Original Paper

MicroRNA-124 (MiR-124) Inhibits Cell Proliferation, Metastasis and Invasion in Colorectal Cancer by Downregulating Rho-Associated Protein Kinase 1(ROCK1)

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Key Words

MiR-124 • ROCK1 • Metastasis • CRC

Abstract

Background/Aims: MiR-124 inhibits neoplastic transformation, cell proliferation, and metastasis and downregulates Rho-associated protein kinase (ROCK1) in Colorectal Cancer (CRC). The aim of this study was to further investigate the roles and interactions of ROCK1 and miR-124 and the effects of knockdown of ROCK1 and miR-124 in human Colorectal Cancer (CRC). **Methods:** Three Colorectal cancer cell lines (HCT116, HT29 and SW620) and one Human Colonic Mucosa Epithelial cell line (NCM460) were studied. The protein expression of ROCK1 was examined by Western-blot and qRT-PCR were performed to examine the expression levels of ROCK1 mRNA and miR-124. Furthermore, We performed transfection of cancer cell line (SW620) with pre-miR-124(mimics), anti-miR-124(inhibitor), ROCK1 siRNA and the control, then observed the affects of ROCK1 protein expression by western-blot, cell proliferation by EDU (5-ethynyl-2'-deoxyuridine assay) and expression levels of ROCK1mRNA by qRT-PCR . A soft agar formation assay, Migration and invasion assays were used to determine the effect of regulation of miR-124 and ROCK1, and survivin on the transformation and invasion capability of colorectal cancer cell. **Results:** MiR-124 expression was significantly downregulated in CRC cell lines compare to normal (P < 0.05). In contrast, ROCK1 protein expression was significantly increased in CRC cell lines compared to the normal (P < 0.05), whereas the gene (ROCK1mRNA) expression remained unaltered (P > 0.05). ROCK1 mRNA was unaltered in cells transfected with miR-124 mimic and miR-124 inhibitor, compared to normal controls. There was a significant reduction in ROCK1 protein in cells transfected with miR-124 mimic and a significant increase in cells transfected with miR-124 inhibitor (P < 0.05). Cell proliferation, transformation and invasion of cells transfected with miR-124 inhibitor were significantly increased compared to those in normal controls (P<0.05). However, cell proliferation, transformation and invasion of

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cells transfected with ROCK1 siRNA were significantly decreased compared to control ($P < 0.05$). **Conclusions:** In conclusion, our results demonstrated that miR-124 not only promoted cancer cell hyperplasia and significantly associated with CRC metastasis and progression, but also downregulated ROCK1 protein expression. More importantly, increased ROCK1 expression or inhibited miR-124 expression may constitute effective new therapeutic strategies for the treatment of renal cancer in the future.

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Introduction

Colorectal cancer (CRC) is one of the most common malignances and the third leading cause of cancer-related death in the world, with an estimated incidence of 1 million new cases and a mortality of >600,000 deaths annually [1-2]. Recent progress in diagnosis and therapy has helped to save the lives of many patients at early stages of this malignancy, but the prognosis for patients with advanced disease or metastasis is still very poor. Therefore, further investigation into the molecular pathogenesis of CRC and the consequential development of novel targeted therapeutics are needed.

A series of studies have revealed that microRNAs (miRNAs) can regulate the expression of a variety of genes pivotal for tumor development and highlight a novel mechanism participating in CRC pathogenesis [3-5]. MicroRNAs (miRNAs) are non-protein-coding small RNAs of approximately 19–25 nucleotides (nt) in length that are cleaved from 70 to 100 nt-long hairpin pre-miRNA precursors by the enzyme Drosha [6, 7]. MiRNAs bind to complementary sequences in the 3'-untranslated regions of their target mRNAs and induce mRNA degradation or translational repression [8]. Recent evidence has shown that abnormal expression levels of miRNAs are associated with a variety of human cancers, and that they play crucial roles in cell proliferation, differentiation and apoptosis [9-11]. Volinia et al. studied the miRNA expression pattern in solid cancers (2,532 samples, 31 cancer types, 120 miRNAs including miR-124), and they found that miR-124 was low expressed in many solid cancers [12]. In some present study, the researchers found that the expression of miR-124 was decreased and acted as a tumor suppressor in many cancers [13-14]. A subset of miRNAs was found to be aberrantly expressed in CRC, and most of the miRNAs are related to cell proliferation, apoptosis and tumor metastasis [12]. Xi et al. demonstrated that miR-124 inhibited proliferation and induced apoptosis by targeting ROCK1 in CRC and the expression of miR-124 was associated with CRC metastasis [15]. Therefore, identification and characterization of miR-124 in CRC may provide new insight into understanding the molecular mechanisms of disease development and is therefore crucial for the development of new therapy.

ROCK1 is a member of the Rho-associated serine/threonine kinase family, which facilitates the reorganization of the actin cytoskeleton during motion [16]. ROCK1 has been found to be increased in many cancers, including glioma, osteosarcoma, prostate cancer, and GC [14, 17]. ROCK1 functioned as an oncogene, and possessed a wide range of functions, including migration, invasion, and metastasis [18]. ROCK1 was targeted by several miRNAs, including miR-135a, miR-145, and miR-148a [13, 18, 19]. In the present study, Hu et al. found that ROCK1 was a direct target of miR-124 in GC cells. Silencing of ROCK1 by shRNA inhibited proliferation, migration, and invasion of GC cell line (SGC-7901) [20]. Furthermore, restoration of ROCK1 may significantly reverse the tumor suppressive role of miR-124 [20]. Xi et al. demonstrated that ROCK1 was high expression in CRC and negatively regulated by miR-124 [15].

However, only little is known regarding the underlying mechanisms through which miR-124 regulates ROCK1 expression in CRC. Therefore, the objectives of this study were to investigate the roles and interactions between ROCK1 and miR-21 in CRC, and the effects of knockdown of ROCK1 and MiR-124 and function in Colorectal Cancer.

Materials and Methods

Cell Culture

Three Colon cancer cell lines (HCT116, HT29 and SW620) and one Human Colonic Mucosa Epithelial cell line (NCM460) were obtained from Xiehe Cell Bank of the Chinese Academy of Medical Sciences (Beijing, China) as a commercial source. HCT116 and HT29 were cultured in McCoy's 5A medium (Invitrogen; Life Technologies, Carlsbad, CA, USA), and SW620 was cultured in RPMI 1640 containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA). All the cells were cultured in a humidified 37°C incubator supplemented with 5% CO₂. The study was approved by the Ethics Committee of Henan University of Science and Technology (A140066).

Western blot

Total proteins were extracted from tissues using a total protein extraction kit (Keygen, Nanjing, China) according to the manufacturer's recommendations. The concentrations of total proteins were measured using a BCA Protein Assay Kit (Keygen). A total of 80 µg protein was separated using SDS-PAGE and transferred onto PVDF membranes; the membranes were then blocked in 5% fat-free milk at room temperature for 2 h. After incubation with rabbit or goat primary antibodies against ROCK1 (Abcamab80590, Cambridge, UK) at a dilution of 1:10,000 or GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:200 at 4°C overnight, the membranes were probed with goat anti-rabbit or mouse anti-goat secondary antibodies at a dilution of 1:5,000 at room temperature for 2 h. The signals were detected using a Super ECL plus Kit (Keygen) determined by quantitative analysis using UVP software. The ratio of IOD ROCK1/IODGAPDH indicated the relative expression of ROCK1 protein.

Total RNA isolation and cDNA synthesis

TRIzol reagent (CWbio. Co. Ltd., Beijing, China) was used to isolate total RNA from the snap frozen tissues. The isolated RNA was treated with DNase I (Invitrogen). The RNA concentration and purity were determined using a NanoDrop® ND-1000 (Nanodrop Products, Wilmington, DE). The ratio of 28S/18S was analyzed by GlykoBandscan 5.0. RNA quality and quantity were determined spectrophotometrically at 260 and 280 nm, respectively. Reverse transcription of RNA was performed using the NCode miRNA First-Strand cDNA Synthesis Kit (Invitrogen, Cat#: MIRC-50).

Quantitative real time PCR (qRT-PCR)

qRT-PCR was performed using the Light Cycler 2.0 Real-Time PCR System (Roche Germany) in a total volume of 20 µL in glass capillaries containing 2 µL of cDNA, 0.8 µL of each primer, and 10 µL of Light Cycler TaqMan Master Mix (Invitrogen, Cat#: MIRC-50). The PCR reaction for the miR-124 gene was initiated using a 10 min denaturation step at 95°C followed by termination with a 30 s cooling step at 40°C. The cycling protocol consisted of denaturation at 95°C for 15 s and annealing at 60°C for 60 s; 40 cycles. Fluorescence detection was performed at the end of each extension step. The PCR reaction for the ROCK1 gene was initiated with a 10-min denaturation at 95°C. Amplification was carried out for 40 cycles of 15 s at 95°C and 60 s at 60°C. An additional extension step of 5 min at 72°C was added following the completion of 40 cycles. All PCR reactions were performed in duplicate. The PCR products were confirmed by melting curve analysis. We used the mathematical delta-delta method ($\text{ratio} = 2^{-\Delta\Delta CT}$) developed by PE Applied Biosystems (Perkin Elmer) to compare relative expression results between treatments in qRT-PCR.

RNAi assay

SW620 cells were incubated in a six-well tissue culture dish without antibiotics for 24 h prior to transfection, when they had reached 60 – 80% confluence. Negative control (NC) siRNA, specific miR-124 inhibitor and mimic siRNA transfection reagent complexes were mixed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations and then added to the cells. After 6 h at 37°C, the medium was changed and the cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum for various times. Silencing of miRNA124 and ROCK1 was determined by qRT-PCR and Western blot.

5-ethynyl-2' deoxyuridine (EdU) proliferation assay

Transfected SW620 cells were plated in 24-well plates at 4×10^4 cells/well, allowed to adhere, washed with PBS, and incubated in serum free RPMI containing 10 $\mu\text{mol/L}$ 5-ethynyl-2' deoxyuridine (Guangzhou RiboBio Co., Ltd, Guangzhou, China) for 2 h. The cells were then washed with PBS, fixed, and permeabilized in PBS containing 2% formaldehyde, 0.5% Triton X100, and 300 mmol/L sucrose for 15 min. After washing with PBS, cells were blocked using 10% FBS in PBS for 30 min, and incorporated EDU was detected by incubation with a fluorescent azide coupling solution (Apollo; Guangzhou RiboBio Co. Ltd) for 30 min. The cells were washed three times with PBS containing 0.05% Tween20 (PBST), incubated with the DNA staining dye Hoechst 33342 for 30 min, and washed in PBS. Images were captured using a fluorescent microscope, and the average nuclear fluorescent intensity was calculated from at least 50 non-S phase cells randomly selected in five different fields of view.

Soft agar colony formation assay

A bottom layer (0.6% low-melt agarose) was prepared with RPMI 1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. A top layer (0.3% low-melt agarose) was prepared with the same RPMI 1640 medium as described above plus 5000 of the indicated cells. Plates were incubated at 37.8°C in 5%CO₂ in a humidified incubator for approximately 2 weeks. The plates were then scanned and photographed, and the number of colonies was quantified using Quantity one v4.0.3 software (Bio-Rad, Hercules, CA).

Migration and invasion assays

Cell migration and invasion assays were performed using transwell chamber. For migration, 2×10^4 transfected cells in serum-free medium were plated into the top chamber (BD, Bedford, MA, USA). For invasion, the same density of cells was seeded into the top chamber, which was pre-coated with Matrigel (BD, Bedford, MA, USA). After incubation for 24 h, the membranes were fixed, stained with 0.1% crystal violet. Cells passing through the membranes were counted under microscope (Olympus, Tokyo, Japan).

Statistical analysis

SPSS 13.0 software was employed for the analyses of all data. Each assay was performed a minimum of three times. The data were expressed as the mean $\pm$ S.D. and the Student's t-test and one-way analysis of variance were used to determine the significance of the difference in multiple comparisons. The Mann-Whitney *U* test was used to determine the associations of miR-124 expression and Colorectal Cancer clinicopathological features. An asterisk represents a *P* value < 0.05, a value which indicated a statistically significant result.

Result

Expression of MiR-124 and ROCK1 in Colorectal cancer cell lines

We next determined the expression levels of miR-124 and ROCK1 (mRNA and protein) in Three Colorectal cancer cell lines (HCT116, HT29 and SW620) and one normal Colorectal cell line (NCM460) (Fig. 1). In Colorectal cancer cell lines with low endogenous miR-124 according to qRT-PCR analysis (*P* < 0.05) (Fig. 1C), a high level of ROCK1 protein was observed (*P* < 0.05) (Fig. 1A and B) by western blot analysis, whereas the normal cell line (NCM460) with high levels of miR-124 demonstrated low levels of ROCK1 protein. For ROCK1 mRNA, however, there was no significant difference (*P* > 0.05) (Fig. 1D).

MiR-124 downregulates ROCK1 in cultured SW620 cell

We determined whether transfection of SW620 cells with miR-124mimic or miR-124inhibitor affects ROCK1expression. In SW620 cell characterized by low miR-124 expression, down-regulation of endogenous miR-124 with miR-124inhibitor (Fig. 2) led to a significant increase in ROCK1 protein levels compared to the control according to westernblot results (*P* < 0.05) (Fig. 2A and B). In contrast, there was a significant decrease in ROCK1 protein levels in miR-

Fig. 1. Expression of MiR-124 and ROCK1 in Colorectal cancer cell lines. (All experiments were repeated three times). (A) Expression of ROCK1 protein in the one Human Colonic Mucosa Epithelial cell line (NCM460) and in Colorectal cancer cell lines (HCT116, HT29 and SW620), detected by western blot. (B) Relative densitometry analysis in the one Human Colonic Mucosa Epithelial cell line (NCM460) and in Colorectal cancer cell lines (HCT116, HT29 and SW620). Data are presented as ratios of ROCK1 to corresponding GAPDH. $P < 0.05$ compared with that of NCM460. * $P < 0.05$. (C) qRT-PCR data for miR-124 in the Human Colonic Mucosa Epithelial cell line (NCM460) and in Colorectal cancer cell lines (HCT116, HT29 and SW620). Y-axis, miR-124 qRT-PCR values as ratios to the NCM460 specimen. * $P < 0.05$. (D) mRNA qRT-PCR for ROCK1. X-axis: the Human Colonic Mucosa Epithelial cell line (NCM460) and Colorectal cancer cell lines (HCT116, HT29 and SW620). Y-axis: ROCK1 mRNA qRT-PCR value relative to NCM460 specimen. $P > 0.05$.

Fig. 2. (A) Proteins from SW620 cells transfected with negative control siRNA (NC), miR-124 mimic and miR-124 inhibitor were used to detect ROCK1 by Western blot. (B) Analysis of the relative density of SW620 cells transfected with negative control siRNA (NC), miR-124 mimic and miR-124 inhibitor. Data are presented as ratios of ROCK1 to corresponding GAPDH. $P < 0.05$ compared with that of NC. * $P < 0.05$. (C) qRT-PCR for miR-124. X-axis: SW620 cells were transfected with negative control siRNA, miR-124 mimic and miR-124 inhibitor. Y-axis: miR-124 qRT-PCR value relative to NC specimen. $P < 0.05$. (D) mRNA qRT-PCR for ROCK1. X-axis: SW620 cells were transfected with negative control siRNA, miR-124 mimic and miR-124 inhibitor. Y-axis: ROCK1 mRNA qRT-PCR value relative to NC specimen. $P > 0.05$.

Fig. 3. MiR-124 gene knockdown promotes cell proliferation. (All experiments were repeated three times). (A) The new generation cells were detected by EDU (red). Hoechst 33342 was used for nuclear staining of all cells (blue). Overlay shows merging of the EDU and DAPI. (B) Proliferative ability data for SW620 cells transfected with negative control siRNA (NC) and miR-124 inhibitor. Y-axis, the cell number of EDU staining as ratios to the cell number of nuclei staining. P-values were obtained by Student's unpaired t test, $P < 0.05$, * $P < 0.05$.

124 mimic-transfected cells ($P < 0.05$) (Fig. 2A and B). ROCK1 mRNA showed the same trend in miR-124-transfected cells ($P > 0.05$) (Fig. 3D).

Knockdown of miR-124 gene induces cell proliferation

We determined the effect of knockdown of miR-124 genes on cell viability and proliferative ability using an EDU assay (Fig. 3). To confirm the increased number of SW620 cells following treatment with miR-124 inhibitor and negative control siRNA, cells were labeled with EDU to measure active DNA synthesis (red) and Hoechst 33342 to show all cell nuclei (blue) (Fig. 3A). According to the results of fluorescent microscopic analysis, the mean percentage of new cells that incorporated EDU was 27.1% in the negative control siRNA group, 39.8% in miR-124 inhibitor transfected cells ($P < 0.05$) (Fig. 3A and B). Furthermore, we observed that the proliferative ability of SW620 cells transfected with miR-124 inhibitor decreased with increasing concentrations of miR-124 inhibitor over the range from 80 nmol/ μ L to 120 nmol/ μ L with a time of transfection from 24 h to 48 h ($P < 0.05$).

Knockdown of miR-124 gene induces metastasis, invasion and clonogenic survival of CRC cells

To study the role of miR-124 in the regulation of growth and invasion of CRC cells, a colony formation assay was performed to further evaluate whether miR-124 knockdown synergistically

Fig. 4. MiR-124 Inhibited Growth and Invasion of SW620 Cells. (A) The result of colony formation assay for the SW620 cells transfected with negative control siRNA (NC) miR-124 inhibitor was obtained on the 14th day. (B) The colony-forming ability of SW620 cells was significantly increased in the miR-124 inhibitor-transfected group at 2 weeks compared with the negative control group ($P < 0.05$). (C and D) SW620 cells were transfected with negative control siRNA (NC) and miR-124 inhibitor, and Migration and Invasion Assays were performed to detect the ability of invasion and migration of SW620 cells. Experiments were performed in triplicate. * $p < 0.05$, compared with control.

Fig. 5. Silencing of ROCK1 inhibited cell proliferation. (All experiments were repeated three times). (A) The new generation cells were detected by EDU (red). Hoechst 33342 was used for nuclear staining of all cells (blue). Overlay shows merging of the EDU and DAPI. (B) Proliferative ability data for SW620 cells transfected with negative control siRNA (NC) and ROCK1 siRNA. Y-axis, the cell number of EDU staining as ratios to the cell number of nuclei staining. P-values were obtained by Student's unpaired t test $P < 0.05$. * $P < 0.05$.

promote SW620 cell transformation ability (Fig. 4A). Similarly, the colony formation assay demonstrated that the total number of colonies generated by miR-124 inhibitor-treated cells was increased compared to negative control siRNA-treated cells (NC) ($P < 0.05$) (Fig. 4A and B).

Fig. 6. Silencing of ROCK1 inhibited Growth and Invasion of SW620 Cells. (A and B) SW620 cells were transfected with negative control siRNA (NC) and ROCK1 siRNA, and Migration and Invasion Assays were performed to detect the ability of invasion and migration of SW620 cells. (C) The result of colony formation assay for the SW620 cells transfected with negative control siRNA (NC) and ROCK1 siRNA was obtained on the 14th day. (D) The colony-forming ability of SW620 cells was significantly decreased in the ROCK1 siRNA-transfected group at 2 weeks compared with the negative control group ($P < 0.05$). Experiments were performed in triplicate. * $p < 0.05$, compared with control.

The results of Migration and invasion assays showed that down-expression of miR-124 remarkably increased the proliferation of SW620 cells ($P < 0.05$) (Fig. 4C and D).

Silencing of ROCK1 inhibited tumor cell growth, invasion and clonogenic survival in CRC

We investigated whether silencing of ROCK1 resulted in effects on CRC cell proliferation. ROCK1 siRNA and the control were transfected into SW620 cells. To confirm the new increased number of SW620 cells transfected with ROCK1 siRNA and the control, the cells also were labeled with EDU to measure active DNA synthesis (red) and Hoechst 33342 to show all cell nuclei (blue) (Fig. 5A). We found that silencing of ROCK1 significantly inhibited cell proliferation, similar to the effects of miR-124 overexpression. According to the results of fluorescent microscopic analysis, the mean percentage of new cells which incorporated EDU was 28.6% in the negative control siRNA group and 11.3% in ROCK1 siRNA transfected cells ($P < 0.05$) (Fig. 5B).

A colony formation assay was performed to determine whether ROCK1 knockdown synergistically inhibited SW620 cell transformation ability (Fig. 6C). The colony formation assay demonstrated that the total number of colonies generated by ROCK1 siRNA-treated cells was less compared to control-treated cells (NC) ($P < 0.05$) (Fig. 6D). Furthermore, the results of migration and invasion assays showed that silencing of ROCK1 markedly reduced both the migration and invasion of SW620 cells compared to the control group ($P < 0.05$) (Fig. 6A and B).

Discussion

Although many miRNAs had been aberrantly altered in CRC, their underlying molecular mechanisms in CRC development and progression still remain poorly understood [21]. Thus exploring the function of miRNAs specifically involved in CRC development and progression would greatly help expand our knowledge on CRC, and provide new targets for its diagnosis and therapy. Investigation of these miRNAs would expand our view to better understand carcinogenesis by analyzing mRNA target associated and miRNA-mediated pathways in CRC. Zhang et al. demonstrated a tumor suppressive role of miR-140-5p in CRC tumorigenesis and

progression by targeting VEGFA [22]. Xu et al. found that miR-149 was an independent prognostic factor and could inhibit migration and invasion of CRC cells, at least partially by targeting FOXM1 [23]. Fang et al. indicated that miR-301a promotes CRC progression by directly downregulating SOCS6 expression, and miR-301a may represent a novel biomarker for the prevention and treatment of CRC [24]. In some study, the expression of miR-124 was detected decreasing in many cancers, and acted as a tumor suppressor. Study by Fowler et al. has reported that transfection of commercialized miR-124 precursor in GBM cell A172 resulted in diminished cell migration and invasion [25]. Xia et al. reported the enhanced miR-124 expression significantly inhibited glioma cell invasion using matrigel invasion assay and tumor xenografts in nude mice [26]. In Gastric Cancer, Xie et al. reported that miR-124 inhibited proliferation and induced apoptosis by targeting enhancer of zeste homolog 2 (EZH2) [27]. Xia J et al. found that miR-124 suppressed the proliferation of gastric Cancer cell via targeting sphingosine kinase type 1 (SphK1) [28]. Xi et al. demonstrated that miR-124 inhibited proliferation by targeting ROCK1 in CRC cell line (HCT116, HT29) and the expression of miR-124 was associated with CRC metastasis [15].

In our study, we investigated the inverse correlation between miR-124 and ROCK1 protein was also observed in another CRC cell line SW620. We found that miR-124 expression was significantly downregulated in CRC cell line once again. In contrast, ROCK1 protein expression was significantly decreased, whereas ROCK1 mRNA expression showing results consistent. Furthermore, in miR-124mimic-transfected SW620 cell, we observed a significant reduction in ROCK1 protein levels and mRNA, but a significant increase in miR-124inhibitor-transfectedcell. The results suggest that miR-124 may post-transcriptionally and negatively regulate ROCK1 by repressing translation in CRC once again. Since other studies in different cell types have shown that ROCK1 is a target of miR-124, we confirmed that ROCK1 is the target gene of miR-124 in another CRC cell line. Furthermore, as the effect in other cell lines, the SW620 cell transfected with miR-124 inhibitor showed increased cell proliferation and transformation capacity, according to EdU and soft agar formation assays.

A variety of targets have been found to be regulated by miR-124, including proliferation-related genes [28–31], invasion/metastasis-related genes [32–34] and so on. ROCKs have been shown to be a central player in the formation of stress fibers via phosphorylation of myosin light chain [35–37]. The ROCK2 transcript is highly expressed in muscle and brain tissues, whereas the ROCK1 is localized in nonnerves tissues [37]. RhoA/ROCK pathway has been shown to play an important role in neurite growth inhibition from CSPG after CNS injury [38]. Several preclinical and clinical studies have utilized inhibitors of Rho/ROCK signaling pathway for anticancer therapeutics in prostate, lung, melanoma, and many other tumor types with remarkable success [39–41]. Liu et al. found that Dex suppressed neutrophil release through ROCK1-independent mechanisms and inhibited the adhesion of U937 mononuclear cells through ROCK1-dependent non-genomic mechanisms that did not involve RhoA [42]. Hu et al. found that silencing of ROCK1 by shRNA inhibited proliferation, migration, and invasion of GC cell line [20]. Restoration of ROCK1 may significantly reverse the tumor suppressive role of miR-124 [13]. In our study, we determined whether silencing of ROCK1 had similar effects on miR-124 overexpression. ROCK1 shRNA was transfected into SW620 cells, and PDCD4 mRNA and protein levels were significantly decreased. The silencing of ROCK1 significantly inhibited cell proliferation, migration and invasion, similar to the effects of miR-124 overexpression.

In conclusion, our present study suggests that miR-124 and ROCK1 may play an important role not only in tumor growth, but also in tumor metastasis and invasion. This, together with our correlative results in previous clinical studies on miR-124 and ROCK1, suggests that rescue strategies against miR-124 or strategies interfering with the miR-124/ROCK1 interaction, or inhibit ROCK1 expression, will provide a strong rationale for therapeutic applications in CRC in the future.

Disclosure Statement

The authors declare that no conflicts of interest exist.

References

- Parkin DM, Bray F, Ferlay J, Pisani P: Global cancer statistics.2002. CA Cancer J Clin 2005;55:74-108.
- Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D: Global cancer statistics. CA Cancer J Clin 2011;61:69-90.
- Bhatti I, Lee A, Lund J, Larvin M: Small RNA: a large contributor to carcinogenesis?. J Gastrointest Surg 2009;13:1379-1388.
- Schetter AJ, Harris CC: Alterations of microRNAs contribute to colon carcinogenesis. Semin Oncol 2011;38:734-742.
- Wu W, Law P, Lee C, Cho CH, Fan D, Wu K, Yu J, Sung JJ: MicroRNA in colorectal cancer: from benchtop to bedside. Carcinogenesis 2011;32:247-253.
- Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281-297.
- Zeng Y: Principles of micro-RNA production and maturation. Oncogene 2006;25:6156-6162.
- Engels BM, Hutvagner G: Principles and effects of microRNA-mediated post-transcriptional gene regulation. Oncogene 2006;25:6163-6169.
- Esquela-Kerscher A, Slack FJ: Oncomirs - micrornas with a role in cancer. Nature Rev Cancer 2006;6:259-269.
- Lu Z, Liu M, Stribinskis V, Klinge CM, Ramos KS, Colburn NH, Li Y: MicroRNA-21 promotes cell transformation by targeting the programmed cell death 4 gene. Oncogene 2008;27:4373-4379.
- Medina PP, Slack FJ: microRNAs and cancer: an overview. Cell Cycle 2008;7:2485-2492.
- Volinia S, Galasso M, Costinean S, Tagliavini L, Gamberoni G, Drusco A, Marchesini J, Mascellani N, Sana ME, Abu Jarour R, Despons C, Teitell M, Baffa R, Aqeilan R, Iorio MV, Taccioli C, Garzon R, Di Leva G, Fabbri M, Catozzi M, Previati M, Ambs S, Palumbo T, Garofalo M, Veronese A, Bottoni A, Gasparini P, Harris CC, Visone R, Pekarsky Y, de la Chapelle A, Bloomston M, Dillhoff M, Rassenti LZ, Kipps TJ, Huebner K, Pichiorri F, Lenze D, Cairo S, Buendia MA, Pineau P, Dejean A, Zanesi N, Rossi S, Calin GA, Liu CG, Palatini J, Negrini M, Vecchione A, Rosenberg A, Croce CM: Reprogramming of miRNA networks in cancer and leukemia. Genome Res 2010;20:589-599.
- Li J, Song Y, Wang Y, Luo J, Yu W: MicroRNA-148a suppresses epithelial-to-mesenchymal transition by targeting ROCK1 in non-small cell lung cancer cells. Mol Cell Biochem 2013;380:277-282.
- Zhang H, Wang Q, Zhao Q, Di W: MiR-124 inhibits the migration and invasion of ovarian cancer cells by targeting SphK1. J Ovarian Res 2013;6:84.
- Xi ZW, Xin SY, Zhou LQ, Yuan HX, Wang Q, Chen KX: Downregulation of rho-associated protein kinase 1 by miR-124 in colorectal cancer. World J Gastroenterol 2015;21:5454-5464.
- Wen X, Ding L, Wang J, Qi M, Hammonds J, Chu H, Chen X, Hunter E, Spearman P: ROCK1 and LIM kinase modulate retrovirus particle release and cell-cell transmission events. J Virol 2014;88:6906-6921.
- Zhou P, Huang G, Zhao Y, Zhong D, Xu Z, Zeng Y, Zhang Y, Li S, He F: MicroRNA-363-mediated downregulation of S1PR1 suppresses the proliferation of hepatocellular carcinoma cells. Cell Signal 2014;26:1347-1354.
- Shin JY, Kim YI, Cho SJ, Lee MK, Kook MC, Lee JH, Lee SS, Ashktorab H, Smoot DT, Ryu KW, Kim YW, Choi IJ: MicroRNA 135a suppresses lymph node metastasis through down-regulation of ROCK1 in early gastric cancer. PLoS One 2014;9:e85205.
- Wan X, Cheng Q, Peng R, Ma Z, Chen Z, Cao Y, Jiang B: ROCK1, a novel target of miR-145, promotes glioma cell invasion. Mol Med Rep 2014;9:1877-1882.
- Hu CB, Li QL, Hu JF, Zhang Q, Xie JP, Deng L: miR-124 inhibits growth and invasion of gastric cancer by targeting ROCK1. Asian Pac J Cancer Prev 2014;15:6543-6546.
- Hong L, Han Y, Yang J, Zhang H, Zhao Q, Wu K, Fan D: MicroRNAs in gastrointestinal cancer: prognostic significance and potential role in chemoresistance. Expert Opin Biol Ther 2014;14:1103-1111.
- Zhang W, Zou C, Pan L, Xu Y, Qi W, Ma G, Hou Y, Jiang P: MicroRNA-140-5p inhibits the progression of colorectal cancer by targeting VEGFA. Cell Physiol Biochem 2015;37:1123-1133.
- Xu K, Liu X, Mao X, Xue L, Wang R, Chen L, Chu X: MicroRNA-149 suppresses colorectal cancer cell migration and invasion by directly targeting forkhead box transcription factor FOXM1. Cell Physiol Biochem 2015;35:499-515.
- Fang Y, Sun B, Xiang J, Chen Z: MiR-301a promotes colorectal cancer cell growth and invasion by directly targeting SOCS6. Cell Physiol Biochem 2015;35:227-236.

- 25 Fowler A, Thomson D, Giles K, Maleki S, Mreich E, Wheeler H, Leedman P, Biggs M, Cook R, Little N, Robinson B, McDonald K: miR-124a is frequently down-regulated in glioblastoma and is involved in migration and invasion. *Eur J Cancer* 2011;47:953-963.
- 26 Xia H, Cheung WK, Ng SS, Jiang X, Jiang S, Jiang S, Sze J, Leung G.K.K, Lu G, Chan D.T, Bian X, Kung HF, Poon WS, and Lin MC: Loss of brainenriched miR-124 microRNA enhances stem-like traits and invasiveness of glioma cells. *J Biol Chem* 2012;287:9962-9971.
- 27 Xie L, Zhang Z, Tan Z, He R, Zeng X, Xie Y, Li S, Tang G, Tang H, He X: MicroRNA-124 inhibits proliferation and induces apoptosis by directly repressing EZH2 in gastric cancer. *Mol Cell Biochem* 2014;392:153-159.
- 28 Xia J, Wu Z, Yu C, He W, Zheng H, He Y, Jian W, Chen L, Zhang L, Li W: miR-124 inhibits cell proliferation in gastric cancer through down-regulation of SPHK1. *J Pathol* 2012;227:470-480.
- 29 Pierson J, Hostager B, Fan R, Vibhakar R: Regulation of cyclin dependent kinase 6 by microRNA 124 in medulloblastoma. *J Neurooncol* 2008;90:1-7.
- 30 Silber J, Lim DA, Petritsch C, Persson AI, Maunakea AK, Yu M, Vandenberg SR, Ginzinger DG, James CD, Costello JF, Bergers G, Weiss WA, Alvarez-Buylla A, Hodgson JG: miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. *BMC Med* 2008;6:14.
- 31 Huang TC, Chang HY, Chen CY, Wu PY, Lee H, Liao YF, Hsu WM, Huang HC, Juan HF: Silencing of miR-124 induces neuroblastoma SK-N-SH cell differentiation, cell cycle arrest and apoptosis through promoting AHR. *FEBS Letters* 2011;585:3582-3586.
- 32 Fowler A, Thomson D, Giles K, Maleki S, Mreich E, Wheeler H, Leedman P, Biggs M, Cook R, Little N, Robinson B, McDonald K: miR-124a is frequently down-regulated in glioblastoma and is involved in migration and invasion. *Eur J Cancer* 2011;47:953-963.
- 33 Hunt S, Jones AV, Hinsley EE, Whawell SA, Lambert DW: MicroRNA- 124 suppresses oral squamous cell carcinoma motility by targeting ITGB1. *FEBS Letters* 2011;585:187-192.
- 34 Zheng F, Liao Y, Cai M, Liu Y, Liu T, Chen S, Bian X, Guan X, Lin M, Zeng Y, Kung HF, Xie D: The putative tumor suppressor microRNA-124 modulates hepatocellular carcinoma cell aggressiveness by repressing ROCK2 and EZH2. *Gut* 2012;61:278-289.
- 35 Katoh K: Stress fiber organization regulated by MLCK and Rho-kinase in cultured human fibroblasts. *Am J Physiol Cell Physiol* 2001;280:C1669-C1679.
- 36 Tang AT, Campbell WB, Nithipatikom K: ROCK1 feedback regulation of the upstream small GTPase RhoA. *Cell Signal* 2012;24:1375-1380.
- 37 Riento K, Ridley AJ: Rocks: multifunctional kinases in cell behaviour. *Nat Rev Mol Cell Biol* 2003;4:446-456.
- 38 Lu X, Zheng J, Tang L, Huang B, Li K, Tao Y, Yu W, Zhu R, Li S, Li L: MiR-133b Promotes neurite outgrowth by targeting RhoA expression. *Cell Physiol Biochem* 2015;35:246-258.
- 39 Spencer C, Montalvo J, McLaughlin SR, Bryan BA: Small molecule inhibition of cytoskeletal dynamics in melanoma tumors results in altered transcriptional expression patterns of key genes involved in tumor initiation and progression. *Cancer Genomics Proteomics* 2011;8:77-85.
- 40 Somlyo AV, Bradshaw D, Ramos S, Murphy C, Myers CE, Somlyo AP: Rhokinase inhibitor retards migration and in vivo dissemination of human prostate cancer cells. *Biochem Biophys Res Commun* 2000;269:652-659.
- 41 Rattan R, Giri S, Singh AK, Singh I: Rho/ROCK pathway as a target of tumor therapy. *J Neurosci Res* 2006;83:243-255.
- 42 Liu D, Xiong R, Chen X, Li P, Ning Y, Peng Y, Zhao Y, Yang N, Zhou Y: The glucocorticoid dexamethasone inhibits U937 cell adhesion and neutrophil release via RhoA/ROCK1-dependent and independent pathways. *Cell Physiol Biochem* 2014;33:1654-1662.