RESEARCH ARTICLE

miR-19a Promotes Cell Growth and Tumorigenesis through Targeting SOCS1 in Gastric Cancer

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Abstract

Accumulating evidence has shown that microRNAs are involved in cancer development and progression. However, it remains unknown about the potential role of miR-19a in the pathogenesis of gastric cancer. Here, we report that suppressor of cytokine signaling 1 (SOCS1) is a novel target of miR-19a in gastric cancer cells and that miR-19a expression is inversely correlated with SOCS1 expression in gastric cancer cells and a subset of gastric cancer tissues. Ectopic expression of miR-19a dramatically promoted proliferation and tumorigenicity of gastric cancer cells both *in vitro* and *in vivo*. Moreover, we showed that silencing of SOCS1 promoted cell growth and colony formation resembling that of miR-19a overexpression, whereas re-introduction of SOCS1 (without the 3'-UTR) attenuated the pro-tumorigenic functions. Taken together, our findings suggest that the SOCS1 gene is a direct target of miR-19a, which functions as an oncogenic miRNA in gastric cancer by repressing the expression of tumor suppressor SOCS1.

Keywords: SOCS1 - miR-19a - gastric cancer - cell growth

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Introduction

Gastric cancer is the fourth most common cancer and the second leading cause of global cancer mortality (Jemal et al., 2011). Despite great progress in surgical technique, diagnostic method and new chemotherapy regimens, no effective targeting therapy is available for gastric cancer (Shi et al., 2010). Thus, understanding the molecular mechanism underlying gastric cancer is still needed.

MicroRNAs (miRNAs) are a new class of small non-coding RNAs involved in the regulation of gene expression by triggering either mRNA degradation or translational repression through binding to the target sites of messenger RNAs (mRNAs) (Filipowicz et al., 2008; Bartel, 2009; Friedman et al., 2009). A large body of evidence has demonstrated that miRNAs can function as tumor suppressors or oncogenes during the initiation and progression of cancer including gastric cancer (Esquela-Kerscher et al., 2006). To date, some miRNAs have been shown to be dysregulated in gastric cancer, such as miR-21, miR-141, miR-150, miR-218, miR-331-3p and miR-375 (Zhang et al., 2008; Du et al., 2009; Ding et al., 2010; Gao et al., 2010; Guo et al., 2010; Wu et al., 2010). These findings suggest that the pathogenesis of gastric cancer may be attributed to miRNAs. A recent study has shown that miR-19a is upregulated in gastric cancer tissues compared with adjacent normal tissues by miRNA microarray analysis (Ueda et al., 2010). However, the role of miR-19a in gastric tumorigenesis has not yet been defined.

In the present study, we identified suppressor of cytokine signaling 1 (SOCS1) as a novel target of miR-19a in gastric cancer cells. SOCS1 functions as a tumor suppressor which plays important roles in many cancers, including gastric cancer (Clevenger, 2004; Oshimo et al., 2004; Davey et al., 2006; Lee et al., 2006; Souma et al., 2012). Furthermore, we found that overexpression of miR-19a was able to significantly promote gastric cancer cell proliferation, colony formation, and xenograft tumor growth. Finally, we show that the oncogenic effect of miR-19a was mediated by repression of SOCS1 in gastric cancer cells. Our finds provide significant clues regarding the role of miR-19a as an oncogenic miRNA in gastric cancer.

Materials and Methods

Tissue specimens and cell lines

18 pairs of gastric cancer tissues and adjacent normal tissues were obtained from First Affiliated Hospital of Xinxiang Medical University (Weihui City, China). Tissues were snap-frozen immediately after resection and stored at liquid nitrogen till use. Written informed consent for tissue donation (for research purposes) was obtained from the patients and the protocol was approved by the Institutional Ethical Board of First Affiliated Hospital of Xinxiang Medical University. Human embryonic kidney cell line HEK293T, gastric cancer cell lines MGC-

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803, BGC-823, MKN28, SGC-7901 and nonmalignant gastric cell line GES-1 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The four gastric cell lines and GES-1 cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 with RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), HEK293T were cultured in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum.

RNA extraction and qRT-PCR

Total RNA was extracted from cell lines and tissue samples using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Detection of the mature form of miR-19a was done by the TaqMan stem-loop RT-PCR method with a mirVana miRNA Detection Kit and gene-specific primers (Applied Biosystems, Foster city, CA). The U6 small nuclear RNA was used as an internal control. For detection of SOCS1 and β-actin transcript levels, cDNA was synthesized from 1 μg of total RNA with a reverse reaction kit (Promega, Madison, WI, USA). Real-time PCR was performed using SYBR Premix Ex Taq II (TaKaRa, Da-Liang, China). Expression of β-actin was used as internal control. The PCR primers for SOCS1 were 5'- AGCCGACAATGCAGTCTCCA-3' (forward) and 5'-TTCACGCTAAGGGCGAAAAA-3' (reverse). The relative expression levels of each gene were calculated and normalized using the $2^{-\Delta\Delta Ct}$ method relative to β-actin or U6 snRNA. All of the reactions were run in triplicate.

Lentivirus production and infection

The pre-miR-19a sequence was amplified with the following primers: 5'-CGCGAATTCGCAGTCCTCTGT TAGTTTTGCATAGTT-3' (forward) and 5'-GCGGGATC CCAGGCCACCATCAGTTTTGCATAGATT-3' (reverse). SOCS1 shRNA was purchased from GeneChem (Shanghai, China). These sequences were separately cloned into the lentivirus-based expression plasmid pCDH-CMV-MCS-EF1-coGFP constructs (System Biosciences, California, USA). Virus particles were harvested 48 h after pCDH-CMV-miR-19a transfection with the packaging plasmid pRSV/pREV, pCMV/pVSVG and pMDLG/pRRE into 293T cells using Lipofectamine 2000 reagent (Invitrogen). MGC-803 and SGC-7901 cells were infected with recombinant lentivirus-transducing units plus 10 μg/ml polybrene (Sigma, St Louis, Missouri, USA).

Oligonucleotide transfection

The miR-19a mimic, a nonspecific miR-control, anti-miR19a, and a nonspecific antimiR control were all purchased from Ambion (Austin, TX, USA). Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were collected 48 h after transfection.

Plasmids construction

The coding sequences with or without 3' untranslated region (3'UTR) of SOCS1 were amplified and cloned into pcDNA3.1 (+) to generate SOCS1 expression vectors pSOCS1-3' UTR and pSOCS1. The full-length SOCS1 3'-UTR was cloned into pGL3 luciferase vector (Ambion). The primers for SOCS1 3'-UTR were 5'-GAGCTCGTGCACGCAG CATTAACTGG -3' (forward) and 5'-AAGCTTTGCACAGCAGAAAAATA AAGCCAGA -3' (reverse). The mutant SOCS1 3'-UTR construct was designed to mutate three intermittent nucleotides complementary to the miR-19a seed-region by site-directed mutagenesis kit (TaKaRa, Dalian, China).

Luciferase reporter assay

HEK293T cells were seeded in 24-well plates at 2×10^4 cells/well and transiently transfected with appropriate reporter plasmid and miRNA using Lipofectamine 2000. After 48 h, the cells were harvested and lysed, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Renilla-luciferase was used for normalization. The experiments were performed independently in triplicate.

Cell proliferation analysis

To evaluate cell proliferation, 2×10^3 cells/well were separately plated in 96-well plates. After transfection, cell proliferation was assessed by MTT assay. Briefly, a 20 μl of MTT solution was added to each well and continued incubation for 4 h. Then, the supernatant was removed and 150 μl of DMSO was added to stop the reaction. Finally, the optical density was determined with a microplate spectrophotometer (ELx800, Bio-TEK, Winooski, VT, USA) at a wavelength of 570 nm. The cell growth curve was drawn based on absorbance and time.

Colony formation assay

Cells were infected with L/miR-19a to stably overexpress miR-19a. After 48 hours of transfection, cells were placed in a fresh six-well plate at 200 per well and maintained in RMPI 1640 containing 10% FBS for 2 weeks. Colonies were stained with 0.1% crystal violet (Amresco, Solon, OH) in 50% methanol and 10% glacial acetic acid for counting.

Western blotting

Protein was extracted from gastric cancer cell lines using RIPA lysis buffer with proteinase inhibitor. Total cellular protein concentrations were determined using a BCA assay kit (Beyotime, China). 20 μg of protein mixed with 2×SDS loading buffer was loaded per lane, separated by 10% SDS-PAGE and then transferred onto the nitrocellulose membrane (Bio-Rad, Munich, Germany). Antibodies to SOCS1 and β-actin (Abcom, Cambridge, UK) were incubated with the membranes overnight at 4°C. The membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. The bands were visualized by using the enhanced chemiluminescence (ECL) system according to the instructions of the manufacturer.

Tumor xenograft animal model

BALB/c nude mice (aged 4-5 weeks, 18-20 g) were purchased from Animal Center of Xinxiang Medical

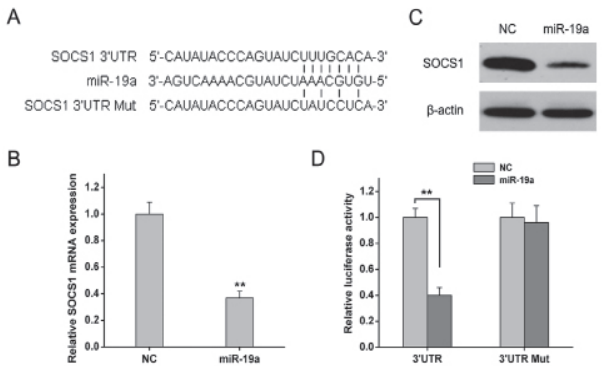

Figure 1. SOCS1 is a Target of miR-19a in Gastric Cancer Cells. (A) The wild type and mutant complementary sequences of the SOCS1 mRNA 3'UTR are shown with the miR-19a sequence. (B) SOCS1 mRNA levels were examined by qRT-PCR in MGC-803 cells transfected with miR-19a mimics or control RNA. (C) SOCS1 protein levels were examined by Western blotting in miR-19a–overexpressed and control cells, and β-actin served as an internal reference. (D) Luciferase reporter assays in HEK293T cells, with cotransfection of wt or mut SOCS1 3'UTR and miRNAs as indicated. Data are expressed as mean ± SD from 3 experiments. $^{**}P < 0.01$

University and were housed in barrier facilities on a 12 h light/dark cycle. All experimental procedures were approved by the Animal Center of Xinxiang Medical University. To evaluate the role of miR-19a in tumor formation, SGC-7901 cells stably overexpressing miR-19a or scramble control were propagated and injected subcutaneously into the dorsal flanks of nude mice (2×10^6 cells in 0.2 ml volume). Tumor diameter was measured and documented every 5 days for 25 days. Tumor volume (mm^3) was estimated by measuring the longest and shortest diameter of the tumor and calculating as follows: volume = (shortest diameter)2×(longest diameter)×0.5. The mice were killed and the tumors were weighed 25 days after inoculation.

Statistical Analysis

Data are presented as mean ± SD from a minimum of 3 replicates. Difference between groups was evaluated by SPSS 13.0 statistical software with one-way analysis of variance. The relationship between SOCS1 and miR-19a expression was explored by Pearson's correlation. A two-tailed P-value of less than 0.05 was considered statistically significant.

Results

SOCS1 is a direct target of miR-19a in gastric cancer cells

To explore the function and mechanism of miR-19a in the development of gastric cancer, we used bioinformatics tools (miRanda and TargetScan) to predicte the mRNA targets of miR-19a. The gene for SOCS1, predicted by both miRanda and TargetScan, is the theoretical target gene of miR-19a (Figure 1A). We next investigated whether miR-19a could directly down-regulate SOCS1 expression in gastric cancer cells. qRT-PCR and Western blotting analyses showed that ectopic expression of miR-19a in MGC-803 cells substantially decreased the levels of SOCS1 mRNA and protein (Figure 1B and C). To further

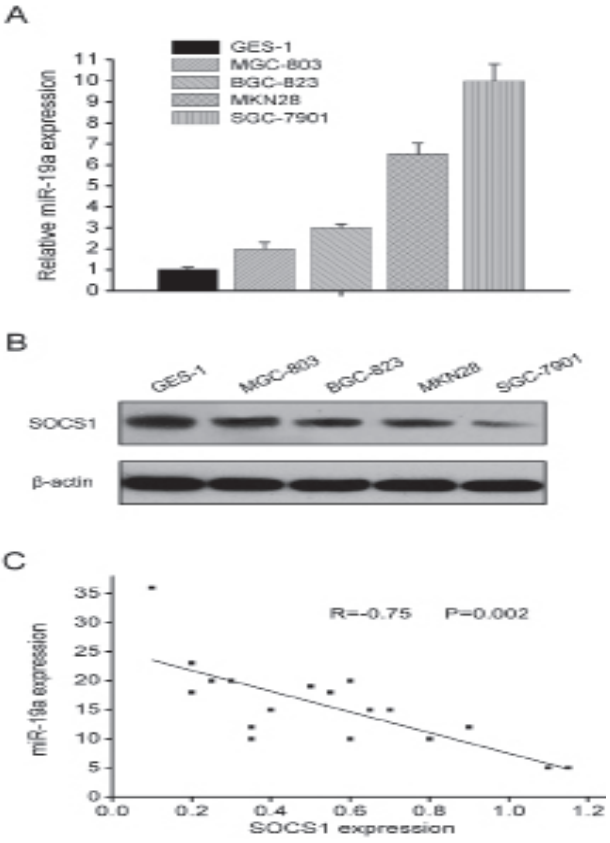

Figure 2. Expression of SOCS1 and miR-19a in Gastric Cancer Cells and Clinical Specimens. (A) qRT-PCR analysis of miR-19a levels in 4 gastric cancer cell lines. (B) Western blotting analysis of SOCS1 protein levels in gastric cancer cell lines. (C) Correlation analysis of miR-19a and SOCS1 mRNA levels in gastric cancer specimens

analyze whether miR-19a could interact with the putative binding site, we cloned the 3'-UTR region of SOCS1 downstream of a luciferase reporter vector. As illustrated in Figure 1D, up-regulation of miR-19a significantly decreased the SOCS1 3'UTR luciferase reporter activity compared with the negative control. Moreover, this effect was abolished when the nucleotides in the miR-19a seed binding site of the SOCS1 3'UTR were mutated. These results demonstrate that SOCS1 is a direct target of miR-19a in gastric cancer cells.

miR-19a is inversely correlated with SOCS1 levels in gastric cancer

To further confirm that SOCS1 is a target of miR-19a, we initially measured the expression levels of miR-19a and SOCS1 in 4 gastric cancer cell lines. As shown in Figure 2A, the expression of miR-19a was higher in all gastric cancer cells than that in nonmalignant gastric GES-1cells. In contrast, SOCS1 protein levels were relatively lower in 4 gastric cancer cell lines (Figure 2B). A significant negatively correlation was observed between the expression of miR-19a and SOCS1 protein in gastric cancer cell lines. Furthermore, we examined miR-19a and SOCS1 expression in 18 pairs of gastric cancer tissues and adjacent normal tissues, and investigated the relationship between miR-19a and SOCS1 expression levels. We found that miR-19a levels were significantly inversely correlated with the expression of SOCS1 mRNA in gastric cancer

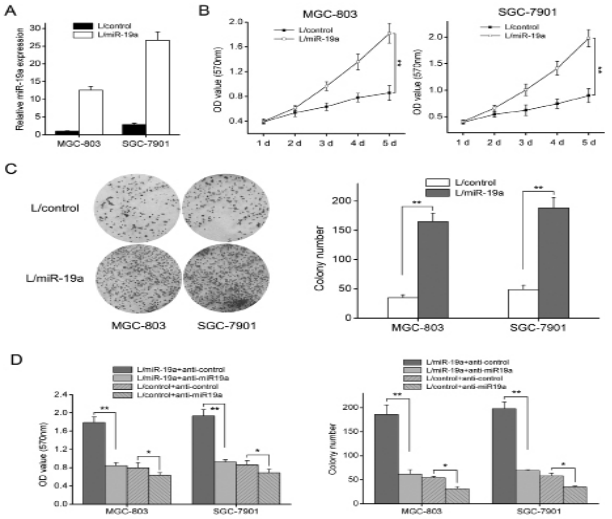

Figure 3. miR-19a Promotes Gastric Cancer Cell Proliferation and Colony Formation in Vitro. Gastric cancer cell lines (ie, MGC-803 and SGC-7901) were infected with miR-19a lentivirus (indicated as L/miR-19a) and miRNA-scramble control lentivirus (indicated as L/control), respectively, and the stably transduced cells were analyzed for proliferation and colonogenic potential as described in the Materials and Methods section. (A) miR-19a levels in cells infected with miR-19a lentivirus and control lentivirus were measured by qRT-PCR. (B) Cell proliferation assay. (C) Colonogenic assays. (D) Inhibition of miR-19a prevents gastric cancer cell proliferation and colonogenic potential. miR-19a–overexpressed or miRNA-scramble control cells were transfected with anti–miR-19a or scramble control, respectively; 48h after transfection, cells were analyzed for proliferation and colonogenic formation. Data are expressed as mean ± SD from 3 experiments. $*P < 0.05$; $**P < 0.01$

tissues (R=–0.75, P=0.002; Figure 2C). Taken together, these results suggest that miR-19a expression is inversely correlated with SOCS1 expression in gastric cancer cells and a subset of gastric cancer tissues.

miR-19a promotes the proliferation and tumorigenicity of gastric cancer cells

To explore the effect of miR-19a on cell growth, two gastric cancer cell lines, MGC-803 and SGC-7901, were established to stably overexpress miR-19a. The qRT-PCR analysis verified that the expression of mature miR-19a was significantly increased in the established cell lines (Figure 3A). These cells were then used to examine their growth curve and colony-formation capacity. As shown in Figure 3B, MTT assay showed that overexpression of miR-19a significantly increased cell growth of both MGC-803 and SGC-7901 cells compared with their corresponding controls. Furthermore, colony formation assay showed that upregulation of miR-19a also significantly increased colony-formation efficiency in the MGC-803 and SGC-7901 cells (Figure 3C). Accordingly, MGC-803 and SGC-7901 cells with miR-19a depletion showed decreased cell growth and colony-formation capacity (Figure 3D).

To further evaluate the effect of miR-19a on gastric cancer cell growth in vivo, SGC-7901 cells with modulated miR-19a expression were subcutaneously inoculated into both flanks of nude mice and the animals were closely monitored for tumor growth. As shown in

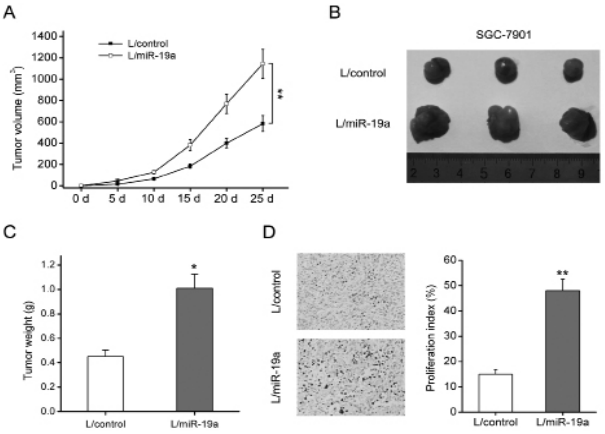

Figure 4. miR-19a Promotes Tumourigenicity of Gastric Cancer Cells in Vivo. (A) Tumour volumes measured on the indicated days. Data points are presented as the mean tumour volume ± SD. (B, C) Representative graph of tumour growth (B) and mean tumour weights (C) 25 days after inoculation. (D) Representative immunohistochemical staining of Ki-67-stained cells in indicated tumours. $*P < 0.05$; $**P < 0.01$

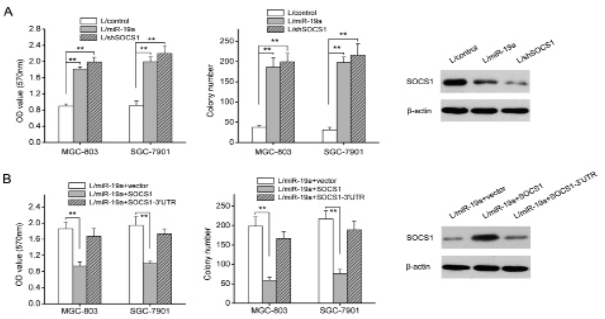

Figure 5. SOCS1 is Involved in miR-19a–induced Growth Promotion in Gastric Cancer Cells. (A) MGC-803 and SGC-7901 cells were infected with L/miR19a or L/shSOCS1. Cell growth and colonogenic potential were measured by MTT assay and colony formation assay. (B) MGC-803 and SGC-7901 cells were infected with L/miR19a for 72 hours, followed by infection with pSOCS1, pSOCS1-3'UTR, or control vector, and MTT assay and colony formation assay were then performed. $**P < 0.01$

Figure 4A, the volume of miR-19a-overexpressed tumors was significantly larger than that of the control. At the end of the experiment, an approximately 2-fold increase in tumor weight was observed in miR-19a-overexpressed tumors compared with the control (Figure 4C). In addition, immunohistochemical staining for the cell proliferation marker Ki67 showed higher percentages of Ki67-positive tumor cells in miR-19a-overexpressed tumors compared with the control tumors (Figure 4D). These results suggest that miR-19a promotes proliferation and tumourigenicity of gastric cancer cells both in vitro and in vivo.

SOCS1 is involved in the regulation of cell growth by miR-19a

To investigate whether the oncogenic effect of miR-19a was mediated by repression of SOCS1 in gastric cancer cells, we silenced SOCS1 to investigate whether the reduced expression of SOCS1 could mimic the oncogenic effect of miR-19a. As shown in Figure 5A, SOCS1 knockdown induced cell growth and colony

formation, which was consistent with the effect of miR-19a overexpression. Furthermore, we performed rescue experiments to further validate the role of SOCS1 in miR-19a-mediated oncogenesis in gastric cancer. The effects of SOCS1 (without 3'UTR) and SOCS1-3'-UTR (with 3'UTR) were examined in the miR-19a-infected cells. The data clearly showed that ectopic expression of SOCS1 led to significant cell growth inhibition, but this growth-suppressive effect was attenuated when SOCS1-3'-UTR was overexpressed (Figure 5B). Taken together, our results suggest that miR-19a promotes gastric cancer cell growth by downregulating SOCS1 expression.

Discussion

Recent studies have shown that miRNAs are frequently deregulated in many human cancers, including gastric cancer, but their function and mechanisms in tumorigenesis remain elusive. Identification of cancer-specific miRNAs as well as their target genes is important for elucidating the roles of miRNAs in tumorigenesis and may provide promising therapeutic targets. In this study, we focused on miR-19a, which has been suggested to promote tumor growth in several human cancers, such as breast cancer (Ouchida et al., 2012), multiple myeloma (Pichiorri et al., 2008), cervical cancer (Xu et al., 2012) and B-cell lymphomas (Liu et al., 2011). Recently, Ueda and colleagues found that miR-19a were upregulated in gastric cancer tissues, and overexpression of miR-19a was also associated with progression and prognosis of gastric cancer (Ueda et al., 2010), yet the detailed mechanisms remain unclear.

In this study, we provide the first evidence that miR-19a enhances gastric cancer progression by targeting SOCS1. Our data indicate that miR-19a overexpression significantly promoted gastric cancer cell proliferation in vitro and the tumorigenesis in vivo. We identified SOCS1 as a direct functional target of miR-19a in gastric cancer cells, and this conclusion is supported by several lines of experimental evidence from this study. First, complementary sequence of miR-19a is identified in the 3'UTR of SOCS1 mRNA. Second, miR-19a overexpression significantly reduced SOCS1 mRNA and protein. Third, overexpression of miR-19a decreased SOCS1 3'UTR luciferase report activity and this effect was abolished by mutation of the miR-19a seed binding site of the SOCS1 3'UTR. In addition, SOCS1 was naturally downregulated in gastric cancer cells and tissues, and inversely correlated with miR-19a levels, further confirming that SOCS1 is a target of miR-19a. To reveal the functions of SOCS1 in gastric cancer, we found that SOCS1 knockdown induced cell growth and colony formation similar to the effect of miR-19a overexpression, whereas SOCS1 overexpression could attenuate the tumorigenic function of miR-19a in gastric cancer cells. These results indicate that miR-19a functions as an oncogenic miRNA in gastric cancer, promoting tumor cell growth through targeting SOCS1. Our findings are consistent with a previous study by Pichiorri et al showing that miR-19a regulates expression of SOCS1 in human multiple myeloma cell lines (Pichiorri et al., 2008).

Suppressors of cytokine signaling (SOCS) family proteins are known to be important negative feedback inhibitors of the Janus tyrosine kinase (JAK)/signal transducers and activators of transcription (STAT) pathway, and activation of JAK/STAT pathway has been recently described to play a crucial role in human cancer development (Jiang et al., 2010; Slattery et al., 2013). SOCS family consists of 8 proteins (SOCS1-7 and a cytokine-inducible SH2-containing protein or CIS), each of which containing a central Src homology-2 (SH2) domain and a COOH-terminal SOCS box (Krebs et al., 2001). Interestingly, a recent study reported that SOCS6 was frequently down-regulated in gastric cancer tissues and cell lines and ectopic expression of SOCS6 suppressed gastric cancer cell growth and colony formation (Lai et al., 2010). These results suggest that SOCS6 may act as a tumor suppressor, and that further investigations are necessary to determine whether miR-19a could regulate the expression of other SOCS members in gastric carcinogenesis. Previous study demonstrated that SOCS1 gene promoter hypermethylation led to downregulation of SOCS1 gene in many human cancers, including gastric cancer (Oshimo et al., 2004). Here, our findings that SOCS1 expression is suppressed by miR-19a provide new insight into the mechanism of loss of SOCS1 function in cancer.

In conclusion, for the first time, our study demonstrates that miR-19a promotes gastric cancer cell proliferation, and an important tumor suppressor SOCS1 is identified as a target of miR-19a. Our findings suggest that miR-19a might have a potential role in gene therapy and may be a promising target for the treatment of gastric cancer in future.

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