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Angiogenesis, Metastasis, and the Cellular Microenvironment

Molecular Cancer Research

miRNA-223 Promotes Gastric Cancer Invasion and Metastasis by Targeting Tumor Suppressor EPB41L3

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Abstract

Traditional research modes aim to find cancer-specific single therapeutic target. Recently, emerging evidence suggested that some micro-RNAs (miRNA) can function as oncogenes or tumor suppressors. miRNAs are single-stranded, small noncoding RNA genes that can regulate hundreds of downstream target genes. In this study, we evaluated the miRNA expression patterns in gastric carcinoma and the specific role of miR-223 in gastric cancer metastasis. miRNA expression signature was first analyzed by real-time PCR on 10 paired gastric carcinomas and confirmed in another 20 paired gastric carcinoma tissues. With the 2-fold expression difference as a cutoff level, we identified 22 differential expressed mature miRNAs. Sixteen miRNAs were upregulated in gastric carcinoma, including miR-223, miR-21, miR-23b, miR-222, miR-25, miR-23a, miR-221, miR-107, miR-103, miR-99a, miR-100, miR-125b, miR-92, miR-146a, miR-214 and miR-191, and six miRNAs were downregulated in gastric carcinoma, including let-7a, miR-126, miR-210, miR-181b, miR-197, and miR-30aa-5p. After examining these miRNAs in several human gastric originated cell lines, we found that miR-223 is overexpressed only in metastatic gastric cancer cells and stimulated nonmetastatic gastric cancer cells migration and invasion. Mechanistically, miR-223, induced by the transcription factor Twist, posttranscriptionally downregulates EPB41L3 expression by directly targeting its 3′-untranslated regions. Significantly, overexpression of miR-223 in primary gastric carcinomas is associated with poor metastasis-free survival. These findings indicate a new regulatory mode, namely, specific miRNA, which is activated by its upstream transcription factor, could suppress its direct targets and lead to tumor invasion and metastasis. *Mol Cancer Res; 9(7); 824–33. ©2011 AACR.*

Introduction

Metastasis is a process whereby cancer cells spread from a primary site and form tumors at distant sites (1, 2). It occurs through a specific series of steps, starting with local invasion, followed by entrance of cancer cells into the bloodstream (intravasation), survival in the circulation, exit from blood vessels (extravasation), initiation, and maintenance of micrometastases at distant sites and finally, vascularization of the resulting tumors (3).

Although the incidence of gastric cancer declined from the 1940s to the 1980s, it remains the most common epithelial malignancy and leading cause of cancer-related death (4). Micro-RNAs (miRNA) are a wide class of small, noncoding RNAs that negatively regulate protein expression at the posttranscriptional level. Through the specific targeting of the 3′-UTR (untranslated region) of multicellular eukaryotic mRNAs, miRNAs downregulate gene expression by either inducing degradation of target mRNAs or impairing their translation (5). The expression of some miRNAs has been shown to be temporally and spatially regulated, whereas the disruption of their physiologic expression patterns was associated with several examples of human tumorigenesis, suggesting that they may play a role as a novel class of oncogenes or tumor suppressor genes (6). However, these studies are all focused on the non-physiologic modulation of miRNA expression in different cancer types, thus making the comprehension of miRNA function in cancer malignant progression, especially in metastasis, an important goal.

Here we show that human miR-223, which was regulated by the transcription factor Twist, stimulated gastric cancer cell migration and invasion *in vitro* and *in vivo*, and that certain cancer cell lines depend on endogenous miR-223 activity to migrate efficiently. Mechanistically, the migration phenotype of miR-223 can be explained to inhibit erythrocyte membrane protein band 4.1-like 3 (EPB41L3).

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We also confirmed that overexpression of miR-223 is associated with poor distal metastasis-free survival. Taken together, our findings indicate that miRNAs are involved in tumor migration and invasion, and implicate miR-223 as a metastasis-promoting miRNA.

Materials and Methods

Tissue specimens and TaqMan real-time PCR

Thirty formalin-fixed paraffin-embedded specimens of gastric cancer tissues, the matched normal tissues, and information about the patients were collected from Gastrointestinal Surgery in Xijing hospital, Xi′an, China. Primary gastric cancer in these patients was diagnosed and treated at Xijing hospital from 1990 to 1997. The matched "normal gastric tissue" were obtained from a 5 centimeter distance from the tumor margin, which were further confirmed by pathologist that they do not have tumor cells. The protocols used in the study were approved by the Hospital's Protection of Human Subjects Committee. Total RNA, with efficient recovery of small RNAs, was isolated from 20-μm sections from formalin-fixed, paraffin-embedded tissue blocks, using the Recover All Total Nucleic Acid Isolation Kit (Ambion). Stem-loop reverse transcription for mature miRNA was done as described previously (7). All reagents were obtained from Applied Biosystems. The TaqMan Human miRNA Array v1.0 (Early Access) containing 368 TaqMan miRNA Assays enabling accurate quantitation of 365 human miRNAs in less than 3 hours were used in this study. Briefly, 5 ng of total RNA were reverse transcribed to cDNA with stem-loop primers and the TaqMan miRNA Reverse Transcription Kit. Quantitative real-time PCR (qRT-PCR) was done by using an Applied Biosystems 7500 Real-time PCR System and a TaqMan Universal PCR Master Mix. All PCR primers were from the TaqMan miRNA Assays. miRNAs expression was quantified in relation to the expression of small nuclear U6 RNA as previously reported (8, 9).

Cell lines

The HEK293 cell, GPG29 cell, spontaneously immortalized normal gastric mucosa GES cell, normal stomach fibroblastic cell NSFC, nonmetastatic gastric cancer SUN-1, KATO-III, NUGC-3 cells, and metastatic gastric cancer XGC-9811L (10), AGS, and N87 cells were used in this study and maintained according to the supplier's protocols. All transfections were done by using Lipofectamine 2000 (Invitrogen).

Generation of retrovirus, miRNA-expressing cells, and knockdown cells

The plasmid vectors encoding the miR-223 were constructed as R. Agami (11) previously described. The production of amphotropic viruses and infection of NUGC-3 cells were described previously (12). For *in vitro* miRNA inhibition studies, XGC-9811L cells were transfected with antagomirs (25 nmol/L; Ambion) based on the manufacturer's recommendations. Forty-eight hours after transfection, cells were plated for migration and invasion assays, or harvested for the luciferase reporter assay. For *in vivo* miRNA metastasis studies, transfected cells were inoculated into immunodeficient mice via tail-vein injection 4 days after transfection.

EPB41L3 plasmids construction and cell transfection

The EPB41L3 expression vector pcDNA-3.1-EPB41L3 was constructed by inserting its open reading frame sequence into the pcDNA-3.1 vector (Invitrogen). pSilencer3.0 (Ambion) was used for construction of human EPB41L3 siRNA vector psiEPB41L3 according to manufacturer's protocol. One pair of specific oligonucleotide (EPB41L3, EPB41L3′) was annealed and then subcloned into the *Bam*HI/*Hind*III cloning site of pSilencer3.0. Cell transfection was done with Lipofectamine 2000 (Invitrogen) as described in manufacturer's protocol.

RNA isolation and miRNA detection

Total RNA from cultured cells, with efficient recovery of small RNAs, was isolated by using the mirVana miRNA Isolation Kit (Ambion). Detection of the mature form of miR-223 was done by using the mirVana qRT-PCR miRNA Detection Kit and qRT-PCR Primer Sets, according to the manufacturer's instructions (Ambion). The U6 small nuclear RNA was used as an internal control.

Migration and invasion assay

For transwell migration assays, 1×10^5 cells were plated in the top chamber with the noncoated membrane (24-well insert; 8-mm pore size; Corning Costar Corp). For invasion assays, 2×10^5 cells were plated in the top chamber with Matrigel-coated membrane (24-well insert; 8-mm pore size; Corning Costar Corp). In both assays, cells were plated in medium without serum. Medium supplemented with serum was used as a chemoattractant in the lower chamber. The cells were incubated for 24 hours and cells that did not migrate or invade through the pores were removed by a cotton swab. Cells on the lower surface of the membrane were fixed with methanol and stained with hematoxylin.

Validation of metastasis-promoting activity of miR-223 *in vivo*

Mice were handled by using best humane practices and were cared for in accordance with NIH Animal Care and Use Committee guidelines. Mice were injected with 1×10^6 cells in 0.1 mL PBS through tail vein. The mice were then monitored for overall health and total body weight. After 4 weeks of injection, the mice were sacrificed. The liver tissues were observed with naked eyes and the number of visible tumors in liver surface was counted. The liver tissues were made into serial sections before being HE dyed and observed under a light microscope. Each experimental group contained 6 to 10 mice.

Luciferase reporter assay

The binding site for miR-223, the EPB41L3–3′-UTR sequence were cloned into the pMIR-REPORT luciferase construct (Ambion). Cells of 50% confluence in 24-well

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plates were transfected. Firefly luciferase reporter gene construct (200 ng) and 1 ng of the pRL-SV40 *Renilla* luciferase construct (for normalization) were cotransfected per well. Luciferase activity was measured 48 hours after transfection by using the Dual-Luciferase Reporter Assay System (Promega).

Chromatin immunoprecipitation assay

Myc-tagged Twist was provided by L-H. Wang. Chromatin immunoprecipitation (ChIP) was done with HEK293 cells transfected with a vector expressing Myc–Twist or the Myc tag alone, using a ChIP Assay Kit (Upstate), according to the manufacturer's instructions. Protein–DNA complexes were precipitated with control immunoglobulin G or anti-Myc antibody (Covance). PCR was done with primers specific for human miR-223.

Immunoblotting

Total cell lysate was prepared in 1×SDS buffer. Proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were then blotted with individual antibodies, including antibodies against EPB41L3 (Santa Cruz Biotechnology) diluted 1:100 and β-actin (Sigma) diluted 1:5,000. The bands were visualized by using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech) according to the instructions of the manufacturer.

Statistical analysis

Student's *t* test was used to investigate the significance of the difference between the covariates. The Kaplan–Meier method was done to calculate survival durations of the patients. Survival durations in the patient groups were calculated with the log-rank test. All statistical analyses were conducted by using SPSS 11.05 software. The value of $P < 0.05$ was considered as statistically significant and error bars represent SEM. All experiments were repeated 3 times and a representative experiment result was shown with SEM.

Results

miR-223 is highly expressed in metastatic gastric cancer cells

Initially, miRNA expression signature was analyzed in 10 pairs of primary gastric cancers and corresponding noncancerous gastric tissues by a stem-loop real-time PCR based miRNA expression profiling method by using the TaqMan Human miRNA Array v1.0 Early Access Kit from Applied Biosystems, which includes 365 identified human miRNAs. For more accurate and reliable results, the U6 small nuclear RNA was used to normalize the miRNA expression data.

When we compared miRNA expression among gastric cancer tissues versus corresponding noncancerous gastric tissues, 32 of the 365 miRNAs showed differential expression between the 2 groups, with *P* values derived from the nonparametric Wilcoxon/Kruskal–Wallis test being less than 0.05. To validate overall differences in miRNA expression between tumor and nontumorous tissue, we measured the expression levels of these 32 miRNAs in tumor and paired nontumorous tissue in the independent validation cohort of 20 patients with incident gastric cancer recruited from another cancer center in Xijing hospital. The expression of the miRNAs identified in the primary testing set was almost fully confirmed in this new set of samples. Data analysis of the combined 30 paired tumor samples indicated that 22 of the 32 miRNAs were significantly differentially expressed between the tumor and normal groups by using U6 small RNA for normalization following correction for multiple testing by using the Tukey–Kramer method (Fig. 1A).

To identify miRNAs that specifically regulate gastric cancer metastasis, we investigated the expression of these candidate miRNAs in a series of human gastric cells and tumor cell lines by real-time PCR (RT-PCR; data not shown). Out of 22 upregulated miRNAs, miR-223 was found to be highly expressed only in metastatic gastric cancer cells when compared with the either spontaneously immortalized normal gastric mucosa GES cells, normal stomach fibroblastic cells, or nonmetastatic gastric cancer cells (Fig. 1B and Supplementary Table S1). The expression level of miR-223 was 80-fold higher in XGC-9811L cells, which are capable of metastasizing to liver, than in cells of the NUGC-3, which have little metastatic potency (Fig. 1C). This specific expression mode indicated that miR-223 might play initiated role in gastric cancer metastasis.

miR-223 promotes gastric cancer cells metastasis and significantly correlates with gastric cancer metastasis-free relapse

Cell proliferation, migration, and invasion are among the common functions required by tumor cells for metastatic progression in target microenvironments. We first carried out *in vitro* function analyses by silencing the miRNAs with antisense oligonucleotides or upregulating of miR-223 expression with retrovirus. We assessed the level of miRNA silencing by a reporter assay, in which the predicted miRNA-binding site was cloned into the 3′-UTR of a luciferase reporter gene. We found that transfection of the antisense inhibitor for miR-223 in XGC9811L cells caused a 2- to 3-fold increase in the luciferase activity (Fig. 2A), suggesting that the transfected antisense RNA achieved a greater than 50% inhibition of the actions of miR-223. We then used the retrovirus vector to express miR-223 in immortalized GES and in the NUGC-3 cells line. The miR-223 expression level was gauged by RT-PCR (Fig. 2B).

Then, we examined the cell proliferation rates in miR-223 silenced or overexpressed cell lines and found that silencing or upregulating the expression of miR-223 did not alter the proliferation of gastric cancer cells (Fig. 2C). Next, to evaluate the effect of miR-223 on cell adhesion, we investigated the adhering ability of XGC-9811L–anti-miR-223 and NUGC-3–miR-223 with the Matrigel adhesive assay. All the gastric cancer cells bound to Matrigel in a time-dependent manner. However, downregulation of

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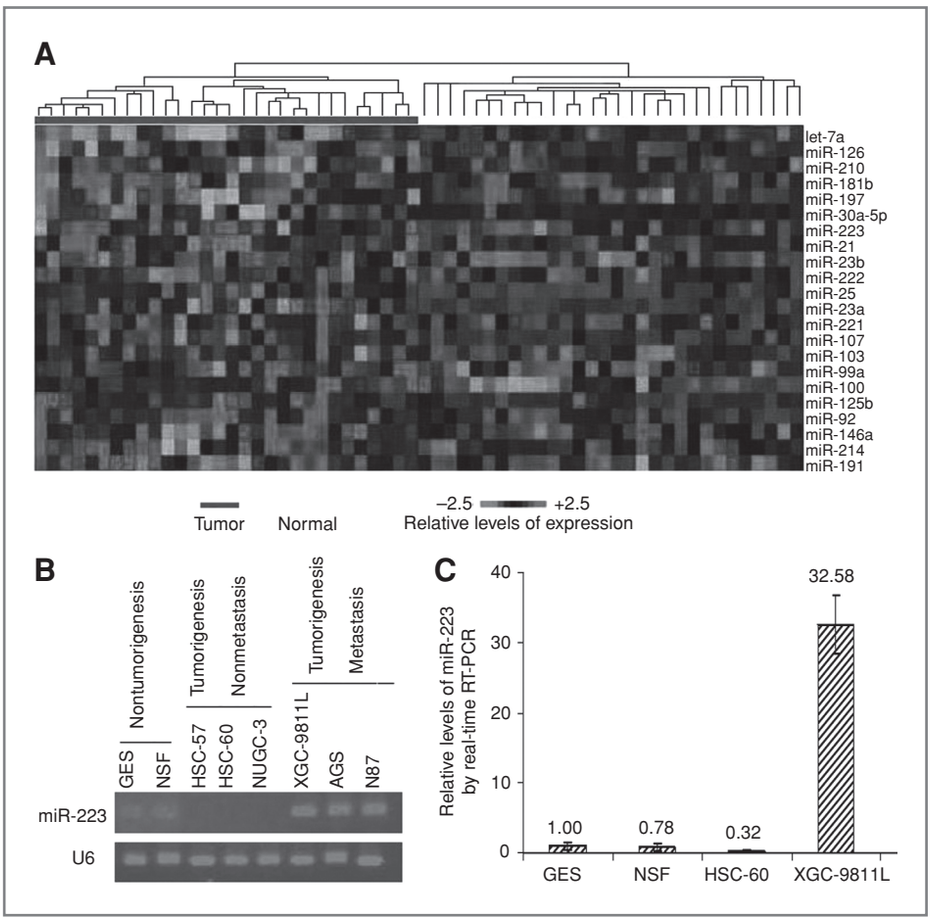

Figure 1 miR-223 is highly expressed in metastatic gastric cancer cells. A, heat map summarizing the patterns of expression for 22 miRNA loci that were differentially expressed in gastric cancers and their paired noncancerous gastric tissues. B, RT-PCR of miR-223 in a series of human gastric tissue originated cells. C, real time RT-PCR of miR-223 in 2 normal gastric tissue cell lines and 2 gastric cancer cell lines.

miR-223 in XGC-9811L decrease their adhesive ability to Matrigel, whereas upregulation of miR-223 in NUGC-3 showed the reverse results (Fig. 2D). Because invasive potential is a common feature in the process of tumor metastasis, we then studied the influence of miR-223 on the invasive ability of gastric cancer cells in *in vitro* invasion assay. As shown in Figure 2E, with the Boyden chamber assay, we found that miR-223 antisense oligonucleotide transfection produced a marked inhibition of invasion of XGC-9811L through Matrigel, whereas miR-223 overexpression significantly promoted the invasion of NUGC-3 through Matrigel. Taken together, these observations suggested that miR-223 function is required for *in vitro* motility and invasiveness but not for viability of these metastatic cells.

Next, the migration and invasion-promoting activity of miR-223 was measured *in vivo*. To address this possibility, an experimental gastric cancer nude mice model was adopted to examine the *in vivo* metastatic ability of XGC-9811L–anti-miR-223 and NUGC-3–miR-223. Compared with control cells transfected with empty vector, i.v. inoculation of XGC-9811L–anti-miR-223 cells led to significantly less visible tumors in liver surface (Fig. 2F and G). Furthermore, we overexpressed miR-223 in otherwise nonmetastatic human gastric cancer cells NUGC-3 to assay the miR-223 metastasis property *in vivo*. The results showed that miR-223–transduced tumor cells metastasized to regional lymph nodes and the liver, whereas control tumor cells only produced localized small tumors (Fig. 2F and G). In conclusion, both *in vitro* invasion assay and *in vivo* nude mice assay suggested that miR-223 had a potential to promote metastasis of gastric cancer.

To determine whether miR-223 is associated with human gastric cancer metastasis, those 30 tumors were stratified based on clinical progression, we found that high miR-223 levels correlated strongly with increased distant disease-free survival relative to tumors with low miR-223 (median survival of 30 months vs. not reached, $P = 0.0122$; Fig. 2H). These results suggested that overexpression of miR-223 is closely related to the increase of gastric cancer metastasis and may play an important role in the pathologic process.

miR-223 is directly regulated by the transcription factor Twist

Now, we want to know how miR-223 expression is regulated. Recently, miRNA and transcription factors have become the hot spot of the molecular biology field. When we focused the metastasis-related transcription factors, we found that several transcription factors previously known as

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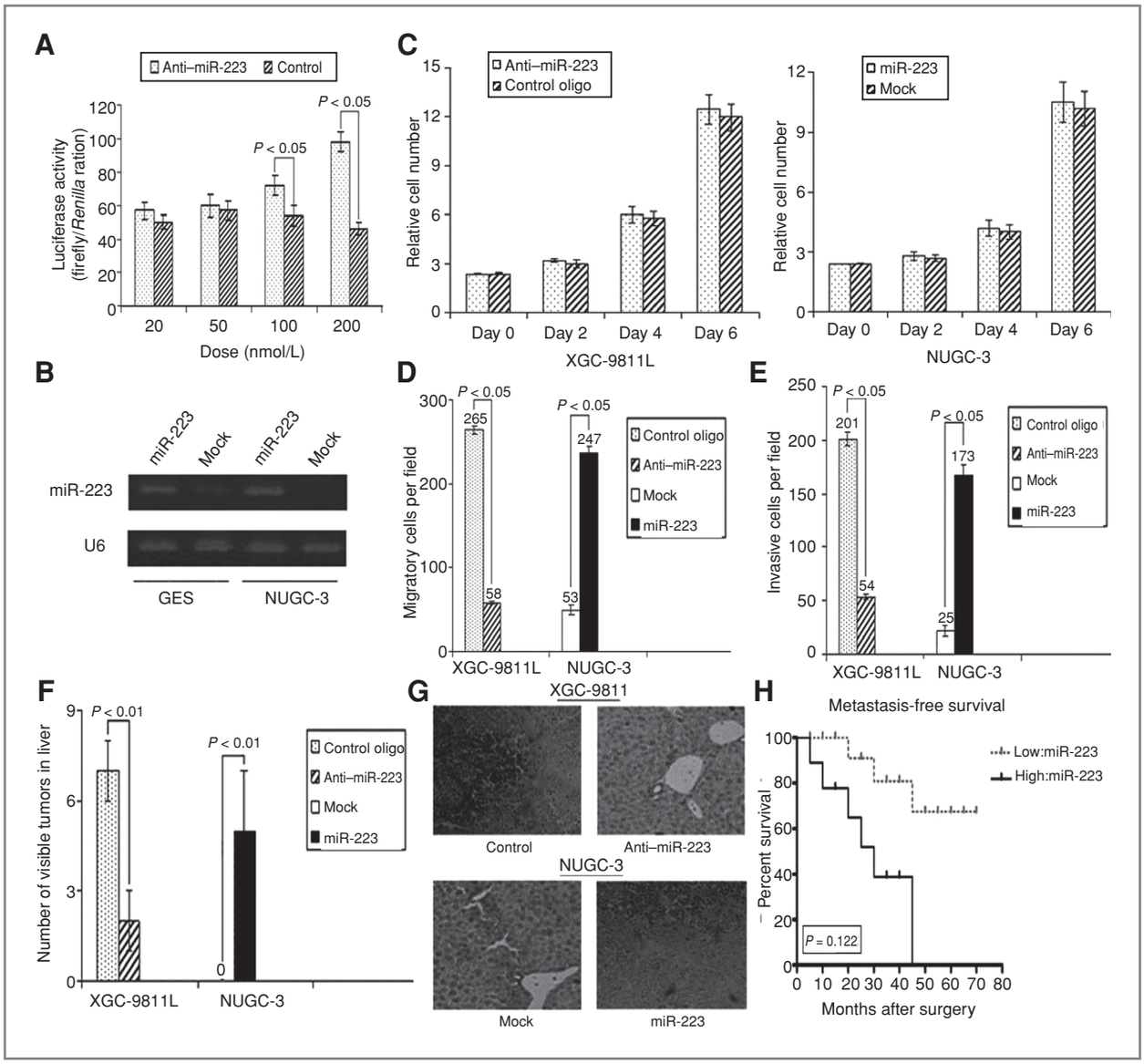

Figure 2. miR-223 promotes gastric cancer cells metastasis and significantly correlates with gastric cancer metastasis-free relapse. A, miRNA inhibitor function for miR-223 was assayed in XGC-9811L cells 48 hours after transfection of anti–miR-223 or control oligonucleotide, using a dual-luciferase reporter system. B, RT-PCR of miR-223 in GES and NUGC-3 cells infected with the miR-223–expressing or empty vector. C, *in vitro* growth curves of XGC-9811L (left) and NUGC-3 (right) transfected cells. D and E, transwell migration assay (D) and Matrigel invasion assay (E) of XGC-9811L and NUGC-3 transfected cells. F and G, mice were injected with 1×10^6 target cells through tail vein. Four weeks later, the mice were sacrificed. The liver tissues were observed with naked eyes and the number of visible tumors in liver surface was counted (F). The liver tissues were made serial sections before being HE dyed and observed under a light microscope (G). H, Kaplan–Meier curve depict metastasis-free survival of patients whose primary tumors contained low or high levels of miR-223. $n = 30$; P value was obtained using a log-rank test.

master regulators of embryogenesis, have been shown to highly express in metastatic cells and seem to have causal roles in tumor metastasis (13–16). Our observation that miR-223 is highly expressed in metastatic gastric cancer cells led us to ask whether any of these transcription factors might function to activate miR-223 expression. In these reported transcription factors, we focused the newly identified transcription factor Twist, which was recently reported to transcriptionally regulate miR-10b. In our previous research, we have reported that Twist was upregulated in gastric cancer and correlated with the metastasis of gastric cancer (17). We therefore explored whether Twist could also activate the miR-223 in the metastasis of gastric cancer. We first expressed either Twist or control transcription factor Slug (Twist and Slug bind to the same E-box sequences-CANNTG) in the nontumorigenic, immortalized GES, which had been found to express a low level of miR-223. Western blot confirmed the Twist and Slug upregulation

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level after transfection (Supplementary Fig. S1A). In contrast to the control transcription factor Slug, which reduced miR-223 expression by 53%, ectopic expression of Twist led to a 3.46-fold increase in the level of this miRNA in these GES (Fig. 3A and B, left). We then used Twist-siRNA (previously constructed by our lab; ref. 17) or control Slug-siRNA (previously constructed by our lab; ref. 17) to inhibit Twist or Slug expression in tumorigenesis and metastasis XGC-9811L cells, which had been found to express a high level of miR-223. Western blot confirmed the Twist and Slug inhibition efficiency after transfection (Supplementary Fig. S1B). We found that compared with the control Slug-siRNA, which increased miR-223 expression by approximately 45%, Twist-siRNA led to an approximately 50% decrease in the level of this miRNA in XGC-9811L cells (Fig. 3A and B, right).

How Twist regulates miRNA-223 is still unknown. To explore the possible regulating mechanism, we chose the ChIP assays to investigate whether Twist regulates miR-223 expression by binding directly to the prompter of miR-223. Twist has been proved to regulate the special genes by binding to E-box sequences (CANNTG) present in those genes (18). We first searched the possible promoter region of miR-223 and found the region between −101 bp and −450 bp could be the putative prompter of miR-223 (19). Then, we examined the 1-kb genomic sequence upstream of the human miR-223 stem loop and identified 5 conserved E-boxes, at −221 bp (E-box 1), −637 bp (E-box 2), −691 bp (E-box 3), −714 bp (E-box 4), and −965 bp (E-box 5), respectively (Fig. 3C). We determined to choose the E-box 1(included in the putative promoter region) and E-box 2, E-box 3, E-box 4, and E-box 5 (used as control sites). We

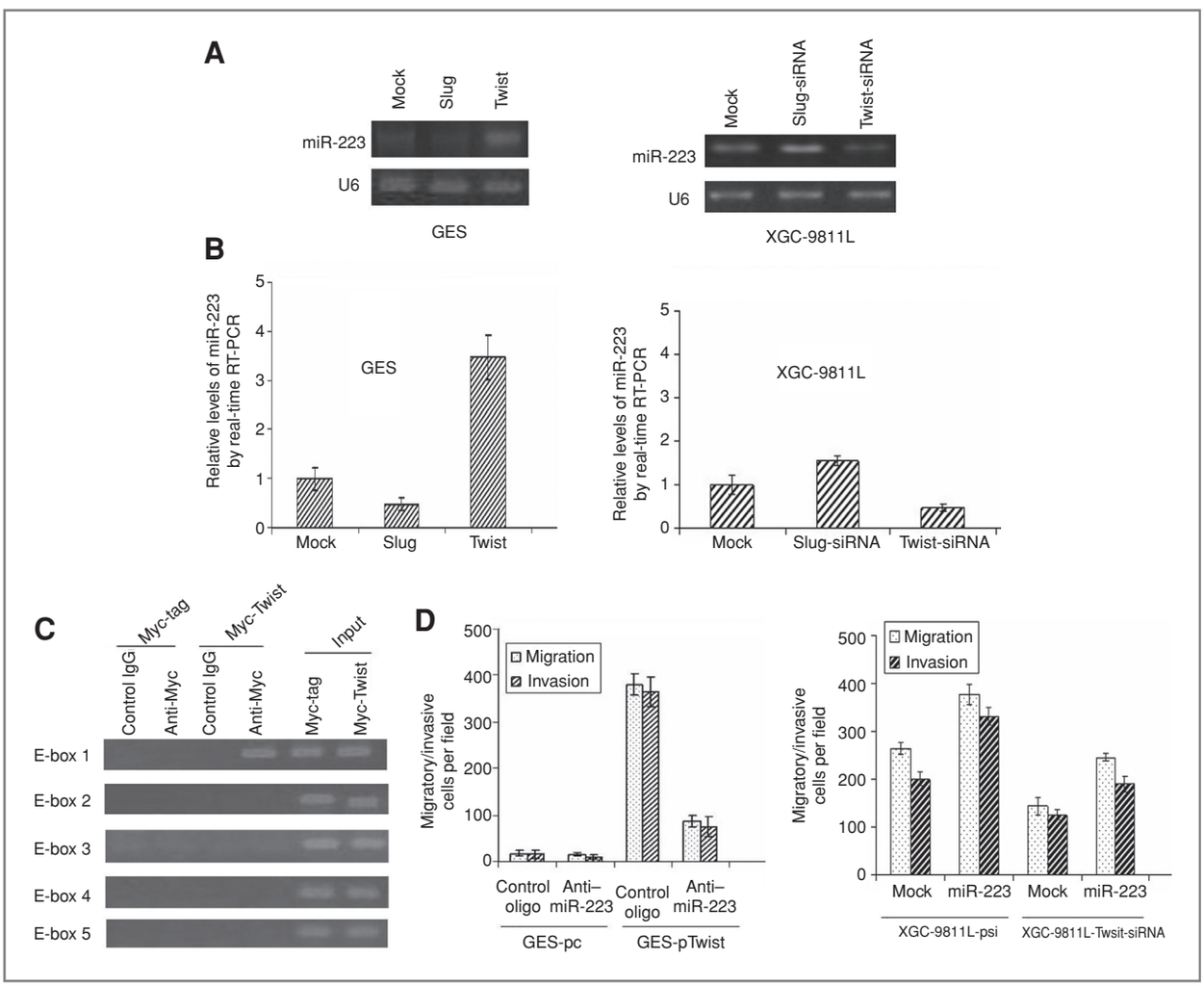

Figure 3. miR-223 is directly regulated by the transcription factor Twist. A and B, left: RT-PCR (A) and real-time RT-PCR (B) of miR-223 in GES transduced by Slug, Twist, or the empty vector. A and B, right: RT-PCR (A) and real-time RT-PCR (B) of miR-223 in XGC-9811L transduced by Slug-siRNA, Twist-siRNA, or the control siRNA. C, ChIP assay in HEK293 cells transfected with a vector expressing Myc-Twist or the Myc tag alone. PCR was performed with primers specific for human miR-223 E-boxes, respectively. D, left: transwell migration assay and Matrigel invasion assay of Twist1 or control pc transduced GES cells that were transfected with the inhibitor for miR-223 or the control oligonucleotide. Right: Transwell migration assay and Matrigel invasion assay of Twist1-siRNA or control siRNA transduced XGC-9811L cells that were transfected with the miR-223 or the mock control.

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designed PCR amplicons to assay for the presence of these putative binding sites in chromatin immunoprecipitates. The experiments revealed that Twist bound to E-box 1 but not to other E-box sites (Fig. 3C). Thus, Twist specifically binds to the putative promoter of miR-223, providing strong evidence that miR-223 can be directly regulated by Twist.

miR-223 has been proved not to affect the cell motility of highly metastatic cancer cells (Fig. 2C). Here, we wanted to further explore whether miR-223 was required for Twist-induced migration and invasion in otherwise poorly motile cells. We first introduced the antisense oligonucleotide for miR-223 into Twist-overexpressing GES (GES-pTwist, previously constructed by our lab). We found that overexpression of Twist led to a strong increase in the motility and invasiveness of these cells (Fig. 3D, left). However, miR-223 inhibition could partly reduce the motility and invasiveness of GES-pTwist cells (Fig. 3D, left). We then introduced the miR-223 into Twist-downregulating XGC-9811L cells. We found that knockdown Twist led to a significant decrease in the motility and invasiveness of these cells (Fig. 3D, right). However, miR-223 restoration could partly promote the motility and invasiveness of XGC-9811L-Twist-siRNA cells (Fig. 3D, right). The data showed that miR-223 may be one of the multielement in Twist regulated gastric cells motility and invasiveness.

miR-223 posttranscriptionally downregulates EPB41L3 expression by directly targeting its 3′-UTR

To identify the mechanism of action of miR-223 which induces tumor invasion and metastasis, the target prediction program TargetScan and PicTar were used to search for predicted direct target genes of miR-223. Among the approximately 180 targets predicted, 2 genes—EPB41L3 and ALCAM—were previously implicated in suppression of cancer metastasis (20, 21). EPB41L3 was of particular interest, because its expression has been found to be progressively lost in gastric cancers, showing increasing degrees of malignancy (22). Bioinformatics analysis of the 3′-UTR of EPB41L3 revealed EPB41L3 have at least 6 nucleotides of sequence complementary to the miR-223 seed region and carries the identical sequence in the human, mouse, and rat mRNA orthologues (Fig. 4A).

To determine whether the altered expression of EPB41L3 in metastatic cells is, in part, mediated through its 3′-UTR, we cloned the 3′-UTRs of EPB41L3 downstream of a luciferase gene as a reporter, and assayed EPB41L3 expression in NUGC-3 cells (low miR-223) and XGC-9811L cells (high miR-223). Transient transfection of NUGC-3 cells, with a low endogenous miR-223 expression, with the EPB41L3–3′-UTR reporter construct and miR-223, led to a significant decrease of reporter activity as compared with the control (Fig. 4B). However, the activity of the reporter construct mutated at the specific miR-223 target site at nucleotide 89 to 95 was unaffected by a simultaneous transfection with miR-223 (Fig. 4B). Further experiments were carried out by using anti–miR-223, which binds to endogenous miR-223 and thereby antagonizes its activity (23). When XGC-9811L cells with a high endogenous miR-223 expression were transfected with anti–miR-223 and the EPB41L3–3′-UTR reporter construct, a significant increase in activity of the EPB41L3 reporter was observed (Fig. 4C). This was abolished when the same cell lines were transfected with the reporter construct mutated at the miR-223 target site instead (Fig. 4C). To further confirm that EPB41L3 is a functional target of miR-223, we used Western blot to examine the EPB41L3 protein level after overexpression or knockdown of miR-223. Compared with the control, inhibition of miR-223 level in XGC-9811L cells (with a high endogenous miR-223 expression) could increase EPB41L3 protein level (Fig. 4D, left). Similarly, in NUGC-3 cells (with a low endogenous miR-223 expression), upregulation of miR-223 level could decrease EPB41L3 protein level (Fig. 4D, right). Taken together, these data suggested that EPB41L3 is a direct target of endogenous miR-223.

EPB41L3 is a functional target of miR-223

To better understand the potential role of EPB41L3 in miR-223–mediated tumor invasion and metastasis, XGC-9811L cells were stable infected with pcDNA-3.1-EPB41L3 or pcDNA-3.1 vector alone, and NUGC-3 cells were stable infected with EPB41L3-specific siRNA or control alone. The transfection efficiency of EPB41L3 was confirmed through Western blot analysis (Fig. 5A). Remarkably, transwell assays indicated that overexpression of EPB41L3 can strongly inhibit XGC-9811L cell migration and invasion and downregulation of EPB41L3 can significantly promote NUGC-3 cell migration and invasion *in vitro* (Fig. 5B and C). Furthermore, we also stable transfected EPB41L3-specific siRNA and control vector into miR-223 knockdown XGC-9811L cell line and transfected pcDNA-3.1-EPB41L3 and control vector into miR-223 upregulated NUGC-3 cell line. Our results showed that knockdown of EPB41L3 significantly prompted miR-223 inhibition, XGC-9811L cells migration and invasion, and inhibited miR-223 upregulation, NUGC-3 cells migration and invasion *in vitro* (Fig. 5D and E), suggesting that EPB41L3 is a direct and functional target for miR-223. In conclusion, these data hinted us that Twist/miR-223/EPB41L3 pathway is involved in the miR-223–regulated gastric cancer metastasis process and may be a potential therapeutic target for gastric cancer metastasis.

Discussion

Little is known about the expression levels or function of miRNAs in normal and gastric cancer cells, although it is becoming clear that miRNAs play major roles in the regulation of gene expression during development (24, 25). Recent gene expression profiling studies have identified miRNAs associated with gastric-related disease. In a study of gastric cancer development and progression, miRNA expression was shown to be markedly different in normal stomach, chronic gastritis, and gastric cancer, with miR-106b-25 cluster contributing to changes in tumor growth and apoptosis

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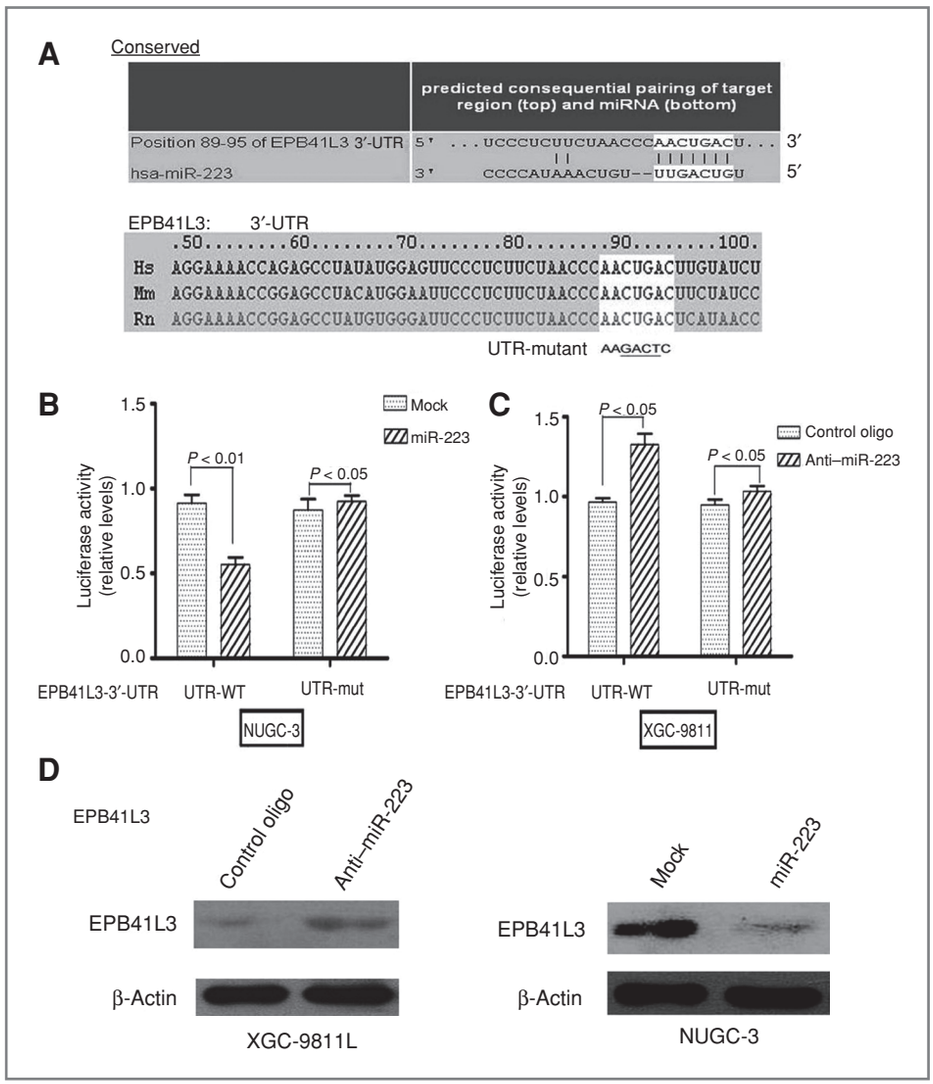

Figure 4. miR-223 posttranscriptionally downregulates EPB41L3 expression by directly targeting its 3′-UTR. A, top: predicted duplex formation between human EPB41L3 3′-UTR and miR-223. Sequence of the miR-223–binding site within the EPB41L3 3′-UTR of human (H.s.) and EPB41L3 3′-UTR of mouse (M.m.), and rat (R.n.). B and C, UTR reporter assays of miR-223–regulated metastasis inhibition gene EPB41L3 in NUGC-3 and XGC-9811L cells. Reporter constructs consisting of the luciferase sequence fused to the 3′-UTR of the EPB41L3 was transfected into the NUGC-3 and XGC-9811L cell lines. Luciferase activity of cells was assayed at 48 hours after transfection and the values were normalized to the NUGC-3 or XGC-9811L cell line. B, luciferase activity of wild-type (UTR-WT) or mutant (UTR-mut) EPB41L3 3′-UTR reporter gene in NUGC-3 cells infected with the miR-223–expressing or empty vector. C, luciferase activity of wild-type (UTR-WT) or mutant (UTR-mut) EPB41L3 3′-UTR reporter gene in XGC-9811L cells infected with the anti–miR-223 or empty vector. D, Western blot examined the EPB41L3 protein level after overexpression of miR-223 in NUGC-3 cells (with a low endogenous miR-223 expression) or knockdown of miR-223 in XGC-9811L cells (with a high endogenous miR-223 expression).

(26). In another recent study research, the association of miR-21 and gastric clinical outcome showed that miR-21 could serve as an efficient diagnostic marker for gastric cancer but does not affect the clinical prognosis of gastric cancer patients (27). We reported here that the genome-wide expression profiling of miRNAs was significantly different among primary gastric cancers and corresponding noncancerous gastric tissues. Twenty-two miRNAs were differentially expressed in tumor tissues by miRNA expression signature analysis in the testing and validation cohorts. The discriminatory power of 22 miRNAs to differentiate between tumor and nontumorous tissue suggests that predictable and systematic changes of miRNA expression patterns may occur during tumorigenesis and may be representative of sporadic gastric cancers. In a comparison of the 19 miRNAs found by Petrocca and colleagues (27) with the 140 tumor versus nontumor miRNAs found in our analysis, only 11 miRNAs overlap. The difference in miRNAs could be due to the differing platforms and particular samples used in either study, but it is affirming that several miRNAs are present in both signatures.

Interestingly, miR-223 in our signature have not been associated with the progression of any human malignancies reported and may therefore be uniquely associated with gastric cancer progression. We found that miR-223 may significantly impact metastasis and survival of gastric cancer patients. In *in vitro* assays, we found that overexpression of miR-223 in otherwise nonmetastatic gastric cancer cells enables them to acquire invasive and metastatic behavior; whereas silencing of miR-223 inhibits metastatic gastric cancer cells migration and invasion. It remains confirmed that miR-223 promotes gastric cancer metastasis process *in vivo*. Bioinformatics analysis showed that miR-223 could posttranscriptionally downregulate its target EPB41L3 expression by directly targeting its 3′-UTR. EPB41L3; protein 4.1B/differentially expressed in adenocarcinoma of the lung-1 (Dal-1), a member of the band 4.1 family of cytoskeletal proteins, is considered to function as a linker between

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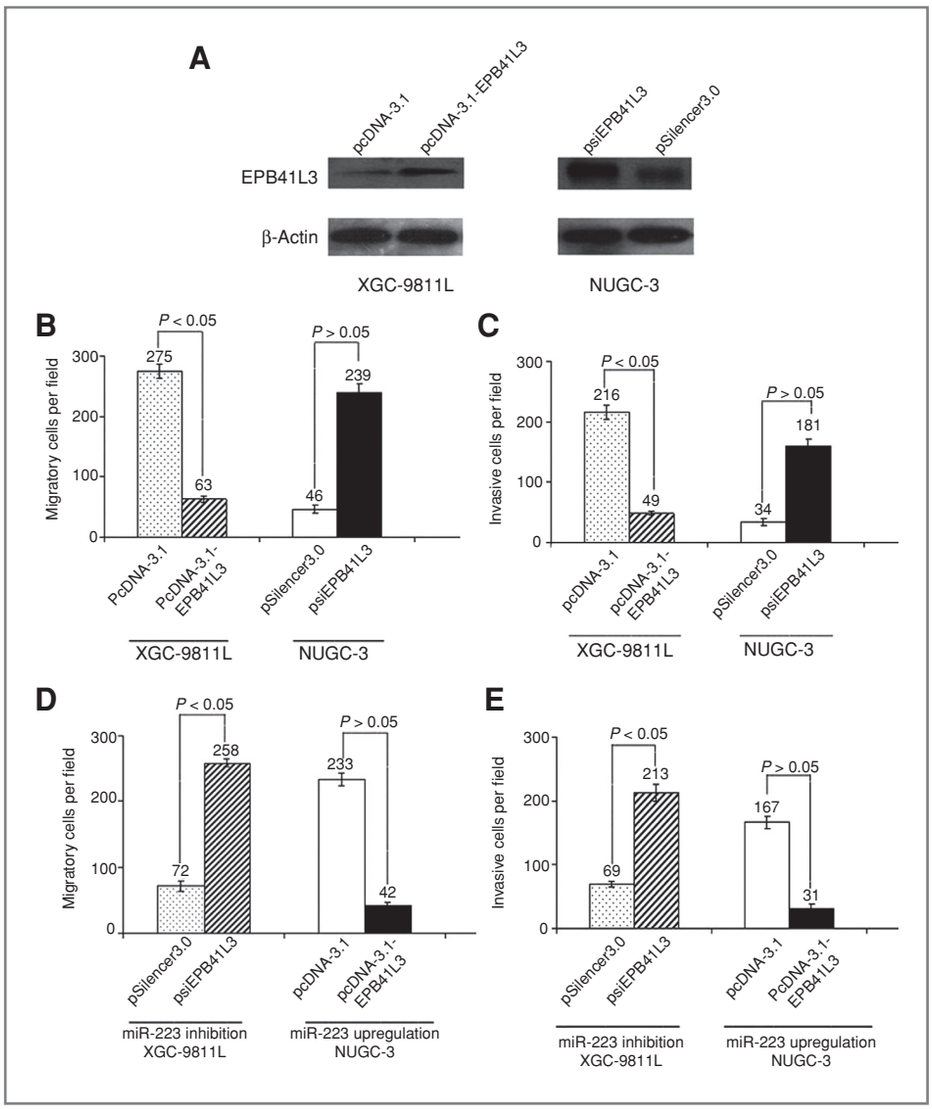

Figure 5. EPB41L3 is a functional target of miR-223. A, immunoblotting of EPB41L3 in HMECs and NUGC-3 and XGC-9811L cells infected with the pcDNA-3.1-EPB41L3, psiEPB41L3 or empty control vectors. B–E, transwell migration assay (B and D) and Matrigel invasion assay (C and E) of XGC-9811L and NUGC-3 transfected cells, respectively.

the actin cytoskeleton and transmembrane proteins (28). EPB41L3 can regulate cell shape, cell–cell and cell–substrate adhesion, cell motility, and take part in the organization of the actin cytoskeleton (29). Decreased motility and increased adhesion due to EPB41L3 re/overexpression has been shown in several different cancer cell lines (29, 30). Furthermore, siRNA knockdown of EPB41L3 in nonmetastatic cell lines stimulated migration and the loss of actin stress fibers (31). As detachment of tumor cells from the primary tumor and migration into the surrounding tissue is the first step of the metastatic cascade (32), these functions of EPB41L3 indicate a possible metastasis suppressor activity (33). Then, we first examined the metastasis suppressor function of the EPB41L3 in XGC-9811L cells and NUGC-3 cells and found that EPB41L3 acts as a metastasis suppressor in gastric cancer cells *in vitro*. To further confirm the potential role of EPB41L3 in miR-223–mediated tumor invasion and metastasis, we also stable transfected EPB41L3-specific siRNA and control vector into miR-223 knockdown XGC-9811L cell line and transfected pcDNA-3.1-EPB41L3 and control vector into miR-223 upregulated NUGC-3 cell line. Our results showed that knockdown of EPB41L3 significantly prompted miR-223 inhibition, XGC-9811L cells migration and invasion, and inhibited miR-223 upregulation, NUGC-3 cells migration and invasion *in vitro*, suggesting that EPB41L3 is a direct and functional target for miR-223. Our results showed that a newly identified transcription factor Twist-induced miRNA (miR-223) can directly inhibit synthesis of the EPB41L3 protein, in turn, causing cell migration and invasion.

These findings suggest the workings of an undescribed regulatory mode, in which a pleiotropic transcription factor induces expression of a specific miRNA, which suppresses its direct targets and, in turn, regulates gastric cancer cells invasion and metastasis. Future studies should be conducted to understand the entire complement of miRNAs and their

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mRNA targets to elucidate more fully the contributions of these miRNAs to high-grade malignancy. Furthermore, our studies showed that suppression of cell migration and invasion by an anti–miR-223 oligonucleotide may serve as a basis for the development of new therapies against human gastric cancer metastasis.

In conclusion, our results showed that human gastric cancer has extensive alterations of miRNA expression. Furthermore, gastric cancer patients with overexpression of miR-223 are associated with poor metastasis-free survival. This may have prognostic or therapeutic implications for the future management of gastric cancer patients.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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