Activation of PAX3-MET pathways due to miR-206 loss promotes gastric cancer metastasis

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Abstract

MicroRNAs (miRNAs) are thought to have an important role in tumor metastasis by regulating diverse cellular pathways. Here, we describe the function and regulation network of miR-206 in gastric cancer (GC) metastasis. MiR-206 expression was downregulated in GC cells especially in high metastatic potential cells and was also significantly decreased in metastatic lesions compared with their corresponding primary tumor samples. Both gain- and loss-of-function studies confirmed that miR-206 significantly suppressed GC cell invasion and metastasis both in vitro and in vivo. Mechanistically, paired box gene 3 (PAX3) was identified as a functional target of miR-206 in GC cells. MiR-206 inhibited GC metastasis by negatively regulating expression of PAX3. In addition, PAX3 expression was markedly higher in GC tissues than in adjacent non-cancerous tissues. GC patients with positive PAX3 expression had shorter overall survival times. Transwell assays and in vivo metastasis assays demonstrated that overexpression of PAX3 significantly promoted the invasiveness and pulmonary metastasis of GC cells. On the other hand, downregulation of PAX3 markedly reduced cell metastatic potential. Mechanistic investigations indicated that prometastasis function of PAX3 was mediated by upregulating downstream target MET. Moreover, we found that levels of PAX3 and MET were positively correlated in matched human GC specimens, and their coexpression was associated with poor prognoses. In conclusion, our results reveal that miR-206-PAX3-MET signaling is critical to GC metastasis. Targeting the pathway described here may open new therapeutic prospects to restrict the metastatic potential of GC.

Introduction

Gastric cancer (GC) is a common malignancy with a high incidence in eastern Asia and ranks the second leading cause of cancer mortality worldwide (1,2). Metastasis is the most frequent reason of primary reason for the malignant progression and high mortality of GC patients. However, the molecular mechanisms underlying GC metastasis have not yet been fully unraveled.

MicroRNAs (miRNAs) are known to regulate gene expression by inducing translational inhibition and/or mRNA degradation of target genes at the posttranscriptional level (3). Over the last decade, many miRNAs have been reported to regulate diverse biological processes such as cellular proliferation, differentiation and apoptosis (4), which are usually deregulated in
tumorigenesis. Given their critical role in malignant progressions of cancers, it was no surprise that some miRNAs were found to promote (5,6) or suppress (7,8) tumor metastasis, acting as pro- or anti-metastatic metastamir (9). To date, however, relatively little is known about the miRNA regulatory network implicated in GC metastasis.

MiR-206, which usually functions as a tumor suppressor, was reported to inhibit GC cell proliferation by repressing CCND2 in previous study (10). Interestingly, the expression of miR-206 in GC samples indicated that deregulation of miR-206 may contribute to GC metastasis. It has been reported that miR-206 expression is downregulated in different types of cancers (11–14), and decreased or loss of miR-206 is associated with human lung cancer (15), colon cancer (13) and breast cancer (16) metastasis, indicating that miR-206 acts as an anti-metastatic metastamir. However, whether miR-206 contributes to GC metastasis remains unknown. Therefore, the aim of this study is to further explore the miR-206 tumor suppressor network in GC metastasis.

Materials and methods

Cell lines

The human gastric invasive cell lines SGC7901-M, MKN28-M and non-invasive cell lines SGC7901-NM and MKN28-NM were established in our laboratory from the human gastric cell lines SGC7901 (obtained from the Academy of Military Medical Science, Beijing, China) and MKN28 (purchased from ATCC, Rockville, MD) using the repeated transwell approach, the results of metastatic examination in vitro and in vivo showed that the established cell sublines had distinct invasive and metastatic capabilities (17). Before this study, metastatic potentials of each cell line were validated in vitro and in vivo again, and no obvious differences in cell proliferation rate or cell-cycle distribution were observed among these cell lines (Supplementary Figure S1, available at Carcinogenesis Online). SGC7901-NM, SGC7901-M, MKN28-NM, MKN28-M and GES cells were routinely cultured in RPMI-1640 medium (GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum (GIBCO), 100 U/ml penicillin sodium, and 100 mg/ml streptomycin sulfate at 37°C in a humidified atmosphere with 5% CO₂.

Clinical samples

This study was approved by the ethics committee of Xijing Hospital, the Fourth Military Medical University. A total of 75 patients who underwent radical gastrectomy between 2005 and 2006 at the Xijing Hospital of Fourth Military Medical University (Xi’an, China) were enrolled in this study. All patients did not receive chemotherapy prior to surgery and were followed up. Overall survival was the primary endpoint of this analysis. Survival time was calculated from the date of surgery to the date of death or to the last follow-up. Tissue microarrays were constructed by Amoebio (Xi’an, China), including the GC samples and matched non-cancerous gastric mucosa >5 cm from the tumoral margins. The clinical characteristics of the participants are summarized in Supplementary Table S1, available at Carcinogenesis Online.

In addition, 40 pairs of primary and matched lymph node metastatic GC specimens were collected from archived paraffin-embedded tissues. Paired of fresh frozen primary GC tissue and lymph node metastatic tissues from 20 surgical patients were obtained for mRNA detection.

Quantitative real-time reverse transcription–PCR

For detection of miR-206 expression, the TaqMan stem-loop reverse transcription–PCR (RT–PCR) method was performed as previously described (10).

For paired bone tissue 3′ (PAX3) and MET mRNA detection, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and was reverse transcribed into cDNA using the PrimeScript RT reagent Kit (TaKaRa, Dalian, China). SYBR green real-time RT–PCR was performed using SYBR Premix Ex Taq II (TaKaRa) with following primers: the PCR primers for PAX3 were 5′-TACTCAAGAGGGGCTGCTGATC-3′ (forward) and 5′-ATTGCCCAAGCCTTGCTT-3′ (reverse); the MET primers were 5′-TCGGTCCTGTATTTACCTTG-3′ (forward) and 5′-ACTGGTTGGGGCTTCAT TATC-3′ (reverse).

GAPDH was used as an internal control with primers: 5′-AGGCTTCT CATGATTGGTGA-3′ (forward) and 5′-ATCACCATCTTTCCAGGAGGA-3′ (reverse).

Human GAPDH was used as an endogenous reference control. The relative fold-changes were calculated using the 2⁻ΔΔCt method. All quantitative RT–PCR reactions were performed in triplicate.

Oligonucleotide construction and lentivirus production

MiR-206 mimic, miR-206 inhibitor and the corresponding control oligonucleotides (purchased from RiboBio, Guangzhou, China) were transfection into cells as described previously (10). The MET siRNA (sc-29397; Santa Cruz, CA) and control siRNA (sc-37007; Santa Cruz) transfections were conducted as recommended by the manufacturer. Cells were allowed to grow for 48 h and lysates were tested by western blotting.

For stable transfection, lentivirus-mediated miR-206 was constructed as previously reported (10). The pGCSil-GFP lentiviral vectors encoding the human PAX3 CDS or siRNA sequence were purchased from GeneChem (Shanghai, China). Empty lentiviral vectors that expressed GFP alone were used as negative controls. After transfection of lentiviral vectors according to the manufacturer’s instructions, GFP-expressing cells were purified using FACSscan flow cytometer (Becton Dickinson, San Jose, CA). Both the upregulation and knockdown of PAX3 expression were confirmed by western blot.

Immunohistochemistry

Immunohistochemical staining was performed as previously described (18), using antibodies: PAX3 (1:200; Invitrogen) and MET (1:1000; Abcam, Cambridge, UK). Images were acquired using Soft Imaging System GmbH (Olympus Deutschland GmbH, Hamburg, Germany).

The staining results of all samples were scored independently by two pathologists blinded to the clinical data. The intensity of staining was scored as 0 (negative), 1 (weak) or 2 (strong). The extent of staining was scored based on the percentage of positive tumor cells: 0 (negative), 1 (1–25%), 2 (26–50%), 3 (51–75%) and 4 (76–100%). Each case was finally considered negative if the final score was 0–1 (–) or 2–3 (+) and positive if the final score was 4–5 (+) or 6–7 (+ +). The two scores were added to obtain the final results: Negative (−), 0–2; Positive (+), 3–6.

Luciferase reporter assay

For dual luciferase assays, the 3′-untranslated region (UTR) fragment (466 bp) of PAX3 containing miR-206 binding site (WT-PAX3-3′-UTR) or the mutated binding site (MUT-PAX3-3′-UTR) were amplified by RT–PCR and cloned into the XhoI/NsiI site of psiCHECK™ vector (Promega). The following primers were used for RT–PCR: PAX3XhoF: 5′-CCGGTCGAGGCTTCTTTTCATGAAAGGCGAGG-3′; PAX3NsiR: 5′- ATAGAATTGGCGCGCAATTTTTGTAATTGTTATTT-3′; mutPAX3F: 5′-GAATGAGGTAGGCACAATGCACATTTTTAGTAAGGAACATAT-3′; mutPAX3R: 5′-ATAGAATTGGC CACATTTTTAGTAAGGAACATAT-3′; mutPAX3: 5′-ATAGAATTGGCC ACATTTTTAGTAAGGAACATAT-3′. GUS luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

Abbreviations

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<tr>
<td>BLI</td>
<td>bioluminescent imaging</td>
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<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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<td>GC</td>
<td>gastric cancer</td>
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<td>IHC</td>
<td>immunohistochemistry</td>
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and detected by the GloMax™ 20/20 detection system (ES331; Promega). Activities were normalized to Renilla luciferase.

Similarly, for promoter analyses, a MET promoter luciferase reporter construct which contains 297 bp upstream of the transcriptional start site and 220 bp of exon 1 was transfected into cells with or without PAX3 expression constructs. After 48 h, the cells were harvested and lysed, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

Immunoblotting and immunofluorescence
Cellular proteins were extracted by complete cell lysis (Roche, Mannheim, Germany) with protease inhibitor, and western blotting was performed as previously described (10). The primary antibodies used were PAX3 (1:200; Abcam), MET (1:500; Abcam) and β-actin (1:5000; Sigma).

For immunofluorescence experiments, cells grown in Lab-Tek II eight-well chamber slides (Thermo Fisher Scientific) were fixed in 4% paraformaldehyde for 15 min, permeabilized in phosphate-buffered saline with 0.1% Triton X-100 and incubated with blocking solution for 30 min at room temperature. Then cells were incubated with antibodies against PAX3 (1:100; Invitrogen), MET (1:200; Abcam) at 4°C overnight, followed by Texas Red-conjugated anti-rabbit secondary antibody (1:100; ThermoScientific) and Alexa Fluor 488-conjugated anti-rabbit secondary antibody (1:100; ThermoScientific), respectively. Immunostaining signal and DAPI-stained nuclei were visualized by the Fluoview laser scanning confocal microscope (Olympus Corp.).

In vitro migration and invasion assays
Cell migration and invasion assays were performed using a 24-well Transwell plate (8 mm pore size; Corning) precoated or not coated with Matrigel (BD Biosciences, San Jose, CA) diluted to a concentration of 200 mg/ml. For migration assays, 5 × 104 cells were plated in the upper chamber with non-coated membrane (24-well insert; 8 mm pore size; Corning Costar Corp.). For invasion assays, 1 × 105 cells were added in the upper chamber with Matrigel-coated membrane. In both assays, cells were suspended in medium without serum or growth factors, and medium supplemented with serum was used as a chemoattractant in the lower chamber. After 24 h, cells that did not migrate or invade through the pores were removed by a cotton swab. The invading cells on the lower surface of the membrane were fixed in 4% paraformaldehyde, stained with 0.1% crystal violet and counted under a microscope.

In vivo metastatic assay and bioluminescent imaging
Animal procedures were performed in accordance with the Institutional Animal Care and Use Committee guidelines of the Experiment Animal center of the Fourth Military Medical University.

Male BALB/c nude mice aged 4–6 weeks were obtained from Shanghai Laboratory Animal Center of China and housed under standard conditions. Each mouse was injected via tail vein with 2 × 106 cells suspended in 200 µl phosphate-buffered saline. For in vivo tracking, different groups of mice were stably transsected with Firefly luciferase. In vivo quantitation of lung metastasis was imaged by bioluminescence. Mice to be imaged were injected via tail vein with 2 × 106 cells suspended in 200 µl phosphate-buffered saline. For in vivo tracking, different groups of mice were stably transsected with Firefly luciferase.

Figure 1B, miR-206 levels were significantly increased in metastatic lymph nodes from 20 GC patients with lymph node metastasis. As shown in Figure 1B, miR-206 levels were significantly decreased in metastatic lymph nodes.

The biological role of miR-206 in human GC was further investigated by gain- and loss-miR-206 function assays. Transwell assays showed that ectopic expression of miR-206 suppressed the migration and invasion abilities of MKN28-M cells (Figure 1C1). In reciprocal experiments, transfection of miR-206 inhibitor in MKN28-NM cells significantly increased tumor cell migration and invasion (Figure 1C2). Quantitative RT-PCR was used to demonstrate miR-206 transfection (Supplementary Figure S2, available at Carcinogenesis Online). Similar results were also observed in SGC7901-M and SGC7901-NM cells (Supplementary Figure S3, available at Carcinogenesis Online). To further test whether miR-206 could inhibit GC metastasis in vivo, MKN28-M cells stably transfected with LV-miR-206 or LV-control were injected into nude mice through the tail vein. Tumor metastases were monitored by non-invasive bioluminescent imaging (BLI) (Figure 1D1). H&E staining of lung sections confirmed that the incidence of lung metastases in the LV-miR-206 group was significantly decreased compared to the control group (Figure 1D2 and D3), and the number of lung metastatic nodules in the LV-miR-206 group was reduced when compared with the control group (Figure 1D4).

MiR-206 negatively regulates PAX3
To identify transcription factors (TFs) responsible for GC metastasis, we first compared activities of TFs in MKN28-M cells with that of MKN28-NM cells using the TranSignal™ protein/DNA array. Of the 15 upregulated TFs (Supplementary Table S2, available at Carcinogenesis Online), PAX3 was right target of miR-206 according to the prediction tools (miRanda, PicTar and TargetScan).

To confirm direct targeting of PAX3 by miR-206, we performed luciferase reporter assay integrating sequences of the PAX3 3′-UTR into a luciferase reporter vector. Luciferase assays showed that miR-206 significantly suppressed the luciferase activity of the Luc-PAX3 3′-UTR compared to the vector control (Figure 2B, P < 0.001), while mutation of the miR-206 binding sites blocked this suppression (Figure 2B).

Furthermore, we detected the endogenous mRNA level of PAX3 in GC cells. The results showed a negative correlation between miR-206 mRNA levels and PAX3 mRNA expression in GC cells and tissue samples (n = 20) (Figure 2C, P = 0.001). As shown
in Figure 2D, overexpression or inhibition of miR-206 had no significant effect on the mRNA level of PAX3 (all \( P > 0.05 \)). However, PAX3 protein levels increased when miR-206 was downregulated in response to transfection of SGC7901-NM and MKN28-NM cells with miR-206 inhibitor (\( P = 0.019 \) and \( P < 0.001 \) respectively). In contrast, upregulation of miR-206 level in SGC7901-M and MKN28-M decreases PAX3 protein levels (both \( P < 0.001 \)).

To determine whether PAX3 was involved in miR-206-mediated GC invasion and metastasis, we next transfected the miR-206 mimic into MKN28-M cells with or without a lentiviral vector encoding the CDS region of PAX3. Migration and invasion assays showed that restoration of PAX3 could rescue the suppression effect of miR-206 in GC cell migration and invasion (Figure 2E). Similar results were observed in vivo metastasis assay (Figure 2F and G).

**PAX3 is overexpressed in GC, and high PAX3 expression predicts poor prognosis in GC patients**

To explore the role of PAX3 in GC development, we examined its expression in a tissue array of 75 paired GC samples. Immunohistochemistry (IHC) showed that PAX3 was primarily localized in the nucleus in GC specimens (Figure 3A). PAX3 expression was found in 48 of 75 (64.0%) primary GC tissues, compared with only 22 of 75 (29.3%) adjacent non-tumor tissues (Figure 3B, odds ratio: 4.3, 95% confidence interval: 2.2–8.5; \( P < 0.001 \)). The relationship between PAX3 expression and the patients’ clinicopathological data including gender, age, lymph node metastasis and TNM stage are presented in Supplementary Table S1, available at Carcinogenesis Online. Increased PAX3 expression in GC was associated with advanced clinical stage, lymph node metastasis. Moreover, the survival rate of patients with PAX3 staining was determined by the log-rank test, as shown in Figure 3C, positive PAX3 expression had shorter overall survival than those showing negative PAX3 expression (\( P = 0.023 \)). Multivariate Cox regression analysis also revealed that PAX3 expression was an independent predictor of survival (\( P = 0.004 \)) in GC after curative resection (Supplementary Table S3, available at Carcinogenesis Online).

Furthermore, we compared PAX3 expression in 40 pairs of primary and matched lymph node metastatic GC specimens by IHC staining. Overall, 23 pairs of GCs (57.5%) showed higher levels of PAX3 expression in metastatic lesions, compared with the corresponding primary tumor samples (Figure 3D). Correspondingly, upregulation of PAX3 was observed in various human GC cell lines, especially in invasive cell lines MKN28-M and SGC7901-M (Figure 3E).

**PAX3 promoted GC invasion and metastasis**

To determine the role of PAX3 in the migration and invasion of GC cells, we transfected MKN28-NM cells which expressed low PAX3 protein level with a lentiviral vector encoding PAX3 or empty vector to perform migration and invasion assay in vitro. Transwell assay showed that upregulation of PAX3 increased...
the migration and invasion of MKN28-NM cells (Figure 4A). In contrast, downregulation of PAX3 by stably transfected with PAX3 siRNA lentiviral vector in MKN28-M cells resulted in a reduction in migration and invasiveness (Figure 4B). Similar results were also observed in SGC7901-M and SGC7901-NM cells (Supplementary Figure S4, available at Carcinogenesis Online). Furthermore, consistent results were obtained from in vivo experimental metastasis assays. Representative BLI of the different groups is shown in Figure 4C. BLI quantification of lung colonization ability of control or MKN28-NM-PAX3 cells confirmed that upregulation of PAX3 increases GC metastasis to the lungs (Figure 4D1). Conversely, silencing endogenous PAX3 expression in MKN28-M cells markedly reduced cell metastatic potential (Figure 4D2). Histological analysis (Figure 4E) further confirmed that the incidence of lung metastasis and the number of lung metastatic nodules in the MKN28-NM-PAX3 group was significantly increased, compared to that in the MKN28-NM control group (7/10 versus 1/10, respectively, P = 0.020). However, incidence of lung metastasis in MKN28-M-siPAX3 group was apparently reduced, compared to that in the MKN28-M control group (3/10 versus 9/10, respectively, P = 0.020).

**PAX3 regulation of MET**

To further explore the molecular mechanism of PAX3 in GC metastasis, we next search for possible downstream effector of PAX3. Previous studies reported that MET, which plays an important role in metastasis, was a downstream target of PAX3 in melanoma (20). We wonder whether MET is directly regulated by PAX3 at the transcriptional level in GC.

To test this hypothesis, we first examined activation of the MET promoter using the luciferase reporter assays. MET promoter constructs were transfected into MKN28 cells with or without PAX3 expression construct. As shown in Figure 5A, expression of PAX3 significantly induced the MET promoter activity, indicating PAX3 could mediate MET mRNA upregulation through direct transactivation of its promoter. Real-time PCR (Figure 5B) and western blot (Figure 5C) assays also demonstrated that upregulation of PAX3 in MKN28-NM cells increased MET expression. However, the knockdown of PAX3 expression significantly decreased MET expression in MKN28-M cells. Immunofluorescence staining of PAX3 and MET (Figure 5D) clearly showed that upregulation of PAX3 could lead to its increase of nuclear localization accompanying with strong positive membrane staining of MET. Downregulation of PAX3 exhibited the opposite effect.

**MET expression is positively associated with PAX3 expression in human GC tissues, and their coexpression indicates poorer prognosis**

To analyze the association between PAX3 and MET expression in GC tissues, we performed IHC assay to detect MET expression
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in the same tissue array of 75 paired GC samples as previously described. Overexpression of MET in GC samples was significantly associated with clinicopathologic features, such as lymph node metastasis and TNM (Supplementary Table S1, available at Carcinogenesis Online). The staining results revealed that there was a significant positive association between PAX3 and MET expression in 75 human GC tissues (P = 0.010, Figures 6A and Supplementary Table S4, available at Carcinogenesis Online).

The patients were subsequently divided into three distinct groups depending on their combined expression levels of PAX3 and MET: both high, one high (PAX3 or MET high) and both low. The Kaplan–Meier analysis revealed that patients with both high had a shorter survival time than in the two other groups (P < 0.001, log-rank test; Figure 6B). Taken together, these results suggest that coexpression of PAX3 and MET was indicative of poor prognosis in GC patients.

Discussion

Here, we further presented the miR-206 tumor suppressor network in GC. We found that loss of miR-206 induces PAX3 overexpression, which in turn enhances MET-mediated GC metastasis.

As mentioned earlier, miR-206 was originally identified as a skeletal muscle-specific miRNA involved in muscle development (21,22). Although, reduced expression levels of miR-206 is associated with several types of cancer metastasis (15,16), its role in GC metastasis has not been reported. In this study, we found that miR-206 expression was downregulated in GC cells especially in high metastatic potential cells and was also significantly decreased in metastatic lesions compared with their corresponding primary tumor samples, indicating miR-206 may be involved in GC metastasis. Subsequent function analysis confirmed that miR-206 significantly suppressed GC cell invasion and metastasis both in vitro and in vivo. These findings have highlighted the metastasis suppressor of miR-206 in GC.

To clarify molecular basis of miR-206 function in GC metastasis, we further searched for the downstream target genes regulated by miR-206. On the basis of TFs microarray and bioinformatics analysis, the target gene PAX3, which harbors migration-promoting activity, may be responsible for metastasis suppression of miR-206 in GC metastasis. We initially demonstrated that miR-206 binds to the 3′-UTR of PAX3, dramatically decreasing the level of PAX3 protein expression. The following experiments showed an inverse relationship between miR-206 and PAX3 in both GC cell lines and primary GC tissues. The functional experiments indicated that introduction of PAX3 could reverse miR-206-mediated suppression of GC cell invasion and metastasis. All these data support the notion that miR-206 acts as a metastatic suppressor by negatively regulating PAX3 in GC.

PAX3 is a member of the Pax family of TFs that regulate cell proliferation, migration, survival and cell-lineage specification during embryogenesis (23). PAX genes are frequently expressed in various types of cancer and implicated in carcinogenesis (24–26). PAX genes are classified into four subgroups (PAX1/PAX9, PAX2/PAX5/PAX8, PAX3/PAX7 and PAX4/PAX6) according to the presence or absence of an octapeptide region, and a complete or truncated version of a homeodomain (26,27). Among the nine numbers of PAX genes, PAX3 was characterized as a cancer-promoting gene, acting as a mediator of tumor progression and a marker of unfavorable outcome (26). It is well documented that PAX3 plays an essential role in differentiation and migration of muscle precursor cells and neural crest cells, thus it is often implicated in the tumorigenesis of these tissues, including rhabdomyosarcoma (28), glioma (29), melanoma (30,31) and neuroblastoma (32,33). Beyond that, consistent expression of
PAX3 was also observed in other cancer cell lines, such as breast cancer (24). However, the expression and roles of PAX3 in GC have not been elucidated.

In the present study, upregulation of PAX3 was observed in human GC tissues compared with their adjacent non-tumor tissues, and the increased expression was significantly correlated with the progression and poor prognosis of patients with GC. Furthermore, we found that PAX3 expression was also markedly higher in metastatic lesions compared to their non-metastatic ones. Consistently, PAX3 protein levels were correlated with the metastatic potential of the GC cell lines. These data suggest that PAX3 may be involved in tumorigenesis and progression of GC.

To further explicit the role of PAX3 in GC metastasis, the biological function of PAX3 in human GC was further investigated by gain- and loss-PAX3 function assays. Ectopic expression of PAX3 in low metastatic potential cancer cells significantly promoted cell migration and invasion. This effect was further confirmed by in vivo metastasis assay. Conversely, knockdown of PAX3 by siRNA in high metastatic potential cells suppressed the cell migration and invasion. Therefore, these findings demonstrated that PAX3 could promote GC cells invasion and metastasis, which expanded the contributions of PAX genes in carcinogenesis of gastrointestinal tumor (34,35).

We further uncovered that prometastasis function of PAX3 was related to the upregulation of MET, a proto-oncogene that encodes a protein known as hepatocyte growth factor receptor (36). Once activated, MET evokes multiple cellular responses leading to promote cancer cell growth, survival, invasion and metastasis (37,38). Mechanisms of MET activation are gene amplification, activating point mutations or hepatocyte growth factor autocrine/paracrine stimulation. Among them, the most common cause for the activation is overexpression of MET (39–41). Increased expression of MET has been observed in various types of human cancers (42–44), especially in gastric carcinoma (45,46), conferring prometastatic effects on tumor cells (47,48). Here we found that PAX3 could upregulate MET expression by direct transactivation of its promoter in GC cells, as demonstrated by luciferase reporter assay. The upregulation of MET by PAX3 was further confirmed by real-time PCR, western blot and immunofluorescence. Therefore, the upregulation of MET induced by PAX3 may explain the prometastatic effect exerted by PAX3 in GC. In addition, we also observed that PAX3 expression was positively correlated with MET expression, and the coexpression of these genes was associated with poor prognosis in GC patients. Taken together, these results suggest a possible mechanism that PAX3 promoted GC metastasis by upregulating MET expression. The precise downstream molecules of PAX3 are worthy of future studies.

It was previously reported that MET is a direct target of mir-206 in several tumors (11,49). However, we analyzed MET protein levels in MKN28-M and SGC7901-M cells and found no difference or a slight decrease in cells transfected with either miR-206 mimics or miR-206 inhibitor (Supplementary Figure S5, available at Carcinogenesis Online), implying that MET is a low
Figure 5. PAX3 regulated MET. (A) The MET promoter luciferase construct was cotransfected with PAX3, and promoter activities were detected using a luciferase reporter assay. Analysis of MET mRNA (B) and protein (C) levels in response to transfection with PAX3-expression lentiviral vectors or PAX3 siRNA vectors in GC cells. *P < 0.05 (D) Immunofluorescence staining of PAX3 and MET in GC cells when transfected with PAX3-expression lentiviral vectors or PAX3 siRNA vectors.

Figure 6. MET expression is positively correlated with PAX3 expression, and their coexpression indicates poor prognosis. (A) Immunohistochemical analysis of coexpression of PAX3 and MET in 75 paired GC tissues. (B) The Kaplan–Meier survival analysis in GC subgroups according to the expression profile of PAX3 and MET in primary tumors.
affinity target of miR-206 in GC cells and other mechanisms may contribute to posttranscriptional regulation of MET.

It is worth mentioning that several proteins associated with tumor progression, such as Cyclin D2 (10), Notch3 (12), estrogen receptor alpha (14) and Connexin 43 (21) etc. had been identified as downstream effectors of miR-206. It is not clear yet whether these proteins are also involved in invasion and metastasis of GC regulated by miR-206. However, it can be suspected that the profound impacts of miR-206 on the invasion and metastasis phenotype can not be solely explained by PAX3 and MET.

In summary, we demonstrated miR-206 acts as a metastatic suppressor by negatively regulating PAX3 in GC. Decreased expression of miR-206 in GC cells induced overexpression of PAX3, which resulted in upregulation of MET, thereby contributing to tumor cell invasion and metastasis. This network provides a new regulatory mechanism underlying GC metastasis, with opportunities for intervention.

Supplementary material

Supplementary Tables 1–4, Figures 1–5 and Supplementary Methods can be found at http://carcin.oxfordjournals.org/

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References


