Epigenetic modulation and repression of miR-200b by cancer-associated fibroblasts contribute to cancer invasion and peritoneal dissemination in gastric cancer

Junji Kurashige1,2, Kosuke Mima1,2, Genta Sawada1,3, Yusuke Takahashi1,3, Hidefumi Eguchi1, Keishi Sugimachi1, Masaki Mori1, Kazuyoshi Yanagihara4, Masakazu Yashiro5, Kosei Hirakawa5, Hideo Baba6 and Koshi Mimori1,7

1Department of Surgery, Kyushu University Beppu Hospital, 4546 Tsurumihara, Beppu, Oita 874-0838, Japan, 2Department of Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto, Kumamoto 860-8556, Japan, 3Department of Gastroenterological Surgery, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan, 4Department of Translation Research, Exploratory Oncology Research and Clinical Trial Center, National Cancer Center, 6-5-1, Kashiwanoha, Kashiwazaki, Chiba 277-8577, Japan and 5Department of Surgical Oncology, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan

*To whom correspondence should be addressed. Tel: +81-977-27-1650; Fax: +81-977-27-1651; Email: kmimori@beppu.kyushu-u.ac.jp

Cancer-associated fibroblasts (CAFs) have recently been linked to the invasion and metastasis of gastric cancer. In addition, the microRNA (miR)-200 family plays a central role in the regulation of the epithelial–mesenchymal transition process during cancer metastasis, and aberrant DNA methylation is one of the key mechanisms underlying regulation of the miR-200 family. In this study, we clarified whether epigenetic changes of miR-200b in CAFs stimulate cancer invasion and peritoneal dissemination in gastric cancer. We evaluated the relationship between miR-200b and CAFs using a coculture model. In addition, we established a peritoneal metastasis mouse model and investigated the expression and methylation status of miR-200b. We also investigated the expression and methylation status of miR-200b and CAFs expression in primary gastric cancer samples. CAFs (CAF-37 and CAF-50) contributed to epigenetic changes of miR-200b, reduced miR-200b expression and promoted tumor invasion and migration in NUGC3 and OCUM-2M cells in coculture. In the model mice, epigenetic changes of miR-200b were observed in the inoculated high-frequency peritoneal dissemination cells. In the 173 gastric cancer samples, the low miR-200b expression group demonstrated a significantly poorer prognosis compared with the high miR-200b expression group and was associated with peritoneal metastasis. In addition, downregulation of miR-200b in cancer cells was significantly correlated with alpha-smooth muscle actin expression. Our data provide evidence that CAFs reduce miR-200b expression and promote tumor invasion through epigenetic changes of miR-200b in gastric cancer. Thus, CAFs might be a therapeutic target for inhibition of gastric cancer.

Recent studies have established the importance of the tumor stroma in cancer progression and metastasis (3). Stromal fibroblasts are the major cellular constituents of tumor stroma, and are often referred to as cancer-associated fibroblasts (CAFs). They often display the phenotypes of myofibroblasts, which are characterized by the expression of α-smooth muscle actin (α-SMA) and strong contractility (4). Moreover, CAFs play an important role in the malignant progression of several cancers such as breast, prostate, pancreatic, esophageal and lung cancer, among others, including the initiation, proliferation, invasion and metastasis of cancer cells (5–7). A previous report indicated that gastric fibroblasts play an important role in the progression, growth and spread of scirrhous gastric cancers (8,9).

Recent evidence has emerged that directly or indirectly associates several microRNAs (miRNAs) with the epithelial–mesenchymal transition (EMT), contributing to the now extensive list of EMT-associated transcription factors (10,11). Gregory et al. and Park et al. demonstrated the clear involvement of the miR-200 family in this process, which consists of five members that can be divided into two clusters: miR-200a/b/429 and miR-200c/141, which map to human chromosomes 1 and 12, respectively (12). The miR-200 family has been suggested to play a central role in the regulation of the EMT process during cancer progression and metastasis. The most prominent gene targets of the miR-200 family are ZEB1 and ZEB2, which are direct repressors of the EMT marker E-cadherin (13). We reported previously that miR-200b was an important regulator of EMT through inhibition of migration and invasion via targeting the miRNAs of ZEB1 and ZEB2 in gastric cancer cells (14). Several studies have shown that the miR-200 family inhibits translation of ZEB1 and ZEB2 mRNA in several types of cancers (15–17). Moreover, in addition to the epigenetic regulation of ZEB1 and ZEB2 by miR200, previous reports have clarified that aberrant DNA methylation is observed in the promoter region of miR200 family themselves, which occurs subsequent to the induction of EMT by the reactivation of ZEB1 and ZEB2 in various cancers (18–20).

In the current study, we demonstrated that the epigenetic mechanisms involved in the regulation of the miR-200 family are not only restricted to malignant cells but are also apparent in CAFs. We found that miR-200b is epigenetically regulated and demonstrated a link between the epigenetics status of miR-200b and the presence of CAFs in gastric cancer cell lines, model mice and cancer tissue samples from patients. The purpose of this study was to evaluate how CAFs are involved in the progression and invasion of the corresponding adjacent cancer cells via epigenetic changes of miR-200b in gastric cancer.

Materials and methods

Human gastric cancer cell lines (NUGC3, NUGC4, AGS, MKN1, MKN7, MKN28, MKN45 and AZ521) were obtained in 2012 from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Japan. Human gastric cancer cell lines (OCUM-2M and OCUM-2M-D3) and human gastric fibroblast cell lines (CAF-37 and CAF-50) were obtained from gastric carcinoma population maintained at Osaka City University (21,22). The fibroblasts were used in the 3rd through 12th passage in culture and mainly at the 5th passage. To examine the incubating myofibroblast content of orthotopic fibroblasts, immunohistochemical staining was performed as described previously (9). HSC-58 cells were established previously from a patient with scirrhous gastric cancer. HSC-58 cells inoculated into BALB/c nude mice led to dissemination of the tumor cells to the greater omentum, mesentery, peritoneum and so on and caused ascites in a small number of animals. Cycles of isolation and orthotopic inoculation of the ascitic tumor cells were repeated in the mice for a total of 12 cycles. We obtained two cell lines (58AS1Luc and 58AS9) that possessed high metastatic potential and showed strong capability of inducing ascites (23). HSC-58, 58AS1Luc and 58AS9

Abbreviations: CAF, cancer-associated fibroblast; EMT, epithelial–mesenchymal transition; PBS, fetal bovine serum; miRNA, microRNA; PBS, phosphate-buffered saline; RT–PCR, reverse transcription–PCR; SMA, smooth muscle actin; TGF, transforming growth factor.
have no KRAS mutation. NUGC3, NUGC4, AGS, MKN1, MKN7, MKN28, MKN45, AZ521, HSC-58, 58As1Luc and 58As9 cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS); Life Technologies, Grand Island, NY) with 100 IU/ml penicillin and 100 mg/ml streptomycin. OCUM-2M, OCUM-2MD3, CA15-37 and CA45 cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco, Invitrogen, Carlsbad, CA) with 10% heat-inactivated FBS. 100 IU/ml penicillin, 100 mg/ml streptomycin and 0.5 mM sodium pyruvate. Cells were cocultured in Transwell chambers separated by 8 μm pore filters. Gastric cancer cells (3.0 × 10^5 cells/700 μl) were placed in the bottom chamber, and CA15-37 and CA45 (2.0 × 10^5 cells/300 μl) were placed in the top chamber. After 5 days, the top chamber was removed, and RNA was isolated from cancer cells. NUGC3, OCUM2M, AZ521, HSC-58, 58As1Luc and 58As9 were authenticated by short tandem repeat-PCR analysis. DNA was extracted by each cell line with QiAamp DNA Mini Kit (QIAGEN) and characterized by short tandem repeat-PCR analysis using GenePrint® 10 System (Promega).

Clinical samples

Primary gastric carcinoma tissue and matched normal gastric epithelium samples were obtained from 173 patients who underwent gastric resection without preoperative treatment at Oita Prefectural Hospital, Kyushu University Beppu Hospital between 1993 and 2003. All tissue samples were immediately cut and frozen instead of 10% formalin. From these tissues, formalin-fixed, paraffin-embedded tissue samples from Kyushu University Beppu Hospital were used in this study. Written informed consent was obtained from all patients, and the study protocol was approved by the local ethics committee. Clinicopathological information, including age, gender, pathology, differentiation and tumor-node-metastasis classification, was available for all patients.

Total RNA isolation and first-strand complementary DNA synthesis

Total RNA was isolated from frozen tissue samples by means of the modified acid-guanidine-phenol-chloroform method and isolated from cultured cell lines by using the miRNeasy Mini Kit (QIAGEN), as described previously (24,25). The purity and concentration of all RNA samples were evaluated by the absorbance ratio at 260/280 nm with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE). Total RNA was reverse transcribed to complementary DNA with M-MLV RT (Invitrogen, Carlsbad, CA).

Quantitative real-time reverse transcription–PCR

The expression of miR-200a, b, miR-429 was determined by TaqMan quantitative real-time reverse transcription–PCR (qRT–PCR) using TaqMan microRNA assay kits (Ambion) according to the manufacturer’s protocols, as described previously (14). miR-200a, b, miR-429 expression was normalized to that of the small nuclear RNA RN16B. The expression of CDH1, ZEB1, ZEB2 and Vimentin was determined using a LightCycler 480 probes master kit (Roche Diagnostics,) according to the manufacturer’s instructions. Primers and TaqMan assays are listed in Supplementary Table 1, available at Carcinogenesis Online. All qRT–PCRs were run in a LightCycler 480 System II (Roche Diagnostics, USA). The relative amounts of miR-200a, b, miR-429, CDH1, ZEB1, ZEB2 and Vimentin were measured using the 2^(-ΔΔCT) method. All qRT–PCRs were performed in triplicate.

miRNA microarray of HSC-58, 58As1Luc and 58As9

miRNA was extracted from each cell line, and RNA samples were dephosphorylated and labeled with Cyanine 3- or 5-Cp using T4 RNA ligase by incubating at 16°C for 2h. After the labeling reaction, the samples were completely dried using a vacuum concentrator at 55°C for 4h. The dried samples were treated with GE blocking agent. The SurePrint G3 Human v1.0 miRNA 8 x 60K array, which contains probes for 1205 human and 144 human viral miRNAs, was used for miRNA profiling. The blocked samples were hybridized to the probes on the microarray at 55°C with constant rotation at 20 r.p.m. in the Agilent microarray hybridization chamber for 2h. The microarray slide was washed and scanned using the Agilent scanner to obtain the microarray image. The numerical data for the miRNA profiles were extracted from the image using the Feature Extraction program. These data were analyzed with the aid of GeneSpring GX software, version 7.3 (all from Agilent Technologies). We normalized the observed expression levels of miRNA through the procedure of quantile normalization, and miRNAs displaying an increase or decrease >2-fold were selected for further analysis. miRNAs that displayed increased and decreased expression are listed in Supplementary Table 2, available at Carcinogenesis Online, and Table I, respectively.

DNA methylation analysis and 5-aza-2′-deoxycytidine treatment

CpG islands were identified in silico using Methyl Primer Express v.1.0 software (Applied Biosystems, Carlsbad, CA). DNA methylation status was established by bisulfite genomic sequencing of multiple clones and methylation-specific PCR. The primer sequences used in the DNA methylation analysis are listed in Supplementary Table 1, available at Carcinogenesis Online. Cells were treated with 2.5 or 5.0 μM 5-aza-2′-deoxycytidine (5-aza-dC; Sigma–Aldrich, St Louis, MO) for 48h.

Migration and invasion assays

Cell migration and invasion were assessed using the BD Falcon FluoroBlok™ 24 Multiwell Invert System (BD Bioscience, San Jose, CA) using 8 mm pore-sized membranes with Matrigel (for invasion assays) or without Matrigel (for migration assays). In brief, 2 × 10^5 cells/750 μl of medium containing 10% FBS in the lower chamber. The medium was changed every 2 days, but no cells were added to the lower chamber of control wells. The NUGC3 cells (1 x 10^5) were placed in the upper chamber of a 24-well plate with serum-free medium. The cell plate was incubated in a humidified atmosphere (37°C and 5% CO2). After 48h incubation, the upper chamber was transferred to a second 24-well plate containing 500 μl/well of 4 μg/ml calcine AM in Hanks’ balanced salt solution and incubated for 1h (37°C and 5% CO2). Invasive cells that migrated through the membrane were evaluated in a fluorescence plate reader at excitation/emission wavelengths of 485/535 nm. Each independent experiment was performed three times.

Immunohistochemistry and quantitative analysis of α-SMA

Immunohistochemical studies of α-SMA were performed on formalin-fixed, paraffin-embedded surgical sections obtained from patients with gastric cancer. Tissue sections were deparaffinized and boiled in 0.01 mol/l sodium citrate buffer in a microwave for 10min at 500W for antigen retrieval. Rabbit anti-α-SMA (ab5694; Abcam, Cambridge, UK), diluted 1:100, was used as the primary antibody. All tissue sections were immunohistochemically stained with the avidin-biotin-peroxidase method (LSAB System HRP; Dako, Tokyo, Japan) and were counterstained with hematoxylin. α-SMA expression in cancer-associated stroma was quantified as the relative percentage of the α-SMA-stained area to the selected field area using an imaging processor, as described previously (26,27). Slides were observed under light microscopy at ×100 magnification, and five regions were selected for every slide at random. The expression was independently evaluated by two of the authors (J.K. and K.M.)
using a blinded protocol design (the observers had no information on clinical outcome or any other clinicopathological data). ImageJ software was used to analyze the positive area percentage and staining intensity in the stroma tissue of every region, and then the average value was calculated from the amount of α-SMA on every slide (Supplementary Figure 1, available at Carcinogenesis Online). The muscle layer region was considered for this assessment because muscle fibers ubiquitously express α-SMA.

**Immunoblotting for E-cadherin**

For immunoblotting, sodium dodecyl sulfate–polyacrylamide gel electrophoresis of proteins was performed using NuPAGE 4-12% Bis-Tris Gel electrophoresis (Invitrogen), an XCell Sure Lock Mini-Cell (Invitrogen), and a Power PAC HC (Bio-Rad). The resolved proteins on the gel were transferred to a nitrocellulose membrane using iBlot Dry Blotting System (Invitrogen). The resulting membranes were blocked with 5% iBlot (Applied Biosystems) and 0.1% Tween-20 (Bio-Rad) in phosphate-buffered saline (PBS) (T-PBS) for 1 h. Membranes were then incubated with primary antibodies. Next, the membranes were washed twice for 5 min in T-PBS and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h and washed twice for 5 min in T-PBS.

Chemiluminescence detection reagents were incubated with the membranes for 1–5 min, followed by image acquisition using an Image Quant LAS500 (GE Healthcare). Primary antibodies targeted pan actin (NeoMarkers) and a monoclonal antibody against E-cadherin (1:200, BD Bioscience) diluted in T-PBS.

**Orthotopic in vivo models**

Six-week-old female BALB/c nu/nu mice were purchased from Kyudo Japan and maintained under specific pathogen-free conditions and provided with sterile food, water and cages. Ambient light was controlled to provide regular cycles of 12 h of light and 12 h of darkness. A total of 1 × 10^6 HSC-58, 58As1Luc and 58As9 cells were inoculated into the gastric wall of each mouse after laparotomy, as described previously (23,28,29). For, assessment of miR-200b influence, a total of 1 × 10^6 cancer cells (58As9 with Pre-miR™ miRNA Precursor Molecule Negative Control and Pre-miR™ miRNA Precursor Molecule pre-200b (Applied Biosystems, Foster City, CA)) were inoculated into the gastric wall of each mouse. The method of transfection of miRNA was followed by the past manuscript (14). At 28 days after inoculation, the mice were sacrificed and dissected and peritoneal dissemination, liver metastasis and ascites formation were examined. The number of mesentery nodules >5mm in diameter was also determined. All animal procedures were performed in compliance with the Guidelines for the Care and Use of Experimental Animals established by the Committee for Animal Experimentation of Kyushu University; these guidelines conform to the ethical standards required by Japanese law and also comply with the guidelines for the use of experimental animals in Japan.

**Statistical analysis**

All experiments were performed at least three times. Continuous variables are expressed as the means ± standard deviations. The relationship between the expression of miR-200b and the patient clinicopathological characteristics was analyzed using the Student’s r-test or a chi-square analysis. The overall survival curves were plotted according to the Kaplan–Meier method, and the generalized log-rank test was applied to compare the survival curves. The findings were considered to be significant for P-values < 0.05. All tests were performed using JMP software, eighth edition (SAS Institute, Cary, NC).

**Results**

**CAFs stimulated the invasion and migration of gastric cancer via epigenetic change of the miR-200b promoter**

We used the coculture system to determine whether CAFs secrete factors that could stimulate the invasion and migration of gastric cancer cells in vitro. NUGC3 cells treated with CAFs (CAF-37 and CAF-50) showed significantly high migratory behavior (P < 0.01; Figure 1A, left) and were significantly invasive (P < 0.01; Figure 1A, right) compared with NUGC3 cells only (NUGC3 control). We examined miR-200b expression in the gastric cancer cell lines cocultured with CAFs. The miR-200b expression in NUGC3 and OCUM-2M cells (which showed high miR-200b expression and low methylation) treated with CAFs was lower than those of control cells (P < 0.05; Figure 1B). Similarly, the levels of CDH1 mRNA and protein, an EMT marker, of NUGC3 and OCUM-2M cells treated with CAFs were lower than those of control cells (Figure 1C and E). Recent evidence suggests that expression of miR-200 family members can be epigenetically regulated through methylation of their promoter regions (18,19,30). Therefore, we evaluated the methylation status to identify the mechanism of downregulation of miR-200b by CAFs in gastric cancer. The miR-200b/200a/429 transcription start sites have been determined previously to be located within canonical CpG islands in chromosome 1 (31,32). The CpG islands of the miR-200b promoter in gastric cancer cells treated with CAFs showed an increased amount of partially methylated changes than those of control cell lines (Figure 1D). Moreover, we examined miR-200a and 429 expression in the gastric cancer cell lines cocultured with CAFs. Similarly to miR-200b, the expression of both miR-200a and 429 in NUGC3 cells treated with CAFs were lower than those of control cells (Supplementary Figure 2, available at Carcinogenesis Online).

Cpg island hypermethylation-associated silencing of miR-200b in cancer cells

miR-200b is suggested to target ZEB1/2, thereby preventing the repression of E-cadherin expression by ZEB1/2. We evaluated the correlation between miR-200b and CDH1, Vimentin and ZEB1/2 mRNA expression in 14 gastric cancer cell lines. As shown in Supplementary Figure 3A–E, available at Carcinogenesis Online, the high miR-200b expression cell line, OCUM-2M, MKN45, KATOIII and OCUM-8, showed low ZEB1, ZEB2 and Vimentin expression, whereas the low miR-200b expression cell line, AZ521 and MKN1, showed high ZEB1, ZEB2 and Vimentin expression and low CDH1 expression. The CpG islands of the miR-200b/200a/429 cluster were almost completely methylated in AZ521 cells, which showed the lowest miR-200b expression, whereas OCUM-2M and NUGC3 cells, which showed high miR-200b expression, were found to be almost completely unmethylated (Supplementary Figure 3F, available at Carcinogenesis Online). To further understand the functional significance of promoter hypermethylation of miR-200b, we treated AZ521 cells with the DNA-demethylating agent 5-aza-2’-deoxycytidine. Indeed, treatment of AZ521 with 5-aza-2’-deoxycytidine restored the expression of miR-200b (Supplementary Figure 3G, available at Carcinogenesis Online).

**The epigenetic change of miR-200b in scirrhous cancer with high peritoneal dissemination**

Two highly metastatic cell lines (58As1Luc and 58As9) were also established from the HSC-58 cells. When 58As1Luc or 58As9 cells were implanted orthotopically, bloody ascites began to form ~3 weeks after the inoculation, accompanied by tumor dissemination to the greater omentum, mesentery, parietal peritoneum, diaphragm and so on, and the mice died soon thereafter (Figure 2A and B). We performed miRNA microarray profiling between HSC-58, 58As1Luc and 58As9 cells. As a result, the miR-200 family was significantly downregulated in 58As1Luc and 58As9 cells compared with HSC-58 cells (Table 1). We next performed quantitative RT–PCR to confirm the microarray results. Similarly, miR-200b expression was downregulated in 58As1Luc and 58As9 cells compared with HSC-58 cells (Figure 2C). The CpG islands were partially methylated in 58As1Luc and 58As9 cells, which showed low miR-200b expression; in contrast, CpG islands of HSC-58 cells, which showed high miR-200b expression, were methylated at a much lower frequency (Figure 2D). Treatment with 5-aza-2’-deoxycytidine in 58As1Luc and 58As9 cells restored the expression of miR-200b (Figure 2E). Next, we orthotopically inoculated 58As9 with miR-200b upregulated in the stomach wall of nude mice. After twenty-eight days of orthotopic transplantation, we confirmed miR-200b upregulated markedly were shown that reduction number of disseminated metastasis (Figure 2F).

**Association of miR-200b expression with clinicopathological characteristics and survival**

Expression of miR-200b was examined in 173 clinical gastric cancer samples using qRT–PCR, with quantified values used to calculate miR-200b/RNU6B ratios. The mean expression of miR-200b in 173 clinical gastric cancer samples using qRT–PCR, with quantified values used to calculate miR-200b/RNU6B ratios. The mean expression of miR-200b
in cancerous tissue specimens was significantly lower than those in non-cancerous tissues \((P < 0.01; \text{Figure 3A})\). Moreover, the mean expression of miR-200b in the cancerous tissue specimens of patients with peritoneal metastasis was significantly lower than those without peritoneal metastasis \((P < 0.01; \text{Figure 3B})\). We divided the 173 gastric cancer patients into two groups according to the median miR-200b expression level: 87 of the cases were placed in the high miR-200b expression group and the remaining 86 cases were placed in the low miR-200b expression group. The association between patient clinicopathological characteristics and miR-200b expression is summarized in Table II. miR-200b expression was significantly associated with cancer differentiation \((P = 0.002)\), depth of tumor invasion \((P = 0.010)\), venous invasion \((P = 0.017)\), peritoneal metastasis \((P = 0.001)\), distant metastasis \((P = 0.002)\), and cancer staging \((P = 0.005)\).

Analysis of 5-year overall survival showed that the low miR-200b expression group had significantly poorer prognosis than the high expression group \((P = 0.015; \text{Figure 3C})\).

**Expression of CAFs in gastric cancer stroma and association with miR-200b in gastric cancer specimens**

Next, we verified the relationship between the epigenetic status of miR-200b and CAFs surrounding cancer cells in clinical gastric cancer samples. Stromal fibroblasts in 53 gastric cancer samples were quantified using a computer-assisted image analysis system as described in the Materials and methods. A representative photograph stained for α-SMA and the corresponding image treated with an imaging processor are shown in Supplementary Figure 1A–F, available at Carcinogenesis Online. The α-SMA staining localized in the cytoplasm of stroma fibroblasts, whereas tumor cells were negatively stained. The α-SMA scores varied from 0.31 to 9.47% \((P = 0.002)\). Two investigators (J.K. and K.M.) independently evaluated α-SMA staining and obtained similar results. Next, we examined the correlation between α-SMA staining in gastric cancer stroma
and miR-200b expression in gastric cancer. There was a significant inverse correlation between miR-200b expression and the α-SMA score (Figure 4A). The gastric cancer samples were then divided into two groups, a high α-SMA group (n = 26) and a low α-SMA group (n = 27), according to α-SMA expression in stroma at a cutoff point at the median mean value. The patients with high α-SMA expression had significantly lower miR-200b expression than the low α-SMA patients (P < 0.05; Figure 4B). When α-SMA expression was compared with the various clinical and pathologic variables listed in Supplementary Table 3, available at Carcinogenesis Online, no significant associations were found. Moreover, we chose three low miR-200b/high α-SMA scoring patients and three high miR-200b/low α-SMA scoring patients for further analysis (Supplementary Figure 4, available at Carcinogenesis Online). The CpG islands were more significantly methylated in the low miR-200b/high α-SMA group than in the high miR-200b/low α-SMA group (Figure 4C).

Discussion
In this study, we demonstrated that CAFs reduced miR-200b expression and induced the hypermethylation of the miR-200b promoter regions. Furthermore, there was a negative correlation between miR-200b expression in gastric cancer specimens and α-SMA
expression in the stroma of gastric cancer, and the patients with high α-SMA expression showed methylation of the miR-200b promoter. These findings suggest that CAFs stimulate cancer invasion and migration via epigenetic changes of miR-200b in gastric cancer. Moreover, model mice with peritoneal dissemination showed methylated miR-200b and low miR-200b expression. Similarly, we found that patients with low miR-200b expression had a significantly poorer prognosis than those with high miR-200b expression, and low miR-200b expression was associated with peritoneal dissemination.

This is the first study to directly analyze the role of CAFs to regulate the expression of miRNA via epigenetic changes to the best of our knowledge. The CAFs populations in tumor-associated stroma are known to include both fibroblasts and myofibroblasts. Myofibroblasts are endowed with the ability to promote tumor growth and are associated with higher grade malignancy and poorer prognosis in patients with several cancers (26,33,34). Indeed, the CAFs prepared and examined in our study contained a subpopulation of α-SMA-expressing fibroblasts, as indicated by immunohistochemistry (Figure 4 and Supplementary Figure 2, available at Carcinogenesis Online). CAFs can promote cancer progression, invasion and metastasis by modulating multiple components in the cancer niche to build a permissive and supportive microenvironment for tumor growth and invasion through the secretion of growth factors including hepatocyte growth factor, stromal cell-derived factor-1, several chemokine factors, platelet-derived growth factor, fibroblast growth factor and transforming growth factor (TGF-β) (35–38). In particular, TGF-β from tumor-associated stroma is an important factor for the induction and functional activation of EMT-related pathways (39–41). Interestingly, TGF-β was shown to induce the expression of DNA methyltransferases, which function in DNA methylation, in several cancers (42–44). Moreover, TGF-β also mediates these effects through the action of epigenetic switches such as CD133 and tristetraprolin, as well as miR-200 CpG island methylation events (18,42,45). Thus, in this study, some signals from CAFs, such as TGF-β, might be related to the corresponding methylation changes observed in miR-200b. This aspect remains to be investigated in future research.

Members of the miR-200 family are being increasingly recognized as important players for regulating epithelial characteristics of cells through direct targeting of ZEB1 and ZEB2, which are EMT-inducing transcription factors, via transcriptional repression of E-cadherin expression (13,46); our present results in gastric cancer cell lines confirm this role of miR-200 (Supplementary Figure 2A–E, available at Carcinogenesis Online). Based on the EMT hypothesis of cancer metastasis, low expression of the miR-200 family would lead to increased metastasis through the targeted induction of ZEB1 and ZEB2 expression, resulting in repressed E-cadherin expression and the adoption of mesenchymal characteristics. This EMT process has been shown to occur in several types of cancer cells, whereby lower levels of the miR-200 family have been associated with a higher frequency of invasive and metastatic tumors and a poorer prognosis (16,46–48). However, several studies have also shown the opposite effect of high expression of miR-200 family members enhancing distant metastases through promoting secondary cancer colonization (30,49,50) in the mesenchymal–epithelial transition process. This has been interpreted as a potential requirement for EMT to accomplish the first steps of metastasis, and a reversion (mesenchymal–epithelial transition) to accomplish the final step of colonization. EMT is first acquired in the onset of transmigration and then reversed mesenchymal–epithelial transition occurs in the new colony; this process is described as epithelial–mesenchymal plasticity. However, because peritoneal dissemination is the most common cause of death in gastric cancer, a better understanding of the EMT mechanism is critical for developing new treatments that can improve the survival of gastric cancer patients with peritoneal dissemination. During EMT, methylation-induced downregulation of miR-200b allows upregulation of several of its direct target genes, including ZEB1 and ZEB2, as they increase invasive and metastatic potential, involving the simultaneous loss of E-cadherin and enhancement of Vimentin expression at peritoneal dissemination sites. Our results demonstrated that restoration of miR-200b expression is a potential candidate approach for miRNA-based therapy against peritoneal dissemination of gastric cancer.

In conclusion, this study provides important insight supporting the roles of miR-200b during peritoneal dissemination in gastric cancer. Our discovery of the pivotal role that miR-200b plays in the metastatic behavior of gastric cancer indicates that this miRNA has potential value as a diagnostic and prognostic biomarker. These results may also have implications for the clinical management of patients with peritoneal dissemination.
Table II. miR-200b expression and clinicopathological features

<table>
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<th>Total (n = 173)</th>
<th>miR-200b</th>
<th>P-value</th>
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<td></td>
<td></td>
<td>High expression, n = 87 (%)</td>
<td>Low expression, n = 86 (%)</td>
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<tr>
<td>Age (years)</td>
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<td>68</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td></td>
<td>121</td>
<td></td>
</tr>
<tr>
<td>Peritoneal metastasis</td>
<td></td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td></td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td></td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Distant metastasis</td>
<td></td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td></td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td></td>
<td>127</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>I–II</td>
<td></td>
<td>55</td>
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</tr>
<tr>
<td>III–IV</td>
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<td>32</td>
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</tr>
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</table>

Staging was classified by Union for International Cancer Control, seventh edition.

*P-value < 0.05.

Fig. 4. Relationship of methylation status of miR-200b and CAFs expression in gastric cancer specimens. (A) There was a significant inverse correlation between miR-200b expression and the α-SMA score. (B) Patients with high α-SMA expression had significantly lower miR-200b expression than low α-SMA patients. (C) We chose three low miR-200b/high α-SMA score patients and three high miR-200b/low α-SMA score patients for methylation analysis. The CpG islands were more significantly methylated in the low miR-200b/high α-SMA group than the high miR-200b/low α-SMA group.
Supplementary material

Supplementary Tables 1–3 and Figures 1–4 can be found at http://carcin.oxfordjournals.org/

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References


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