40 cycles of 95 ℃ for 15 s, and 60 ℃ for 1 min) was performed with the ABI Step OnePlus™ system (Applied Biosystems). For miRNA analysis, real time PCR was performed as above. All miRNA data were expressed relative to a U6 small nuclear (sn) RNA (RiBoBio Company, Guangzhou, China). The $2^{-\Delta\Delta Ct}$ method was employed to process the data relative to U6 or GAPDH.

The PCR primer sequences were: RhoE: Forward: 5'-ATAGAGTTGAGCCTGTGGGACAC-3'; Reverse: 5'-AGGGTCTCTGGTCTACTGATGTC-3'; GAPDH: Forward: 5'-TGCACCACCAACTGCTTAGC-3'; Reverse: 5'-

GGCATGGACTGTGGTCATGAG-3'.

miRNA target prediction and Luciferase reporter assay

miR-200b targets were predicted based on miRanda (http://www.microrna.org), TargetScan (http://www.Targetscan.org), and PITA (http://genie.weizmann.ac.il/pubs/mir07) [15–17]. Many different target genes were predicted. Among these targets, we focused on one gene, RhoE (*RND3*), which has been shown to have a major role in the control of the actin cytoskeleton, influencing migration by changing cell motility [18].

A luciferase reporter assay was employed to validate whether RhoE was the direct target gene of miR-200b. The 3'-UTRs of RhoE were amplified by PCR using human genomic DNA. These were cloned into the *XhoI* site downstream of the luciferase coding region in the pGL3 vector (Promega, Madison, WI, USA). A pGL3 construct containing 3'-UTRs of RhoE with mutant seed sequences of miR-200b was also synthesized. For the luciferase assay, human HEK293T cells were seeded in DMEM supplemented with 10% FBS in 96-well plates and transfected with either pGL3 or pGL3-miR-200b vector (100 ng) and wild type or mutant 3'-UTR of RhoE (10 ng) using Lipofectamine-2000. Cells were harvested 48 hours after transfection. The Dual-Luciferase reporter assay system (Promega, WI, USA) was utilized for luciferase activity assay. All experiments were performed 3 times and the relative reporter activity was obtained following normalization to Renilla control luciferase activity.

Establishment of RhoE knockdown stable cell lines

To establish stable RhoE knockdown cell lines, SW620 and HT-29 cells were seeded in 6-well plates and transduced with RhoE-knockdown- or negative-control-lentiviruses (GenePharma, Shanghai, China). Lentiviral infection was carried out according to operation instructions.

Immunofluorescence assay

The cells were fixed with 4% paraformaldehyde, blocked with 3% bovine serum albumin (BSA) for half an hour, and then incubated with anti-E-cadherin primary antibody for 2 hours at room temperature. After rinsing washing times with PBS, the cells were incubated with second antibody conjugated with fluorescein isothiocyanate (FITC) for 1 hour at room temperature. Subsequently, the nuclei were counterstained with DAPI. Images were captured under a fluorescent microscope (Olympus BX51, Olympus, Japan) and analyzed using ImageJ software (National Institute of Health, MA, USA) [14].

Cell migration assay

For migration assay, Transwell migration chambers with 8 μm pore size (Corning, NY, USA) were used. Briefly, suspended cells in serum-free medium (1×10^5) were placed on each upper chamber, and 500 μL medium with 10% FBS was added to the bottom chamber in a 5% CO_2 humidified incubator. After incubation for 24 hours at 37 ℃, the cells on the upper surface of the trans-well membrane were removed using a cotton swab. The migrated cells on the reverse side were fixed in methanol, stained with crystal violet and photographed under a microscope (100 × magnification). Six random fields from each triplicate membrane were imaged and the number of migrant cells counted for each experimental group.

Statistical analysis

All data were expressed as mean ± SEM. The difference among treatment groups was analyzed by Student's *t*-test or one-way ANOVA followed by Student-Newman-Keuls (SNK) test (SPSS 19.0 statistical software). *P*-value < 0.05 was regarded statistically significant.

Results

miR-200b regulated expression of E-cadherin and vimentin in CRC cells

Loss of E-cadherin expression and increased vimentin expression are important markers of EMT. To validate whether miR-200b regulates EMT of CRC cells, we characterized the expression levels of E-cadherin and vimentin in SW620 and HT-29 cell lines by transfecting with miR-200b mimics, miR-200b inhibitors, or miR-n.c. (negative control). As shown in Fig. 1, overexpression of miR-200b (treatment with miR-200b mimics) led to up-regulation of E-cadherin and down-regulation of vimentin compared to negative control in SW620 and HT-29 cell lines ($P < 0.05$). Moreover, transfection with miR-200b inhibitor resulted in reduction of E-cadherin and elevation of vimentin ($P < 0.05$). These data showed that miR-200b regulates the expression of E-cadherin and vimentin, and inhibited EMT of CRC cells.

Metalloproteinase (MMP)-9 and MMP-2 are involved in ECM remodeling and angiogenesis and associated with cancer progression [19]. We also investigated the effects of miR-200b on MMP-2/9 in our study. We found that MMP-9 and MMP-2 were dramatically decreased in CRC cells after transfection with miR-200b mimics ($P < 0.05$). On the contrary, miR-200b inhibitor treatment significantly increased the expression MMP-9/2 protein ($P < 0.05$), suggesting that miR-200b also regulates the expression of MMP-9/2.

miR-200b targeted RhoE

Using online miRNA target prediction databases (Targetscan, miRNA.org and PITA), we predicted a great number of target genes of miR-200b. Among these target

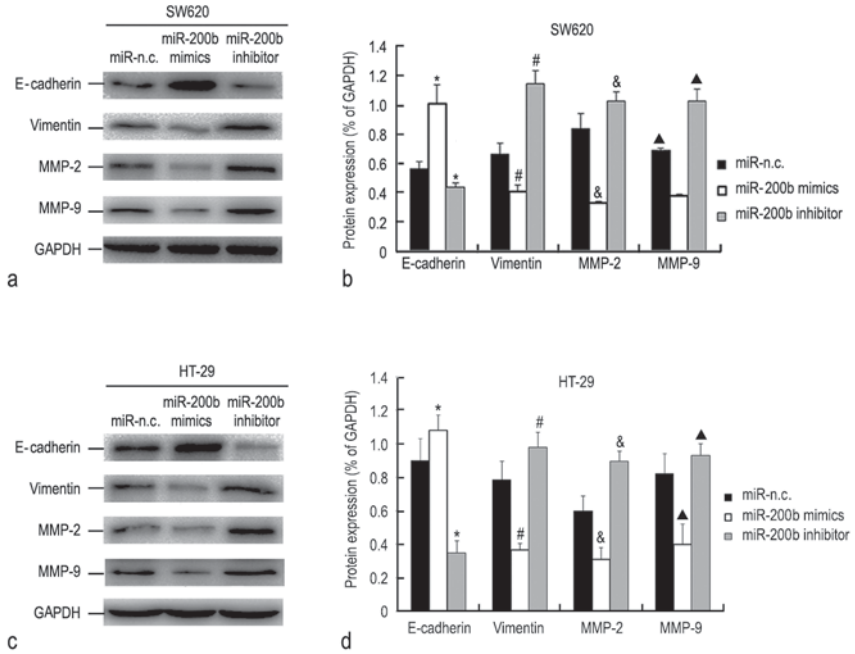

Fig. 1 Effect of miR-200b on E-cadherin, vimentin, MMP-2 and MMP-9 in SW620 and HT-29 cells. (a and c) SW620 and HT-29 cells were transfected with miR-200b n.c., miR-200b mimics and miR-200b inhibitor. Western blot analysis detected protein expression of E-cadherin, vimentin, MMP-2, MMP-9 and control GAPDH protein levels. (b and d), Densitometry analysis of E-cadherin, vimentin, MMP-2 MMP-9 and control GAPDH protein levels in SW620 and HT-29 cells. * $P < 0.05$, compared with negative control of E-cadherin; # $P < 0.05$, compared with negative control of vimentin; & $P < 0.05$, compared with negative control of MMP-2; ▲ $P < 0.05$, compared with negative control of MMP-9

genes, we identified on RhoE, which is involved in cell motility and migration [20]. To validate whether RhoE was the direct target gene of miR-200b, we cloned luciferase reporter vectors containing the full length 3'-UTR of RhoE. Relative luciferase activity was significantly suppressed in HEK293T cells co-transfected with miR-200b and wild type 3'-UTR of RhoE ($P < 0.05$, Fig. 2). The data confirm that RhoE is the target gene of miR-200b.

Moreover, as shown in Fig. 2a, we also identified two predicted target sites of miR-200b: the site 1 was from nucleotides 1584 to 1591, and site 2 was from nucleotides 1729 to 1735 in 3'-UTR of RhoE. To analyze which predicted site was targeted by miR-200b, we generated a wild type RhoE 3'-UTR reporter construct and 2 mutated RhoE 3'-UTR reporter constructs. The first one contained predicted target site 1 and the second one contained site 2. As shown in Fig. 2b, luciferase activity was significantly suppressed in HEK293T cells co-transfected with miR-200b and the wild type 3'-UTR of RhoE. In contrast, the first mutant reporter construct led to a smaller decrease in luciferase activity in miR-200b-transfected HEK293T cells ($P < 0.05$). The second mutant reporter construct led to slightly decreased luciferase activity in miR-200b-transfected HEK293T cells, although this did not reach statistical significance (Fig. 2b, $P > 0.05$). These results reveal that both predicted target site 1 and 2 in 3'-UTR of RhoE are target sites of miR-200b.

The effect of miR-200b on endogenous expression of RhoE was subsequently examined by western blot (Fig. 3) in CRC cells lines. Transfection of miR-200b mimics resulted in a strong decrease of RhoE protein in SW620 and HT-29 cells ($P < 0.05$). However, transfection of miR-200b inhibitor up-regulated RhoE expression ($P < 0.05$). These findings demonstrate that miR-200b negatively regulates the RhoE expression.

RhoE knockdown inhibits EMT in CRC cell lines

Next, to test whether RhoE expression affected miR-220b regulated EMT in SW620 and HT-29 cells, we knocked down the expression of RhoE in SW620 and HT-29 cells. As shown in Fig. 4, the expression level of RhoE was significantly reduced in RhoE-knockdown cells compared to the negative control in the two cell lines ($P < 0.05$). RhoE knockdown resulted in increased E-cadherin and decreased vimentin expression ($P < 0.05$). Furthermore, miR-200b mimic transfection in RhoE-knockdown cells resulted in further down-regulated RhoE, up-regulated E-cadherin and down-regulated vimentin expression compared to the negative control ($P < 0.01$). The results in SW620 and HT-29 cell lines were consistent.

Immunofluorescence labeling with an anti-E-cadherin antibody demonstrated similar results (Fig. 3b). When RhoE expression was silenced in SW620 cells, E-cadherin expression was markedly increased compared with the negative control. Furthermore, miR-200b mimic transfection led to more obviously decreased E-cadherin expression in RhoE-knockdown SW620 cells. Taken together, these data demonstrate that RhoE expression was negatively regulated by miR-200b, which might regulate EMT process by targeting RhoE.

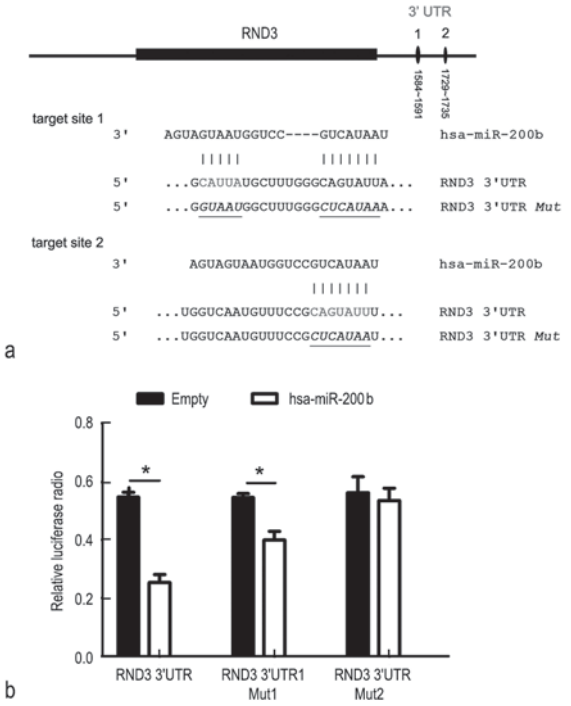

Fig. 2 miR-200b targets RhoE. (a) Two putative binding sites of miR-200b at position of 1584–1591 and 1729–1735 regions in RhoE 3'-UTR region were predicted by TargetScan. The mutated versions by sites mutagenesis were also shown. (b) Effect of 2 mutated RhoE 3'-UTR reporter constructed on luciferase activity. Luciferase reporter assay detected the luciferase activity in HEK293T cells co-transfected with miR-200b, and wild type or mutant 3'-UTR of RhoE. * $P < 0.05$, compared with negative control

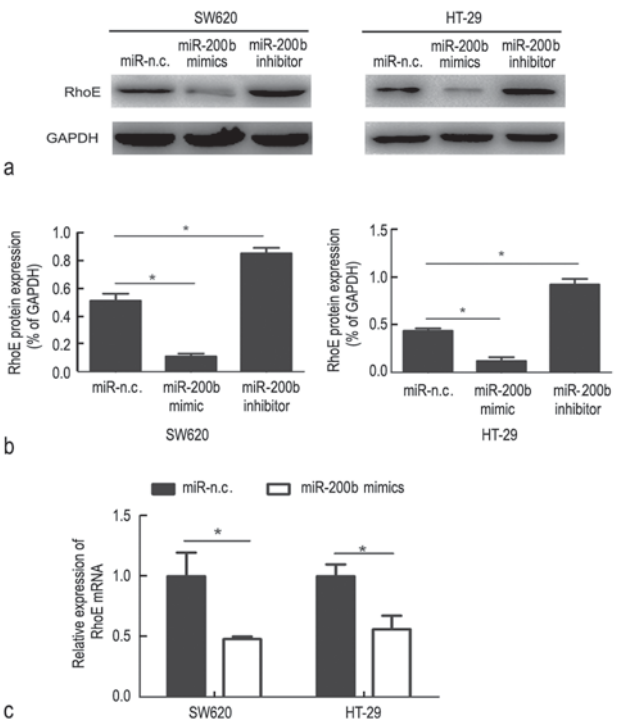

Fig. 3 Effect of miR-200b on endogenous expression of RhoE in SW620 and HT-29 cells. (a) Detecting the protein expression of RhoE in SW620 and HT-29 cells transfected with miR-200b-n.c., miR-200b mimics and miR-200b inhibitor by western blot. (b) Densitometry analysis of RhoE protein in SW620 and HT-29 cells transfected with miR-200b-n.c., miR-200b mimics and miR-200b inhibitor, respectively. RhoE protein was significantly down-regulated by miR-200b mimics, but was elevated by miR-200b inhibitors. * $P < 0.05$, compared with miR-n.c. (c) Detecting the expression of RhoE mRNA in SW620 and HT-29 cells transfected with miR-n.c. and miR-200b mimics, respectively. * $P < 0.05$, compared with miR-n.c.

Reduced miR-200b levels induced EMT in result of control CRC cells migration

We further investigated the influence of miR-200b and RhoE on CRC cell migration by using trans-well migration assay. As shown in Fig. 5, we found that miR-200b inhibitor transfection of SW620 cells promoted cell migration compared to negative control ($P < 0.05$). RhoE knockdown dramatically decreased SW620 cell migration compared to the negative control ($P < 0.05$), indicating that down-regulation of RhoE expression suppresses cell migration. Moreover, treatment with miR-200b mimics further reduced migrated RhoE-knockdown-SW620 cells compared to the negative control transfected RhoE knockdown-SW620 cells ($P < 0.05$). Our data demonstrate that miR-200b overexpression and RhoE knockdown inhibit cell migration. miR-200b may suppress CRC cell migration by down-regulating RhoE.

Discussion

Cancer metastasis remains a primary reason of colorectal cancer-related mortality. EMT is recognized as the initiation step of cancer metastasis and is linked to malignant conversion of cancer cells [21]. EMT is characterized by the decreased E-cadherin and increased of vimentin expression. Although it is known that miR-200b, as a member of miR-200 family, is a powerful regulator of EMT in several cancer types, there little data regarding miR-200b in CRC. In the current study, we found that treatment of cells with miR-200b mimics led to up-regulation of E-cadherin and down-regulation of vimentin, whereas treatment of miR-200b inhibitor exhibited opposing effects on expression of E-cadherin and vimentin. We also found that miR-200b suppressed EMT of CRC cell. This finding is in agreement with a previous report showing that miR-200b suppresses EMT in intestinal epithelial cells [9].

MMP-9/2 play positive roles in remodeling of extracellular matrix and angiogenesis, thus they are closely associated with cancer progression [22–23]. MMP-9/2 have reported to be elevated in CRC tissue compared to normal

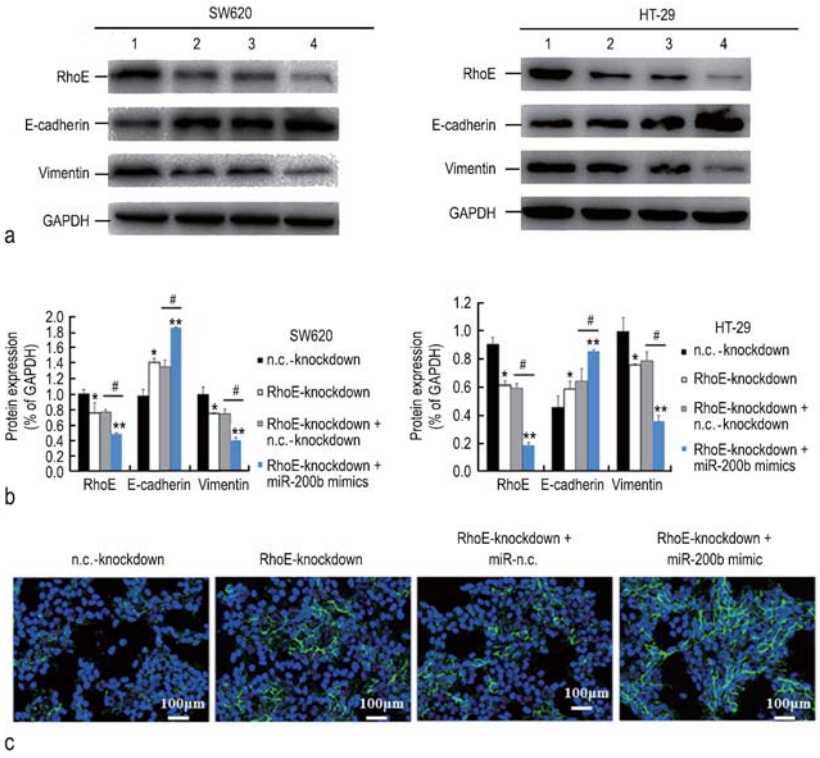

Fig. 4 Effect of RhoE silencing on E-cadherin and vimentin in SW620 and HT-29 cells. (a) Western blot analysis of RhoE, E-cadherin, vimentin after RhoE knockdown with lentivirus transfection. RhoE knockdown cells were transfected with miR-200b mimics or miR-200b n.c. (1, n.c.-knockdown; 2, RhoE-knockdown; 3, RhoE-knockdown + miR-n.c.; 4, RhoE-knockdown + miR-200b mimics). (b) Densitometry analysis of RhoE, E-cadherin and vimentin. * $P < 0.05$, ** $P < 0.01$, compared with negative control. (c) Immunofluorescent analysis with anti-E-cadherin antibody. RhoE knockdown SW620 cells were transfected with miR-200b mimics or miR-200b n.c. Expression of E-cadherin was observed by phase contrast microscopy (x 200)

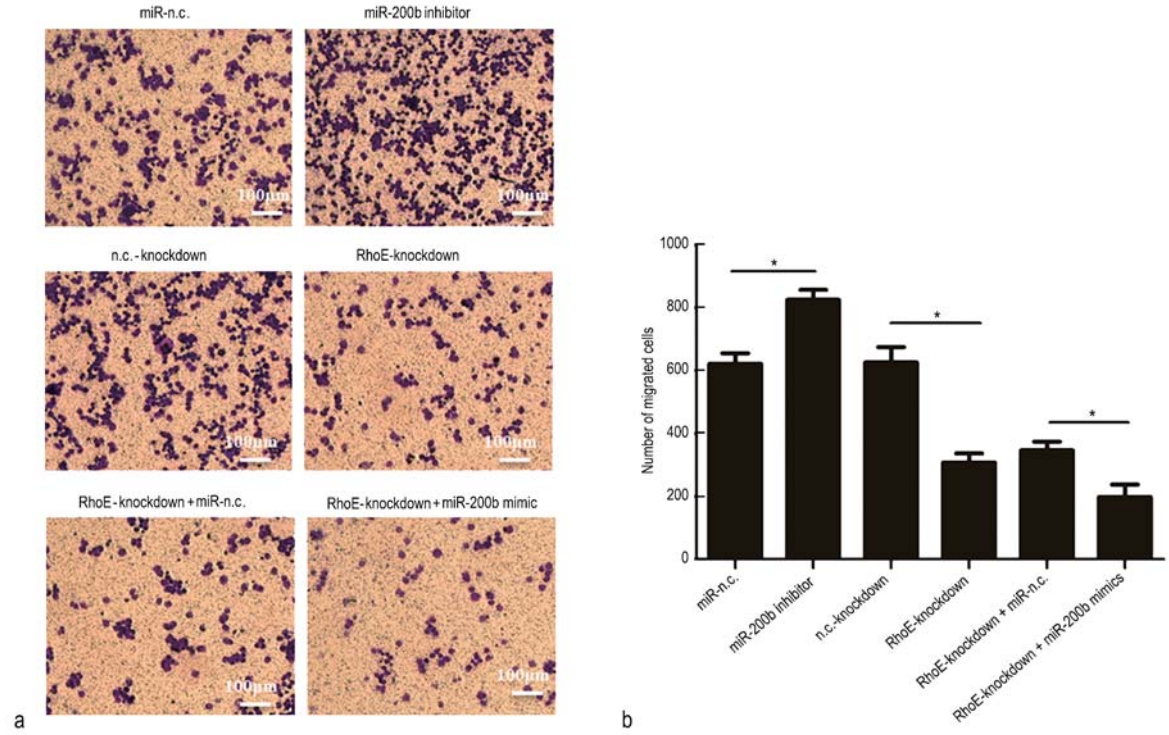

Fig. 5 Influence of miR-200b on migration of SW620 and HT-29 cells. (a) Transwell migration system was used to evaluate cell migration. The migrated cells were observed by phase contrast microscopy (x 100). (b) Quantitative analysis of average numbers of migrated cells for each condition. * $P < 0.05$, compared with negative control

tissue [24]. In our study, the expression of both MMP-9 and MMP-2 was decreased in response to miR-200b mimics, but were increased in response to miR-200b inhibitor treatment. This indicates that expression of MMP-9/2 might be negatively regulated by miR-200b, potentially preventing EMT via regulation of MMP-9/2.

RhoE, an atypical Rho protein, is intimately involved with cell migration [25–26]. Its expression is significantly correlated with cancer cell invasion, lymph node metastasis, and distant metastasis of CRC, and a poor prognosis for patients with CRC. Additionally, the positive rate of RhoE is higher in CRC patients than that in normal subjects [27]. Our study showed that RhoE is specifically targeted by miR-200b. In line with our observations, RhoE expression has been reported to be reduced by miR-200b transfection in HeLa cells [28]. Furthermore, we found two target sites of miR-200b in 3'-UTR of RhoE: site 1 from nucleotides 1584 to 1591, and site 2 from nucleotides 1729 to 1735.

Analysis of western blot results showed that miR-200b mimic transfection reduced RhoE expression to a greater extent in RhoE knockdown cells, confirming that miR-200b negatively regulates RhoE expression. Moreover, RhoE knockdown combined with miR-200b mimic transfection further up-regulated E-cadherin and down-regulated vimentin compared to treatment of miR-200b mimic transfection alone, as indicated by consistent results of western blot and immunofluorescence analysis. These findings reveal that miR-200b suppresses EMT of CRC cells by targeting RhoE.

It has been previously demonstrated that RhoE expression is reduced in CRC tissues compared to normal tissues and adenomas, and may function as a tumor suppressor gene to suppress CRC cell proliferation and growth [29]. Our study revealed that RhoE also prohibits cell migration, as indicated by the observation that RhoE knockdown decreases migration of SW620 cells. Furthermore, miR-200b mimic treatment promoted the effect of RhoE-knockdown to decrease migration of cells. Our findings indicate that miR-200b inhibits migration of cancer cells via down-regulating RhoE.

Our findings showed that miR-200b plays a suppressive role in EMT and cellular migration, at least in part by inhibiting expression of RhoE. There is evidence that RhoE inhibits invasion of cancer cells mediated by RhoA-ROCK (Rho-associated coil-containing protein kinase) pathway [30]. Moreover, overexpression of dominant-negative N-terminally truncated ROKα, functions as a downstream target for RhoA in induced cell spreading of HeLa and 3T3 cells [31]. These studies reveal that RhoA-ROCK and ROKα might be possible downstream targets of RhoE in CRC. More experiments are required to validate and extend the results of this study.

Conclusion

In summary, we provide *in vitro* evidence that miR-200b suppresses EMT and cell migration, via inhibition of RhoE expression in CRC. The study deepens our understanding of the role of miR-200b in CRC. These data collectively suggest that miR-200b and RhoE may serve as potential therapeutic targets to reduce CRC migration.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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