Proliferin-related protein overexpression in SGC-7901 gastric cancer cells inhibits *in vitro* cell growth and tumorigenesis in nude mice

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Abstract. As reported in the literature, the worldwide 5-year overall survival rate for patients with gastric cancer receiving surgical treatment in the progressive stage is less than 25%. Therefore, there is an urgent need for the development of novel therapeutic strategies. Our preliminary studies demonstrated that proliferin-related protein (PRP) inhibits the proliferation of TM3 Leydig testicular cells. To evaluate whether PRP has antitumor effects *in vitro* and *in vivo*, we stably expressed PRP in SGC-7901 gastric carcinoma cells. PRP inhibited the proliferation and cell cycle progression of SCG-7901 cells, as determined by cell growth and cell cycle assays. Transwell experiments demonstrated that PRP inhibited the cell migration and invasion of SCG-7901 cells. Western blotting demonstrated that PRP-overexpressing cells had upregulated matrix metalloproteinase 9 (MMP-9) and downregulated tissue inhibitor of metalloproteinases-1 (TIMP-1). In a xenograft tumor formation assay using nude mice, tumors formed by PRP-overexpressing cells had significantly lower weights than those formed by control cells, and the tumor inhibitory rate reached 71.9%. We demonstrated for the first time that PRP inhibits gastric carcinoma cell proliferation, motility, and tumorigenicity *in vivo*, suggesting that PRP may become an important target for the development of gastric cancer gene therapy.

Introduction

Gastric carcinoma is one of the most common malignant tumors with high incidence and mortality rates. The 5-year overall survival rate for patients with early stage gastric carcinoma following treatment is higher than 90%. However, the 5-year postoperative survival rate for patients with gastric carcinoma in progressive stages is only 30-40%, and the overall rate of disease relapse is 50-70%. Current treatments for gastric carcinoma include the removal of the primary tumors and affected lymph nodes in combination with radiotherapy, chemotherapy, biological therapy and gene therapy (1).

Radical gastric cancer therapy is only applicable to early stage tumors, whereas radiotherapy and chemotherapy lack selectivity, have marked toxic side effects, and are often not tolerated by patients. Thus, there is a need for novel tumor therapies. Gene therapy aims to introduce an exogenous normal gene or sequence into target cells to replace or compensate for the function of a defective gene or to suppress the overexpression of an abnormal gene, thereby treating disease (2). As the understanding of the molecular mechanisms of tumor pathology has advanced, gene therapy as a novel strategy has been shown to have a therapeutic advantage for treating several types of tumors, including gastric carcinoma (3-6).

Proliferin-related protein (PRP), a prolactin (PRL) family member (7-10), is secreted by mammalian placenta (11-15). Placenta-secreted proliferin (PLF) can induce the formation of decidual new blood vessels, whereas PRP inhibits the effects of PLF and other angiogenic factors, thereby blocking the formation of new blood vessels. Jackson *et al* (12) found that PRP inhibits endothelial cell migration and blood vessel formation. This inhibitory role may affect tumorigenesis and cancer progression.

Our previous studies demonstrated that the PRP gene is not only expressed in the female reproductive system but is also expressed in the male reproductive system, where it is located in Leydig cells, and its expression increases with age (16). To elucidate the function of PRP, we performed experiments to examine the consequences of lentiviral-mediated PRP knockdown on cell growth, cell cycle, testosterone production and the expression of several Leydig genes. Our results revealed that PRP indirectly participates in testosterone production. In addition, we found that another marked change in TM3 Leydig cells, which was induced by PRP silencing, was related to the growth rate. Cell growth and cell cycle analyses demonstrated that the inhibition of PRP expression resulted in a decrease in the TM3 cell doubling time and an increase in the number of cells in S-phase, suggesting that PRP plays a negative role in cell proliferation.

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Based on the anti-proliferative effects of PRP, we aimed to ascertain whether PRP also inhibits the growth of SGC-7901 gastric carcinoma cells. Thus, we stably expressed PRP in SGC-7901 cells to evaluate the effects of PRP on proliferation and invasion. We also established an *in vivo* xenograft tumor model by subcutaneously injecting SGC-7901 cells in nude mice to evaluate the effects of PRP on xenograft tumor growth.

Materials and methods

Materials. RPMI-1640 medium and fetal bovine serum (FBS) were purchased from HyClone (Logan, UT, USA). A protein extraction kit and BeyoECL Plus western blotting detection reagent were purchased from Beyotime Biotechnology (Jiangsu, China). Anti-PRP (rat monoclonal IgG2a, sc-80531), anti-MMP-9 (sc-6840) and anti-TIMP-1 (sc-5538) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other chemicals were purchased from Sangon Biotech (Shanghai, China).

Cell culture. The human gastric cancer cell line SGC-7901 was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI-1640 medium supplemented with 10% FBS at 37˚C in a humidified atmosphere containing 5% CO_2.

EGFP-C1-PRP eukaryotic expression vector construction and transfection. The PRP full-length open reading frame was cloned into the pEGFP-C1 vector to generate the pEGFP-C1-PRP expression vector. Following the manufacturer's instructions, the pEGFP-C1-PRP vector or the empty vector control was transfected into SGC-7901 cells using Lipofectamine 2000, and the cells were subsequently selected with G418 to screen for stably transfected cell lines, which were named SGC-7901-PRP and SGC-7901-C1, respectively.

Calculation of the cell doubling time. Parental SGC-7901, SGC-7901-PRP and SGC-7901-C1 cells in the log growth phase were trypsinized, separated into single-cell suspensions and seeded into 24-well plates. Three wells were randomly selected at 24, 48, 72, 96, 120, 144 and 168 h before being trypsinized. Cell numbers were counted, and an average was obtained from three calculations. A growth curve was graphed with the cell number plotted on the y-axis and time on the x-axis. Doubling time was calculated from the viable cell number versus time (linear zone) graph using the following formula: Doubling time (T_D) = t * lg2/($1gN_t$ - lgN_0), where t is the time of culture and IgN_0 and $1gN_t$ are the cell numbers at the time of seeding and at t, respectively.

Invasion and migration assay. Cell invasion assays were performed using Transwells (8-mm pore size; Corning-Costar Corp., Acton, MA, USA). Filters were coated with 120 μl Matrigel, and the upper chambers were seeded with 200 μl of the cell suspension containing $1x10^5$ cells/ml, and the lower chambers were seeded with 500 μl of complete media. The Transwell plates were incubated at 37˚C in the presence of 5% CO_2 for 24 h. Cells that infiltrated and adhered to the filter membrane surface facing the lower chamber were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Five to 10 views were randomly selected, and an average cell number per view was counted to evaluate the cancer cell invasive ability. Cell migration assays were performed using the same protocol with non-coated filters.

Cell cycle analysis. Synchronized SGC-7901, SGC-7901-PRP and SGC-7901-C1 cells were trypsinized, separated into single-cell suspensions and seeded into 6-well plates at $1x10^5$ cells/ml. Cells were cultured at 37˚C in the presence of 5% CO_2 for 24 h to allow the cells to adhere to the plates prior to replacement with fresh media. After 48 h, cells were trypsinized, collected by centrifugation at 1,000 rpm for 5 min, washed twice with phosphate-buffered saline (PBS) and fixed in 1 ml pre-chilled 70% ethanol at 4˚C overnight. The next day, the cells were washed 3 times with PBS to remove the ethanol, collected by centrifugation at 1,000 rpm for 5 min and resuspended in 0.5 ml PBS. Subsequently, protease inhibitor and RNase inhibitor were added at a final concentration of 50 μg/ml. The cells were then incubated in a 37˚C waterbath for 30 min, filtered through a 75-nm filter and analyzed by flow cytometry for cell cycle analysis. Three independent experiments were performed.

Immunofluorescence. To determine the PRP expression level in the pEGFP-C1-PRP-transfected stable SGC-7901 cells and to detect PRP protein in the xenografts, an immunofluorescence assay was performed using a rat anti-PRP primary monoclonal antibody. Slides were analyzed by observation under a fluorescence microscope.

Western blotting. For protein isolation, tissues or cells were lysed with RIPA buffer. Insoluble material was removed by centrifugation at 12,000 x g for 10 min at 4˚C, and the supernatants were collected. The protein concentration was determined using a bicinchoninic acid (BCA) assay. Proteins were separated in 10% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes followed by incubation with the primary antibody. After washing 3 times with Tris-buffered saline and Tween-20 (TBST), the membranes were incubated with a secondary IgG antibody for 2 h (at room temperature) and washed again. The antigen-antibody complex was detected using the BeyoECL Kit following the manufacturer's protocol.

Nude mouse xenograft experiments. Nude mice were grouped to receive an injection of parental SGC-7901, SGC-7901-C1 or SGC-7901-PRP cells. Cells in the log growth phase were collected in serum-free RPMI-1640, spun down and resuspended in PBS at $3x10^6$ cells/ml. Cell suspensions (0.2 ml per animal) were injected into the right front armpit of nude mice. The xenografts were observed every 4 days for 32 days.

The maximum (A) and minimal diameters (B) of the xengrafts were measured. The volume of the tumors was calculated as Volume = A x $B^2/2$ and plotted onto a xenograft growth curve. Mice were euthanized by cervical dislocation at the end of the experiment. The tumors were removed under sterile conditions and weighed. The tumor inhibitory rate was calculated as follows: Tumor inhibitory rate = (1 - experimental group average weights/control group average weights) x 100%. The tumors were fixed in methanol and stored in liquid

Figure 1. PRP protein expression in SGC-7901-PRP cells (harboring the pEGFP-C1-PRP vector). The pEGFP-C1-PRP vector was transfected into SGC-7901 cells using Lipofectamine 2000. These cells were later harvested to analyze the PRP protein expression. (A) Representative PRP western blotting for parental SGC-7901, SGC-7901-C1 and SGC-7901-PRP cells. (B) Relative PRP protein expression levels for the different groups based on β-actin normalization. The values represent the means ± SD (n=3).

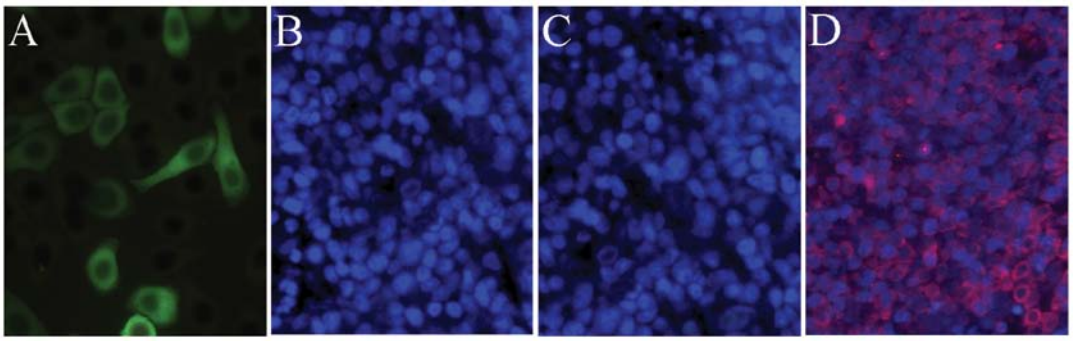

Figure 2. Immunofluorescence PRP protein staining. (A) Subcellular localization of the PRP protein (green) in the cytoplasm of SGC-7901 cells bearing the EGFP-C1-PRP vector. Immunofluorescence staining of PRP protein (red) in tumor tissues from mice injected with (B) SGC-7901, (C) SGC-7901-C1 and (D) SGC-7901-PRP cells. The nuclei are non-specifically stained with DAPI (blue).

nitrogen for subsequent immunofluorescence staining and protein extraction. The animal protocols conformed to those approved by the Chongqing Medical University Animal Care and Use Committee.

Statistical analysis. All data are presented as means ± SE. For comparisons between two groups, the Student's t-test was performed. For comparisons among multiple groups, an ANOVA test was performed followed by a Student-Newman-Keuls test. Differences were considered significant at $P<0.05$.

Results

PRP is expressed in the SGC-7901-PRP cells. To investigate the potential effects of the mouse PRP activity on cell growth and proliferation, pEGFP-C1-PRP or the pEGFP-C1 empty vector (as a control) were transfected into SGC-7901 cells. Stably transfected cells were selected using G418 and subsequently analyzed for PRP protein expression. Western blot analysis results confirmed the detectable expression of PRP in cells stably transfected with the pEGFP-C1-PRP vector (Fig. 1).

Immunofluorescence staining demonstrates that PRP is expressed in the cytoplasm of SGC-7901-PRP cells (Fig. 2A). Immunofluorescence staining of tumor slices revealed that PRP was expressed in the cytoplasm of tumor cells in the SGC-7901-PRP group, while there was no PRP expression in tumors from the SGC-7901-C1 and SGC-7901 groups (Fig. 2B-D).

PRP overexpression alters the doubling time and cell cycle distribution of SGC-7901 cells in vitro. The doubling times were calculated by cell counting. Compared with the SGC-7901-C1 and SGC-7901 cells, the SGC-7901-PRP cells exhibited a longer doubling time, suggesting a role for PRP in human tumor cell growth (Fig. 3A). To address this observation further, the cell cycle profiles were assessed by flow cytometric analysis. The data demonstrated that PRP overexpression caused a marked decrease in the number of cells in the S-phase (Fig. 3B-E).

Effect of PRP overexpression on SGC-7901 cell migration and invasion. The results of the Transwell cell migration and invasion assays are shown in Fig. 4 and Table I. PRP overexpression resulted in significant suppression of SGC-7901 cell migration and invasion.

Matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinases-1 (TIMP-1) are involved in PRP-regulated SGC-7901 cell invasion. Our finding that PRP has an inhibitory effect on cell invasion prompted us to examine its effects on MMP-9 and TIMP-1 expression in SGC-7901 cells. As shown in Fig. 5, western blot analysis revealed that MMP-9 expression was significantly decreased ($P<0.05$), while TIMP-1 expression was significantly increased ($P<0.05$) in the SGC-7901-PRP cells. These results suggest that the inhibitory effect of PRP on the invasiveness of SGC-7901 cells was at least partially mediated by MMP-9 downregulation and TIMP-1 upregulation, which may contribute to degradation of the extracellular matrix.

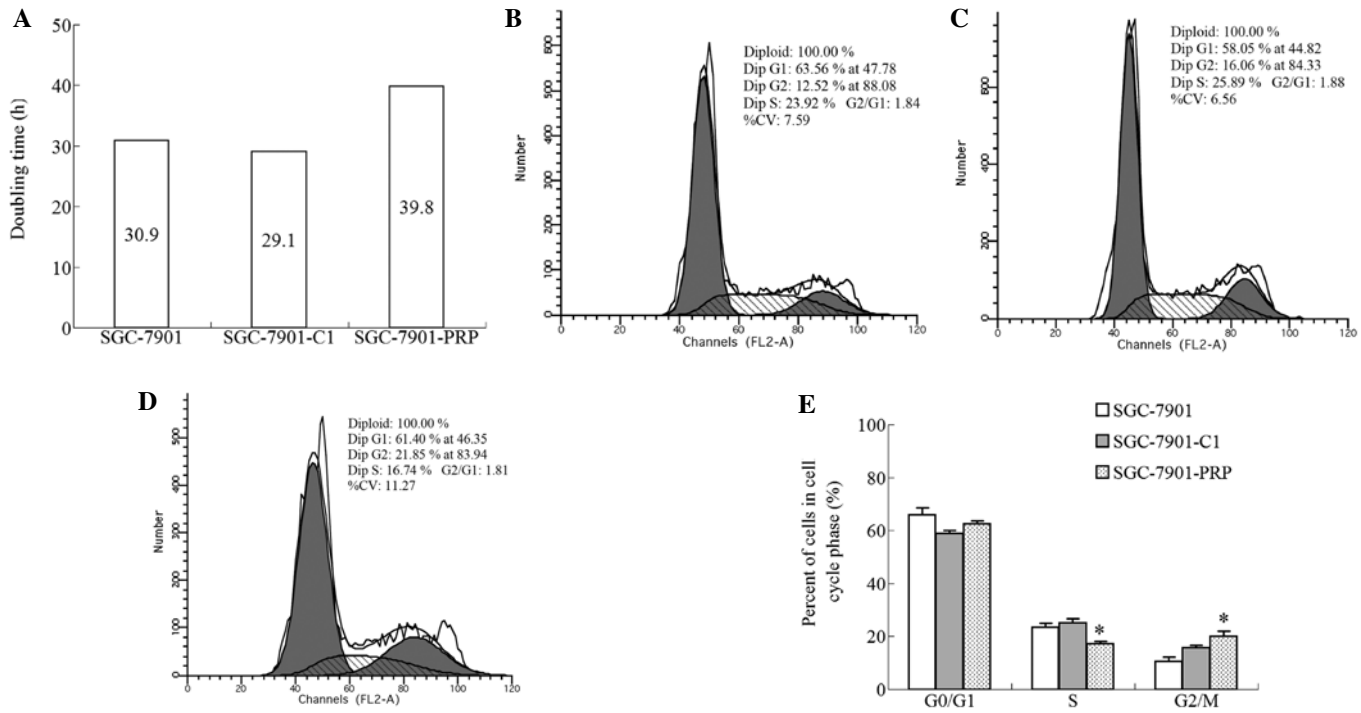

Figure 3. PRP overexpression alters the doubling time and cell cycle distribution of SGC-7901 cells. (A) Doubling time of the parental SGC-7901, SGC-7901-C1 (transfected with pEGFP-C1) and SGC-7901-PRP cells (transfected with pEGFP-C1-PRP). A representative cell cycle analysis plot of (B) SGC-7901, (C) SGC-7901-C1 and (D) SGC-7901-PRP cells. Cells were fixed with 70% ethanol and stained with propidium iodide (PI) followed by flow cytometric analysis. (E) The relative frequency of the different cell phases. The data are expressed as the means ± SD (n=3). *P<0.05 compared with parental SGC-7901 or SGC-7901-C1 cells.

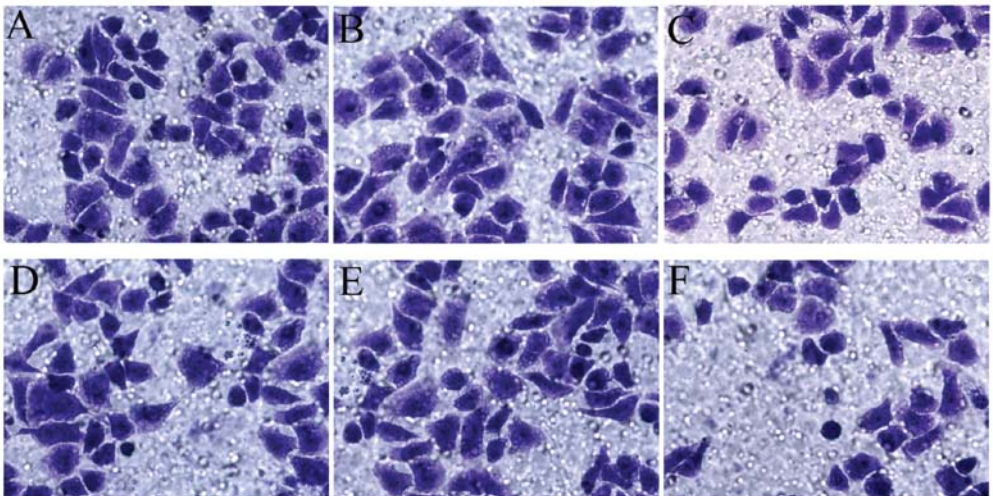

Figure 4. Representative images of SGC-7901, SGC-7901-C1 and SGC-7901-PRP cell migration and invasion. (A) SGC-7901 cell migration; (B) SGC-7901-C1 cell migration; (C) SGC-7901-PRP cell migration; (D) SGC-7901 cell invasion; (E) SGC-7901-C1 cell invasion; (F) SGC-7901-PRP cell invasion.

Growth-inhibitory effect of the overexpression of PRP in gastric tumor xenografts. We performed a subcutaneous tumor formation assay in nude mice to evaluate the growth suppressive effects of PRP *in vivo*. Nude mice were subcutaneously injected with parental SGC-7901, SGC-7901-C1 or SGC-7901-PRP cells. The tumor volume and the tumor weight at 32 days were measured after subcutaneous injection. Among the 8 mice that were injected with SGC-7901-PRP cells, one did not develop a tumor. The tumor formation rate was 87.5% for all mice. The tumor formation rate for the groups of mice that received parental SGC-7901 or SGC-7901-C1 cells was 100%. As shown in Fig. 6, the tumor volume and tumor weight induced by PRP-overexpressing SGC-7901 cells were significantly reduced when compared with the tumor xenografts from SGC-7901-C or parental SGC-7901 cells (P<0.01). The inhibitory rate of tumor growth of the mouse group injected with PRP-overexpressing SGC-7901 cells was 71.9%.

Discussion

PRP has been previously reported to be expressed only in female mammals. The main function of PRP was believed

Table I. Effect of the overexpression of PRP on SGC-7901 cell migration and invasion.

Cell group	No. of invading cells	No. of migrating cells
SGC-7901	65.6±4.0	70.2±4.9
SGC-7901-C1	60.0±3.5	68.4±7.2
SGC-7901-PRP	31.8±6.1[a]	46.6±7.6[a]

Data, which are representative of three independent experiments, are expressed as means ± SE. [a]P<0.05 vs. the SGC-7901 or SGC-7901-C1 cell group.

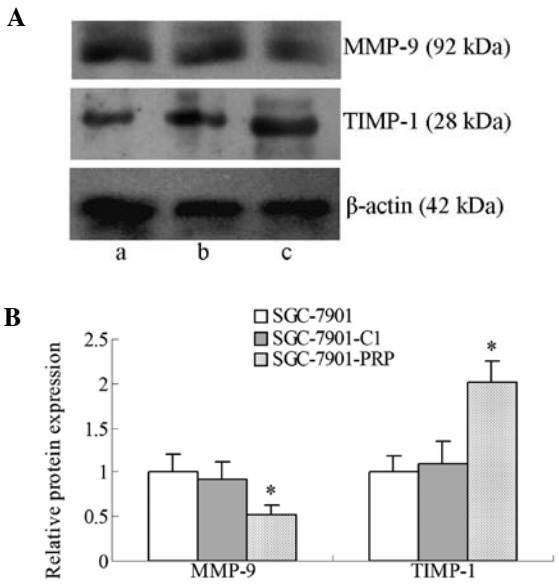

Figure 5. Effect of PRP on MMP-9 and TIMP-1 expression in SGC-7901 cells. (A) The expression levels of MMP-9 and TIMP-1 were evaluated by western blotting. Lane a, parental SGC-7901; lane b, SGC-7901-C1; lane c, SGC-7901-PRP cells. A representative image of three experiments is shown. (B) The relative PRP protein expression in the different cells as normalized to β-actin. The values represent the means ± SD (n=3). β-actin was used as an internal control. *P<0.05 compared to the control.

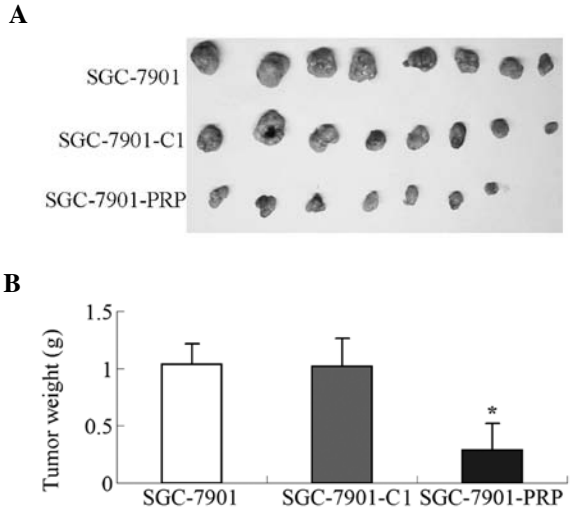

Figure 6. PRP overexpression inhibits SGC-7901 tumor cell growth *in vivo*. (A) Tumor images 32 days after the subcutaneous injection of the indicated cells. (B) The mean tumor volume after the subcutaneous injection of nude mice with parental SGC-7901, SGC-7901-C1 or PRP-overexpressing SGC-7901 PRP cells was measured at the indicated time points. The data represent the mean tumor weights of the xenografts (mean ± SD, n=8). *P<0.01 vs. the SGC-7901 or SGC-7901-C1 group.

to be anti-angiogenesis, and it was reported to play a role in pregnancy (12). However, the present study provides evidence demonstrating that in addition to the anti-angiogenesis function (12,17), PRP also possesses *in vitro* and *in vivo* antitumor effects through the inhibition of cell growth, proliferation, migration and invasion.

Our results demonstrated that PRP significantly inhibited cell proliferation. Compared with the controls, SGC7901-PRP cells exhibited a longer doubling time, suggesting a role for PRP in human tumor cell growth. Cell proliferation proceeds through a series of events in which the synthesis and organization of DNA and other cellular components occur prior to mitosis. Since PRP inhibits cell proliferation and an altered cell cycle is a common cancer characteristic, we aimed to ascertain whether PRP plays a role in regulating cell cycle progression. Flow cytometric results demonstrated that PRP overexpression caused a significant decrease in the cell number in the S-phase of the cell cycle.

Numerous studies have demonstrated an association between cell cycle progression and cancer, and targeting the cell cycle has become a recognized strategy for tumor treatment (18,19). The potency of PRP in inducing a decrease in the number of S-phase cells in human tumor cells suggests that it may lead to new strategies for treating human gastric cancer.

Metastasis is a major cause of the high mortality rate of patients with gastric cancer, and gastric cancer has a high frequency of recurrence, even after treatment. Cancer cell migration and invasion are the major events that occur during metastasis. Thus, blocking cancer cell migration and invasion may enhance the efficacy of gastric cancer treatments. Targeting particular mechanisms in these events may become a novel strategy to treat gastric cancer. By evaluating the migration and invasion capabilities of SGC-7901 cells using a Transwell chamber assay, our study demonstrated that PRP significantly decreased the migration and invasion of SGC-7901 cells. These data suggest that PRP not only inhibits cell proliferation, it also antagonizes cancer cell migration and invasion.

A major event that occurs during invasion and metastasis is the degradation of the extracellular matrix (ECM). MMP-9 degrades type-IV collagen, which is the major component of the ECM; therefore, it plays an important role in tumor invasion into the surrounding tissue and the subsequent distal metastasis through blood vessels and the lymph vascular system. TIMP-1, an MMP-9 inhibitor (20-22), inhibits metastasis.

To investigate further the molecular mechanism underlying the anti-migratory and anti-invasive properties of PRP, we assessed the effect of the overexpression of PRP on MMP-9 and TIMP-1 expression in SGC-7901 cells. Our results demonstrated that PRP overexpression resulted in a significant decrease in MMP-9 expression and an increased in TIMP-1 expression. Sier *et al* (23) and Bando *et al* (24) confirmed that the MMP-9 expression level significantly increases in

gastric tumors. Patients with higher MMP-9 expression have a lower survival rate, and MMP-9 expression may be used as an independent prognostic indicator for gastric cancer. The downregulation of MMP-9 expression inhibits tumor invasion, angiogenesis and growth (25). The orally available MMP inhibitor marimastat has demonstrated a trend toward survival benefit in a small phase III gastric cancer study and was proposed as a maintenance therapy for patients with advanced gastric cancer (4). PRP overexpression decreased the MMP-9 and TIMP-1 ratio, suggesting that PRP could antagonize metastasis by modulating the expression of MMP-9 and TIMP-1.

Our assessment of the biological effects of PRP on human gastric carcinoma cells focused on the tumorigenicity of SGC-7901 cells in a xenotransplantation nude mouse model. Our results demonstrated that tumors from the PRP-overexpressing group had a lower growth rate. There were significant differences in the volume and weight of tumors between the PRP and control groups, suggesting that PRP was able to reverse the tumorigenic effects of SGC-7901 cells *in vivo*, which was also supported by *in vitro* experiments in which PRP overexpression caused a decreased in proliferation, motility and invasion.

Theoretically, gene therapies should effectively inhibit tumor growth to various extents; however, the clinical outcomes of these therapies are not ideal, which may be because tumor formation is a multistep, multistage, complex process. Abnormalities in the function of multiple genes have been implicated in cancer and when these genes interact with each other, they synergistically stimulate proliferation and inhibit apoptosis, altering the ability of cells to adhere and migrate. To achieve an optimal therapeutic effect, multiple tumorigenesis-related factors must be targeted. In addition to its previously reported anti-angiogenic role during pregnancy, we provide evidence that PRP has multiple inhibitory effects on gastric tumor growth, including suppressing cell proliferation and reducing proliferative activity by blocking the cell cycle and inhibiting cell migration and invasion. Furthermore, we revealed that, at least partially, the anti-invasive and anti-migratory effects of PRP are mediated via MMP-9 downregulation and TIMP-1 upregulation. The *in vitro* and *in vivo* antitumor effects of PRP suggest that it may be an effective target for the gene therapy of gastric carcinoma.

Acknowledgements

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