PRL-3 siRNA Inhibits the Metastasis of B16-BL6 Mouse Melanoma Cells In Vitro and In Vivo

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Phosphatase of regenerating liver-3 (PRL-3) has been proposed to promote the invasion of tumor cells to metastasis sites. However, the effect of PRL-3 on spontaneous metastasis has not been clearly demonstrated, and whether PRL-3 could become a new therapeutic target in malignant tumor is still unknown. In this study, we used PRL-3 siRNA as a molecular medicine to specifically reduce the expression of PRL-3 in B16-BL6 cells, a highly metastatic melanoma cell line. In vitro, PRL-3 siRNA significantly inhibited cell adhesion and migration, but had no effect on cell proliferation. In the spontaneous metastatic tumor model in vivo, PRL-3 siRNA treatment remarkably inhibited the proliferation of primary tumor, prevented tumor cells from invading the draining lymph nodes, and prolonged the life span of mice. Therefore, our results indicate that PRL-3 plays a critical role in promoting the whole process of spontaneous metastasis and tumor growth initiation, and that inhibiting PRL-3 will improve malignant tumor therapy.

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INTRODUCTION

Metastasis is a leading reason for cancer mortality and involves a complex, multistep progress by which tumor cells disseminate to distant sites to establish discontinuous secondary colonies (1,2). A large number of factors are validated in the process of metastasis, such as cytokines, chemokines, hormones, growth factors, cell adhesion molecules, matrix metalloproteinase, and hypooxygen microenvironment (3-5). However, mechanisms of regulating the multistep progression from primary tumor initiation to proliferation in metastatic sites remain poorly elucidated.

Recent studies suggest that phosphatase of regenerating liver (PRL), a newly identified tyrosine phosphatase family, plays a causative role in tumor metastasis. The PRLs (PRL-1, -2, and -3) are relatively small proteins of ~22 kDa with at least 75% amino acid sequence similarity, containing the protein tyrosine phosphatase (PTP) active domain (CX5R) and a C-terminal CAAX sequence for prenylation (6). Among these PRLs, PRL-1 is overexpressed in breast, prostate, ovarian, and pancreatic cancers, while PRL-2 can lead to epithelial cell transformation and forms tumors in nude mice (6-8). PRL-3 plays a more notable role in metastatic cancer cells. Kato et al. (9) indicate that downregulation of endogenous PRL-3 in human DLD-1 cells, treated with PRL-3 small interfering RNA, inhibits the cells' motility and metastasis formation in liver. Overexpression of PRL-3 in mouse low-metastatic B16 cells and CHO cells not only promotes their migration and invasion in vitro, but also enhances their metastatic ability to lung and liver in experimental passive metastatic models, which are established by injecting tumor cells into mice via tail vein (10,11). Although these data validate the function of PRL-3 in tumor metastasis, most of them were obtained by simulating the partly metastatic process from tumor cells to distant tissues through the bloodstream. Therefore, the role of PRL-3 in the whole metastatic progress from primary tumor to distant sites is an untilled field.

Accumulating evidence in clinical trials indicates that PRL-3 gene not only is commonly overexpressed in colorectal cancer metastasis (9), liver carcinomas (10), and ovarian tumors, but is also associated with clinic stages of cancer progression (12). Bardelli et al. (13) reported that PRL-3 could be widely detected in metastatic sites from colorectal cancer, such as lymph nodes, liver, brain, and ovary. More recently, PRL-3 was found in endothelial cells besides malignant cells, which suggests that PRL-3 may play a functional role in endothelial compartment of tumors, such as promoting angiogenesis (8). These data support the fact that PRL-3 can be used as an attractive target for innovative anticancer therapeutics.

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RNA interference (RNAi), a sequence-specific, posttranscriptional gene silencing mechanism, is triggered by small interfering double-stranded RNA (siRNA) with degradation of mRNA homologous in sequence to the siRNA (14,15). With its efficient and specific ability to downregulate gene expression, RNAi has been widely used for analysis of gene function (16,17) and in vivo treatment of many tumors by injecting naked siRNA or siRNA expression vector (18,19).

The present work, therefore, aims at confirming the role of endogenous PRL-3 in the whole metastatic process of B16-BL6 cells from footpad to draining lymph node in C57BL/6J mice. Furthermore, we evaluated the clinical ability of PRL-3 siRNA to prolong the survival time of mice in the spontaneous metastasis model.

MATERIALS AND METHODS

Cell Lines

Highly metastatic B16-BL6 cells and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies), 100 U/mL penicillin, and 100 μg/mL streptomycin and incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air.

Animals

C57BL/6J mice (6 to 8 weeks old) were obtained from the Shanghai Laboratory Animal Center (Shanghai, China). Throughout the experiments, mice were maintained with free access to pellet food and water in plastic cages at 21 ± 2°C and kept on a 12-h light-dark cycle. Animal welfare and experimental procedures were performed strictly in accordance with the care and use of laboratory animals (National Research Council, Washington, DC, USA) and the related ethics regulations of our university. All efforts were made to minimize the animals' suffering and to reduce the number of animals used.

Construction of Vectors and Transient Transfection in Cells

Myc-PRL-3 was generated by RT-PCR with the following primers: 5′-GCGGATCCACCATGGAGCAGAAGCTGATCTCCGAGGAGGACCTCGCCCGCATGAACCGGCCTGCGCCTG-3′ and 5′-CTGGTACCCTACATGACGCAGCATCTGGTC-3′ from muscle total RNA of C57BL/6J mouse. The PCR fragments were cloned into *Bam*HI/*Kpn*I sites of pTARGET (Promega).

Oligonucleotides encoding four different PRL-3 siRNAs shown in Figure 1A were commercially synthesized (Invitrogen, Shanghai, China) and cloned into *Bam*HI/*Hind*III sites of pRNA-U6.1/Neo (Genescript). pRNA-U6.1/Neo-Luciferase-siRNA (Genescript) was used as the monitor and a negative control.

Transient transfections were performed with polyethylenimine (PEI) liposome complex (kindly provided by Dr. Zi-Chun Hua, Nanjing University). Briefly, 4 μL PEI (1 mg/mL) and plasmids were diluted in 50 μL MEM (Life Technologies) and mixed, then incubated at room temperature for 20 min before addition of 900 μL MEM. The mixture was spread onto cells. Cells were switched to 10% FBS DMEM after 6 h and incubated at 37°C for another 18 h, then collected and prepared for the experiments.

Semiquantitative RT-PCR and Real-Time PCR

First-strand cDNAs were generated by reverse transcription using oligo(dT) from RNA samples treated by DNase. The specific primers and cycle number for each gene we used are as follows:

Gene	Product length	Primer	Cycle no.
PRL-1	472 bp	Forward: 5′-CAACCAATGCGACCTTAA-3′ Reverse: 5′-CAATGGCATCAGGCACCC-3′	30
PRL-2	339 bp	Forward: 5′ATTTGCCATAATGAACCG-3′ Reverse: 5′-ACAGGAGCCCTTCCCAAT-3′	30
PRL-3	468 bp	Forward: 5′-CTTCCTCATCACCCACAACC-3′ Reverse: 5′-TACATGACGCAGCATCTGG-3′	28
GAPDH	191 bp	Forward: 5′-AACGACCCCTTCATTGAC-3′ Reverse: 5′-TCCACGACATACTCAGCAC-3′	28

PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining. The gel images were captured and analyzed by the Gel Imaging and Documentation Digi-Doc-It System (v. 1.1.23; UVP, Upland, CA, USA). Real-time quantitative PCR was performed with the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) using EvaGreen dye (Biotium, USA), and threshold cycle numbers were obtained using ABI Prism 7000 SDS software v. 1.0. The primer sequences used in this study were the same as above. Conditions for amplification were one cycle of 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 30 s.

Western Blot

The Western blot was performed as described (20). The cells, cotransfected with pTARGET-Myc-PRL-3 (1 μg) and pRNA-U6.1/Neo-Luc-siRNA or pRNA-U6.1/Neo-PRL3-siRNA-1, -2, -3, or -4 (1 μg), were collected and lysed (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 5 mM EDTA, 0.1 mM PMSF, 0.15 U/mL aprotinin, 1 μg/mL pepstatin, and 10% glycerol). Anti-Myc (clone 9E10) and anti-actin (Santa Cruz Biotechnology) antibodies were used for Western blot.

Cell Proliferation Assay

B16-BL6 cells were transiently transfected with or without PRL-3 siRNA (1 and 2 μg), or Luc-siRNA (2 μg) for

24 h, then 2×10^4 cells in 1 mL of culture medium were added into each well of 24-well plates in triplicate for proliferation assay on day 0. Cells were trypsinized and counted on days noted in the figure legends.

Cell Cycle Analysis

B16-BL6 cells, transiently transfected with or without PRL-3 siRNA (1 and 2 μg) or Luc-siRNA (2 μg) for 24 h, were trypsinized and fixed in 75% ethanol at 4°C for 2 h. Cells were stained with 50 μg/mL propidium iodide (Sigma) and assayed by flow cytometry (Becton Dickinson, San Jose, CA, USA).

Cell Adhesion Assay

The cell adhesion assay was performed essentially as described (21) with some modifications. In brief, 96-well flat-bottom plates were coated with 50 μL fibronectin (10 μg/mL; Sigma) or laminin (10 μg/mL; Calbiochem) in PBS overnight at 4°C and blocked with 0.2% BSA for 2 h at room temperature followed by washing three times. Next, B16-BL6 cells, either untreated or transiently transfected with Luc-siRNA (2 μg) and PRL-3 siRNA (1 and 2 μg) for 24 h, were added to each well in triplicate and incubated for 30 min at 37°C. Plates were then washed three times with PBS to remove unbound cells. Cells remaining attached to the plates were fixed and stained with a solution containing 0.5% crystal violet and 2% ethanol in 100 mM borate buffer (pH 9.0). After washing, 100 μL SDS (1% wt/vol) was added, and the absorbance of the color substrate was measured with an ELISA reader (TECAN, Austria) at 592 nm. After subtraction of the background cell binding to BSA-coated wells, the percentage of adherent cells was calculated by dividing the optical density of the adherent cells by that of the initial input cells.

Cell Migration Assay and Invasion Assay

Cell migration and invasion assays were performed using 8.0-μm pore size Transwell inserts (Costar, Cambridge, MA, USA) as described (10) with some modifications. For all migration essay, in brief, the undersurface of the membrane was coated with fibronectin (10 μg/mL) in PBS at 37°C for 2 h. The membrane was washed in PBS to remove excess ligand, and the lower chamber was filled with 0.6 mL DMEM with 10% FBS. Cells were serum-starved overnight (0.5% FBS), harvested with trypsin/EDTA, and washed twice with serum-free DMEM. Then, cells were resuspended in migration medium (DMEM with 0.5% FBS), and 1×10^5 cells in 0.1 mL were added to the upper chamber. After 24 h at 37°C, the cells on the

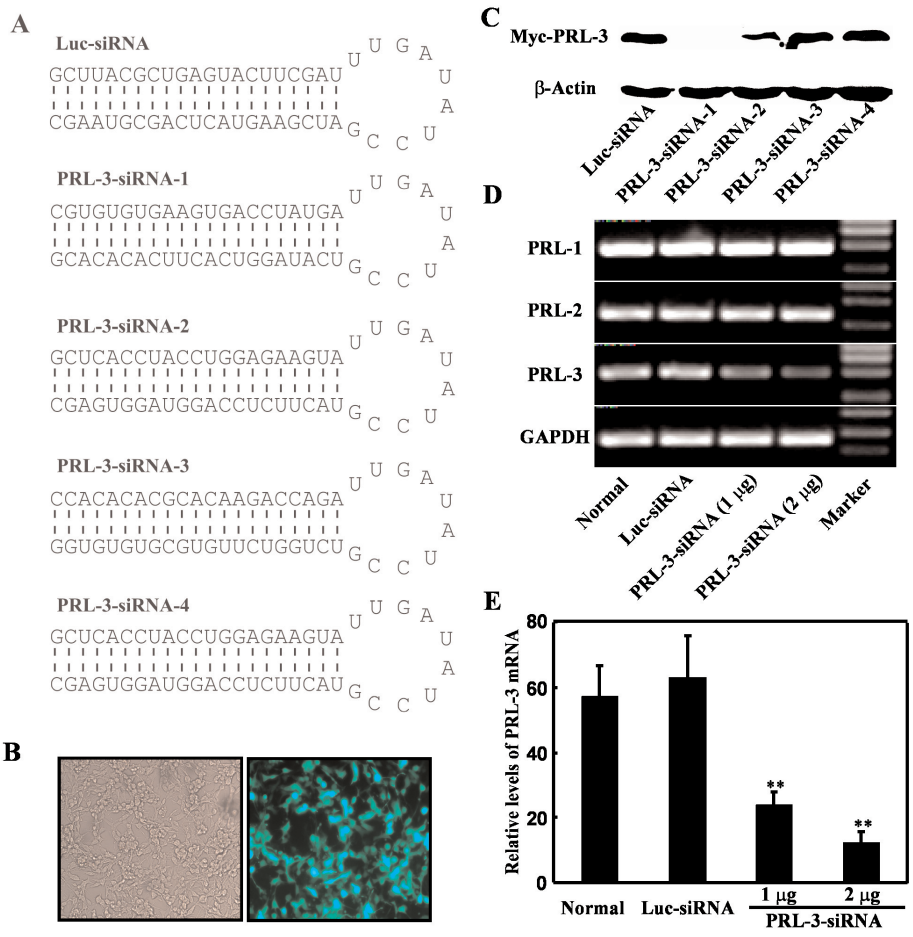

Figure 1. Effect of PRL-3 siRNA on PRL-3 expression. (A) The secondary hairpin structure of pRNA-U6.1/Neo-PRL-3 siRNA transcripts. (B) Photographs of transfection efficiency of B16-BL6 cells. B16-BL6 cells were transfected with pEGFP-N1 for 24 h and then photographed under fluorescent microscopy (right) and light microscopy (left). (C) Expression of Myc-PRL-3 protein was examined by Western blot in 293T cells cotransfected with Myc-PRL3 expression vector (pTARGET) and Luc siRNA, PRL-3 siRNA-1, -2, -3, or -4 RNAi vector (pRNA-U6.1/Neo). (D) Effect of PRL-3 siRNA on endogenous PRL-1, PRL-2, and PRL-3 expression in B16-BL6 melanoma cells. B16-BL6 cells were untreated or transfected with Luc siRNA and PRL-3 siRNA-1 (1 and 2 μg). After treatment at 37°C for 24 h, PRL-1, PRL-2, and PRL-3 mRNAs were studied by RT-PCR; levels of GAPDH expression are shown as an internal control. (E) Real-time quantitative PCR evaluation of PRL-3 siRNA's effect on endogenous PRL-3 expression in B16-BL6 melanoma cells. Data are mean ± SEM of three independent experiments, and each experiment includes triplicate sets. $^{**}P < 0.01$ vs. Luc siRNA control.

upper surface of the membrane were removed using cotton swabs. The migrant cells attached to the lower surface were fixed in methanol at room temperature for 30 min and stained for 20 min with a solution containing 0.5% crystal violet and 2% ethanol in 100 mM borate buffer (pH 9.0). The number of migrated cells on the lower surface of the membrane was counted under a microscope in five fields at ×100. For cell invasion assay, all procedures were carried out as in the migration assay except that Matrigel was coated beforehand on the upper surface of the chambers (BD Biosciences) according to the manufacturer's protocol.

In Vivo Metastasis Assay

B16-BL6 cells in exponential growth phase were harvested by trypsinization and washed twice before injection. Cell vitality was >95% as determined by trypan blue dye exclusion. B16-BL6 cells (5×10^4 cells in 20 μL PBS) were injected into the right hind footpads of C57BL/6 mice (100% of injected mice formed tumors). Ten days after injection, the mice were distributed into four groups with five mice each according to tumor size. PRL-3 siRNA (3 or 6 μg) and Luc-siRNA (6 μg) with PEI complexes (30 μL in each mouse) or PBS were injected into tumors four times every 4 days. Tumor volumes were measured every 4 days from day 10 to 22 and calculated by the following formula: $0.5236 \times L1 \times (L2)^2$, where L1 is the long axis and L2 is the short axis of the tumor. Twenty-six days later, mice were killed. The right footpads and draining popliteal lymph nodes were resected, and photos were taken (Nikon Coolpix 4500). Survival tests were made using groups of mice ($n = 10$) treated as above and monitored daily until all the mice died.

Statistical Analysis

Data are expressed as mean ± SEM. Student's t test was used to evaluate the difference between two groups. Kaplan-Meier method was used to evaluate the survival test. $P < 0.05$ was considered to be significant.

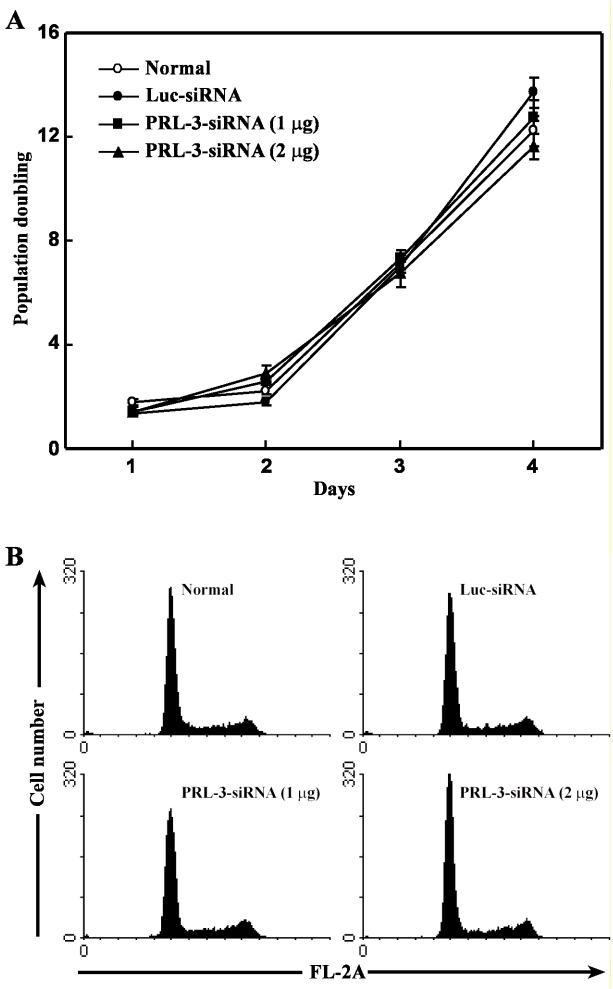

Figure 2. Effect of PRL-3 siRNA on the proliferation of B16-BL6 melanoma cells. B16-BL6 cells were untreated or transfected with Luc siRNA or PRL-3 siRNA (1 and 2 μg) for 24 h. (A) Cell proliferation: 2×10^4 cells per well were seeded in 24-well plates. Cells were trypsinized and counted to quantify cell proliferation. Data are mean ± SEM. (B) Cell cycle analysis after PRL-3 knockdown.

RESULTS

Identification of an Efficient and Specific PRL-3 siRNA Sequence against the Expression of PRL-3

Four PRL-3 siRNA expression plasmids (PRL-3 siRNA-1, -2, -3, and -4) targeting different regions of mouse PRL-3 mRNA and firefly luciferase siRNA (Luc siRNA) as an independent control were constructed using the pRNA-U6.1/Neo vector (Figure 1A). To test the efficiency of the selected sequences, we cotransfected 293T cells with Myc-tagged PRL-3 expression vector (pTARGET-Myc-PRL-3) and PRL-3 siRNA expression plasmids. Cells were lysed 24 h after transfection, and protein extracts were analyzed with anti-Myc antibodies by Western blot. As shown in Figure 1B, PRL-3 siRNA-1 and -2 significantly downregulated the expression of Myc-PRL-3, whereas such an effect was not apparent with PRL-3 siRNA-3 and -4, compared with the control Luc siRNA. Considering the remarkable silencing effect of PRL-3 siRNA-1, we used it for the

following experiments. Because PRL-1, -2, and -3 share at least 75% sequence similarity, the specific inhibitory effect on PRL-3 was tested by RT-PCR. With treatment of PRL-3 siRNA-1, the mRNA level of endogenous PRL-3 in B16-BL6 cells decreased in a dose-dependent manner, whereas that of endogenous PRL-1 and -2 were not influenced (Figure 1C).

No Effect of PRL-3 siRNA on the Proliferation of B16-BL6 Cells In Vitro

B16-BL6 cells were transfected with PRL-3 siRNA or Luc siRNA as control to investigate whether PRL-3 affected the tumor cell proliferation in vitro. We counted the cell number over a 4-day interval. The results shown in Figure 2A and B indicate that downregulation of PRL-3 does not affect the proliferation and cell cycle of B16-BL6 cells.

Inhibition of Adhesion, Migration, and Invasion of B16-BL6 Cells by PRL-3 siRNA

B16-BL6 cells were transfected with PRL-3 siRNA for 24 h, and their capabilities of adhesion, migration, and invasion were analyzed. As shown in Figure 3A and B, after 30-min incubation, compared with untreated cells, B16-BL6 cells transfected with PRL-3 siRNA (1 or 2 μg) exhibited a significant decrease in adhesion capability to fibronectin and laminin, by 38% and 55% for fibronectin and 29% and 45% for laminin, respectively. On the contrary, Luc siRNA had little effect on cell adhesion to both fibronectin and laminin (Figure 3). Similar to the results in the adhesion assay, cells migrating to the undersurface in the migration assay were reduced by 53% and 68%, and those traversing the Matrigel in the invasion assay were reduced by 38% and 60% in B16-BL6 cells transfected with PRL-3 siRNA (1 or 2 μg), respectively, after 24 h compared with untreated cells (Figure 4A and B). Luc siRNA did not have such significant effects.

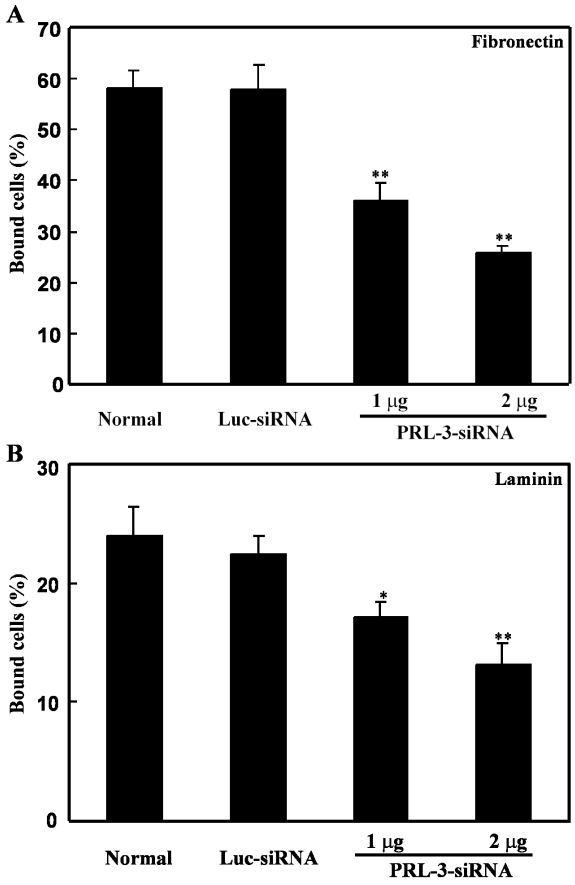

Figure 3. Effect of PRL-3 siRNA on adhesion of B16-BL6 melanoma cells to fibronectin (A) and laminin (B). B16-BL6 cells untreated or transfected with Luc siRNA or PRL-3 siRNA (1 and 2 μg) for 24 h were added to 96-well plates coated with fibronectin (10 μg/mL) or laminin (10 μg/mL). After incubation at 37°C for 30 min, nonadherent cells were gently washed off, and the amount of the adherent cells was determined at 592 nm after crystal violet staining. Data are mean ± SEM of three independent experiments, and each experiment includes triplicate sets. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Luc siRNA control.

Inhibition of Tumor Growth and Spontaneous Metastasis by Intratumoral Injection of PRL-3 siRNA

With the above findings of PRL-3 siRNA's effects in vitro, we next investigated whether PRL-3 plays a critical role in tumor formation in vivo, and whether it can be used in clinical gene therapy. B16-BL6 cells were subcutaneously injected into footpads of C57BL/6J mice. Ten days after injection, PBS, PEI/Luc siRNA, or PEI/PRL-3 siRNA (3 or 6 μg) was injected into the tumors four times every 4 days. As shown in Figure 5B, compared with PBS, PRL-3 siRNA significantly inhibited tumor growth in mice, especially at a higher dose (6 μg), whereas Luc-siRNA did not influence tumor growth. After 26 days, one of five mice died in each group treated with PBS, Luc-RNAi, or PRL-3 siRNA (3 μg). The mice were killed and the footpads inoculated with B16-BL6 cells were collected and photographed (Figure 5A). Strikingly, the popliteal lymph nodes from PBS- or Luc siRNA–treated groups showed high ratios of visible metastases, with about 75% for both PBS and Luc siRNA groups. In contrast, no metastasis was observed in the draining lymph nodes of PRL siRNA (3 and 6 μg) groups (Figure 5C). There were no detectable

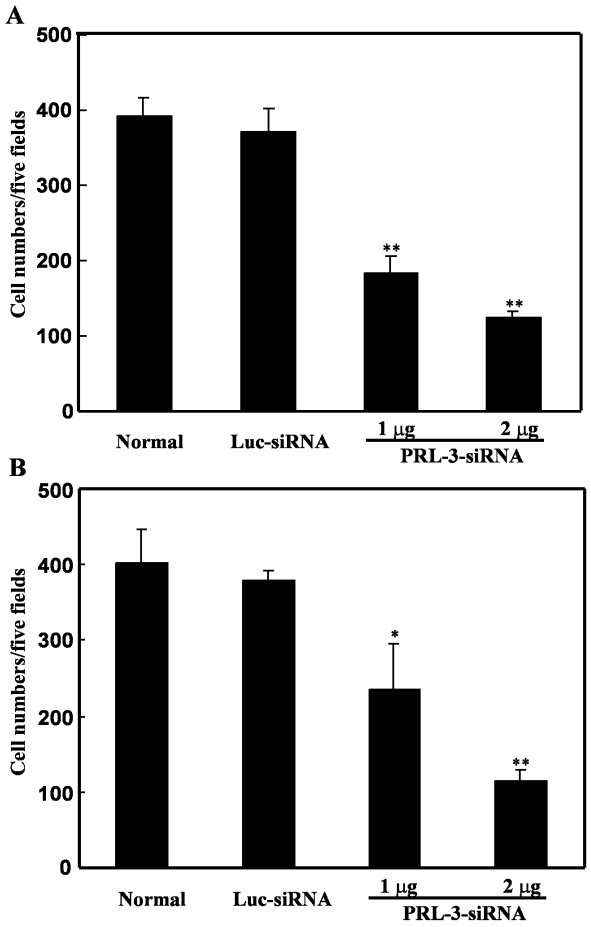

Figure 4. Effect of PRL-3 siRNA on migration and invasion of B16-BL6 melanoma cells. In both assays, B16-BL6 cells were untreated or transfected with Luc-siRNA or PRL-3 siRNA (1 and 2 μg) for 24 h. Each cell suspension (100 μL, 1×10^6 cells/mL) was plated into the upper wells of Transwell inserts containing 8-μm pore polycarbonate membranes precoated with fibronectin (10 μg/mL) on the undersurface or also with Matrigel on the upper surface in the invasion assay. Cells were allowed to migrate for 24 h at 37°C, then those on the upper wells were removed gently and those that migrated to the undersurface were fixed and stained. (A) Quantitative analysis of the number of the cells that migrated to the lower side of the membrane in the migration assay. (B) Histogram of the invasion assay performed with B16-BL6 cells and the relative transfectants. Data are mean ± SEM of three independent experiments. $^{**}P < 0.01$ vs. Luc siRNA control.

metastases in lungs and livers of all the groups (data not shown). Figure 5D shows the survival time of the mice. Downregulation of PRL-3 by PRL-3 siRNA (3 and 6 μg) prolonged the lifespan of mice bearing B16-BL6 tumor cells in a dose-dependent manner, whereas Luc-siRNA treatment showed no such effect.

DISCUSSION

Recent studies have indicated that PRL-3 plays a causative role in malignant transformation and metastasis, and inhibition of PRL-3 expression may provide an effective means for inhibiting tumor formation and metastasis and prolonging survival. Our previous study also indicated that highly metastatic B16-BL6 melanoma cells express more elevated PRL-3 than less metastatic B16 cells (10). In this study, we took B16-BL6 cells to establish a metastatic model and investigated its characteristics against vector-based siRNA, which was used to directly knock down the endogenous expression of PRL-3 in B16-BL6 cells (Figure 1). Although PRL-1 and PRL-2, which share at least 75% homologous protein sequence with PRL-3 (22,23), were also detected in B16-BL6 cells, PRL-3 siRNA treatment only specifically reduced the endogenous expression of PRL-3 in B16-BL6 cells, with no influence on the expression of PRL-1 and PRL-2 (Figure 1C). Nevertheless, some reports have shown that overexpression of PRL-1 and PRL-2 prompts the transformation of epithelial cells and enhances the metastatic ability of cancer cells (8), and results that demonstrate the role of PRL-3 in various tumor metastases are accumulating. Recently, several studies using in vitro assays showed that reduced PRL-3 expression inhibited tumor growth, invasion, and metastasis in human colorectal cancer cells and ovarian cancer cells (8,9,12). In addition, Saha et al. (24) have compared the global gene expression profile of metastatic colorectal cancers with that of primary tumors, benign tumors, and normal colorectal epithelium and found that PRL-3 is the only gene highly expressed in all 18 metastases examined. Overall, this evidence suggests that PRL-3 may be a potential therapeutic target for the treatment of malignant tumor.

Tumor metastasis is a complex process with prominent stages as follows: detachment of cancer cells from a primary tumor, entry into the circulatory system, and adherence and migration of the tumor cells to distant sites. The first stage of tumor cell detachment from the original site depends on adhesiveness to extracellular matrix and migration to blood capillaries and lymphatic vessels (25). Therefore, we detected these capabilities of PRL-3 in B16-BL6 cells. Although knockdown of PRL-3 by specific siRNA had little influence on the proliferation and cell cycle of B16-BL6 cells in vitro (Figure 2), it did inhibit B16-BL6 cells' adhesive ability to fibronectin and

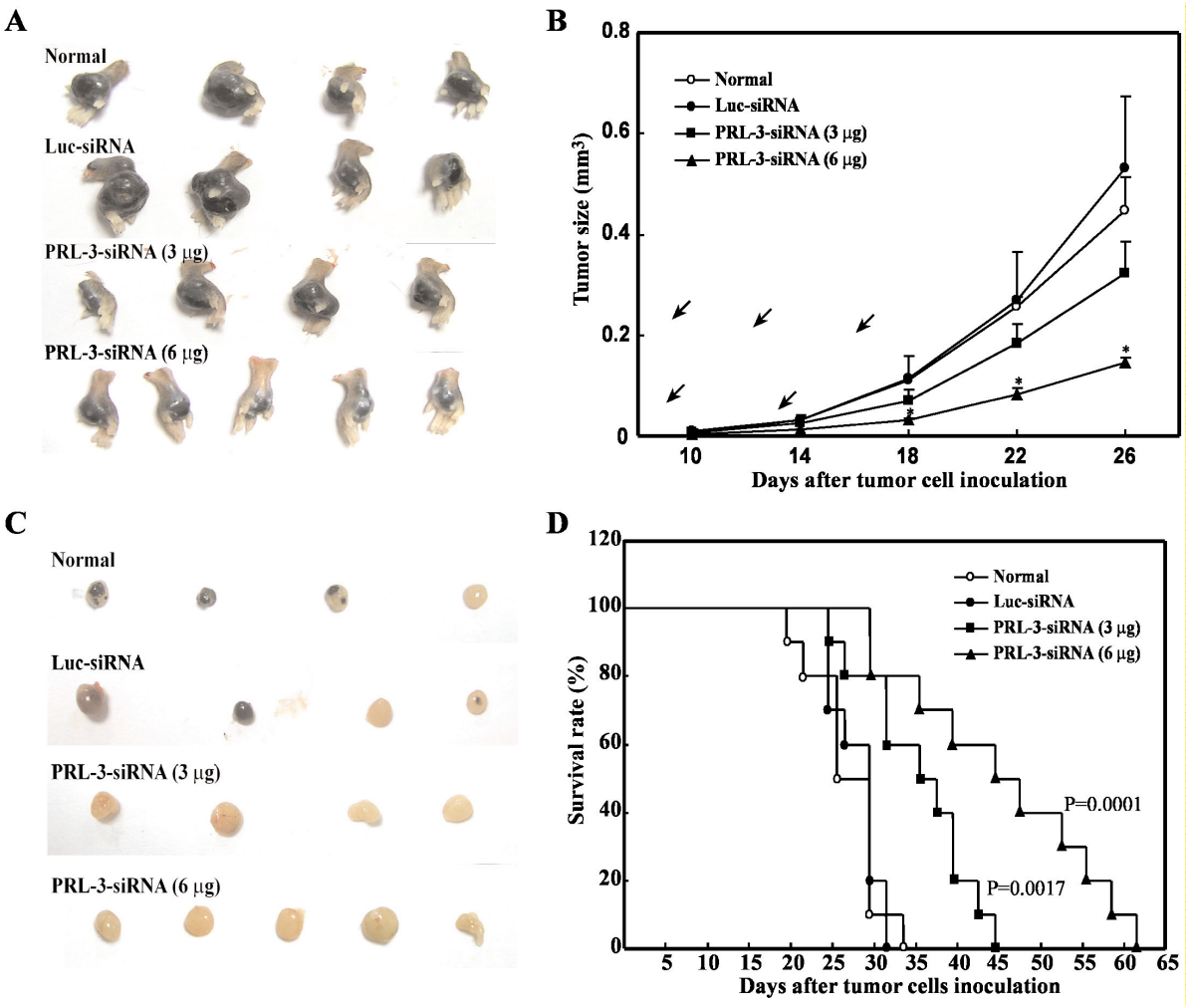

Figure 5. Effect of PRL-3 siRNA on the spontaneous metastasis of B16-BL6 melanoma cells in vivo. B16-BL6 cells (20 μL, 2.5 × 10^6 cells/mL) were injected subcutaneously into right footpads of C57BL/6J mice (100% of injected mice formed tumors). Ten days after injection, the mice were distributed into four groups according to tumor size. The tumors were injected with PBS, Luc siRNA, and PRL-3 siRNA (3 or 6 μg) four times every 4 days. (A) After 26 days, mice were killed, and the footpads inoculated with B16-BL6 cells were resected. (B) Time course of tumor growth. Tumor volumes were measured every 4 days from day 10 to 26 after inoculation. Data are mean ± SEM of five mice in each group. A significant difference was shown compared with the PBS control group. *$P < 0.05$ vs. Luc siRNA control. (C) Draining popliteal lymph node from injected footpads. The arrow indicates the spontaneous metastatic B16-BL6 tumor. (D) Survival curve of mice ($n = 10$) treated with PBS, Luc-siRNA, or PRL-3 siRNA (3 or 6 μg). Results were evaluated by the Kaplan-Meier method. The differences between the Luc-siRNA control group and the two treatment groups are statistically significant, *$P < 0.01$.

laminin, and also their migration and invasion ability under the Transwell-based assay (Figure 3 and 4). The adhesion, migration, and invasion ability of tumor cells may be regulated by multiple signaling cascades, including integrin-mediated signaling, mitogen-activated protein kinase signaling, and cytoskeletal reorganization (26,27). This hypothesis was recently substantiated by the fact that PRL-3 directly interacted with integrin α1, decreased the tyrosine phosphorylation of integrin β1, and activated the Erk1/2 signal pathway (28). These findings further confirm that PRL-3 plays a key role in tumor cell acquisition of metastatic potential.

On the other hand, although previous studies suggest that PRL-3 regulates tumor metastasis in vivo, the function of PRL-3 in the whole process of metastasis is not totally clear. Experimental passive models, which mimic the metastatic events after tumor cells enter the circulatory system by injecting tumor cells to tail veins, have been used to investigate the function of PRL-3 in vivo (10,11), but PRL-3's part in cancer cell detachment from the original site, such as adhesion to the extracellular matrix, digestion of the matrix, and intravasation, has not been explored. Our previous study (10) indicated that PRL-3 transfectants could

attach well to fibronectin, a common matrix for adhesion and migration assays. The present work reconfirmed those observations and showed that PRL-3 siRNA could inhibit the adhesion, migration, and invasion ability of B16-BL6 cells in vitro (Figures 3 and 4). With these results, we further examined whether PRL-3 siRNA can suppress the metastasis of B16-BL6 cells in vivo and prolong lifespan. B16-BL6 cells were implanted into mouse footpads to form primary tumors. Ten days later, intratumoral injections with specific siRNA were carried out to observe that parental B16-BL6 cells with endogenous expression of PRL-3 grow more rapidly than PRL-3 knockdown cells (Figure 5B). In addition, treatment with PRL-3 siRNA remarkably reduced the metastatic ability of B16-BL6 cells to popliteal lymph nodes, where no visible metastatic sites were detected in that group (Figure 5C). Furthermore, all mice treated with PBS or Luc siRNA vector died within 32 days; animals with 6 μg PRL-3 siRNA treatment survived through 60 days (Figure 5D). These results suggest that the in vivo proliferation of B16-BL6 cells in the primary tumor site, the development of macroscopic metastases, and the survival of mice implanted with B16-BL6 cells were highly dependent on the expression level of PRL-3. Untreated B16-BL6 cells with normal endogenous expression of PRL-3 showed more apparent metastasis to draining lymph nodes than PRL-3 siRNA–treated cells (Figure 5C), whereas there were no metastatic sites visible to the lung or liver (data not shown).

Our data extend observations of PRL-3 having substantial function in spontaneous metastasis in vivo. Although this result was consistent with the reports that PRL-3 promoted metastasis in mice models in vivo (11), we found that endogenous PRL-3 tended to initiate spontaneous local lymph metastasis through lymphatic circulation but not lung or liver metastasis through blood circulation. Actually, the lymphatic system is optimally suited for the entry and transportation of tumor cells, as the smallest lymphatic vessels are much larger than blood capillaries (29). When tumor cells released from the primary tumor mass, it is easier for them to enter lymphatic vessels than the blood system. Data from clinic metastatic samples also support this hypothesis. Bardelli et al. (13) have found that PRL-3 is detected in all colorectal metastasis to the lymph nodes. These data indicate that PRL-3 plays a critical role in regional lymph node metastasis. At the mechanistic level, lymph node metastasis is closely associated with lymphangiogenesis, which facilitates the dissemination of tumor cells to transfer to regional lymph nodes through the lymphatic drainage network. Further studies will be necessary to uncover whether PRL-3 regulates the expression of these factors in the process of lymph node metastasis.

In this study, B16-BL6 cells treated with PRL-3 siRNA showed slow growth compared with those treated with Luc siRNA in primary tumor in vivo (Figure 5). This result contrasted with observations of cell growth under in vitro culture conditions, in which the level of PRL-3 did not affect proliferation rate (Figure 2). Similar effects of PRL-3 on cell growth have also been reported for human colon cancer DLD-1 cells in vitro, whose endogenous PRL-3 was abrogated by PRL-3 siRNA (9). These data indicate that the promotion of tumor growth by PRL-3 in vivo is associated with tumor microenvironment, which can provide many survival and growth factors for tumor cells. This is further supported by a recent report that PRL-3 enhanced the activation of Rho family GTPases RhoA and RhoB, following the initiation of the Rho/ROCK signal pathway necessary for invasion and motility (30). In addition, this signal pathway also facilitates angiogenesis by inducing the expression of VEGF (31). Pille et al. (32) have reported that the angiogenesis of breast cancer, treated with RhoA-siRNA, was remarkably reduced and tumor growth was also inhibited. These data suggest that the promotion of B16-BL6 cell proliferation by PRL-3 in vivo may also be associated with angiogenesis.

In conclusion, our present work reproduced the role of PRL-3 in the whole process of spontaneous metastasis from primary tumor to distant site. We also found that specific reduced expression of PRL-3 inhibited the growth of B16-BL6 cells in vivo and prevented detectable metastasis from primary tumors by decreasing adhesive ability to extracellular matrix such as fibronectin and laminin, and by reducing the migratory ability of B16-BL6 cells. PRL-1 and PRL-2 have roles in cancer cells (9), but we found, at least in B16-BL6 cells, that PRL-3 played a more pivotal role in tumor formation and metastasis. The current results extend potential therapeutic applications of PRL-3 inhibitors to the clinical treatment of malignant tumor.

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