MicroRNA-148a Suppresses Tumor Cell Invasion and Metastasis by Downregulating ROCK1 in Gastric Cancer

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**AUTHOR CONTRIBUTIONS**

B.Z., X.H., and Y.S. designed the experiments, interpreted the data and wrote the manuscript. B.Z., L.L., S.H., L.L., D.J., Q.T., J.W., Y.Y. performed experiments. R.Z. and Q.W. conducted the animal experiments. C.W., Z.L., Y.Z., X.C., C.D. collected the human samples and clinical data.

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Translational Relevance

Gastric cancer (GC) is the second leading cause of cancer-related death worldwide. Understanding the molecular mechanisms underlying GC progression contributes to develop novel avenues for targeted therapy. Previous study shows that miR-148a is specific to be overexpressed in healthy gastric tissues. In this study, we found that miR-148a expression was suppressed by more than 4 folds in GC compared with their corresponding non-tumorous tissues, and the downregulated miR-148a was significantly associated with TNM stage and lymph-node metastasis. Functional studies revealed that miR-148a acted as a new tumor metastasis suppressor in GC. Moreover, we found that miR-148a could target RhoA/ROCK signaling pathway through downregulating ROCK1. By understanding the function and molecular mechanism of miR-148a in GC, miR-148a may have a therapeutic potential to suppress GC metastasis.
Abstract

**Purpose:** MicroRNAs (miRNAs) have been documented playing a critical role in cancer development and progression. In this study, we investigate the role of miR-148a in gastric cancer (GC) metastasis.

**Experimental design:** We examined miR-148a levels in 90 gastric cancer samples by qRT-PCR and analyzed the clinicopathologic significance of miR-148a expression. The gastric cancer cells stably expressing miRNA-148a were analyzed for migration and invasion assays in vitro and metastasis assays in vivo, the target genes of miR-148a were further explored.

**Results:** We found that miR-148a expression was suppressed by more than 4 folds in GC compared with their corresponding non-tumorous tissues, and the downregulated miR-148a was significantly associated with TNM stage and lymph-node metastasis. Functional assays demonstrated that overexpression of miR-148a suppressed GC cell migration and invasion *in vitro* and lung metastasis formation *in vivo*. In addition, overexpression of miR-148a in GC cells could reduce the mRNA and protein levels of ROCK1, whereas miR-148a silencing significantly increased ROCK1 expression. Luciferase assays confirmed that miR-148a could directly bind to the two sites of 3′ untranslated region of ROCK1. Moreover, in GC tissues, we observed an inverse correlation between miR-148a and ROCK1 expression. Knockdown of ROCK1 significantly inhibited GC cell migration and invasion resembling that of miR-148a overexpression. We further found that ROCK1 was involved in miR-148a-induced suppression of GC cell migration and invasion.

**Conclusions:** miR-148a functions as a tumor metastasis suppressor in GC, and downregulation of miR-148a contributes to GC lymph-node metastasis and progression. miR-148a may have a therapeutic potential to suppress GC metastasis.
Introduction

Gastric cancer (GC) is the second leading cause of cancer-related death worldwide, with an estimated one million new cases per year. Approximately 50% of cases occur in Eastern Asia (mainly in China) (1). In most patients, GC is diagnosed at advanced stage accompanied by extensive invasion and lymphatic metastasis, successful therapeutic strategies are limited and the mortality is high (2, 3). Therefore, investigations into the molecular mechanisms involving in GC progression have major importance and may tend to develop novel avenues for targeted therapy.

MicroRNAs (miRNAs), a class of small non-protein-coding RNAs, have been identified as a new kind of gene expression regulators through binding to the 3′ untranslated regions (UTRs) of target mRNA, thereby resulting in mRNA degradation or the blockade of mRNA translation (4). Emerging evidence shows that miRNAs are abnormally expressed in various cancers, and deregulated miRNAs are associated with tumor initiation, promotion and progression through regulating many oncogenes and tumor suppressors (5, 6). Human cancers show a general downregulation of miRNAs, and the miRNAs loss can promote tumorigenesis (7, 8). Gastric cancer has unique dysregulated miRNAs, among them, miR-148a is one of the most downregulated miRNAs (9, 10). In addition, tchernitsa et al using miRNAs microarray compare the miRNA expression patterns between GC with and GC without lymph node metastasis, and find that six miRNAs (miR-103, miR-21, miR-145, miR-106b, miR-146a and miR-148a) are associated with GC lymph node metastasis, of them, miR-146a and miR-148a are downregulated in GC (11). It has been reported that miR-146a has the capacity to inhibit GC cell migration and invasion(12). However, for miR-148a, the possible roles and related target genes in GC metastasis are still not well elucidated.

In this study, we found that the expression of miR-148a was suppressed in about 79% of primary GC and was highly associated with lymph-node metastasis in an
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independent GC cohort (n=90). Further studies demonstrated that overexpression of miR-148a suppressed GC cell migration and invasion in vitro and lung metastasis formation in vivo. Moreover, ROCK1, a potential metastasis promoter, was identified as a direct and functional target of miR-148a.

Materials and Methods

Human Samples

Human gastric cancer and their corresponding nontumorous gastric tissues were collected at the time of surgical resection from 90 patients with gastric adenocarcinoma from 2008 to 2009 at the Department of Gastric Cancer and Soft Tissue Sarcomas, Shanghai Cancer Center of Fudan University. Human tissues were immediately frozen in liquid nitrogen and stored at -80°C refrigerator. Signed informed consent was obtained from all patients and the study was approved by the Clinical Research Ethics Committee of Fudan University Shanghai Cancer Center.

Cell Culture

Human gastric cancer cell lines (MGC-803, AGS, HGC-27), and HEK293T cells were purchased from the Cell Resource Center, Shanghai Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences. Cells were maintained at 37°C in a humidified air atmosphere containing 5% carbon dioxide in RPMI1640 (MGC-803, HGC-27), F12 (AGS) or DMEM (HEK293T) medium supplemented with 10% fetal bovine serum.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction

Total RNA from tissue samples and cultured cells was extracted using TRIzol reagent (Invitrogen, CA, USA). Quantitative RT-PCR assays were performed to detect
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mRNA expression using the PrimeScript RT reagent Kit (TaKaRa, Dalian, China) and SYBR Premix Ex Taq (TaKaRa) according to the manufacturer’s instructions. The levels of ROCK1 transcript were measured by forward primer: AGGAAGGCGGACATATTAGTCCCT, and reverse primer: AGACGATAGTTGGTCCCGGC. β-Actin was used as an internal control and amplified with forward primer: AGTGACGTGGACATCCGCAAAG, and reverse primer: ATCCACATCTGCTGGAAGGTGGAC. The expression level of mature miR-148a was measured by TaqMan miRNA assays (Applied Biosystems, CA, USA) according to the provided protocol, and U6 small nuclear RNA was used as an internal control.

Vector Constructs

The pri-miR-148a sequence was amplified from normal human genomic DNA and constructed into the lentivirus expression vector pWPXL to generate pWPXL-miR-148a. The 3′UTR sequence of ROCK1 was amplified from normal human genomic DNA and subcloned into the region directly downstream of a cytomegalovirus (CMV) promoter-driven firefly luciferase cassette in a pCDNA3.0 vector (pLuc). With appropriate primers, a series of 3′UTR sequences were generated and inserted into the pCDNA3.0 vector. All constructs were verified by sequencing.

Lentivirus Production and Transduction

A mixture of pWPXL-miR-148a or pWPXL, psPAX2 and pMDG2 were transfected into HEK293T cells using lipofectamin 2000 reagent to generate lentivirus. MGC-803 and AGS cells were infected with the recombinant lentivirus-transducing units plus 6 μg/ml Polybrene (Sigma, MO, USA).

Oligonucleotide Transfection

miR-148a inhibitors and siRNA against ROCK1 were synthesized by Ribobio (Guangzhou, China). The miR-148a inhibitors are 2′-O-methyl-modified, single stranded nucleic acids which can specifically bind to and inhibit endogenous
miR-148a. The sequence of siRNA targeting ROCK1 is as follows: CAGCAAAUCCUAAUGAUAA. Oligonucleotide transfection was performed using Lipofectamine 2000 reagents according to the manufacturer’s protocol.

**Cell Migration and Invasion Assays**

For the migration assays, $5 \times 10^4$ cells were added into the upper chamber of the insert (BD Bioscience, 8-μm pore size, NJ, USA). For the invasion assays, $1 \times 10^5$ cells were added into the upper chamber of the insert precoated with Matrigel (BD Bioscience, MA, USA). In both assays, cells were plated in medium without serum, and medium containing 10% fetal bovine serum in the lower chamber served as chemoattractant. After several hours of incubation, the cells that did not migrate or invade through the pores were carefully wiped out with cotton wool. Then the inserts were stained with 20% methanol and 0.2% crystal violet, imaged, and counted with an IX71 inverted microscope (Olympus, Tokyo, Japan).

**Luciferase Assays**

HEK293T cells were seeded in 96-well plates at 8000 cells per well the day before transfection. A mixture of 100 ng indicated pLUC-3'UTR, 200 ng of pWPXL or pWPXL-miR-148a and 20 ng Renilla plasmid (containing no 3'UTR) was transfected into HEK293T cells with Lipofectamine 2000 in each well. 48 hours later, Firefly and Renilla luciferase activities were measured with a Dual-Luciferase Reporter System (Promega, Madison, WI). The Renilla luciferase activities were used as an internal control for transfection efficiency (6).

**In vivo Metastasis Assays**

For in vivo metastasis assays, MGC-803 cells infected with either the miR-148a-overexpressing lentivirus or the mock lentivirus were transplanted into nude mice (five-week-old BALB/c-nu/nu, six per group, $1 \times 10^6$ cells for each mouse) through the lateral tail vein. After 7 weeks, mice were sacrificed. Their lungs were removed and subjected to hematoxylin & eosin (H&E) staining. All research
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involving animal complied with protocols approved by the Shanghai Medical Experimental Animal Care Commission.

**Western Blot Analysis**

According to standard western blot procedures, briefly, proteins were separated by 8% SDS–PAGE and then transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). After blocking in 5% non-fat milk, the membranes were incubated with the following primary antibodies: rabbit anti-ROCK1 monoclonal antibody (mAb) (1:500) (Novus Biologicals, CO, USA), mouse anti-β-actin mAb (1:10000) (Sigma, St. Louis, MO). The proteins were visualized with enhanced chemiluminescence reagents (Pierce, Rockford, IL).

**Immunohistochemical Staining**

Immunohistochemical staining was performed according to standard procedures. For incubation with primary mAb, Tissue slides were incubated at 4°C overnight with rabbit anti-ROCK1 mAb (1:200) (Novus Biologicals, CO, USA). Negative controls were treated identically, but without the primary antibody. Scoring was measured by the cell cytoplasm staining pattern of tumor or non-tumorous tissues as described (13): scored 0, absent cell cytoplasm staining; scored 1, week cell cytoplasm staining; scored 2, moderate cell cytoplasm staining; scored 3, strong cell cytoplasm staining.

**Statistical Analysis**

Data were shown as mean ± SEM. Unless otherwise noted, Student's t-test was used for statistical analysis, with \( P < 0.05 \) considered significant.
Results

miR-148a is downregulated in GC and associated with advanced clinical stage and lymph-node metastasis

miR-148a has been reported to be downregulated in various cancers including GC (10, 14, 15). Recently, Tchernitsa et al using miRNA microarray find that miR-148a is associated with lymph-node metastasis in GC (11). However, chen et al have not found the relationship between miR-148a expression and lymph-node metastasis in GC patients (14). To further understand the relationship between miR-148a expression and GC metastasis, we determined the miR-148a expression levels in 90 pairs of primary GC and their corresponding non-tumorous tissues by qRT-PCR. The relationship between the miR-148a expression levels and clinicopathologic parameters of GC was summarized in Table 1. The results showed that no significant correlations were observed between the miR-148a expression and age, gender, tumor size, location, differentiation and local invasion. However, we found that miR-148a expression was suppressed by more than 4 folds in GC compared with their corresponding non-tumorous tissues (median=0.1997 and 0.8443, respectively) (Fig. 1A). Moreover, 79% (71/90) of the GC had at least 2-folds reduced expression of miR-148a compared with their corresponding non-tumorous tissues (Fig. 1B). We also found that GC with advanced stages (stage III and IV) had a lower miR-148a expression than GC with early stages (stage I and II) (Table 1 and Fig. 1C). Interestingly, when 90 GC samples were stratified based on the status of lymph-node metastasis, we found that miR-148a expression was further significantly downregulated in GC that had lymph-node metastasis, when compared with those that did not have (P=0.0003) (Fig. 1D). Collectively, the above findings suggest that loss of miR-148a expression may play an important role in GC metastasis.

miR-148a suppresses GC cell invasion in vitro and metastasis in vivo
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Since we observe that the downregulation of miR-148a in GC is a frequent event and closely associated with GC metastasis, we postulated that overexpression of miR-148a in GC cells can exert inhibitory effects of cell invasion and metastasis. Thus, we established miR-148a stably expressing cell lines of MGC-803 and AGS by lenti-virus infection, as MGC-803 and AGS cells had low basal levels of miR-148a in GC cell lines (Supplementary Figure S1A). Successful overexpression of mature miR-148a was confirmed by qRT-PCR (Supplementary Figure S1B). We found that miR-148a had no obvious effect on GC cell proliferation (Supplementary Figure S1 C, D and E). Intriguingly, overexpression of miR-148a significantly suppressed the migratory and invasive abilities of GC cells (Fig. 2A and 2B). Conversely, we transiently transfected miR-148a inhibitor into HGC-27 cells, which had relatively high endogenous miR-148a expression among GC cell lines (Supplementary Figure S1A). We found that knockdown of miR-148a could increase GC cell migration and invasion (Fig. 2C). To further explore the role of miR-148a on tumor metastasis in vivo, MGC-803 cells stably expressing miR-148a were transplanted into nude mice through the lateral tail vein. Histological analysis on the lungs of mice confirmed that miR-148a could suppress lung metastasis formation. The numbers and size of lung metastasis nodules were significantly decreased in MGC-803-miR-148a group when compared with MGC-803-vector group (Fig. 2D). Taken together, our results suggest that miR-148a is a negative regulator for GC metastasis.

miR-148a posttranscriptionally reduces ROCK1 expression by directly targeting its 3’UTR

To explore the molecular mechanism of miR-148a in GC metastasis, we employed TargetScan algorithm to search for putative protein-coding gene targets of miR-148a, especially for those that have the abilities to promote tumor cell invasion and metastasis. Basing on this rationale, five candidate genes (ITGA5, MET, ROCK1, SP1, WNT1) were selected. We performed qRT-PCR to screen the genes that could be downregulated by miR-148a. The results revealed that the expression of ROCK1 mRNA was most downregulated by miR-148a in the miR-148a stably expressing cell
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lines of MGC-803 and AGS (Fig. 3A and Supplementary Figure S2). ROCK1 acts as an essential effector kinase of Rho GTPases, and plays vital roles in regulation of cancer invasion and metastasis (16, 17), so ROCK1 might be one target of miR-148a. In addition, Western Blot analysis showed that enforced expression of miR-148a triggered a silencing effect on the endogenous ROCK1 protein expression (Fig. 3B). Furthermore, after transfection with miR-148a inhibitor in HGC-27 cells, the expression of ROCK1 was obviously increased (Fig. 3C). Those results suggest that the ROCK1 expression is regulated by miR-148a in GC.

Analysis of the 3'UTR sequence of ROCK1 using TargetScan reveals two possible binding sites for miR-148a, indicating that ROCK1 gene transcript may be a strong target for miR-148a. To test whether ROCK1 is a direct target of miR-148a, a series of 3'UTR fragments of ROCK1 including full length, binding site 1, binding site 2 and their corresponding mutant counterparts were directly fused to the downstream of the firefly luciferase gene (pLuc) (Fig. 3D). The luciferase-3'UTR construct (pLuc-3'UTR) as described was cotransfected into HEK-293T cells with Renilla plasmid and pWPXL-miR-148a or pWPXL control. The transfection efficacy was normalized by cotransfection with Renilla reporter vector. As shown in Fig. 3E, miR-148a could decrease the relative luciferase activity of full length-ROCK1 3'UTR construct, whereas in the counterpart with the sites both mutated the luciferase activity was not significantly changed, indicating such regulation was dependent on specific sequence. In addition, our results further found that miR-148a could target each of the binding sites of ROCK1 3'UTR. Moreover, when one of two sites was mutated, the luciferase activity was reduced compared with both sites mutated. Taken together, these results indicate that miR-148a downregulates ROCK1 expression by directly targeting its 3'UTR.

Upregulation of ROCK1 is inversely correlated with miR-148a expression in GC

As miR-148a is downregulated in GC and targets ROCK1 by binding to its 3'UTR, we next determined whether ROCK1 protein expression is negatively associated with miR-148a levels in the GC tissue samples. Analysis of ROCK1 protein expression in
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GC and their corresponding non-tumorous tissues by immunohistochemical staining showed that ROCK1 was significantly upregulated in GC ($P<0.0001$) (Fig. 4A and 4B). Furthermore, we found that high expression of ROCK1 was more likely to be seen in GC with low levels of miR-148a ($P=0.0041$) (Fig. 4C), suggesting that the upregulation of ROCK1 may result from repression of miR-148a in GC.

ROCK1 is involved in miR-148a-induced suppression of GC cell migration and invasion

It has been reported that ROCK1 is closely associated with tumor invasion and metastasis (16, 17). However, the effects of ROCK1 on GC cells have not been characterized. To explore the functions of ROCK1, specific siRNAs against ROCK1 were exploited to knockdown ROCK1 expression. As shown in Fig. 5A and 5B, si-ROCK1 significantly reduced the expression of ROCK1 protein. Migration and invasion assays showed that si-ROCK1 could inhibit GC cell migration and invasion (Fig. 5C and 5D), which resembled the inhibitory effects of miR-148a on the GC cell migration and invasion (Fig. 2A and 2B). As miR-148a is downregulated in GC, and miR-148a silencing enhances ROCK1 expression in GC cells. To determine whether deregulation of ROCK1 is involved in regulation of cell migration and invasion by miR-148a, we co-transfected HGC-27 cells with miR-148a inhibitor and si-ROCK1 as described in Fig. 5E, the expression of ROCK1 was confirmed by Western Blot. Interestingly, we found that the migration- and invasion-promoting effects of anti-miR-148a were partially attenuated by si-ROCK1 (Fig. 5F), suggesting ROCK1 is involved in miR-148a-induced suppression of GC cell migration and invasion.

Discussion

In this study, we showed that miR-148a was frequently downregulated in human GC and the downregulated miR-148a was significantly associated with advanced clinical stage and lymph-node metastasis. Further studies showed that overexpression of
miR-148a suppressed GC cell migration and invasion in vitro and metastasis in vivo. ROCK1 was identified as a direct and functional target of miR-148a. The data from the current study suggest that miR-148a acts as a novel metastasis suppressor in GC and that downregulated miR-148a contributes to lymph-node metastasis and tumor progression in GC patients.

Downregulation of miR-148a is a frequent event in various cancers (10, 14, 15), suggesting that miR-148a may play an important role in tumorigenesis and tumor progression. Hanoun et al show that miR-148a is repressed not only in pancreatic ductal adenocarcinoma but also in preneoplastic lesions (18), indicating downregulation of miR-148a is an early event in pancreatic carcinogenesis. In this study, we also found that miR-148a was frequently downregulated in GC, and 79% (71/90) of the GC had at least 2-folds reduced expression of miR-148a compared with their corresponding non-tumorous tissues. Intriguingly, we found that lower expression of miR-148a tended to have more advanced TNM stage (stage I/II vs stage III/IV, \( P=0.0049 \)), suggesting that low expression of miR-148a is associated with GC progression. Recently, it is reported that miR-148a is subjected to epigenetic regulation in various tumors including GC (18-20), which may explain the downregulation of miR-148a in GC. Interestingly, Ribeiro-dos-Santos et al using next-generation sequencing technology reveal that miR-148a is specific to be overexpressed in healthy gastric tissues (21). Together with our results, these data suggest that miR-148a is likely to play a crucial role in gastric tissue homeostasis, when dysregulated, may contribute to the development of a stomach neoplasia.

Lymph-node metastasis is an initial step of GC metastasis, and is a crucial factor in the determination of the clinical staging, prognosis and survival of GC patients (2). Therefore, identifying metastatic factors and elucidating the molecular mechanisms underlying GC lymph-node metastasis become critical issues. Recent studies document that miRNAs play important roles in GC initiation and progression (22-24). It is intriguing that miRNAs are associated with lymph-node metastasis of GC, such as miR-218 (25), miR-107 (26), miR-146a (12), miR-429 (27) and miR-370 (28), which may provide new insights for designing better therapeutic strategies to treat GC.
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patients with lymph-node metastasis. In accordance with our results, it has been reported that miR-148a is one of the most downregulated miRNAs in GC (9, 10). However, its possible roles in GC have not been fully elucidated. Although Guo et al have shown that miR-148a promotes GC cell proliferation by targeting p27 (29), miR-148a appears to act as an oncogene in GC. Other studies suggest that miR-148a acts as a potential tumor suppressor in cancers by inhibiting cancer cell growth, invasion and multidrug resistance (30-32). Therefore, we speculate that the downregulated miR-148a may have other important functions in GC. However, the relationship between miR-148a expression and GC metastasis is largely unknown.

Intriguingly, in this study, we demonstrated that miR-148a expression was significantly associated with lymph-node metastasis, as the GC with lymph-node metastasis (n=52) had a much lower miR-148a expression than those without lymph-node metastasis (n=38) (P=0.0003). Supporting this notion, Tchernitsa et al using miRNAs microarray (three GC patients with and three GC patients without lymph-node metastases) also find that miR-148a is associated with lymph-node metastasis (11), thus suggesting that miR-148a may have the potential as a risk biomarker for judging GC metastasis status. In addition, our functional studies further found that miR-148a played an important role in GC metastasis, overexpression of miR-148a could suppress GC cell migration and invasion in vitro and metastasis in vivo. Taken together, these data indicate miR-148a has a close relationship with lymph-node metastasis and acts as a metastasis suppressor in GC.

Cell motility and invasion are required for the spreading of tumor cells from their primary tumor to lymph or blood vessels in the process of metastasis. ROCK1 as an essential effector kinase downstream of Rho GTPases participates in regulation of cytoskeletal reorganization and is crucial for cell motility (17). Increasing evidence have shown that upregulation of Rho-ROCK signaling in cancers contributes to invasive and metastatic behavior (16, 17). It has been reported that activation of RhoA-ROCK signaling pathway is involved in GC cell migration, invasion and GC progression (33). Together with this, in this study, we found that miR-148a could target this signaling pathway through downregulating ROCK1, and high ROCK1
expression was associated with low miR-148a levels in GC, bringing a new insight about the essential mechanisms of regulating the Rho-ROCK pathway in GC. In addition, miR-146a and miR-584 are also proved to target ROCK1 in prostate cancer and clear cell renal cell carcinoma respectively (34, 35). miR-139 and miR-124 are demonstrated to modulate hepatocellular carcinoma cell aggressiveness by repressing ROCK2 (36, 37). Valastyan et al have demonstrated that miR-31 inhibits local invasion, extravasation and metastatic colonization of breast cancer cells by targeting RhoA (38). These evidence suggest that dysregulation of RhoA/ROCK signaling pathway by miRNAs is an important mechanism underlying cancer metastasis, and those miRNAs may serve as potential treatments for modulating this pathway in the metastatic diseases.

In summary, our results show that miR-148a as an important anti-metastatic miRNA is downregulated and associated with lymph-node metastasis in GC. Enforced expression of miR-148a suppresses GC cell invasion and metastasis through directly targeting ROCK1. These findings suggest that the frequently downregulated miR-148a in GC contributes to GC metastasis and progression and that miR-148a may have a therapeutic potential to suppress GC metastasis.

References

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### Tables

Table 1. The relationship between miR-148a expression and clinicopathological parameters in primary gastric cancer

<table>
<thead>
<tr>
<th>Clinicopathologic parameters</th>
<th>Number of cases</th>
<th>Median expression of miR-148a</th>
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<tr>
<td>Age (years)</td>
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<tr>
<td>≥60</td>
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<td>0.1902±0.0235</td>
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<tr>
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<td>Female</td>
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<td>0.2279±0.0349</td>
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<td>Diameter (cm)</td>
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<td></td>
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<td>≥5</td>
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<td>Middle and proximal</td>
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<td>Well and moderately</td>
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Figure legends

Figure 1. miR-148a is downregulated in GC and associated with advanced clinical stage and lymph-node metastasis.

(A) Expression of mature miR-148a was determined by way of qRT-PCR in 90 paired human gastric cancers (GC) and their corresponding nontumorous samples (NT) and normalized against an endogenous U6 RNA control. (B) The downregulation of miR-148a was found in 79% (71/90) of primary GC compared with corresponding non-tumorous tissues, the fold change of relative miR-148a expression (NT/GC) >2 or <1/2 was defined significant. (C) miR-148a expression in different clinical stages of GC. Patients were staged in accordance with the 7th Edition of the AJCC Cancer’s TNM Classification. (D) Downregulation of miR-148a in GC was associated with lymph-node metastasis, patients were classified into lymph-node metastasis negative group (LN-Negative) and positive group (LN-Positive). Statistical analysis was performed by paired t-test (A) and Student’s t-test (C, D).

Figure 2. miR-148a suppresses gastric cancer cell invasion in vitro and metastasis in vivo.

Over-expression of miR-148a significantly impeded abilities of cell migration (A) and invasion (B) in MGC-803 and AGS cells after infection with miR-148a-expressing or vector lentivirus. (C) miR-148a inhibitor enhanced cell migration and invasion in HGC-27. (D) Representative H&E stained sections of the lung tissues isolated from mice that injected with MGC-803-Vector or MGC-803-miR-148a cells through the lateral tail vein, Arrow head points to the tumor focus formed in the lung. The numbers of metastases in the lungs were counted.
Figure 3. miR-148a negatively regulates ROCK1 by binding to the ROCK1 3'UTR.

(A) The mRNA levels of ROCK1 were determined by qRT-PCR in stably miR-148a-expressing cell lines of MGC-803 and AGS. β-Actin served as an internal control. ***P<0.001. (B) Western blot analysis was used to detect the expression level of endogenous ROCK1 in MGC-803 and AGS cells after infection with miR-148a expressing or control lentivirus. β-Actin served as an internal control. (C) Western blot analysis was used to detect the expression level of endogenous ROCK1 in HGC-27 cells after transfection with NC inhibitor or miR-148a inhibitor. β-Actin served as an internal control. (D) Two putative miR-148a binding sites in ROCK1 3'UTR and the two corresponding mutant binding sites (underlined) were shown. (E) A series of 3'UTR fragments of ROCK1 including full length, binding site 1, binding site 2 and their corresponding mutant counterparts were directly fused to the downstream of the firefly luciferase gene in the luciferase reporter plasmids (pLuc). Relative luciferase activity was analyzed after the described reporter plasmids (pLuc-3'UTR) or mock reporter plasmid (pLuc) was cotransfected with Renilla plasmid and pWPXL or pWPXL-miR-148a into HEK293T cells. The luciferase activity was normalized to Renilla luciferase activity. The normalized luciferase activity of pLuc group was set as 1.

Figure 4. Upregulation of ROCK1 is inversely correlated with miR-148a expression in GC. (A) Immunohistochemical staining of ROCK1 protein in 70 paired of GC and their corresponding non-tumorous tissues. The staining intensities were evaluated and represented as follows: (a) non-tumorous case 1, score 0; (b) GC case 1, score 0; (c) GC case 2, score 1; (d) GC case 3, score 2; (e) GC case 4, score 3. Original magnification, 200x. (B) Statistical analysis of ROCK1 expression according to the scoring (paired t-test). (C) Correlation between ROCK1 expression and miR-148a levels in the 70 GC tissue samples. The expression levels of ROCK1 were classified into low (scores of 0 and 1) and high groups (scores of 2 and 3) according to the
scores of ROCK1 immunohistochemical staining.

Figure 5. ROCK1 is involved in miR-148a-induced suppression of GC cell migration and invasion.

(A, B) Silencing of ROCK1 was confirmed by Western blot in MGC-803 and AGS cells after transfection with specific si-ROCK1. β-Actin served as an internal control. (C, D) Migration and invasion assays were performed in MGC-803 and AGS cells after transfection with negative control (NC) or si-ROCK1. (E) Western blot analysis was used to detect the ROCK1 expression in HGC-27 cells after transfection with miR-148a inhibitor, si-ROCK1 or NC. β-Actin served as an internal control. (F) HGC-27 cells after transfection with miR-148a inhibitor, si-ROCK1 or NC were subjected to migration and invasion assays. **P<0.01; ***P<0.001.
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