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Lentiviral-mediated miRNA against liver-intestine cadherin suppresses tumor growth and invasiveness of human gastric cancer

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Liver-intestine cadherin (CDH17) represents a novel type of cadherin within the cadherin superfamily, and is distinguished from other cadherins by its distinct structural and functional features. Our previous studies had identified that increased CDH17 was significantly associated with tumor differentiation and lymph node metastasis in gastric cancer. In this study, we tested the hypothesis that CDH17 was associated with proliferation and invasiveness in gastric cancer using recombinant lentivirus-mediated miRNA targeting to CDH17 both *in vitro* and *in vivo*. We also detected the activity of matrix metalloproteinase (MMP)-2 and MMP-9 with gelatin zymography to explore the mechanisms underlying the inhibition of the *CDH17* gene. Our results showed that a well-differentiated gastric cancer cell line had higher CDH17 expression. Down-regulation of CDH17 inhibited proliferation, adherence, and invasion of the poorly differentiated BGC823 gastric cancer cells *in vitro*, and induced cell cycle arrest. The activities of MMP-2 and MMP-9 were lower in the CDH17-miRNA-transfected cells compared to the control cells. Using an *in vivo* tumor growth assay, we confirmed that CDH17 silencing could obviously slow the growth of gastric cancer derived from BGC823 cells. Taken together, we have demonstrated that CDH17 maybe a positive regulator for proliferative, adhesive, and invasive behaviors of gastric cancer. (*Cancer Sci* 2010; 101: 1807–1812)

Gastric cancer (GC) is one of the most prevalent and lethal malignancies worldwide owing to the difficulty of early detection and high postsurgical recurrence rate.[1] Almost two-thirds of the cases occur in developing countries, with 42% in China alone.[2] Patients afflicted with GC are often asymptomatic, and the lack of sensitive and reliable biomarkers for early detection of GC means that diagnosis normally occurs late, when surgical intervention is not an option. Today, there are no effective drugs for curing gastric cancer.

Using integrative genomic and proteomic approaches, researchers have begun to identify novel oncogenes and tumor suppressors in GC. Previous studies using clinical cohorts identified a cell surface adhesion molecule, cadherin-17 (CDH17), also known as liver-intestine cadherin, as a potential disease marker for GC.[3–5] In contrast to the conserved cytoplasmic domain of classical cadherins such as E-, P-, and N-cadherin with a length of 150–160 amino acid residues, that of the novel cadherin has only 18 amino acids.[6,7] The independence of its adhesive function from cytoskeletal anchorage clearly distinguishes CDH17 from E-cadherin and other classical cadherins.

In humans, CDH17 is expressed exclusively in the intestinal epithelial cells and a fraction of pancreatic ductal epithelial cells.[8] It is not found in the healthy adult liver and stomach,[6,9–12] while altered CDH17 has been reported in colorectal, liver, and gastric cancer,[5,6,9,13] which means that CDH17 may play an important role in tumor progression. In our earlier studies, we identified that the expression of CDH17 was significantly higher in chronic atrophic gastritis with intestinal metaplasia than in gastric cancer and was lower in poorly differentiated tumors than in well and moderately differentiated tumors.[4] Furthermore, we demonstrated a significant inverse correlation between expression levels of CDH17 and Galectin-3 and suggesting CDH17 as an independent factor associated with lymph node metastasis.[5] However there has been some inconsistency in previous research, as some researchers found that CDH17 was positively correlated to T and N stages; while others have reported that CDH17 was negatively correlated to T and N stages. So it is still uncertain whether CDH17 is a negative or positive regulator for cancer progression. In order to explore the role of CDH17 in GC, we analyzed the effects of stable CDH17 silencing with recombinant lentivirus-mediated miRNA both *in vitro* and *in vivo*.

Materials and Methods

Cell lines and animals. Four gastric cancer cell lines MKN28 (well differentiated), SGC7901 (moderately differentiated), BGC823 (poorly differentiated), and HGC27 (undifferentiated) were purchased from the Cell Bank of Shanghai Institute for Biological Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and incubated in a humidified (37°C, 5% CO_2) incubator. The 293FT cell line (Invitrogen, Carlsbad, CA, USA) was cultured in complete medium containing geneticin according to the manufacturer's instructions.

A total of 18 BALB/c-nu mice, 5–6 weeks old and 18 g in weight, provided by the Central Laboratory of Animal Science at Wuhan University (Wuhan, China), were bred in laminar-flow cabinets and kept at a constant humidity and temperature (25–28°C) according to standard guidelines under an approved protocol of Wuhan University.

Construction and transfection of lentiviral vectors with specific miRNAs for CDH17. Four precursor miRNA sequences (SR1-4) targeting to CDH17 (GenBank accession number NM_004063) and its mismatch mutants (SRneg) as negative control were designed using an Internet application system (Invitrogen). These double-stranded oligonucleotides were inserted into pcDNA 6.2-GW/EmGFP-miR expression vector (Invitrogen) and then transiently transfected into BGC823 cells according to the Lipofectamine 2000 operating manual (Invitrogen). Real-time PCR and western blot analysis showed that the SR4 yielded

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the best suppression efficiency (data not shown); therefore, this construct was chosen to package the recombinant lentiviral vector for CDH17 RNAi with pDONR 221 vector and pLenti6/V5-DEST (Invitrogen) using Gateway Technology (Invitrogen). The new miRNA expression vector (pLenti6/V5-GW/Em GFP-miR) was then transfected into 293FT to assemble the lentiviral stocks named lenti-CDH17. The lentivirus was used to transduce BGC823 cells and the stable transfectants named lenti-CDH17-miR-B were selected using blasticidin for 2 weeks. Flow cytometry was performed to detect transfection efficiency. Two controls were included; BGC823 cells received no treatment or an RNAi vector with a mismatch sequence (Lenti-CDH17-miR-neg, Mock).

Immunofluorescence staining. Approximately 10 000 cells cultured on coverslips were fixed by 4% paraformaldehyde for 5 min and permeabilized with 0.1% Triton X-100 for 15 min at room temperature. Cells were incubated with monoclonal antibody against CDH17 (1:200; Sigma, St. Louis, MO, USA) for 1 h at room temperature. After three washes with PBS, the cells were incubated with rhodamine phalloidin-conjugated secondary antibody, counterstained with DAPI (diamidino phenylindole) and then examined by fluorescence microscopy.

Real-time polymerase chain reaction. We extracted RNA using Trizol (Invitrogen), and generated single-stranded cDNA with the cDNA synthesis kit (Takara, Kyoto, Japan) according to standard protocols. We performed real-time PCR with the Applied Biosystems SYBR Green master mix kit using a Roter-Gene 3000 thermal cycling instrument (Corbett Life Science, Sydney, Australia). Human β-actin gene was amplified as endogenous control. The PCR conditions consisted of 40 cycles, with 5 s denaturation at 95°C, 30 s annealing at 60°C, and 60 s primer extension at 72°C. The primes used were: CDH17 forward primer 5′-GGACAGAGAAGCCGGAAGTC-3′, CDH17 reverse primer 5′-GAACAAGCCCGTGTAGTCCTT-3′ (GenBank accession number NM_004063); β-actin forward primer 5′-CAACTGGGACGACATGGAGAAAAT-3′, β-actin reverse primer 5′-CCAGAGGCGTACAGGGATAGCAC-3′ (GenBank accession number NM_001101). All reactions were performed in triplicate. For relative quantification, the expression levels of genes were calculated and expression based on the method of $2^{-\triangle\triangle Ct}$.

Western blotting analysis. Total proteins were prepared by standard procedures and quantified by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Thirty micrograms of protein per sample were loaded onto a 10% SDS-polyacrylamide gel. After electrophoresis, the protein was transferred onto nitrocellulose paper, incubated with 5% non-fat milk for 1 h and then incubated overnight at 4°C with a 1 μg/mL of CDH17 antibody (Sigma). Protein bands were visualized with an enhanced chemiluminescence (ECL) kit (Pierce). Quantification of the western blots was performed using Bio-Rad Software Quantity One.

Detection of cellular proliferation by MTT assay. The cellular proliferation of transfected cells was measured via MTT assay. Briefly, after an additional incubation for 4 h with10 μL of MTT (5 mg/mL) (Sigma), the resulting formazan was dissolved in 100 μL of isopropanol with 40 mM hydrochloric acid. Optical densities at 570 nm were measured using extraction buffer as a blank. Each assay was repeated three times.

Cell cycle analysis. For cell cycle analysis, cells were fixed in 70% ethanol on ice, stained with 50 μg/mL propidium iodide and 0.1 μg/mL RNase A, then analyzed by flow cytometry using a FACStar Plus. Data were analyzed to calculate the percentage of cell population in each phase using CellQuest software.

Cell invasion assay. The ability of cells to invade through a Matrigel-coated filter was measured in Transwell chambers (Corning, New York, NY, USA) following the manufacturer's instructions. In the upper chambers, 5×10^5 cells were grown in serum-free medium on 8-μm porous polycarbonate membranes, which were coated with diluted Matrigel basement membrane matrix. The lower chambers were filled with RPMI-1640 medium containing 10% fetal calf serum. After 24-h incubation, non-invading cells remaining on the upper surface of the filter were removed using cotton tips, and the cells that migrated to the underside of the membrane were fixed with 4% paraformaldehyde and stained with Giemsa (Sigma). Cells in 10 random fields of view at ×200 magnifications were counted and expressed as the average number of cells/field of view. These data were represented as the average of the three independent experiments with the SD of the average indicated.

Cell adhesion assay. Pre-coating of 96-well microtiter plates was performed by incubating wells with 20 mg/L fibronectin at 4°C overnight. To reduce non-specific binding, 2% bovine serum albumin (BSA) was added to each well and incubated for 60 min at 37°C, then rinsed twice with sterile PBS. Cells (4×10^4/well) were added to each well. After 30 min, nonadherent cells were removed by washing three times with sterile PBS. The results of adherent cells were measured by the MTT method as previously described.

Detection of matrix metalloproteinase (MMP)-2 and MMP-9 activity by gelatin zymography. To evaluate the effect of CDH17 on the activity of MMP-2 and MMP-9, cells were incubated in a serum-free medium at 37°C for 18 h. The medium was collected to detect the protein concentrations using BioRad assay and were loaded onto zymographic SDS gel containing gelatin (1 mg/mL). Then the gel was incubated in renaturing buffer and developing buffer (Invitrogen) according to the manufacturer's instructions. The enzyme activity was visualized by staining the gel with Coomassie Blue R-250.

***In vivo* tumor growth assay.** After alcohol preparation of the skin, a 0.2-mL cell suspension of 1×10^6 cells was inoculated into nude mice subcutaneously in the dorsal area using a sterile 22-gauge needle. The animals were monitored every other day, and tumor volume (cm^3) was calculated as follows: volume = (shortest diameter)^2 × (longest diameter)/2. Tumors were also evaluated with H&E (hematoxylin–eosin) and the expression of CDH17 were examined by immunohistochemistry as described previously.[4]

Statistical analysis. Statistical analyses were performed using SPSS version 11.0 (SPSS, Chicago, IL, USA). Values are expressed as the mean ± SD. Statistically significant differences between groups in each assay were determined by ANOVA, followed by post-hoc Dunnett's *t*-tests. $P < 0.05$ was considered as statistically significant.

Results

Expression of CDH17 in four distinct differentiated gastric cancer cell lines. We measured the CDH17 mRNA levels in a panel of human gastric cancer cell lines with distinct differentiation by way of quantitative polymerase chain reaction analysis. The control cell line HGC27 is an undifferentiated human gastric cancer cell line that expressed little or undetectable CDH17. SGC7901, BGC823 expressed some CDH17 transcript, whereas strong expression was seen in the well-differentiated cell line MKN28 (Fig. 1a). The results were strengthened by western blot analysis (Fig. 1b).

In order to investigate the biological significance of CDH17 in gastric cancer, not only do we want to explore the biological role of CDH17 in the present research, we also want to find global protein changes in response to CDH17 knockdown and overexpression in the same GC cell line with proteomic screening strategy in our further research. So we chose BGC823 which expressed CDH17 moderately, instead of MKN28 with the highest levels of CDH17 natively, in our present study.

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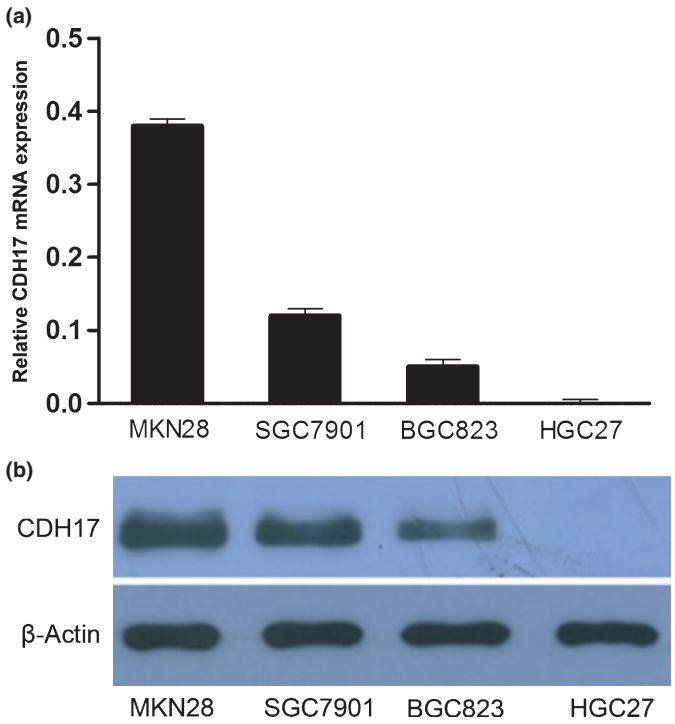

Fig. 1. Liver-intestine cadherin (CDH17) expression in four distinct differentiated gastric cancer cell lines. (a) Real-time PCR validated different CDH17 mRNA expressions in gastric cancer cell lines. (b) Western-blot analysis also identified different CDH17 protein levels in gastric cancer cell lines. Data shown are mean (±SD) from three independent experiments.

Lentiviral stably transfected BGC823 cells and efficiently suppressed CDH17 expression. The lentiviral vector expression cassette allowed for the permanent expression of GFP and CDH17 miRNA in transduced cells. Green fluorescent protein (GFP) expression and the percentage of GFP expression cells were determined by flow cytometry analysis. Figure 2(a) indicates the efficient transduction of lenti-CDH17 (98.8%) after 3 days transfection. To test the knockdown efficiency, we also examined the expression of CDH17 at both mRNA and protein levels. Data showed that after being transfected with a MOI of 10 for 72 h, the mRNA and protein levels of CDH17 were down-regulated by 95% and 78% respectively in lenti-CDH17-miR-B cells (Fig. 2b,c) compared with the control cells (lenti-CDH17-miR-neg). Next, the surface expression of CDH17 in the cell lines was further confirmed at the protein level by immunofluorescence staining (Fig. 2d). CDH17 was high in both BGC823 and lenti-CDH17-miR-neg cells, while it was significantly down-regulated in lenti-CDH17-miR-B cells.

Effect of down-regulation of CDH17 on *in vitro* proliferation and invasion of BGC823 cells. To assess the potential effects of RNAi-mediated CDH17 silencing on cell proliferation and survival, we investigated cell growth *in vitro*. RNAi with a mismatch sequence had no effect on the proliferative ability of BGC823 cells, whereas RNAi specific to BGC823 caused a dramatic reduction in the proliferation of cells ($P < 0.01$) (Fig. 3a).

In the Matrigel assays, the migrated cell numbers of lenti-CDH17-miR-B cells (24.4 ± 4.4/HP) were much lower than that of the mock cells lenti-CDH17-miR-neg (115 ± 9.5/HP) and the parental cells (113.0 ± 14.0/HP) ($P < 0.01$), which suggests that lenti-CDH17 could significantly suppress the invasion of BGC823 cells. No significant difference was found between BGC823 and the lenti-CDH17-miR-neg cells. (Fig. 3b,c).

To assess the role of CDH17 in cell adhesion to ECM substrates, cells were plated on plastic plates coated with fibronectin. The lenti-CDH17-miR-B cells showed a significant reduction in their ability to adhere to fibronectin compared with untreated BGC823 cells or the mock cells lenti-CDH17-miR-neg, as presented in Figure 3(d).

Cell cycle analysis was executed to determine whether the effect of miR-CDH17 on cell proliferation of BGC823 was due to cell cycle alterations. The result showed that compared to the parental cells and the mock cells lenti-CDH17-miR-neg, the percentage of cells at the G1 phase increased from 57.1% and 60.7% to 72.5% ($P < 0.01$) (Fig. 3e). These results indicated that down-regulation of CDH17 induced a G1 cell cycle arrest in lenti-CDH17-miR-B cells.

Gelatin zymography analysis showed that the activities of MMP-2 and MMP-9 were much lower in lenti-CDH17-miR-B cells compared to those in BGC823 and lenti-CDH17-miR-neg cells (Fig. 3f).

Knockdown of CDH17 decreased the growth of the tumor derived from BGC-823 cells. The tumorigenicity of BGC823 cells after transfection was compared in the nude mice model. Each mouse was inoculated with 1×10^6 cells. At 28 days' post inoculation, obvious tumors were formed in the two control groups of mice inoculated with BGC823 cells or the lenti-CDH17-miR-neg cells, while tumor growth was nearly completely suppressed in mice inoculated with lenti-CDH17-miR-B cells. As shown in Figure 4(a,b), we observed only small tumors in mice inoculated with lenti-CDH17-miR-B cells. We sacri-

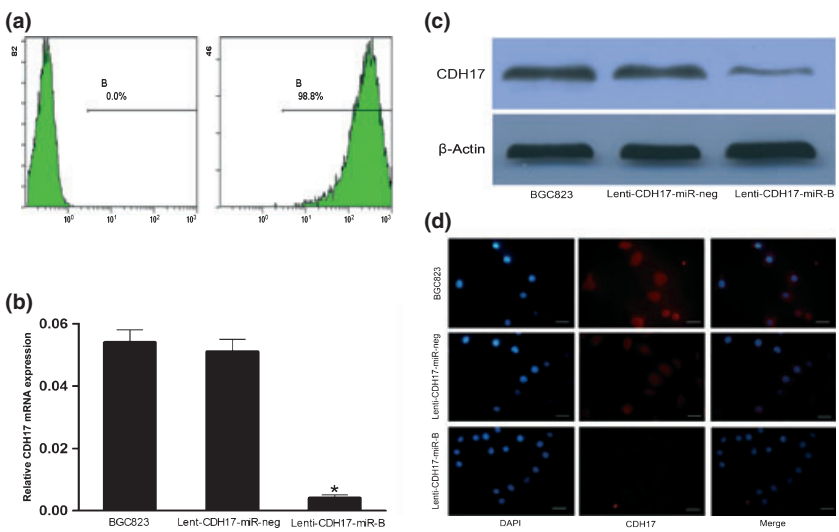

Fig. 2. Construction of lentiviral-mediated miRNA against liver-intestine cadherin (CDH17) and their impact on CDH17 expression levels. (a) Flow cytometry assay showed that the transduction rate was 98.8% when the MOI was 10. (b) Real-time PCR analysis showed the inhibition of CDH17 at the mRNA level in lenti-CDH17-miR-B cells (*$P < 0.01$). (c) Western blot analysis showed the inhibition of CDH17 at the protein level in lenti-CDH17-miR-B cells ($P < 0.01$). (d) Immunofluorescence staining showed the reduction of CDH17 in lenti-CDH17-miR-B cells. All the nuclei were stained with DAPI (shown in blue). The expressions of CDH17 are shown in red. Scale bar, 100 μm.

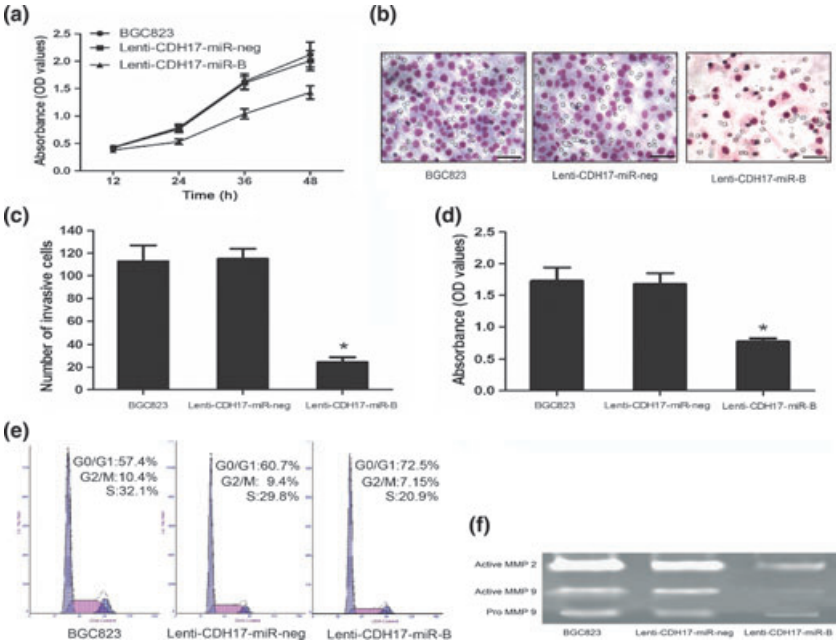

Fig. 3. Down-regulation of liver-intestine cadherin (CDH17) inhibited proliferation, invasion, and adherence of BGC823 cells *in vitro*. (a) Knockdown of CDH17 led BGC823 cell growth inhibition ($P < 0.05$ *vs* BGC823). (b) The cells that invaded through the Matrigel-coated inserts were stained, counted, and photographed under a microscope. Scale bar, 200 μm. (c) Quantification of invasive cells showed more than 50% decrease in invasion of lenti-CDH17-miR-B cells. Data were calculated from three independent experiments (*$P < 0.05$ *vs* BGC823). (d) Down-regulation of CDH17 inhibited adherence of BGC823 cells. Data shown are mean (±SD) from three independent experiments (*$P < 0.05$ *vs* BGC823). (e) Cell cycle analysis by flow cytometry demonstrated that knockdown CDH17 induced G0/G1 phase arrest ($P < 0.01$). (f) The activities of matrix metalloproteinase (MMP)-2 and MMP-9 were much lower in lenti-CDH17-miR-B cells analyzed by gelatin zymography.

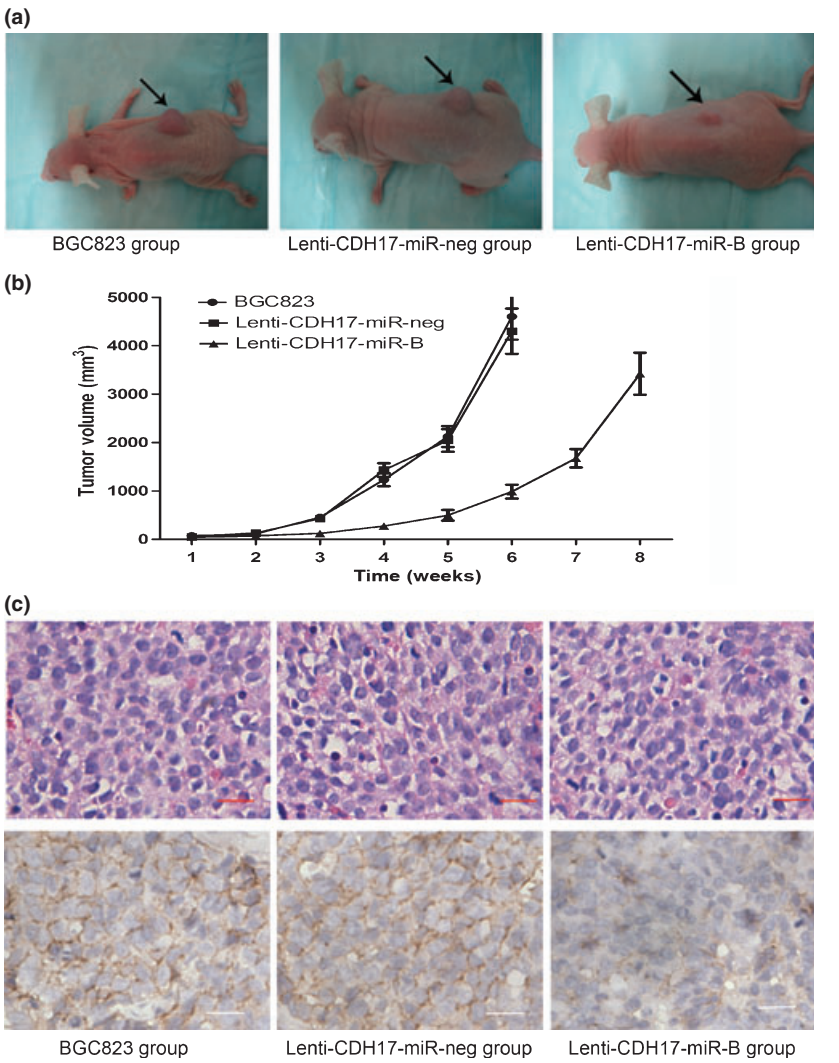

Fig. 4. Tumorigenicity was inhibited by suppression of liver-intestine cadherin (CDH17) using lenti-miRNA in BGC823 cells. (a) Representative photographs of the mice in each group post inoculation. The mice in BGC823 and lenti-CDH17-miR-neg groups were sacrificed and photographed 6 weeks' post inoculation because of their large tumor size according to the guidance of the Animal Experimental Ethics Committee of Wuhan University. Mice in the lenti-CDH17-miR-B group were sacrificed and photographed 8 weeks' post inoculation because very small tumors were not observed until 8 weeks had passed. (b) Tumor sizes and tumor volumes of the nude mice in each group. Data is presented as mean ± SD ($n = 6$). (c) The hematoxylin–eosin (H&E) staining and immunohistochemistry showed that CDH17 was positive in BGC823 mice and lenti-CDH17-miR-neg mice tumor and was weak positive in lenti-CDH17-miR-B tumor. (SP, ×400). We did not find any significant differences in morphology between these groups. Scale bar, 100 μm.

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ficed the mice in control groups 6 weeks' post inoculation because of their tumor size, whereas in the lenti-CDH17-miR-B group, we could still examine tumor growth *in vivo* for another 2 weeks.

To confirm the effect of CDH17 knockdown *in vivo*, we dissected the tumors and then conducted H&E staining and immunohistochemical analysis. The immunohistochemistry study confirmed that CDH17 protein expression was significantly suppressed in the lenti-CDH17-miR-B group compared with the BGC823 and lenti-CDH17-miR-neg groups (Fig. 4c).

Discussion

The cadherin family is one of the major intercellular adhesion molecules particularly essential for the formation and intermediation of tight junctions between epithelial cells.[14–16] Among the described subclasses of cadherin molecules, CDH17 is a novel cadherin with an extracellular domain consisting of seven homologous repeated domains instead of the five characteristic of the classical cadherins. Moreover, the novel cadherin mediates Ca^{2+}-dependent intercellular adhesion, despite the structural differences that make this molecule unique among the known members of the cadherin family.[8] A growing body of literature has investigated the relationship between CDH17 expression and the clinicopathological factors of many human cancers. Despite these significant clinical findings, the molecular pathogenesis of CDH17 remains unknown, and its role in GC has not yet been confirmed. Previous studies were not consistent on its value in the metastasis and prognosis of tumor diseases.[3–5,9–13,17–19] Some papers have shown that low CDH17 expression correlated with advanced tumor stage and that high CDH17 expression correlated with good survival,[20] while other researchers have proposed that CDH17 is a positive regulator in cancer progression and that the prognosis of patients with positive CDH17 expression was significantly poorer than in negative cases.[21] To determine the role and mechanism of CDH17 in the process of invasive growth in gastric cancer, miRNA-based CDH17 targeting pre-miRNA pol II lentivirus vectors were constructed in our study. We found that lentiviruses could efficiently deliver CDH17 miRNAs into BGC823 cells and the CDH17 protein and mRNA levels were significantly decreased through transfection of CDH17 miRNA.

Our present data shows that infection with the recombinant lentivirus expressing CDH17-specific miRNA dramatically reduced the proliferation of the gastric cancer cells BGC823 by MTT assay. This powerful effect was associated with a strong decrease in the expression of CDH17 in cells treated with the miRNA. Our adhesion and invasion assays showed that knockdown of CDH17 expression contributed to the significant reduction of ECM adhesion and invasion potency through the Matrigel of BGC823 cells. Furthermore, our tumor growth assay *in vivo* demonstrated that knockdown of CDH17 expression can clearly slow the growth of gastric cancer derived from BGC823 cells. Previous study reported that the down-regulation of CDH17 by siRNA reduced invasion and metastasis of hepatocellular carcinoma.[22] Our results were consistent with theirs. Thus we proposed that CDH17 may be a positive regulator for the proliferative, adhesive, and invasive behaviors of GC. In GC, CDH17 may mediate carcinoma cell interaction with gastric stroma and be involved in the promotion of GC metastasis by facilitating carcinoma cell migration through the gastric stroma and reestablishing homophilic cell–cell adhesion in metastasis. Whether CDH17 contributes to a stroma-oriented cellular adhesion profile with increased gastric tumor cell invasion still awaits further research in different GC cell lines.

Earlier studies reported that lentivirus-mediated expression of CDH17 shRNA in human hepatocellular xenografts *in vivo* resulted in relocalization of β-catenin to the cytoplasm with concomitant reduction in cyclin D1 and increased immunostaining of caspase 3, Bax, and Bcl-XL levels, which in turn, promotes apoptosis in hepatocellular carcinoma.[23] In our research, we demonstrated that knockdown of CDH17 induced cell cycle arrest. CDH17 silencing cells showed G0/G1 phase arrest and S phase reduction, suggesting that the reducing growth of the cells and tumor may associate with the cell cycle arrest. In our research we also examined the activities of MMP-2 and MMP-9, which belong to the gelatinase subfamily of the MMPs and were related to tumor invasion and metastasis by their capacities of tissue remodeling via ECM as well as basement membrane (BM) degradation.[24,25] We found that the activities of MMP-2 and MMP-9 were significantly lower in the lenti-CDH17-miR-B group than that in the control groups, suggesting that decreased activities of MMP-2 and MMP-9 could be achieved by inhibiting CDH17. These observations, together with the ability of RNAi targeting for CDH17 to inhibit the invasion and growth of gastric cancer cells, suggested the main role of CDH17 in induction of invasiveness, and the knockdown of CDH17 might result in marked suppression of the proliferation and invasive potential of GC cells. All the experiments should be reexamined with other GC cells to investigate the biological significance of CDH17 in gastric cancer.

Although, we have not demonstrated clearly the mechanisms underlying the inhibition of the *CDH17* gene here, there has been evidence that knockdown of CDH17 could inactivate Wnt signaling which in turn suppresses the invasive activity of cancer cells.[23] On the other hand, loss of CDH17 results in the up-regulation of placental growth factor (PIGF) and metal-responsive transcription factor-1, which might contribute to tumor aggressiveness, thereby regulating angiogenesis in human intrahepatic cholangiocarcinoma.[26] Thus, we speculate *CDH17* gene silencing may inhibit invasion and proliferation of GC in multitude mechanisms, and CDH17 may be a rational molecular therapeutic target in GC. More research should be done to explore the regulation of CDH17.

Taken together, our results indicate that the silencing of CDH17 expression by RNAi suppressed the proliferation and invasion of gastric cancer cells both *in vitro* and *in vivo*. CDH17 could be taken as an important determinant of malignant cellular behavior and maybe a promising target for therapeutic intervention of GC.

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Disclosure Statement

The authors have no conflict of interest.

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