Colorectal Cancer Migration and Invasion Initiated by microRNA-106a

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Abstract
MicroRNAs have been implicated in the regulation of several cellular signaling pathways of colorectal cancer (CRC) cells. Although emerging evidence proves that microRNA (miR)-106a is expressed highly in primary tumor and stool samples of CRC patients; whether or not miR-106a mediates cancer metastasis is unknown. We show here that miR-106a is highly expressed in metastatic CRC cells, and regulates cancer cell migration and invasion positively in vitro and in vivo. These phenotypes do not involve confounding influences on cancer cell proliferation. MiR-106a inhibits the expression of transforming growth factor-β receptor 2 (TGFBR2), leading to increased CRC cell migration and invasion. Importantly, miR-106a expression levels in primary CRCs are correlated with clinical cancer progression. These observations indicate that miR-106a inhibits the anti-metastatic target directly and results in CRC cell migration and invasion.

Introduction
Metastases lead to approximately 90% of colorectal cancer (CRC)-related mortalities, yet the underlying mechanisms remain largely unclear [1]. Metastasis is a complex, multiple process whereby CRC cells invade surrounding tissues, intravasate into the vasculature, translocate through the systemic circulation, extravasate into the parenchyma of distant tissues (liver and lungs), establish micrometastases, and form macroscopic secondary tumors [2]. In recent years, multiple studies have been conducted to investigate the genes and their products that are involved in CRC progression, particularly as it relates to invasion and metastasis.

MicroRNAs (miRRNs) comprise an evolutionarily-conserved class of small RNAs that can silence gene expression post-transcriptionally through sequence-specific interactions with the 3’ untranslated regions (UTRs) of cognate mRNA targets [8]. Recently, the role for miRNAs in the establishment and progression of CRC has become evident. Greater than 50% of miRNA genes are situated in fragile chromosomal regions that are altered during tumor progression [9], and some miRNAs have been identified as oncogenes or tumor suppressor genes in CRC [10–14]. In addition, expression profiling analysis has revealed characteristic miRNA signatures that can predict the clinical outcomes of CRC [15,16].

miR-106a (miRBase: MIMAT0000103) has been shown to be highly expressed in cancer tissues and stool samples of CRC patients[16–18]; however, the precise role played by miR-106 in CRCs is unknown. We therefore correlated miR-106a with CRC migration and invasion, hoping that such an association might provide insight into the mechanisms by which CRC cells metastasize.

Results
miR-106a is Highly Expressed in Metastatic CRC Cells
We first determined the levels of miR-106a expression in a variety of human CRC cells. In agreement with previous reports [16–18], miR-106a was up-regulated in all six human CRC cell lines when compared to spontaneously immortalized, normal human colon epithelial cells (NCM640; Fig. 1A). miR-106a was more highly expressed in more invasive cells relative to cells with less invasive capacity (Fig. 1A and B). For example, the level of expression of miR-106a was 9-fold higher in the more invasive SW480 cell line than the less invasive HT29 cell line.

Furthermore, the expression of epithelial-mesenchymal transition (EMT) markers (E-cadherin, N-cadherin, vimentin, and ZEB1) was determined by Western blot analysis. EMT can be considered a pathologic process that contributes to cancer progression, particularly as it relates to invasion and metastasis. As shown in Fig. 1C and D, the levels of expression of E- and N-cadherin varied among seven cell lines. E-cadherin was expressed in NCM640 and five CRC cell lines, but not SW480, and more strongly expressed in NCM640, HT29, SW620, and LOVO. N-cadherin, vimentin, and ZEB1 expression was lower in the four cell lines.
The two CRC cell lines, RKO and SW480, which have the highest invasion potential, exhibited consistently low expression of E-cadherin and high expression of N-cadherin, vimentin, and ZEB1.

miR-106a Initiates CRC Cell Migration and Invasion in vitro

We next undertook in vitro loss-of-function analyses to determine the role played by miR-106a in CRC cell migration and invasion. miR-106a was silenced in vitro via anti-sense oligonucleotides, then the levels of expression were determined by real-time RT-PCR [19]. We found that transfection of miR-106a anti-sense oligonucleotides in SW480 cells resulted in 7.3-fold lower levels of miR-106a expression (Fig. 2A). Anti-sense inhibitors of miR-106a led to a 2.5-fold decrease in the migration of SW480 cells, as determined by migration assays in vitro, relative to the control oligonucleotides (Fig. 2B). miR-106a inhibitors caused a 2.7-fold reduction in the invasive capacity of SW480 cells measured by invasion assays in vitro compared with control oligonucleotides (Fig. 2C). Importantly, this reduction could not be attributed to the destruction of cellular viability (Fig. 2D, P>0.05). Thus, these observations indicate that miR-106a is necessary for the migration and invasion of these more metastatic CRC cells in vitro, but not required for viability.

To determine whether or not overexpression of miR-106a conferred migratory and invasive potential on non-metastatic or less metastatic CRC cells, we cloned the genomic sequence of the human miR-106a gene into a mouse stem cell retrovirus-derived vector [20]. We then used the resulting vectors to express miR-106a in immortalized NCM640 and HT29 cells with less metastatic potential. Successful transductions of miR-106a were ascertained by real-time RT-PCR (Fig. 2E).

In both of these two lines, ectopic expression of miR-106a caused a significant increase in cellular migration and invasion (Fig. 2F and 2G). Such an increase cannot be due to the increased proliferative rates of these cells (Fig. 2H, P>0.05 for days 0, 2, 4, and 6). These observations suggest that overexpression of miR-106a is sufficient to initiate migration and invasion of CRC cells in vitro.

miR-106a Promotes CRC Invasion in vivo

We next determined whether or not miR-106a induced invasion in vivo. To this end, we overexpressed miR-106a in CRC cells that are normally less invasive. First, we implanted HT29 cells transduced with miR-106a or infected with control vectors into nude mice subcutaneously. Recipient mice grew remarkable tumors within 2 weeks after injection, and became moribund at week 8 post-injection due to tumor burden when the experiment was terminated.

At week 4 after implantation, tumors of miR-106a-overexpressing HT29 cells and tumors of control infected cells were similar in size, suggesting that miR-106a had a minimal effect on primary tumor growth (Fig. 3A). As anticipated, tumors of control cells were non-invasive, shown as tumor masses confined within fibrous capsules (Fig. 3B, left panel). In contrast, tumors of miR-106a-overexpressing cells had islands of epithelial cancer cells invading into adjacent stromal tissues (Fig. 3B, left panel). Thus, ectopic expression of miR-106a conferred invasive capacity on cancer cells that are otherwise non-invasive.

TGFB2 is a Direct Target of miR-106a

To understand the mechanism by which miR-106a induce cancer cell migration and invasion, we used two algorithms (PicTar and TargetScan) to identify targets of human miR-106a.
Figure 2. miR-106a initiates CRC cell migration and invasion in vitro. A, Levels of miR-106a expression in SW480 cells after transfection with inhibitors of miR-106a, as determined by real-time RT-PCR analysis. Data were normalized with U6 small nuclear RNA. A representative experiment in triplicate, along with standard errors is shown. B, Transwell migration assays of SW480 cells transfected with inhibitors of miR-106a or control vectors. A representative experiment is shown in triplicate and the bars denote standard errors. Also shown are phase-contrast images of SW480 cells migrating through membranes. C, Matrigel invasion assays of SW480 cells transfected with oligonucleotides against miR-106a or control...
Among 990 targets predicted by TargetScan, the LAMA3 (Gene ID: 3909) and TGFBR2 genes (Gene ID: 7048) have previously been implicated in the regulation of cellular migration and/or invasion [23–27]. TGFBR2 is particularly interesting because the expression of TGFBR2 has been shown to be lost progressively during malignant progression of diverse types of cancer [23,25,27,28]. In addition, TGFBR2-encoding mRNA has a 3′UTR element that is complementary to miR-106a (Fig. 4A). Indeed, overexpression of miR-106a led to degradation of TGFBR2 mRNA, as measured by real-time RT-PCR (Fig. 4B).

We next determined whether or not TGFBR2 is a direct target of miR-106a-mediated inhibition by assaying the activity of a luciferase reporter gene fused into the 3′UTR of the wild-type TGFBR2 gene. miR-106a reduced the activity of the luciferase reporter significantly (P<0.05, Fig. 4C). Inhibition of TGFBR2 mediated by miR-106a depends on the presence of miR-106a homologous binding sites in the 3′UTR of the TGFBR2 gene. Because the luciferase reporter carries a mutant TGFBR2 3′UTR, substitution of 4 nucleotides within the miR-106a binding sites were not inhibited by miR-106a ectopic expression (Fig. 4D, P>0.05). Collectively, these observations indicate that TGFBR2 may function as a direct target of miR-106a-mediated silencing.

**TGFR2 is a Functional Target of miR-106a**

We next determined whether or not reduced expression of the TGFBR2 gene is necessary for the induction of cellular migration and invasion observed after miR-106a overexpression.

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**Figure 3. miR-106a promotes CRC invasion in vivo.** A, Growth curves of tumors formed by HT29 cells infected with miR-106a-expressing or control vectors. Every data dot represents the mean ± standard error of 3–4 mice. B, Haematoxylin and eosin-stained sections of CRCs formed by HT29 cells infected with miR-106a-expressing or control vector 8 weeks after implantation.

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We overexpressed miR-106a and a construct expressing TGFBR2 constitutively. This construct encodes the entire encoding sequence of TGFBR2, while lacking the 3' UTR element, thus yielding a type of mRNA resistant to miR-106a mediated inhibition. Remarkably, the resulting constitutively-expressed TGFBR2 abrogated the previously elevated migration and invasion initiated by miR-106a (Fig. 5A), but had no effect on cell viability (Fig. 5B, P>0.05), suggesting that TGFBR2 is indeed a functional target of miR-106a.

We also wanted to clarify whether or not degradation of TGFBR2 is sufficient for the miR-106a-mediated increase of migration and invasion. Transfection of TGFBR2 small interfering RNA (siRNA), which resulted in a >90% reduction in the levels of TGFBR2 protein (Fig. 5C), led to a remarkable, but not complete induction of cell migration and invasion when compared to the induction caused by miR-106a overexpression (Fig. 5D). Collectively, TGFBR2 appears to be a necessary, but not sufficient target of miR-106a.

miR-106a Expression is Increased in Metastatic Primary CRC

Importantly, a recent microarray analysis showed that miR-106a is among those miRNAs that are highly expressed in primary CRC when compared to normal colorectal epithelial tissue [16,18]. We determined whether or not miR-106a expression in metastasis-positive tumors is higher than metastasis-free tumors, and whether or not miR-106a expression is associated with clinical outcomes in CRC patients. We recruited 28 CRC patients who were long-term metastasis-free survivors after complete surgical resection of the primary tumor, and compared the patients to a control cohort of patients with metastasis at time of diagnosis or metastasis in follow-up (<60 months) (Table S1). Furthermore, the metastasis-free survival rates of patients without metastasis at diagnosis were stratified based on miR-106a expression status. Indeed, metastasis-positive patients had significantly higher levels of miR-106a in their primary tumors relative to the patients with no metastases (Fig. 6A, P<0.05). Notably, among all of these patients, those who had lower levels of miR-106a had better clinical outcomes (Fig. 6B, p<0.05). Collectively, these results indicate that miR-106a has an important role in CRC metastasis.

Discussion

The present work identified miR-106a as a pro-metastasis miRNA leading to the promotion of migration and invasion of CRC cells. Reduction of this miRNA in CRC cells that are otherwise metastatic decreased the migratory and invasive potential of the CRC cells. In contrast, up-regulation of miR-106a in CRC cells with less migration and invasion potential.
increased cellular migration and invasion. We showed that miR-106a does not significantly influence the proliferative rates of CRC cells in vitro and in vivo. Such evidence suggests that miR-106a might only play a role in CRC cell invasion.

Interestingly, although from the same patient, SW480 and SW620 cells had different invasion potential and miR-106a expression (Fig. 1A and B). It is possible that this finding was because SW480 cells are from a primary cancer undergoing EMT and SW620 cells are from a secondary cancer. The decline in expression of ZEB1 in SW620 cells compared with SW480 cells may result in restoration of E-cadherin levels and induce a reverse process of EMT, or mesenchymal-epithelial transition (MET) [29], in which miR-106a may play a role as an upstream or downstream factor of ZEB1.

Our findings report for the first time that as a target gene of miR-106a, TGFBR2 may be negatively regulated by miR-106a at the transcriptional level via binding of the 3′ UTR of TGFBR2 mRNA in CRC. TGFBR2 is a major TGF-β signaling molecule often found to be one of the genes altered in cancer. The TGF-β signaling pathway plays a complex role, which remains controversial, in the malignant progression of tumors. It has been shown that TGF-β can induce the EMT and promote tumor cell invasion [30], whereas another study has shown that reduced TGFBR2 expression is associated with aggressive features of hepatocellular carcinoma [25]. In CRC, TGF-β may induce growth inhibition of cancer cells, and TGFBR2 was a tumor suppressor protein that is required for TGF-β signaling [31]. The present study showed that down-regulation of TGFBR2 increases cellular migration and invasion of HT29 (Fig. 2G and 5D). Therefore, TGF-β signaling may be an important approach by which miRNAs regulate the development of tumors. A recent study reported that miR-17-92, activated by c-Myc, attenuates the TGFβ signaling pathway, thereby stimulating angiogenesis and tumor cell growth [32]. Although TGFBR2 appears to be a necessary, but not sufficient target of miR-106a in the current study, the relationship between miR-106a and TGF-β signaling deserves further study.

Clinicopathologic analysis further demonstrated the connection between miR-106a and metastasis. Long-term survivors without metastasis had significantly lower levels of miR-106a in their primary tumors relative to patients with disease recurrence, and lower levels of miR-106a suggests a higher metastasis-free survival rate (Fig. 6). This finding strongly supports the notion that overexpression of miR-106a is intimately involved in the metastasis of CRC.

In conclusion, we showed that the expression of miR-106a was positively correlated with the migration and invasion potential of CRC cells, and down-regulation of TGFBR2, as one of the target genes for miR-106a, could increase cell migration and invasion. More importantly, the levels of miR-106a expression in primary CRCs are correlated with clinical cancer progression, which

Figure 5. TGFBR2 is a functional target of miR-106a. A, Matrigel invasion assays of miR-106a-transduced or control-infected HT29 cells transiently transfected with TGFBR2. A representative experiment is shown in triplicate along with standard errors. B, Trypan blue exclusion assay of miR-106a-expressing HT29 cells transduced TGFBR2 or control vector. Shown is a representative experiment in triplicate, along with standard errors. C, Immunoblotting for TGFBR2 in HT29 cells transiently transfected with TGFBR2 siRNA. A representative experiment is shown in triplicate, along with standard errors. D, Comparison of the increased migration and invasion potential between HT29 cells transduced with miR-106a and HT29 cells transiently transfected with β siRNA. A representative experiment is shown in triplicate. Bars denote standard errors.
suggests that miR-106a may represent a novel target for therapeutic intervention to prevent CRC metastasis.

**Materials and Methods**

**Cell Lines**

The immortalized human colon epithelial cell line, NCM640, was a gift from J.Q. Zhang and cultured in DMEM supplemented with 10% FBS HT29, SW480, LOVO, SW1116, and SW480 cell lines were purchased from the ATCC (Manassas, VA, USA) and cultured under conditions in accordance with the manufacturer’s instructions.

**RNA Isolation and miRNAs Assays**

Total RNA of cultured cells was extracted with mirVana Isolation Kit (AM1560, Ambion, Austin, TX, USA). miRNAs assays were performed with mirVana qRT-PCR miRNA Detection Kit (AM1550, Ambion) together with qRT-PCR Primer

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**Figure 6. miR-106a expression is increased in metastatic primary CRCs.**

A. Levels of miR-106a expression in primary CRCs with or without metastasis. There was a significant difference in miR-106a expression in these two groups. B, Kaplan-Meier metastasis-free survival for 28 CRC patients without metastasis at diagnosis, stratified based on the levels of miR-106a expression in their primary tumors. A χ² test was used for statistical analysis and a P < 0.05 was considered significant.

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Sets (4395280, Ambion) in accordance with manuals of manufacturers. U6 small nuclear RNA was used as internal control.

Oligonucleotide Transfection
The miRIDIAN microRNA inhibitor, miRIDIAN Mimic hsa-miR-106a and siRNA of TGFBR2 was purchased from Dharmacon (H-300526-08, C-300526-07, and D-003930, Dharmacon, Lafayette, CO, USA). Cells were transfected with 200 nM of the indicated oligonucleotides using Oligofectamine Reagent (12252011, Invitrogen, Carlsbad, CA, USA). Cells were used for subsequent experiments 48 h after transfection.

Gene Cloning and Ectopic Expression
The human miRNA gene was PCR-amplified from normal genomic DNA and cloned into the MDH1-PGK-GFP 2.0 retrovirus-derived vector. TGFBR2 cDNAs lacking the 3′UTR was PCR-amplified from normal genomic DNA and expressed from pWZL-blasticidin vector. Viral generation and infection of target cells have been described previously [33]. Infected cells were selected with 2 μg/ml of puromycin (96290, Sigma, St. Louis, MO, USA), 200 μg/ml of hygromycin (H9773, Sigma), and 10 μg/ml of blasticidin (sc-205604, Santa Cruz, Santa Cruz, CA, USA).

In vitro Migration and Invasion Assays
The migration and invasion potential of CRC cells was evaluated, as described previously with slight modifications [34]. CRC cells (2.5×105) were plated on uncoated upper chambers (24-well inserts; pore size, 8 μm; BD Bioscience, Bedford, MA, USA) for transwell migration assays. CRC cells (2.5×105) were placed on Matrigel-coated upper chambers (24-well inserts; pore size, 8 μm; BD Bioscience) for invasion assays.

The medium in the top component was aspirated and replaced with serum-free media 2 h after seeding, and medium containing 20% serum was used as a chemottractant in the lower chambers. Cells were allowed to migrate for 24 h, and then the cells on both sides of the chamber were fixed with 30/50% acetone/methanol. Cells at the bottom of the chamber were stained with crystal violet (C3886, Sigma). Then, the picture of each well was taken to image the nuclei of the cells with the 10 objective, and the cell nuclei in each field was counted using Image-J software (NIH; http://rsb.info.nih.gov/ij/).

Animal Experiments
Six-to-eight week old nude mice were used for the study, and all the experimental procedures were approved by the Animal Care Committee of Shanghai Jiaotong University. 106 HT29 cells in 200 μl DMEM were injected into the flanks of mice subcutaneously. The diameters of tumors were measured twice a week with precision calipers. Three-to-four mice per group were euthanized at each of the 4 time points (weeks 2, 4, 6, and 8 post-transplantation). The tumors were removed and weighed. Three-to-four mice per group were cutaneously. The diameters of tumors were measured twice a week with precision calipers. Three-to-four mice per group were euthanized at each of the 4 time points (weeks 2, 4, 6, and 8 post-transplantation). The tumors were removed and weighed.

SYBR Green Real-time RT-PCR
The total RNA of cells was extracted and reverse-transcribed, as described previously [35]. The upstream and downstream primers for the TGFBR2 gene were obtained from Primerbank (http://pga.mgh.harvard.edu/primerbank/): 5′-GTAGCTCTGAT-GAGTGCATGAC-3′ and 5′-CAGATATGGCAACTGC-

CAGTG-3′. SYBR green real-time RT-PCR and the corresponding data analysis have been described previously [35]. β-actin mRNA were used as an internal control (primers: 5′-CATG-TAGCTTGCTATCCAGGC-3′ and 5′-CTCCITTAATGT-CACCCACAGAT-3′).

Immunoblotting
Cells were harvested in RIPA hys buffer. Proteins from cellular lysates were resolved with PAGE gel, transferred to PVDF membranes (Millipore Corp., Billerica, MA, USA) then blocked with 5% non-fat milk in PBS/Tween-20, followed by incubation with antibody against N-cadherin (sc-271380, Santa Cruz), E-cadherin (sc-21791, Santa Cruz), vimentin (sc-373717, Santa Cruz), ZEB1 (sc-25388, Santa Cruz) or TGFBR2 (sc-17799, Santa Cruz). The quantity of target protein was calibrated using β-actin (sc-130656, Santa Cruz), and relative intensities were obtained.

Construct
TGFBR2 3′UTR sequences were cloned into a pMIR-REPORT luciferase construct (AM5795, Ambion) [36]. Mutant TGFBR2 3′UTR was generated with a QuickChange Site-Directed Mutagenesis Kit (200519, Stratagene, Palo Alto, CA, USA).

Luciferase Reporter Assay
Cells at 50% confluence in 24-well plates were transfected with Fugene6 (E2691, Promega, Madison, WI, USA). The luciferase reporter gene construct (200 ng) and pRL-SV40 Renilla luciferase construct (1 ng [used for normalization]) were co-transfected in each well. Cell extracts were prepared 24–48 h after transfection and measured with a Dual-Luciferase Reporter Assay System (E1910, Promega).

Clinical Colorectal Tumors
Primary colorectal tumors and corresponding normal colorectal tissue were collected between 2004 and 2006, and processed in accordance with a protocol approved by the Ethical Committee of Ruijin Hospital of the Shanghai Jiaotong University School of Medicine. The TNM stage of the tumor was determined according to the classification proposed by the AJCC Cancer Staging Manual [37]. Follow-up data were summarized at the end of 2011 with a median follow-up of 60 months. Fresh tissue was harvested from patients, snap-frozen, and preserved at −80°C. Partial sections from the same tissues were then stained with haematoxylin-eosin to validate the presence of tumor and the tissues containing >90% tumor cells were used for qRT-PCR as tumors. Total RNA was isolated from frozen tissue using TRizol extraction (15596, Ambion) and a mirVana miRNA Isolation Kit (AM1360, Ambion). miRNA assays were performed with a mirVana qRT-PCR miRNA Detection Kit (AM1558, Ambion) together with qRT-PCT Primer Sets (4395280, Ambion) in accordance with the manufacturer’s manuals. U6 small nuclear RNA was used as an internal control. Primary tumors were stratified as miR-106a lower or higher to discern whether or not miR-106a levels correlated with metastasis. Tumors were considered miR-106a lower or higher if the normalized expression of miR-106a resided in the bottom or top 50% of the tumors in the group, respectively.

Cell Growth and Viability assay
To determine growth rates, 2.5×105 cells were plated in each well of a 12-well plate. Twenty-four hours later, the cells were harvested and counted on a hemocytometer every 2 days until day...
6. To determine cell viability, cells were trypsinized and diluted with 0.4% trypan blue (302643, Sigma) staining solution and counted on a hemocytometer.

Statistical Analysis
Data are expressed as the mean ± standard errors. A Student t-test (two-tailed) was used to compare two groups (a P < 0.05 was considered significant) unless indicated otherwise (χ² test).

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