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miR-374a promotes cell proliferation, migration and invasion by targeting SRCIN1 in gastric cancer

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ABSTRACT

MicroRNAs (miRNAs) play a prominent role in gastric cancer (GC) initiation and progression. In this study, we found that miR-374a expression was up-regulated in human GC cell lines and tissues. Inhibition of miR-374a suppressed GC cell proliferation, migration and invasion in vitro and slowed tumor growth in vivo. SRC kinase signaling inhibitor 1 (SRCIN1) was identified as a direct target of miR-374a. Silencing of SRCIN1 significantly enhanced cell proliferation, migration and invasion, whereas SRCIN1 reintroduction partially abrogated the oncogenic effects of miR-374a. Taken together, these findings suggest that miR-374a functions as a candidate oncogene in GC by directly targeting SRCIN1. miR-374a may therefore be useful as a promising therapeutic target for malignant GC.

1. Introduction

Gastric cancer (GC) is the fourth most common human cancer and the second most prevalent cause of cancer-related mortality in the world [1]. It is estimated that approximately 1 million new cases of GC are diagnosed per year worldwide [2]. Although there have been recent advances in surgery and chemotherapy, the prognosis for GC remains poor, with an overall 5-year survival rate below 30% [3,4]. Thus, it is of great significance to further elucidate the molecular mechanisms of GC and look for new therapeutic targets for this disease [5].

MicroRNAs (miRNAs) are a class of small non-coding, endogenous, single-stranded RNA with 21–23 nucleotides, which regulate gene expression by influencing post-transcriptional processing or mRNA degradation [6,7]. miRNAs play important roles in a range of biological processes such as cell proliferation, migration and invasion [8]. Accumulating evidence shows that miRNAs also have significant regulatory functions in various tumors including GC [9,10]. For example, miR-27a acts as an oncogene, and promotes cell proliferation by targeting prohibition in gastric adenocarcinoma [11]. miR-423-5p elevates GC cell proliferation and suppresses cell migration and invasion by repressing trefoil factor 1 [12]. On the other hand, miR-126 is down-regulated in GC cells and inhibits cell growth in vitro and in vivo, reduces the capabilities of migration and invasion, and increases apoptosis though Crk [13]. Therefore, it is important to explore the roles of miRNAs in the tumor biology of GC.

MicroRNA microarray results have shown that miR-374a expression is significantly up-regulated in GC tissues compared with adjacent normal tissues [14]. The expression of miR-374a was previously reported to be elevated in osteosarcoma cell lines and in colon cancer [15,16]. Functional assays revealed that miR-374a directly targets Wnt5a, and regulates the proliferation, gefitinib-induced apoptosis, epithelial-to-mesenchymal transition (EMT), migration and invasion of non-small cell lung cancer in vitro and in vivo [17]. Furthermore, ectopic expression of miR-374a activates Wnt/β-catenin signaling to promote breast cancer EMT and metastasis in vitro and in vivo by suppressing WIF1, PTEN or WNT5A expression [18]. However, the exact functions and precise molecular mechanisms of miR-374a in GC progression remain undefined.

In this study, up-regulation of miR-374a in GC cells and tissues was confirmed by real-time PCR. Further investigation demonstrated that miR-374a inhibition could repress GC cell proliferation, migration and invasion, and attenuate the tumorigenic ability of GC cells in animal models. We experimentally confirmed that miR-374a targets the 3′UTR of SRC kinase signaling inhibitor 1 (SRCIN1). Knockdown of SRCIN1 exerted opposite effects of miR-374a inhibition, while reintroduction of SRCIN1 partially

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counteracted miR-374a-induced proliferation, invasion and migration of GC cells. Thus, our results revealed that miR-374a is an oncogene and a potential therapeutic target of GC.

2. Materials and methods

2.1. Cells and clinical tissues

GC cell lines MKN-45, HGC-27, BGC-823, and SGC-7901 and the immortalized normal gastric epithelial cell line GES-1 were obtained from the American Type Culture Collection (ATCC; Manassas, VA). All cells were cultured in RPMI 1640 medium (GIBCO, Carlsbad, CA) supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in 5% CO_2. Human GC tissues and adjacent non-cancerous tissues were collected from 18 GC patients who underwent surgical resection at the Shanghai Changzheng Hospital. All materials were collected after written informed consent was obtained, and this study was approved by the Ethics Committee of Changzheng Hospital.

2.2. RNA extraction and real-time PCR

Total RNA was extracted from tissue samples and cell lines using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The concentration and quality of total RNA were assessed via spectrophotometric and electrophoretic assay. First-strand cDNA was synthesized using the SuperScript® III first-strand synthesis system (Invitrogen). Real-time PCR was carried out in the ABI PRISM 7300 system (Applied Biosystems, Foster City, CA) using the SYBR Premix Ex Taq™ (TaKaRa, Dalian, China). Relative expression levels of miR-374a and SRCIN1 were calculated by the $2^{-\Delta\Delta CT}$ method after normalization with reference to expression of U6 and β-actin. All reactions were carried out in triplicate.

2.3. Vectors and cell transfection

The wild-type 3′UTR of SRCIN1 containing the putative miR-374a target site was amplified and cloned into the pGL3-control luciferase reporter vector (Promega, Madison, WI). The mutant constructs were generated using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The coding sequence of SRCIN1 was amplified by PCR and inserted into the pcDNA3.1(+) vector (Invitrogen). All constructs were finally confirmed by DNA sequencing. AgomiR-374a, antagomiR-374a and si-SRCIN1 were obtained from RiboBio (Guangzhou, China) and transfected according to the manufacturer's instructions. Cells were transfected with vectors using Lipofectamine 2000 reagent (Invitrogen).

2.4. Cell proliferation assay and colony formation assay

Cell proliferation was assayed using the Cell Counting Kit-8 (CCK-8). Cells (1.5×10^3) were plated into 96-well plates and cultured for 1, 2, 3 or 4 days, after which 10 μl CCK-8 was added to each well, and cells were incubated for a further 2 h at 37 °C. The absorbance was measured at a wavelength of 450 nm. For the colony formation assay, cells were seeded onto a six-well plate at a density of 500 cells per well after transfection. Approximately 10 days later, the colonies were stained with 1% crystal violet for 5 min after fixation with methanol for 10 min, and the number of colonies was counted.

2.5. Cell cycle and apoptosis assays

Transfected cells (1×10^6) were harvested and washed in cold PBS, followed by fixation in 70% ice-cold ethanol for 30 min. After washing, cells were pelleted by centrifugation using a chilled centrifuge and resuspended in 0.8 ml of PBS with 40 μg of propidium iodide and 0.1 μg of RNase A for 30 min at 37 °C. Samples were analyzed for DNA content using a FACSCaliber flow cytometer (BD Bioscience, San Jose, CA). For apoptosis assay, transfected cells were stained with AnnexinV/PI Apoptosis Detection kit (Promega) according to the manufacturer's protocol. Apoptotic cells were analyzed using flow cytometry.

2.6. Cell migration and invasion assays

BGC-823-antagomiR-374a or SGC-7901-antagomiR-374a cells (1×10^5 cells/well) were plated on the top side of transwell chambers coated without or with Matrigel (BD Bioscience). Medium containing 20% FBS in the lower chambers functioned as the chemo-attractant. After 24 h, the cells adhering to the lower side of the membrane were fixed, stained with crystal violet and counted under a microscope (Olympus Corp., Tokyo, Japan).

2.7. Xenograft model in nude mice

SGC-7901-antagomiR-374a cells (2×10^6) in 0.2 ml serum-free medium were subcutaneously inoculated into the left flank of nude mice ($n = 4$). Tumor volume (V) was assessed every week by measuring the length (A) and the width (B) using a slide caliper, and calculated by the formula: $V = 0.5 \times A \times B^2$. After 5 weeks, all mice

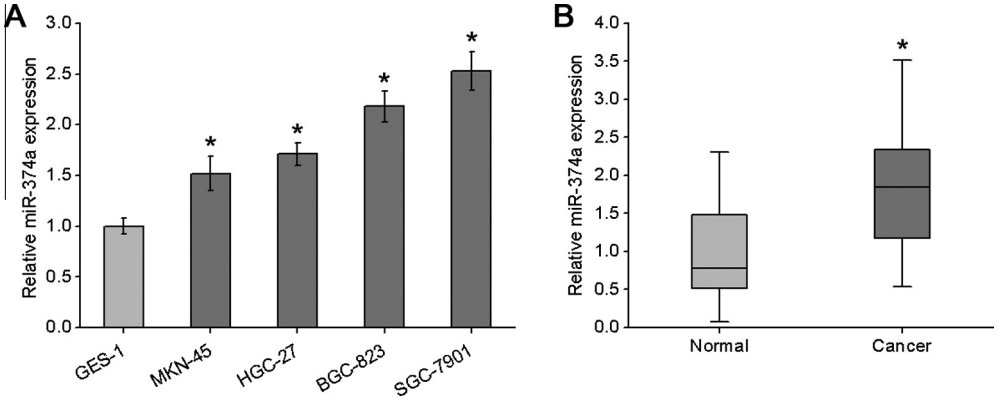

Fig. 1. miR-374a is up-regulated in human GC cell lines and tissues. (A) Relative expression of miR-374a was examined by real-time PCR in four GC cell lines (MKN-45, HGC-27, BGC-823 and SGC-7901) and the immortalized normal gastric epithelial cell line (GES-1). (B) Real-time PCR analysis of miR-374a expression in 18 pairs of GC tissues and their adjacent non-cancerous tissues. Each sample was analyzed in triplicate and normalized to U6, $^*P < 0.05$.

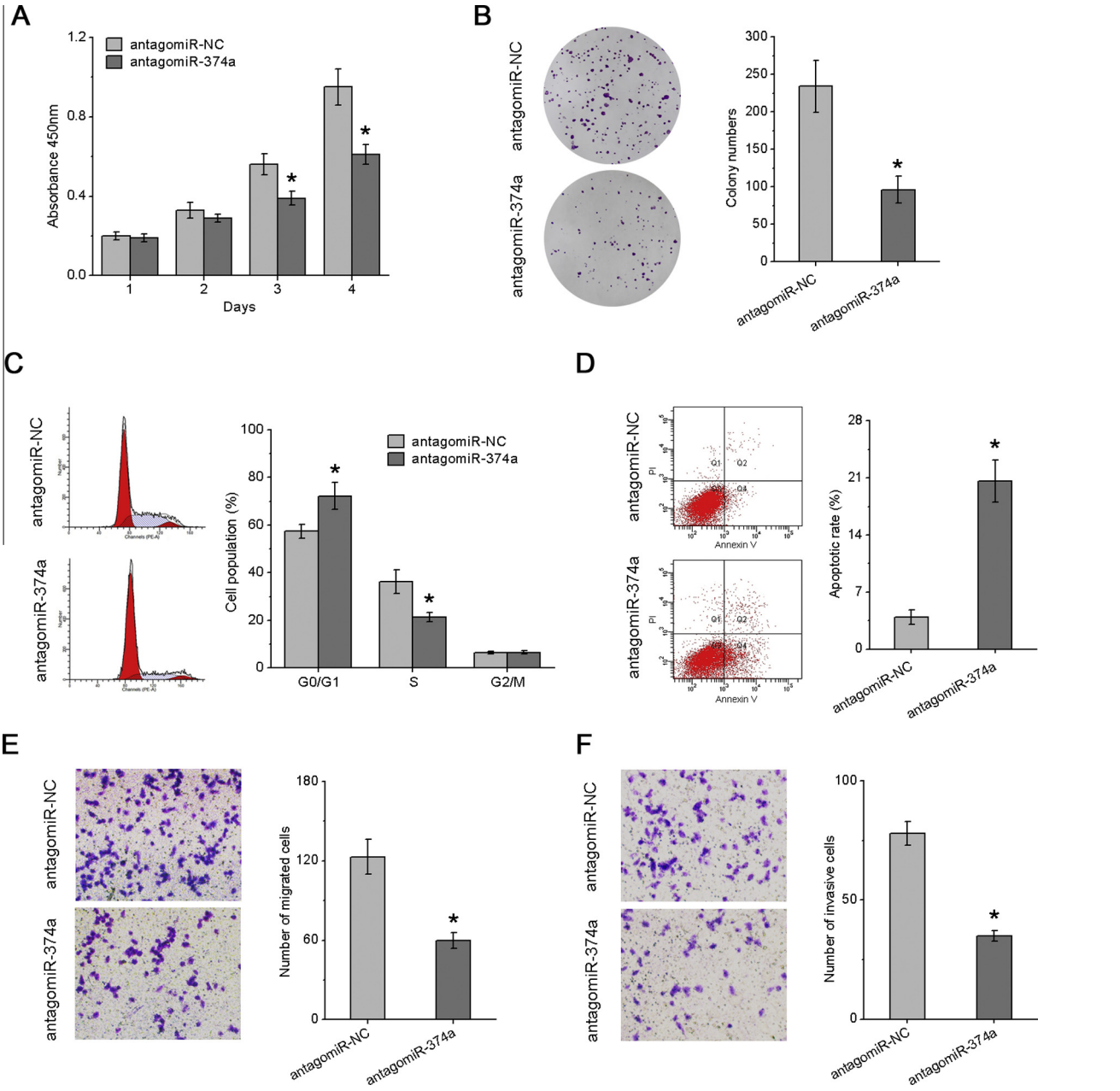

Fig. 2. miR-374a inhibition suppresses GC cell proliferation, migration and invasion. (A) SGC-7901 cells were transfected with antagomiR-NC or antagomiR-374a for 48 h, and cell viability was analyzed by CCK-8 assay. (B) Colony formation assay. (C, D) Flow cytometric analysis of cell cycle and apoptosis of the transfected cells. (E) Transwell migration assay was used to evaluate cell migration. (F) Transwell invasion assay, $^{*}P < 0.05$.

were killed and the tumors were excised. Tumor weights were measured and recorded. Tumor tissues were stored for further analysis.

2.8. Immunohistochemical analysis

Tumor tissues were fixed in 10% neutral buffered formalin, embedded with paraffin, and sliced into 4-μm sections. After mounting on slides the paraffin sections were baked, deparaffinized and dehydrated, antigen retrieval was carried out by boiling in 0.1 M citrate buffer (pH 6.0) for 15 min. The slides were incubated in 3% H_2O_2 for 10 min to suppress endogenous peroxidase activity, then sections were blocked with 10% goat serum in PBS for 30 min and incubated with rabbit anti-Ki67 polyclonal antibody (Abcam, Cambridge, UK) for 1 h at room temperature. Finally, the percentage of Ki67 positive cells were calculated under a microscope and photos were taken for analysis.

2.9. Luciferase assay

pGL3-SRCIN1-3′UTR-WT or pGL3-SRCIN1-3′UTR-MUT constructs were transfected into HEK293T cells, along with agomiR-374a or agomiR-NC and pRL-TK vector (Promega). Forty-eight hours post-transfection, luciferase activities were assayed using the Dual Luciferase Reporter Assay kit (Promega) according to the manufacturer's protocol. Renilla luciferase activity was used for endogenous normalization.

2.10. Western blotting

Total proteins were extracted and measured using the BCA protein assay kit (Thermo Scientific, Rockford, IL). Equal amounts of protein (30 μg) were loaded, separated by 8% SDS–PAGE, and transferred to PVDF membranes (Millipore, Danvers, MA). After blocking with 5% non-fat milk in TBS, membranes were probed with primary antibodies against SRCIN1 (Cell Signaling Technology, Danvers, MA) or GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), followed by peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). Bands were visualized using enhanced chemiluminescence (Thermo Scientific).

2.11. Statistical analysis

Results are expressed as the mean ± S.D. The differences between two groups were assessed by Student's *t*-test. The relationship between miR-374a and SRCIN1 expression was explored by Spearman's correlation. $P < 0.05$ was considered significant.

3. Results

3.1. miR-374a expression is up-regulated in human GC cell lines and tissues

We initially examined the expression level of miR-374a in four GC cell lines (MKN-45, HGC-27, BGC-823 and SGC-7901) and the immortalized normal gastric epithelial cell line GES-1. As shown in Fig. 1A, the expression level of miR-374a was significantly increased in GC cell lines compared with GES-1. Real-time PCR was then conducted to analyze 18 pairs of GC tissues and their matched normal tissues. The result showed that miR-374a expression was significantly higher in GC tissues than in their paired corresponding non-cancerous tissues. These results provide strong evidence that the expression of miR-374a is elevated in GC cell lines and tissues.

3.2. miR-374a inhibition suppresses GC cell proliferation, migration and invasion

miR-374a expression was increased in GC cell lines and tissues, which prompted us to investigate the biological significance of miR-374a in tumorigenesis. As an initial step, we selected SGC-7901 and BGC-823 cells for transfection experiments. After SGC-7901 and BGC-823 cells were transfected with miR-374a specific inhibitor antagomiR-374a or antagomiR-NC for 48 h, the CCK-8 assay was used to evaluate their growth rate. The result showed that cell viability was remarkably reduced upon antagomiR-374a treatment (Fig. 2A and S1A). Accordingly, the colony formation rate of these cells transfected with antagomiR-374a was significantly decreased compared to the control group (Fig. 2B and S1B). We also analyzed cell-cycle progression by flow cytometric analysis and found that SGC-7901-antagomiR-374a and BGC-823-antagomiR-374a cells showed a decrease in the S-phase peak and a dramatic cell cycle arrest at the G1/G0 phase compared to negative controls (Fig. 2C and S1C). In addition, the rate of apoptosis was significantly higher in antagomiR-374a-transfected cells (Fig. 2D and S1D). Moreover, transwell assays showed that antagomiR-374a significantly suppressed the migration and invasion of SGC-7901 and BGC-823 cells (Fig. 2E, F and S1E). Collectively, these data suggest that miR-374a inhibition reduces the proliferation, migration and invasion of GC cells.

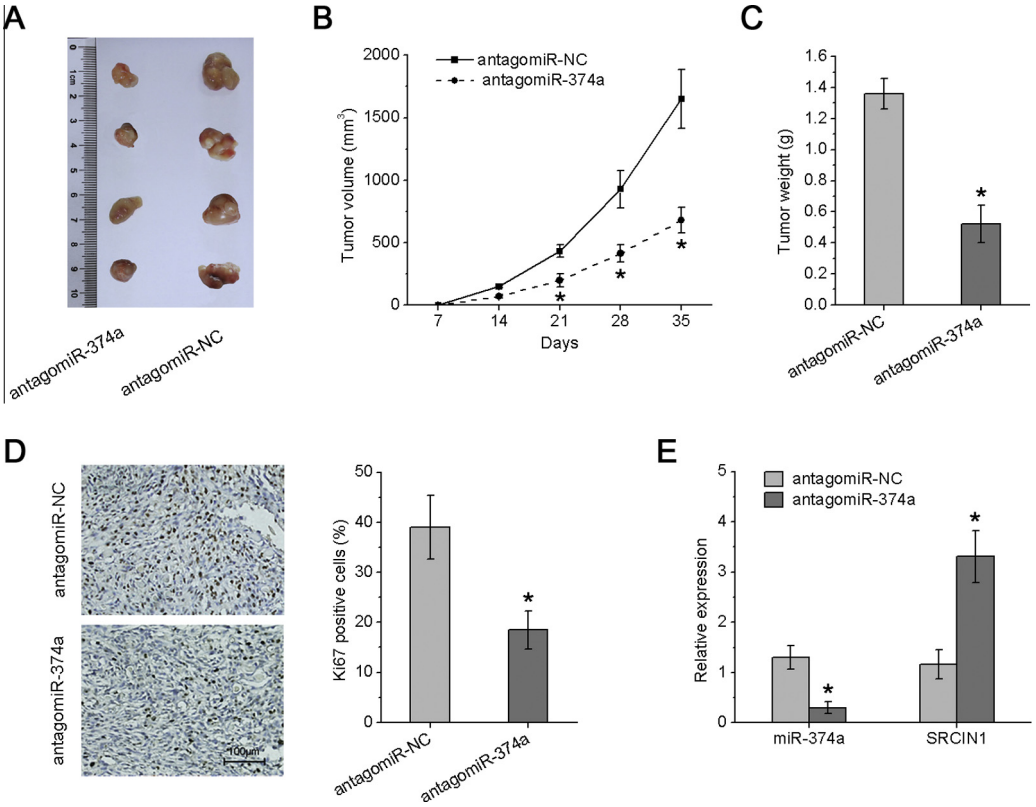

Fig. 3. Inhibition of miR-374a suppresses tumorigenicity in vivo. SGC-7901 cells transfected with antagomiR-NC or antagomiR-374a were injected subcutaneously into nude mice. After 35 days, the nude mice were killed. Images of representative tumors are shown in (A). (B) Growth curve of tumors in nude mice, the volume of each tumor was calculated each week after injection. (C) Average weight of tumors at the end of the experiment. (D) Percentage of positive cells by IHC for Ki-67 in tumor tissues. (E) Real-time PCR analysis of the expression of miR-374a and SRCIN1 mRNA in xenograft tumor tissues, $^{*}P < 0.05$.

3.3. miR-374a inhibition suppresses tumorigenicity in nude mice

We next examined whether miR-374a inhibition could affect the growth of gastric tumors in vivo. SGC-7901-antagomiR-374a or SGC-7901-antagomiR-NC cells were injected subcutaneously into nude mice. Five weeks after injection, SGC-7901-antagomiR-374a cells formed smaller tumors than control cells (Fig. 3A and B). The mean weight of tumors transfected with miR-374a inhibitor was markedly lower than that of the negative control group (Fig. 3C). Furthermore, IHC confirmed that the percentage of Ki-67 positive cells in the tumors with miR-374a inhibitor markedly decreased compared with that in the negative control group (Fig. 3D). Hence, a decreased proliferation rate caused by miR-374a inhibition is responsible for tumor suppression in vivo.

3.4. SRCIN1is a direct target of miR-374a

As miRNAs often regulate the expression of target genes, we searched for putative candidate targets of miR-374a using different bioinformatics algorithms such as TargetScan, Microrna and PicTar. Among the predicted miR-374a targets, SRCIN1, an inhibitor of Src activity and signaling, was chosen for further experimental verification. The 3′UTR of SRCIN1 mRNA contains a complementary site for the seed region of miR-374a (Fig. 4A). To investigate whether miR-374a directly interacts with SRCIN1, we constructed Luc-SRCIN1-3′UTR-wild-type (WT) and its 3′UTR mutant (MUT) plasmids. Luciferase reporter assays showed that agomiR-374a suppressed transcriptional activity of the Luc-SRCIN1-3′UTR-WT reporter by more than 50% compared with agomiR-NC, whereas the SRCIN1-3′UTR-MUT, in which the putative miR-374a binding site had been mutated, was refractile to the inhibition (Fig. 4B). We also confirmed that SRCIN1 mRNA and protein expression was decreased by the introduction of agomiR-374a, whereas it was significantly elevated by the transfection of antagomiR-374a in SGC-7901 and BGC-823 cells (Fig. 4C and D). Moreover, we examined the endogenous mRNA level of SRCIN1 in 18 pairs of GC tissues and matched non-tumor tissues, and found an inverse correlation between miR-374a and SRCIN1 mRNA levels (Fig. 4E). Real-time PCR analysis of the xenograft subcutaneous tumors also confirmed reduced miR-374a with enhanced SRCIN1 mRNA in antagomiR-374a-treated tumors (Fig. 3E). Taken together, these results suggest that miR-374a directly targets SRCIN1 and inhibits its expression.

3.5. Downregulation of SRCIN1 promotes GC cell proliferation, migration and invasion

It has been reported that SRCIN1 is essential for the regulation of cell proliferation and motility [12]. However, the effects of SRCIN1 on GC cells have not been characterized. To explore the roles of SRCIN1, we transfected SGC-7901 cells with specific siRNAs against SRCIN1, and Western blotting analysis revealed that SRCIN1 expression was significantly reduced after si-SRCIN1 transfection (Fig. 5A). Functional assays showed that si-SRCIN1 could promote GC cell proliferation, migration and invasion (Fig. 5B and C), suggesting a tumor suppressor role of SRCIN1 in GC cells.

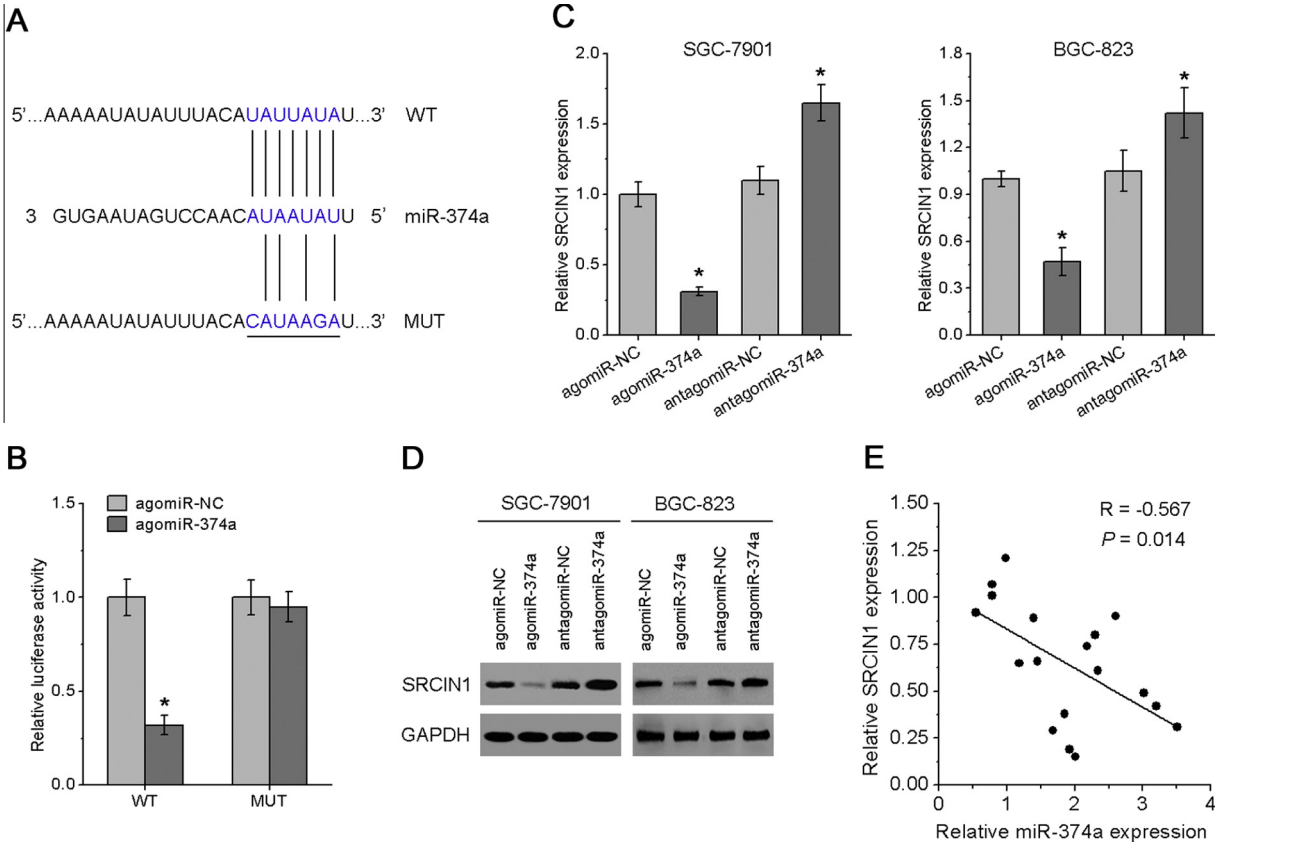

Fig. 4. SRCIN1 is a direct target of miR-374a. (A) Wild-type and mutant sequences of the putative miR-374a target sequences of SRCIN1 3′UTR. (B) Luciferase assays were performed after HEK293T cells were transfected with luciferase reporter plasmids containing SRCIN1-3′UTR-WT or SRCIN1-3′UTR-MUT, along with agomiR-374a or agomiR-NC. Renilla luciferase activity was used for endogenous normalization. The mRNA (C) and protein (D) expression of SRCIN1 in SGC-7901 and BGC-823 cells transfected with agomiR-374a or antagomiR-374a were analyzed by real-time PCR and Western blot, respectively. (E) Spearman's correlation analysis demonstrated that miR-374a expression level was inversely correlated with SRCIN1 mRNA level in GC tissues, $^{*}P < 0.05$.

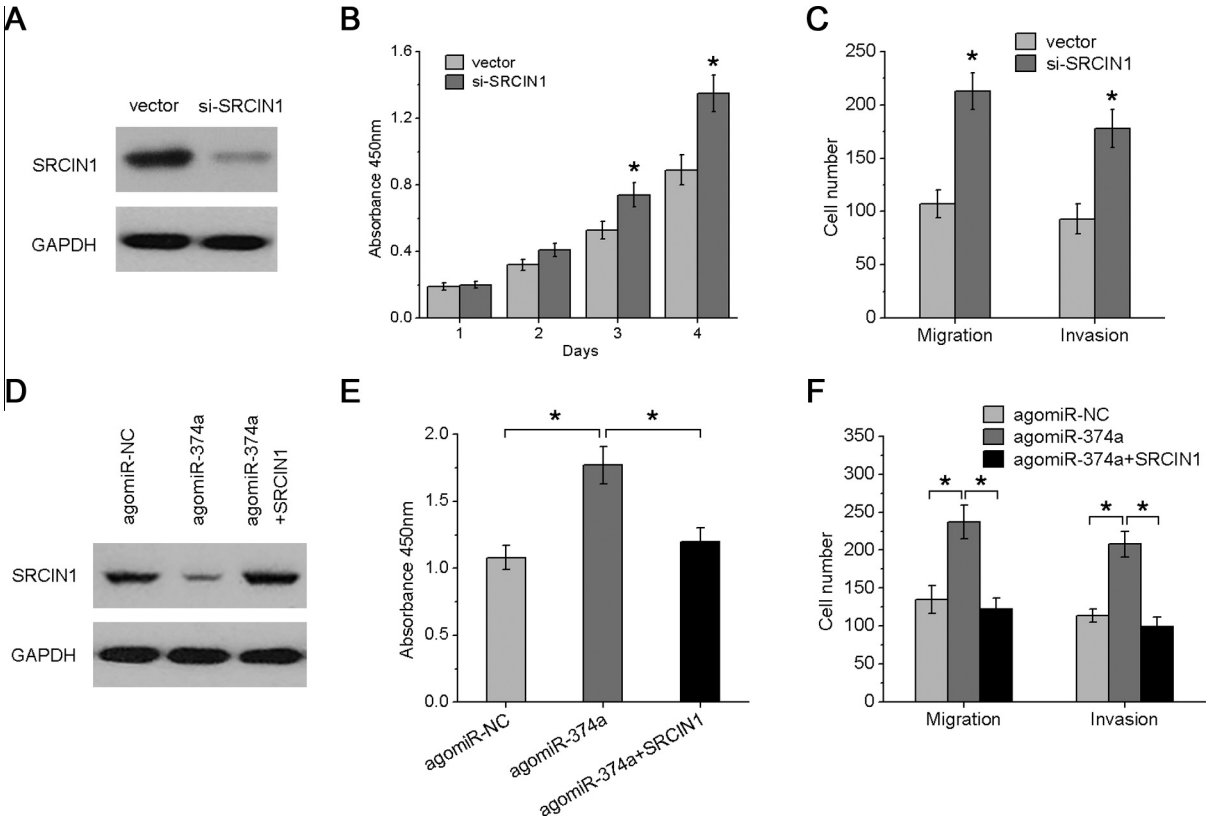

Fig. 5. Suppression of SRCIN1 promotes GC cell proliferation, migration and invasion while restoration of SRCIN1 reverses the biological phenomena induced by miR-374a. (A) Western blotting was performed to detect the protein level of SRCIN1 after transfection with si-SRCIN1 or vector into SGC-7901 cells. Representative histograms depict cell proliferation (B), migration and invasion (C). (D) The protein level of SRCIN1 in SGC-7901 cells after transfection with agomiR-NC, agomiR-374a or agomiR-374a and SRCIN1 vector. (E) Cell proliferation. (F) Cell migration and invasion, $^{*}P < 0.05$.

3.6. Reintroduction of SRCIN1 reverses the biological phenomena induced by miR-374a

We further evaluated whether reintroduction of SRCIN1 could reverse the effects of miR-374a. SGC-7901 cells were transfected with agomiR-NC, agomiR-374a, or cotransfected with agomiR-374a and SRCIN1 vector (lacking 3′UTR) (Fig. 5D). As shown in Fig. 5E and F, overexpression of miR-374a significantly promoted cell proliferation, migration and invasion, whereas reintroduction of SRCIN1 abrogated miR-374a-dependent effects. These data suggest that SRCIN1 is a functional mediator for miR-374a in GC cells.

4. Discussion

miRNAs serve as crucial regulators of gene expression, regulating cellular physiology and development [19]. Accumulating evidence has demonstrated that aberrant expression of miRNAs is related to tumorigenesis, progression and prognosis of various types of human cancers including GC [20–22], and that miRNAs can bind directly to their target genes to regulate cellular processes in GC. For example, miR-196a/-196b expression is up-regulated in GC tissues. Its expression inversely regulates GC invasion, migration and lung metastasis [23]. miR-296-5p increases GC proliferation and decreases GC apoptosis, and inhibition of both processes could provide beneficial GC treatments [24]. Thus, identifying and elucidating the exact roles and underlying mechanisms of miRNAs in GC may help us to understand the etiology and pathogenesis of GC and provide novel therapeutic targets. In this study, we focus on the potential oncogene miR-374a.

We first confirmed that miR-374a expression is markedly higher in GC cell lines and tissues than in GES-1 and normal gastric tissues. At the cytological level, inhibition of miR-374a suppresses proliferation, invasion and migration of GC cells and markedly reduces tumorigenesis in vivo. Furthermore, luciferase reporter assays show that miR-374a directly targets SRCIN1 though its 3′UTR.

SRCIN1, which is also known as p140CAP (p140 cas-associated protein), contains two coiled-coil domains, two proline-rich regions and two regions of highly charged amino acids [25,26]. According to the characteristic domain structure, SRCIN1 is thought to act as an adaptor protein [27]. Recently, SRCIN1 has been shown to participate in tumor progression, as an inverse regulator of integrin and growth factor-dependent tumor cell properties [25,28]. Screening of mammary breast cancers showed a positive interaction between the expression of SRCIN1 and malignancy [29]. SRCIN1 was shown to suppress the spreading, migration and invasion of breast cancer cells [30]. Furthermore, overexpression of SRCIN1 has also been shown to reverse the oncogenic effects of miR-150 on proliferation, migration and invasion in lung cancer cells [31]. These results suggest that SRCIN1 functions as a tumor suppressor gene and may slow tumor progression. However, the way in which SRCIN1 acts at the molecular and cellular levels in GC still remains unclear. In this study, we found that silencing of SRCIN1 promotes GC cell proliferation, invasion and migration. Reintroduction of SRCIN1 is able to abrogate the oncogenic effects of miR-374a. Furthermore, SRCIN1 expression inversely correlates with miR-374a in clinical samples. These results suggest that SRCIN1 is a functional mediator for miR-374a in GC.

In conclusion, we have shown for the first time that miR-374a promotes GC cell proliferation, migration and invasion by directly targeting SRCIN1. This study therefore has provided a novel promising therapeutic target for GC.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.12.027.

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