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Inhibition of PRL-3 gene expression in gastric cancer cell line SGC7901 via microRNA suppressed reduces peritoneal metastasis

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Abstract

High expression of PRL-3, a protein tyrosine phosphatase, is proved to be associated with lymph node metastasis in gastric carcinoma from previous studies. In this paper, we examined the relationship between PRL-3 expression and peritoneal metastasis in gastric carcinoma. We applied the artificial miRNA (pCMV-PRL3miRNA), which is based on the murine miR-155 sequence, to efficiently silence the target gene expression of PRL-3 in SGC7901 gastric cancer cells at both mRNA and protein levels. Then we observed that, *in vitro*, pCMV-PRL3miRNA significantly depressed the SGC7901 cell invasion and migration independent of cellular proliferation. *In vivo*, PRL-3 knockdown effectively suppressed the growth of peritoneal metastases and improved the prognosis in nude mice. Therefore, we concluded that artificial miRNA can depress the expression of PRL-3, and that PRL-3 might be a potential therapeutic target for gastric cancer peritoneal metastasis.

Keywords: PRL-3; Gastric cancer; Peritoneal metastasis; RNA interference; MicroRNA; Plasmid vector

Peritoneal carcinomatosis, a common feature of the natural history of gastric cancer, is a major problem for gastric cancer management. Despite advances in therapeutic modalities for peritoneal diseases such as combination chemotherapy and chemohyperthermia, the results are unsatisfactory [1,2]. The mechanisms involved in gastric cancer peritoneal metastasis have not been fully clarified. Although previous studies have identified that many molecular factors are involved, the metastatic process still needs to be clarified in order to facilitate the development of a new therapeutic target for peritoneal metastases.

The protein tyrosine phosphatase (PTP) superfamily of phosphatases, which are defined by the signature $(X)_5R$ active site motif, consists of a large group of enzymes which play an important regulation role in both cellular physiologic and pathogenic processes [3]. The PRL phosphatases (PRL-1, PRL-2, and PRL-3), three closely related PTP, have been identified throughout the past few years [4]. These PRLs represent a novel class of PTP with a unique COOH-terminal prenylation motif. Recent reports showed PRL-3 significantly higher level in colorectal cancer and ovarian cancer with metastasis. PRL-3 level was further proved to be correlated with cancer progression and pathological stage [5–8]. PRL-3's role in gastric cancer metastasis has also been proposed by Miskad [9]. They concluded that PRL-3 expression in gastric cancer was associated with invasion of lymphatic vessels and extent of lymph node metastasis. Furthermore, high expression of PRL-3 was detected in other metastatic sites, such as peritoneum. Although these studies demonstrated that PRL-3 plays a causative role in lymph node metastasis, no study has yet been done to understand the function of PRL-3 in gastric cancer peritoneal metastasis.

RNA interference is a powerful method to suppress gene expression in mammalian cells. Post-transcriptional gene

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expression can be mediated by small noncoding RNAs such as short interfering RNA (siRNA) and microRNA (miRNA). The mature miRNAs regulate gene expression by mRNA cleavage [10] or translational repression [11–13]. Target cleavage can be induced artificially by altering the target or the miRNA sequence to obtain complete hybridization [13–15]. Furthermore, microRNA-based shRNAs inhibit gene expression more potently than traditional stem-loop shRNAs [16].

In this study, we applied the artificial miRNA, which is based on the murine miR-155 sequence [17], to produce specific and long-term silencing of PRL-3 in SGC7901 gastric cancer cells [18]. We designed our experiment as a loss-of-function study. Western blot analysis was used to measure the extent and stability of PRL-3 knockdown in our clones, which stably express miRNA against PRL-3. We evaluate the metastatic phenotype of PRL-3-silenced SGC7901 clones using standard migration and invasion assays *in vitro*, and the commonly used model for experimental peritoneal metastases *in vivo*. These results indicated that PRL-3 enhanced gastric cancer peritoneal metastasis by improving tumor cell invasion and migration independent of cellular proliferation.

Methods

Immunohistochemical staining. PRL-3 expression levels of cancer cells were compared between peritoneal metastasis and primary gastric carcinoma using an immunohistochemical method. Gastric cancer specimens were obtained from 25 patients. Both primary and metastatic lesions were sampled from the same patients. Four-micron-thick sections were cut from each block, deparaffinized with xylene, and rehydrated with graded ethanol solutions in deionized distilled water. The avidin–biotin-peroxidase complex technique was used for immunohistochemical staining. The deparaffinized sections were treated for 30 min at room temperature with 3% hydrogen peroxide in methanol to inhibit endogenous peroxidase activity. They then were blocked with 1% bovine serum albumin for 1 h. The primary polyclonal rabbit antibody, anti-PRL-3 (1:300 dilution; Sigma, Saint Louis, Missouri, USA), was applied to sections and incubated overnight at 4 °C in a moist chamber. Subsequently, sections were incubated with biotinylated goat antirabbit secondary antibody for 30 min and streptavidin conjugated to horseradish peroxidase (Maixin, Fuzhou, China) for 30 min. 3,3-Diamino-benzidine tetrahydrochloride (Maixin, Fuzhou, China) was used to make the reaction visible at room temperature for 5 min until a distinct reaction was evident microscopically. Counterstaining for nuclei was performed with Mayer's hematoxylin. Negative control sections were prepared using normal rabbit immunoglobulins instead of the PLR-3 antibodies. PRL-3 expression levels in primary carcinomas and metastatic peritoneal lesions in the same individual were compared. Staining was scored semiquantitatively. Scores were ranked as follows: weak, no immunoreactive tumor cells detectable or <10% of tumor cells positive with a weak staining intensity; moderate, 10–50% of tumor cells positive; and strong, >50% of tumor cells positive with strong staining intensity.

Cell culture. Human gastric cancer cell line, SGC7901, was purchased from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Tumor cells were cultured in RPMI-1640 medium (GIBCO, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) and 1% antibiotics (i.e., 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate) in an atmosphere of 37 °C in 5% CO_2, and passaged by treating with 0.02% EDTA in phosphate-buffered saline (PBS) and 0.25% trypsin when achieving confluence.

Plasmid construction. The engineered pre-miRNA sequence structure is based on the murine miR-155. The pcDNA™6.2-GW/EmGFP-miR plasmid (Invitrogen, Carlsbad, CA, USA) with spectinomycin resistance gene was used for the cloning of small synthetic oligonucleotides (Fig. 2). Three different miR155-based PRL-3 targeting sequences were designed by using Invivogen's RNAi design algorithm on line. (https://rnaidesigner.invitrogen.com/rnaiexpress/setOption.do?designOption=mirna). Basic local alignment search tool (BLAST) was used on all miRNA sequences to avoid off-target gene silencing. These sequences are shown in Table 1.

Transfection of stable miRNA plasmids. Harvesting of SGC7901 cells using trypsin was done 24 h prior to transfection and plated at a density of 3×10^5 cells/well in 6-well plates (Corning, NY, USA) in RPMI-1640 −10% FBS without antibiotics. Four micrograms of purified pcDNA™6.2-GW/EmGFP-miR expression vectors containing either the PRL-3 miRNA insert (pCMV-PRL3miRNA-29, pCMV-PRL3miRNA-477, and pCMV-PRL3miRNA-1249) or the negative-control mismatch sequence (pCMV-PRL3miRNA-neg) (Invitrogen, Carlsbad, CA, USA) was transfected into SGC7901 cells with the Lipofectamine2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Western blot and RT-PCR were performed, 48 h after transfection, to assess the selectivity of PRL-3 knockdown. Successfully transfected cells clones were obtained by a long-term culture in a selection medium containing 6 μg/ml Blasticidin.

RT-PCR. Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration and quality were assessed spectrophotometrically at wavelengths 260 and 280 nm. Reverse transcription reaction was performed with random hexamer primers and a SuperScript Reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA). The sequences of specific primers were as follows: PRL-3 mRNA forward, 5′-GGGACTTCTCAGGTCGTG TC-3′, and PRL-3 mRNA-reverse, 5′-AGCCCCGTACTTCTTCAGGT-3′. As a control, the levels of β-actin expression were also analyzed, using the following primers: β-actin mRNA forward, 5′-TCATCACCATTG GCAATGAG-3′, and β-actin mRNA reverse, 5′-CACTGTGTTGG CGTACAGGT-3′. The cDNAs were amplified by PCR for 35 cycles (94 °C for 15 s, 60 °C for 30 s, and 72 °C for 1 min) using Taq PCR MasterMix (Tianwei, Beijing, China). The resultant PCR products were 198 bp (PRL-3) and 155 bp (β-actin). PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining. For the semi-quantification, an image of the gel was captured, and the intensity of the bands was quantitated using the AlphaImager gel analysis system (Alpha Innotech, San Leandro, CA).

Western blotting. Tumor cells were cultured until subconfluence, and whole-cell proteins were extracted according to mammalian cell lysis kit (Bio Basic Inc., Ontario, Canada). In brief, cells were washed three times with PBS and then lysed in lysis buffer for 5 min. After removal of cell debris by centrifugation (12,000*g*, 10 min), supernatants containing

Table 1
The oligonucleotide sequences of shRNA driven by CMV promoter in pcDNA™6.2-GW/EmGFP-miR

	Top oligo	Bottom oligo
pCMV-PRL3miRNA-29 (29–59 nt)	5′-TGCTG***AAGTAAAGCGGGCAACTCCAAG***TTTTG GCCACTGACTGACTTGGAGTTCCGCTTTACTT-3′	5′-CCTGAAGTAAAGCGGAACTCCAAGTCAGTCAG TGGCCAAAACTTGGAGTTGCCCGCTTTACTTC-3′
pCMV-PRL3miRNA-477 (477–497 nt)	5′-TGCTG***TGTCATAGGTCACTTCACACAG***TTTTGG CCACTGACTGACTGTGTGAAGACCTATGACA-3′	5′-CCTGTGTCATAGGTCTTCACACAGTCAGTCAGT GGCCAAAACTGTGTGAAGTGACCTATGACAC-3′
pCMV-PRL3miRNA-1249 (1249–1269 nt)	5′-TGCTG***TTAAGGTGCCGAGAACAGGTCG***TTTTGG CCACTGACTGACGACCTGTTCGGCACCTTAA-3′	5′-CCTGTTAAGGTGCCGAACAGGTCGTCAGTCA GTGGCCAAAACGACCTGTTCTCGGCACCTTAAC-3′

proteins were stored frozen until use. Fifty micrograms of proteins of different groups were boiled for 5 min in sample buffer, separated in 10% SDS–PAGE, and transferred onto PVDF membrane (Invitrogen, Carlsbad, CA, USA). Five percent of skim milk (blocking solution) was loaded over the membrane and incubated for 1 h at room temperature with agitation. The membranes were then incubated with the rabbit anti-human PRL-3 antibody (Sigma, Saint Louis, Missouri, USA) for 1 h at 37 °C with agitation. After being washed with 0.1% Tween 20 in Tris-saline three times, the membranes were incubated with biotin-labeled anti-rabbit IgG for 1 h at room temperature with agitation. Reactive protein was detected using ECL chemiluminescence system (Pierce, Rockford, USA).

Invasion assay. The ability of cells to invade through a Matrigel-coated filter was measured in transwell chambers (Corning, NY, USA). Polyvinylpyrrolidone free polycarbonate filters (pore size 8 mm) were coated with basement membrane Matrigel (50 μl/filter) (BD, Bedford, USA) as described in the standard protocol [19]. The membrane was washed in PBS to remove excess ligand, and the lower chamber was filled with 0.6 ml of RPMI-1640 medium containing 10% fetal bovine serum (FBS). Cells were serum-starved overnight (0.5% FBS), harvested with trypsin/EDTA, and washed twice with serum-free RPMI-1640 medium. Cells were resuspended in migration medium (RPMI-1640 medium with 0.5% FBS), and 0.1 ml migration medium containing 1×10^5 cells was added to the upper chamber. After 24 h in 5% CO_2–95% air at 37 °C, the cells on the upper surface of the membrane were removed using cotton tips. The migrant cells attached to the lower surface were fixed in 10% formalin at room temperature for 30 min and stained with hematoxylin. The number of migrated cells on the lower surface of the membrane was counted under a microscope in five fields at 100×.

Scratch wound-healing motility assays. Gastric cancer cells were seeded on 60 mm plates and allowed to grow to confluence. Confluent monolayers were scratched with a pipette tip and maintained under standard conditions for 24 h. Plates were washed once with fresh medium to remove nonadherent cells and then photographed. The cell migration was evaluated by counting cells that migrated from the wound edge.

Peritoneal metastatic model by intraperitoneal inoculation in vivo. Five-week-old BALB/c nude mice were purchased from the Center of Experimental Animal Sun Yat-San University (Guangzhou, China). All of the *in vivo* experimental protocols were approved by the Animal Care Committee of Sun Yat-San University. The mice were allocated to three groups: SGC7901, pCMV-PRL3miRNA-neg-A1, and pCMV-PRL3miRNA-1249-A2. Cultured tumor cells were removed by trypsinization, washed twice with PBS, suspended in RPMI-1640 medium, and inoculated into the peritoneal cavities of nude mice (5×10^6 cells/1 ml/mouse). On day 21, all mice were sacrificed, and the number of macroscopic nodules on the peritoneal surface was counted as described previously [20]. Using the same methods, the survival of mice was evaluated for each group up to day 120.

Statistical analysis. The χ^2 test was used to analyze the difference in PRL-3 expression between the primary gastric cancer and the peritoneal metastases. Differences between control and experiment groups were analyzed using the ANOVA test. Survival was analyzed by the Kaplan–Meier method. A *P*-value of less than 0.05 was considered statistically significant.

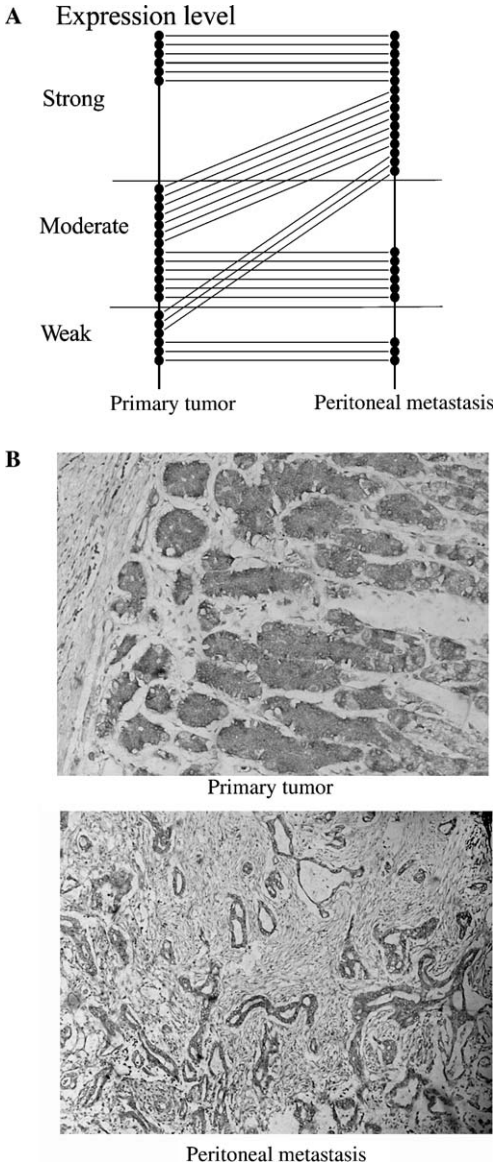

Fig. 1. Expression of PRL-3 in human gastric tumors and metastasis. (A) A comparison of PRL-3 expression in primary gastric tumor with that in peritoneal metastatic lesions in the same individual. Primary and metastatic lesions were obtained from 25 cases. A data matrix was prepared by plotting the degree of PRL-3 expression in primary tumors on the left and that in the corresponding peritoneal metastasis on the right. Expression levels of PRL-3 in the metastasis were higher than those in the primary lesion. Expression levels were scored semiquantitatively as described in Methods. (B) Representative positive immunoactivity of PRL-3 in gastric tumors and metastases.

Results

Expression levels of PRL-3 in the metastasis were higher than those in the primary lesion

Fig. 1 provides a comparison of PRL-3 expression between the primary tumors and peritoneal metastases in the same 25 individuals. The primary tumors showed strong, moderate, and weak staining in 6, 13, and 6 cases, respectively. In contrast, the peritoneal metastases showed strong, moderate, and weak staining in 16, 6, and 3 cases, respectively. Although the entire primary lesion showed more than a moderately strong stain of PRL-3, most of the peritoneal metastatic lesions demonstrated strong staining ($P < 0.05$).

Construction of recombinant plasmids targeting PRL-3

Three plasmids were constructed and designated as pCMV-PRL3miRNA-29, pCMV-PRL3miRNA-477, and

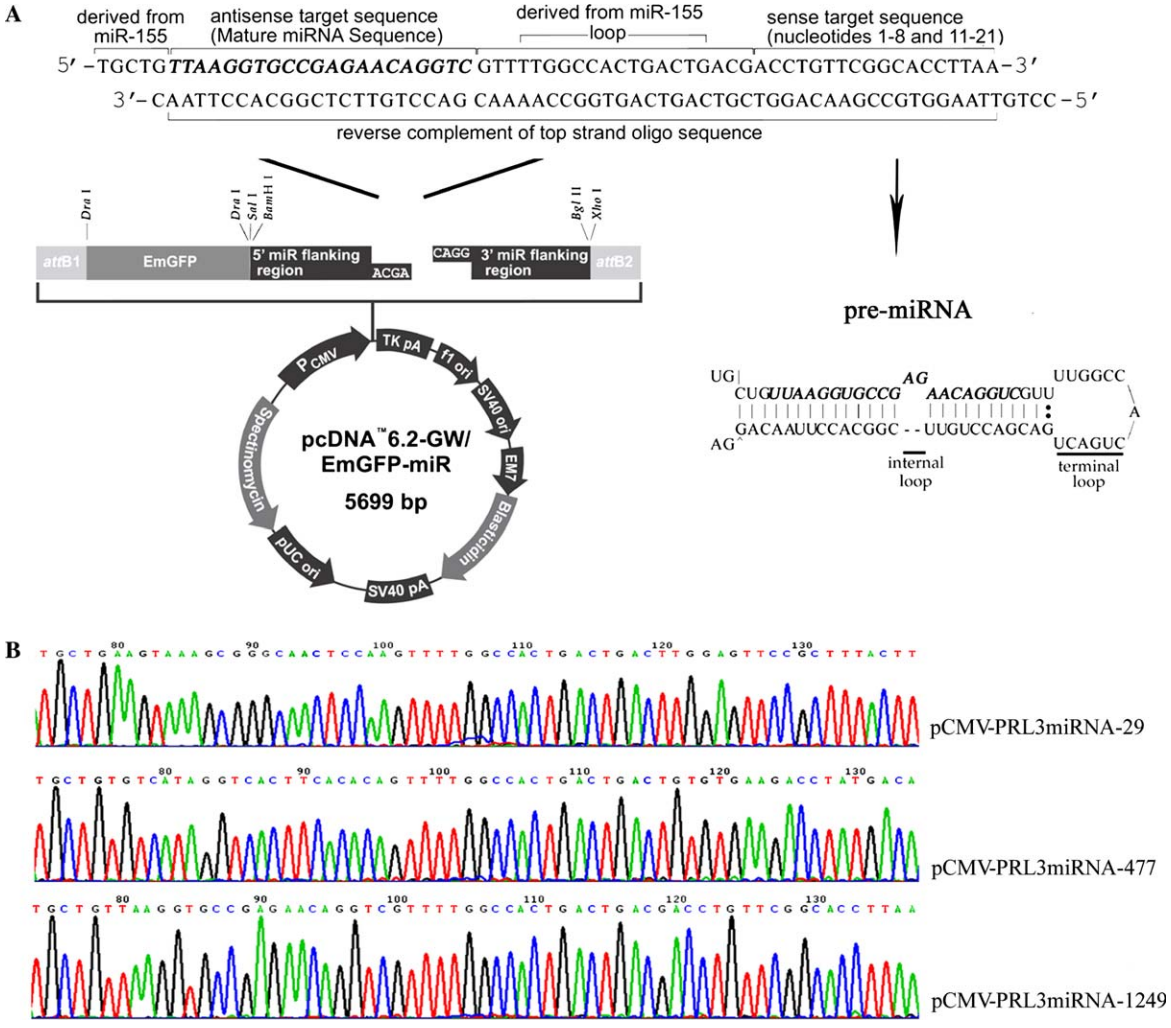

Fig. 2. Scheme and identification of recombinant plasmids. (A) The linearized pcDNA™6.2-GW/EmGFP-miR plasmid contains 4 nucleotide overhangs derived from miR-155 sequences. The ds oligo coding for the mature miR155 miRNA was cloned into the vector. Upon transcription, the mature miRNA sequence and its complement form a stem of the pre-miRNA with a short internal loop, separated by a larger terminal loop (mature miRNA sequence in bold). (B) The oligonucleotides of inserts were confirmed by DNA sequencing.

pCMV-PRL3miRNA-1249, respectively. In order to identify successful construction of recombinant plasmids, the miRNA forward sequencing primer (5′-GGCATGGACG AGCTGTACAA-3′, Invitrogen, Carlsbad, CA, USA) and miRNA reverse sequencing primer (5′-CTCTAGAT CAACCACTTTGT-3′, Invitrogen, Carlsbad, CA, USA) were designed to perform PCR. Then the PCR product, which contained the miRNA insert fragments, was verified by DNA sequencing. Finally, the mutant was excluded from this experiment (Fig. 2B). Based on the computer analysis, these inserted oligonucleotides would specifically bind to homologous sites of PRL-3 mRNA, and thus might interfere with PRL-3 expression in cultured cells.

MicroRNA mediated RNAi inhibits PRL-3 expression

The recombinant plasmids (pCMV-PRL3miRNA-29, pCMV-PRL3miRNA-477, and pCMV-PRL3miRNA-1249) were transfected into SGC7901 cells. To examine miRNA-induced gene silencing, at 48 h after transfection, total protein was extracted from SGC7901 cells of transient transfection to perform Western blotting. The protein levels of PRL-3 were compared among parental, neg-transfected control, and miRNA-knockdown cells. As shown in Fig. 3, PLR-3 proteins were strongly expressed in both parental SGC7901 cells and neg-transfected cells expressed at similar level. However, as expected, pCMV-PRL3miRNA-477 and pCMV-PRL3miRNA-1249 demonstrated a decreasing PRL-3 protein expression level of 2.7-fold ($P < 0.05$) and 3.5-fold ($P < 0.05$), respectively, in comparison with parental SGC7901 cells. This RNAi mediated effect was specific, as β-actin levels did not differ significantly amongst the treated cells and controls. To evaluate inhibition of PRL-3 mRNA expression, RT-PCR was performed. The mRNA expression in pCMV-PRL3miRNA-477 or pCMV-PRL3miRNA-1249 transfected SGC7901 cells was reduced by more than 80% as compared with parental SGC7901 cells. In addition, no difference was observed among

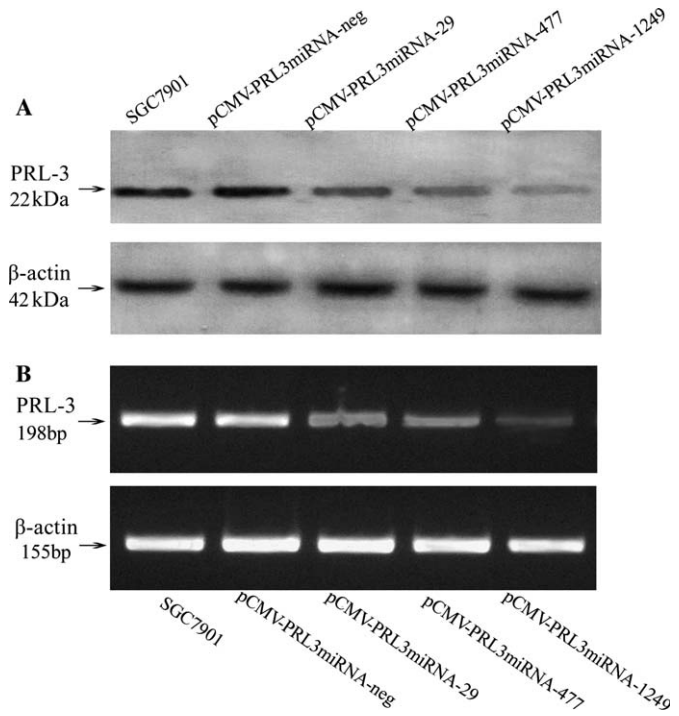

Fig. 3. Transfection with pCMV-PRL3miRNA of two independent oligonucleotide sequences using Lipofectamine 2000 efficiently silences PRL-3 expression in SGC7901 gastric cancer cells at 48 h. (A) A representative Western blot image for the extracted cytosol of PRL-3 gastric cancer cells from three independent experiments illustrating that pCMV-PRL3miRNA-477 or pCMV-PRL3miRNA-1249, rather than pCMV-PRL3miRNA-neg or pCMV-PRL3miRNA-29, reduces cytosolic PRL-3 protein in SGC7901 cells. (B) A representative RT-PCR image from three independent experiments illustrating that pCMV-PRL3miRNA-477 or pCMV-PRL3miRNA-1249, rather than pCMV-PRL3miRNA-neg or pCMV-PRL3miRNA-29, reduces PRL-3 mRNA in SGC7901 cells.

pCMV-PRL3miRNA-29, pCMV-miRNA-neg, and parental SGC7901 cells. Therefore, pCMV-PRL3miRNA-477 and pCMV-PRL3miRNA-1249 are specific RNAi targets.

In order to assess the phenotype of tumor cells in which PRL-3 expression was inhibited over a long-term period, we directed our attention to generating stable, PRL-3-downregulated clonal cell lines. Based on the higher efficiency of pCMV-PRL3miRNA-1249 compared with pCMV-PRL3miRNA-477, pCMV-PRL3miRNA-1249 cells were cultured for a long-term period in a selection medium containing Blasticidin. At 2 months, Western blot analysis of the clones demonstrated decreased PRL-3 protein expression of 2.0-fold (pCMV-PRL3miRNA-1249-A1), 3.7-fold (pCMV-PRL3miRNA-1249-A2), 3.5-fold (pCMV-PRL3miRNA-1249-A3), comparedwith parental SGC7901 cells ($P < 0.05$). Clones pCMV-PRL3miRNA-neg-A1 and pCMV-PRL3miRNA-neg-A2 showed similar PRL-3 protein levels compared to parental SGC7901 cells (Fig. 4; $P > 0.05$). Protein levels of β-actin, a housekeeping gene, were similar between different cell lines, indicating that the RNAi-mediated knockdown of PRL-3 was specific and did not result from a global decrease in gene expression (Fig. 4). In parallel, the PRL-3 expression at mRNA lever was evaluated by RT-PCR. The mRNA expression in pCMV-PRL3miRNA-1249 transfected SGC7901 cells clones (A1, A2, and A3) was reduced by more than 80% as compared with parental SGC7901 cells.

PRL-3 knockdown inhibits gastric cancer cell migration and invasion in vitro

The effects of PRL-3 expression on cell migration *in vitro* are shown in Fig. 5. PRL-3 knockdown cells (pCMV-PRL3miRNA-1249-A2) exhibited significant decrease in migration (Fig. 5A). The number of pCMV-PRL3miRNA-1249-A2 cells migrated to the scratched area was 5 ± 1.6 cells/mm^2, which was significantly smaller than those of SGC7901 control (23 ± 2.6 cells/mm^2) and pCMV-PRL3miRNA-neg-A1 (20.8 ± 1.9 cells/mm^2) ($P < 0.05$) (Fig. 5B).

An *in vitro* cell invasion assay was performed based on the principle of the Boyden chamber assay. The Matrigel matrix served as a reconstituted basement membrane *in vitro*. The number of cells migrating through the Matrigel matrix was counted, and the result is presented in Fig. 6. The mean numbers ± SD of three separate experiments of these cells attached to the lower surface of the filters were as follows: SGC7901, 109 ± 23/HP; pCMV-PRL3miRNA-neg-A1, 112 ± 15/HP; and pCMV-PRL3miRNA-1249-A2, 32 ± 10/HP. The PRL-3 knockdown cells showed significantly reduced invasiveness compared to parental SGC7901 and pCMV-PRL3miRNA-neg-A1 cells ($P < 0.05$). These data indicated that the inhibition of PRL-3 expression in SGC7901 cells was associated with reduced invasive ability.

PRL-3 knockdown reduced peritoneal metastasis of SGC7901 cells in vivo

Finally, we examined whether PRL-3 knockdown resulted in the inhibition of peritoneal dissemination. Mice received i.p. injections of SGC7901, pCMV-PRL3miRNA-neg-A1 or pCMV-PRL3miRNA-1249-A2 cells. The macroscopic nodules of peritoneal dissemination were then counted 3 weeks after tumor inoculation. Metastatic nodules developed preferentially around the vessels of the intestinal mesentery (Fig. 7A).

The total number of metastatic nodules was 107 ± 23.5 and 110 ± 27.6 in the SGC7901 and pCMV-PRL3miRNA-neg-A1 groups, respectively. However, the PRL-3 knockdown cell (pCMV-PRL3miRNA-1249-A2) groups showed comparatively fewer nodules (37 ± 7.3, $P < 0.05$) (Fig. 7B). The survival of these mice is shown in Fig. 6C. Consistent with the finding of macroscopic peritoneal metastasis, the outcome showed a significant difference among the three groups. Median survival was significantly prolonged in the PRL-3 knockdown group ($P < 0.05$ versus SGC7901 and pCMV-PRL3miRNA-neg-A1 groups). This indicates that PRL-3 knockdown can effectively suppress the growth of peritoneal

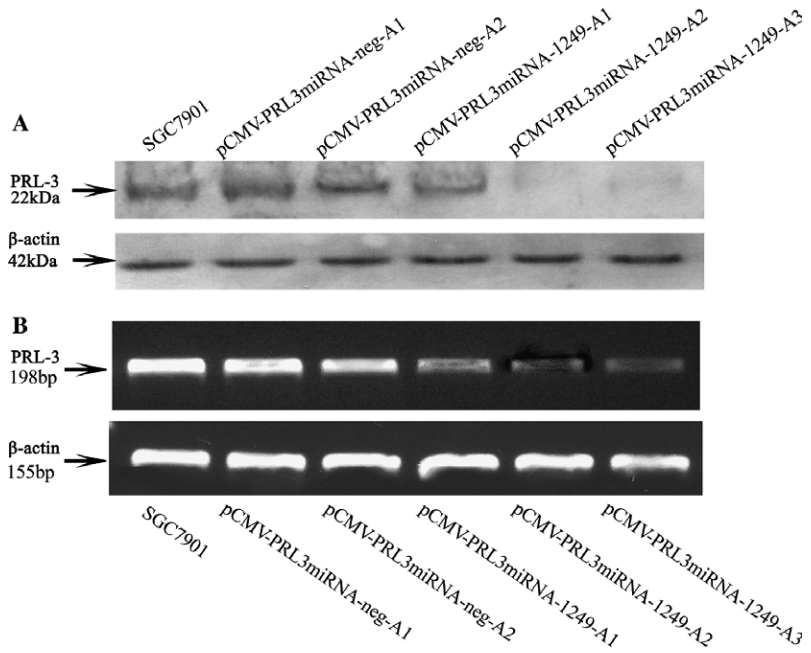

Fig. 4. Western blot and RT-PCR showing the extent of PRL-3 protein and mRNA in cells stably transfected with pCMV-PRL3miRNA-1249 at 2 months after initial transfection. (A) Western blot shows that pCMV-PRL3miRNA-1249, rather than pCMV-PRL3miRNA-neg, decreased the PRL-3 protein in SGC7901 cells. Moreover, the pCMV-PRL3miRNA-1249-A2 is the clone that reduced the PRL-3 protein most. (B) RT-PCR shows that pCMV-PRL3miRNA-1249, rather than pCMV-PRL3miRNA-neg, decreased the PRL-3 mRNA in SGC7901 cells. Moreover, the pCMV-PRL3miRNA-1249-A2 is the clone that reduced the PRL-3 mRNA most.

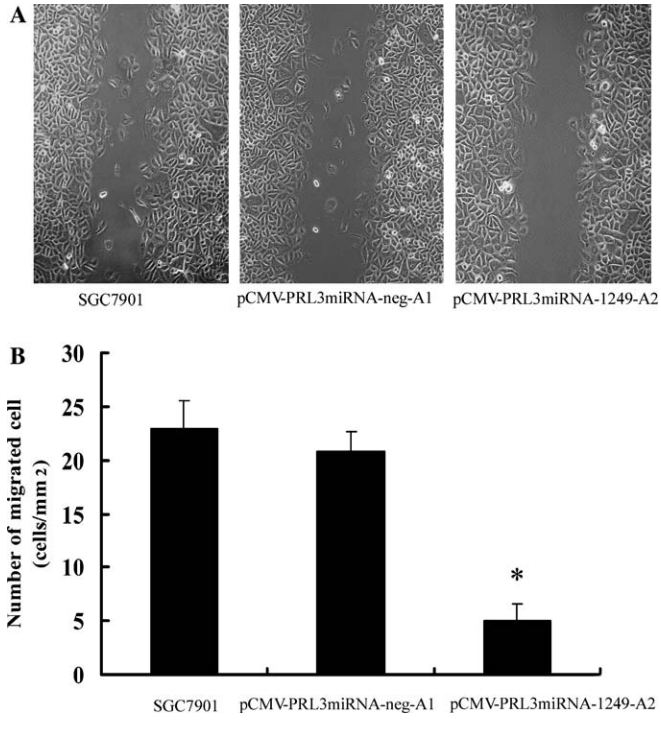

Fig. 5. (A) Scratch-wound healing assay. Movement of cells into the wound is shown for SGC7901 control, pCMV-PRL3miRNA-neg-A1 and pCMV-PRL3miRNA-1294-A2 cells at 24 h. (B) The number of pCMV-PRL3miRNA-1294-A2 migrating 24 h after scratching was 5 ± 1.6 cells/mm^2, which was significantly smaller than those of SGC7901 (23 ± 2.6 cells/mm^2) and pCMV-PRL3miRNA-neg-A1 groups (20.8 ± 1.9 cells/mm^2) ($^*P < 0.05$).

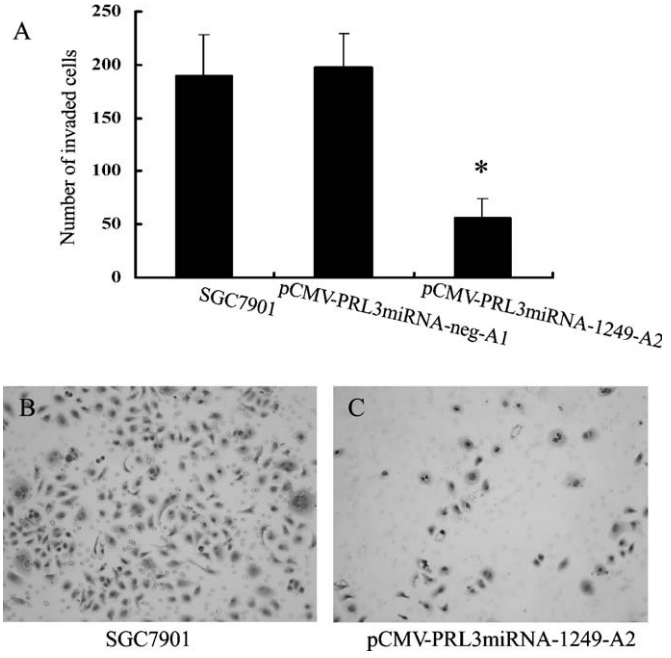

Fig. 6. The invasion ability of PRL-3 on cells was assayed by modified Boyden chamber. (A) After 24 h, the number of cells, which had migrated through the membrane, was counted under a microscope in five random fields at a magnification of 100×. The pCMV-PRL3miRNA-1249-A2 cells showed significantly reduced invasiveness as compared with parental SGC7901 and pCMV-PRL3miRNA-neg-A1 cells ($^*P < 0.05$). (B) SGC7901 cell invasion picture. (C) pCMV-PRL3miRNA-1249-A2 cell invasion picture. The slides were stained with hematoxylin and eosin, and visualized 100×.

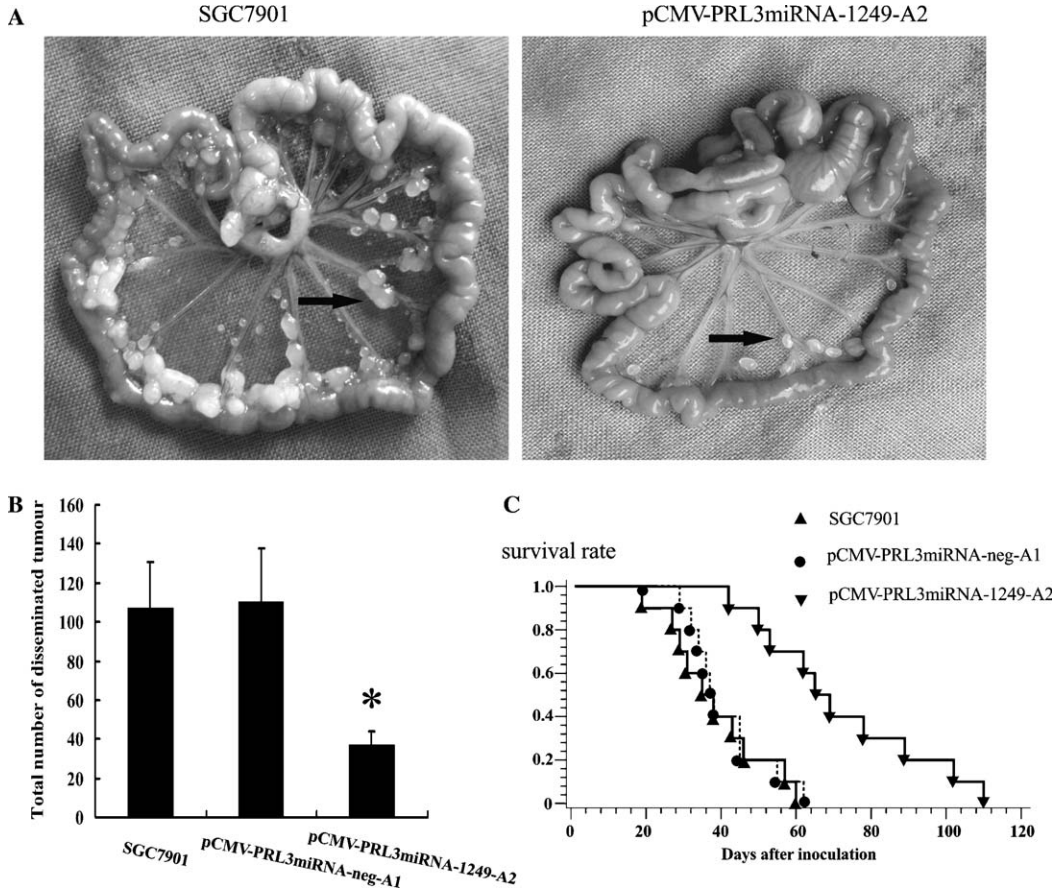

Fig. 7. Knockdown of PRL-3 in SGC7901 cells suppressed growth of peritoneal metastases and prolonged murine survival. (A) Metastatic nodules formed on the intestinal mesentery. The arrows show the nodules. (B) Total number of metastatic nodules on peritoneal surface. Bars show means ± SD ($n = 10$ for each group). The total number of metastatic nodules was reduced in PRL-3 knockdown cells. Compared with control: $^*P < 0.05$. The difference between SGC7901 and pCMV-PRL3miRNA-neg-A1 cells was not significant. (C) The effects of PRL-3-knockdown on murine survival. 5×10^6 SGC7901 (▲), pCMV-PRL3miRNA-neg-A1 (●) or pCMV-PRL3miRNA-1249-A2 (▼) cells were injected into nude mice. The survival rate of the pCMV-PRL3miRNA-1249-A2 group was significantly better than those of the SGC7901 group and pCMV-PRL3miRNA-neg-A1 group ($P < 0.05$; $n = 10$ for each group).

metastases and improve the prognosis in this experimental condition.

Discussion

PRL-3 belongs to a recently discovered family of protein phosphatases. The first evidence that the phosphatase was linked to metastasis came from genome-wide transcriptional analysis of colorectal cancer samples [6]. More studies have been performed on PRL-3 in the cancer field as it has been reported to correlate with lymphatic metastasis of gastric cancer. In order to provide more insight into the molecular mechanism of gastric metastasis, we assessed the expression levels of PRL-3 in peritoneal metastasis and primary tumors from 25 gastric cancer patients with peritoneal metastasis. We found that expression levels of PRL-3 in the metastasis were higher than those in the primary lesion. This suggested to us that PRL-3 could play a important role in gastric peritoneal metastasis.

In this paper, microRNA-induced RNA silencing was utilized. RNA interference (RNAi) has become a commonly used tool for the analysis of gene function in animals and plants [21]. MicroRNAs (miRNAs) are endogenously expressed small ssRNA sequences of ~22 nucleotide in length, which naturally direct gene silencing through components shared with the RNAi pathway [22]. Unlike shRNAs, however, the miRNAs are found embedded, sometimes in clusters, in long primary transcripts (pri-miRNAs) of several kilobases in length containing a hairpin structure and driven by RNA polymerase II [23]. Drosha, a nuclear RNase III, cleaves the stem-loop structure of the pri-miRNA to generate small hairpin precursor miRNAs (pre-miRNAs) which are ~70 nucleotides in length [24]. The pre-miRNAs are exported from the nucleus to the cytoplasm by exportin-5, a nuclear transport receptor [25,26]. Following the nuclear export, the pre-miRNAs are processed by Dicer into a ~22 nucleotides miRNA (mature miRNA) molecule, and incorporated into a miRNA-containing RNA-induced silencing complex (miRISC) [27].

The mature miRNAs regulate gene expression by mRNA cleavage or translational repression [10–13]. Target cleavage can be induced artificially by altering the target or the miRNA sequence to obtain complete hybridization [14]. Cullen and colleagues designed a vec-

tor for RNAi in which a synthetic miRNA is expressed from a synthetic stem-loop precursor based on the miR-30 miRNA precursor [14,28]. Subsequently, other groups have developed additional miR-30-based vectors for RNAi [13,29–31]. Recently, a new RNA polymerase II expression vector based upon murine miR-155 sequence came into use [17,32]. In this study, three different miR155-based PRL-3 targeting pre-miRNA pol II vectors were constructed. We found that PRL-3 protein and mRNA levels significantly decreased in pCMV-PRL3miRNA-1249 and pCMV-PRL3miRNA-477 groups. Although initial reports suggested that down-regulation by partially complementary miRNAs was due entirely to decreased translation [22], recent studies indicated that miRNAs can also reduce the cellular concentration of the mRNAs that they regulate, both *in vitro* and *in vivo* [33–36].

Moreover, the pCMV-PRL3miRNA-1249 targeting to 3′-untranslated region (UTR) of mRNA more efficiently depressed the PRL-3 protein expression than pCMV-PRL3miRNA-477 targeting to ORF (open reading frame). Our findings are consistent with the previous reports, in terms of miRNA band to 3′-UTR of mRNA, which is a more efficient knockdown function. However, very little is known about the mechanism by which they do so. Jing et al. [36] found that AU-rich elements (AREs) in the 3-untranslated region degradation (UTR) of unstable mRNAs dictate their degradation. MiRNA targeting of ARE appears to be an essential step in ARE-mediated mRNA degradation.

To confirm the association between PRL-3 expression and peritoneal metastasis, we generate the stable transfectants of PRL-3 knockdown in SGC7901 cell, which is a cell line derived from a patient with advanced gastric adenocarcinoma. Using an *in vivo* model of peritoneal metastasis in nude mice, we tested the ability of PRL-3 knockdown cells to form tumor nodules in the peritoneal cavity. For the experiments, mismatch sequence-transfectants and the parental cells were used as controls. Compared to control cells, PRL-3 knockdown SGC7901 cells had a decreased ability to form peritoneal metastatic nodules. To identify the mechanisms regulating the decreased ability of PRL-3 knockdown cells to form peritoneal metastasis, the ability of migration and invasion *in vitro* was analyzed. We found that PRL-3 knockdown cells exhibited marked decrease in migration. In addition, the ability of SGC7901 cells to invade into Matrigel also decreased after PRL-3 knockdown. To confirm that the data from the *in vitro* migration and invasion assays did not result from differences in cellular proliferation amongst our cell lines, we measured growth rates using an *in vitro* cellular proliferation assay. No significant difference in the rate of proliferation was observed amongst cell lines at 24, 48, and 72 h (data not shown). Therefore, it is concluded that inhibition of PRL-3 expression resulted in decreased cell motility and invasiveness, independent of cellular proliferation in SGC7901 cells.

Taken together, our results indicate that artificial miRNA can inhibit endogenous PRL-3 expression. Knockdown of PRL-3 significantly depressed the SGC7901 gastric cancer cell invasion and migration ability independent of cellular proliferation. This report provides supportive evidence that PRL-3 facilitates the peritoneal metastasis in gastric cancer cells, and suggests that PRL-3 gene might be a potential therapeutic target for gastric cancer peritoneal metastasis.

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