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RESEARCH

Semaphorin 5A, an Axon Guidance Molecule, Enhances the Invasion and Metastasis of Human Gastric Cancer through Activation of MMP9

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Abstract Semaphorin 5A, a member of semaphorin family, was originally identified as axonal guidance factor functioning during neuronal development. Previously, we showed that the expression of semaphorin 5A might contribute to the metastasis of gastric cancer. However, its functional roles and mechanism(s) in invasion and metastasis of gastric cancer remain unclear. By using human gastric caner cell lines Parental SGC7901, SGC7901-siScrambled and SGC7901-siSema 5A, we found that semaphorin 5A significantly promoted the invasive and metastatic abilities of gastric cancer cell in vitro. Semaphorin 5A increased the expression of MMP9 by activating phosphorylated ErK1/2 in gastric cancer cell. Furthermore, MEK inhibitor PD98059 and MMP9 antibody (Ab) significantly inhibited in vitro invasive and metastatic abilities induced by semaphorin 5A. Taken together, the present work revealed a novel function of semaphorin 5A that the existence of semaphorin 5A could promote invasion and metastasis of gastric cancer by regulating MMP9 expression, at least partially, via the MEK/ERKs signal transduction pathway. Semaphorin 5A and its regulated molecules could be the potential targets for cancer therapy.

Keywords Semaphorin 5A · Gastric cancer · Invasion and metastasis · MMP9 · ErK1/2

Abbreviations

MMPs	matrix metalloproteinase
ERK	extracellular signal-regulated kinase
RT-PCR	reverse-transcription polymerase chain reaction
PBS	phosphate buffer solution
PVDF	polyvinylidene difluoride
TBS	tris-buffer saline
FBS	fetal bovine serum
Ab	antibody
ELISA	enzyme linked immuno sorbent assay
ECM	extracellular matrix
RGDS	Arginine-Glycine-Aspartic acid-Serine

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Introduction

Gastric carcinoma is one of the most frequent causes of cancer-related deaths in the world. With approximately 876,000 new cases diagnosed each year, it ranks second in incidence following lung cancer [1]. Although great achievements have been achieved in high-resolution imaging, surgery techniques, chemotherapy, and radiotherapy, patients with advanced gastric carcinoma still face poor prognosis, 5-year survival rate lower than 20 % [2]. One of main reasons is that the exact molecular events leading to its development and progression are not fully understood. Therefore, it is of great clinical value to further understand the molecular mechanism(s) of gastric cancer and find novel therapeutic strategies that specifically suppress this process.

The semaphorin represents a large family of genes that currently contain more than 30 members, all of which share a conserved N-terminal domain called the 'sema' domain, a segment of approximately 400–500 amino. Based on sequence similarity and distinctive structural features, these genes have been grouped into eight subclasses (1–8) [3]. Semaphorin 5A belongs to the class V of semaphorin family, and is an integral membrane protein with characteristic seven thrombospondin specific repeats (TSP-1) [4, 5], which was originally identified as axonal guidance factor functioning during neuronal development. In our previous studies, we have shown that the expression of semaphorin 5A might contribute to the metastasis of gastric cancer [6]. However, very little is known about its roles and molecular mechanism(s) in the invasion and metastasis of gastric cancer.

In this study, by using gastric cancer cell lines Parental SGC7901, SGC7901-siScrambled and SGC7901-siSema 5A [7], in vitro experiments revealed that semaphorin 5A enhanced the invasive and metastatic abilities of gastric cancer cell in vitro. Semaphorin 5A promoted the activity of matrix metalloproteinase-9 (MMP-9) along with phosphorylation of extracellular signal-regulated kinase (ERK) 1/2. Moreover, MEK inhibitor PD98059 and MMP9 antibody (Ab) significantly inhibited in vitro invasive and metastatic abilities induced by semaphorin 5A. Therefore, semaphorin 5A could promote invasive and metastatic abilities of gastric cancer cell through activation of the MEK/ERKs pathway and consequent transactivation of MMP9.

Materials and Methods

Cell Culture

The gastric cancer cell lines Parental SGC7901, SGC7901-siScrambled which was transfected with the Scrambled plasmid, stably expressing semaphorin 5A, and SGC7901-siSema 5A which was transfected with siRNA plasmid, stably expressing semaphorin 5A siRNA were maintained in DMEM (GIBCO, Carlsbad, CA) containing 10 % heat inactivated fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air. Among these gastric cancer cell lines, Parental SGC7901, SGC7901-siScrambled cells were used as control groups.

Western Blotting Analysis

The cells were homogenized in lysis buffer. Equal amounts of protein were subjected to 10 % SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5 % nonfat milk and incubated with primary antibody. After three washes for 15 min in Tris-buffered saline (TBS) supplemented with 0.1 % Tween 20 (TBST), the membranes were incubated with horseradish peroxidase conjugated rabbit anti-mouse secondary antibody, followed by enhanced chemiluminescence (KPL, Gaithersburg, USA). Primary antibodies against MMP2, MMP9, pERK1/2, tERK1/2 and β-actin were applied at the optimized concentrations (Santa Cruz Biotechnology).

RT-PCR Analysis

Total RNA was extracted from specimens using Trizol reagent (Invitrogen). After RNA was quantified, cDNA was synthesized from 5 μg of total RNA according to the manufacturer's guidelines in a total volume of 20 μl (Fermentas Maryland, USA). Primers used for RT-PCR were as follows: MMP2-f, 5′- G C T A C G A T G G A G G C C C T A A T G -3′, and MMP2-r, 5′- T C T C C T T G G G G C A G C C A T -3′; MMP9-f, 5′-C A C T G T C C A C C C C T C A G A G C-3′, and MMP9-r, 5′-G C C A C T T G T C G G C G A T A A G G-3′; β-actin-f, 5′-C A C G C A C G A T T T C C C G C T C G G-3′, and β-actin-r, 5′-C A G G C T G T G C T A T C C T G T A C-3′. The PCR was performed at 94 °C for 5 min followed by 30 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 40 s with a PCR Thermal Cycler Dice (Takara, Otsu, Japan). β-actin was used as an internal control.

ELISA

The supernatant of the examined cells was centrifuged for 10 min at 2,000×*g* and collected, and stored in -80 °C until use. In order to activate the samples, 100 μl of each sample were incubated with 20 μl of 1 N HCl for 10 min at room temperature. The sample was then neutralized by adding 20 μl of 1.2 N NaOH/0.5 mol/l HEPES. Active MMP2 and MMP9 levels were measured by ELISA kit

(QuantikineTM, R&D, Minnepolis, MN, USA) according to the instructions of the manufacturer.

Wound Closure Assay

Gastric cancer cell mobility was assessed using a wound healing assay. The examined cells (5×10^4/per well) were seeded in a six-well plate coated with fibronectin and cultured until confluent to get cell monolayers, which were then carefully wounded using sterile pipette tips and any cellular debris was removed by washing with PBS. Photos were captured at 0, 36 h after wounding.

Invasion and Migration Assays

To determine the invasion ability, the examined cells were plated on cell culture inserts coated with Metrigel in 24-well plates (8-μm pore size; Becton Dickinson Labware) in serum-free medium, and 700 μl of DMEM containing 10 % bovine serum was added into the lower compartment. Migration assays were done using the same procedure but with uncoated Metrigel. In some inhibitory experiments, inhibitors and antibody were also added to the upper chambers. The cells were allowed to invade for 24 h at 37 °C in a 5 % CO_2 humidified incubator. Next, the cells were removed from the upper surface of the filter with the cotton swab, and the cells that had invaded the bottom surface of the filter were fixed with methanol and stained with hematoxylin. The invasiveness was determined by counting of the penetrating cells under a microscope at × 200 magnification on 10 random fields in each well.

Statistical Analysis

All experiments were performed at least three times, and the results were from representative experiments. The results were presented as means±SD. The data were analyzed by ANOVA. The statistical analysis was done using SPSS 11.0 software (SPSS) and $P<0.05$ was considered significantly.

Results

The Expression of Semaphorin 5A in Parental SGC7901, SGC7901-siScrambled, SGC7901-siSema 5A cells

To investigate molecular mechanism(s) by which semaphorin 5A enhances the invasion and metastasis of gastric cancer, we first examined the expression status of semaphorin 5A in gastric cancer cells Parental SGC7901, SGC7901-siScrambled, SGC7901-siSema 5A. Western blotting analysis showed that semaphorin 5A expression in SGC7901-siSema 5A cell was lower than that in Parental SGC7901, SGC7901-siScrambled cells (Fig. 1A).

Semaphorin 5A Promoted In Vitro Migratory, Invasive Abilities of Gastric Cancer Cells

In order to explore the functional roles of semaphorin 5A in the invasion and metastasis of gastric cancer, in vitro studies were done to compare the ability of Parental SGC7901, SGC7901-siScrambled, SGC7901-siSema 5A cells in migration, invasion. The effect of semaphorin 5A on the motility capability of gastric cancer cells was analyzed using a wound healing assay, in which cells were scratched and allowed to migrate into the wound area, the amount of migration or wound closure was enumerated 36 hours after disruption. As shown in Fig. 1B, D, Parental SGC7901 and SGC7901-siScrambled cells had wound closures of 95 %, 97 % by 36 hours, respectively while SGC7901-siSema 5A cell had a wound closure of 48 % in the same period.

A critical event in tumor invasion and metastasis is the ability of tumor cells to invade through the extracellular matrix, allowing tumor cells to move beyond the confines of the primary tumor environment. To examine the influence of semaphorin 5A on cell invasion, a modified Transwell chamber assay was carried out to determine the ability of gastric cancer cells to invade through biological matrices in vitro. As shown in Fig. 1C, E, we observed that SGC7901-siSema 5A cells capable of invading through the filter coated with Matrigel were decreased by 53.5 % as compared to Parental SGC7901 and SGC7901-siScrambled cells. These data, collectively, indicate that semaphorin 5A expression promotes the invasion and metastasis of gastric cancer cells.

Integrins Were Involved in Migration of Gastric Cancer Mediated by Semaphorin 5A

It is generally accepted that RGD actions are linked to its interaction with integrins. To determine whether integrins were involved in invasion of gastric cancer mediated by semaphorin 5A, migration assay was used to explore the effect of anti-integrins antibody on the migration ability of gastric cell. SGC7901-siSema 5A and Parental SGC7901cells were serum starved for 48 h and pretreated with 30 μg/mL anti-integrin-$\alpha_v\beta_3$, -$\alpha_5\beta_1$ antibody [8] for 4 h before the addition of 50 μM RGDS peptide (Bachem) for 16 h. As shown in Fig. 2A, anti-$\alpha_v\beta_3$ completely abolished the effect of RGDS peptide on the migration of SGC7901-siSema 5A and Parental SGC7901cells. Same result was obtained from the treatment of anti-$\alpha_5\beta_1$ (data no shown). These data indicate that different integrins may be involved in invasion of gastric cancer mediated by semaphorin 5A.

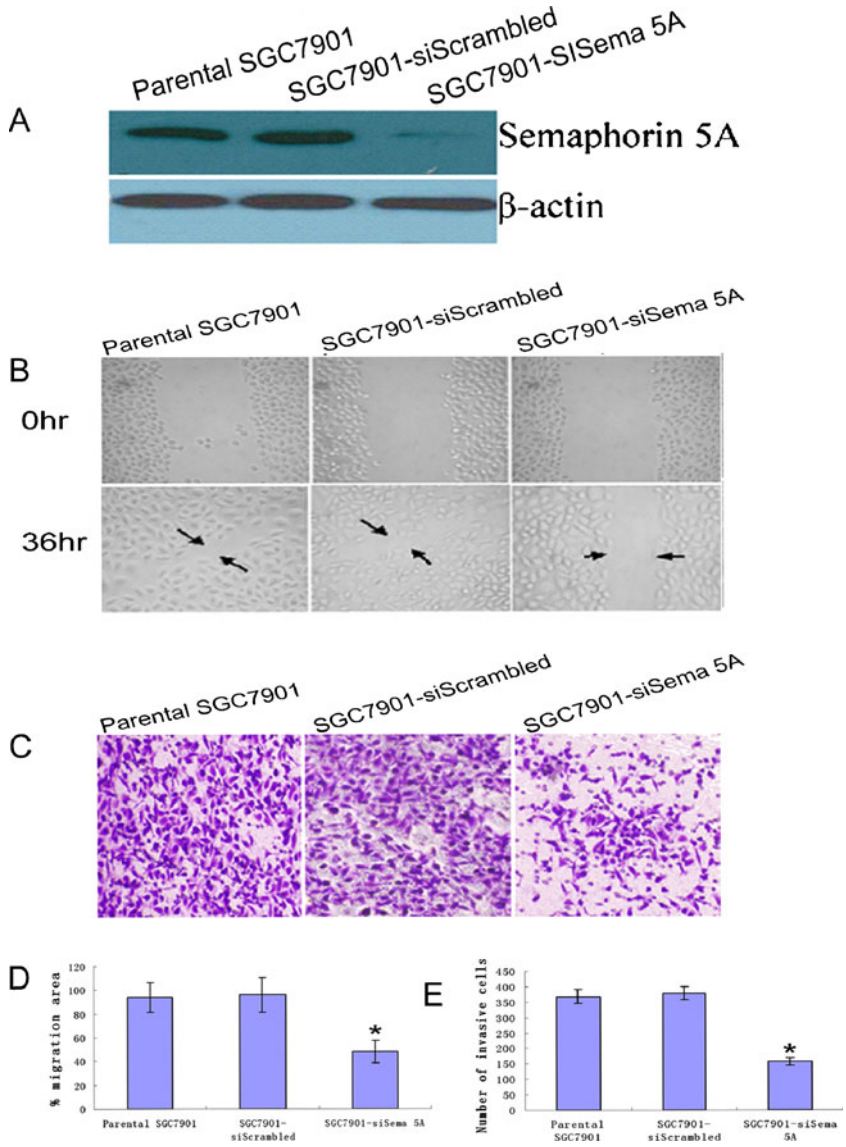

Fig. 1 Effect of semaphorin 5A knockdown by siRNA on gastric carcinoma cells migration and invasion. (**A**) The expression of semaphorin 5A in gastric cancer cells Parental SGC7901, SGC7901-siScramble and SGC7901-siSema 5A. (**B**) Effect of semaphorin 5A on the migration of gastric cancer cells Parental SGC7901, SGC7901-siScramble and SGC7901-siSema 5A by using the wound-healing scratch assay. (**C**) Effect of semaphorin 5A on the invasion of gastric cancer cells Parental SGC7901, SGC7901-siScramble and SGC7901-siSema 5A by using the Transwell chamber assay. (**D**) Statistical plot of migration assay. (**E**) Statistical plot of invasion assay. *$P<0.05$ vs controls

MMP9 was Involved in Invasion of Gastric Cancer Regulated by Semaphorin 5A

Tumor cell invasion through matrix and tissue barriers requires the combined effects of increased cell motility and regulated proteolytic degradation of matrix. Elevated levels of the MMPs such as MMP2 and MMP9 in tumor tissue have been generally correlated with cancer cell invasion and metastasis. In order to determine the cellular components involved in semaphorin 5A-induced invasion and metastasis, we initially focused on MMP2 and MMP9. Therefore, we examined the expression of MMP2 and MMP9 in Parental SGC7901, SGC7901-siScrambled, SGC7901-siSema 5A cells. The results from ELISA assay showed that MMP9 expression in the supernatant of SGC7901-siSema 5A cells was significantly down-regulated with compared to Parental SGC7901, SGC7901-siScrambled cells whereas the expression level of MMP2 protein was not altered (Fig. 2B). On the basis of RT-PCR and Western blotting assays, we also found that the expression level of MMP9 was decreased in SGC7901-siSema 5A cell (Fig. 2C). To explore the possible role of MMP9, MMP2 in semaphorin 5A-mediated invasion and metastasis, SGC7901-siSema 5A and Parental SGC7901 were treated with MMP9 and MMP2 Ab (15 μg/ml), respectively before performing invasion assay. The results demonstrated that treatment with MMP9 Ab could inhibit the invasive activities of those cell lines. Inhibiting rates caused by MMP9 Ab in Parental SGC7901 (49.1 %) were higher than those in SGC7901-siSema 5A (30.9 %) (Fig. 3A, B) whereas MMP2 Ab had no effect on the invasive activities of those cell lines (data no shown). All together, these data showed that enhancing the effect of semaphorin 5A on invasiveness of gastric cancer was at least partially mediated by MMP9 rather than MMP2, and possibly the consequent degradation of ECM.

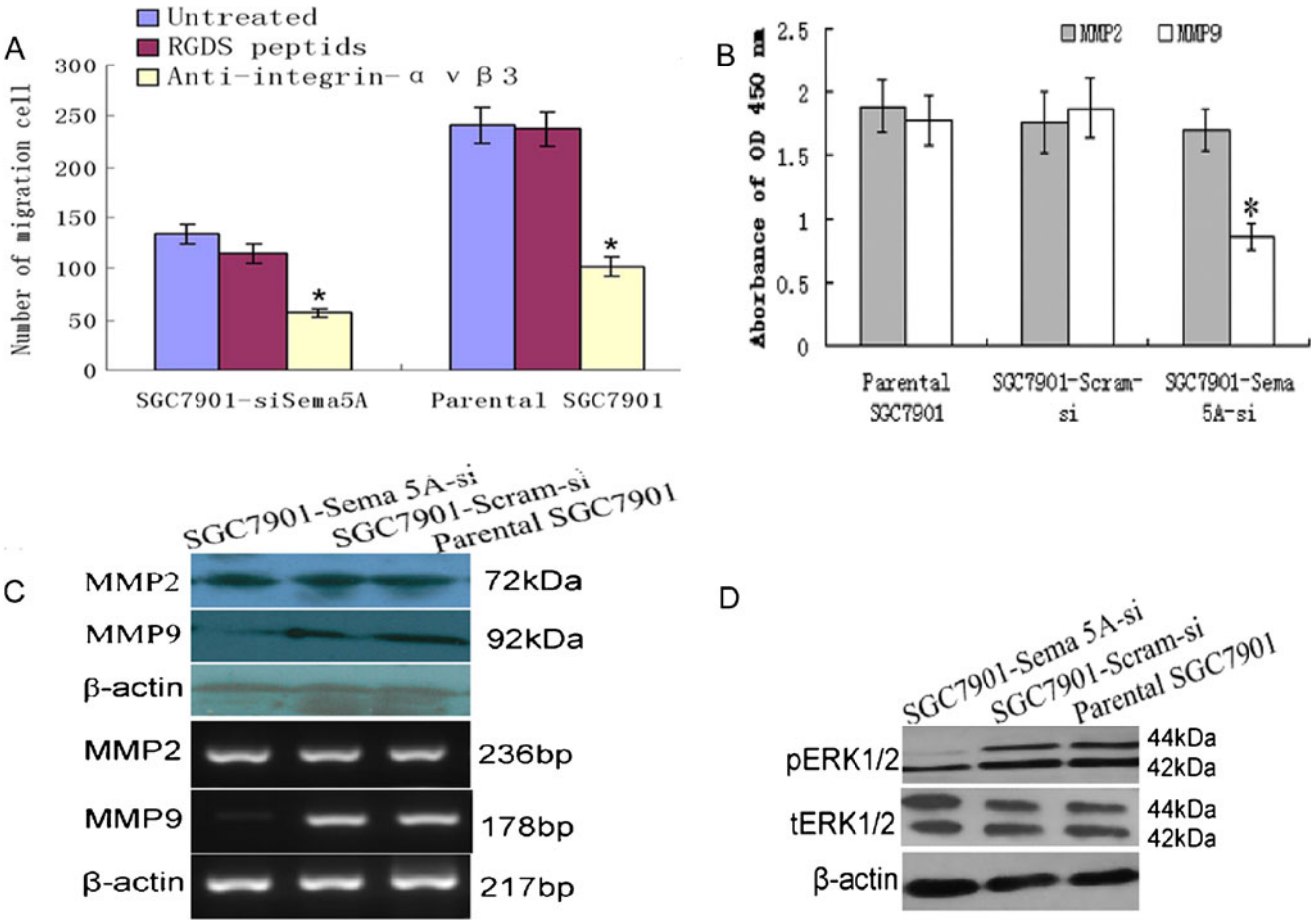

Fig. 2 Anti-integrin-$\alpha_v\beta_3$ antibody affects migration of gastric cancer and effect of semaphorin 5A knockdown on the expression of MMP2, MMP9 and pERK1/2. (**A**) Anti-$\alpha_v\beta_3$ antibody affects migration of Parental SGC7901 and SGC7901-siSema 5A. (**B**), (**C**) Effect of semaphorin 5A on the expression of MMP2, MMP9 in gastric cancer cells Parental SGC7901, SGC7901-siScramble and SGC7901-siSema 5A by using the ELISA, Western blotting and RT-PCR assays, respectively. (**C**) Effect of semaphorin 5A on the expression of pERK1/2, tERK1/2 in gastric cancer cells Parental SGC7901, SGC7901-siScramble and SGC7901-siSema 5A by using Western blotting. *$P<0.05$ vs controls

ERK1/2 Signal Was Involved in Gastric Cancer Invasion and MMP9 Transactivation Regulated by Semaphorin 5A

As downstream effectors of oncogene Ras, the MEK/ERKs pathway has been demonstrated to be an important mediator of tumor progression and invasion [9]. To elucidate the signaling pathway responsible for the semaphorin 5A-mediated invasion and metastasis in gastric cancer, we first examined the phosphorylated status of ErK1/2 in Parental SGC7901, SGC7901-siScrambled and SGC7901-siSema 5A by Western blotting. The results showed that phosphorylated ErK1/2 in SGC7901-siSema 5A cell was markedly down-regulated, whereas total ErK 1/2 protein was not altered when compared with control groups (Fig. 2D). To further understand the mechanism of semaphorin 5A-induced MMP-9 activation, we investigated the effect of PD98059, an MEK specific inhibitor, on the expression of MMP9 and the invasive ability of gastric cancer cells. After treatment with PD98059 at a concentration of 15 μM, both the protein expression and transcription level of MMP9 in gastric cancer cells were significantly down-regulated (Fig. 3C). These alteration was more significant in control group (Parental SGC7901) than in Sema 5A-siRNA transfected cell (SGC7901-siSema 5A), suggesting that semaphorin 5A enhanced the expression of MMP9 by activating phosphorylated ErK1/2. Similarly, PD98059 could also inhibit the invasive abilities of Parental SGC7901 cells with or without Semaphorin 5A siRNA transfection. The inhibiting rate of PD98059 on invasive abilities of Parental SGC7901 was 51.3 %, higher than 28.3 % of SGC7901-siSema 5A cell (Fig. 3A, B). All these results suggested that ErK1/2kinases of MAPK family were specifically involved in semaphorin 5A-related invasion and mediated transactivation of MMP9 induced by semaphorin 5A.

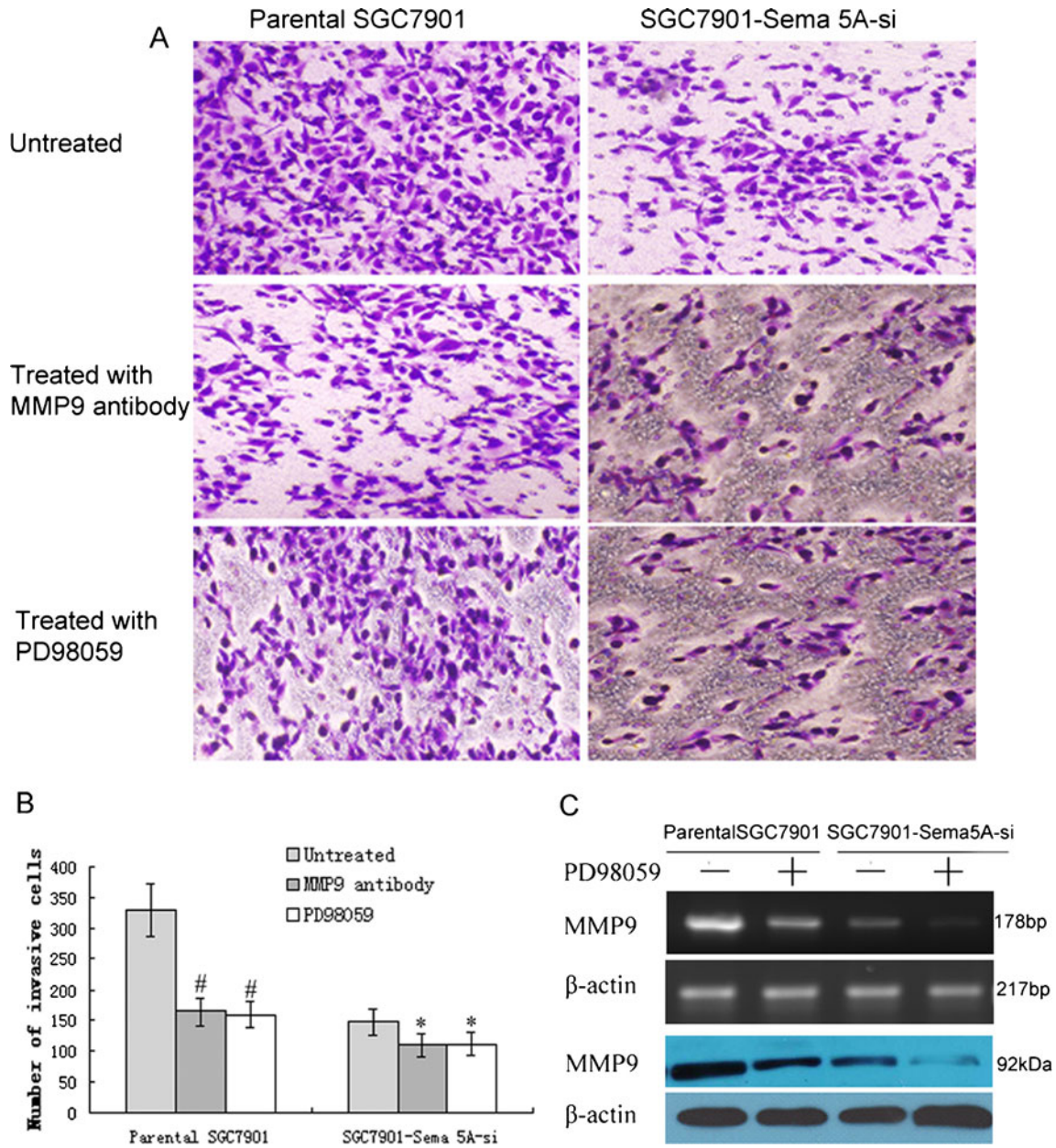

Fig. 3 Effect of MMP9 antibody and PD98059 on invasion and expression of MMP9 in gastric carcinoma cells. (**A**) MMP9 antibody and PD98059 inhibited invasion of Parental SGC7901 and SGC7901-siSema 5A. Inhibiting invasion rate was higher in Parental SGC7901 than in SGC7901-siSema 5A cells. (**B**) Statistical plot of invasion assay. (**C**) PD98059 reduced expression of MMP9 in gastric carcinoma cells Parental SGC7901 and SGC7901-siSema 5A. Inhibiting invasion rate was higher in parental SGC7901 than in SGC7901-siSema 5A cells. *$P<0.05$ vs controls, #$P<0.01$ vs controls.

Discussion

Most cancer-related mortality is not caused by the primary tumor but is the result of metastasis of malignant cells to distant organs. Therefore, better understanding the mechanism(s) regulating gastric cancer metastasis is a main objective of cancer research. In the present study, we presented the first evidence that semaphorin 5A could promote invasion and metastasis of gastric cancer by regulating MMP9 expression, at least partially, via the MEK/ERKs signal transduction pathway.

Semaphorin 5A, a member of class-5 semaphorins, is a membrane-bound protein that is located in chromosome 5p15.2. Initially, the gene was characterized as constituents of the complex regulatory system responsible for the guidance of growing axons to their targets during the development of the central nervous system [10]. However, cumulative data have indicated that certain semaphorins

implicated in axonal pathfinding in the developing nervous system were found to be expressed by multiple types of cancer cells, modulate the behaviour of cancer cells and promote tumor angiogenesis and tumor progression by multiple mechanisms [11–13]. In our previous study, we have shown that semaphorin 5A might contribute to the development and progression of gastric carcinoma [6]. However, its roles in the invasion and metastasis of gastric carcinoma have not been fully characterized yet so far. By using gastric cancer cells, we found that semaphorin 5A significantly enhanced the invasion and metastasis of gastric cancer cells, indicating its important roles in the development of gastric cancer.

Cancer progression involves a sequential series of critical genetic and molecular alterations inducing the deregulation of cell proliferation, adhesion, migration, and invasion and leading to the lethality associated with metastatic spread of malignant tumors. It is well known that matrix degradation by MMPs is critical for tumor invasion and metastasis. The importance of degradation of the ECM by matrix metalloproteinase during tumor invasion and metastasis has long since been established. In gastric cancer, two MMPs subunits, MMP2 and MMP9 play an important role in invasion and metastasis of tumor, and the extent of MMP2 and MMP9 expression has been found to correlate with tumor grade and stage [14]. In this study, we found that MMP9 expression at mRNA and protein levels was down-regulated by semaphorin 5A siRNA, and no change was showed in MMP2. Additionally, the in vitro assay showed that the blockage of MMP9 by antibody could partially reverse the enhancing effect of semaphorin 5A on the invasion in gastric cancer cell. Those results implied that the semaphorin 5A might be an upstream molecule of MMP9 in gastric cancer, through which semaphorin 5A promotes the invasiveness and metastasis of gastric cancer.

However, through which downstream signaling intermediates could semaphorin 5A regulate the expression of MMP9 in gastric cancer? Previous studies have shown that as downstream effectors of oncogene Ras, the MEK/ERKs pathway is an important mediator of tumor progression and invasion. To elucidate the signaling mechanism(s) by which semaphorin 5A exerts biological functions in invasion and metastasis of gastric cancer, we initially examined the association of the expression of semaphorin 5A with the activation of MEK-ERKs pathway. From the experiments, we found that phosphorylated Erk1/2 was down-regulated in semaphorin 5A siRNA cells when compared to control groups, suggesting that the biological effects of semaphorin 5A on invasion and migration of gastric cancer go through the MEK/ERKs pathway. The effect was verified by the fact that PD98059, a specific inhibitor for MEK/ERKs, could inhibit the invasive and migratory abilities of gastric cancer cells, which further showed that semaphorin 5A promotes metastasis and invasion of gastric cancer, at least partially, via the MEK/ERKs signal transduction pathway. To determine whether MEK-ERKs pathway, which was regulated by semaphorin 5A in gastric cancer, was responsible for regulation of the MMP9 expression, we treated cells with PD98059 before performing invasive experiments. Evidence that PD98059 could reduce the expression level of MMP9 indicated that the signal transduction pathway of MEK-ERKs was required for activation of MMP9.

Those inhibitory experiments had confirmed the regulatory function of semaphorin 5A on MMP9 through the MEK/ERKs signaling pathway. Taken them together, we may conclude that the expression of semaphorin 5A significantly companied with the development and progresses of gastric cancer, and that semaphorin 5A enhanced invasion and metastases of gastric cancer cells through activation of the MEK/ERKs pathway and consequent transactivation of MMP9. However, a recent study showed that Plexin B3 is identified as a high-affinity receptor for semaphorin 5A [15]. In our previous study, we have shown that the expression of semaphorin 5A might contribute to the metastasis of gastric cancer through Plexin B3. Therefore, it is possible that semaphorin 5A activates the MEK/ERKs pathway by binding to Plexin B3 and consequent the transactivation of MMP9, thereby exerting its biological functions in gastric cancer. In addition, some studies showed that some semaphorins can modify the behavior of cells through interacting with integrins[16, 17]. Whether semaphorin 5A in cooperation with integrins affects the invasion of gastric cancer cell remains unclear. The results from migration assay showed that anti-$\alpha_v\beta_3$, -$\alpha_5\beta_1$ completely abolished the effect of RGDS peptide on the migration of SGC7901-siSema 5A and Parental SGC7901cells, indicating that different integrins may be involved in invasion of gastric cancer mediated by semaphorin 5A.

To our knowledge, this is the first time to reveal the functional roles of semaphorin 5A and its molecular mechanism in the invasion and metastasis of gastric cancer, which not only displays a novel function of semaphorin 5A outside the nervous system but adds more weight to our knowledge of semaphorin 5A. Most importantly, since gastric cancer is one of the most risky cancers, this study may provide some useful information for the development of biomarkers in the clinical early diagnosis and also for the design of an appropriate therapeutical strategy of gastric cancer.

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Disclosure of Potential Conflicts of Interest No potential conflicts of interest were disclosed.

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