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MicroRNA-92a Promotes Colorectal Cancer Cell Growth and Migration by Inhibiting KLF4

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Colorectal cancer (CRC) is the third most common malignancy with high mortality around the world. However, the biological mechanism of CRC carcinogenesis is not completely known. In the present study, we determined the role of miR-92a in the regulation of CRC cell motility. Expression of miR-92a is aberrantly upregulated in human CRC tissues and cultured cells, as shown by RT-PCR analysis. The effects of miR-92a on the proliferation and migration of human CRC SW620 and LoVo cells were measured by CCK-8 and Transwell assay, respectively. Results showed that the proliferation and migration capacity of both SW620 and LoVo cells were significantly increased by miR-92a mimic transfection but reduced by miR-92a inhibition. Additionally, KLF4 was identified as a direct target of miR-92a in CRC cells through bioinformatics and luciferase reporter analysis. KLF4 overexpression attenuated the effects of miR-92a on the regulation of CRC cell motility. Further studies suggested that the cell cycle inhibitor p21 was aberrantly downregulated in CRC cells, whereas this inhibition was reversed by miR-92a inhibitor. In conclusion, our data demonstrated that miR-92a may play a positive role in the colorectal carcinogenesis by promoting the proliferation and migration of CRC cells through targeting KLF4 as well as downstream p21. This could be an alternative therapeutic target for CRC.

Key words: miR-92a; Colorectal cancer (CRC); KLF4; p21; Proliferation; Migration

INTRODUCTION

Colorectal cancer (CRC) is the third most common malignancy with high mortality around the world. The chromosomal instability and sequential mutations of oncogenes and tumor-suppressor genes have been considered as the main molecular pathogenesis of CRC (1). According to the way that genetic alteration is transmitted, CRC can be classified as sporadic, familial, and hereditary forms with a different proportion in CRC cases. Most CRC correspond to the sporadic form; 10–30% of CRC cases are the familial form, and the hereditary form has the least number of cases (2). So far, early detection and surgical resection are the primary treatment approaches for CRC (1,3). Despite significant improvements in diagnostic method and surgical therapy, the cure rate of CRC is very low. Hence, knowledge about the molecular mechanisms underlying CRC progression is urgently necessary to improve the understanding of, and therapeutic strategies for, human CRC.

MicroRNAs (miRNAs) are noncoding, 19–22 nucleotides, single-stranded RNAs that comprise the key family of small endogenous regulators for gene expression

(4). It has been recognized that miRNA can regulate various target genes with negative effects by binding to the specific targets in the 3'-UTR of mRNA. Through the inhibition of translation or degradation of target mRNA, miRNA plays a regulatory role at the posttranscriptional level and contributes to the regulation of diverse biological processes, including cellular development, differentiation, growth, proliferation, and apoptosis (5,6). Accumulating evidence suggests that the dysregulation of miRNAs is involved in the pathogenesis of multiple human diseases (7). In cancer, aberrant regulation of miRNA expression has been reported widely and proven to be associated with cancer progression in glioma, metastatic prostate cancer, hepatocellular carcinoma, and others (8,9). In terms of CRC, various studies also reported the differential expression of miRNAs in patients with colorectal cancer, including miR-222, miR-135b, miR-17-3p, miR-31, miR-18b, miR-29a, and miR-124 (10–12).

Dysregulation of the miR-92a family expression has been detected in various cancers and was proven to be correlated with the biological mechanism of tumor

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development (13). He et al. reported that miR-92a expression is upregulated in human pancreatic cancer cells with a correlation to malignant degree of pancreatic cancer cells and plays a regulatory role in the proliferation of pancreatic cancer cells via DUSP10/JNK signaling pathway (14). In human neuroblastoma, miR-92a can promote the proliferation and migration of neuroblastoma cells by downregulating the TrkA protein expression (15). Additionally, the increased level of microRNA-92a in the plasma of patients with acute myeloid leukemia is related to the progression of disease and may represent a diagnostic and prognostic indication of myeloid leukemia (16). In CRC, miRNA-92 was found to be significantly elevated and might be a potential screening biomarker for colorectal cancer (17). Tsuchida et al. reported that miR-92 plays an oncogenic role in colon cancer as a key component of the miR-17-92 cluster (18); however, the regulatory mechanism of miR-92 in CRC progression is still unknown.

Krüppel-like factor 4 (KLF4), a zinc finger transcription factor enriched in a variety of tissues, has a critical role in cell differentiation and development. Growing evidence indicates that KLF4 is a tumor suppressor in the tumorigenesis of various cancers (19). Dysfunction of KLF4 was also reported to be involved in CRC metastasis by regulating the expression of matrix metalloproteinase 2 and E-cadherin (20). However, how KLF4 expression is regulated in the progression of CRC is still not well known.

In this study, we investigated the role of miR-92a in regulating colorectal cancer cell proliferation and migration. miR-92a was found to be significantly upregulated in CRC and promotes colorectal cancer cell motility by inhibiting KLF4. Identification of this mechanism might provide to be a novel understanding and therapeutic approach for CRC.

MATERIALS AND METHODS

Colorectal Cancer Samples and Cells

Primary cancerous biopsies and adjacent normal colonic tissues were collected from 30 patients with CRC undergoing surgery resection at Linyi People's Hospital. Informed consent was obtained from all patients. This study was approved by the Ethics Committee of Linyi People's Hospital.

Human CRC cell lines SW480, SW620, LoVo, and HCT-116 were purchased from ATCC (Manassas, VA, USA). SW480 and SW620 cells were cultured in DMEM medium. LoVo cells were maintained in F12-K, and HCT-116 cells were cultured in McCoy's 5a medium. Normal colon epithelial cell line NCM460 was purchased from Rongbai (Shanghai, China) and maintained in DMEM-H medium containing 10% FBS at 37°C in a 5% CO₂ atmosphere. All cells were maintained in medium supplemented

with 10% fetal bovine serum (FBS; Invitrogen) at 37°C in a 5% CO₂ atmosphere.

Quantitative RT-PCR

TRIzol reagent (Sigma-Aldrich) was used to isolate the total RNA from tissues and cells. Expression of miR-92a was measured using MicroRNA First-Strand Synthesis and miRNA Quantitation kits (Takara, Dalian, China) according to the manufacturer's instructions. CellAmp Direct RNA Prep kit for qPCR and a Protein Analysis kit (Takara) were used to detect the KLF4 and p21/CIP1 expression. The Ct values of U6 and GAPDH were used as the internal control to normalize the relative expression of miR-92a and KLF4, respectively. The reaction was performed as follows: 10 min at 95°C; 40 cycles of 1 min at 95°C, 2 min at 63°C, 1 min at 72°C; final annealing at 72°C for 10 min. All PCRs were performed in triplicate.

Western Blotting

Proteins were isolated from cell lysates and performed with bicinchoninic acid (BCA) assay to determine the concentration of proteins. An equal amount of protein was then separated by precast SDS-PAGE gels (Invitrogen) and electrophoretically transferred to PVDF membrane. After blocking, the blots were probed with primary antibodies [rabbit anti-KLF4, 1:1,000 (Abcam); mouse anti-β-actin, 1:1,000 dilution (ABclonal)] overnight at 4°C. Specific secondary antibodies were then incubated with blots for 1 h at room temperature, followed by visualization with ECL (Amersham Pharmacia, Piscataway, NJ, USA).

Cell Transfection

The miR-92a mimic, inhibitor, and negative control miRNA were purchased from RiboBio (Guangzhou China) and transfected into cells at 100 nM concentrations via Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. KLF4 plasmid (1 μg; Origene, Rockville, MD, USA) was also transfected into cells in the presence or absence of the miR-92a mimic by Lipofectamine 2000. After transfection twice in 48 h, cells were used in the subsequent experiments. RT-PCR and Western blotting were used to evaluate the transfection efficacy.

Cell Proliferation Assay

Cell proliferation was determined by Cell Counting Kit (CCK-8/WST-8) (Bioroot, Shanghai, China) according to the kit's instructions. Briefly, 10 μl of CCK-8 solution was added to the transfected cells that were planted in the 96-well plates (5 × 10³ cells/ml) at 12, 24, 48, and 72 h and incubated for 4 h at 37°C. The absorbance of cells was detected at 450 nm using ELISA plate reader (Bio-tek, Winooski, VT, USA).

Cell Migration Assay

Cell migration was measured in the Transwell Boyden chamber. Briefly, after 2 days of transfection, cells were incubated in the growth factor-free DMEM-H and then moved into the upper chamber of Boyden chambers coated with gelatin. The lower compartment of the chamber was filled with 600 μ l of DMEM-H medium supplemented with 10% FBS. Cells were incubated at 37°C for 4 h, followed by fixation with 90% ethanol and staining with 0.05% crystal violet for 15 min. Nontransmigrated cells were gently scraped off using cotton swab. Migrated cells were determined by counting the cells on the lower membrane surface within five fields per chamber under a microscope.

Luciferase Assay

Site-directed mutagenesis was introduced into the miR-92a binding site of KLF4 mRNA using QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene). The 3'-UTR fragment of KLF4 mRNA was then subcloned into the pGL3 luciferase vector (Promega, Madison, WI, USA) by PCR method and cotransfected with miR-92a mimic into CRC cells for 36 h in 96-well plates using Lipofectamine 2000. Dual Luciferase Assay (Promega) was then performed to analyze the luciferase assays. Renilla (Promega) activity was used as the internal control.

Statistical Analysis

All data are expressed as mean \pm SEM. The differences between groups were analyzed using SPSS15.0 by one-way ANOVA. A value of $p < 0.05$ was considered statistically significant. All experiments were performed in triplicate.

RESULTS

miR-92a Is Aberrantly Upregulated in Human Colorectal Cancer Samples and Cells

We first investigated the expression levels of miR-92a in sample tissues of patients with colorectal cancer to determine the role of miR-92a in human CRC. As shown in Figure 1A, miR-92a was expressed at low levels in the adjacent normal colonic tissues, while it was significantly upregulated in the colorectal cancer tissues.

Furthermore, we investigated the expression of miR-92a in human CRC cell lines SW480, SW620, LoVo, and HCT-116. Compared to the normal colon epithelial cells NCM460, the expression levels of miR-92a were aberrantly increased in the SW480, SW620, LoVo, and HCT-116 cells to different extents (Fig. 1B). Taken together, these data suggest that miR-92a may play a positive role in the regulation of human CRC progression.

miR-92a Promotes the Proliferation and Migration of Human Colorectal Cancer Cells

We then introduced miR-92a mimic and inhibitor to determine the possible effects of miR-92a on the proliferation and migration of human colorectal cancer cells in vitro. Herein, the SW620 and LoVo cell lines were chosen as the cell models according to the difference of miR-92a expression. The efficiency of miR-92a mimic and inhibitor was confirmed by RT-PCR. Compared with the control miRNA, the miR-92a expression was significantly enhanced by the mimic but reduced by the inhibitor in both SW620 and LoVo cell lines (Fig. 2A).

A CCK-8 assay was performed to examine the effect of miR-92a on the proliferation of SW620 and LoVo cells. As seen in Figure 2B, miR-92a mimic transfection

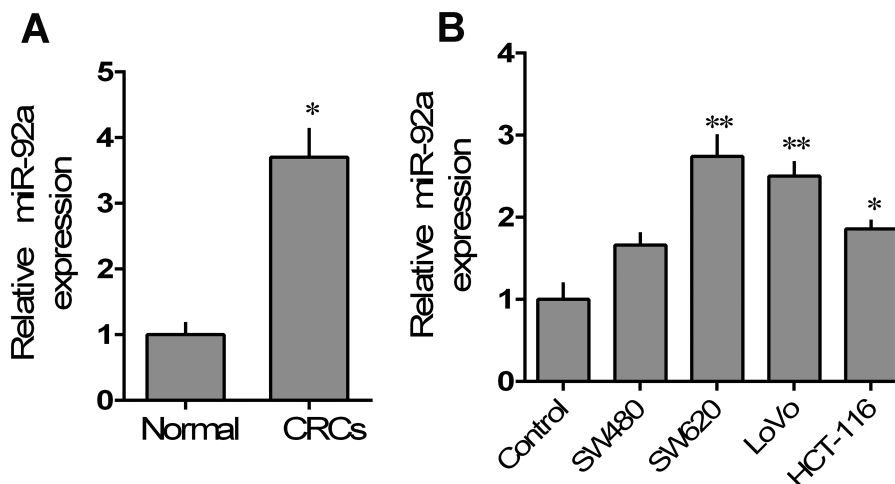


Figure 1. miR-92a is upregulated in human CRC tissues and cells. (A) The expression of miR-92a was measured in human CRC tissues and adjacent normal colonic tissues (normal) by RT-PCR assay ($*p < 0.05$ vs. normal). (B) The levels of miR-92a were examined in human CRC cell lines SW480, SW620, LoVo, HCT-116, and the normal colon epithelial cells NCM460 (control) ($**p < 0.01$, $*p < 0.05$ vs. control).

remarkably increased the proliferation of both SW620 and LoVo cells compared with the control group. On the contrary, the proliferation capacity of these two cell lines was significantly restrained by miR-92a repression induced by inhibitor. We also confirmed the alteration of SW620 and LoVo cell migration using Transwell assay. Results showed that the migration of SW620 and LoVo cell lines was significantly increased by miR-92a overexpression, but repressed by miR-92a silencing (Fig. 2C). These results suggest that high levels of endogenous miR-92a may play a regulatory role in the development of CRC by promoting CRC cell proliferation and migration.

KLF4 Is the Direct Target of miR-92a in Colorectal Cancer Cells

KLF4 has been considered to be a tumor suppressor in CRC by previous studies (20). To further elucidate the underlying mechanism of miR-92a regulating colorectal cancer cells, we predicted the possible targets of miR-92a using TargetScan and miRDB databases. Online analysis

suggested that KLF4 is a potential direct target of miR-92a with a binding site in the 3'-UTR. To further confirm whether or not this prediction is right, we performed the luciferase reporter assay in SW620 and LoVo cells. The 3'-UTR regions of KLF4 containing the predicted binding site of miR-92a or the mutant site were cloned into a luciferase vector (Fig. 3A). It was found that miR-92a overexpression significantly inhibited the luciferase activities of KLF4-3'-UTR-wt reporter in two cell lines, whereas miR-92a mimic transfection exhibited no inhibitory effects on the luciferase activities of KLF4-3'-UTR-mu reporter in cells (Fig. 3B).

In addition, we confirmed that the protein level of KLF4 was strongly downregulated by miR-92a mimic, but increased by miR-92a inhibitor in SW620 and LoVo cells (Fig. 3C). However, there was no effect of miR-92a mimic and inhibitor on the mRNA expression of KLF4. In summary, these data indicate that KLF4 expression is directly regulated by miR-92a at the posttranscriptional level in colorectal cancer cells.

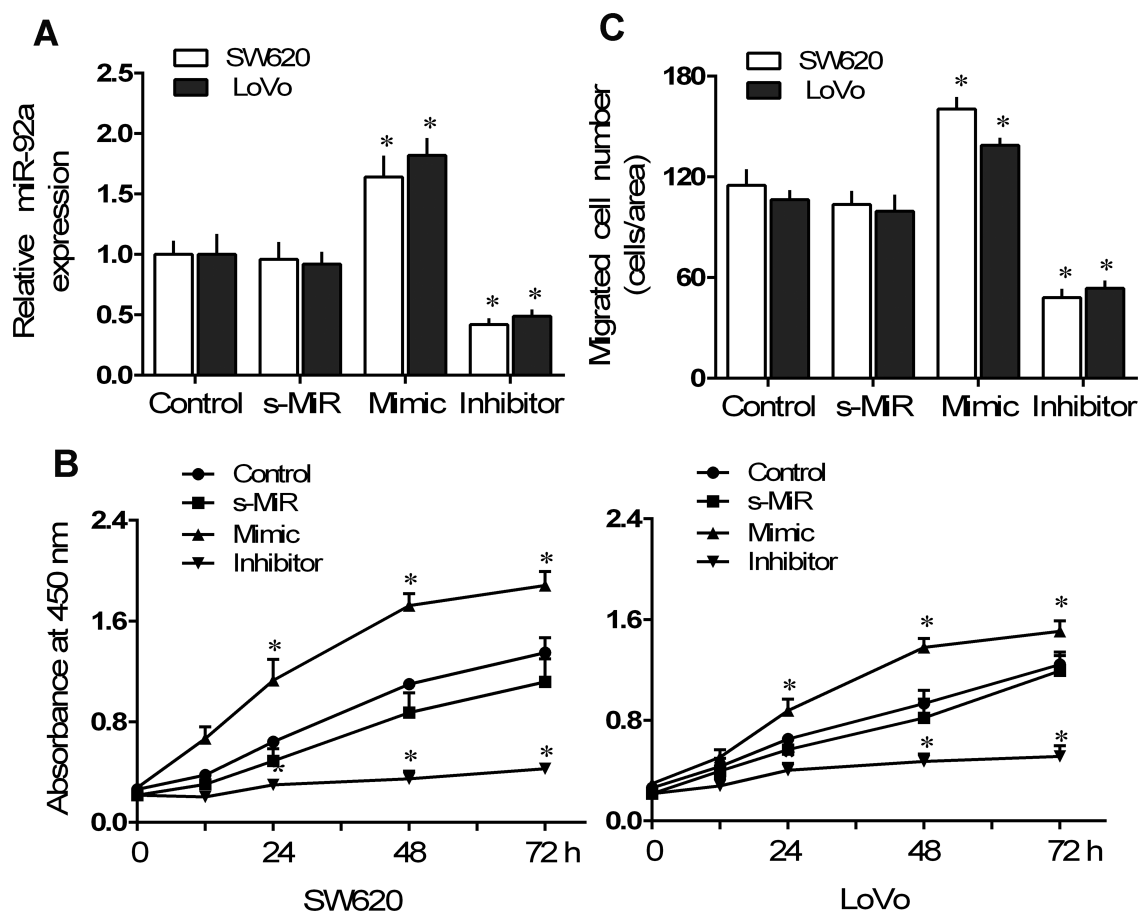


Figure 2. miR-92a promotes the proliferation and migration of human CRC cells. Cultured SW620 and LoVo cells were transfected with miR-92a mimic, inhibitor, or negative control miRNA (s-MiR). (A) The levels of miR-92a in cells were evaluated after 48 h of transfection using RT-PCR. (B) Cell proliferation was detected at 12, 24, 48, and 72 h via CCK-8 assays. (C) Cell migration was measured after 48 h of transfection by Transwell assay. Nontransfected cells were used as the control (* $p < 0.05$ vs. control).

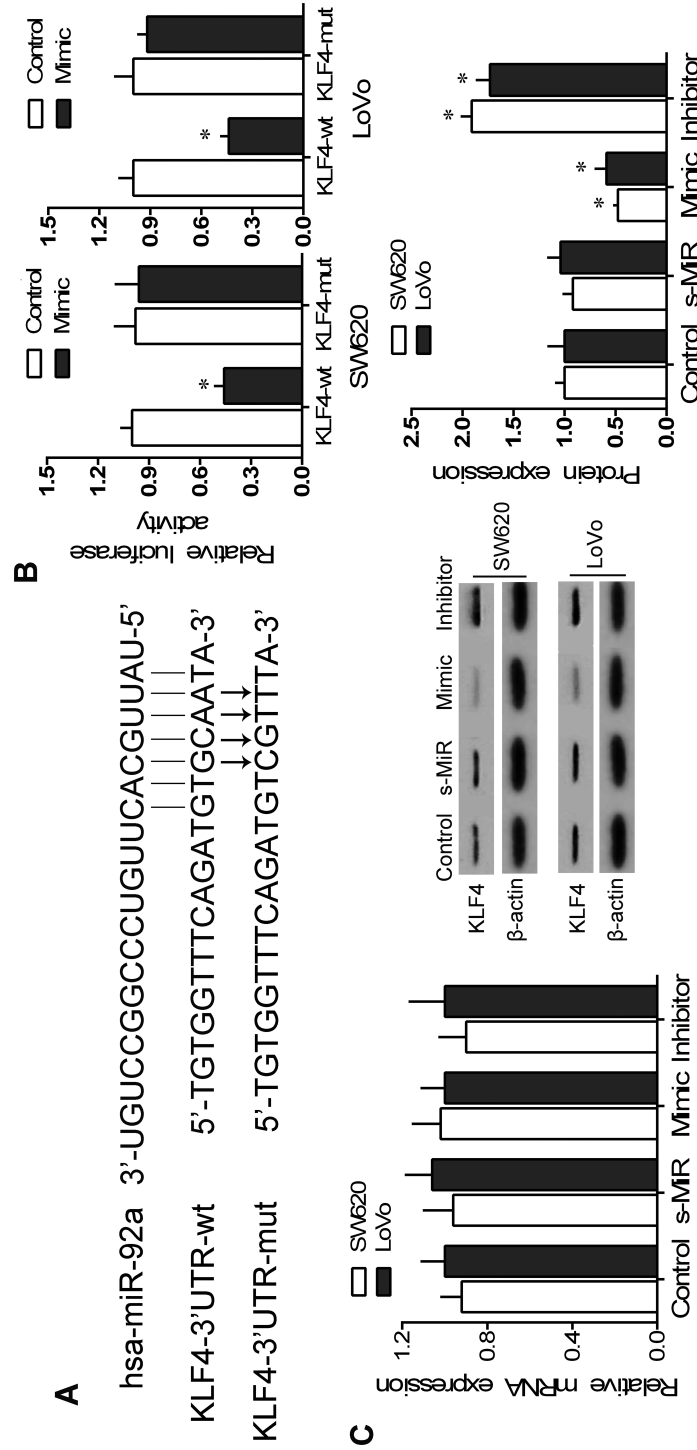


Figure 3. KLF4 is the target of miR-92a in CRC cells. (A) The predicted binding site of miR-92a in the 3'-UTR of KLF4. (B) Luciferase activities were analyzed in the cells transfected with KLF4-3'-UTR-wt (KLF4-wt) or KLF4-3'-UTR-mut (KLF4-mut). (C) The expression of KLF4 in cells was examined via qRT-PCR and Western blot assay. Nontransfected cells were used as the control (* $p < 0.05$ vs. control).

miR-92a Serves as a Positive Regulator of Colorectal Cancer Cell Motility by Targeting KLF4

miRNAs play a role in the regulation of cellular function by inhibiting target genes. To explore that KLF4 is indeed the downstream mediator of miR-92a in promoting colorectal cancer cell motility *in vitro*, we employed a KLF4 plasmid to specifically induce the expression of KLF4 in cells (Fig. 4A). The upregulation of KLF4 expression reversed the promoting effect of miR-92a on the proliferation capacity of SW620 and LoVo cells (Fig. 4B). The migration of cells induced by miR-92a mimic was also inhibited by KLF4 overexpression as shown in Figure 4C, indicating that miR-92a promotes the proliferation and migration of colorectal cancer cells by targeting KLF4.

Cell cycle inhibitor p21 is a downstream signal molecule of KLF4. Previous studies indicate that KLF4 inhibits cellular proliferation and migration by directly targeting p21 (19). To further confirm the involvement of KLF4 in miR-92a-mediated effects, we investigated the expression of p21. Compared with the normal colon epithelial cells NCM460, p21 expression was aberrantly downregulated in SW620 and LoVo cells, whereas this inhibition was reversed by miR-92a inhibitor (Fig. 4D). These findings imply that miR-92a plays a positive role in the regulation

of colorectal cancer cell motility by repressing the inhibitory effect of KLF4 on cell cycle progression.

DISCUSSION

Differential expression of microRNAs in patients with colorectal cancer was reported in numerous studies, such as miR-222, miR-135b, miR-17-3p, miR-31, miR-18b, miR-29a, and miR-124 (21–23). Although over- or underexpression of microRNAs occurring in CRC has been considered to contribute to colorectal carcinogenesis, the potential mechanism by which miRNAs regulate colorectal progression needs to be further explored. Previous reports demonstrate that miRNA-92 expression is significantly upregulated in CRC and may present a novel screening biomarker for early diagnosis of CRC (17). However, the precise role of miR-92a in the pathogenesis of CRC remains unknown. In the present study, we determined that miR-92a is aberrantly upregulated in the primary cancerous biopsies of patients with CRC as well as in four human CRC lines *in vitro*, implying that miR-92a may play a positive role in the regulation of human CRC progression.

Emerging evidence indicates that cell malignant proliferation and invasion are the main causes that contribute

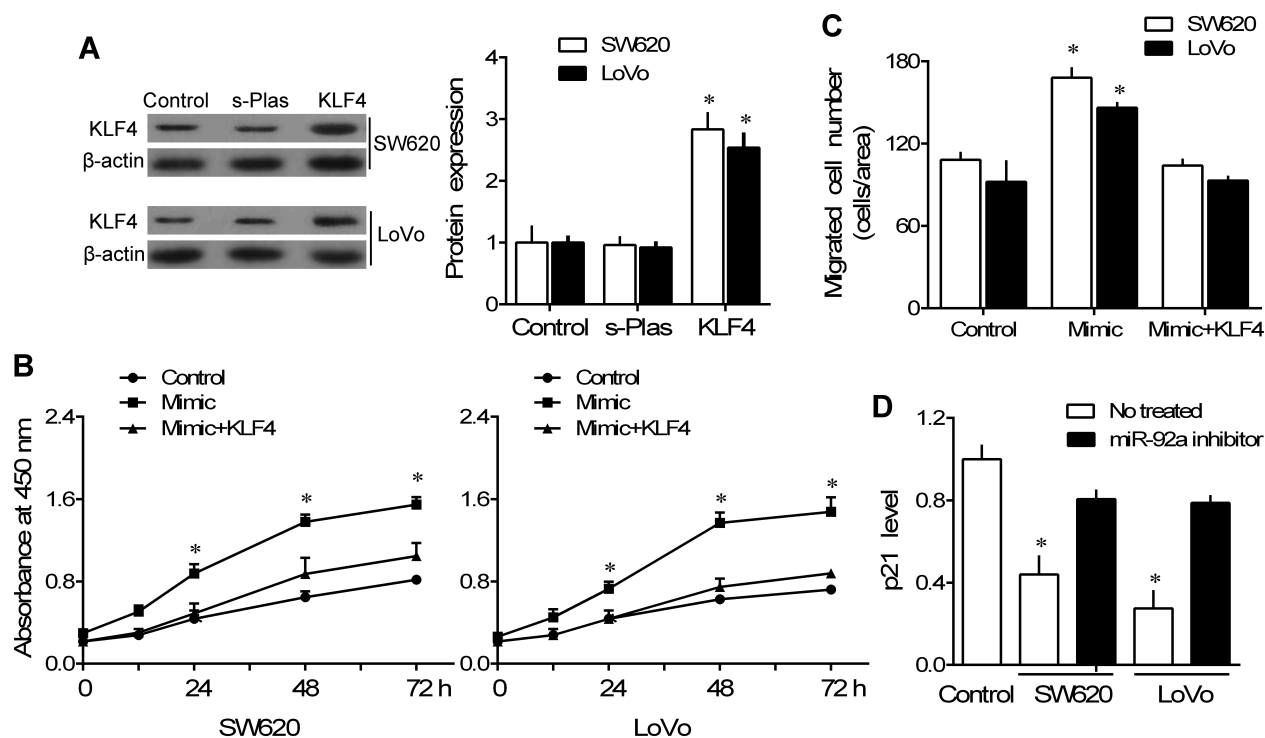


Figure 4. miR-92a regulates CRC cells motility by targeting KLF4. (A) The protein levels of KLF4 were detected via Western blot in SW620 and LoVo cells transfected with KLF4 plasmid or negative control plasmid (s-Plas). After cotransfection with miR-92a mimic and KLF4 plasmid, cell proliferation (B) and migration (C) were then tested. (D) The level of p21 in SW620, LoVo, and NCM460 cells (control) was measured by RT-PCR (* $p < 0.05$ vs. control).

to tumorigenesis and carcinogenesis in humans (24). In CRC, it is reported that dysregulation of miRNAs has diverse effects on colorectal cancer cell motility. miR-29a was found to promote colorectal cancer cell invasion by regulating the expression of matrix metalloproteinase 2 and E-cadherin via targeting KLF4 (20). Zhang et al. also reported that miR-124 is significantly downregulated in CRC tissues and can suppress the proliferation of CRC cells by directly targeting STAT3 (25). Consistently, our results revealed the positive role of miR-92a in CRC tumorigenesis. Increased expression of miR-92a increased the proliferation and migration capacity of cultured CRC cells, whereas miR-92a repression exhibited an inhibitory effect on CRC cell motility *in vitro*.

KLF4 is a member of KLF zinc finger transcription factor family that is enriched in a variety of tissues with a critical role in the regulation of cell differentiation and development. Accumulating evidence determined that KLF4 is expressed at a low level and exerts a tumor-suppressive effect in many types of cancer (20,26–28). In CRC, KLF4 expression is also remarkably downregulated in CRC tissues compared with matched normal tissues (29). Tang et al. reported that KLF4 has an effect as a target of miR-29a on the modulation of miR-29a to colorectal cancer metastasis by regulating the expression of matrix metalloproteinase 2 and E-cadherin (20). Here we demonstrated that KLF4 is a direct target of miR-92a and can be negatively regulated by miR-92a at the post-transcriptional manner in CRC cells. Notably, we found that the overexpression of KLF4 reverses the effect of miR-92a on the proliferation and migration of CRC cells, indicating that miR-92a may regulate the motility of CRC cells by directly targeting KLF4.

As the downstream target of KLF4, p21 has been identified as a negative regulator in cell cycle progression. It is reported that p21 transactivation is induced by the binding of KLF4 to its promoter, contributing to the inhibitory role of p21 in cell cycle (19,27,28). We investigated the effects of miR-92a-KLF4 on downstream p21 in CRC cells. Compared with normal colon epithelial cells, p21 expression is significantly downregulated in cultured CRC cells, which is consistent with the findings in previous studies (30,31). In contrast, miR-92a inhibitor significantly increased the expression of p21 in parallel with an enhanced KLF4 level, indicating that miR-92a may promote colorectal cancer cell growth and invasion by regulating the activity of KLF4 as well as downstream p21.

In summary, we provide a novel understanding about the pathogenesis of human CRC. Increased miR-92a level plays a positive role in colorectal carcinogenesis by promoting the proliferation and migration of CRC cells through targeting downstream KLF4 and p21. This may offer an alternative therapeutic target for CRC.

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