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Establishment and characterization of a GES-1 human gastric epithelial cell line stably expressing miR-23a

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Abstract. MicroRNAs (miRNAs/miRs) are highly conserved, endogenous, small and single-stranded RNA molecules that promote the degradation and translational inhibition of specific target mRNAs in order to regulate cell proliferation and differentiation, and organism growth and development. MiR-23a has been demonstrated to function as an oncogene in certain types of tumor. The aim of the present study was to provide a tool for elucidating the mechanisms of action of miR-23a in gastric cancer, and identify the function of miR-23a in a human gastric epithelium cell line, by establishing a human gastric epithelial GES-1 cell line that stably expressed miR-23a. A plasmid was constructed for the expression of miR-23a by inserting the miR-23a primary sequence into a pcDNA3 vector (pcDNA3/pri-23a). PcDNA3/pri-23a or the empty pcDNA3 vector (EV), which was then transfected into human gastric epithelium GES-1 cells using Lipofectamine to produce GES-1/miR-23a cells and GES-1/EV cells, respectively. G418 (Geneticin) was used to select and expand the G418-resistant colonies, and miR-23a expression was assessed by reverse transcription-semi-quantitative polymerase chain reaction. The proliferation of the cells was assessed using cell counting and MTT assays. The invasive ability of the cells was evaluated using a Transwell assay. The colony-forming ability of the cells was assessed using a colony formation assay. A human gastric epithelium GES-1/miR-23a cell line with the stable expression of miR-23a was successfully established. Compared with the control GES-1 and GES-1/EV cells, the mRNA expression of the miR-23a gene in GES-1/miR-23a cells was significantly increased ($P<0.05$). The proliferation rate, invasive ability and colony-forming ability of the GES-1/miR-23a cells were significantly higher compared with those of the control GES-1/EV cells and the parental GES-1 cells ($P<0.05$). Additionally, the results of the present study demonstrated that miR-23a enhanced the cell proliferation rate, invasive ability and cell colony forming ability of GES-1 cells. This data provides a solid experimental foundation for further studies on the function of miRNAs in the development and progression of gastric cancer.

Introduction

MicroRNAs (miRNAs/miRs) are a class of endogenous, small and single-stranded RNA molecules, ~22 nucleotides in length and highly conserved, encoded by the genome (1,2). MiRNAs promote the degradation and translational inhibition of specific target mRNAs in order to regulate various processes, including cell proliferation and differentiation, and organism growth and development (3-5). MiRNAs have been identified as a mode of gene expression regulation in tumor development via the modulation of oncogenes or tumor suppressor gene expression levels (6-9). Certain studies have identified that miRNAs are expressed at abnormal levels in a variety of cancer tissues, and similar to the role of proto-oncogenes or tumor suppressor genes, aberrantly expressed miRNAs may affect tumor proliferation and apoptosis (10,11).

Gastric cancer is the fourth leading type of cancer worldwide, and is endemic in certain Asian countries, including China, Japan, Iran and India (12). In particular, gastric cancer

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has a high incidence in China, where the morbidity and mortality rates are approximately twice the world average (13). The abnormal expression of a number of miRNAs, including miR-23a and miR-27a, has already been identified in gastric cancer; these miRNAs may function as oncogenes, based on their upregulated expression in gastric cancer (14,15). Consequently, the mechanism of action of miRNAs in gastric cancer has been a focus for research in recent years. In our previous study, it was identified that miR-27a functions as an oncogene in gastric adenocarcinoma by targeting prohibitin (15).

In the present study, in order to further clarify the role of miR-23a in gastric cancer, gene recombination technology was used to construct a eukaryotic expression vector, pcDNA3/pri-23a, for miR-23a. This vector was transfected into the human gastric epithelial GES-1 cell line followed by continuous clonal selection with G418 (Geneticin). The expression of miR-23a was assessed by reverse transcription-semi-quantitative polymerase chain reaction (RT-sqPCR) until a human gastric epithelial monoclonal cell line was obtained, and this cell line stably expressed miR-23a. Furthermore the expression of miR-23a in gastric adenocarcinoma and normal gastric tissues was considered, and whether miR-23a promotes the growth and invasion of the human gastric epithelium GES-1 cell line was assessed.

Materials and methods

Human gastric cancer tissue samples. In September 2004, four pairs of human gastric cancer tissue samples (males; 26, 45, 46 and 62 years old) and adjacent non-cancerous tissue samples were obtained from the Tumor Bank Facility of Tianjin Medical University Cancer Institute and Hospital and the National Foundation of Cancer Research (Tianjin, China). All samples were confirmed by pathological analysis. The study was performed with the written informed consent of all participants, was approved by the Tianjin Ethics Committee, and was in accordance with the ethical standards of the Declaration of Helsinki and its later amendments.

The miRNA profiles of the tissue samples and matched normal gastric tissue samples were determined using an oligonucleotide microarray. Carcinoma tissue RNAs were labeled with cyanine 5 (Cy5) and normal tissue RNAs were labeled with cyanine 3 (Cy3). The labeled samples were hybridized in a microarray (Version 3.0.0.0016; PerkinElmer, Inc., Waltham, MA, USA) containing 243 *Homo sapiens* miRNA probes. The ScanArray™ Express Microarray scanner (PerkinElmer, Inc.) to scan the hybridized microarray, and the figures were processed, normalized and analyzed using the ScanArray® Express Microarray Analysis system. The data were analyzed using the ScanArray® Express Microarray Analysis System, and the Cy5/Cy3 value was calculated (16), and the value of miR-23a in every pair of samples was calculated.

Materials and reagents. The normal human gastric epithelial GES-1 cell line and 293 cells were purchased from the Beijing Institute of Cancer Research (Beijing, China). GES-1 cells were maintained in RPMI-1640 with 10% fetal bovine serum at 37˚C in a humidified chamber supplemented with 5% CO_2. The pcDNA3 plasmid was provided by Professor Kenzo Takada (Japan Tumor Virology Department, Institute of Genetic Medicine, Hokkaido University School of Medicine, Sapporo). Lipofectamine™ 2000, Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 medium were purchased from Thermo Fisher Scientific, Inc. (Gibco; Waltham, MA, USA). Fetal bovine serum was purchased from Tianjin Saierbio (Tianjin, China). The restriction enzymes, T4 DNA ligase, PCR kit, plasmid mini preparation kit and gel extraction purification kit were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). G418 (Geneticin) reagents and primers were synthesized by Shanghai Biology Engineering Technology Service Ltd. (Shanghai, China).

Construction of plasmid pcDNA3/pri-23a. The miR-23a precursor (pri-23a) sequence, which refers to its coding site in the human genome, was identified in the Rfam database (17). Simultaneously, the gene sequence for pri-23a was retrieved from the GenBank database provided by National Center for Biotechnology Information (18). Primer Premier 5.0 software (version 5.0; Premier Biosoft International, Palo Alto, CA, USA) was used to analyze these pri-23a sequences from the Rfam and GenBank databases, and to design specific PCR primers to amplify the miR-23a sequence. Specific PCR primers to amplify the miR-23a sequence and to introduce restriction sites for cloning were designed using Primer Premier5 and were as follows: Upstream of pri-23a-*Bgl* II, the forward sequence was, 5'-CTCATATGCAGGAGCCAGATC TCGC-3' and the reverse sequence was, 5'-GCGAGATCT GGCTCCTGCATATGAG-3'; downstream of pri-23a-*Eco*RI, the forward sequence was, 5'-CCGAAGCCTGTGCCTGAA TTCATC-3' and the reverse sequence was, 5'-GATGAATTC AGGCACAGGCTTCGG-3'. The *Bgl*II and *Eco*RI restriction enzyme sites were introduced into the upstream and downstream primers, respectively. HEK293 cell genomic DNA was used as a template for PCR reactions and purification. The PCR products and pcDNA3 were digested with restriction endonuclease, the fragments were recovered and they were ligated with T4 ligase. These connecting fragments were transformed into competent XL1-blue bacteria and white, medium-sized colonies were selected on ampicillin plates. The selected colonies were cultured overnight with agitation in lysogeny broth medium (Takara Biotechnology Co., Ltd.) us ampicillin, and plasmids were extracted. The recombinant plasmid, pcDNA3/pri-23a, was verified by restriction enzyme digestion with 1% gel electrophoresis and ethidium bromide staining, followed by gene sequencing.

Cell transfection and screening of stable GES-1 cell derivatives. GES-1 cells in the logarithmic phase were harvested 1 day prior to transfection and were seeded at a density of $1x10^6$ cells/ml onto 24-well plates with DMEM, prior to being incubated in a humidified incubator supplemented with 5% CO_2 at 37˚C. When the cells reached 70% confluence, they were transfected with Lipofectamine 2000 according to the manufacturer's protocol. The cells were seeded onto 96-well plates following transfection for 48 h. G418, also known as Geneticin, is a 2-deoxystreptamine antibiotic from *Micromonospora echinospora*, which inhibits protein synthesis by preventing the elongation step in prokaryotic and eukaryotic cells. Complete medium (Takara Biotechnology

Co., Ltd.) with 300 g/ml G418 was used to select clones for between 20 to 25 days. The monoclonal culture was selected, the cultivation was further expanded, and the stable GES-1/miR-23a (transfection group) and GES-1/EV (empty vector group) cell lines were established.

Detection of miR-23a expression by RT-sqPCR. Total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), reverse transcribed to cDNA using a QuantiTect Reverse Transcription kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, and amplified with the PTC 200 machine (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The cDNA was amplified using the following PCR primers: Pri-23a forward, 5'-GCGAGATCTGGCTCCTGC ATATGAG-3' and reverse, 5'-GATGAATTCCAGGCACAG GCTTCGG-3'; U6 forward, 5'-TGCGGGTGCTCGCTTCGG CAGC-3' and reverse, 5'-CCAGTGCAGGGTCCGAGGT-3'. The length of the amplification product was 324 bp. The reaction conditions of PCR were as follows: 94˚C for 4 min, followed by 33 cycles of 94˚C for 30 sec, 42˚C for 1 min and 72˚C for 30 sec. The PCR products were detected by 3% agarose gel electrophoresis and the images were assessed using Quantity One software version 4.6.2 (Bio-Rad Laboratories, Inc.). The ratio of intensity of the target band versus the internal reference, U6 rRNA, was used to measure the levels of miR-23a expression.

Cell counting assay. GES-1/miR-23a, GES-1/EV and GES-1 cells in the logarithmic growth phase were seeded onto 48-well plates at a density of $1x10^3$ cells/well. Three wells were selected each day for 7 days to count the total number of cells, and the means were calculated for each group in order to generate growth curves.

MTT assay. GES-1/miR-23a, GES-1/EV and GES-1 cells in the logarithmic growth phase were seeded onto 96-well plates at a density of $1x10^3$ cells/well and cultured in a humidified incubator with 5% CO_2 at 37˚C. Following incubation for 24, 48 or 72 h, the culture plates were incubated with 20 μl MTT/well, with a final concentration of 5 mg/ml for a further 4 h. The culture plates were centrifuged at 2,000 x g for 5 min at room temperature, the supernatant was carefully removed, and 100 μl DMSO was added to each well to end the reaction. Blue-violet formazan particles were dissolved for ~10 min, in the dark at 25˚C. The absorbance at 570 nm was detected using a μQuant Universal microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). The experiment was repeated three times in the same conditions.

Transwell invasion assay. At 48 h after transfection, GES-1/miR-23a, GES-1/EV and GES-1 cells were seeded at a density of $1x10^5$ cells/ml onto 24-well plates. RPMI-1640 medium supplemented with 5% fetal bovine serum and without 100 IU/ml penicillin and 100 mg/ml streptomycin was placed into the upper chamber of an insert coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 mg/ml streptomycin was placed into the lower chamber. The cells were incubated in a humidified incubator supplemented with 5% CO_2 at 37˚C for 5 h. Cells that invaded through the membrane were fixed with 75% methanol and 25% glacial acetic acid at 37˚C for 30 min, and were then stained with 0.1% crystal violet at 37˚C for 5 min. The cells were imaged and counted in four random fields/well using an inverted microscope at x100 magnification (Olympus Corporation, Tokyo, Japan). The experiment was repeated independently three times in the same conditions.

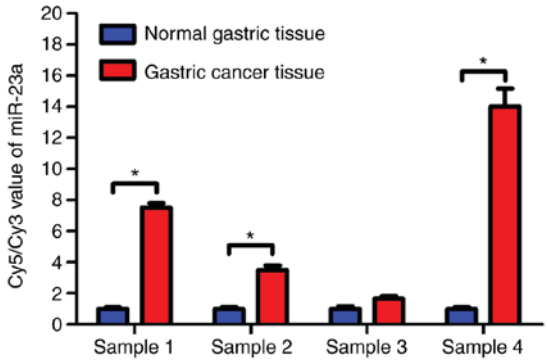

Figure 1. miR-23a is overexpressed in human gastric cancer tissues, as determined by oligonucleotide microarray analysis. *P<0.05 vs. normal gastric tissue. miR, microRNA; Cy5, cyanine 5; Cy3, cyanine 3.

Colony formation assay. GES-1/miR-23a, GES-1/EV and GES-1 cells were evaluated using colony formation analysis. Cells were harvested at 3,000 x g at 37˚C for 10 min and seeded onto 12-well plates at a density of $1x10^2$ cells/well. The plates were incubated in a humidified incubator supplemented with 5% CO_2 at 37˚C for 2 weeks. During colony growth, the culture medium was replaced every 3 days. The culture medium was removed and the cells were stained with 0.1% crystal violet at 37˚C for 5 min at the end of the 2 weeks. The colonies were counted using an inverted microscope at x100 magnification. Each assay was performed in triplicate.

Statistical analysis. SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) were used for statistical analysis. All data are expressed as the mean ± standard deviation. One-way analysis of variance followed by a Student Newman-Keuls post hoc test was used to compare the data measurement groups. A two-tailed P<0.05 was considered to indicate a statistically significant difference.

Results

miR-23a is overexpressed in gastric cancer. Based on the oligonucleotide microarray analysis of four pairs of cancer and normal gastric tissues, miR-23a was overexpressed in the gastric cancer tissues (Fig. 1). Compared with the normal gastric tissue samples, miR-23a was significantly upregulated in gastric cancer tissues (P<0.05). These results indicated that miR-23a may promote gastric cancer development and influence the gene regulation of gastric cancer cells.

Preparation of the miR-23a expression plasmid. A plasmid expressing miR-23a was prepared by PCR amplification of the miR-23a sequence and cloning into pcDNA3 to yield

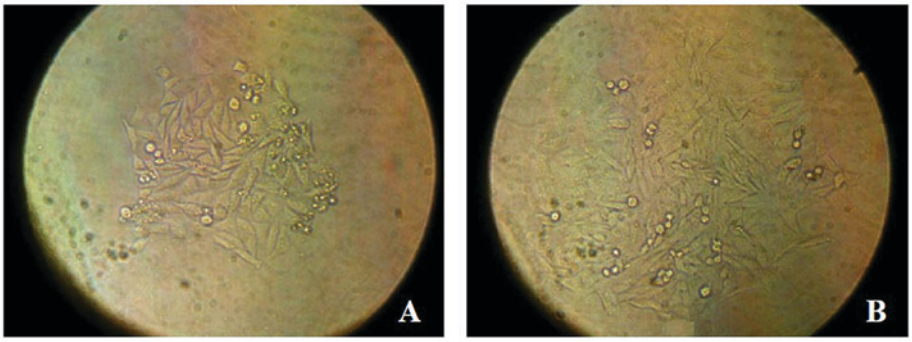

Figure 2. Clonal cell populations isolated following plasmid transfection, dilution and selection. (A) GES-1/EV was isolated following transfection with the pcDNA3 EV; (B) GES-1/miR-23a was isolated following transfection with pcDNA3/miR-23a. Magnification, x100. EV, empty vector; miR, microRNA.

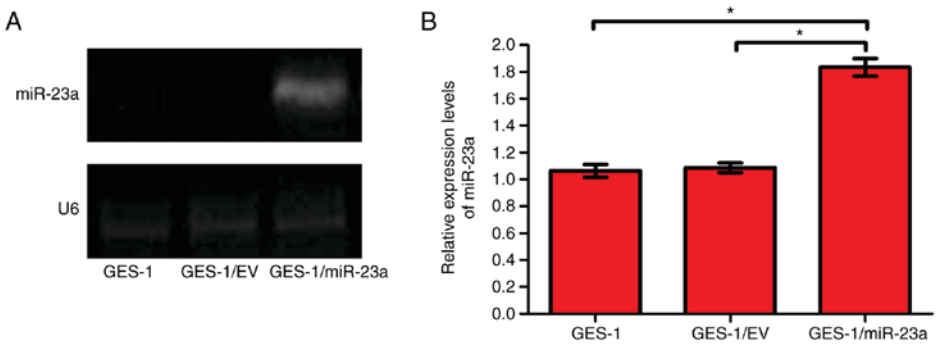

Figure 3. Expression of miR-23a in GES-1/miR-23a cells. (A) Gel electrophoresis following reverse transcription-polymerase chain reaction with miR-23a-specific primers. U6 rRNA was amplified as an internal control. (B) Relative expression levels of miR-23a in GES-1/miR-23a cells. *P<0.05 vs. control group (include GES-1 cell and GES-1/EV cell). EV, empty vector; miR, microRNA.

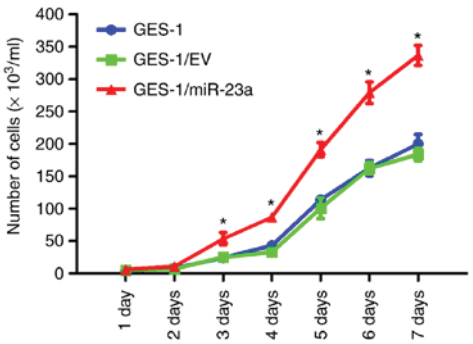

Figure 4. Effects of miR-23a on the proliferation of GES-1 cells. Cells were counted each day for 7 days to generate growth curves. Results are representative of three independent experiments. *P<0.05 vs. control groups (includes GES-1 cell and GES-1/EV cell). EV, empty vector; miR, microRNA.

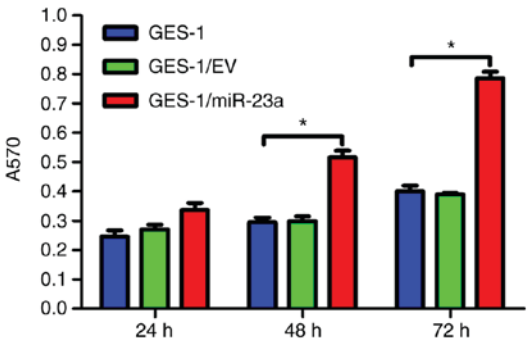

Figure 5. Effect of miR-23a on the viability of GES-1 cells. Mean A_{570} readings from the MTT assay are presented as an indicator of cellular viability. Results are representative of three independent experiments. *P<0.05 vs. control groups (includes GES-1 cell and GES-1/EV cell). EV, empty vector; miR, microRNA.

recombinant plasmid pcDNA3/pri-23a. The recombinant plasmid pcDNA3/pri-23a and the empty vector were identified by restriction endonuclease *Bgl*II and *Eco*RI. For the pcDNA3/pri-23a plasmid, a pcDNA vector fragment of 5,337 bp and a miR-23a precursor fragment of 324 bp was obtained. For the empty vector, only a vector fragment of 5,337 bp was obtained. The presence of the 324 bp miR-23a precursor gene fragment demonstrated that the recombinant pcDNA3/pri-23a plasmid was successfully constructed.

Selection of stable GES-1 cells expressing miR-23a. To prepare stable control and miR-23a expressing cell lines, the pcDNA3 and pcDNA3/pri-23a plasmids were transfected into GES-1 cells. Following selection with G418, stable clones with the empty pcDNA3 vector (GES-1/EV cells) and pcDNA3/pri-23a (GES-1/miR-23a cells) were selected (Fig. 2). The expression of miR-23a in GES-1, GES-1/EV and GES-1/miR-23a cells was measured by RT-sqPCR. The gel electrophoresis image and quantification demonstrated that miR-23a was expressed in GES-1/miR-23a cells, but not at observable levels in parental GES-1 cells or the GES-1/EV control (Fig. 3A). The relative expression level of miR-23a in GES-1/miR-23a cell was significantly higher compared

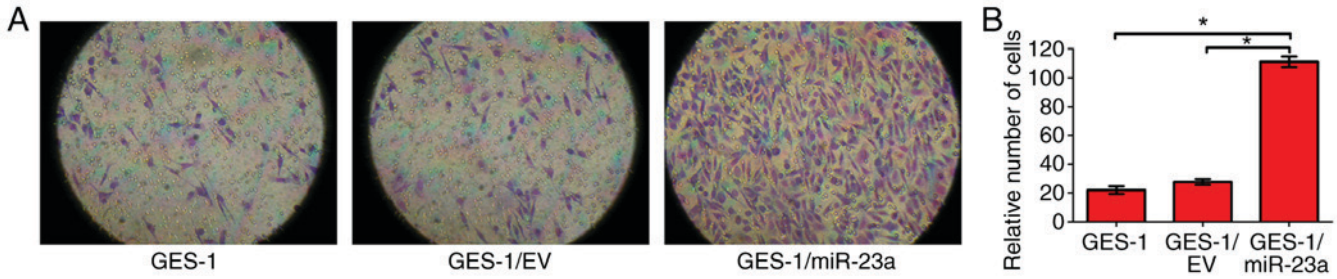

Figure 6. Effects of miR-23a on the invasive ability of GES-1 cells. (A) Representative images of cell invasive ability (magnification, x100); (B) quantification and statistical analysis of cell invasive ability. *P<0.05 vs. the control group (include GES-1 cell and GES-1/EV cell). EV, empty vector; miR, microRNA.

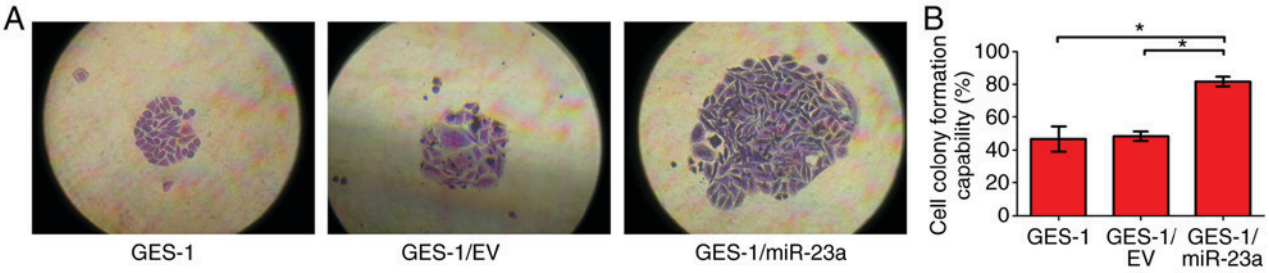

Figure 7. Effects of miR-23a on the colony forming ability of GES-1 cells. (A) Representative images of the colony-forming ability (magnification, x100); (B) quantification and statistical analysis of the colony-forming ability. *P<0.05 vs. the control group (include GES-1 cell and GES-1/EV cell). EV, empty vector; miR, microRNA.

with that in the GES-1and GES-1/EV cells (P<0.05; Fig. 3B). This indicated that the GES-1/miR-23a cell line effectively expressed miR-23a.

miR-23a expression promotes cell proliferation and viability. To determine whether miR-23a expression affects the proliferation of GES-1 cells, cell counting and MTT assays were performed. Cell counting demonstrated that GES-1/miR-23a cells proliferated significantly faster compared with cells in the control groups (P<0.05) (Fig. 4). Furthermore, compared with the control groups, the viability of GES-1/miR-23a cells was significantly increased at 48 and 72 h (P<0.05), as determined with an MTT assay (Fig. 5).

miR-23a expression promotes cell invasion. To identify whether miR-23a expression affects the invasive ability of GES-1 cells, a Transwell invasion assay was performed. The results indicated that the invasive ability of GES-1/miR-23a cells was significantly enhanced compared with that of the control groups (P<0.05; Fig. 6). It was concluded that miR-23a expression enhanced the invasive ability of GES-1 cells.

miR-23a expression promotes colony-forming ability. A colony formation assay was used to detect the effect of miR-23a on the colony-forming ability of GES-1 cells. After growth for 2 weeks, the cell clones were counted. The results revealed that the colony forming capability of GES-1/miR-23a was significantly enhanced compared with that of the control groups (P<0.05; Fig. 7). It was concluded that miR-23a enhanced the colony forming ability of GES-1 cells.

Discussion

miRNAs are a novel class of endogenous and non-coding small RNAs, which function predominantly as sequence-targeted modifiers of gene expression through translational repression (19-21), and were first described as a regulator of gene expression in *Caenorhabditis elegans* (22,23). It has since been identified that miRNAs are prevalent among plants and animals, including in humans. Increasing evidence has demonstrated that the alteration of miRNA expression profiles is associated with several human diseases, including diabetes (24), liver disease (25), inflammation (26), and cardiac development and pathologies (27). miRNAs serve an essential role in various biological and pathological processes, including cell proliferation, stem cell differentiation, tumorigenesis, neuronal development, apoptosis and carcinogenesis (28,29). These studies suggest that miRNAs serve an important role during the growth and development of an organism.

Emerging studies have shown that miRNA regulation is associated with the development of a number of types of malignant tumor. Therefore, an increasing focus of research is the screening of tumor-associated miRNAs, the identification of miRNA target genes and the mechanism of their regulation. The understanding of the role of miRNAs in gastric cancer is an emerging area of research. miR-34 may be involved in the negative regulation of p53 in gastric cancer (30). miR-125a-5p is an independent positive prognostic factor in gastric cancer and inhibits proliferation *in vitro* (31). miRNA-27a may regulate the growth of gastric cancer cells by functioning as an oncogene through its effects on prohibitin (15). miR-650 promotes the proliferation and growth of gastric cancer cells (32). miR-663 contributes to hyperplasia, leading to

the development of gastric cancer (33). Furthermore, miR-9, miR-16 and miR-21 may regulate the growth of gastric cancer cells in human gastric cancer pathogenesis (34,35). miR-29 inhibits the proliferation, migration and invasion of gastric cancer cells by targeting cell division cycle 42 (36). In addition, miR-544 is an essential regulator of cell cycle control in gastric cancer (37). miR-223 appears to regulate apoptosis, proliferation and invasion in gastric cancer (38). In addition, miR-200b regulates zinc finger E-box binding homeobox 2 expression and metastasis in gastric cancer (39). The trio of miR-23a/27a/24 has growth-promoting and anti-apoptotic roles (40).

The miRNA expression differences were analyzed in four pairs of gastric cancer tissue samples and matched adjacent normal tissue samples to identify candidate miRNAs that may be associated with gastric cancer. Previous studies have demonstrated that miR-23a was significantly upregulated in gastric cancer tissues (14,40). In the present study, of the differentially expressed miRNAs identified, miR-23a exhibited the highest upregulation fold in gastric cancer tissues compared with normal tissues, and thus, we hypothesized that it may have a significant role. In our previous study, it was identified that the genes coding miR-23a and miR-27a were located in the same cluster, and that miR-27a acted as an oncogenic miRNA in gastric cancer cells through targeting prohibitin (14). Hence GES-1, a normal human gastric epithelial cell line, was selected for determining the role of miR-23a, using the pcDNA3/pri-23a plasmid to introduce exogenous expression.

The stable expression of miR-23a in GES-1 cells was confirmed following cloning and selection. The primary transcript (pri-23a), which was overexpressed in GES-1/miR-23a cells, was processed in the cell to obtain mature miR-23a, thereby facilitating its ability to serve a negative regulatory role in the expression of target genes. Using cell counting and MTT methods, it was identified that the overexpression of miR-23a significantly increased the proliferation rate and viability of gastric epithelial cells. In addition, the results of the Transwell assay demonstrated that the invasive ability of GES-1/miR-23a cells was significantly enhanced compared with the controls. Furthermore, a colony formation assay was used to detect the effect of miR-23a on the colony-forming ability of GES-1 cells. The colony-forming ability of GES-1/miR-23a was significantly increased compared with the controls. Based on these observations, the present study demonstrated that miR-23a may serve a role as an oncogene in gastric carcinogenesis.

With further research, additional tumor-associated miRNAs and target genes, as well as downstream regulatory pathways, will be identified. These studies will facilitate the further understanding of the specific mechanisms associating miRNAs and tumor formation. In addition, the establishment of miRNA-expressing cell lines will assist in the discovery of novel methods for the prevention and treatment of malignant tumors.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

LC designed the experiments and edited the manuscript; YG conducted the statistical analyses; LHZ and HS conceived and designed the experiments; LLZ and AL performed the experiments; GZ and GS collected the data and provided the technical assistance. All authors have read and approved the manuscript.

Ethics approval and consent to participate

The study was performed following written informed consent from all participants; the study was approved by the Tianjin Ethics Committee in accordance with the ethical standards of the Declaration of Helsinki and its later amendments.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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