

PRL-3 promotes breast cancer progression by downregulating p14^{ARF}-mediated p53 expression

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Abstract. Prior studies have demonstrated that phosphatase of regenerating liver-3 (PRL-3) serves avital function in cell proliferation and metastasis in breast cancer. However, the molecular mechanisms underlying the function of PRL-3 in breast cancer remain unknown. PRL-3 expression was analyzed in 24 pairs of breast cancer and normal tissues using the reverse transcription-quantitative polymerase chain reaction assay. The results of the present study identified that the expression of PLR-3 in breast cancer tissues was increased 4.2-fold, compared with normal tissues. Notably, overexpression of PRL-3 significantly promoted the proliferation of cancer cells and inhibited endogenous p53 expression by downregulating the expression level of p14 alternate reading frame (p14^{ARF}). In addition, decreased expression levels of PRL-3 resulted in decreased breast cancer cell proliferation and increased expression level of p14^{ARF}. These results suggested that PRL-3 enhances cell proliferation by downregulating p14^{ARF} expression, which results in decreased levels of p53. The results of the present study demonstrated that PRL-3 promotes tumor proliferation by affecting the p14^{ARF}-p53 axis, and that it may serve as a prognostic marker for patients with breast cancer.

Introduction

Breast cancer is the most common type of invasive cancer in females worldwide (1). Despite diverse screening programs and novel therapeutic strategies implemented to markedly decrease mortality rates, the physiological mechanism underlying the pathogenesis of breast cancer remains unknown (2). Therefore, the identification of biomarkers is required to improve the early diagnosis of breast cancer and decrease cancer mortality rates.

Phosphatase of regenerating liver-3 (PRL-3), also known as PTP4A3, is a member of the protein tyrosine phosphatase superfamily (3). Protein tyrosine phosphatases exert crucial functions in modulating cellular processes, including cell growth, cell cycle progression and apoptosis, by regulating a number of proteins (4,5). PRL-3 is associated with tumor proliferation and metastasis, and is overexpressed in various types of cancer, including colorectal (6) and gastric cancer (7,8), ovarian carcinoma (9,10), multiple myeloma (11) and squamous cell carcinoma of the uterine cervix (12). Wang *et al* (13) demonstrated that PRL-3 was overexpressed in breast cancer and predicts a poor clinical outcome for patients. In the present study, PRL-3 was identified to function in tumor proliferation by acting on the p14^{ARF}-p53 axis, suggesting that PRL-3 may be involved in the tumorigenesis of breast cancer.

Materials and methods

Tissues specimens and cell lines. A total of 24 breast cancer and adjacent normal tissues were obtained from the Sichuan Cancer Hospital (Chengdu, China). The tissues from each individual were frozen in liquid nitrogen and stored at -80°C until use. The tissues were fixed with 10% formalin overnight at room temperature and the fixed-tissues were embedded in paraffin. The 4- μ m-thick sections of the tissues were validated using H&E staining and immunohistochemical detection. Briefly, sections were stained for nuclei with hematoxylin for 5 min at room temperature, and then sections were washed with tap water and counterstained with eosin for 1 min and washed. The following primary antibodies were used to perform immunohistochemistry: anti-estrogen receptor (cat. no. ab180900; dilution, 1:200; Abcam, Cambridge, UK), anti-progesterone receptor (cat. no. ab32085; dilution, 1:100; Abcam) and anti-HER2 (cat. no. ab194979; dilution, 1:200; Abcam). Briefly, sections were blocked using 2% PBS + bovine serum albumin for 15 min at room temperature. Subsequently, primary antibody incubation was performed overnight at 4°C. Next, a biotinylated anti-rabbit immunoglobulin (Ig)G (cat. no. ab150088; dilution, 1:2,000; Abcam) was used as a secondary antibody. The antibody staining in the tissue sections was observed using a light microscope (magnification, x40). Written informed consent was obtained from all patients and all protocols were approved by the Sichuan Cancer Hospital Ethics Committee (Chengdu, China).

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Two breast cancer cell lines (MDA-MB-231 and MCF-7) were purchased from the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) at 37°C in a humidified chamber containing 5% CO₂.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol[®] reagent (Invitrogen, Thermo Fisher Scientific, Inc.) was used to extract total RNA from the breast cancer tissues or two breast cancer cell lines (MDA-MB-231 and MCF-7) and subsequently reverse-transcribed into cDNA using a First Strand cDNA Synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol with the following temperature protocol: -25°C for 10 min and then at 42°C for 60 min, followed by 99°C for 5 min and then cooling to 4°C for 5 min. Subsequently, RT-qPCR was performed on the ABI 7500 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with FastStart[™] Universal SYBR[®] Green Master (ROX) reagents (Roche Diagnostics GmbH). The temperature protocols for the qPCRs were as follows: 95°C for 10 min, 40 cycles of 95°C for 30 sec (inactivation) and 56°C for 1 min (annealing). A melt curve was constructed following each reaction and data were analyzed using a 7500 Fast System SDS (version 1.4.0.25; Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences for PRL-3 were as follows: Forward, 5'-CTTCCTCATCACCACAACC-3'; reverse, 5'-GTCTTGTGCGTGTGTGGGTC-3'. The primer sequences for p14^{ARF} were as follows: Forward, 5'-GCCACATTCGCTAAGTGCTC-3' and reverse, 5'-GCCACATTCGCTAAGTGCTC-3'. The primer sequences for GAPDH were as follows: Forward, 5'-AACGACCCCTTCATTGAC-3' and reverse, 5'-TCCACGACATACTCA GCAC-3'. All reactions were conducted in triplicate. GAPDH was used as the normalization control and the relative levels were quantified using the 2^{-ΔΔC_q} method (14).

Plasmid construction and transfection. Total RNA was extracted from the MDA-MB-231 cells using TRIzol buffer (Invitrogen; Thermo Fisher Scientific, Inc.). Then the reverse transcription was performed using a Transcript First Strand cDNA Synthesis kit (Roche Diagnostics GmbH). The cDNA of PRL-3 was amplified by PCR using the following primers: Forward, 5'-TACCGACTCAGATCTCGAGCGCCACC ATGGCTCGGATGAACCGC-3' and reverse, 5'-GATCCC GGGCCCGCGGTACCGTCATAACGCAGCACCGGG TCT-3'. The reverse transcription temperature protocol used was as follows: 42°C for 60 min and 70°C for 5 min. The PCR program for the amplification of cDNA was started at 94°C for 5 min, followed by 40 cycles at 94°C for 30 sec, 58°C for 30 sec, 72°C for 5 min and completed with a final extension at 72°C for 5 min. The amplified product and pcDNA3 vector (Invitrogen, Thermo Fisher Scientific, Inc.) were digested with *EcoRI* and *XhoI* enzymes to construct pcDNA3/PRL-3. The short hairpin (sh)RNAs against PRL-3 (target sequence, 5'-AAA TCTCGTTTCTCTTGGACA-3') and p14^{ARF} (target sequence, 5'-GAACAUGGUGCGCAGGUUCTT-3') were annealed and cloned into the *BamHI* and *HindIII* restriction sites of the

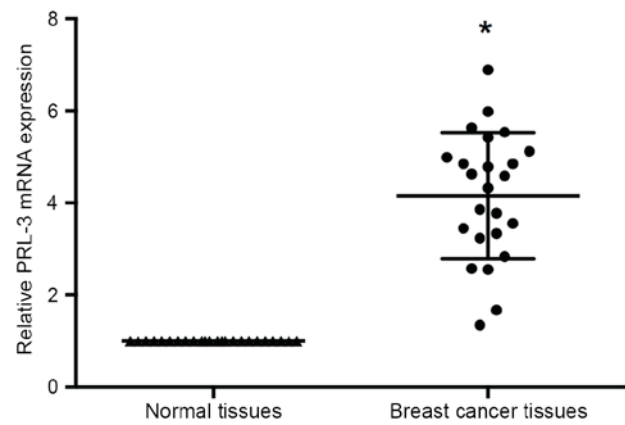


Figure 1. PRL-3 expression in breast cancer and adjacent normal tissues. The results of quantitative polymerase chain reaction indicate that the expression of PRL3 is increased in breast cancer tissues compared with adjacent normal tissues. Data are presented as the mean \pm standard deviation from triplicate procedures; * $P < 0.05$. PRL-3, phosphatase of regenerating liver-3.

pSilencer vector (Ambion; Thermo Fisher Scientific, Inc.) to construct pSilencer/ShR-PRL-3, and pSilencer/ShR-p14^{ARF}. Empty vectors with a scramble shRNA sequence were used as a negative controls (pSilencer/NC). The shRNA sequences were as follows: PRL-3shRNA sense, 5'-ACAAACACATGC GCTTCCTCA-3' and antisense, 5'-TGAGGAAGCGCATGT GTTTGT-3'; p14^{ARF}shRNA sense, 5'-CCGATTGAAAGA ACCAGAGAG-3' and antisense, 5'-CTCTCTGGTTCTTTC AATCGG-3'; and scrambled shRNA (pSilencer/NC) sense, 5'-UAAUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'. The transfection was performed with Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Briefly, MDA-MB-231 or MCF-7 cells were seeded into 12-well plates (3x10⁵ or 4x10⁵ cells/well) the day prior to transfection and then transfected with 100 nM constructed pcDNA3/PRL-3, pcDNA3, pSilencer/ShR-PRL-3, pSilencer negative control (pSilencer/NC) and pSilencer/ShR-p14^{ARF}. At 48 h after transfection, the mRNA/protein were collected and subject to RT-qPCR (as aforementioned) and western blot analysis.

Western blot analysis. Proteins were extracted from the cell lines using Radio-immunoprecipitation Assay lysis buffer (Thermo Fisher Scientific, Inc.) for 20 min at 4°C with occasional agitation. Equal amounts of protein extracts (determined using the BCA method) were subjected to 10% SDS-PAGE and subsequently transferred to a polyvinylidene fluoride membrane (0.45 μ m pore size; EMD Millipore, Billerica, MA, USA). Followed by blocking with 5% skimmed milk for 1.5 h at room temperature, the membrane was incubated with primary antibodies anti-PRL-3 (dilution, 1:500; cat. no. ab50276; Abcam), anti-p53 (dilution, 1:500; cat. no. ab31333; Abcam), anti-p14^{ARF} (dilution, 1:500; cat. no. ab3642; Abcam) and anti-GAPDH antibody (dilution, 1:500; cat. no. SAB4300645-100UG; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 4°C overnight and rinsed twice with PBS, followed by incubation with peroxidase-conjugated secondary anti-rabbit (dilution, 1:2,000; cat. no. ab6721; Abcam) or anti-mouse IgG antibodies (dilution, 1:2,000; cat. no. ab6709; Abcam) for 1 h at room

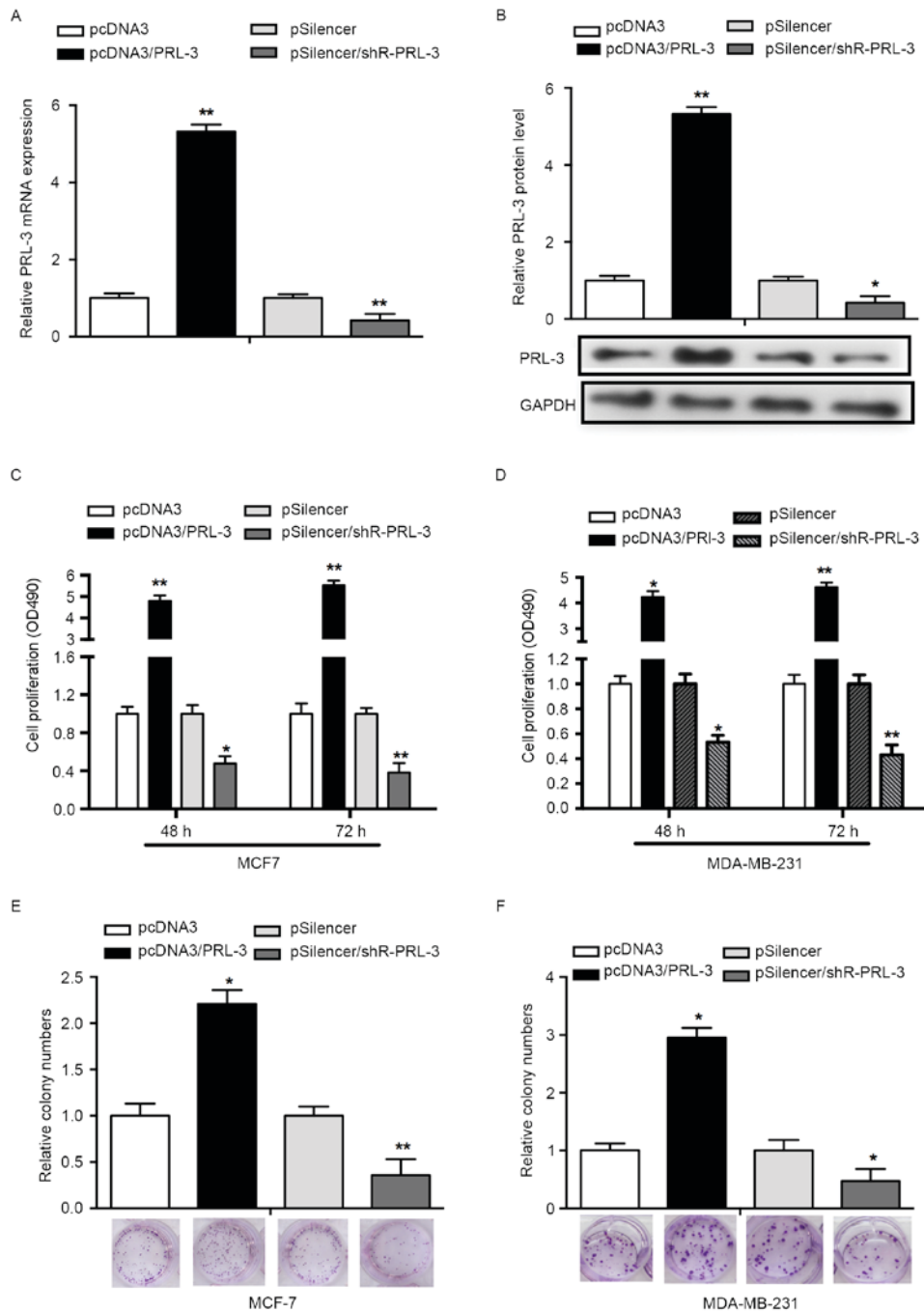


Figure 2. Function of PRL-3 in two breast cancer cell lines. MCF-7 and MDA-MB-231 cells were transfected with pcDNA3/PRL3 or pcDNA3/shR-PRL3 and their corresponding controls. (A) The PRL-3 level was determined using quantitative polymerase chain reaction. (B) Western blot analysis of PRL-3 protein following transfection with pcDNA3/PRL3 or pcDNA3/shR-PRL3 and their corresponding controls. An MTS assay was performed to determine the proliferation of (C) MCF-7 and (D) MDA-MB-231 cells. A colony formation assay was performed to determine the proliferation of (E) MCF-7 and (F) MDA-MB-231 cells transfected with pcDNA3/PRL3 or pcDNA3/shR-PRL3 and their corresponding controls. The images of MCF-7 or MDA-MB-231 cell colonies were captured using a camera and GAPDH was used as the loading control for western blot analysis. Data are presented as the mean \pm standard deviation from triplicate procedures. *P<0.05, **P<0.01. PRL-3, phosphatase of regenerating liver-3; shR, short hairpin RNA.

temperature. The membrane was subsequently incubated with an enhanced chemiluminescent substrate kit (Thermo Fisher Scientific, Inc.) and exposed to X-ray film. ImageJ (version 1.41; National Institutes of Health, Bethesda, MD, USA) was used for the quantitative analysis of band densities. GAPDH was used as a control (primers as aforementioned).

Colony formation assay. MDA-MB-231 and MCF-7 cells were seeded into 12-well plates (3×10^5 or 4×10^5 cells/well) the day prior

to transfection and were then transfected with 100 nM constructed pcDNA3/PRL-3, pSilencer/ShR-PRL-3, pSilencer/ShR-p14^{ARF} and corresponding controls (pSilencer/NC). After 2 weeks of culture, 0.2% crystal violet was used to stain the cell colonies at room temperature for 30 min. Subsequently, the colonies were counted using an inverted fluorescence microscope (magnification, x40; IX71; Olympus Corporation, Tokyo, Japan). Each experiment was performed in triplicate (1 colony, >50 cells) and images of MCF-7 or MDA-MB-231 cell colonies were captured.

MTS assay to determine cell proliferation. Transfected MDA-MB-231 and MCF-7 cell lines [(3-5) $\times 10^3$ cells/well] were plated in 96-well plates for 48 and 72 h and subsequently, the proliferation was determined using the CellTiter 96[®] Aqueous cell proliferation assay kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. The absorbance was determined at a wavelength of 490 nm using a Tecan microplate reader (Tecan Group, Ltd., Mannedorf, Switzerland).

Statistical analysis. Experiments were performed in triplicate. The results are presented as the mean \pm standard deviation. Differences between two groups were determined with unpaired Student's t-test using SPSS (version 12.0; SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used for multiple comparisons followed by a Bonferroni Comparison post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

PRL-3 is overexpressed in breast cancer tissue samples. RT-qPCR was performed to investigate the expression manner in the 24 pairs of breast cancer and adjacent non-cancerous tissues. The results of RT-qPCR identified that PRL-3 expression levels were significantly increased in the tumor tissues compared with adjacent normal tissues ($P < 0.05$; Fig. 1).

PRL-3 promotes the proliferation of breast cancer cells. To determine whether PRL-3 affects cell proliferation, an MTS assay was performed using MCF-7 and MDA-MB-231 cells. First, overexpression of PRL-3 was achieved via transfection with pcDNA3/PRL-3, whereas the suppression of PRL-3 was achieved by transfection with pSilencer/PRL-3. RT-qPCR and western blot analysis was used to validate the efficiency of the vectors. The expression of PRL-3 mRNA was significantly increased in the pcDNA3/PRL-3 group while decreased in the pSilencer/PRL-3 group, compared with the control groups ($P < 0.05$; Fig. 2A). The expression of PRL-3 protein was consistent with the expression of PRL-3 mRNA ($P < 0.01$; Fig. 2B). An MTS assay results revealed that PRL-3 overexpression promoted cell proliferation 3.8- and 4.5-fold at 48 and 72 h post-transfection, respectively, compared with NCs. Downregulation of PRL-3 with pSilencer/PRL-3 in MCF-7 cells decreased the rate of cell proliferation by 52 and 68% at 48 and 72 h post-transfection, respectively ($P < 0.05$; Fig. 2C). Concordant with the results observed in the MCF-7 cell line, PRL-3 served a similar role in the MDA-MB-231 cell line ($P < 0.05$; Fig. 2D).

To assess the effects of PRL-3 on cell growth, a colony formation assay was performed. Overexpression of PRL-3 in MCF-7 cells revealed a significant increase in the number of colonies, compared with the control group ($P < 0.05$; Fig. 2E). Knockdown of PRL-3 (pSilencer/shR-PRL-3) in MCF-7 cells resulted in a significant decrease in the number of colonies, compared with the control group (Fig. 2E). In addition, a similar effect was observed in MDA-MB-231 cells ($P < 0.05$; Fig. 2F). The results of the present study indicated that PRL-3 promotes the proliferation of breast cancer cell lines.

PRL-3 negatively regulates p53 and p14^{ARF} protein expression in MCF-7 cells. As previous studies have demonstrated that PRL-3 was involved in the regulation of p53 and p14^{ARF} (14-16), the present study aimed to determine the association between PRL-3, p53 and p14^{ARF} in breast cancer cells. The results of the present study revealed that the overexpression of PRL-3 in MCF-7 cells results in an inhibited p53 expression (48% decrease) compared with the negative control, whereas the inhibition of PRL-3 promoted the level of p53 1.8-fold ($P < 0.05$; Fig. 3A). The effect of PRL-3 on the expression level of p14^{ARF} was investigated. The results demonstrated that p14^{ARF} was downregulated when PRL-3 was overexpressed, whereas inhibition of PRL-3 promoted the level of p14^{ARF} in MCF-7 cell lines ($P < 0.05$; Fig. 3B).

PRL-3 promotes cell proliferation via the p14^{ARF}-p53 axis. An MTS assay was performed to determine whether PRL-3 affected cell proliferation via p14^{ARF}-p53 and the knock-down efficiency of pSilencer/shR-p14^{ARF} was validated in MCF-7 cells. The RT-qPCR results demonstrated that the p14^{ARF} expression levels in the pSilencer/shR-PRL-3 group decreased by $\sim 70\%$ ($P < 0.01$; Fig. 4A). Suppression of p14^{ARF} with pSilencer/shR-p14^{ARF} (pSilencer/shR-p14^{ARF}+pSilencer NC) promoted cell proliferation and inhibition of PRL-3 (pSilencer/shR-PRL-3+ pSilencer NC) lead to suppressed cell proliferation in comparison with 3×10^2 or 4×10^2 cells/well controls (pSilencer NC+ pSilencer NC). The promoted cell proliferation induced by suppression of p14^{ARF} can be rescued by co-transfection with pSilencer/shR-PRL-3 (pSilencer/shR-p14^{ARF}+ pSilencer/shR-PRL-3) (72 h, $P < 0.05$; Fig. 4B). Furthermore, the colony formation assay was used to evaluate the effects of p14^{ARF} and PRL-3 on cell proliferation. MCF-7 cells transfected with pSilencer/shR-p14^{ARF} revealed a significant increase in the number of the colonies, whereas cells transfected with pSilencer/shR-PRL-3 revealed a significant decrease in the number of colonies compared with the control (pSilencer NC+ pSilencer NC). Additionally, promoted cell proliferation induced by the knockdown of p14^{ARF} can be rescued by co-transfection with pSilencer/shR-PRL-3 (pSilencer/shR-p14^{ARF}+ pSilencer/shR-PRL-3) ($P < 0.05$; Fig. 4C).

To determine the function of p53 in PRL-3-induced cell proliferation, p53 protein expression was evaluated in human breast cancer cells. The results revealed that the p53 level was significantly decreased in cells transfected with pSilencer/shR-p14^{ARF} while significantly increased in cells transfected with pSilencer/shR-PRL-3. The decreased expression of p53 induced by pSilencer/shR-p14^{ARF} can be rescued by the knockdown of PRL-3 ($P < 0.05$; Fig. 4D). Therefore, the results of the present study demonstrated that PRL-3 down-regulated p53 by affecting the expression of p14^{ARF} in the progression of breast cancer cell proliferation.

Discussion

PRL-3 has previously been identified as an oncogene (8). The present study hypothesized that PRL-3 participates in p53-dependent cell proliferation. The results of the present study suggested that PRL-3 promotes breast cancer progression via the p14^{ARF}-p53 axis that is concordant with the hypothesis. In

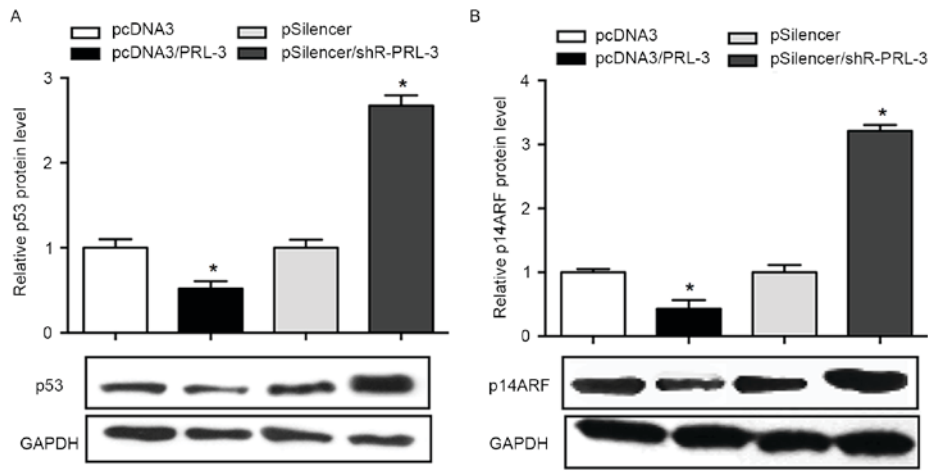


Figure 3. Western blot analysis of p53 and p14^{ARF} proteins in different groups. (A) The expression of p53 was decreased in cells transfected with pcDNA3/PRL3 while the expression of p53 was increased in cells transfected with pSilencer/shR-PRL-3; (B) The expression of p14^{ARF} was decreased in cells transfected with pcDNA3/PRL3 while the expression of p14^{ARF} was increased in cells transfected with pSilencer/shR-PRL-3. GAPDH was used as the loading control. Data are presented as the mean standard deviation from triplicate procedures. *P<0.05. ARF, alternate reading frame; PRL3, phosphatase of regenerating liver-3; shR, short hairpin RNA.

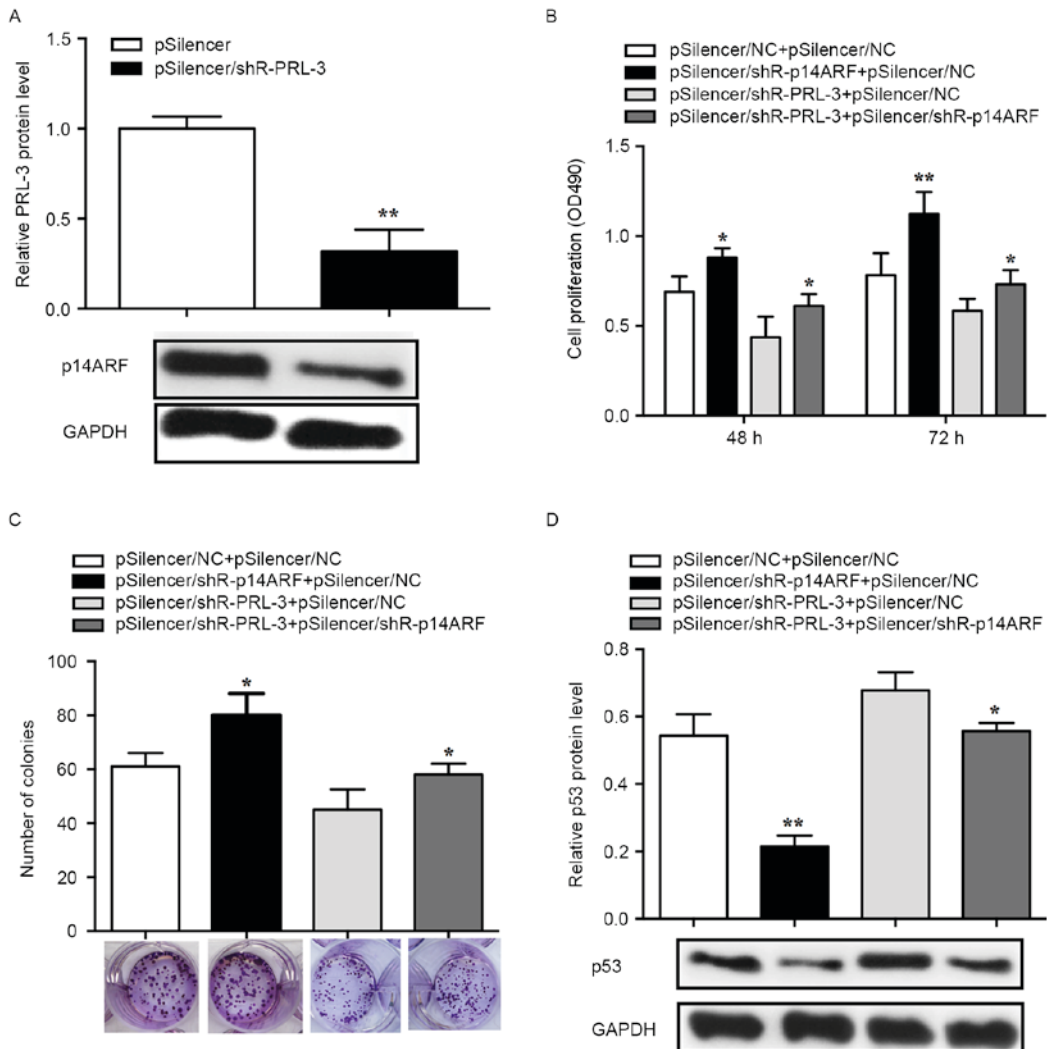


Figure 4. PRL-3 promotes the proliferation of breast cancer cells via the p14^{ARF}-p53 axis. (A) The p14^{ARF} protein level in MCF-7 cells transfected with pcDNA3/shR-p14^{ARF} or the control was determined using western blot analysis. (B) The proliferation of cells transfected with pcDNA3/shR-p14^{ARF} or pcDNA3/shR-PRL3 and their corresponding controls was determined using an MTS assay. (C) Colony formation assay analysis of MCF-7 cells transfected with pcDNA3/shR-p14^{ARF} or pcDNA3/shR-PRL3 and their corresponding controls. The clones were imaged using a camera. (D) The expression levels in cells transfected with pcDNA3/shR-p14^{ARF} or pcDNA3/shR-PRL3 and their corresponding controls. GAPDH was used as the loading control for the western blots. *P<0.05, **P<0.01. ARF, alternate reading frame; PRL-3, phosphatase of regenerating liver-3; shR, short hairpin RNA; NC, negative control.

addition, the results of the present study demonstrated that PRL-3 inhibits p53 and p14^{ARF} protein expression, indicating that PRL-3 may serve a key function in breast cancer progression.

Previous studies have identified that PRL-3 is associated with breast tumor progression (15), which prompted the present study to investigate the function of PRL-3 in breast cancer through examining the mRNA levels of PRL-3 in breast cancer and adjacent normal tissues. The results of the present study revealed that the expression of PRL-3 was increased in breast tumors compared with adjacent normal tissues. p14^{ARF} and p53 protein have been previously identified to function as tumor suppressors (16-18); in the present study, overexpression of PRL-3 resulted in decreased levels of p14^{ARF} and p53 protein in breast cancer cell lines. Studies have demonstrated that p14^{ARF} induces apoptosis to inhibit cancer progression and that it is downregulated during breast cancer progression (19-21). The results of the present study revealed that suppression of p14^{ARF} promotes cell proliferation, which may be rescued by co-transfection with pSilencer/shR-PRL-3. Additionally, p14^{ARF} has been identified to regulate the levels of p53 in breast cancer cells (22,23). In the present study, it was revealed that the suppression of p14^{ARF} inhibits the expression of p53 in breast cancer cell lines. Furthermore, p53 is regulated by the expression of p14^{ARF} (24-26) and PRL-3 (27), via mouse double minute 2 homolog. The results of the present study revealed that PRL-3 decreases the p14^{ARF}-mediated expression of p53 in breast cancer cells. In summary, PRL-3 regulates the level of p14^{ARF} in order to inhibit the expression of p53. These results may indicate an underlying molecular mechanism involved in breast cancer development, and enable the identification of novel therapeutic targets for breast cancer.

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