

# RNAi-mediated knockdown of PRL-3 inhibits cell invasion and downregulates ERK 1/2 expression in the human gastric cancer cell line, SGC-7901

YI CAO<sup>1\*</sup>, YI TU<sup>2\*</sup>, JINHONG MEI<sup>2</sup>, ZHENGRONG LI<sup>1</sup>, ZHIGANG JIE<sup>1</sup>, SHAN XU<sup>2</sup>,  
LINLIN XU<sup>2</sup>, SHANSHAN WANG<sup>2</sup> and YIFENG XIONG<sup>2</sup>

Departments of <sup>1</sup>General Surgery and <sup>2</sup>Pathology, The First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi 330006, P.R. China

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**Abstract.** The deregulated expression of members of the phosphatase of regenerating liver (PRL) family is important in the metastatic progression of multiple human cancers; however, the underlying mechanisms are not well understood. Previous studies have demonstrated that PRLs are able to enhance the activation of extracellular signal-regulated kinase 1/2 (ERK 1/2) in cancer cells, which may contribute to tumor metastasis. However, the effect of PRL-3 activation in gastric cancer (GC) remains unclear. The present study aimed to investigate whether the downregulation of PRL-3 by small interfering RNA (siRNA) was able to inhibit cell motility and affect ERK 1/2 expression in human GC. The results demonstrated that the downregulation of PRL-3 expression by siRNA in human GC cells significantly inhibited cell invasion and migration *in vitro*; accordingly, inhibition of PRL-3 also prevented ERK1/2 protein and mRNA expression, and reduced the mRNA level of matrix metalloproteinase-7 (MMP-7), the downstream target of ERK 1/2 signaling. Our data demonstrated that RNAi-mediated downregulation of PRL-3 expression leads to potent antitumor activity in human GC. Furthermore, ERK 1/2 and MMP-7 may contribute to the carcinogenesis and development of human GC in combination with PRL-3.

## Introduction

Gastric cancer (GC) is one of the most common types of cancer globally. It is considered to be the second most frequent cause of cancer-related mortality worldwide (1-3). The invasion and migration of GC are important factors leading to tumor recurrence and affecting prognosis; however, the molecular pathogenesis of GC metastasis is not well understood. For this reason, understanding the mechanisms underlying GC as well as the identification of novel molecular targets are of great importance.

Phosphatase of regenerating liver-3 (PRL-3), a metastasis-associated protein, belongs to the PRL family of protein tyrosine phosphatases (PTP), which includes two other members, PRL-1 and PRL-2 (4). The PTP superfamily of phosphatases includes a large group of enzymes important for the regulation of a wide variety of cell mechanisms, including signal transduction, the cell cycle, differentiation, cell transformation, adhesion and motility (5,6). Evidence has accumulated for the association of PRL-3 with oncogenic states, and several studies have linked its expression to human cancer progression and metastasis, such as in the case of malignant melanoma, as well as pancreatic, ovarian, breast and nasopharyngeal cancer (7-11). In a previous study, we examined the expression of PRL-3 in primary GC tissues and in peritoneal metastases, and found that PRL-3 expression was significantly higher in primary gastric carcinoma with peritoneal metastasis than in peritoneal metastasis-negative gastric carcinoma (12). However, little is known regarding the molecular mechanisms by which PRL-3 promotes motility, invasion and metastasis.

The extracellular signal-regulated kinase 1/2 (ERK 1/2), part of the mitogen-activated protein kinase (MAPK) family, is well-known for its role in numerous cell processes, such as cell migration, invasion and proliferation (13,14). One suggested mechanism whereby the ERK 1/2 pathway promotes invasiveness in tumor cells is through the upregulation of matrix metalloproteinases (MMPs), for extracellular matrix remodeling (15), which are important in tumor metastasis (16). A decrease in cell adhesion and proteolytic degradation of collagen by MMPs promotes the invasive migration of cells through the extracellular matrix (17). PRL-3 has previously

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*Correspondence to:* Professor Jinhong Mei, Department of Pathology, The First Affiliated Hospital of Nanchang University, 17 Yong Wai Zheng Road, Nanchang, Jiangxi 330006, P.R. China  
E-mail: jxwck@126.com

Professor Zhigang Jie, Department of General Surgery, The First Affiliated Hospital of Nanchang University, 17 Yong Wai Zheng Road, Nanchang, Jiangxi 330006, P.R. China  
E-mail: jiezg123@126.com

\*Contributed equally

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been described as one of the PRLs that are also capable of degrading the extracellular matrix; however, the molecular details remain unclear (7). Previously, it was demonstrated that an increased PRL-1 expression results in the activation of ERK1/2, which stimulates MMP production, and increases cell invasion and migration (18). It is known that PRL-3 has  $\geq 75\%$  amino acid sequence similarity with PRL-1 and PRL-2 (19). Therefore, it is also necessary and crucial to determine the relationship between PRL-3, ERK 1/2 and MMP expression in human GC.

In the present study, we employed siRNA targeting PRL-3 to explore the potential of new therapeutic targets in the treatment of GC. Our results suggested that knockdown of PRL-3 was able to inhibit GC invasion and migration, and significantly decrease ERK 1/2 and MMP-7 expression, which provides novel insights into tumorigenesis and may ultimately lead to more effective therapies.

## Materials and methods

**Gastric tissues, cell lines and cultures.** Human gastric adenocarcinoma cancer cell lines, SGC-7901, MKN-45, MKN-28 and BGC-823, were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The human gastric mucosa cell lines, GES-1 and HFE-145, were preserved in our central laboratory. Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Gibco-BRL, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100  $\mu\text{g}/\text{ml}$ ). Cells were incubated at 37°C in a humidified incubator with 5% CO<sub>2</sub>. All human gastric tissue samples, including eight gastric tissues and 20 malignant tissues, were obtained from the General Surgery Department of the First Affiliated Hospital of Nanchang University (Nanchang, China). All samples were obtained with informed consent and approved by the hospital institutional review board. The tissues were stored in liquid nitrogen following removal from patients.

**Plasmids and transfection.** The human PRL-3-specific siRNA was based on NCBI reference sequences (GenBank: PRL-3, NM\_032611). The siRNA sequences used for PRL-3 gene silencing were designed based on published data, as follows: Sense: 5'-GATCCGTGACCTATGACAAAACGCTTCAA GAGAGCGTTTTGTTCATAGGTCACCTTTTTGGAAA-3' and antisense: 5'-AGCTTTTCCAAAAAGTGACCTATGACAAA ACGCTCTTTGAAGCGTTTTGTTCATAGGTCACG-3' (20). The negative control sequences were as follows: Sense: 5'-GAT CCGTTATGTACAGGAACGCATTCAAGAGATGCGTT CCTGTACATAACCTTTTTGGAAA-3' and antisense: 5'-GCCAATAGATGTCCTTTCGTAAGTTCTCTACGCAA GACATGTATTGGAAAAACCTTTTCGA-3'. All siRNA transfections were performed using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) in Opti-MEM (Invitrogen Life Technologies) according to the manufacturer's instructions, with a final siRNA concentration of 100 nM.

**Quantitative real-time polymerase chain reaction (qRT-PCR) analysis.** Total RNA from tumor cells or tissues was isolated with a total RNA extraction kit (Sangon Biotech, Shanghai,

China) according to the manufacturer's instructions. The primer sequences for qRT-PCR analysis were designed and synthesized as follows: Forward: 5'-CACGCTCAGCACCTTCATTG-3' and reverse: 5'-GGTGAGCTGCTTGCTGTTGA-3' for PRL-3; forward: 5'-CGCTACACGCAGTTGCAGTACA-3' and reverse: 5'-AAGCGCAGCAGGATCTGGA-3' for ERK 1; forward: 5'-TGTTCCCAAATGCTGACTCCAA-3' and reverse: 5'-TCGGGTGCGTAATACTGCTCCAGATA-3' for ERK 2; forward: 5'-TGGACGGATGGTAGCAGTCT-3' and reverse: 5'-TCTCCATTTCCATAGGTTGGAT-3' for MMP-7; forward: 5'-GGCGGCACCACCATGTACCCT-3' and reverse: 5'-AGGGGCCGGACTCGTCATACT-3' for  $\beta$ -actin. RNA was first retrotranscribed using the TaqMan<sup>®</sup> Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA), and then RT-PCR was carried out using the TaqMan SYBR-Green master mix (Applied Biosystems). The relative expression of the mRNA was normalized to  $\beta$ -actin mRNA. The threshold cycle value (Ct) was defined as the fractional cycle number at which the fluorescence passed an invariable threshold. The comparative Ct method was used to calculate the relative abundance of mRNA compared with that of  $\beta$ -actin expression (21).

**Western blot analysis.** The cells were harvested, washed twice with 1X PBS and lysed in 100  $\mu\text{l}$  radioimmunoprecipitation assay (RIPA) lysis buffer. Protein concentrations were determined using a bicinchoninic acid (BCA) kit (Tiangen, China). Proteins were resolved by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Roche Diagnostics GmbH, Mannheim, Germany), which were then blocked with 5% non-fat milk in Tris-buffered saline for 3 h, and incubated overnight with primary antibodies. The secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit or -mouse IgG from Tiangen (Beijing, China). Membranes were incubated with the secondary antibodies for 1 h at room temperature, and proteins were detected using an ECL Western Blotting Analysis system (Amersham Biosciences Corp., Piscataway, NJ, USA).

**Cell migration and invasion analysis.** Cell migration assays were performed using 8.0- $\mu\text{m}$  pore size Transwell inserts (Costar, Cambridge, MA), with certain modifications. Cell invasion was investigated using Matrigel-coated 8.0- $\mu\text{m}$  filter invasion chambers (BD Biosciences, San José, CA, USA). Cells were incubated for 24 h (for migration assay) or 48 h (for invasion assay) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Cells on the upper surface of the membrane were removed using cotton tips after the indicated incubation times. The migrant cells attached to the lower surface were stained with crystal violet (500  $\mu\text{l}$  of 5 mg/ml crystal violet dissolved in 20% methanol) and incubated for 30 min. Cells were then soaked in 33% ice-cold acetic acid and oscillated for 10 min. The ice-cold acetic acid was then assessed by measuring the absorbance at 570 nm using a microplate reader (Tecan, Shanghai, China).

**Statistical analysis.** Statistical analyses were carried out using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). The data

are expressed as the means  $\pm$  standard deviation, and the significance of the data was determined by one-way ANOVA analysis.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Gastric cell lines express variable levels of PRL-3.** We first examined the protein and mRNA expression levels of PRL-3 in six gastric cell lines, using western blot analysis and qRT-PCR. The six gastric cell lines included two normal gastric cell lines, GES-1 and HFE-145, and four malignant human GC cell lines with different degrees of cell differentiation, BGC-823, MKN-28, SGC-7901 and MKN-45 (SGC-7901 and MKN-45 were low-grade cell lines, and BGC-823 and MKN-28 were high-grade cell lines). Among the six gastric cell lines that expressed PRL-3 at various levels, SGC-7901 cells demonstrated the highest level of PRL-3 protein (Fig. 1A) and mRNA (Fig. 1B) expression, while GES-1 cells exhibited the lowest PRL-3 protein and mRNA expression; SGC-7901 cell PRL-3 mRNA expression was 4.602-fold greater compared with the GES-1 cell line ( $P < 0.05$ ). Therefore, we selected the SGC-7901 cell line for subsequent RNA interference studies.

**ERK 1/2 is highly expressed in GC samples.** We examined GC cell lines and tissues to elucidate whether ERK 1/2 was expressed in GC. The results revealed that the expression of ERK 1/2 protein was upregulated in both GC tissues and cell lines (Fig. 2A and B, respectively), compared with normal gastric tissues and cell lines. The qRT-PCR analysis revealed that the SGC-7901 cell line exhibited strong ERK 1/2 mRNA expression; ERK 1 and ERK 2 mRNA were 3.968- and 2.525-fold greater, respectively, compared with the GES-1 cell line ( $P < 0.01$ ; Fig. 2B). Consistently, ERK 1/2 mRNA was over-expressed in malignant tissues compared with normal gastric tissues ( $P < 0.05$ ; Fig. 2A).

**Identification of the efficiency of PRL-3 siRNA.** As the PRL-3 levels were significantly higher in tumor cells compared with normal cells, we aimed to determine whether synthetic PRL-3 siRNA inhibited the expression of the PRL-3 gene in GC cells. To examine the efficiency of the specific PRL-3 siRNA, we used scrambled siRNA as the control. Following 48 h of siRNA transfection, PRL-3 mRNA and protein expression levels were measured by qRT-PCR and western blot analysis, respectively. As shown in Fig. 3, PRL-3 siRNA was capable of specifically and efficiently suppressing PRL-3 expression at the mRNA and protein level, compared with cells treated with siCONTROL and untreated cells ( $P < 0.05$ ; Fig. 3). Considering the significant silencing effect of PRL-3 siRNA, we used this siRNA for subsequent experiments.

**Downregulation of PRL-3 inhibits invasion and migration of SGC-7901 human GC cells.** To investigate the molecular mechanisms by which PRL-3 promotes cell invasion and migration in human carcinoma cells, following transfection with PRL-3 siRNA or the negative vector (siCONTROL) for 48 h, SGC-7901 cells were subjected to invasion or migration assays. As demonstrated in Fig. 4, cells transfected with PRL-3 siRNA exhibited a 66.7% decrease in invasion and a 68.1%

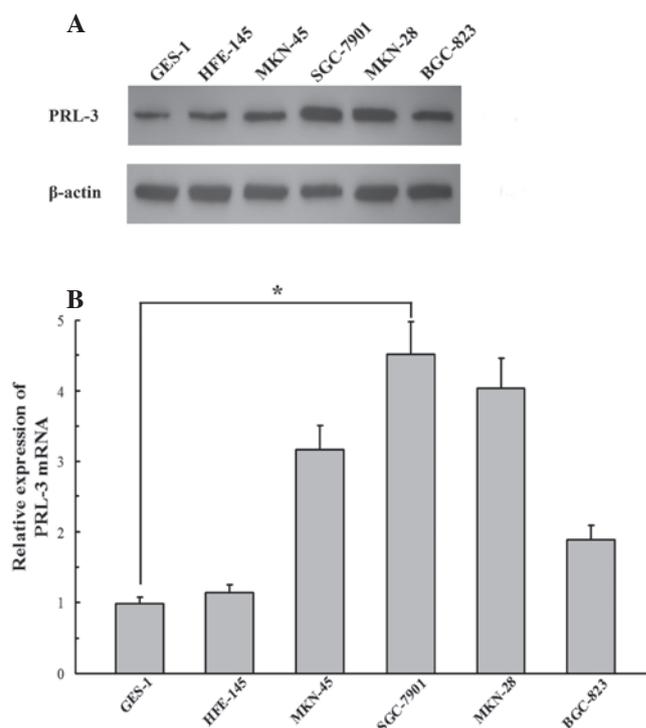


Figure 1. (A) Western blot analysis of phosphatase of regenerating liver-3 (PRL-3) protein and (B) quantitative real-time polymerase chain reaction (qRT-PCR) analysis of PRL-3 mRNA, showing expression in four human gastric cancer (GC) cell lines and in two normal human gastric epithelial cell lines. The GES-1 and HFE-145 cell lines demonstrate low levels of PRL-3 protein and mRNA. The four GC cell lines express relatively high levels of PRL-3 protein and mRNA, particularly the SGC-7901 cell line ( $P < 0.05$ ).

decrease in migration, compared with control vector cells and untreated cells ( $P < 0.05$ ; Fig. 4A and B, respectively).

**Inhibiting PRL-3 downregulation of ERK 1/2 and MMP-7 expression in SGC-7901 cells.** ERK 1/2 is a member of the MAPK family and has been demonstrated to be associated with cell motility and invasion (13,14). MMP-7, the downstream target of ERK 1/2, contributes to cell motility by remodeling the extracellular matrix. In the present study, we used western blot analysis and qRT-PCR to examine the changes in ERK 1/2 and MMP-7 protein and mRNA expression post-transfection with PRL-3 siRNA. The western blot analysis revealed that 72 h post-transfection, the PRL-3 siRNA resulted in significant decreases in ERK 1/2, phosphorylated ERK 1/2 (pERK 1/2) and MMP-7 protein levels, compared with untreated controls or cells transfected with siCONTROL (Fig. 5A). The qRT-PCR results also demonstrated that ERK 1/2 and MMP-7 mRNA levels were similarly affected 72 h post-transfection ( $P < 0.05$ ,  $P < 0.01$  and  $P < 0.05$ , respectively; Fig. 5B-D).

## Discussion

The majority of cancer-related mortalities are due to tumor metastases rather than primary tumors (22,23). Failure of treatment for GC is mainly caused by metastasis and invasion of the tumor cells to the neighboring organs. However, the specific molecular changes in GC cells that promote the metastatic process are largely unclear. Understanding the

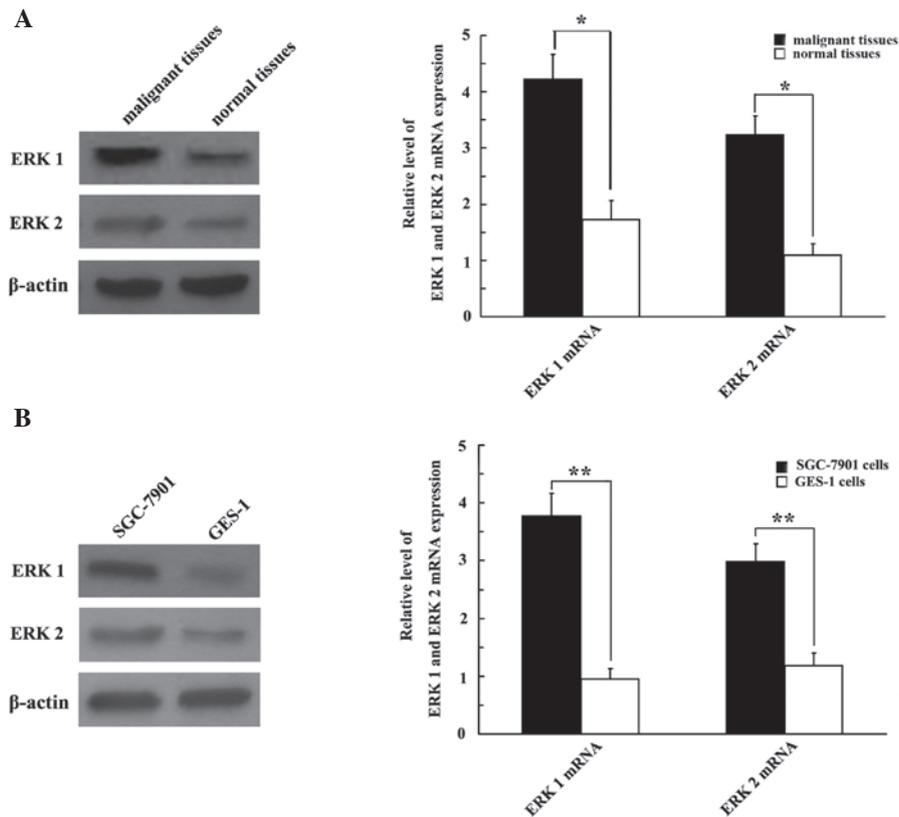


Figure 2. Expression of extracellular signal-regulated kinase 1/2 (ERK 1/2) in gastric cancer (GC) samples and normal gastric samples. (A) ERK 1/2 protein and mRNA levels in the GC tissues and normal tissues.  $\beta$ -actin was used as an internal loading control. (B) ERK 1/2 protein and mRNA levels in the SGC-7901 GC cell line and normal gastric cell line, GES-1 (normalized vs.  $\beta$ -actin). Compared with the normal gastric samples, ERK 1/2 protein and mRNA are overexpressed in the GC samples ( $P < 0.05$  and  $^{**}P < 0.01$ ). Each bar shows the mean  $\pm$  standard deviation from three independent experiments.

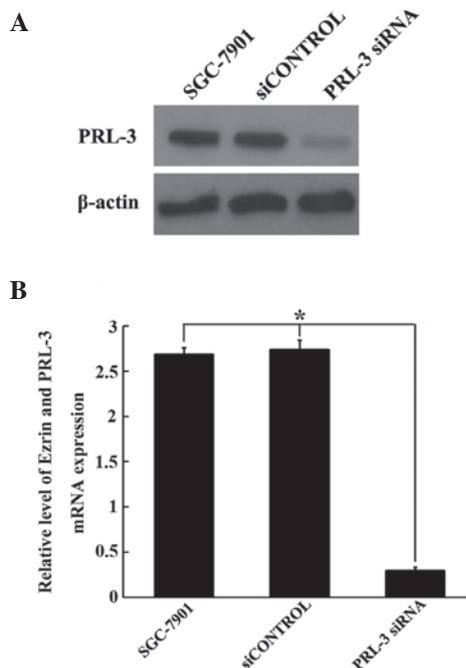


Figure 3. Specific downregulation of phosphatase of regenerating liver-3 (PRL-3) protein and mRNA expression by PRL-3 small interfering RNA (siRNA). (A) The protein expression levels of PRL-3 were determined by western blot analysis. (B) The mRNA expression levels of PRL-3 were quantified by quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Introduction of PRL-3 siRNA into SGC-7901 gastric cancer cells downregulates endogenous PRL-3 ( $P < 0.05$ ). Data were normalized using  $\beta$ -actin as an internal standard.

metastatic mechanisms is important for aiding and improving the success of treatment for this cancer.

Inhibiting specific gene expression by RNAi has become an important method of cancer treatment (24,25). Recently, PRL-3 was demonstrated to be overexpressed in GC cells, and has been proposed to be a novel marker of poor outcome in GC (26,27). To explore whether PRL-3 may become a potential molecular target for gene therapy of GC, we employed RNAi technology to downregulate PRL-3 expression in a human GC cell line, SGC-7901. Compared with the control group, the mRNA and protein expression levels were significantly decreased in the cells transfected with PRL-3 siRNA. These results indicated that PRL-3 siRNA was able to effectively and specifically silence the expression of PRL-3 in SGC-7901 cells.

It is known that PRL-3 is associated with the progression and eventual metastasis of several types of human cancer (28). Thus, we examined the effect of PRL-3 suppression on the invasion of SGC-7901 cells by mobility assays. The results demonstrated that the migratory ability was significantly reduced through Matrigel-coated chamber membranes compared with the control group. Therefore, there is a strong correlation between PRL-3 expression levels and the invasion or migration ability of human GC cells. These results are consistent with previous studies demonstrating that inhibition of PRL-3 signaling reduced the migration and invasion ability of tumor cells (29,30).

Although the mechanism whereby PRL-3 affects cell invasion remains undefined, several studies have clarified

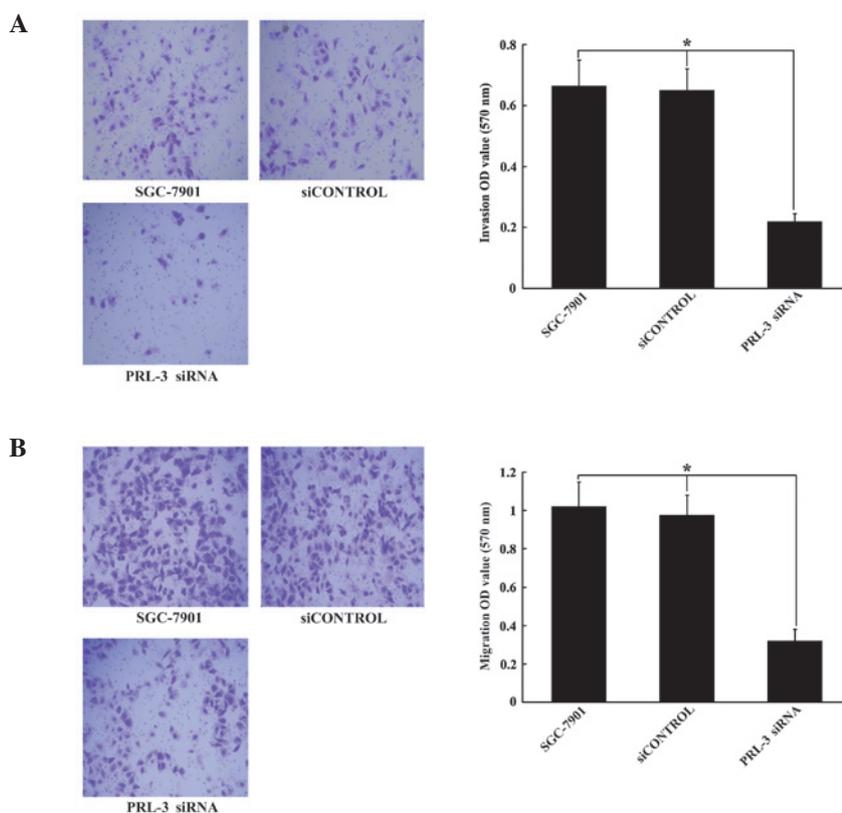


Figure 4. Downregulation of phosphatase of regenerating liver-3 (PRL-3) inhibits the mobility of SGC-7901 cells. SGC-7901 cells were transfected with PRL-3 small interfering RNA (siRNA) or siCONTROL, or they remained untreated. The cells were then subjected to Transwell assays. Representative photographs of (A) invading and (B) migrating cells. Average optical density (OD)<sub>570</sub> values ( $\pm$ standard deviation) for invasion and migration from three independent experiments. Original magnification,  $\times 200$ ; \* $P < 0.05$ .

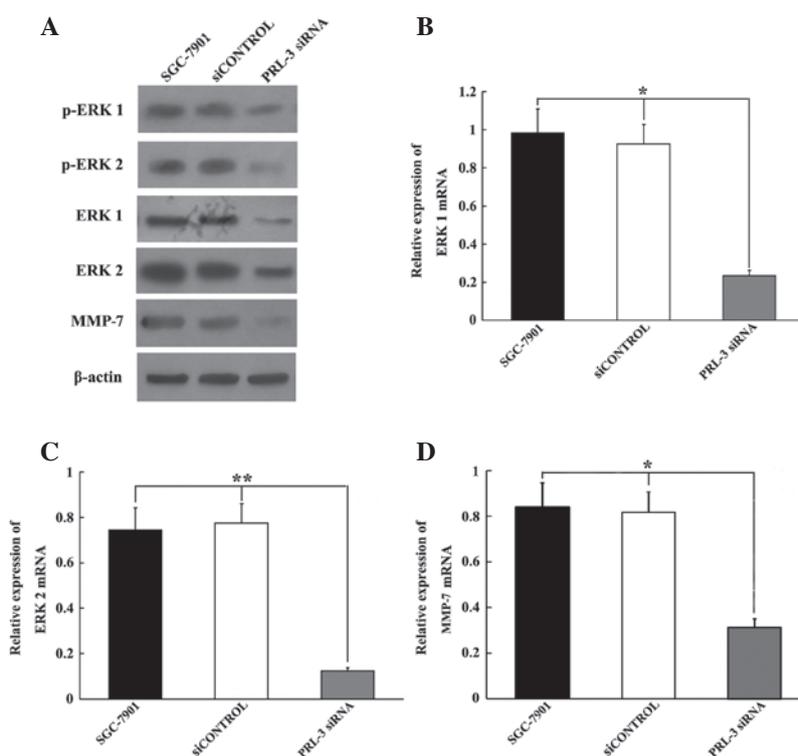


Figure 5. Inhibition of phosphatase of regenerating liver-3 (PRL-3) decreases the expression of extracellular signal-regulated kinase 1/2 (ERK 1/2) and matrix metalloproteinase-7 (MMP-7). (A) ERK1/2, phosphorylated ERK 1/2 (pERK 1/2) and MMP-7 protein levels in SGC-7901 cells were determined by western blot analysis 72 h post-transfection.  $\beta$ -actin was used as an internal loading control. (B and C) ERK1/2 and (D) MMP-7 mRNA levels in the SGC-7901 cell lines were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) 72 h post-transfection of PRL-3 small interfering RNA (siRNA) and were significantly decreased (vs. siCONTROL and untreated cells; normalization was performed versus  $\beta$ -actin). \* $P < 0.05$  and \*\* $P < 0.01$ . Error bars show the standard deviation and were obtained from three independent experiments.

the PRL-3 target molecules, a number of which are known to be involved in cell motility (31). ERK 1/2 are crucial for various cell activities including proliferation, migration and invasion (32,33). MMPs are downstream target proteins of ERK 1/2. Among >20 MMPs, MMP-7 appears to be one of the most important MMPs in human GC, as it is highly over-expressed in GC (34,35). Luo *et al* demonstrated that PRL-1 promoted motility and invasion in HEK293 cells by increasing MMP-2 and MMP-9 expression via the ERK 1/2 pathway (18). As the amino acid sequences of PRL-1 and PRL-3 are >75% identical, and the two sequences contain the C-terminal prenylation motif CAAX to be prenylated (36,37), it is reasonable that PRL-3 may possess similar abilities to PRL-1. Therefore, we hypothesized that PRL-3 is capable of influencing the protein synthesis or activities of ERK 1/2-MMP-7, leading to the facilitation of cell motility and invasion.

To test this hypothesis, we examined the effect of PRL-3 on the cell activities of ERK1/2, and found that the expression of ERK 1/2 decreased significantly following transfection with PRL-3 siRNA. Additionally, to investigate whether PRL-3 siRNA had any effect on MMP-7, we used qRT-PCR and western blot analysis to detect the expression levels of MMP-7. The results demonstrated that the level of MMP-7 expression also decreased significantly. To the best of our knowledge, this study provides the first evidence that MMP-7 expression may be downregulated via the inhibition of PRL-3 in GC. However, whether PRL-3 activates MMP-7 through the ERK 1/2 pathway requires further investigation.

The present study has demonstrated that PRL-3 and ERK 1/2 proteins were highly expressed in GC cells and tissues, and that downregulation of PRL-3 expression by siRNA inhibited the invasion and migration of SGC-7901 GC cells. Furthermore, knockdown of PRL-3 suppresses the expression levels of ERK 1/2 and MMP-7. These findings suggest that further investigation of the regulation of the PRL-3-ERK1/2-MMP-7 pathway may have therapeutic implications for the prognosis and treatment of GC metastasis.

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