Carcinogenesis vol.34 no.12 pp.2851–2860, 2013
doi:10.1093/carcin/bgt226
Advance Access publication June 26, 2013

Metallopanstimulin-1 regulates invasion and migration of gastric cancer cells partially through integrin β4

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MPS-1 (metallopanstimulin-1), also known as ribosomal protein S27, was overexpressed in gastric cancer cells. However, how MPS-1 contributes to gastric carcinogenesis has not been well characterized. Here, we show that high expression of MPS-1 was observed in gastric cancer tissues and associated with gastric cancer cell metastasis. Alteration of MPS-1 expression regulates invasion and migration of gastric cancer cells both *in vitro* and *in vivo*. Furthermore, by using Signal-Net and cluster analyses of microarray data we identified integrin β4 (ITGB4) as a downstream target of MPS-1 that mediates its effects on cell metastasis. Knockdown of MPS-1 expression in gastric cancer cells led to significant reduction of ITGB4 expression at both the RNA and protein levels. Mechanically, we found that overexpression of ITGB4 in MPS-1 knockdown cells largely recovers the ability of invasion and migration. Conversely, knockdown of ITGB4 partially reduced cell invading/migrating ability induced by MPS-1 overexpression. Moreover, MPS-1 and ITGB4 expressions are positively correlated in gastric cancer cell lines and tissues. Finally, the survival analyses show that the expression of MPS-1 and ITGB4 is associated with poor outcomes in gastric cancer patients. Collectively, our findings suggest that MPS-1 regulates cell invasiveness and migration partially through ITGB4 and that MPS-1/ITGB4 signaling axis may serve as therapeutic targets in the treatment of gastric cancer.

Introduction

Gastric cancer is one of the most common cancers worldwide, with the second highest mortality rate among all cancers (1). Approximately 1 million individuals are diagnosed with gastric cancer yearly, ~ 10% of these patients will die, and the situation is even worse for gastric cancer patients with metastasis (2). Therefore, it is of great significance to identify the mechanisms underlying cancer metastasis in gastric cancer.

Metastasis is a complicated biological cascade that starts with local invasion by tumor cells and continues with migration to distant tissues and ultimately colonization (3,4). This process requires multiple factors, including abnormal gene expression, defects in cell surface adhesion molecules and altered expression of matrix components (5–7). Among these factors, the abnormally expressed genes that execute the metastatic cascade may play pivotal roles (8,9); therefore, the identification of these genes will elucidate the molecular mechanisms of metastasis. Certain ribosomal protein genes that belong to this group of abnormally expressed genes are closely related to cancer metastasis (10). Liu *et al.* (11) showed that RPL23 played a role in cell movement and metastasis, overexpression of L23 altered lung cancer cell morphology and enhanced invasiveness. Using 2D liquid chromatography and mass spectrometry, Kreunin *et al.* (12) identified 45 unique ribosomal proteins, among which RPL13, RPL40 and 40S ribosomal proteins were differentially expressed in metastatic M4A4 cells. Interestingly, in the ribosomes of human embryonic lung fibroblasts, 40S ribosomal subunits appeared to associate preferentially with integrin β3 in focal adhesions at the leading edges of spreading cells, which facilitates cell migration. Therefore, ribosomal proteins may be important regulators of metastasis (13). However, as a component of the 40S ribosomal protein, the biological function of metallopanstimulin-1 (MPS-1) in human cancer, especially in cancer metastasis, has not yet been fully elucidated.

Integrins have well-defined roles in cancer cell invasion and migration (14,15), and integrin β4 (ITGB4), which forms dimers with integrin α6, promotes invasion and migration in various cancer cells (16–18). As a receptor for the laminins, ITGB4 is essential for the organization and maintenance of epithelial structure and plays a pivotal role in the metastasis of various cancer cells (19,20). In many epithelia, this integrin mediates the formation of stable adhesive structures termed hemidesmosomes that link the intermediate filament cytoskeleton with the extracellular matrix (21,22). Moreover, Rabinovitz *et al.* (23) demonstrated that ITGB4 mediates the migration of invasive colon carcinoma cells on laminin-1 through its ability to associate with the actin cytoskeleton and promotes the formation and stabilization of filopodia and lamellae.

In the current study, we showed that MPS-1 regulates invasion and migration in gastric cancer cells partially through ITGB4. ITGB4 expression was greatly depressed in MPS-1 knockdown cells. Overexpression of ITGB4 in MPS-1 knockdown cells largely recovers the ability of invasion and migration. Conversely, knockdown of ITGB4 partially reduced cell invading/migrating ability induced by MPS-1 overexpression. Additionally, ITGB4 and MPS-1 are correlatively expressed in gastric cancer cells and tissues, and the survival analyses show that the expression of ITGB4 and MPS-1 is associated with poor outcomes in gastric cancer patients. Taken together, our study provides the novel link between MPS-1 and ITGB4 in regulation of cell invasiveness and migration in gastric cancer and suggests that targeting the MPS-1/ITGB4 signaling axis may be more effective in the treatment of gastric cancer patients.

Materials and methods

Cell culture

Gastric cell line AGS (ATCC: CRL-1739) was originally obtained from American Type Culture Collection. Gastric cancer cell lines SGC-7901, MKN45, MKN28 and the human embryonic kidney cell line HEK293T were preserved by our institute (24,25). The immortalized gastric mucosal epithelial cell line GES-1 was a gift from Prof. Feng Bi (Institute of Digestive Disease, Xi'jing Hospital, The Fourth Military Medical University, People's Republic of China) (26). All cell lines were grown in RPMI-1640 or Dulbecco's modified Eagle's medium (Sigma–Aldrich, St Louis, MI) supplemented with 10% fetal bovine serum (FBS).

Patients and gastric cancer specimens

We selected 75 patients with gastric cancer who underwent surgery at hospitals that cooperated with Shanghai Outdo Biotech (27) during 2006–07. This

Abbreviations: FBS, fetal bovine serum; ITGB4, integrin β4; MPS-1, metallopanstimulin-1; NC, negative control; NF-κB, nuclear factor-kappa B; qRT–PCR, quantitative realtime reverse transcription–polymerase chain reaction; shRNA, short hairpin RNA.

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sample included 51 men and 24 women, ranging from 37 to 81 years of age (median: 65 years) with 1–61 months of follow-up information. Paraffin-embedded diagnostic tumor biopsy specimens and their adjacent non-tumor specimens (≥5 cm away from the tumor) were collected before any treatments. Patients were only included in the study if they provided written consent to participate in the study. Approval for the study was received from the Ethics Committee of the host institution.

RNA interference

Short hairpin RNA (shRNA) sequences targeting MPS-1 (5′-aaaccatct-caataaacacat-3′), ITGB4 (5′-AAGAACCGGATGCTGCTTATT-3′) and a negative control (NC) sequence (5′-TGCGTTGCTAGTACCAAC-3′) were synthesized, annealed and ligated into the retroviral pSIREN-RetroQ vector (Clontech, Palo Alto, CA). Retrovirus packaging and infection were conducted as described previously (28). Virally infected cells were cultured in medium containing 2 μg/ml puromycin for 7 days, and drug-resistant clones were collected and expanded. Gastric cancer cells infected with NC sequence or shRNAs targeting MPS-1 were termed NC or P4, respectively, as reported previously (29), and cells with ITGB4-targeting shRNA was termed shITGB4.

Plasmids

Human MPS-1 complementary DNA was amplified from SGC-7901 cells by PCR (forward primer: 5′-ATGCCTCTCGCAAAGGATCTCC-3′ and reverse primer: 5′-TTAGTGCTGCTTCCTCCT-3′) and subcloned into the pQCXIN retroviral vector to generate the pQCXIN-MPS-1 construct for transfection. The pcDNA3.0/Myc-ITGB4 plasmid was purchased from Addgene (Cambridge, MA).

Quantitative real-time reverse transcription–polymerase chain reaction

Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with ribonuclease-free deoxyribonuclease. Reverse transcription was performed with the RNA PCR kit (Takara). The double-stranded DNA dye SYBR Green PCR Master Mixture reagents were used for quantitative real-time reverse transcription–polymerase chain reaction (qRT–PCR) as described previously (30). The specific primers are listed in Supplementary Table 1, available at *Carcinogenesis* Online.

Tumorigenesis and metastasis of xenografts

In vivo experiments were performed with 4-week-old male nonobese diabetic/severe combined immunodeficiency mice in accordance with the guidelines of the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine. Mice received lateral tail vein injections of 1.5×10^6 cells. After 8 weeks, the mice were killed under anesthesia. Metastatic foci on the lung surface were counted macroscopically. Lung tissues were collected and fixed in 10% formalin. For the tissue morphology evaluation, hematoxylin and eosin staining was performed on sections from embedded samples. Immunohistochemical staining for MPS-1 and ITGB4 was performed on sections from the metastatic tumor nodes.

Immunoblotting

Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). The primary antibodies recognized MPS-1 (Abcam, Cambridge, UK; ab4385) and ITGB4 (M126) (Abcam, Cambridge, UK, ab29042), and β-actin antibody (Cell Signaling Technology, Beverly, MA) was used as a loading control. Quantitative changes in luminescence were estimated with LAS1000 UV mini and Multi Gauge Ver. 3.0 (Fuji Film, Tokyo, Japan).

Immunofluorescence

Gastric cancer cells were seeded onto cover slips, fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton X-100 for 10 min. Slides were blocked with 1% bovine serum albumin and incubated with anti-ITGB4 monoclonal antibody (1:100, Abcam, Cambridge, UK; ab29042) overnight at 4ºC or F-actin probe Phalloidin-FITC (P5282) (Sigma–Aldrich, 1.5 μg/ml) for 40 min at room temperature. After washing in phosphate-buffered saline, the cells were stained with secondary antibodies and incubated for 1 h at room temperature, followed by nuclear counterstaining with 4',6-diamidino-2-phenylindole.

Flow cytometry

Direct immunofluorescence flow cytometric analysis was performed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA). The expression of ITGB4 was measured with purified PE-rat anti-Human ITGB4 (BD Pharmingen, San Diego, CA). Data were acquired from 10 000 events, and staining was compared with a matched isotype control antibody PE-rat IgG2a, κ. The data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

Immunohistochemistry

Tissue slide was heated at 60°C for 1 h, followed by treatment with xylene, 100% ethanol and then decreased concentrations of ethanol. After the antigen retrieval, tissues were blocked with 5% bovine serum albumin and stained with antibodies against MPS-1 (Abcam, Cambridge, UK; ab4385) and ITGB4 (M126) (Abcam, Cambridge, UK; ab29042), respectively, followed by incubation with biotinylated secondary antibody (1:200), and visualized by standard avidin-biotinylated peroxidase complex method. Tissues were counterstained with hematoxylin, and morphologic images were observed under Olympus BX51 microscope. The expression of MPS-1 and ITGB4 was examined by light microscopy through counting 1000 cells over five fields to obtain the average number of positive cells counted. The percentage of positive cells and the intensity of immunostaining were quantified and classified into five groups as follows: 0, <5% positive cells; 1, 5–24% positive cells; 2, 25–49% positive cells; 3, 50–74% positive cells; and 4, ≥75% positive cells (25,31). The intensity for MPS-1 and ITGB4 protein staining was scored as 1 for yellow staining, 2 for claybank and 3 for sepia staining (32,33). The percentage of positive tumor cells and staining intensity were multiplied to produce a weighted score for each case (34). Cases with weighted scores of 0–5 were termed negative, 6–8 as positive and 9–12 as strong positive.

Scratch-wound healing assay

SGC-7901 or AGS cells were seeded to form a monolayer in six-well plates, 24 h later a wound was made by scratching the cells in a line with a sterile pipette tip in the middle of the plate, then, the media was replaced by RPMI-1640 medium without FBS. After that, a photograph was taken immediately by Olympus BX51 microscope, and the wound distance was calculated as a basic width. After 12 and 24 h, cells were washed three times by phosphate-buffered saline and photographs were taken and the width of the wound distance was calculated. The wound closure (%) was determined as the width migrated after 12 and 24 h relative to the basic width.

Matrigel transwell invasion assay

Indicated SGC-7901 and AGS cells were harvested and resuspended in RPMI-1640 medium without FBS and then, 5×10^5 cells were added in triplicates to the upper compartment of the prehydrated Matrigel-coated invasion chambers (Becton Dickinson Labware, Bedford, MA). The lower compartment of the invasion chamber was filled with RPMI-1640 medium containing 10% FBS as a chemoattractant. After 12 h, the upper surface of the membrane was scrubbed with moistened cotton swabs to remove matrigel and non-invading cells. Then, the lower surface of the membrane was fixed by 100% methanol and stained with crystal violet. The numbers of cells invaded through the matrigel layer was counted in five random fields with Olympus BX51 microscope (28).

Statistical analysis

All experiments were performed at least three times, and the results are shown as the mean ± SD. A paired *t*-test was used for statistical analyses between two groups. The SPSS statistics 20.0 package (SPSS, Chicago, IL) was used for other analyses.

Results

MPS-1 knockdown inhibits the metastasis of gastric cancer cells in an in vivo mouse model

To examine the expression patterns of MPS-1, we performed immunohistochemical staining of MPS-1 on tissue microarrays containing 75 human gastric carcinomas with matched surrounding tissues (Supplementary Figure 1A, available at *Carcinogenesis* Online). Tissue staining was scored based on the MPS-1 staining intensity and the rate of positively stained cells. As illustrated in Supplementary Figure 1B, available at *Carcinogenesis* Online, gastric cancer tissues showed significantly higher MPS-1 expressing compared with the matched surrounding tissues ($P < 0.001$). Importantly, MPS-1 expression has significant correlations with the tumor-node-metastasis stage and metastasis (Supplementary Table 2, available at *Carcinogenesis* Online).

Because MPS-1 is highly expressed in gastric cancer cells, we tested whether knocking down MPS-1 affects tumor metastasis in an *in vivo* metastasis model. After injection into tail veins, SGC-7901 cells injected mice developed distal metastases in lung. By comparing with parental cells and shRNA control group (NC), P4 cells (MPS-1 knockdown cells, as described in Materials and methods) had only two lung metastatic nodules on average (seven nodules on average for the two control groups) with much smaller size (Figure 1A and B). The number and the size of metastatic nodules from parental cells

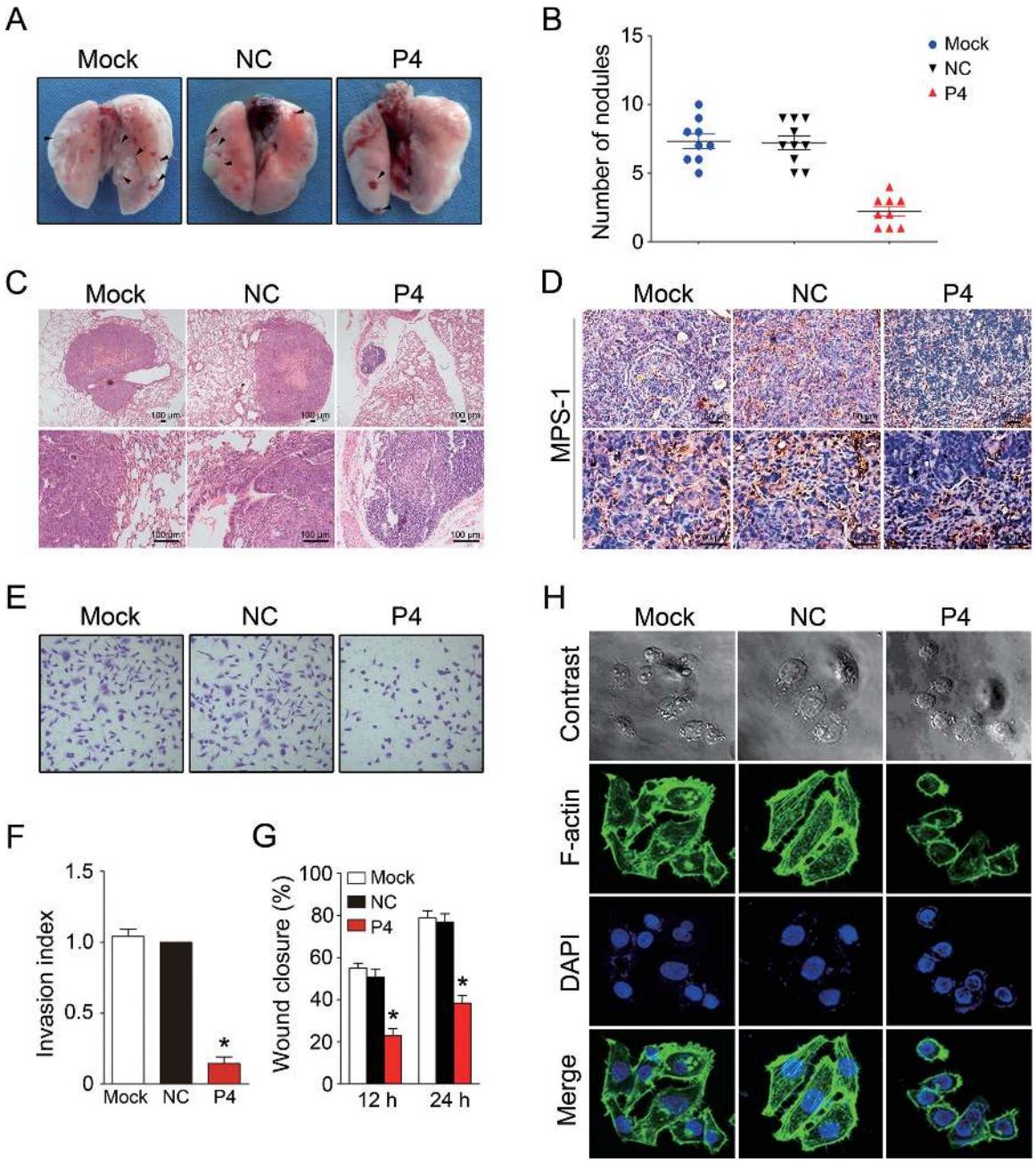

Fig. 1. Knockdown of MPS-1 suppresses the invasion and migration of SGC-7901 cells *in vitro* and *in vivo*. (**A**) Representative photographs of lungs with metastatic nodules are shown (arrow heads). (**B**) Number of metastatic nodules per lung in nonobese diabetic/severe combined immunodeficiency mice ($n = 10$). (**C**) Representative micrographs of lung tissues with metastatic cells are shown by hematoxylin and eosin staining at a magnification of ×40 (top) and ×200 (bottom). (**D**) MPS-1 expression was determined by immunohistochemical staining in metastatic tumors derived from mock, NC and P4 cells (×200, top; ×400, bottom). (**E**) A matrigel invasion assay and scratch-wound assay were performed. The invasion index (**F**) and wound closure (%) (**G**) were calculated (the invasion index between mock and P4 was 1.04 ± 0.071 versus 0.145 ± 0.068 and NC was normalized to 1). *$P < 0.05$. (**H**) Immunofluorescence microscopy of mock, NC and P4 cells with F-actin probe Phalloidin-FITC staining. 4',6-Diamidino-2-phenylindole was used to visualize cell nuclei (magnification: ×400).

were similar to those from the NC group, suggesting that expression of non-specific shRNAs had no effects on tumor metastasis. Metastatic lung nodules were confirmed by hematoxylin and eosin staining (Figure 1C). Immunohistochemical staining of sections from lung metastases showed that the tissues in the mock and NC control groups had strong MPS-1 staining, whereas the P4-derived metastases stained weakly for MPS-1 (Figure 1D). Additionally, Ki67 staining of sections from lung metastases displayed modest reduction in P4-derived metastases compared with the mock and NC groups (Supplementary Figure 2, available at *Carcinogenesis* Online), which implied the modest antiproliferative effect of MPS-1 knockdown.

To understand the molecular bases by which MPS-1 regulates cancer metastases, we examined cell invasiveness and cell migration. Figure 1E shows typical cell images in cell invasiveness assay. MPS-1 knockdown cells had fewer cells passing through matrigel in comparison with either parental cells or the shRNA control cells. After further analysis, we found that MPS-1 cells showed significant reduction in cell invasiveness and migration in scratch-wound healing assay (Figure 1F and G). Similar results were obtained in another gastric cancer cell line AGS (Supplementary Figure 3, available at *Carcinogenesis* Online), indicating that our results are not cell specific. In addition, we found that P4 cells were round and small in

appearance, with few extensions around the circumference of the cell, as shown by F-actin staining (Figure 1H), a morphology associated with reduced cell invasiveness (23).

Taken together, these results indicate that MPS-1 plays a key role in driving the metastasis of human gastric cells *in vitro* and *in vivo*.

Overexpression of MPS-1 promotes the invasion and migration of gastric cells

To determine the effects of MPS-1 overexpression on normal gastric cells, we constitutively expressed MPS-1 in GES-1 cells, in which the expression of MPS-1 is relatively low (25). Ectopically expressed MPS-1 was confirmed by western blotting (Figure 2A). Overexpression of MPS-1 promoted the proliferation (Figure 2B) and colony formation of GES-1 cells (Figure 2C). Additionally, MPS-1 overexpressing cells were significantly more invasive and migratory compared with the control groups (Figure 2D–F). These observations were also extended to human gastric cancer cells. To further confirm this result, MPS-1 was depleted by specific small interfering RNA in GES-1 cells transfected with pQCXIN-MPS-1 (Figure 2G). As seen in Figure 2H–J, the depletion of MPS-1 markedly suppresses the proliferation, invasiveness and migration of GES-1 cells, thus confirming that the enhancement of invasion is specifically mediated by MPS-1

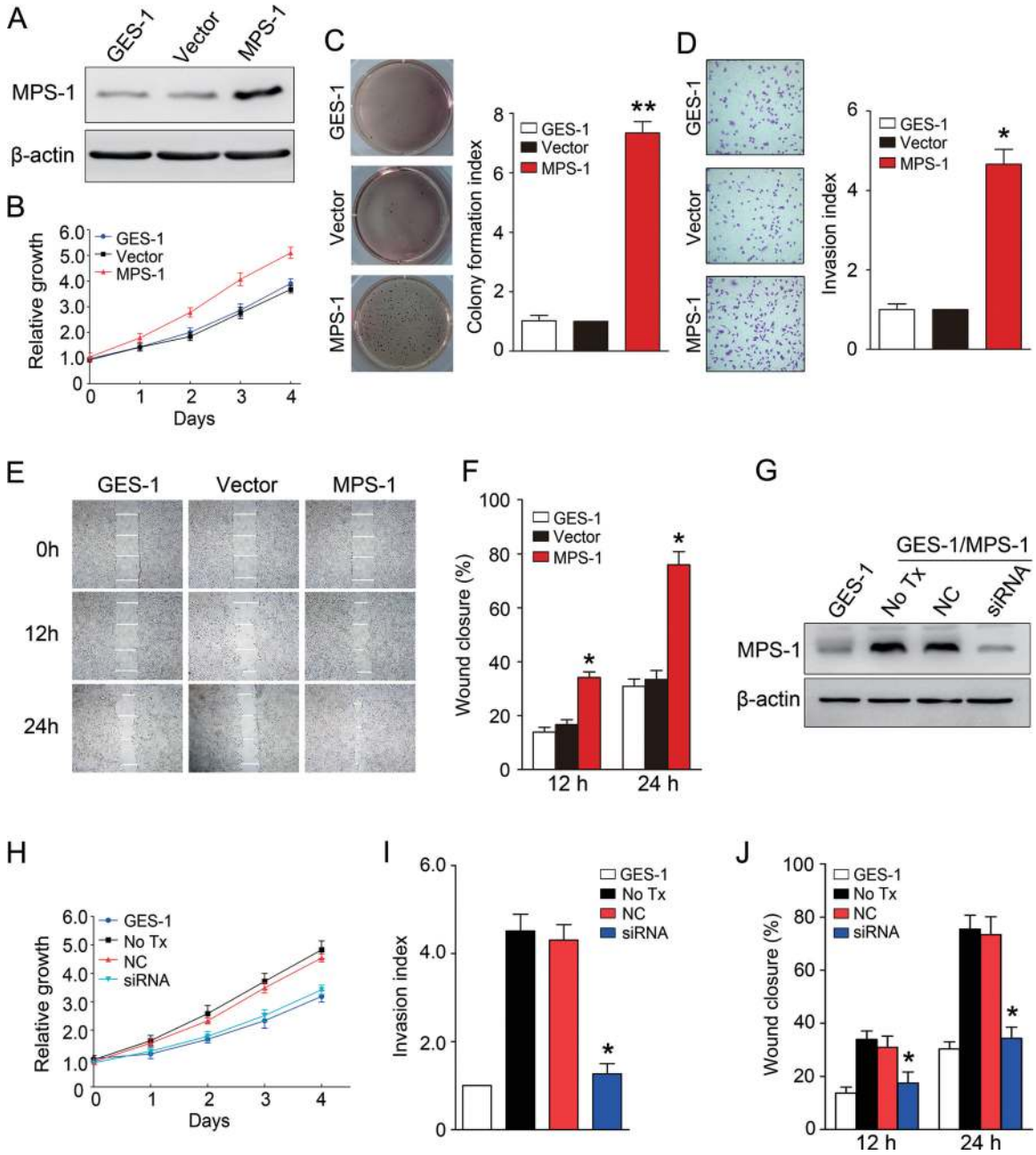

Fig. 2. Overexpression of MPS-1 promotes the invasion and migration of gastric cells. (**A**) Western blotting analysis was used to examine the expression of MPS-1 in GES-1, GES-1 transfected with vector (Vector) and GES-1 transfected with pQCXIN-MPS-1 (MPS-1) cells. (**B**) The growth of GES-1, Vector and MPS-1 cells was measured within 4 days. (**C**) Colony formation assay was performed to examine the colony formation of GES-1, Vector and MPS-1 cells (the colony forming index between GES-1 and MPS-1 was 7.32 ± 0.42 versus 1.13 ± 0.22). Matrigel invasion assay (**D**) and scratch-wound assay (**E**) were performed. The invasion index and wound closure (%) (**F**) were calculated. (**G**) Western blotting analysis of GES-1, no treatment (No Tx), NC and small interfering RNA transfected cells with the detection of MPS-1 antibody. (**H**) Cell growth of GES-1, No Tx, NC and small interfering RNA cells was assessed by the WST-8 method. Matrigel invasion assay (**I**) and scratch-wound assay (**J**) were performed. *$P < 0.05$; **$P < 0.01$.

expression. Taken together, these results indicate that MPS-1 expression is responsible for the invasion and migration enhancement of gastric cells.

Knockdown of MPS-1 expression downregulates the expression of ITGB4

Because we found that the knockdown of MPS-1 inhibits the invasion and migration of gastric cancer cells *in vitro* and *in vivo*, we sought to establish a relationship between the knockdown of MPS-1 and defects in gastric cancer invasion and migration. By comparing the gene expression of P4 cells with control cells using a microarray pathway (29) and Signal-Net analysis, we found that genes responsible for cell-matrix adhesion and cytoskeletal regulation were significantly altered (Supplementary Figure 4 and Supplementary Table 3, available at *Carcinogenesis* Online). We further analyzed the differentially expressed genes related to metastasis and found that the integrin family member *ITGB4* showed remarkable changes both in profiling and qRT–PCR analysis (Figure 3A and B).

We examined expression of ITGB4, and as displayed in Figure 3C, ITGB4 was distinctly reduced in P4 cells at the messenger RNA and protein levels. Consistent with this low expression of ITGB4, immunofluorescence microscopy confirmed a reduced signal at the cell membrane (Figure 3D). Similarly, ITGB4 expression was reduced in P4 cells compared with control cells, as determined by flow cytometry (Figure 3E and F). Similar findings were observed in AGS cells (Supplementary Figure 5, available at *Carcinogenesis* Online), suggesting that this was not a cell line-specific phenomenon. Furthermore, immunohistochemical staining of sections from lung metastases showed that the cells of the control groups had strong ITGB4 staining, whereas P4-derived cells were characterized by weak ITGB4 staining (Figure 3G). Overall, these data demonstrate that the expression of ITGB4 is downregulated by MPS-1 inhibition.

Expression of ITGB4 is correlated with MPS-1 in human gastric cancer

ITGB4 is reported to be overexpressed in many types of human cancers (16,17). Immunohistochemical staining of ITGB4 was performed on tissue microarrays containing 75 matched human gastric carcinomas and surrounding tissues (Figure 4A). We found that ITGB4 was highly expressed in gastric cancer tissues compared with the matched surrounding tissues and correlated with tumor-node-metastasis stage and metastasis (Figure 4B and Supplementary Tables 4 and 5, available at *Carcinogenesis* Online). Furthermore, there was a positive correlation between MPS-1 and ITGB4 expression (Figure 4C and Supplementary Table 6, available at *Carcinogenesis* Online). Additionally, cytological experiments showed that ITGB4 was highly expressed in all four gastric cancer cell lines (SGC-7901, AGS, MKN28, MKN45), whereas GES-1 has low ITGB4 expression. These results are similar to the MPS-1 expression patterns (Figure 4D and E). Together, these data suggest that ITGB4 is highly expressed in gastric cancer and has a close correlation with MPS-1 in gastric cancer at cytology and tissue levels.

Invasion-promoting characteristics of ITGB4 and phenotypes induced by ectopic expression of MPS-1 partially depends on ITGB4 expression

To explore the *in vitro* significance of ITGB4, we used specific shRNAs to suppress ITGB4 expression in gastric cancer cells. When ITGB4 was specifically inhibited in SGC-7901 cells, MPS-1 expression was unchanged (Figure 5A). There was, however, a significant reduction of invasion and migration by SGC-7901 cells after downregulation of ITGB4 (Figure 5B and C). In contrast, GES-1 cells with ectopic expression of ITGB4 showed enhanced colony formation efficiency (Figure 5D and E) and cell invasiveness and migration (Figure 5F and G). These data indicate that ITGB4 expression enhances the invasion and migration of gastric cells and suggest a strong tumorigenic potential.

Next, we sought to determine whether the increase in invasion and migration induced by constitutive MPS-1 expression in gastric cancer cells was ITGB4 dependent. We transfected pcDNA3.0/Myc-ITGB4 into the P4 cells (Figure 5H). There was no obvious effect on the expression of MPS-1, and as expected, the invasiveness and migration of gastric cancer cells were largely restored (Figure 5I and J). Furthermore, we transfected a specific shRNA targeting ITGB4 into SGC-7901 cells constitutively expressing MPS-1 (Supplementary Figure 6A, available at *Carcinogenesis* Online) and found that ITGB4 suppression largely attenuated cell colony formation and the invasion and migration of gastric cancer cells in the presence of MPS-1 overexpression, although not to the basal level (Supplementary Figure 6B–D, available at *Carcinogenesis* Online). These results demonstrate that ITGB4 suppression greatly abrogates phenotypes induced by MPS-1, indicating that MPS-1 regulates the invasion and migration of gastric cancer cells partially through ITGB4.

Combination of MPS-1 and ITGB4 for survival prediction in gastric cancer patients

Because MPS-1 and ITGB4 are critical for cancer cell invasiveness and migration, we hypothesized that high levels of these two genes predict poor patient survival in gastric cancer. To test this hypothesis, the panel of 75 paired gastric cancer/surrounding tissues used in immunohistochemical staining were analyzed for overall survival. We first examined the overall survival of patients with metastasis (M_1) and without metastasis (M_0), and there was a significant difference between these two groups ($P < 0.05$) (Figure 6A). Next, we examined the overall survival to explore whether MPS-1, ITGB4 or the two combined are important risk factors for clinical outcomes. Patients with high MPS-1 or ITGB4 expression in gastric cancer had a significantly poorer prognosis than those with low MPS-1 or ITGB4 expression, with the median survival of 47 versus 23 months or 40 versus 21 months, respectively ($P < 0.01$; Figure 6B and C). Furthermore, patients with both genes highly expressed had the worst prognosis, with a median survival of 51 versus 19 months ($P < 0.01$; Figure 6D). Thus, it appears that high expression of MPS-1 and/or ITGB4 is a significant risk factor for gastric cancer prognosis.

Discussion

Despite high expression of MPS-1 in various types of tumors, little is known about the mechanisms by which MPS-1 regulates tumorigenesis (35–39). There is also little evidence about the role of MPS-1 in regulating tumor cell behavior, let alone the underlying mechanisms. Our previous studies have shown that MPS-1 is overexpressed in primary and metastatic human gastric carcinomas, with a trend toward higher MPS-1 expression with metastasis. In the current study, we provide strong evidence for the first time that the expression of MPS-1 regulates the invasion and migration of gastric cancer cells.

One possible explanation for MPS-1 overexpression in human cancer is that MPS-1 is a p53-responsible target and subjected to transcriptional repression by wild-type p53 but not by mutant p53. Loss of expression of wild-type p53, some human cancers have a higher MPS-1 expression. On the other hand, overexpressed MPS-1 may stabilize p53 by inhibiting MDM2-mediated degradation, which in turn confers growth advantage in some cancer cells (40). Thus, through interacting with p53-MDM2 axis, MPS-1 could regulate cell growth and survival.

Downregulation of MPS-1 obviously suppressed invasion and migration of gastric cancer cells *in vitro* and *in vivo*, whereas upregulation of MPS-1 enhances the invasion and migration of these cells. However, how does the expression of MPS-1 mediates this process? To resolve this puzzle, pathway and Signal-Net analysis of MPS-1 knockdown microarrays were performed and, subsequently, identified ITGB4 as the key gene in MPS-1 regulated invasion and migration. We showed that overexpressed ITGB4 enhanced invasion and migration, and downregulation of ITGB4 greatly inhibited

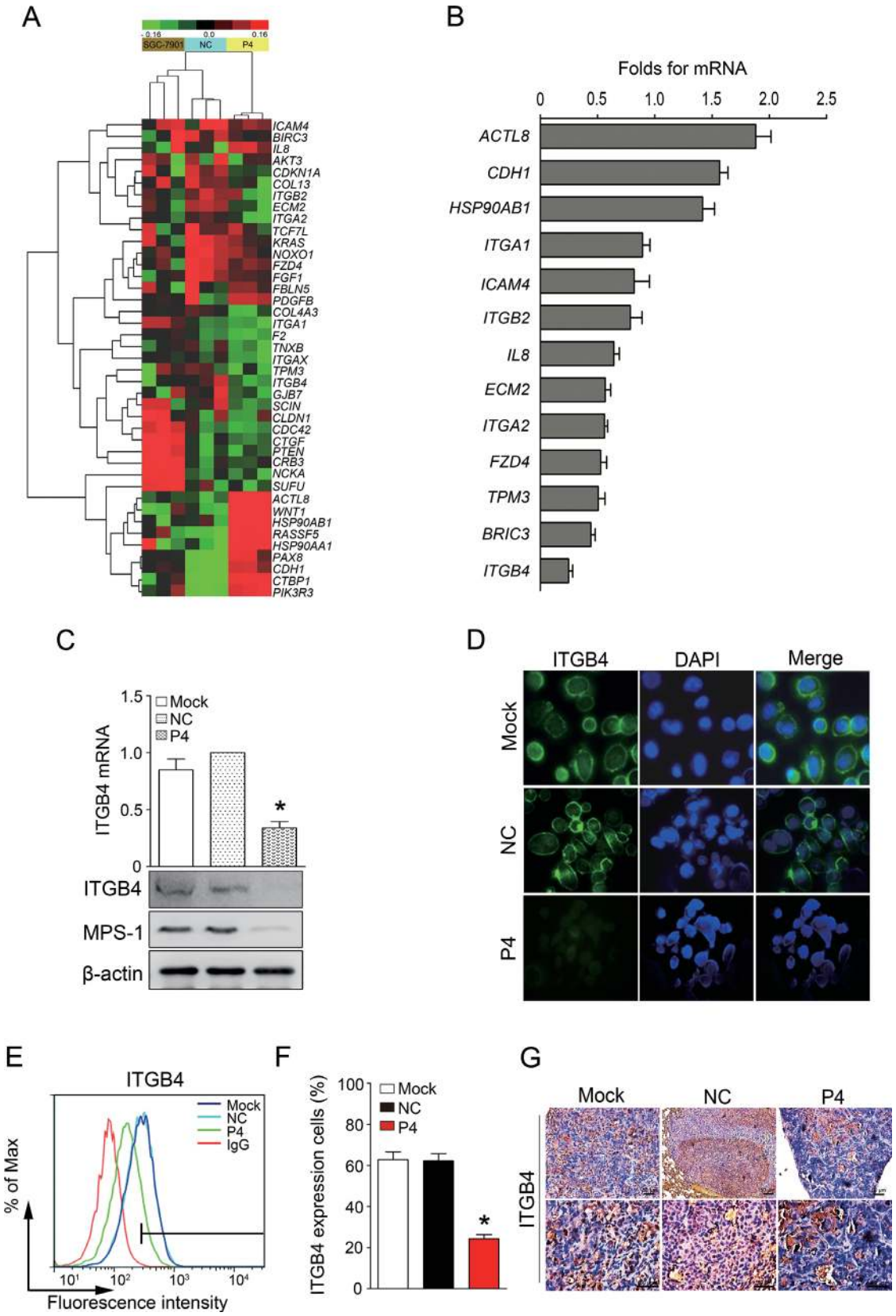

Fig. 3. MPS-1 knockdown inhibits the expression of ITGB4. (**A**) Clustering analysis of differentially expressed metastasis-related genes among SGC-7901, NC and P4 cells. Upregulated genes are shown in red and downregulated genes in green. (**B**) qRT–PCR analysis of selected differentially expressed metastasis-related genes. (**C**) Expression of ITGB4 was examined by qRT–PCR and western blotting in mock, NC and P4 cells. (**D**) Subcellular localization of ITGB4 was examined by immunofluorescence microscopy. The second panel depicts 4',6-diamidino-2-phenylindole nuclear counterstaining. (**E**) Flow cytometry analysis of ITGB4 expression relative to control IgG isotype in mock, NC and P4 cells. (**F**) The percentage of ITGB4-expressing cells is indicated in the column statistics with IgG isotype as a control. *$P < 0.05$. (**G**) ITGB4 expression was determined by immunohistochemical staining in metastatic tumors derived from mock, NC and P4 cells (×200, top; ×400, bottom).

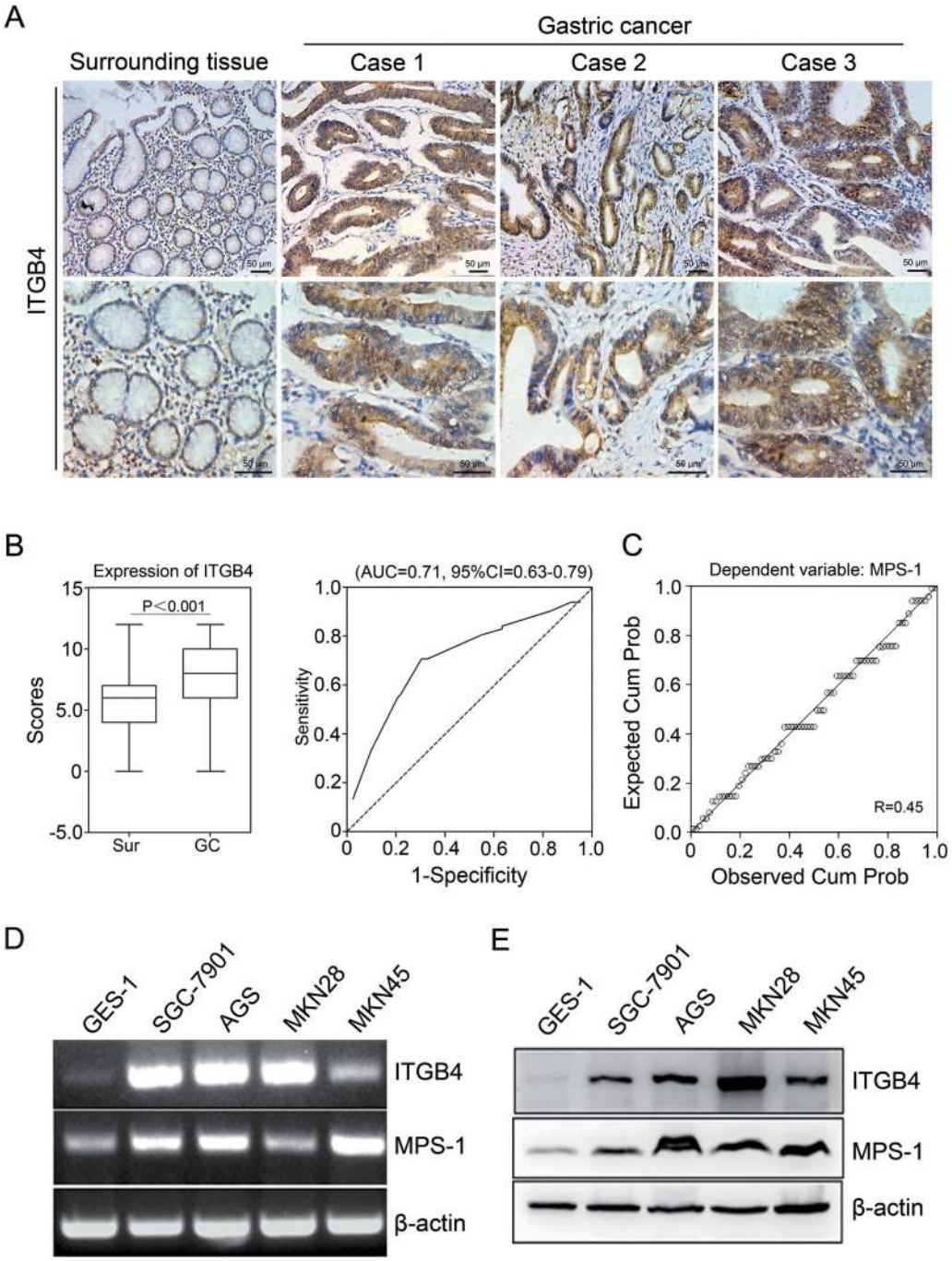

Fig. 4. ITGB4 and MPS-1 are correlatively expressed in gastric cancer tissues and cell lines. (**A**) Immunohistochemical staining of ITGB4 in gastric tissue microarrays. Low (×200, upper) and high (×400, lower) magnification images of representative staining are shown. (**B**) The expression of ITGB4 in gastric cancer (GC) tissues is much higher compared with the matched surrounding (Sur) tissues in the tissue microarray ($P < 0.001$). The receiver operating characteristics (ROC) curve showed that the area under the curve (AUC) was 0.71. (**C**) Normal P-P plot of regression standardized residual between MPS-1 and ITGB4 ($R = 0.45$; $P < 0.01$). Expression of ITGB4 and MPS-1 was examined by RT–PCR (**D**) and western blotting (**E**) in GES-1, SGC-7901, AGS, MKN28 and MKN45 cells with β-actin as an internal control or loading control.

the invasion and migration of gastric cancer cells. ITGB4 structure composed of three prime domains, the extracellular domain, the transmembrane domain and the relatively short cytoplasmic domain (41). Much of the work on signaling through the ITGB4 to date has focused on the cytoplasmic domain, which interacts with the actin cytoskeleton (42). Downregulation of ITGB4 characterizes by cytoskeleton changing with non-polarized lamellar extensions (23). In consistence with this, MPS-1 knockdown-induced morphologic changing of gastric cancer cells has a similar appearance, which discourages invasion abilities. Additionally, during the progression from tumor cell growth to metastasis, specific integrins enable cancer cells to detach from neighboring cells, survive and proliferate in foreign microenvironments (43). For example, introduction of ITGB4 in ITGB4-negative breast carcinoma cells increases the invasive ability of these cells *in vitro* (44). Accordingly, we showed that introduction of ITGB4 expression in gastric cells correlates with enhanced proliferation and their progression to invasive phenotypes. Moreover, our results showed a positive association of MPS-1 and ITGB4

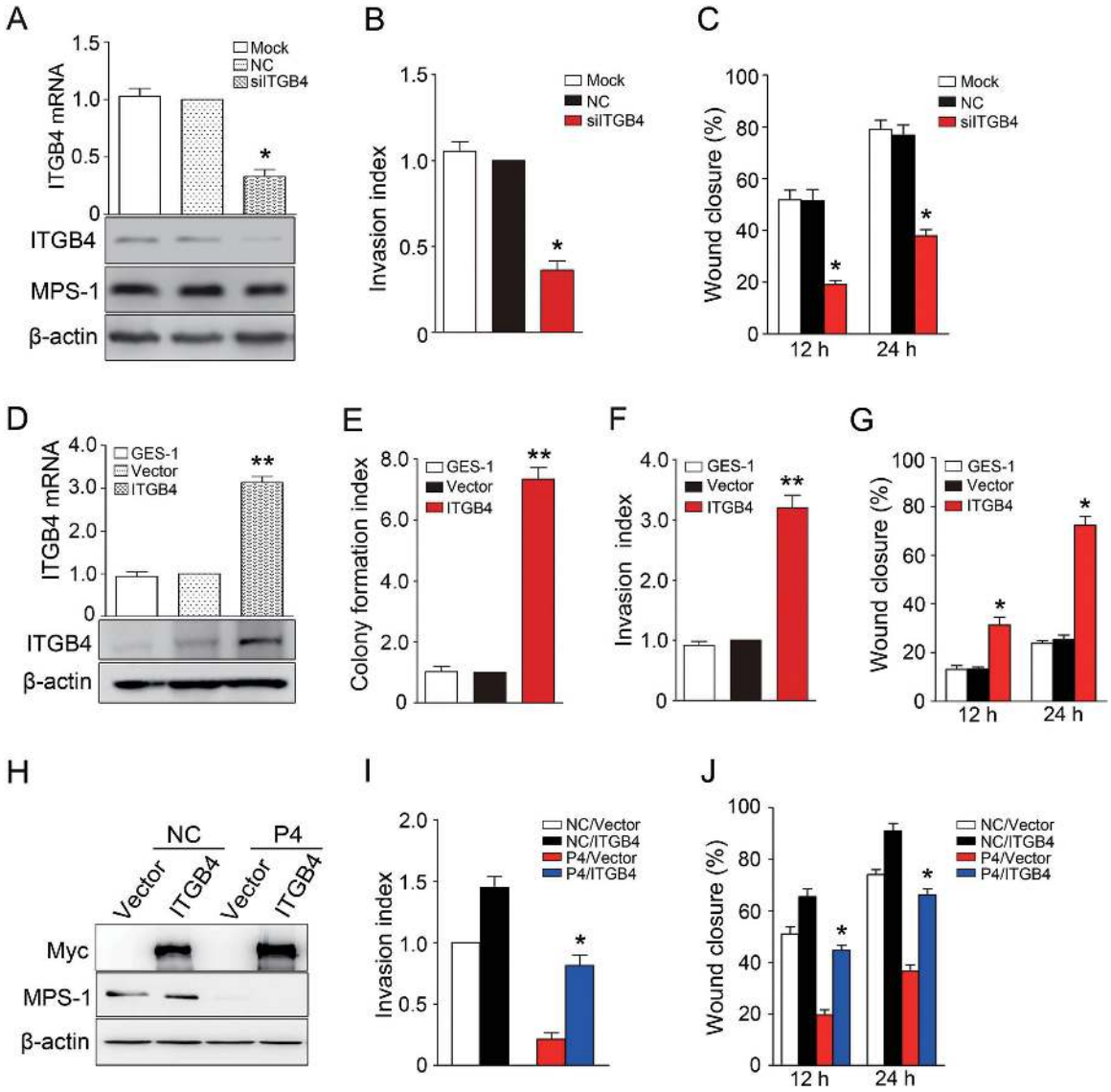

Fig. 5. ITGB4 mediates the invasion and migration of gastric cancer cells regulated by MPS-1. (**A**) ITGB4 and MPS-1 expressions were examined by qRT–PCR and western blotting. (**B**) A matrigel invasion assay of mock, NC and shITGB4 cells was performed and the invasion index was calculated. (**C**) A scratch-wound healing assay was performed and wound closure (%) was determined as the width migrated after 12 and 24 h relative to the initial width. (**D**) Overexpression of pcDNA3.0/Myc-ITGB4 in GES-1 cells. ITGB4 expression in GES-1, GES-1/Vector and GES-1/ITGB4 cells was detected by qRT–PCR and western blotting. (**E–G**) Colony formation assay, Matrigel invasion assay and scratch-wound assay were performed in GES-1, GES-1/Vector and GES-1/ITGB4 cells. (**H**) Western blotting examined the expression of ITGB4 and MPS-1 with Myc and MPS-1 antibodies in NC and P4 cells transfected with vector and pcDNA3.0/Myc-ITGB4. Matrigel invasion assay (**I**) and wound healing assay (**J**) were performed in NC/Vector, NC/ITGB4, P4/Vector and P4/ITGB4 cells, and the invasion index was calculated. $*P < 0.05$; $**P < 0.01$.

expression with the tumor-node-metastasis stage and metastasis in gastric cancers. We wonder if there is any correlation between these two proteins in gastric cancer. Further exploration of the relationship between MPS-1 and ITGB4 based on immunhistochemical staining and western blotting demonstrated that they express uniformly and correlatively in gastric cancer tissues and cell lines (Figure 4 and Supplementary Table 6, available at *Carcinogenesis* Online), which implies that they cooperated to promote the invasion and migration of gastric cells.

p53 is reported to inhibit the survival function of ITGB4 by inducing the caspase-dependent cleavage and inactivation of the serine/threonine kinase AKT/PKB (45). Combined with the report that MPS-1 is a transcriptional repression target of p53, these two genes seem to be negatively regulated by p53. However, in p53-mutant cell line SGC-7901, the function of ITGB4 can be inhibited by the downregulation of MPS-1, and the direct regulation and interaction between these two genes is unclear. Different cell types used may be one reasonable explanation. Further, we tend to explore the regulation and interaction between these two genes. We have previously found that knockdown of MPS-1 expression inhibited nuclear factor-kappa B (NF-κB) activity, and as a transcription factor NF-κB plays important role in the proliferation and metastasis of cancer cells. There are two NF-κB binding sites in the promoter of ITGB4 (within 1000 bp), NF-κB transfection elevated ITGB4 luciferase activity and the two NF-κB binding sites contributed to NF-κB transactivity (Supplementary Figure 7, available at *Carcinogenesis* Online). So we propose that knockdown of MPS-1 reduces NF-κB activity, which in turn inhibits ITGB4 transcription activity and leads to the downregulation of ITGB4 expression. However, the exact mechanism has to be further explored.

In clinical practice, hitherto, there are no studies that have looked into the relationship between MPS-1 expression and cancer prognosis,

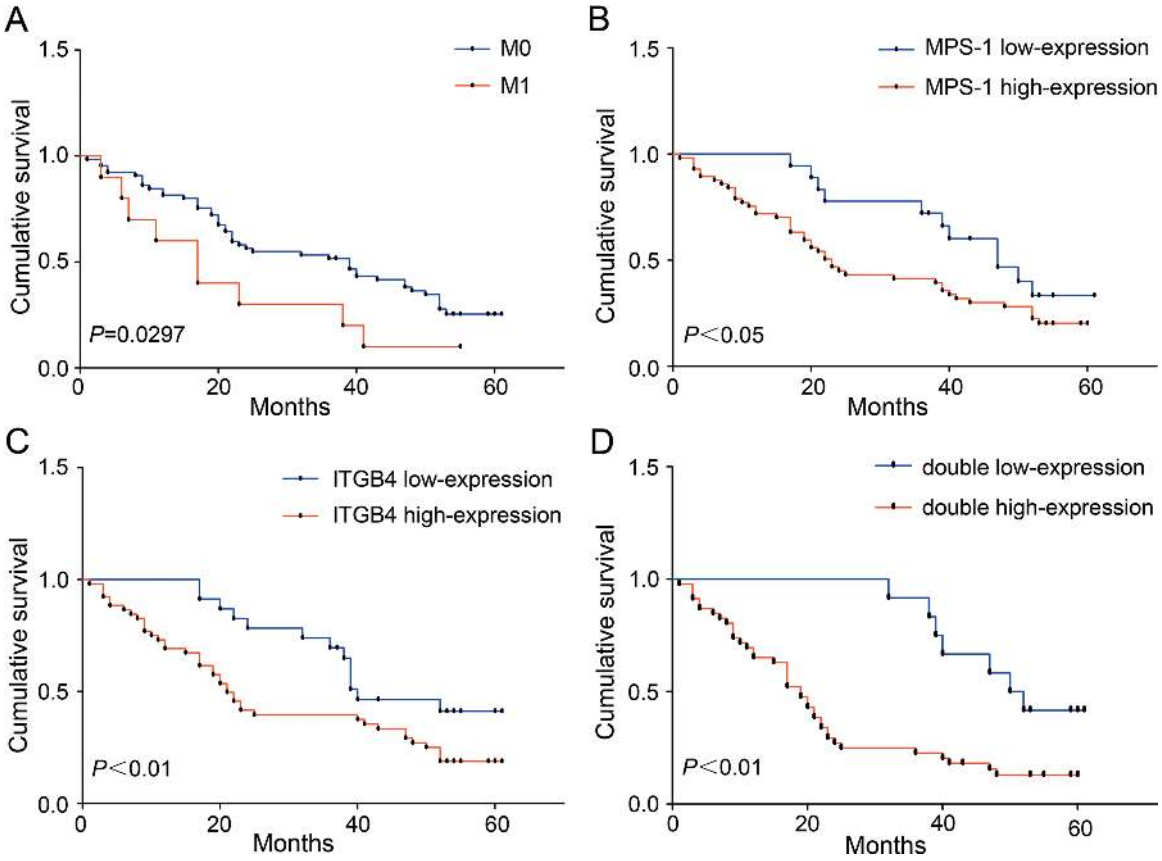

Fig. 6. Combination of MPS-1 and ITGB4 expression for the prediction of survival in gastric cancer patients. (**A**) Kaplan–Meier curves of the cumulative survival of gastric cancer patients with (M_1) and without metastasis (M_0) ($P = 0.0297$). (**B**) Kaplan–Meier curves of the cumulative survival of gastric cancer patients with low and high MPS-1 expression ($P < 0.05$). (**C**) Kaplan–Meier curves of the cumulative survival of gastric cancer patients with low and high ITGB4 expression ($P < 0.01$). (**D**) Kaplan–Meier curves of the cumulative survival of gastric cancer patients with both MPS-1 and ITGB4 low and high expression ($P < 0.01$).

although high expression of ITGB4 is reported to be associated with poor prognosis of some types of carcinomas (46,47). Here, we demonstrated that MPS-1 is highly expressed in gastric cancer patients, and the prognosis is poor in MPS-1 highly expressed gastric cancer patients ($P < 0.05$). The prognosis is still worse for those with both MPS-1 and ITGB4 high expression ($P < 0.01$). These findings suggest that MPS-1, particularly in combination with ITGB4, is a promising target for gastric cancer therapy, although it requires further verification.

In conclusion, we show here that MPS-1 expression is increased in patients with gastric carcinoma and correlates with ITGB4 expression. Importantly, MPS-1 regulates the invasion and migration of gastric cancer cells partially through ITGB4. These results shed new light on MPS-1/ITGB4-based mechanisms of metastasis and suggest a potential use of MPS-1/ITGB4 for targeted therapy in gastric cancer.

Supplementary material

Supplementary Tables 1–6 and Figures 1–7 and can be found at http://carcin.oxfordjournals.org/

Funding

National Natural Science Foundation of China (81201915, 81200643); the Research Fund for the Doctoral Program of Higher Education of China (20120073120098); Special fund for cultivation of outstanding young teachers of Shanghai University (82013011870002); the outstanding doctor cultivation fund of Shanghai Jiao Tong University (YBPY2009007); the National Basic Research Program of China (2010CB912104).

Acknowledgements

We gratefully acknowledge Prof. Ningli Li (Shanghai Institute of Immunology) for helpful review and polishing and Dr Xiaojiang Cui (John Wayne Cancer Institute, USA) for assistance in the manuscript preparation. We would also like to thank the Genminix Informatics for assisting us with data analysis.

Conflict of Interest Statement: None declared.

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Received December 12, 2012; revised June 14, 2013; accepted June 19, 2013