miRNA-223 inhibits epithelial-mesenchymal transition in gastric carcinoma cells via Sp1

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Received March 14, 2016; Accepted April 25, 2016

DOI: 10.3892/ijo.2016.3533

Abstract. Sp1 plays critical roles in epithelial-mesenchymal transition (EMT) of certain cancer. However, few studies have indicated whether Sp1 is involved in the EMT of gastric cancer, and whether abnormal expression of Sp1 in gastric cancer EMT is regulated in a post-transcriptional manner, and the involvement of miRNAs in this regulation. In this study, we selected 20 cases of gastric cancers, their liver metastases and para-carcinoma tissues to examine the levels of Sp1 protein and mRNA by immunohistochemistry and fluorescent PCR, which showed that Sp1 was increased in gastric cancers and their metastases compared with adjacent tissues, but there was no difference in Sp1 mRNA between these three groups, suggesting changes in Sp1 may be attributed to inactivation of post-transcriptional regulation. We verified by a luciferase reporter system that miRNA-223 binds to 3'-UTR of Sp1 gene and inhibits its translation, in agreement with negative correlation between miRNA-223 and Sp1 protein levels in gastric cancer cells. By employing TGF-β1 to induce MGC-803, BGC-823 and SGC-7901, we successfully built cellular EMT model. Then, we overexpressed miRNA-223 in the model by using a lentiviral system, which diminished EMT indicators and suppressed proliferation and invasion ability, and induced apoptosis. Finally, we verified the specificity of the regulation pathway miRNA-223/Sp1/EMT. These findings suggest that low expression of miRNA-223 in gastric cancer cells is an important cause for EMT. miRNA-223 specifically regulates the EMT process of gastric cancer cells through its target gene Sp1. Overexpression of miRNA-223 in these cells inhibits EMT via the miRNA-223/Sp1/EMT pathway.

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Key words: Sp1, miRNA-223, epithelial-mesenchymal transition, gastric cancer, invasion and metastasis

Introduction

The epithelial-mesenchymal transition (EMT) is a normal process of early embryonic development and also contributes pathologically to multiple conditions such as renal fibrosis and cancer progression. In this process, the epithelial cells undergo both structural and phenotypic changes, including changes in adhesion, cytoskeletal and polarity structures, which result in loss of cell polarity, enhanced migration and invasion, and increased resistance to apoptosis (1). EMT plays important roles in tumorigenesis, invasion and metastasis of cancers (2), and has consequently been studied extensively in the cancer research field (3).

The Sp1 transcription factor is critical for many biological processes, including cellular growth and differentiation (4). In cancer growth and metastasis, Sp1 influences a variety of cancer cell types through the regulation of oncogenes, tumor suppressor genes, cell cycle regulators, growth related signal transduction pathways, angiogenic factors and apoptosis (5,6). Sp1 is expressed at high levels in many cancers (7) and is closely associated with EMT (8,9). New therapies targeting Sp1 could, therefore, represent beneficial additions to current clinical chemotherapy strategies.

EMT behavior increases invasiveness and mobility of gastric cancer cells and makes them more resistant to apoptosis (10). A previous study suggests Sp1 is an important target in the study of gastric cancer (11). Sp1 can affect invasive ability of gastric cancer cells through regulating transcription of MTA2 gene (12). miRNA-22 also regulates invasion and migration of gastric cancer via its target gene Sp1 (13). Since Sp1 is closely involved in invasion and migration of gastric cancer, it is worthy to study whether it plays a role in EMT of gastric cancer cells. In addition, whether miRNAs are involved in this process is a focus of this study.

In this study, we immunohistochemically assessed Sp1 expression in selected specimens from primary and metastatic

tissues of gastric cancers and adjacent tissues, and the results showed that Sp1 was higher in metastatic and primary cancer tissues than in normal adjacent tissues, suggesting close association of high Sp1 expression and invasion and metastasis of gastric cancer. Further study showed that the high expression of Sp1 was attributed to inactivation of translation mechanism and low miRNA-223 level was the direct cause for increased Sp1 expression. Therefore, we reasoned that miRNA-223/Sp1/EMT is a pathway regulating metastasis and invasion, and low expression of miRNA-223 increases Sp1 gene expression in gastric cancer, induces EMT, and consequently increases its ability to invade, proliferate and resist apoptosis. Afterwards, we focused our study on verification of this pathway regulating EMT and investigated whether overexpressing miRNA-223 can inhibit EMT in gastric cancer cells.

Materials and methods

Detection of Sp1 protein in gastric cancer tissues and liver metastatic tissues. Gastric cancers, their associated liver metastatic tissues and para-carcinoma tissues from 20 cases were collected from the General Surgery Department of Shanghai Changzheng Hospital, China. Sp1 expression was detected by immunohistochemistry. Tissues were collected and fixed with 4% paraformaldehyde and soaked in 20% saccharose solution at 4˚C overnight. The specimens were embedded in paraffin, sliced and subjected to conventional immunohistochemical detection of Sp1. Briefly, each slice was washed three times with 0.01 M potassium phosphate-buffered saline (PBS) for 5 min each, treated with a 0.3% hydrogen peroxide-methanol solution for 30 min, and then washed with PBS an additional three times. Next, the samples were treated with 0.3% Triton X-100 in PBS for 30 min, washed with PBS three times, and incubated with the primary antibody (Anti-Sp1, 1:200; sc-420, Santa Cruz Biotechnology, USA) in serum diluent solution (1% bovine serum albumin, 0.08% sodium azide in PBS) at 4˚C for 24 h. The slice was then washed with PBS, and incubated with horseradish peroxidase-conjugated secondary antibody (anti-mouse, 1:3,000; #7076, Cell Signaling Technology, USA) at room temperature for 2 h, before washing again in PBS. Avidin-biotin complex was then added and the slice incubated at room temperature for 2 h, after which it was washed three times with PBS followed by an additional three washes with distilled water before addition of developing solution for 15 min. The slice was then rinsed with distilled water and PBS to terminate the reaction. Finally, the slice was dehydrated through a graded series of alcohol washes, sealed and photographed.

Detection of miRNA-223, Sp1 mRNA and protein, EMT-marker proteins in tumor specimens. Tissue samples of ~100 mg each were cut from gastric cancers, liver metastases and adjacent para-carcinoma tissues of 20 cases and stored in liquid nitrogen. For sample processing, tissues were treated with 1 ml TRIzol (Invitrogen, Carlsbad, CA, USA) or protein lysis buffer (Pierce, USA) for the subsequent extraction of RNA and proteins, respectively. After homogenization, total RNA and protein was extracted in accordance with the manufacturer's instructions. The purity of total RNA was measured by ultraviolet spectrophotometry, and miRNA-223 and Sp1 mRNA levels were detected by real-time RT-PCR (for experimental methods, see the the details below). The concentration of each protein sample was measured using the BCA protein assay (Pierce), and the protein expression was determined by western blotting.

Detection of mature miRNA-223. Total RNA (2 μg) was reverse-transcribed into cDNA using primers specific for human U6 snRNA (5'-TACCTTGCGAAGTGCTTAAAC-3'), and miRNA-223 (5'-GTCGTATCCAGTGCGTGTCGTGGA GTCGGCAATTGCACTGGATACGAATGGGGT-3'). The reaction product (2 μl) was used as the template for fluorogenic quantitative PCR to measure miRNA-223. The results were analyzed by the $2^{-\Delta\Delta Ct}$ method using U6 snRNA (NM_004394.1) as the internal reference. The forward and reverse PCR primers for U6 snRNA were 5'-GTGCTCGCTTCGGCAGCACAT-3' and 5'-TACCTTGCGAAGTGCTTAAAC-3', respectively. The forward and reverse primers for miRNA-223 were 5'-GCCG GCGCCCGAGCTCTGGCTC-3' and 5'-TGTCAGTTTGTCA AATACCCCA-3', respectively. The PCR reaction mix (20 μl) included 10 μl of SYBR Premix Ex Taq (Takara, Japan), 0.2 μl each of forward and reverse primers (20 μM stock), 2 μl of the cDNA template, and 7.6 μl dH_2O. The PCR reaction conditions involved 40 cycles of a 95˚C denaturing step, a 58˚C annealing step, and a 72˚C elongation step, each of which was performed for 10 sec.

Detection of Sp1 mRNA. Analysis of Sp1 mRNA expression was performed using real-time PCR and the $2^{-\Delta\Delta Ct}$ method. Briefly, total RNA of patient tissues was reverse-transcribed into cDNA using oligo dT(18) primers (Takara, Japan) and this was used as a template for the PCR reaction with amplification of β-actin providing the internal reference. Forward and reverse primers for the detection of Sp1 mRNA (NM_138473.2) were 5'-AACAGATTATCACAAATCGAGG-3' and 5'-AAG GTAGCTGCAGAAACGCTG-3', respectively. Forward and reverse primers for β-actin mRNA (NM_001101.3) were 5'-GTGCTCGCTTCGGCAGCACAT-3' and 5'-TACCTTGCG AAGTGCTTAAAC-3', respectively. The PCR reaction mix and cycle parameters were as above with the exception that the annealing step was performed at 56˚C.

Western blotting. Protein samples were separated by SDS-PAGE using an 11% polyacrylamide gel and transferred to PVDF membranes. Membranes were blocked at room temperature for 2 h in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% nonfat milk and then incubated overnight at 4˚C with primary antibodies (Sp1, sc-420, 1:300; Vimentin, sc-53464, 1:200; Fibronectin, sc-81769, 1:400; N-cadherin, sc-393933, 1:600; E-cadherin, sc-21791, B actin, sc-130300, 1:1,000; Santa Cruz Biotechnology). Membranes were then rinsed with TBST three times and incubated with horseradish peroxidase-conjugated secondary antibody (rabbit anti-mouse IgG, #7076, 1:3,000; Cell Signaling Technology) for 2 h. ECL chemiluminescence substrate (Pierce) and X-ray film were used to detect protein bands. Relative protein concentration was quantified by densitometry analysis using image processing software by normalizing the intensity of the target band to that of the corresponding β-actin control band.

miRNA-223 binding site prediction and luciferase reporter assays. To predict the miRNA-223 binding site, we used Targetscan (http://www.targetscan.org/) to predict whether a miRNA-223 binding site exists within the 3'-UTR of human Sp1 mRNA (NM_138473.2). The results showed that a seven-base miRNA-223 seed sequence is present in the 3'-UTR of Sp1 mRNA.

To construct luciferase reporter vectors, total RNA was extracted from 293 cells, reverse-transcribed into cDNA, and 2 μl of the reaction product subsequently used as a template for PCR. Primers were designed that targeted the 3'-UTR of the Sp1 gene such that flanking *Xba*I restriction sites were introduced into the 261 base-pair (bp) PCR product containing the 5'-AACTGAC-3' miRNA-223 target site. The forward and reverse primer sequences were 5'-GCTCTAGAGCTAACAG AAATTAATTTAACTG-3' and 5'-GCTCTAGAAAGACGG TGTGGGTTGTTAC-3', respectively. PCR reaction conditions were as follows: 35 cycles of a 94˚C denaturing step for 30 sec, a 55˚C annealing step for 30 sec and a 72˚C elongation step for 10 sec. The PCR product was digested with *Xba*I and cloned into the pGL3-promoter luciferase reporter vector (Promega, USA) to generate the vector pGL3-Pro-WT-Sp1. The miRNA-223 target site in the pGL3-Pro-WT-AP1 vector was mutated from 5'-AACTGAC-3' to 5'-ACCTATC-3' to construct the mutated reporter vector, pGL3-Pro-MT-Sp1. The products of all cloning and mutagenesis reactions were confirmed by DNA sequencing. Endotoxin free DNA was prepared in all cases. The miRNA-223 mimic (5'-UGUCAGUUUGUCAAAU ACCCCA-3'), the miRNA-223 inhibitor (5'-UGGGGUA UUUGACAAACUGACA-3), and negative control miRNA (miRNA-NC, 5'-CUCAUGUCACUGUGACAUAUAC-3') were all chemically synthesized.

A suspension of 293 (SIBCB, China) cells in logarithmic phase growth was prepared and the number of viable cells counted using a hemocytometer in conjunction with trypan blue staining. The cells were seeded into 6-well plates at a concentration of $2x10^5$ cells per well and maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum at 37˚C for 24 h in a 5% CO_2 atmosphere. The transfection of plasmid DNA and RNA was performed using Lipofectamine 2000 (Invitrogen). Transfection of cells with pGL-TK (100 ng) (Invitrogen) served as a reference for luciferase detection. Luciferase activity was measured using the dual luciferase reporter assay system (Promega) 48 h after transfection.

Generation of recombinant lentivirus for miRNA-223 and Sp1 overexpression studies. Genome DNA was extracted from para-carcinoma tissues and the 465-bp DNA sequence containing the miRNA-223 precursor sequence was amplified by PCR. The forward and reverse primer sequences were 5'-CGGAATTCGCCACCTTCTGGTGCTTTGGTTGGTC-3' and 5'-CGGGATCCTTCCATTCCTGGACACTTTATAC-3', respectively. *Eco*RI and *Bam*HI restriction sites were introduced into the forward and reverse primers, respectively, to enable subsequent cloning of the PCR product into the pcDNA lentiviral expression vector resulting in the generation of pcDNA-miRNA-223. To clone human Sp1, total RNA was extracted from human gastric cancer samples using TRIzol, and reverse-transcribed into cDNA. This reaction product was then used for the amplification of Sp1 cDNA by PCR using primers that incorporated an *Eco*RI site and a Kozak sequence (5'-GCCACC-3') upstream, and a *Bam*HI site downstream, of the target sequence. Forward and reverse primers were 5'-CGG AATTCGCCACCATGAGCGACCAAGATCACTCC-3' and 5'-CGGGATCCTCAGAAGCCATTGCCACTGAT-3', respectively. The Sp1 PCR product was cloned into the pcDNA lentiviral expression vector (SystemBiosciences, USA) to generate pcDNA-Sp1. In all cases, successful cloning was confirmed by DNA sequencing. For the generation of lentiviral particles, expression vectors were co-transfected into 293T producer cells (SystemBiosciences) with a lentiviral packaging plasmid mix (SystemBiosciences) using Lipofectamine 2000 (Invitrogen). Culture supernatants were collected 48 h later, clarified by centrifugation, and passed through a 0.45-μm filter (Millipore, USA). Viral titer was evaluated by the gradient dilution method. Lentiviral particles generated using the pcDNA-miRNA-223 and pcDNA-Sp1 vectors were designated Lv-miRNA-223 and Lv-Sp1, respectively. Cells were infected with lentivirus 1 day after seeding using a multiplicity of infection (MOI) of 20.

Measurement of correlation between miRNA-223 and Sp1 protein and effect of overexpression of miRNA-223 on Sp1 protein in gastric cancer cells. GES-1, a normal human gastric mucosal cell line and three gastric cancer cell lines, MGC-803, BGC-823 and SGC-7901, were maintained in DMEM+10% FBS at 37˚C and 5% CO_2. Total RNA and protein were extracted and miRNA-223 and Sp1 contents were examined to determine whether there was abnormal expression or correlation between miRNA-223 and Sp1. MGC-803, BGC-823 and SGC-7901 cells were seeded to 6-well plates, incubated overnight and infected with Lv-miRNA223 or Lv-NC. Cells were collected 72 h after infection, and total RNA and protein were extracted and measured for miRNA-223 and Sp1, to evaluate the effect of overexpression of miRNA-223 on Sp1 protein level.

TGF-β1-dependent in vitro model of gastric cancer EMT. TGF-β1 is commonly used to induce EMT in a variety of cancer cell types (14,15). We therefore established a TGF-β1-dependent model of EMT for gastric cancer cell lines. MGC-803, BGC-823 and SGC-7901 cells were incubated with TGF-β1 for 72 h and subsequently the expression of EMT related markers was evaluated to determine whether the model had been established successfully. Cells in logarithmic phase growth were seeded into 6-well plates at a density of $2x10^5$ cells/well, and then treated with 100 ng/ml TGF-β1 (Pierce) for 72 h. At the same time, the cells treated with or without TGF-β1 were infected with Lv-miRNA-223 (MOI of 20) and then maintained for 48 h under normal culture conditions. The cells were then collected and total RNA and protein extracted and analyzed by real-time PCR and western blotting to measure miRNA-223 and Sp1 protein levels, respectively.

To determine whether EMT could successfully be induced following TGF-β1 treatment, we examined the expression of a group of EMT markers. SGC-7901 cells were stimulated with TGF-β1 at a final concentration of 100 ng/ml for 72 h. The cells were infected with recombinant lentiviruses when

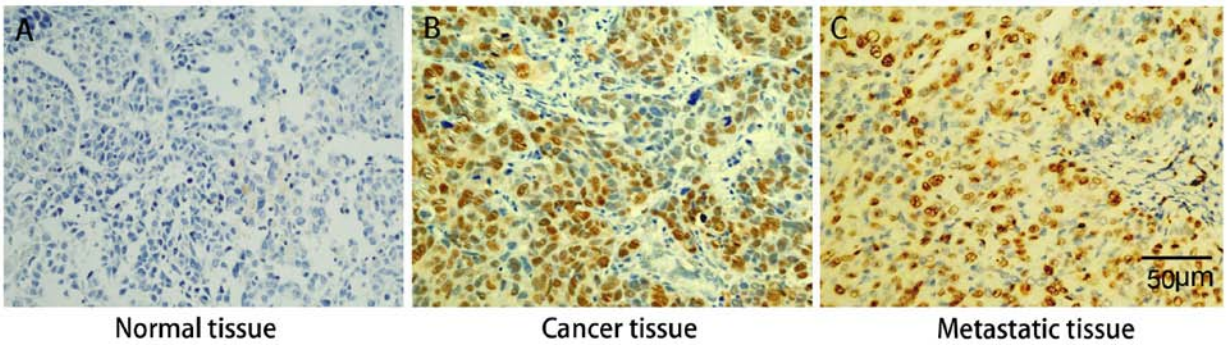

Figure 1. Sp1 protein expression in normal, primary tumor, and metastatic tissues. Representative micrographs showing the immunohistochemical staining of Sp1 in (A) normal adjacent tissue, (B) gastric cancer tissue, and (C) metastatic tissue in the liver.

appropriate and collected 48 h later for protein extraction and western blotting to determine Vimentin, Fibronectin, N-cadherin and E-cadherin expression. Based on this model, we carried out experiments of gene intervention, i.e., 24 h after lentiviral infection, the cells were incubated with TGF-β1 for 72 h and EMT protein markers were measured. Moreover, we analyzed EMT progress by evaluating proliferation, apoptosis and invasion to determine the effect of miRNA-223 on TGF-β1 induced EMT in gastric cancer cells.

Cellular proliferation assay. Increased proliferation is one indicator of EMT and so we measured changes in proliferation to determine whether miRNA-223 overexpression could inhibit EMT. MGC-803 cells infected with recombinant lentiviruses were stimulated with 100 ng/ml TGF-β1 for 72 h, trypsinized, and seeded into 96-well plates at a density of $1x10^4$ cells per well. The cells were cultured under normal conditions and cell viability was examined using CCK-8 at 24-, 48-, and 72-h time-points. Briefly, 10 μl CCK-8 solution was added, and the cells then cultured under normal conditions for an additional 4 h before measurement of absorbance at 450 nm.

Apoptosis assay. Increased apoptosis is a marker of EMT inhibition. BGC-823 cells in logarithmic phase growth were seeded into 6-well plates at a density of $2x10^5$ cells/well, and incubated with 100 ng/ml TGF-β1 for 72 h. At the same time, the cells treated with or without TGF-β1 were infected with Lv-miRNA-223 or Lv-Sp1 (MOI of 10) and then maintained for 48 h under normal conditions. The cells were collected and apoptosis assessed using the Annexin V-FITC Apoptosis Detection Kit II (BD Biosciences, USA). The cells were washed twice by centrifugation in PBS (2,000 x g, 5 min), resuspended in 500 μl binding buffer containing 5 μl of Annexin V-FITC, and incubated for 10 min. The cells were then stained with 5 μl propidium iodide for 5 min. Apoptosis was analyzed at an excitation wavelength of 488 nm using the FL1 channel for Annexin V-FITC and the FL2 channel for PI.

Cell invasion assay. The EMT model was established using SGC-7902 cells which were subsequently infected with lentiviruses. Forty-eight hours later, the cells were seeded into Transwell chambers (Millipore) for the analysis of cell invasion according to the manufacturer's instructions. For quantitative analysis, we conducted DAPI staining and α-SMA immunolabeling to fluorescently stain nuclei and the cell body, respectively. This enabled the number of cells that had transversed the chamber filter to be observed and counted using epifluorescence microscopy (IX81, Olympus, Japan). In addition, in accordance with the kit's instructions, the cells were detached and counted after staining.

Analysis of specificity of miRNA-223/Sp1/EMT pathway. There may be several target genes of miRNA-223, so we had to verify whether the Sp1 effect is critical to miRNA-223 on EMT. We designed the following experiment: we cloned Sp1 coding sequence to construct a recombinant vector pcDNA-Sp1 to express Sp1 in cancer cells via our lentiviral system. In contrast with endogenous Sp1, it lacks the binding site of miRNA-223 due to no wild-type 3'-UTR. Therefore, in the presence of exogenous Sp1, we could determine the specificity of miRNA-223/Sp1/EMT according to the effect of miRNA-223 on EMT indicators.

Statistical analysis. The data, expressed as means ± SD, were analyzed using SPSS software (version 13.0; SPSS). Statistical analysis was determined by one-way ANOVA followed by multiple comparisons with the least significant difference (LSD). P-values <0.05 were considered statistically significant.

Results

Analysis of Sp1 protein expression in clinical gastric cancer tissues and associated liver metastatic tissues. Immunohistochemical analysis revealed that Sp1 protein was expressed at higher levels in primary gastric cancer and liver metastatic tissues than in para-carcinoma tissues. There was no observable difference in Sp1 expression between primary gastric cancer and liver metastatic tissues, suggesting that high expression of Sp1 is associated with both tumorigenesis and metastasis of gastric cancer (Fig. 1).

Detection of miRNA-223, Sp1 mRNA and protein, EMT markers in tumor specimens. Expression of miRNA-223 in normal adjacent tissues, gastric cancer tissues and metastatic tissues negatively correlated with Sp1 protein levels and was lower in the gastric cancer and metastatic tissues than in the adjacent tissues ($P<0.05$; Fig. 2A). Sp1 mRNA and protein expression data (Fig. 2B and C) demonstrate that, in compar-

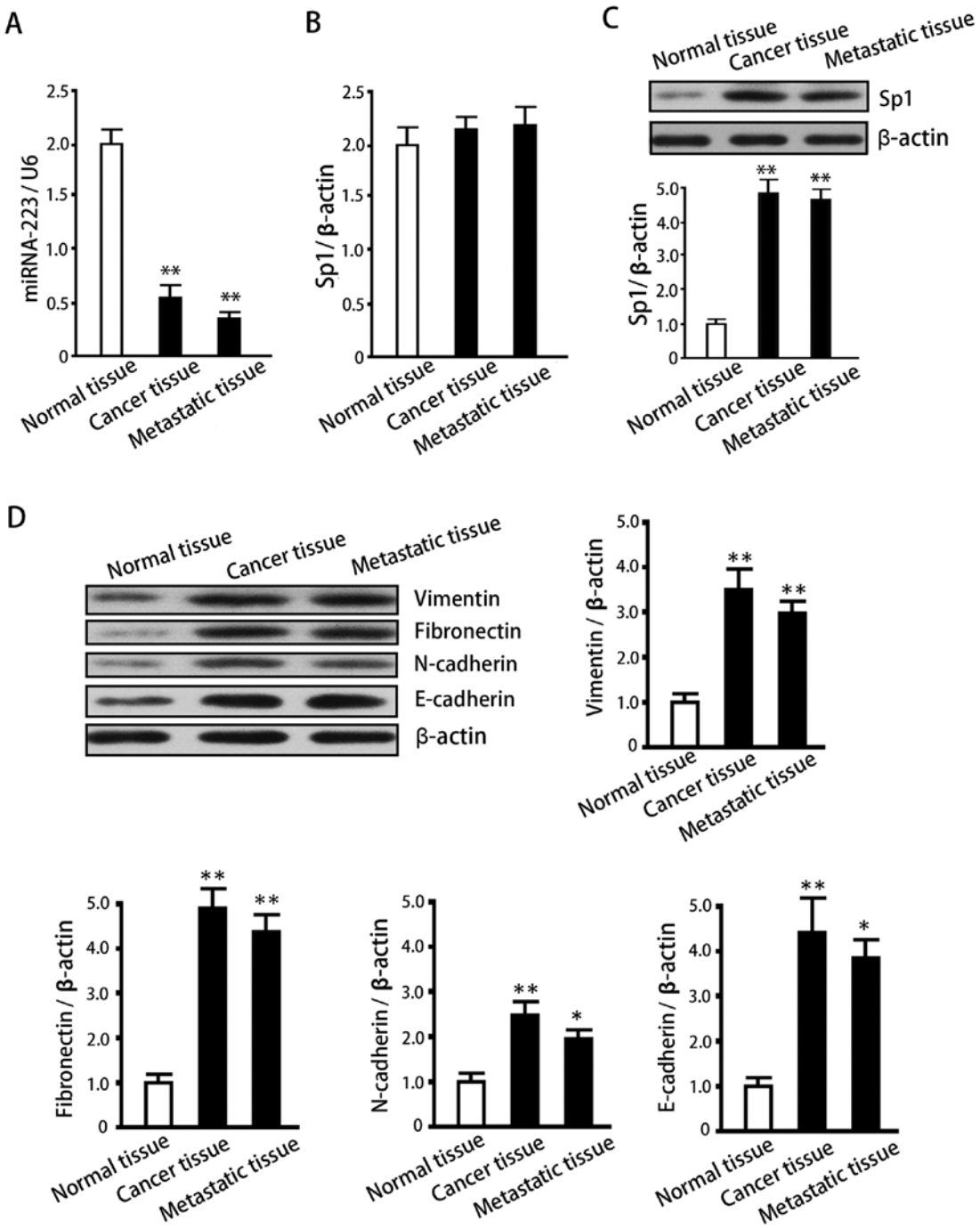

Figure 2. Expression analysis of miRNA-223, Sp1 mRNA, Sp1 protein and EMT-associated proteins in normal adjacent tissue, gastric cancer tissue, and the tissue of liver metastases. (A) miRNA-223 levels in normal adjacent tissue, gastric cancer tissue, and the tissue of liver metastases. (B) Sp1 mRNA expression levels in the same tissues, as measured by quantitative PCR. (C) Sp1 protein levels in normal adjacent tissue, gastric cancer tissue, and the tissue of liver metastases as determined by western blotting. Upper panel, representative western blotting; lower panel, quantification of relative Sp1 protein expression (the intensity of each Sp1 protein band was normalized to that of the corresponding β-actin control and the data then normalized to that of the control tissue group). Data are expressed as means ± SD of at least three independent experiments. (D) Protein levels of Vimentin, Fibronectin, and N-cadherin in normal adjacent tissue, gastric cancer tissue, and the tissue of liver metastases as determined by western blotting. Upper panel, representative western blots; lower panel, quantification of relative protein expression (normalization of data for the comparison of treatments was performed as in (C). Data are expressed as means ± SD of at least three independent experiments. *P<0.05 and **P<0.01, when compared with the normal adjacent group.

ison with normal para-carcinoma tissues, Sp1 protein was elevated in gastric cancer and its metastatic tissues in the liver (P<0.05). However, no significant differences in mRNA levels were observed between the three tissue groups (P>0.05). These results suggest that the high expression of Sp1 is due to an inactivation of post-transcriptional regulation. We also examined the expression of EMT-related proteins in these tissues, and found an increase in Vimentin, Fibronectin, N-cadherin and E-cadherin in gastric cancers and their metastatic tissues when compared with the adjacent tissues (P<0.05; Fig. 2D). These results suggest that EMT is closely associated with the invasion and metastasis of gastric cancer cells and that Sp1

A

Name	Predicted consequential pairing of target region and miRNA
Position 2896-2903 of SP1 3' UTR	5' ...AACAGAAAUUAAUUUAACUGACA...
	|||||||
hsa-miR-223	3' ACCCCAUAAACUGUUUGACUGU

B

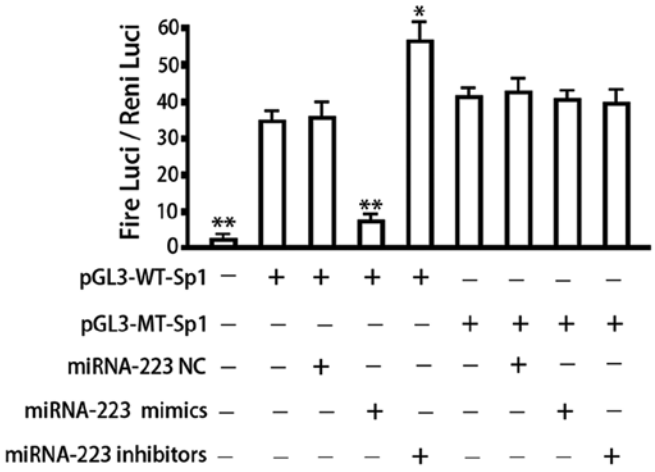

Figure 3. Verification of interaction between miRNA-223 and Sp1. (A) Predicted binding site of miRNA-223 in the 3'-UTR of Sp1 mRNA. (B) Effects of miRNA-223 on the expression of a luciferase cassette encoding the Sp1 3'-UTR. 293 cells were transfected with pGL3-WT or pGL3-MT in the presence or absence of miRNA-223-mimic or miRNA-223-inhibitor. The histogram shows the relative firefly luciferase activity for the different experimental groups. Error bars represent standard deviation and data were obtained from at least three independent experiments. *P<0.05; **P<0.01.

expression positively correlates with EMT and gastric cancer spread.

Prediction and verification of a miRNA-223 binding site within the 3'-UTR of Sp1. The measurements of Sp1 protein and mRNA levels, and the levels of miRNA-223 suggested that miRNA regulation may underlie the increase in Sp1 expression observed in gastric cancers and their metastases. Consequently, to further explore the relationship between Sp1 expression, miRNA regulation and gastric cancer dissemination via the EMT pathway, we next examined whether Sp1 represents one of the target genes of miRNA-223. Our bioinformatics analysis identified a seven-base hsa-miRNA-223 seed sequence in the 3'-UTR of Sp1 mRNA (Fig. 3A). We therefore constructed luciferase reporter vectors to verify whether this site represents a valid miRNA-223 target. Reporter vectors were generated that contained the wild-type Sp1 3'-UTR or a variant in which the miRNA-223 target site within the 3'-UTR had been mutated. Both reporter constructs expressed luciferase at a high level (Fig. 3B). However, the miRNA-223 mimic significantly inhibited luciferase activity in cells transfected with the reporter vector encoding the wild-type 3'-UTR (34.25±3.69 vs. 8.11±1.24; P<0.05), while the miRNA-223 inhibitor significantly increased luciferase activity in these cells (34.25±3.69 vs. 55.71±6.68; P<0.05). Conversely, in cells transfected with the reporter vector encoding the mutated miRNA-223 target site, neither the miRNA-223 mimic nor the miRNA-223-inhibitor had any observable effect on luciferase activity (P>0.05). Co-transfection of miRNA-143-NC (non-targeting control) had no effect on the luciferase activity of either of the vectors (P>0.05). These results verified the presence of a hsa-miRNA-223 target site in the 3'-UTR of Sp1 mRNA and demonstrated that binding of miRNA-223 to this target site downregulates Sp1 expression.

Detection of correlation between miRNA-223 and Sp1 protein in normal cells and gastric cancer cells and effect of over-expression of miRNA-223 via lentiviral approach on Sp1 expression in gastric cancer cells. As compared with normal gastric mucosa cells, MGC-803, BGC-823 and SGC-7901 cells showed lower expression levels of miRNA-223 (P<0.05, vs GES-1) and higher levels of Sp1 protein (P<0.05, vs GES-1), with a significant negative correlation between miRNA-223 and Sp1 (Fig. 4A). The lentiviral system effectively delivered miRNA-223 in MGC-803, BGC-823 and SGC-7901 cells (Fig. 4B) (P<0.01, vs cell control or Lv-NC), which inhibited Sp1 protein in turn (Fig. 4C) (P<0.01, vs cell control or Lv-NC).

Verification of EMT model established by TGF-β1 induction in gastric cancer cells. We measured EMT markers 72 h after incubating MGC-803 cells with 100 ng/ml TGF-β1. The results (Fig. 5A) showed that TGF-β1 significantly increased Vimentin, Fibronectin, cadherin and E-cadherin (P<0.05, vs control), indicating the successful establishment of the EMT model.

Effects of miRNA-223 overexpression on cellular proliferation, apoptosis, and invasion in a gastric cancer model of EMT.

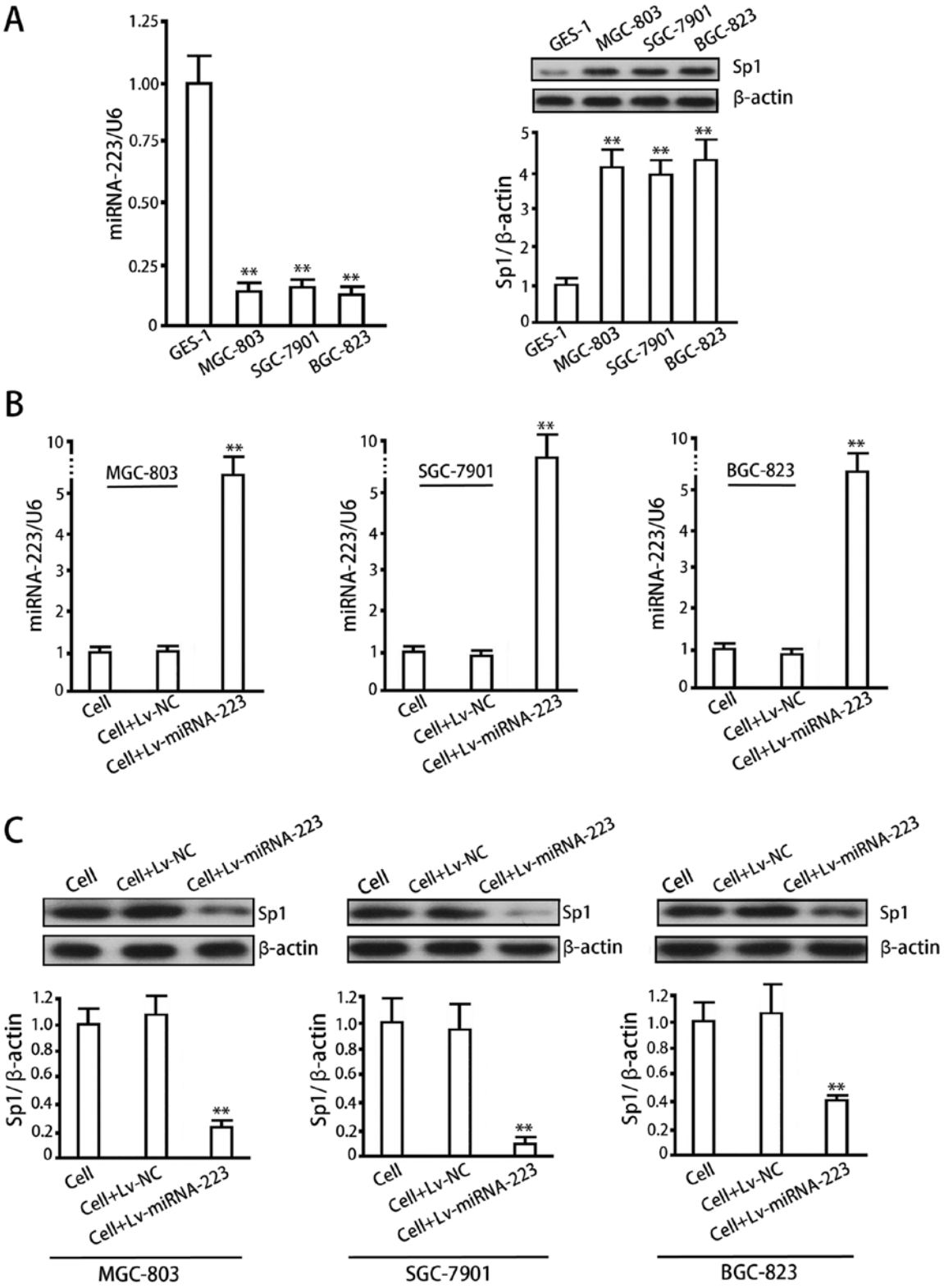

Figure 4. miRNA-223 is negatively correlated to SP1 protein in MGC-803, BGC-823 and SGC-709 cells and expression of miRNA-223 decreases Sp1 protein in these cells. (A) miRNA-223 expression levels and Sp1 protein levels in the indicated cell lines as determined by quantitative PCR or assessed by western blotting; right upper panel, representative western blots; right lower panel, quantification of Sp1 protein expression as determined by densitometric analysis. (B) miRNA-223 levels expressed were evaluated by quantitative PCR in the indicated cell lines infected with or without indicated lentivirus. (C) Protein levels of Sp1 were assessed by western blotting in the indicated cell lines infected with or without indicated lentivirus. Upper panel, quantification of Sp1 protein expression as determined by densitometric analysis; lower panel, representative western blots. The intensity of each Sp1 protein band was normalized to that of the corresponding β-actin control band. Data are expressed as means ± SD of at least three independent experiments. **P<0.01.

TGF-β1 (100 ng) significantly increased vimentin, fibronectin, and N-cadherin protein expression in SGC-7901 gastric cancer cell lines (P<0.05; Fig. 5A), indicating the successful induction of EMT. Overexpression of miRNA-223 significantly

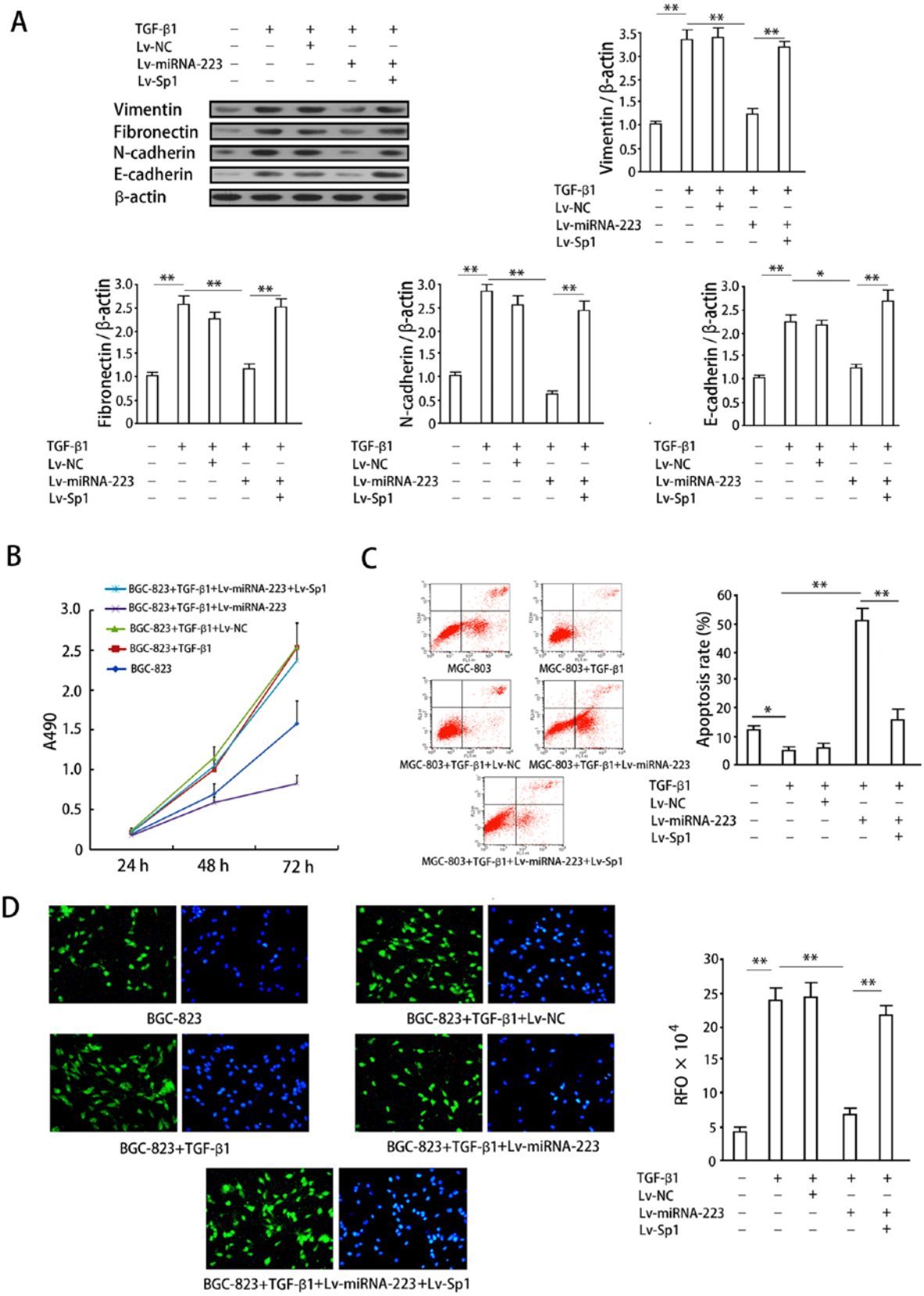

Figure 5. Effects of miRNA-223 overexpression on cellular proliferation, apoptosis, invasion and protein expression in an *in vitro* gastric cancer model of EMT. (A) Vimentin, fibronectin, and N-cadherin protein expression in SGC-7091 cells was determined by western blotting. Upper panel, quantification of protein expression as determined by densitometric analysis. The intensity of each target protein band was normalized to that of the corresponding β-actin control band and the data then normalized to that of the no-treatment control group. Lower panel, representative western blots. (B) Growth curves for BGC-823 cells exposed to the indicated treatments. Data are expressed as means ± SD of at least three independent experiments. (C) Apoptosis in MGC-803 cells. Left panel, representative plots of MGC-803 cell in the presence of the indicated treatments. Right panel, quantification of apoptosis for the indicated treatments. Data represent means ± SD of at least three separate experiments. *P<0.05; **P<0.01. (D) Invasion assay of BGC-823 cells in the presence of the indicated treatments. Epifluorescence images of migrated cells after DAPI staining (left panel) and α-SMA immunofluorescence staining (right panel). Right hand graph, quantification of cell invasion (RFU = relative fluorescence units). Data represent means ± SD of relative fluorescence units from at least three separate experiments, **P<0.01.

impaired the increase in expression of these proteins induced by TGF-β1 (P<0.05, when compared with the induction group). Conversely, co-expression of exogenous Sp1 reversed the inhibition of vimentin, fibronectin, and N-cadherin expression by miRNA-223 (P<0.05).

Stimulation with 100 ng/ml TGF-β1 increased BGC-823 cell proliferation (P<0.05, when compared with the control group), which is an expected response associated with the induction of EMT (Fig. 5B). Overexpression of miRNA-223 significantly inhibited the increase in proliferation induced by TGF-β1 (P<0.05), whereas co-expression of exogenous Sp1 with miRNA-223 reversed this inhibitory effect (P<0.05).

A decrease in apoptosis was also observed in BGC-823 cells following 72-h stimulation with 100 ng/ml TGF-β1 (P<0.05; Fig. 5C), which again is an expected response associated with the induction of EMT. Overexpression of miRNA-223 significantly increased BGC-823 cell apoptosis (P<0.05, when compared with the group treated with TGF-β1 alone). Co-expression of Sp1 with miRNA-223 reversed the increase in apoptosis observed following the overexpression of miRNA-223 alone (P<0.05).

Cell invasion assays were performed in combination with fluorescence imaging to observe and quantify the effects of the TGF-β1-dependent induction of EMT on the invasive properties of gastric cancer cells. Fluorescence imaging enabled the direct observation of cells penetrating through the matrix membrane of the invasion chamber (Fig. 5D, left, DAPI staining; right, α-SMA immunofluorescence). Induction of SGC-7901 cells with 100 ng/ml TGF-β1 resulted in a significant increase in cellular invasion (P<0.05, when compared with the non-induced group), a finding that was in agreement with the decrease in cellular apoptosis observed with the induction of EMT. Overexpression of miRNA-223 significantly inhibited cellular invasion (P<0.05), while co-expression of exogenous Sp1 with miRNA-223 reversed this inhibitory effect (P<0.05). The data obtained from cell counts were in agreement with those obtained using the fluorescent plate reader.

Analysis of specificity of miRNA-223/Sp1/EMT pathway. Based on that miRNA-223 inhibits EMT in gastric cancer cells, we then verified the specificity of miRNA-223/Sp1/EMT pathway. We overexpressed Sp1 protein by using the lentiviral system, which would not be regulated by miRNA-223 due to lack of wild 3'-UTR. The results showed that exogenous expression of Sp1 significantly reversed inhibition of EMT by miRNA-223: exogenous Sp1 revered decrease of EMT markers (Fig. 5A), proliferation inhibition (Fig. 5B), apoptosis (Fig. 5C), and invasion inhibition (Fig. 5D) induced by miRNA-223 in the EMT model. We hence considered that exogenous Sp1 counteracts miRNA-223 in respect of EMT inhibition in gastric cancer cells, i.e., the inhibition of EMT by miRNA-223 specifically mediated by its target gene Sp1.

In summary we have found that TGF-β1 induction decreased miRNA-223 in cancer cells, resulting in an increase in Sp1 protein (a target gene of miRNA-223) and the promotion of EMT. Overexpression of miRNA-223 effectively inhibited EMT-associated processes, and this inhibition could be reversed by the co-expression of exogenous Sp1. Analysis of EMT was performed by observing changes in EMT-associated markers such as the expression of the EMT-related proteins vimentin, fibronectin, and N-cadherin, and behavioral processes including cellular proliferation, apoptosis and invasion.

Discussion

Highly evolutionarily conserved, miRNAs represent endogenous non-coding RNAs of ~20-25 bases in length that regulate gene expression at the post-transcriptional level through binding to the 3'-UTR of target genes (16). miRNAs have been implicated in tumorigenesis and tumor development, and their roles in these processes have been the subject of extensive research (17). Studies show that dysfunction of intrinsic miRNA regulatory mechanisms results in abnormal expression of various oncogenes and tumor suppressor genes in a variety of tumor types (18); therefore, miRNAs may act as both oncogenes and tumor suppressor genes. miRNAs have been implicated in the biology of gastric cancer (19,20): miRNA array analysis has shown that expression of miR-21, miR-191, miR-223, miR-24, miR-107, miR-92 and miR-221 is elevated in gastric cancer (21), while that of miR-128b, miR-129 and miR-148 is decreased (22). Gastric cancer is a disease involving the complex activation and inactivation of multiple genes; miRNAs may therefore assume the role of both oncogene and tumor suppressor in the onset and development of this disease. With progress now being made in our understanding of the relationship between gastric cancer and miRNAs, the search for the target proteins of miRNAs, as well as the molecular mechanisms regulating miRNA expression, remains an important area of study in this field, especially considering the potential implications such research has for the development of gene therapies. miRNAs are also involved in the regulation of EMT: curcumin suppresses EMT in chemoresistant colorectal cancer by upregulating EMT-suppressive miRNAs and mediating chemosensitization to 5-fluorouracil (23). Moreover, miRNA-153 has been shown to be a marker of gastric cancer metastasis associated with EMT (24). The involvement of miRNA-223 in gastric cancer (including EMT associated with this disease) has not been reported previously.

The invasion and metastasis of cancer refers to the dissemination of tumor cells from the primary tumor to discrete target tissues, often of remote organs, where these cells then proliferate into cancers of a similar nature (25). This process depends on the interaction between cancer cells and the local tumor environment which promotes their survival, growth, invasion, and ultimately metastasis (26). EMT, which drives many of these processes, is therefore a major factor contributing to the invasion and metastasis of cancer cells (27-29).

In recent years, an increasing number of studies have shown that EMT is related not only to cancer invasion and metastasis, but also to the acquisition of cancer stem cell characteristics (30,31). Gastric cancer is the second most common cancer in the world. Studies have shown that EMT is closely related to gastric cancer metastasis (including post-operative metastasis). Zhang *et al* reported that Wnt5a, through stimulation of the non-classical Wnt signaling pathway, mediates EMT during the metastasis of cancer cells, and may serve as a target for the inhibition of EMT and gastric cancer metastasis (32). Lu *et al* have shown that miRNA-19a, which is associated with cancer invasion and metastasis and is expressed highly in

gastric cancer cell lines, induces EMT in gastric cancer cells by activating the PI3K/AKT signal pathway (33).

Sp1, a member of the SP/Kruppel-like factor super-family (Sp/KLF family) of transcription factors (34), contains four domains: a DNA binding domain, an Sp1 activation domain, a Btd box and an SP box (4). Sp1 initiates gene transcription by forming a tetramer and binding the gene promoter. Once bound, the tetramer can recruit and promote the binding of additional tetramers to DNA, driving gene transcription via a positive feedback loop. Sp1 is highly expressed in gastric cancer, lung cancer, cervical cancer, breast cancer, and thyroid tumors (35-38). Qiu *et al* have shown that inhibition of Sp1 expression in gastric cancer cells suppresses proliferation, migration and invasion (39). Zhou *et al* demonstrated that high expression of Sp1 could enhance gastric cancer metastasis (12). Previous study by Nam and colleagues on cancer cell EMT has shown that Sp1 plays crucial roles in the integrin α5-dependent induction of EMT and metastasis (8). Given these findings, we sought to determine whether Sp1 is involved in the EMT of gastric cancer, and whether abnormal expression of Sp1 in gastric cancer EMT, invasion and metastasis is regulated in a post-transcriptional manner. We additionally sought to determine whether miRNAs are key players in this regulation.

We found that Sp1 expression is closely associated with gastric cancer metastasis, and that abnormal expression of Sp1 is regulated at a post-transcriptional level. Consequently we explored the possibility that miRNA regulates Sp1 expression. We identified miRNA-223 as a putative regulator of Sp1 using a bioinformatics approach, and verified experimentally that miRNA-223 can negatively regulate Sp1 expression by targeting its 3'-UTR. Sp1 expression negatively correlated with that of miRNA-223. We selected three gastric cancer cell lines and established a model of gastric cell EMT using TGF-β induction. This model was used in conjunction with miRNA-223 overexpression studies to observe possible changes in the expression of EMT-related markers in gastric cancer cells. Our results demonstrated that overexpression of miRNA-223 in BGC823 cells effectively suppressed proliferation. Moreover, high expression of miRNA-223 induced apoptosis and inhibited the invasion of gastric cancer cells. Subsequent analysis of EMT-related proteins verified our conclusion. We also investigated whether miRNA-223 affects EMT through Sp1 by expressing exogenous Sp1 in gastric cancer cells. We found that overexpression of Sp1 impaired the effects of miRNA-223 on EMT-related indicators suggest that there is a specific miRNA-223/Sp1/EMT signaling axis regulating gastric cancer metastasis.

In this study, we found that the direct cause for TGF-β1 to induce EMT in gastric cancer cells is that it further reduces miRNA-223 expression. It is worth additional study to clarify how TGF-β1 inhibits miRNA-223, which will be our future direction. A reasonable hypothesis is that TGF-β1 may change some transcription factors responsible for miRNA-223 regulation. We, therefore, are screening differentially expressed transcription factors in gastric cancer and EMT model focusing on sequence analysis of the promotor region of miRNA-223, which has made some preliminary progress.

Our study demonstrates that low expression of miRNA-223 in gastric cancer cells gives rise to high expression of Sp1, and consequently the invasion and metastasis of cancer cells through EMT. Overexpression of miRNA-223 in gastric cancer cells inhibits expression of Sp1 protein and the invasion and metastasis of tumor cells via EMT. These findings provide new possibilities for gene therapy strategies designed as adjuvant postoperative treatments for gastric cancer and for the development of drugs targeting new genes.

Acknowledgements

This study was supported by Heilongjiang Province Science Funds for Distinguished Young Scientists (JC2015019, H2015079), National Science Foundation of China (81372293, 81273161), Heilongjiang Province Graduate Innovation and Creativity Funds (YJSCX2011-321HLJ), New Century Excellent Talents in Heilongjiang Province University (1254-NECT-023).

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