MicroRNA-202-3p Inhibits Cell Proliferation by Targeting ADP-Ribosylation Factor-like 5A in Human Colorectal Carcinoma

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Introduction

MicroRNAs (miRNA) are small noncoding RNAs (18–22 nt in length) that regulate the expression of target genes at the posttranscriptional level by binding to their target mRNAs (1, 2). Since their discovery in 1993, the number of verified miRNAs has grown rapidly, and the latest version of miRBase (release 20.0, June 2013, http://www.mirbase.org/) has annotated 2,578 mature miRNA sequences in the human genome. miRNAs are estimated to regulate up to one third of human genes at the posttranscriptional level (3). miRNA deregulation has been demonstrated to play a role in the pathologic processes of tumorigenesis (4, 5). Furthermore, miRNAs serve as potential diagnostic markers, prognostics factors, and therapeutic targets (6–9). Although miRNAs have been reported to be involved in human cancers, their biologic functions and molecular mechanisms remain largely unknown.

Colorectal cancer is the third most commonly diagnosed cancer in men and the second most commonly diagnosed cancer in women worldwide. Over 1.2 million new cancer cases occur annually, resulting in greater than 600,000 deaths each year (10). Despite recent advances in diagnostic and therapeutic measures, the prognoses of patients with colorectal cancer remain poor. Therefore, it is necessary to clarify the underlying molecular mechanisms and to identify...
Translational Relevance

MicroRNAs (miRNA) are small, noncoding RNAs that are strongly implicated in carcinogenesis and have recently reshaped our understanding of the role of non–protein-coding RNAs. miRNAs have been argued to be used as potential diagnostic markers, prognostic factors, and therapeutic targets in the pathologic processes of tumorigenesis, while the biologic functions and molecular mechanisms of action of the majority of miRNAs remain largely unknown. In this study, miR-202-3p, which is frequently downregulated in human colorectal cancer, was identified as a potent repressor of proliferation. Reexpression of miR-202-3p expression inhibited colorectal cancer proliferation by silencing its direct target, ADP-ribosylation factor-like 5A (ARL5A), which was closely related to poor prognosis of patients with colorectal cancer. Although miRNA research is still in the early stages, the contribution of miRNAs to disease processes is becoming clear. Novel and expanded insights into miRNA deregulation will deepen our understanding of the complicated molecular pathogenesis of colorectal cancer and delineate new options for colorectal cancer treatment.

specific biomarkers of colorectal cancer. The timely discovery of the importance of miRNAs is fortunate in this regard.

Over the past decade, the contribution of miRNAs (11), such as let-7 (12, 13), miR-17 (14, 15), miR-21 (16–19), miR-31 (20, 21), miR-95 (22), miR-101 (23, 24), miR-135 (25, 26), miR-137 (27), miR-139 (28, 29), miR-143 (30–32), miR-145 (31, 33), miR-155 (34), miR-183 (35), and miR-195 (36), to colorectal carcinogenesis has been examined. In a previous study, we determined that miR-95 is frequently overexpressed in colorectal cancer and acts as an important inducer of proliferation via the negative regulation of SNX1 expression (22). All these data highlight the importance of miRNAs in tumor development and provide new insights into the molecular mechanisms underlying carcinogenesis.

Our previous microarray data showed that miR-202-3p, a member of the let-7 family, was downregulated in colorectal cancer (22). In addition to colorectal cancer, miR-202-3p has also been reportedly deregulated in breast cancer and cervical squamous cell carcinoma (37, 38) and could inhibit cell proliferation in neuroblastomas (39, 40). These data suggest that miR-202-3p may play common roles in tumorigenesis. However, the detailed function and molecular mechanisms of miR-202-3p in human tumors have not been elucidated. In the present work, we confirmed that miR-202-3p expression was significantly decreased in colorectal cancer tissues compared with the noncancerous tissues (NCT) in an expanded colorectal cancer cohort. Moreover, we showed that miR-202-3p could inhibit cell proliferation by directly targeting ADP-ribosylation factor-like 5A (ARL5A) in colorectal cancer, and analysis of clinical tumor samples indicated that ARL5A is a new independent prognostic factor in colorectal cancer.

Materials and Methods

Human tissues and cell lines

A total of 94 pairs of human primary colorectal cancer tissues and their adjacent NCTs were collected between 2005 and 2008 at the Fudan University Shanghai Cancer Center. The tissue samples were immediately snap-frozen in liquid nitrogen and were histologically examined in a timely manner. All of the human materials were obtained with informed consent, and this project was approved by the Clinical Research Ethics Committee of Fudan University Shanghai Cancer Center. The clinical information of the patients with colorectal cancer is included in Supplementary Table S1. HEK-293T cell line and six human colorectal cancer cell lines, including the HCT-8, HCT-116, LoVo, DLD1, SW480, SW620, were purchased from the American Type Culture Collection (ATCC). HCT-8 cells were cultured in RPMI-1640 medium, HCT-116 cells were cultured in McCoy’s 5a medium, and LoVo cells were cultured in F12-K medium. DLD1 and HEK-293T cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM). SW480 and SW620 cells were maintained in Leibovitz’s L-15 medium. All of these media (Invitrogen) were supplemented with 10% FBS (Gibco). The cells were incubated under the conditions recommended by ATCC.

DNA and RNA extraction and quantitative real-time RT-PCR

Tissue genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer’s protocol. Total RNA, including miRNA, was extracted using the TRizol reagent (Invitrogen) according to the manufacturer’s instructions. The concentrations of DNA and RNA were determined using a NanoDrop ND-1000 (NanoDrop), and aliquots of the samples were stored at −80°C. Relative DNA copy numbers were determined by quantitative PCR (qPCR) using SYBR Premix Ex Taq (TaKaRa). An amount of 50 ng DNA was used as template in a final volume of 20 µL with LINE-1 as an internal control (41–43). cDNA was synthesized with the PrimeScript RT Reagent Kit (TaKaRa) using 500 ng total RNA as template. qPCR analyses were conducted to quantify mRNA relative expression using SYBR Premix Ex Taq (TaKaRa) with β-ACTIN as an internal control (44–46). TaqMan microRNA assays (Applied Biosystems) were used to determine the expression levels of miR-202-3p after reverse transcribing by sequence-specific primers (Applied Biosystems), and U6 small nuclear RNA was used as an internal control (47–50). The results of qPCR were defined from the threshold cycle (Ct), and relative expression levels were calculated by using the 2^−ΔΔCt method (51). PCR was performed using an ABI 7900HT instrument (Applied Biosystems). The primers used for PCR analysis were listed in Supplementary Table S2.
**Vector constructs**

The human pri-let-7b-202 sequence was amplified from normal human genomic DNA by nested PCR using PrimerSTAR Premix (TaKaRa). The sequence was then cloned into the lentivirus expression vector pGIPZ (Thermofisher) to generate pGIPZ-pmiR-202. The predicted binding sites in the 3’ untranslated regions (UTR) of the potential target genes of miR-202-3p were amplified by nested PCR and cloned into the region directly downstream of a cytomegalovirus (CMV) promoter–driven firefly luciferase cassette in the pCDNA3.0 vector (pLuc). The mutant-type (MT) 3’ UTR of ARL5A, which carries the mutated sequence in the complementary site of the seed region of miR-202-3p, was generated from the pLuc-ARL5A 3’ UTR–wild-type (WT) plasmid by overlap-extension PCR. The sequences of the WT and MT 3’ UTRs were confirmed by DNA sequencing. The open reading frame (ORF) of ARL5A was amplified via nested PCR and cloned into the plVX-IRES-Neo vector (Clontech). The primers and endonuclease sites used for the vector constructs are shown in Supplementary Table S2.

**Lentivirus production and transduction**

Virus particles were harvested 48 hours after cotransfecting pGIPZ-pmiR-202 and pLVX-ARL5A with the packaging plasmid ps-PAX2 and the envelope plasmid pMD2G into HEK-293T cells using Lipofectamine 2000 Reagent (Invitrogen). HCT-116 and LoVo cells were infected with recombinant lentivirus-transducing units plus 6 μg/mL polybrene (Sigma).

**Oligonucleotide transfection**

Small interfering RNA (siRNA) of miR-202-3p target genes (sequence information; Supplementary Table S3), as well as miR-202-3p/5p mimics and inhibitors (anti-miR-202-3p/5p, chemically modified antisense oligonucleotides designed to specifically target mature miR-202-3p/5p), were synthesised (Ribobio). Oligonucleotide transfection was performed using RNAiMAX reagents (Invitrogen) according to the manufacturer’s instructions. The final concentration of the miR-202-3p/5p mimics or inhibitors in the transfection mixture was 50 nmol/L.

**Cell proliferation and colony formation assays**

Cell proliferation was quantified using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories) according to the manufacturer’s instructions and the optical density (OD) value was obtained for determine the cell numbers. For the colony formation assays, 1,000 cells per well of HCT-116 and 1,500 cells per well of LoVo were plated into 6-well plates and incubated in medium containing 10% FBS for 2 weeks. The colonies were fixed with methanol and stained with 0.1% crystal violet in 20% methanol for 20 minutes. The number of colonies containing more than 30 cells was counted using an inverted microscope.

**Tumor formation assay in a nude mouse model**

Athymic male BALB/c nude mice were maintained under specific pathogen-free conditions in the Experimental Animal Department of Fudan University. The assay protocols were approved by the Shanghai Medical Experimental Animal Care Commission. HCT-116 or LoVo cells stably expressing miR-202 or the vector control were washed from subconfluent cell culture plates with PBS and then were resuspended with DMEM at a concentration of 1 × 10⁷ cells/mL. A 0.1 mL aliquot of HCT-116 or 0.2 mL aliquot of LoVo-suspended cells was subcutaneously injected into the right flank of each mouse age 5 weeks (8 mice for each group). After transplantation, the growth of the subcutaneous tumors was assessed twice a week. Tumor size was monitored by measuring the length and width with callipers, and volumes were calculated with the formula: \( V = \frac{L \times W^2}{2} \), where L is the length and W is the width of each tumor. The mice were sacrificed after a period of 4 to 6 weeks, and the weight of subcutaneous tumors was recorded. HCT-116 cells, 24 hours after transfection with miR-202-3p mimics or negative control (NC), were also used for xenograft experiments following the procedures described above (52). The expression levels of miR-202-3p and ARL5A protein of cells used for tumor formation assay were examined (Supplementary Figs. S2B and S2C).

**Cell migration assay**

For the migration assays, 1 × 10⁵ HCT-116 or 5 × 10⁴ LoVo cells (stably expressed miR-202 or vector control) in 200 μL serum-free medium were placed onto the top chamber of each insert (BD Biosciences). After 36 (HCT-116) or 8 (LoVo) hours of incubation at 37°C, cells adhering to the lower membrane were stained with 0.1% crystal violet in 20% methanol, imaged, and counted using an IX71 inverted microscope (Olympus).

**Microarray analysis**

Genome-wide expression profiling was performed using an Agilent whole human genome oligo microarray chip (4 × 44 K) that contains more than 42,034 genes and transcripts (Agilent). An amount of 5 × 10⁶ cells (HCT-116 or LoVo) were seeded in a 6-cm² tissue culture dish and were transfected with miR-202-3p or control miRNA as described above. After propagating for 48 hours, total RNA was extracted from the cells using TRIzol reagent (Invitrogen). The RNA concentration was determined, and the integrity of RNA was verified using an Agilent 2100 Bioanalyzer (Agilent). Cy3-labeled cRNA was prepared with an Agilent One Color Spike Mix Kit (Agilent) and then hybridized by using a Gene Expression Hybridization Kit (Agilent) at 65°C for 17 hours. Signal intensity was calculated from digitized images captured by Laser Scanner (Agilent) and the chip data were analysed using the GeneSpring GX software (Agilent). An mRNA was designated as “down-regulated” if its expression in miR-202-3p–transfected cells was 2-fold lower than that of the corresponding control cells.

**Luciferase assay**

Approximately 8,000 HEK-293T cells per well and 12,000 HCT-116 cells per well were plated into 96-well plates and
were cotransfected with 50 nmol/L of miR-202-3p mimic (or NC), 50 ng of the luciferase reporter, and 5 ng of the pRL-CMV Renilla luciferase reporter using 0.5 µL per well Lipofectamine 2000 (Invitrogen). LoVo cells, plated into 96-well plates with 12,000 cells per well, were cotransfected with 100 nmol/L of miR-202-3p mimic (or NC), 200 ng of the luciferase reporter, and 20 ng of the pRL-CMV Renilla luciferase reporter using 1.25 µL per well X-tremeGENE Reagent (Roche). After a 48-hour incubation, the Firefly and Renilla luciferase activities were quantified using a dual-luciferase reporter assay (Promega).

**Western blot analysis**

Harvested proteins were first separated by 10% SDS–PAGE and then transferred to nitrocellulose membranes (Bio-Rad Laboratories). The membranes were blocked with 5% nonfat milk and incubated with a mouse anti-ARL5A polyclonal antibody at a dilution of 1:500 (Abgent) or a mouse anti-β-ACTIN monoclonal antibody at a dilution of 1:1,000 (Sigma). The membranes were subsequently incubated with a goat anti-mouse horseradish peroxidase secondary antibody (Sigma). The protein complex was detected using enhanced chemiluminescence reagents (Pierce). Endogenous β-ACTIN was used as the internal control.

**Immunohistochemical staining**

Tissue arrays were constructed using the 94 paired colorectal cancer tissues and NCTs. Immunohistochemical staining was performed on 4 µm sections of paraffin-embedded tissues to determine the expression level of ARL5A protein. In brief, the slides were incubated in ARL5A antibody (Abgent) diluted 1:200 at 4°C overnight. The subsequent steps were performed using the EnVision FLEX High pH visualisation system according to the manufacturer’s instructions (Dako). The ARL5A staining intensity measurements are presented in Supplementary Fig. S2.

**Statistical analyses**

The results are presented as the mean values ± SEM. Overall survival (OS) rates were calculated actuarially according to the Kaplan–Meier method with log-rank test and were measured from the day of surgery. Differences between groups were estimated using the χ² test, the Student t test, the Mann–Whitney U test, and the repeated-measures ANOVA test. Relationships were explored by the Spearman correlation. A value of P < 0.05 was considered statistically significant. SPSS 16.0 package (IBM) and Graphpad prism 5.0 software (GraphPad Software) were used for statistical analyses and scientific graphing, respectively.

**Results**

**Expression of miR-202-3p is decreased in human colorectal cancer tissues**

miRNA expression profiling previously demonstrated that miR-202-3p is downregulated in colorectal cancers compared with NCTs (22). To further confirm this result, the expression level of mature miR-202-3p was examined using qRT-PCR in an expanded cohort of 94 patients with colorectal cancer. Consistent with the microarray data, miR-202-3p expression was remarkably downregulated in 44 of the 94 (46.7%) colorectal cancer tissues when compared with the corresponding NCTs (P < 0.001; Fig. 1A). However, there was no significant association between miR-202-3p expression in colorectal cancer tissues and gender, age, tumor location, tumor size, histologic classification, nodal status, or survival (P > 0.05; data not shown). In addition, miR-202-3p T:N expression ratios were weakly associated with OS of patients with colorectal cancer (Supplementary Fig. S1A).

**Downregulation of miR-202-3p in colorectal cancer tissues caused by DNA deletion**

To confirm the hypothesis that downregulation of miR-202-3p in colorectal cancer tissues was partly caused by copy number variation of its coding sequence, we determined the pre-miR-202 copy number by qPCR in 37 pairs of the colorectal cancer tissues and NCTs. Interestingly, we found that the copy number of pre-miR-202 was lower in 64.9% (24 of 37) and higher in 13.5% (5 of 37) of the colorectal cancer tissues compared with their NCT counterparts (P < 0.05; Fig. 1B). Subsequent analysis showed that the pre-miR-202 copy numbers were related to the expression levels of mature miR-202-3p in both the colorectal cancer and NCT samples (P < 0.05; Fig. 1C and D).

**miR-202-3p represses colorectal cancer cell proliferation in vitro and in vivo**

The consistently low expression of miR-202-3p in colorectal cancer suggests it contributes to tumorigenesis. The expression of miR-202-3p was examined in 6 different colorectal cancer cell lines (Supplementary Fig. S1B). A cell proliferation assay revealed that overexpression of miR-202-3p significantly reduced the growth rate of HCT-116 and LoVo cells (P < 0.01; Fig. 2A), and a colony formation assay confirmed that miR-202 overexpression represses the proliferation of colorectal cancer cells (P < 0.01; Fig. 2B). In contrast, silencing of miR-202-3p expression with siRNA significantly promoted the growth of HCT-116 and LoVo cells (P < 0.01; Fig. 2A). Besides that, miR-202-5p did not show significant effect on the colorectal cancer cell growth (Supplementary Fig. S1C). To assess the function of miR-202 in vivo, a tumor formation assay in a nude mouse model was performed using HCT-116 and LoVo cells stably expressing miR-202 after lentiviral infection. Compared with the empty vector control, overexpression of miR-202 remarkably repressed tumorigenesis in nude mice (P < 0.01; Fig. 2C and D). Furthermore, overexpressed miR-202-3p by mimics transfecting in HCT-116 cells also repressed tumor growth in vivo (Supplementary Fig. S2A). In addition, transwell assay in HCT-116 or LoVo cells indicated that miR-202-3p may not significantly affect colorectal cancer migration (Supplementary Fig. S1D).

**Screening of candidate target genes of miR-202-3p**

To explore the potential mechanism by which miR-202-3p represses the proliferation of colorectal cancer cells, we...
performed a microarray analysis to search for candidate genes regulated by miR-202-3p. Compared with the vector controls, a total of 823 downregulated genes (>2-fold change) were identified in the miR-202-3p–transfected LoVo cells (Supplementary Table S4). By comparing all of the downregulated genes with the candidate genes predicted by the TargetScan and Miranda programs, a total of 18 downregulated genes were selected out (Fig. 3A). Then the expression of these 18 genes was validated using qRT-PCR in both miR-202-3p–transfected HCT-116 and LoVo cells and 10 of these 18 genes were indeed downregulated by miR-202-3p (Fig. 3B). Then the recombinant plasmids with the 3′ UTR sequences of these 10 genes containing the predicted binding site sequence of miR-202-3p were constructed for luciferase assays. A panel of 4 candidate genes (ARL5A, LRIG3, HECTD2, and CPEB4) was selected because of their relatively decreased luciferase activity in miR-202-3p–transfected cells (Fig. 3C). Subsequently, a preliminary cell proliferation assay was performed on HCT-116 and LoVo cells transfected with the pertinent siRNAs (to eliminate the off-target effects of siRNAs by qRT-PCR; Supplementary Fig. S3B). ARL5A, LRIG3, and CPEB4 were found to be
associated with cell growth, and depleting ARL5A expression showed the most obvious growth repression effect (Fig. 3D). In addition, ARL5A mRNA expression was upregulated in colorectal cancer tissues compared with NCTs in small samples, whereas LRIG3 mRNA was not (n = 30; Supplementary Fig. S3C).

**ARL5A is a direct target of miR-202-3p in colorectal cancer**

To determine whether ARL5A is a direct target of miR-202-3p in colorectal cancer, we constructed a recombinant plasmid containing the ARL5A 3’ UTR sequence with a mutated binding site sequence (Fig. 4B). Luciferase activity was no longer reduced in cells (HEK-293T, HCT-116, LoVo) transfected with the mutant 3’ UTR plasmid versus the WT 3’ UTR plasmid (Fig. 4A). In concordance with these results, endogenous ARL5A protein levels were downregulated in miR-202-3p-overexpressing cell lines and were restored when the expression of miR-202-3p was repressed (Fig. 4C).

**Upregulation of ARL5A is closely correlated with miR-202-3p expression and poor prognosis in colorectal cancer**

To study the relationship between miR-202-3p and ARL5A in human colorectal cancer, we measured the mRNA and protein expression levels of ARL5A in the 94 paired colorectal cancer and NCT samples using qRT-PCR and immunohistochemistry methods, respectively. Compared with the matched NCTs, ARL5A mRNA expression was upregulated in colorectal cancer tissues (P < 0.05; Fig. 5A) and inversely correlated with miR-202-3p levels (Supplementary Fig. S4B). ARL5A protein was overexpressed in 56.4% (53 of 94) of the colorectal cancer tissues compared with the matched NCTs (P < 0.05; Fig. 5B) and clearly negatively correlated with miR-202-3p expression levels (P < 0.05; Fig. 5C). More importantly, enhanced immunoreactivity of ARL5A in colorectal cancer tissues was inversely correlated with OS and suggested a poor prognosis for patients with colorectal cancer (P < 0.01; Fig. 5D).
addition, higher ARL5A protein levels in colorectal cancers compared with NCTs also indicated poor prognosis (Supplementary Fig. S4C). No association was observed between the intensity of ARL5A staining and tumor size, grade, stage, or lymph node metastasis \((P > 0.05)\).

**miR-202-3p represses proliferation by directly targeting ARL5A in colorectal cancer**

To further clarify whether targeting of ARL5A might mediate the inhibition of cell proliferation in miR-202-3p-overexpressing colorectal cancer cells, we performed a series of functional restoration assays using HCT116 and LoVo cells. First, we inhibited ARL5A expression with siRNA and found that ARL5A-depleted cells exhibited decreased proliferation, which strongly indicated that silencing ARL5A phenocopied the proliferation-repressing effect of miR-202-3p (Fig. 6A). Next, cotransfection experiments using ARL5A and miR-202-3p siRNAs also showed that miR-202-3p knockdown did not enhance the growth rate of colorectal cancer cells when ARL5A expression had been silenced by siRNA (Fig. 6B). Moreover, ARL5A overexpression (overexpressed ORF without 3’ UTR) promoted cell proliferation, which could not be restored by either exogenous or endogenous overexpression of miR-202-3p (Fig. 6C and D).

**Discussion**

It is well known that miRNAs are key components of tumorigenesis, as they participate in many cellular processes including cell proliferation, differentiation, and death. The effects of miRNAs are based on their regulation of the expression of many cancer-related genes through posttranscriptional repression. As we previously reported, a panel of miRNAs was altered in colorectal cancer tissues, suggesting that variations in the expression of miRNAs are common events in colorectal tumorigenesis (1, 5, 11). We previously reported that miR-95, which seems to be an oncogenic miRNA in colorectal cancer, promoted cell proliferation by directly targeting SNX1 (22). In addition to upregulated miRNAs, downregulated miRNAs also contribute notably to tumorigenesis. miR-139-5p, miR-145, miR-125a, and miR-133a have been reported to be tumor suppressors whose precise underlying mechanisms have been identified.
as they relate to the development of colorectal cancer (31, 53–55).

With the aim of finding new colorectal cancer-associated miRNAs, we focused on miR-202-3p (previously named miR-202 before miRBase release 16.0), a target that was found to be significantly downregulated in colorectal cancer samples in our previous profiling data (22). miR-202-3p is highly conserved across animal species and is now considered a member of the let-7 family, which consists of tumor suppressors that target k-RAS and MYCN (12, 39, 40). Consistent with the role of let-7 family members, in this study, we found that miR-202-3p repressed cell proliferation and colony formation in colorectal cancer via its direct target, ARL5A. In addition to affecting proliferation, the role of miR-202-3p in metastasis is another important aspect that must be considered in future tumor-related research. However, we did not conduct in-depth study on the influence of miR-202-3p on tumor metastasis and only found that it did not significantly affect the migration of HCT-116 or LoVo cells (Supplementary Fig. S1D).

Mature miRNAs are generated from primary precursors encoded by genes involving two subsequent RNA cleavage steps (56). When we overexpressed miR-202-3p by lentiviral overexpression of the pre-miR-202 sequence, the intrinsic mature miR-202-5p was simultaneously overexpressed with a complementary sequence of miR-202-3p. For that reason, we also detected the effect of miR-202-5p on cell growth and found that overexpressing of miR-202-5p did not affect colorectal cancer cell proliferation (Supplementary Fig. S1C). Thus, we excluded the side effect of miR-202-5p overexpression on functional assays using cells that stably expressed miR-202.

Copy number variants (CNV) have been described as notable structural variations in the human genome that affect gene expression and contribute to phenotypic differences (57, 58). However, only a few studies have closely examined the CNVs of miRNAs and recognised their potential consequences. The coding sequence of miR-202-3p is located on chromosome 10q26.3, a region considered a chromosome fragile site that is frequently deleted in

Figure 4. miR-202-3p suppresses ARL5A expression by directly targeting its 3′ UTR. A, relative luciferase activity assays of luciferase reporters with ARL5A WT or MT 3′ UTR were performed in cells (HEK-293T, HCT-116, and LoVo) after cotransfection with pGIPZ-miR-202 plasmids. Luciferase activity was determined 48 hours after transfection and normalized to the Renilla luciferase activity. The luciferase activity generated by pLUC in each experiment was set as 1. B, schematic of the WT or MT 3′ UTRs of the ARL5A vector constructs. The complementary site of the seed region of miR-202-3p was selected for mutation. C, endogenous ARL5A expression was suppressed by miR-202-3p. The protein levels of ARL5A were determined by Western blot assays in HCT-116 and LoVo cells infected with miR-202-3p or control lentiviruses, the miR-202-3p inhibitor (anti-miR-202-3p), or the negative control (anti-NC). β-ACTIN served as an internal control.
endometrial and brain tumors (59, 60). We observed that the copy numbers of pre-miR-202 were decreased in most colorectal cancer or NCT samples and were positively correlated with miR-202-3p expression, most likely leading to the downregulation of miR-202-3p. The analysis of more clinical samples is necessary to confirm these findings.

ARL5A, located on human chromosome 2q23.3, and ARL5A protein in eukaryotes belongs to the ARF family, which are members of the Ras gene superfamily of GTP-binding proteins that are involved in a variety of processes, such as cellular communication, endoplasmic reticulum binding, vesicle transport, and protein synthesis (61, 62). ARL5A is upregulated in liver carcinoma according to a previous report (62). In our study, ARL5A was identified as a direct target of miR-202-3p using a series of assays. We demonstrated that ARL5A is upregulated in colorectal cancer tissues, promotes proliferation, and is a prognostic factor in patients with colorectal cancer. These data suggest, for the first time, that ARL5A is a potential oncogene in colorectal cancer. However, the detailed mechanism by which ARL5A influences colorectal cancer cell growth has not yet been elucidated. Further study about other cell growth–related genes in colorectal cancer (LRIG3 and CPEB4) may be carried through in the future work.

Taken together, miR-202-3p, which is frequently downregulated in human colorectal cancer, was identified as a potent repressor of proliferation. Reexpression of miR-202-3p inhibited colorectal cancer proliferation by silencing its direct target, ARL5A, which was closely related to prognosis of patients with colorectal cancer. Although miRNA research is still at the early stages, the contribution of miRNAs to disease processes is becoming clear. Novel and expanded insights into miRNA deregulation will deepen our understanding of the complicated molecular pathogenesis of colorectal cancer and delineate new options for colorectal cancer treatment.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: Z. Huang, X. He, X. Du
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Acknowledgments
The authors thank Didier Trono (School of Life Sciences, Ecole Polytechnique Federale de Lausanne, 1015 Lausanne, Switzerland) for the generous gift of the psPAX2 and pMD2.G plasmids.

Grant Support
This study was partially supported by grants from the National Natural Science Foundation of China (81071791, 81000867, and 81272299), the Science and Technology Commission of Shanghai Municipality (10DJ1400501), and the Medical Key Professionals Program of Jiangsu Province (RC2011031).

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Received April 16, 2013; revised October 22, 2013; accepted October 23, 2013; published OnlineFirst December 10, 2013.

Figure 6. miR-202-3p represses colorectal cancer cell growth by downregulating ARL5A expression. The cell proliferation rate was determined 96 hours after transfection, and the level of ARL5A protein expression was detected 48 hours after transfection. A, ARL5A knockdown using siRNA significantly repressed colorectal cancer cell proliferation; overexpression of miR-202-3p did not suppress cell proliferation in ARL5A-depleted HCT-116 or LoVo cells. B, miR-202-3p knockdown restored the endogenous expression of ARL5A protein and promoted colorectal cancer cell growth, but did not promote cell proliferation in ARL5A-depleted HCT-116 or LoVo cells. C and D, overexpression of ARL5A (ORF without 3' UTR) significantly promoted cell growth and abrogated the miR-202-3p–induced cell growth inhibition in HCT-116 and LoVo cells. The miR-202-3p was overexpressed by mimics transfection (C) or lentivirus (lenti) transduction. To get ARL5A ORF and/or miR-202 overexpressed stable cells, HCT-116 or LoVo cells were infected with Lentil-ARL5A ORF and/or Lentil-miR-202 virus particles (D).

References


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