Downregulation of tumor suppressor MBP-1 by microRNA-363 in gastric carcinogenesis

Kai-Wen Hsu1, An-Ming Wang1, Yueh-Hsin Ping2,3, Kuo-Hung Huang4,5, Tzu-Ting Huang6, Su-Shun Lo7,8, Kai-Wen Hsu1, Yueh-Hsin Ping2,3, Kuo-Hung Huang4,5, Tzu-Ting Huang6, Su-Shun Lo7,8

1Department of Anatomy and Cell Biology and 2Department and Institute of Pharmacology, School of Medicine, National Yang-Ming University, Taipei 112, Taiwan; 3Department of Surgery, Taipei Veterans General Hospital, Taipei 112, Taiwan; 4Institute of Clinical Medicine and 5Department of Medicine, School of Medicine, National Yang-Ming University, Taipei 112, Taiwan; 6Department of Surgery, National Yang-Ming University Hospital, Yilan 260, Taiwan; 7Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei 112, Taiwan; 8Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei 110, Taiwan

*To whom correspondence should be addressed. Department of Anatomy and Cell Biology, School of Medicine, National Yang-Ming University, 155, Section 2, Li-Nong Street, Taipei 112, Taiwan. Tel: +886 2 2826 7070; Fax: +886 2 2821 2884; Email: tseyh@ym.edu.tw

Gastric carcinoma is one of the most common malignancies and the second most lethal cancer worldwide. The mechanisms underlying aggressiveness of gastric cancer still remain obscure. c-Myc promoter binding protein 1 (MBP-1) is a negative regulator of c-myc expression and ubiquitously expressed in normal human tissues. It is produced by alternative translation initiation of α-enolase gene. Both MBP-1 and α-enolase are involved in the control of tumorigenesis including gastric cancer. MicroRNAs (miRNAs) are involved in tumorigenesis and could have diagnostic, prognostic and therapeutic potential. In this study, whether miRNAs modulate tumorigenesis of gastric cancer cells through targeting MBP-1 was evaluated. We found that miR-363 targets 3′-untranslated region of human MBP-1/α-enolase messenger RNA. The exogenous miR-363 promotes growth, viability, progression, epithelial–mesenchymal transition and tumour sphere formation of SC-M1 gastric cancer cells through downregulation of MBP-1, whereas the knockdown of endogenous miR-363 suppresses tumorigenesis and progression of SC-M1 cells via upregulation of MBP-1. The miR-363/MBP-1 axis is also involved in the control of carcinogenesis in KATO III and SNU-16 gastric cancer cells. Furthermore, miR-363 induces the xenografted tumor growth and lung metastasis of SC-M1 cells through MBP-1 in vivo. Taken together, these results suggest that miR-363 plays an important role in the increment of gastric carcinogenesis via targeting MBP-1.

Introduction

Gastric carcinoma is one of the most common malignancies and the second most lethal cancer in both sexes worldwide (1). At present, gastric cancer with distant metastasis is nearly incurable (2). The risk factors of gastric cancer include diet, Helicobacter pylori infection and specific genetic alterations (3,4). It is critical to dissect the underlying mechanisms responsible for aggressiveness of gastric cancer to identify molecular markers and design therapeutic strategy in the future. c-Myc promoter binding protein 1 (MBP-1) is a negative regulator of c-myc expression and ubiquitously expressed in normal tissues (5). It is produced by alternative translation initiation of α-enolase gene (6,7). Both MBP-1 and α-enolase are involved in controlling tumorigenesis including gastric cancer (8–14). They also participate in regulating epithelial–mesenchymal transition (EMT), which plays an essential role in tumorigenesis (14). Besides targeting c-myc (5) and COX-2 (14) directly, MBP-1 could regulate target genes via the p53–p21 axis (15).

MicroRNAs (miRNAs) are involved in many biological functions (16,17). Recent studies showed that miRNAs could play either oncogenic or tumor-suppressive roles in tumorigenesis and could have diagnostic, prognostic and therapeutic potential (18). Many miRNAs are aberrantly expressed in gastric cancer cells (19,20). Furthermore, increasing lines of evidence reveal that miRNA levels are associated with gastric cancer progression and they could be served as prognostic markers (19,20). In gastric cancer cells, miRNA expressions are downregulated by hypermethylation of CpG islands in their promoters (21–23).

MBP-1 upregulates the expression of miR-29b, which in turn suppresses Mcl-1, collagens and matrix metalloproteinase-2 expressions in prostate cancer cells (24). Thus, miR-29b is regulated by MBP-1 and then could modulate tumorigenesis. It is possible that miRNAs may be controlled by MBP-1 or target MBP-1 to regulate tumorigenesis. To delineate this possibility in gastric carcinogenesis, we first sought to search for the MBP-1-related miRNAs, which are involved in controlling tumor development and progression of gastric cancer cells. We found that the 3′-untranslated region of human MBP-1/α-enolase messenger RNA (mRNA) is a potential target of miR-363. In this study, we evaluated the role of miR-363 in gastric carcinogenesis. Furthermore, we also investigated the underlying mechanism responsible for the control of MBP-1-mediated tumorigenesis by miR-363 in gastric cancer cells.

Materials and methods

Plasmids and plasmid construction

The DNA segment of human MBP-1/α-enolase 3′-UTR [1–356 nucleotide (nt) from the start of 3′-UTR] was amplified by PCR from the genomic DNA of SC-M1 cells with the primers 5′-AAATGATCCCGCATTGTGGCCAG-3′ and 5′-AGATATCTCCATGGGTCACTAC-3′. Then, the PCR products were inserted into the XbaI site downstream of the luciferase gene in pGL3-control vector (Promega) to construct pMBP-1/α-enolase-Luc (1–356) reporter plasmid. The reporter plasmids with various lengths of MBP-1/α-enolase 3′-UTR were also constructed. Plasmids pMBP-1/α-enolase 3′-UTR-Luc (1–110), (111–259) and (260–356) contain the MBP-1/α-enolase 3′-UTR DNA fragments from 1 to 110, 111 to 259 and 260 to 356 nt, respectively. The pMBP-1/α-enolase 3′-UTR-Luc (1–356 d) and (1–356 m) plasmids contain the MBP-1/α-enolase 3′-UTRs from 1 to 356 nt in which the seed sequences, located at 208–216 nt, of miR-363 site were deleted and mutated from CGCTGAATT to GCACCTTAA, respectively.

The pcDNA-HA-MBP-1 and pcDNA-HA-α-enolase expression constructs contain the complementary DNAs of MBP-1 and α-enolase, respectively (25). The small interfering RNA (siRNA) vectors (#22 and #24) constructed in pLKO.1 siRNA vector were used to knockdown the endogenous MBP-1 and α-enolase in this study (25). The pLKO.1-shLuc siRNA vector contains the target sequence against luciferase for the control of knockdown validation.

For the construction of the miR-363-expressing adenoviral plasmid, the precursor sequence of miR-363 was amplified from the genomic DNA of SC-M1 cells by PCR using the specific primers 5′-CTCGAGACCACAAAGTTCACAAAC-3′ and 5′-AGATATCTCCGGGGTCGGCGAGAA-3′. The amplified DNA fragment was cloned into shuttle vector pAdTrack-CMV containing a green fluorescent protein (GFP) tracer to generate pAdTrack-miR-363 construct. The pAdTrack-miR-363 plasmid linearized with Pmel was transformed into BJ5183-AD-1

Abbreviations: EMT, epithelial–mesenchymal transition; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; HPV, human papillomavirus; MBP-1, c-myc promoter binding protein 1; miRNAs, microRNAs; mRNA, messenger RNA; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide; siRNA, small interfering RNA; UTR, untranslated region.

*These authors contributed equally to this work.

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bacterial cells harboring the adenoviral pAdEasy-1 vector (Stratagene) for homologous recombination to construct a recombinant miR-363-expressing adenoviral plasmid. All constructs were verified by sequencing.

Cell culture and transfection
Human stomach carcinoma SC-M1, KATO III, SNU-16 and NCI-N87 cells were cultured in RPMI 1640 with 10% fetal bovine serum. The established SC-M1 cells expressing the HA-MBP-1 fusion protein (SC-M1/HA-MBP-1) and their control cells (SC-M1/pcDNA3) were described previously (14). Cells were transiently transfected by electroporation or transfection reagents such as Lipofectamine™ 2000 (Invitrogen). SC-M1 cells (5 × 10^6) were seeded onto six-well plates and then transiently transfected for luciferase reporter gene assay (14,26). Two days after transfection, luciferase activity was quantified and then normalized with Renilla activity for transfection efficiency using the Dual-Luciferase™ Reporter Assay System (Promega). SC-M1 and SNU-16 cells were transfected with antagomiR-363 or scrambled negative control oligonucleotide (Ambion) at a final concentration of 100 nM using the Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions.

Recombinant adenovirus
The recombinant miR-363-expressing adenoviral plasmid was linearized by PciI and subsequently transfected into AD-293 cells, adenovirus packaging cells, to obtain packaged recombinant adenovirus expressing miR-363 (designated Ad-miR-363). The pAdTrack-CMV empty vector was also used to generate recombinant adenovirus expressing GFP as a control (designated Ad-GFP). The titer and multiplicity of infection of recombinant adenoviruses were determined according to the manufacturer’s protocol (Stratagene).

Quantitative real-time PCR analysis for miR-363
Total RNA of SC-M1 cells was extracted using Trizol reagent (Invitrogen). For the detection of mature miR-363, 10 ng of total RNA was reversely transcribed into complementary DNA using TaqMan MicroRNA Reverse Transcription Kit and the specific primer designed for miR-363 (Applied Biosystems). Then, the miRNA level was determined by real-time PCR using TaqMan Universal PCR Master Mix and TaqMan MicroRNA Assays (Applied Biosystems). The miRNA expression was normalized with the level of RNU48 small nuclear RNA.

Western blot analysis
Whole cell lysates were prepared as described previously (14). Western blot analysis was performed with anti-COX-2 (Cayman Chemical), anti-α-enolase, anti-c-Myc, anti-E-cadherin, anti-plakoglobin, anti-vimentin (Santa Cruz), anti-N-cadherin (BD Biosciences) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Biogenesis) antibodies. Owing to the similarity of amino acid sequences between MBP-1 and α-enolase, these two proteins can be simultaneously detected by polyclonal anti-α-enolase antibody.

Cell growth and viability assays
For the evaluation of cell growth, cells (3 × 10^4) were seeded onto six-well plates in triplicate and then counted by trypan blue exclusion method at the time indicated. Cell viability was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-dimethyl tetrazolium bromide (MTT, Sigma–Aldrich) assay. Briefly, cells were seeded onto 24-well plates at a density of 1 × 10^4 cells per well in triplicate. After incubation for 24 or 48 h, MTT (0.5 mg/ml) was added to each well for additional 3 h. Thereafter, 250 μl of dimethylsulfoxide was added to each well and then the absorbance at 550 nm was measured using a microplate enzyme-linked immunosorbent assay reader (TECAN Infinite 200).

Colony forming assay
As described previously (14), 4000 cells were used for the assay of anchor-age-independent growth in soft agar. After incubating at 37°C for 14 days, cells were stained with 0.005% crystal violet in phosphate-buffered saline and subsequently the colonies were counted from 10 random fields under the microscope.

Migration and invasion assays
As described previously (14), abilities of cellular migration and invasion were determined in 24-well plates for 12 and 20 h, respectively. Cells (1 × 10^4) for migration assay and 5 × 10^5 (for invasion assay) were seeded onto the upper chamber (8 μm pore size), and the lower chamber contained complete medium. After incubation, cells on the upper surface of the membrane were removed with a cotton swab. The migrated or invaded cells on the underside were fixed with methanol and stained with crystal violet. The numbers of migrated or invaded cells were counted under the microscope from 10 random fields.

Tumorsphere formation assay
For the formation of tumorspheres, a total of 150 and 500 or 1000 cells were suspended in serum-free Dulbecco’s modified Eagle’s medium containing 1% N2, 2% B27, 20 ng/ml human fibroblast growth factor-2 and 20 ng/ml epidermal growth factor (Invitrogen) and subsequently seeded onto 96- and 24-well ultra-low attachment plates (Corning), respectively. Then, cells were incubated at 37°C in stem cell-selective medium for 9 days and supplemented with medium every 3 days. Spheres >50 μm in diameter were counted under the microscope.

Xenografted tumorigenicity assay in nude mice
All animal experiment protocols in this study were carried out in accordance with a protocol approved by the institutional ethical committee (Institutional Animal Care and Use Committee of National Yang-Ming University). Five-week-old BALB/c nu/nu mice were purchased from the National Science Council Animal Center (Taipei, Taiwan) and allowed free access to food and water. Mice were subcutaneously injected at both hind limbs with 3 × 10^6 viable cells in a total volume of 0.1 ml of phosphate-buffered saline. The volume of xenografts was estimated every 3 days (14,26). The expression of mature miR-363 in excised tumor samples was detected by miRNA quantitative real-time PCR.

In vivo tail vein metastasis assay
Female non-obese diabetic severe-combined immunodeficiency mice (National Taiwan University, Taipei, Taiwan) aged 6 weeks were injected with 1 × 10^6 viable cells by tail vein injection (14). After 11 weeks, the mice were killed and the metastatic nodules in lungs of mice were counted by gross and microscopic examination.

Statistical analysis
Data analyses were performed using Student’s t-test for simple comparison of two values. The difference of results was considered statistically significant when the P value was <0.05.

Results

miR-363 targets the 3′-UTR of MBP-1/α-enolase mRNA
To identify miRNAs that have the potential to regulate gastric carcinogenesis through targeting MBP-1, we employed widely used software such as MicroCosm Targets and PITA algorithms to search for the putative binding sites of miRNAs in the 3′-UTR of human MBP-1/α-enolase mRNA. Our in silico analyses revealed that a putative miR-363-binding site is located at 208–216 nt from the start of MBP-1/α-enolase 3′-UTR (Figure 1A). Additionally, there is the phylogenetic conservation of the putative miR-363-binding sites within 3′-UTRs of MBP-1/α-enolase miRNAs in mammals including human, chimpanzee, monkey, mouse, rat, dog and cow (Figure 1A). Therefore, miR-363 could be a potential regulator of MBP-1 and α-enolase expressions.

To further evaluate whether MBP-1/α-enolase mRNA is a target of miR-363, the adenoviral system exogenously expressing miR-363 was established. The level of miR-363 was significantly increased in SC-M1 gastric cancer cells infected with miR-363-expressing adenoviruses by quantitative real-time PCR analysis compared with those infected with GFP-expressing adenoviruses (Figure 1B, left). The protein amounts of MBP-1 and α-enolase in SC-M1 cells were decreased by miR-363 as revealed by western blot analysis (Figure 1B, right). Furthermore, the levels of COX-2 and c-Myc, target genes of MBP-1 and α-enolase, were enhanced along with the decreased expressions of MBP-1 and α-enolase in the SC-M1 cells infected with miR-363-expressing adenoviruses.

Next, luciferase reporter gene assay was performed to check whether miR-363 targets 3′-UTR of MBP-1/α-enolase mRNA. Various lengths of DNA fragments containing the human MBP-1/α-enolase 3′-UTR were inserted at the rear of luciferase reporter gene to construct pMBP-1/α-enolase 3′-UTR-Luc reporter plasmids (Figure 1C). In SC-M1 cells infected with miR-363-expressing adenoviruses, reporter gene activities were attenuated after transfection with the reporter plasmids containing MBP-1/α-enolase 3′-UTR-Luc from 1 to 356 and 111 to 259 nt, but not those from 1 to 110 and 260 to 356 nt (Figure 1D). These results suggest that DNA sequence from 111 to 259 nt is the critical region of the MBP-1/α-enolase 3′-UTR targeted by miR-363. There is a putative miR-363-binding site located at 208–216 nt in the DNA sequence of this region (Figure 1A). After the deletion or mutation of miR-363-binding site in the DNA sequence,
miR-363 did not exert its suppressive effect on the activity of reporter gene containing MBP-1/α-enolase 3′-UTR (Figure 1D). The protein levels of MBP-1 and α-enolase in the infected and transfected SC-M1 cells were confirmed by western blot analysis (Supplementary Figure S1, available at Carcinogenesis Online). These data strongly demonstrate that miR-363 regulates MBP-1 and α-enolase levels through its binding site in MBP-1/α-enolase 3′-UTR.

Exogenous miR-363 promotes growth and progression of SC-M1 cells via MBP-1 or α-enolase

To investigate whether miR-363 regulates growth of gastric cancer cells, trypan blue exclusion method was performed first. The cumulative numbers of SC-M1 cells infected with miR-363-expressing adenoviruses were higher than those infected with GFP-expressing adenoviruses after infection for 6 or 9 days (Figure 2A, left). Results of MTT assay showed that the viability of SC-M1 cells was enhanced after infection with miR-363-expressing adenoviruses for 48 h compared with those cells infected with GFP-expressing adenoviruses (Figure 2A, right). Subsequently, it was further addressed whether the miR-363-induced growth and viability of gastric cancer cells were dependent on MBP-1. Compared with SC-M1/pcDNA3 control cells, the augmented effect on growth and viability by miR-363 was attenuated in MBP-1-expressing SC-M1 cells (SC-M1/HA-MBP-1 cells) as determined by trypan blue exclusion method (Figure 2B, left) and MTT assay (Figure 2B, right), respectively.

To evaluate whether miR-363 is also involved in gastric cancer progression through targeting MBP-1, SC-M1 cells were infected with adenoviruses expressing miR-363 or GFP for reporter gene assay. Means of three independent experiments performed at least in triplicate are shown. **P < 0.01; ***P < 0.001 compared with cells infected with adenoviruses expressing GFP.

miR-363 knockdown suppresses tumor progression of SC-M1 cells through MBP-1 or α-enolase

We also sought to check whether the endogenous miR-363 is indeed involved in gastric carcinogenesis. The antagoni-miR-363 reagent, chemically modified antisense RNA oligonucleotides, was employed to inhibit the function of endogenous miR-363. Transient transfection with antagoni-miR-363 significantly knocked down miR-363 expression in SC-M1 cells by analysis of miRNA quantitative real-time PCR (Figure 3A, left). In addition, MBP-1 and α-enolase levels were increased after transfection with antagoni-miR-363 in SC-M1 cells, whereas COX-2 and c-Myc expressions were suppressed (Figure 3A, right). Growth and viability of SC-M1 cells were suppressed after transfection with antagoni-miR-363 (Figure 3B). The suppressive effect of...
miR-363 promotes growth and progression of SC-M1 cells through downregulation of MBP-1. (A) SC-M1 cells infected with adenoviruses expressing miR-363 or GFP were seeded and then counted by trypan blue exclusion method at the time indicated (left). The infected cells were also seeded onto 24-well plates and incubated for 24 or 48 h. Then, cell viability was analyzed by MTT assay (right). **P < 0.01 compared with cells infected with adenoviruses expressing GFP. (B) MBP-1-expressing SC-M1 cells (SC-M1/HA-MBP-1 cells) and control cells (SC-M1/pCDNA3 cells) were infected with adenoviruses expressing miR-363 or GFP and then seeded for evaluation of growth by trypan blue exclusion method (left) and cell viability by MTT assay (right). *P < 0.05; **P < 0.01 compared with control cells infected with adenoviruses expressing GFP. (C) After transfection with miR-363-insensitive MBP-1-expressing construct (pcDNA-HA-MBP-1) or α-enolase-expressing construct (pcDNA-HA-α-enolase), SC-M1 cells infected with adenoviruses expressing miR-363 or GFP were seeded for colony formation (upper), migration (middle) and invasion (lower) assays. *P < 0.05; **P < 0.01; ***P < 0.001 compared with cells transfected with mock and infected with adenoviruses expressing GFP. #P < 0.05; ##P < 0.01 compared with cells transfected with mock and infected with adenoviruses expressing miR-363. (D) SC-M1/HA-MBP-1 cells and SC-M1/pCDNA3 control cells were infected with adenoviruses expressing miR-363 or GFP and then seeded for colony formation (upper), migration (middle) and invasion (lower) assays. Means of at least three independent experiments performed in triplicate are shown. *P < 0.05; **P < 0.01 compared with control cells infected with adenoviruses expressing GFP. *P < 0.05; **P < 0.01 compared with control cells infected with adenoviruses expressing miR-363.

miR-363 knockdown on growth and viability of SC-M1 cells was restored after cotransfection with siRNA vectors against MBP-1 and α-enolase (#22 and #24). Moreover, the reduction of colony formation, migration, and invasion abilities in SC-M1 cells by antagonism-363 was also rescued by knockdown of MBP-1 and α-enolase (Figure 3C and Supplementary Figure S2, available at Carcinogenesis Online). Thus, miR-363 knockdown upregulates MBP-1 and α-enolase expressions and diminishes growth as well as progression of gastric cancer cells via MBP-1 or α-enolase.

miR-363 is also involved in gastric cancer progression of KATO III and SNU-16 cells

Besides SC-M1 cells, we also checked whether miR-363 participates in tumor progression of the other gastric cancer cells. The relative levels of endogenous miR-363 in human gastric cancer cell lines were determined using miRNA quantitative real-time PCR including SC-M1, KATO III, SNU-16, and NCI-N87 cells. Results showed that miR-363 was expressed in SC-M1 and SNU-16 cells but hardly detected in KATO III and NCI-N87 cells (Supplementary Figure S3A, left, available at Carcinogenesis Online). Data of western blot analysis showed that MBP-1 and α-enolase were differentially expressed in these gastric cancer cells (Supplementary Figure S3A, right, available at Carcinogenesis Online). Notably, MBP-1 and α-enolase levels were inversely proportional to miR-363 expressions in these gastric cancer cells.

In this study, the role of miR-363 in gastric cancer progression was further evaluated in KATO III cells scarcely expressing miR-363 and SNU-16 cells expressing miR-363 by overexpression and knockdown, respectively. Using adenoviral system expressing miR-363 or GFP, the growth and viability of KATO III cells were augmented by miR-363 as determined by trypan blue exclusion method and MTT assay, respectively (Supplementary Figure S3B, available at Carcinogenesis Online). The increment of growth and viability in KATO III cells by miR-363 was attenuated by transfection with miR-363-insensitive MBP-1- or α-enolase-expressing constructs. The growth and viability of SNU-16 cells were inhibited after transfection with antagonism-363, and the inhibition was relieved by cotransfection with siRNA vectors against MBP-1 and α-enolase (Supplementary Figure S3C, available at Carcinogenesis Online). Furthermore, the colony formation, migration and invasion abilities of KATO III cells were promoted by miR-363 (Supplementary Figure S3D, available at Carcinogenesis Online). The miR-363-enhanced ability of gastric cancer progression in KATO III cells was suppressed after transfection with the expression constructs of miR-363-insensitive MBP-1 or α-enolase. Ability of gastric cancer progression was attenuated after transfection with antagonism-363 in SNU-16 cells (Supplementary Figure S3E, available at Carcinogenesis Online). The inhibition of gastric cancer progression by miR-363 knockdown in SNU-16 cells was rescued after cotransfection with siRNA vectors against MBP-1 and α-enolase.
miR-363 enhances EMT of SC-M1 cells through MBP-1

We also examined whether miR-363 is involved in the control of EMT. After infection with miR-363-expressing adenoviruses for 48 h, the infected SC-M1 cells grew dispersedly and had a spindle- and fibroblast-like morphology compared with those infected with GFP-expressing adenoviruses (Figure 4A, left). Consistently, the levels of epithelial markers E-cadherin and plakoglobin were decreased by miR-363 along with the enhanced expressions of mesenchymal markers N-cadherin and vimentin in SC-M1 cells, as revealed by western blot analysis (Figure 4A, right). The tightly packed morphology of SC-M1/pcDNA3 control cells became obviously extended and elongated by miR-363 (Figure 4B, left). However, the effect of miR-363 on morphologic change was abolished in MBP-1-expressing SC-M1/HA-MBP-1 cells. Similarly, the blockade of epithelial marker levels along with the increment of mesenchymal marker expressions was exerted by miR-363 in SC-M1/pcDNA3 control cells but not in MBP-1-expressing SC-M1/HA-MBP-1 cells using western blot analysis (Figure 4B, right). These results demonstrate that miR-363 induces EMT of SC-M1 gastric cancer cells depending on MBP-1.

miR-363 elevates ability of tumorsphere formation in gastric cancer cells via MBP-1

The presence of gastric cancer stem-like cells was shown in several gastric cancer cells such as AGS, KATO III and NUGC-3 cells (27,28). Herein, we also checked whether miR-363 affects the maintenance of cancer stem-like phenotype in gastric cancer cells. No definitely specific markers of gastric cancer stem cells have been identified and widely accepted thus far. Moreover, it was found that isolation of cancer stem cells using surface markers did not provide robust stem cells of cancers including gastric and colorectal cancers (29,30). Therefore, we examined the ability of tumorsphere formation in unsorted SC-M1 cells under non-adherent condition with stem cell-selective medium. The tumorspheres of SC-M1 cells were found after incubation for 9 days under stem cell-selective conditions (Supplementary Figure S4, available at Carcinogenesis Online). The mRNA expressions of stemness-associated markers including SOX-2, Oct4, Nanog and CD44 were enhanced in tumorspheres of SC-M1 cells by quantitative real-time PCR analysis compared with parental cells.
miR-363 enhances gastric carcinogenesis through MBP-1

After infection with adenoviruses expressing miR-363 or GFP, the infected SC-M1 cells were seeded and then cultured under stem cell-selective conditions for tumorsphere formation assay. Data showed that miR-363 significantly elevated the ability of formation of the first-, second- and third-generation tumorspheres in SC-M1 cells (Figure 5A). Moreover, the ability of tumorsphere formation in SC-M1/pcDNA3 control cells was enhanced after infection with miR-363-expressing adenoviruses (Figure 5B). The enhanced ability of tumorsphere formation by miR-363 was relieved in MBP-1-expressing SC-M1/HA-MBP-1 cells. Likewise, the miR-363-enhanced ability of tumorsphere formation in SC-M1 cells was suppressed after transfection with miR-363-insensitive MBP-1- or α-enolase-expressing constructs (Figure 5C). Alternatively, the ability of tumorsphere formation in SC-M1 cells was inhibited after transfection with antagonmir-363 and the inhibition was rescued by cotransfection with siRNA vectors against MBP-1 and α-enolase (Figure 5D).

The relative abilities of tumorsphere formation in gastric cancer cell lines were also evaluated under normal medium and stem cell-selective conditions including SC-M1, KATO III, SNU-16 and NCI-N87 cells. No tumorsphere was formed in these cells under normal medium (Supplementary Figure S5, available at Carcinogenesis Online). Among these cells, SNU-16 cells exerted the strongest ability of tumorsphere formation under stem cell-selective conditions, whereas KATO III cells and NCI-N87 cells exhibited the weakest ability (Figure 5E). Interestingly, the abilities of tumorsphere formation in these cells were positively correlated with miR-363 levels, which were inversely associated with MBP-1 and α-enolase expressions (Supplementary Figure S3A, available at Carcinogenesis Online).

The mRNA expressions of SOX-2, Oct4, Nanog and CD44 were also promoted in tumorspheres of KATO III, SNU-16 and NCI-N87 cells compared with parental cells (Supplementary Figure S4, available at Carcinogenesis Online). Furthermore, the miR-363-enhanced ability of tumorsphere formation was suppressed after transfection with the expression constructs of miR-363-insensitive MBP-1 or α-enolase in KATO III cells scarcely expressing miR-363 (Figure 5F). The inhibited ability of tumorsphere formation by miR-363 knockdown was rescued after cotransfection with siRNA vectors against MBP-1 and α-enolase in miR-363-expressing SNU-16 cells (Figure 5G).

miR-363 induces tumor growth and lung metastasis of SC-M1 cells through MBP-1

To further assess the effect of miR-363 on tumor growth of gastric cancer cells in vivo, the xenografted tumor growth in the case of subcutaneous injection with SC-M1 cells infected with adenoviruses expressing miR-363 or GFP was explored in nude mice. The tumor sizes of miR-363-expressing SC-M1 cells were larger than those of control cells (Figure 6A, left and middle). On day 27 after implantation, the mice were killed and then subcutaneous tumors were excised for the detection of miR-363 expression. Results of miRNA quantitative real-time PCR showed that miR-363 level in mice injected with miR-363-expressing SC-M1 cells was higher than those injected with control cells (Figure 6A, right). Additionally, the increment of tumor growth by miR-363 was observed in SC-M1/pcDNA3 control cells (Figure 6B, left and middle). However, the enhanced xenografted tumor sizes by miR-363 were attenuated in MBP-1-expressing SC-M1/HA-MBP-1 cells. On day 27 postinjection of tumor cells,
miR-363 levels in the excised tumors of mice injected with miR-363-expressing SC-M1/HA-MBP-1 or SC-M1/pDNA3 cells were higher than those injected with GFP-expressing cells (Figure 6B, right).

To investigate the effect of miR-363 on metastatic colonization of gastric cancer cells, MBP-1-expressing SC-M1/HA-MBP-1 or SC-M1/pDNA3 control cells were intravenously injected into lateral tail vein of non-obese diabetic severe-combined immunodeficiency mice. Eleven weeks later, mice were killed for evaluation of metastatic nodules in lungs. Data showed that mice injected with miR-363-expressing SC-M1/pcDNA3 control cells have numerous large metastatic nodules in lungs compared with mock and infected with adenoviruses expressing miR-363. SC-M1/pcDNA3 control cells infected with adenoviruses expressing miR-363 were seeded for the subsequent assay of tumorsphere formation as described previously. \( *P < 0.05; \ **P < 0.01 \) compared with control cells infected with adenoviruses expressing GFP. (D) After cotransfection with antisense miR-363 and siRNA vectors against MBP-1 as well as alpha-enolase, SC-M1 cells were seeded for the subsequent assay of tumorsphere formation as described previously. \( *P < 0.05; \ **P < 0.01 \) compared with cells transfected with mock and infected with adenoviruses expressing miR-363. \( \#P < 0.05; \ ##P < 0.01 \) compared with cells transfected with siRNA vector against luciferase and antagonir-363. (E) The assay of tumorsphere formation in SC-M1, KATO III, SNU-16 and NC1-N87 cells was performed as described previously. (F) After transfection with miR-363-insensitive MBP-1- or alpha-enolase-expressing constructs, KATO III cells infected with adenoviruses expressing miR-363 were seeded for the subsequent assay of tumorsphere formation as described previously. \( *P < 0.01; \ **P < 0.001 \) compared with cells transfected with mock and infected with adenoviruses expressing GFP. \( \#P < 0.01; \ ##P < 0.001 \) compared with cells transfected with siRNA vector against luciferase and antagonir-363. (G) After transfection with miR-363-expressing or GFP-expressing cells, MBP-1-expressing SC-M1/HA-MBP-1 cells were seeded for the subsequent assay of tumorsphere formation as described previously. \( *P < 0.05; \ **P < 0.01 \) compared with cells transfected with mock infected with adenoviruses expressing GFP. \( \#P < 0.05; \ ##P < 0.01 \) compared with cells transfected with siRNA vector against luciferase and antagonir-363.

Discussion

The mechanisms regarding the regulation of gastric cancer progression are very complicated and still remain unclear. Previously, we had found that MBP-1 suppresses growth and metastasis of gastric cancer cells (14). It was demonstrated that miRNAs may exhibit oncogenic or tumor-suppressive roles in tumorigenesis (18). To decipher the molecular mechanism for aggressiveness in gastric cancer, we identified the MBP-1-related miRNAs and characterized their roles in gastric carcinogenesis. We proved that miR-363 plays an oncogenic role in gastric cancer progression through MBP-1, which acts as a tumor suppressor. Till date, there were few reports regarding the cross talk between miRNAs and MBP-1/alpha-enolase in tumorigenesis. In breast cancers, miR-17/20 cluster reduces the expression of secreted alpha-enolase that is involved in cell migration and invasion (31). MBP-1 increases the expression of miR-29b that inhibits Mcl-1, collagens and matrix-metalloproteinase-2 in prostate cancer cells (24). Results of this study showed that the functional cross talk between miR-363 and MBP-1 is crucial for the control of tumor development and progression in gastric cancer.

Several studies had revealed the possible regulatory functions of miR-363 in diverse biological processes. The miR-363 is significantly upregulated in human uterine leiomyoma (32) and downregulated in CD4+ T cells of patients with rheumatoid arthritis (33). It is also upregulated in CD4+ and CD8+ cells of cord blood compared with adult peripheral blood (34). The miR-363 is downregulated in human papillomavirus (HPV)-transfected keratinocyte HaCaT cells, but upregulated in HPV-positive pharyngeal squamous cancers (35) and in HPV-positive
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squamous cell carcinoma of the head and neck as well as normal oral keratinocytes compared with those of HPV-negative cells (36). The miR-363 mimic reduces the growth of natural killer lymphoma cells (37). Additionally, miR-363 targets the 3'-UTRs of Hand2 as well as Tbx3 and is involved in limb bud positioning in mouse embryonic mesoderm (38). The miR-363 suppresses head and neck cancer progression

Fig. 6. miR-363 induces tumor growth and metastasis of SC-M1 cells through MBP-1 in vivo. (A and B) After infection with adenoviruses expressing miR-363 or GFP, the viable SC-M1 (A) or SC-M1/HA-MBP-1 cells and SC-M1/pDNA3 control cells (B) were subcutaneously injected into nude mice (n = 5 per group) for measurement of tumor sizes at the time indicated (left). On day 27, the mice were killed and subcutaneous tumors were excised for the detection of miR-363 expression using miRNA quantitative real-time PCR (middle and right). Data are representative of three experiments. Bar, 1.0 cm. *P < 0.05; **P < 0.01 compared with cells infected with adenoviruses expressing GFP. (C) For measurement of metastatic nodules in lungs, non-obese diabetic severe-combined immunodeficiency mice (n = 6 per group) were injected with the viable SC-M1/HA-MBP-1 cells and SC-M1/pDNA3 control cells infected with adenoviruses expressing miR-363 or GFP by tail vein injection. After 11 weeks, the mice were killed and the metastatic nodules in the lungs were counted by gross and microscopic examination. Data are from a representative experiment that was performed three times with identical results. *P < 0.05; **P < 0.01 compared with control cells infected with adenoviruses expressing GFP.
by targeting podoplanin (39). We also show herein that miR-363 contributes to tumorigenesis and progression of gastric cancer via MBP-1.

The miRNAs regulate the stemness of cancer stem cells, which are implicated in tumor progression and metastasis. For example, miR-34a suppresses property of cancer stem cells via CD44 (17). There were multiple markers used to isolate cancer stem cells from gastric cancer cell lines or tumor tissues and peripheral blood of patients with gastric cancer (27,40). Although the isolated cells in these reports exhibit stem cell-like phenotypes, the definitely specific markers of gastric cancer stem cells have not been identified. Our results clearly showed that miR-363 increases the ability of tumorsphere formation in gastric cancer cells via MBP-1 (Figure 5). These results imply that miR-363-MBP-1 axis might also play a critical role in the maintenance of cancer stem-like phenotype in gastric cancer cells.

It is estimated that each miRNA might target a large number of genes (18). The 3′-UTR of an miRNA frequently contains several putative miRNA-binding sites and the combinatorial miRNAs regulate gene expression (41). Besides interacting with 3′-UTRs, miRNAs can bind to the open reading frames as well as 5′-UTRs of the target genes, ribonucleoproteins, and DNAs to carry out their biological functions (18). They also have been found to activate gene expression. Additionally, the miRNA genes contain their own promoters and regulatory elements or are transcribed along with host genes. An aberrant expression of miRNAs in cancer could be the result of deregulation by miRNA processing, epigenetical DNA methylation, histone modification and transcription factors (18). Thus, the control of gene expression by miRNAs could be a complicated network in this scenario. The regulatory mechanism of miR-363 expression still remains obscure and further studies are required to dissect it.

The development of miRNA-based therapy for the treatment of cancers could be foreseeable in the future (18). It is reasonable to restore miRNAs acting as tumor suppressors or downregulate those acting as oncogenes. Effectively, miRNA expression is antagonized by antagonomers in mice (42) and attenuated by an unconjugated locked-nucleic-acid-modified oligonucleotide in the liver of non-human primates (43). Numerous reports had documented that miRNAs may also act as potentially diagnostic and prognostic markers of gastric cancer (19,20). An identified seven-miRNA signature is correlated with overall and relapse-free survival of patients with gastric cancer (44). Possibly, the prognostic signature would be beneficial to decide whether further adjuvant treatment is necessary for gastric cancer patients besides surgical resection (44). However, it might be heterogeneous in expression profile of miRNAs in different sample types and stages of gastric cancer (45). The regulation of gene expression by miRNAs is complex and wide as described previously. Therefore, the development of miRNA-based therapy for gastric cancer remains a scientific and clinical challenge.

MBP-1 blocks c-Myc and COX-2 expressions and is a potential candidate for gene therapy against tumor growth (10,12,14). Both c-Myc (46) and COX-2 (47) are upregulated in gastric cancer and significantly associated with the presence of distant metastasis. Their levels are upregulated by overexpression of miR-363, which targets MBP-1 (Figure 1), whereas downregulated by knockdown of miR-363 (Figure 3A). The miR-363-enhanced biological functions of gastric cancer cells are decreased dramatically by MBP-1 overexpression (Figures 2 and 4–6). Therefore, the miR-363-MBP-1-c-Myc/COX-2 axis may play a critical role in the control of gastric carcinogenesis. The specific inactivation of MYC is a promising therapeutic strategy for various tumors (48,49). Inhibitors of COX-2 also exert a possible role in prevention and treatment of gastric cancer (50), but the susceptibility of gastrointestinal toxicity and increase of cardiovascular risk need consideration. It is possible that gene therapy targeting miR-363, MBP-1 and c-Myc in combination with COX-2 inhibitors could offer a new therapeutic strategy for treatment with gastric cancer in the future.

Supplementary material
Supplementary Figures S1–S5 can be found at http://carcin.oxfordjournals.org/

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References
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