Gene silencing of *hPRLR* mRNA by RNA interference in human breast cancer cells

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Abstract. Up-regulation of the human prolactin receptor (hPRLR) has been implicated in aberrant signaling that may induce abnormal proliferation of breast epithelium. In this study, we evaluated the inhibition of cell proliferation by small-interfering RNA (siRNA) targeting of *hPRLR*, as well as the anti-tumor efficacy of this treatment on the T-47D breast cancer cell line. The *hPRLR*-targeted siRNA were chemically synthesized and constructed in a specific siRNA expression vector (p*Silencer* 2.0), the expression of hPRLR was analyzed by real-time quantitative PCR and Western blot analysis, growth inhibition was measured by MTT assay, and cell cycle analysis was carried out to determine the effect of siRNA treatment on T-47D cells. Our results indicate that the hPRLR siRNA plasmid markedly reduced PRLR gene expression, decreased the growth rate and reduced the frequency of T-47D cells in the G2/M phase, while significantly increasing cells in G0/G1. In summary, RNAi silencing of hPRLR gene expression specifically inhibited the proliferation of T-47D breast cancer cells. This implies that RNAi have therapeutic potential as a treatment for breast cancer by targeting the overexpression of hPRLR, suggesting that this gene product might be a potential therapeutic target.

Introduction

The hormone/cytokine human prolactin (hPRL) is critical to the development and differentiation of the mammary gland. It is also a well-documented tumor promoter of the mammary gland, as revealed by a variety of experimental approaches (1-4). The effects of hPRL on human breast tissue are mediated by the interaction of the ligand with its receptor, hPRLR. hPRLR is up-regulated in mammary tissue during development, and studies have shown that the majority of human breast tumors have higher hPRLR levels than surrounding normal mammary tissue (5,6). As well, it was recently demonstrated that both the degradation and phosphorylation of hPRLR on Ser349 were impaired in breast tumor cells and tissues. This observation was directly correlated with enhanced expression of hPRLR in malignant breast epithelium (7). Therefore, the inhibition or blocking of hPRLR expression might offer a new method for breast cancer biotherapy.

RNA interference has evolved into an excellent approach for the targeted silencing of gene expression, where activation of an intracellular pathway modulated by small-interfering RNA (siRNA) composed of 21 nucleotides leads to the degradation of a specific targeted mRNA (8,9). The specificity and potency of siRNA in cell culture and in animal studies suggest that it can be a powerful therapeutic agent. In this study, we investigated whether hPRLR expression can be effectively and specifically reduced using siRNA technology. We also hypothesized that the inhibition or down-regulation of hPRLR expression might serve as an anti-cancer therapy against certain types of neoplastic breast cells.

Materials and methods

Cell line and cell culture. T-47D human breast cancer cells were acquired from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were maintained at 37˚C in 5% CO_2. FCS and DMEM were purchased from Gibco (USA). All other reagents were purchased from Sigma (USA).

Anti-hPRLR siRNA. Three different siRNA sequences against hPRLR mRNA were designed and chemically synthesized (Invitrogen, USA). The identified target sequences within hPRLR (GenBank accession no. NM_000949.2) were as follows: siRNA-1, 5'-GUUUCUCGGAUGAACUUU AdTdT-3'; siRNA-2, 5'-CACUACAGAGUACGUGAAA dTdT-3'; siRNA-3, 5'-GCACACUGCUUAGUAUUCUd TdT-3'. siRNAc, 5'-UUCUCCGAACGUGUCACGUdTdT-3', served as a negative control with no significant homology to any known human gene sequences. All siRNA sequences were investigated for specificity using a BLAST database search and failed to show homology to any other human genes.

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Abbreviations: siRNA, small-interfering RNA; hPRLR, human prolactin receptor

Key words: human prolactin receptor, small-interfering RNA, breast cancer, T-47D

Construction of plasmid expressing hPRLR-targeted siRNA. Sequences for siRNA corresponding to siRNA-1 were generated by annealing two oligonucleotides (5'-GATCCCGTTTCT CGGATGAACTTTATTCAAGAGATAAAGTTCATCCGA GAAACTTTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAAA GTTTCTCGGATGAACTTTATCTCTTGAATAAAGTTC ATCCGAGAAACGG-3'). The annealed product was cloned into the *Bam*HI and *Hind*III sites of the p*Silencer* 2.0 Neo plasmid (Ambion, USA), which constitutively expresses siRNA from a U6 promoter. As controls, a scrambled version of the siRNA-1 sequence was cloned into the *Bam*HI and *Hind*III sites of the p*Silencer* 2.0 Neo plasmid. The two scrambled-sequence oligonucleotides were 5'-GATCCCTTCTCCGAA CGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAG AATTTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAAATTC TCCGAACGTGTCACGTTCTCTTGAAACGTGACACGT TCGGAGAAGG-3'. The completed plasmids were confirmed by DNA sequencing and designated p*Silencer*-hPRLR and p*Silencer*-control, respectively. The p*Silencer*-hPRLR plasmid contains a U6 promoter that directs synthesis of the oligonucleotide in an inverted repeat with a 9 nt loop and 6 T bases added at the end to serve as a termination signal for RNA polymerase III. The RNA is expected to fold back to form a hairpin loop structure after being transcribed. This hairpin dsRNA can then be further processed by Dicer to generate 21-nucleotide siRNA, the active form for RNAi effect. This siRNA will form dsRNA-endonuclease complexes that bind and destroy *hPRLR* mRNA inside cells.

siRNA and plasmid transfection. To transfect the siRNA, cells were plated at 70% confluence in 6-well plates. After 12 h, cells were transfected with siRNA using the RNAiFect Transfection Reagent (Qiagen, USA) according to the manufacturer's instructions. Briefly, 5 μg siRNA were diluted in culture medium for a final volume of 100 μl, mixed by vortexing, and then 15 μl of RNAiFect Transfection Reagent was added to the diluted siRNA. The samples were mixed by pipetting and incubated for 10 min at room temperature. While complex formation was taking place, the growth medium was gently aspirated from the plate. Cell growth medium (2 ml) containing serum and antibiotics was added to the cells. The transfection complex was added drop-wise to the culture. Cells were incubated with the transfection complex under their normal growth conditions and monitored for gene silencing after 24 h.

To transfect the plasmids, a total of 10^4 cells was seeded into each well of a 6-well culture plate. When the cells reached >60% confluence, they were transfected with p*Silencer*-hPRLR-1 and p*Silencer*-control plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Real-time quantitative polymerase chain reaction

RNA extraction. Poly-A$^+$ RNA was extracted from siRNA-treated cells after 24 h and from plasmid-treated cells after 72 h using the Micro-FastTrack™ 2.0 mRNA Isolation Kit (Invitrogen) according to the manufacturer's instructions. The extracted RNA was quantified with a SmartSpec spectrophotometer (Bio-Rad Laboratories, USA) at wavelengths of 260 nm/280 nm and stored at -80˚C until analysis.

One-step real-time qRT-PCR. The real-time qRT-PCR reaction was performed in Lightcycler glass capillaries in combination with Lightcycler RNA Amplification Kit SYBR-Green I in a total volume of 20 μl, according to the manufacturer's instructions. The specific primer pair for *hPRLR* was designed using Primer Designer 3.2 software. Homology with other human sequences and the formation of template secondary structures were carefully avoided. The *hPRLR* primers consisted of the following sequences: (forward) 5'-GCGACC TTCATTCAGATACC-3' and (reverse) 5'-CCAGCAAGTCCT CATAGTCA-3'. The primer pair for the *ß-actin* housekeeping gene was made up of the following sequences: (forward) 5'-AAAGACCTGTACGC CAACAC-3' and (reverse) 5'-GTC ATACTCCTGCTTGCT GAT-3'. Each sample of poly-A$^+$ RNA (1 μl of 20 ng/μl) was added to the reaction mixture with SYBR-Green I. The reaction was initiated with a 10-min reverse transcription reaction at 55˚C and terminated with a 30-sec denaturation step at 95˚C. The amplification protocol consisted of 35 cycles of the following: a denaturation step at 95˚C for 45 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 60 sec. Melting curve data were collected to verify PCR specificity. Each mRNA sample was analyzed in triplicate. *hPRLR* mRNA levels for each sample were normalized against *ß-actin* mRNA levels, and relative expression was calculated.

MTT assay. On the day before transfection, cells were seeded at a density of $2.5x10^3$ cells/well in 96-well plates in 100 μl DMEM supplemented with 10% FCS. Cultures were grown for 5 days. Each day, 5 wells were selected from each group of cells; 20 μl PBS containing 5 mg/ml MTT (Sigma) was added to each of these wells. The cells were incubated with MTT at 37˚C for 4 h, then the reaction was terminated by the addition of 150 μl/well DMSO and incubated for 15 min. Absorbance was measured spectrophotometrically on a plate reader (Bio-Rad) at 570 nm.

Western blot analysis. Four days post transfection, three groups of cells (untreated, p*Silencer*-hPRLR and p*Silencer*-control) were harvested by two washes in ice-cold PBS, then were scraped and lysed in lysis buffer (50 mM Tris-HCl pH 7.5, 2 mM EDTA, 100 mM NaCl, 1% Triton X-100 and protease inhibitor cocktail) plus 5 mM NaF, 1 mM Na_3VO_4 and 10 mM ß-glycerolphosphate. Cell lysates were incubated for 1 h on ice and centrifuged at 12,000 x g for supernatant collection. Protein concentration in the supernatant was evaluated by the Lowry method. After the addition of SDS-PAGE sample buffer and boiling, 20 μg of denatured proteins were separated on 12% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane (Amersham Biosciences) using a semi-dry apparatus (Bio-Rad). Subsequently, the blots were transferred to a blocking buffer solution (1X PBS, 0.1% Tween-20, 5% w/v non-fat dry milk) and incubated for 1 h. After blocking, the membranes were incubated with the appropriate diluted primary antibody in 5% bovine serum albumin, 1X PBS and 1% Tween-20 at 4˚C overnight in a roller bottle. The antibodies (and dilutions) included anti-hPRLR (1:1000 dilution, Santa Cruz) and anti-ß-actin (1:5000 dilution, Sigma). Following 3-5 washing steps in wash buffer (1X PBS, 1% Tween-20), the blots were incubated with an appropriate secondary antibody diluted in PBS. HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (USA). After 1 h of

gentle agitation, the blots were washed five times for 5 min in wash buffer. Immunolabelling was visualized using the ECL procedure (Amersham Biosciences, USA) and Kodak BioMax MR film.

Flow cytometric analysis of the cell cycle. On the day before transfection, T-47D cells were seeded at a density of $2x10^5$ cells/well into 6-well plates. Experimental groups were as follows: the blank group (DMEM was substituted for transfection complex), the negative group (100 nmol/l non-silencing siRNA), and the siRNA-hPRLR group (100 nmol/l siRNA-hPRLR). After transfection (72 h), cells were harvested, washed twice with 1X PBS, fixed with ice-cold 70% ethanol and stained with 20 μg/ml propidium iodide for 20 min. Cell cycle analysis was performed with a FACS station equipped with the CellQuest software application.

Statistical analysis. All statistical analyses were performed using SPSS 10.0 software. Comparison of all groups was carried out using the Student's t-test, setting the α value for statistical significance at $P<0.05$ and $P<0.01$.

Results

Down-regulating the effects of hPRLR gene expression by anti-hPRLR siRNA. We investigated the effects of three anti-hPRLR siRNA on mRNA levels by real-time qRT-PCR. Non-specific siRNA and medium-only-treated cells were used as controls. Of the three siRNA tested, only one resulted in considerable inhibition (65%) of hPRLR expression. The most effective inhibition was achieved using siRNA-1 at a 200-nM concentration (data not shown). Thus, siRNA-1 was chosen for subsequent experiments. siRNA-2 and -3, which did not give rise to significant silencing of hPRLR relative to non-specific siRNA treatment, were excluded from further experiments. As anticipated, a non-specific siRNA control failed to produce any significant decrease in hPRLR mRNA levels.

Plasmid-encoded siRNA has been shown to be an efficient means of constitutively expressing siRNA in cells. To verify our synthetic siRNA-1 results, p*Silencer*-encoded shRNA corresponding to anti-hPRLR siRNA-1 was constructed and transiently transfected into T-47D cells. This shRNA down-regulated hPRLR mRNA levels similarly to cells treated with the anti-hPRLR siRNA-1 (Fig. 1A). We found that hPRLR protein expression levels were suppressed up to 60% in T-47D cells compared with p*Silencer*-control-transfected cells. This inhibitory effect was specific, because transfection with p*Silencer*-control did not alter hPRLR levels. Furthermore, RNAi did not cause non-specific down-regulation of gene expression, as demonstrated by the ß-actin control (Fig. 1B). These data indicate that vector-based RNAi can effectively suppress hPRLR overexpression.

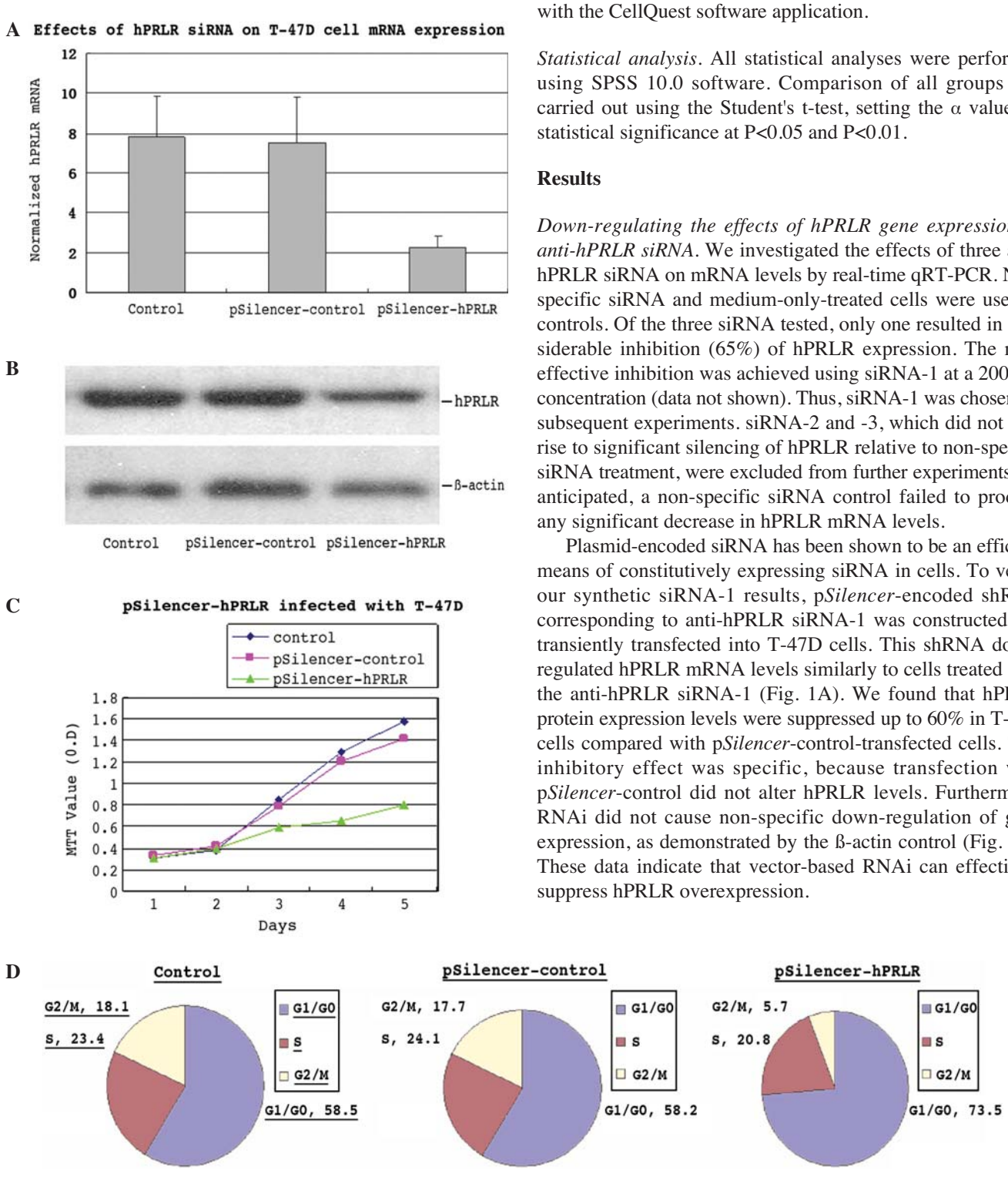

Figure 1. (A) Quantification of hPRLR mRNA in T-47D cells. hPRLR mRNA levels for each sample were normalized against *ß-actin* mRNA levels, and relative expression was calculated. (B) Western blot of cytoplasmic protein lysates from the three T-47D transfection groups. Anti-hPRLR shRNA expression led to reduced hPRLR protein. (C) Cell growth curves. MTT assay was performed and expressed as an O.D. value. (D) The effect of anti-hPRLR siRNA on the cell cycle of T-47D cells. Control, cells without transfection (medium only); pSilencer-control, cells transfected with an unrelated siRNA control; pSilencer-hPRLR, cells transfected with pSilencer-hPRLR plasmid.

Table I. Comparison of cell cycle distribution for all groups of cells (mean ± SD).

Treated types	G0/G1 phase (%)	S phase (%)	G2/M phase (%)
Control	58.5±0.3	23.4±0.5	18.4±0.6
Control-siRNA	58.2±0.5	24.1±0.7	17.7±0.5
pSilencer-siRNA	73.5±2.2	20.8±2.6	5.7±3.2[a]

[a]P<0.05 compared to control.

Inhibition of cell growth by anti-hPRLR siRNA. To investigate whether treatment with p*Silencer*-hPRLR was capable of reducing cell number and viability, an MTT assay was performed. The results confirmed that hPRLR siRNA significantly reduced the number of viable cells. The growth curves of the groups revealed that cell proliferation was reduced in cells transfected with the active p*Silencer*-hPRLR plasmid, as compared to cells transfected with the p*Silencer*-control or treated with medium alone (Fig. 1C).

Changes in the cell cycle caused by anti-hPRLR siRNA. Flow cytometric cell cycle distribution analysis indicated that the proportion of T-47D cells in the G0/G1 phase was significantly higher in the p*Silencer*-hPRLR-transfected group than in the two control groups (P<0.05). There was no significant change in the proportion of S phase cells, while the percentage of G2/M phase cells was significantly reduced. There were no obvious differences in cell cycle distribution between the p*Silencer*-control and the untreated control groups (P>0.05) (Fig. 1D, Table I).

Discussion

The hormone prolactin (PRL) is best known for its effects on mammary gland development. It is required for lobuloalveolar formation of the mammary ducts during pregnancy, for terminal differentiation of the mammary epithelial cells, and for the synthesis of milk components during lactation (10). The binding of PRL to the PRL receptor (PRLR), a member of the class I cytokine receptor family, induces receptor dimerization and activation of cytoplasmic tyrosine kinases (11). Growth and differentiation of breast cancer are regulated by hormones, notably estrogen, progesterone and prolactin. Human prolactin (hPRL) not only promotes the proliferation and differentiation of mammary epithelial cells during mammary gland development, but has also been associated with breast tumor development. In this role, hPRL is a potent mammary epithelial differentiation factor in breast carcinomas, and autocrine/paracrine hPRL signaling is now recognized to contribute to tumor cell viability (12-14). The effect of hPRL on human breast tissue is mediated by its interaction with hPRLR and the primary signaling cascade downstream of hPRLR, which are components of the Janus-activated kinase-2 (JAK2)/signal transducer and activator of transcription-5a. hPRLR is up-regulated in mammary tissue during development. Moreover, studies have shown that the majority of human breast tumors have higher hPRLR levels than surrounding normal mammary tissue, with 70-95% of human breast cancer specimens expressing hPRLR (15-17). Interference of the hPRL autocrine/ paracrine loop in breast cancer cells using PRL and hPRLR antagonists led to the inhibition of cell growth and the induction of apoptosis (18-24). In a rat prolactin (rPRL)-transgenic mouse, the PRLR of breast tissue became more active, leading to a high incidence of breast cancer (25). An antibody raised against PRLR has also been shown to reduce the incidence of carcinogen-induced breast cancer in mice *in vivo* (26). The findings of that study indicated that up-regulation of hPRLR causes defective signaling, and finally induces abnormal proliferation of breast epithelium. Thus, based on the observation that both hPRL and hPRLR are widely expressed in breast cancers, it has been proposed that targeted inhibition of PRLR signaling may have potential as a breast cancer therapy.

RNAi is sequence-specific gene silencing induced by double-stranded RNA (dsRNA). This effect is mediated by short-interfering RNA that are produced from long dsRNA of exogenous or endogenous origin by an endonuclease of the ribonuclease III type, called Dicer. The resulting 21- to 23-nucleotide-long siRNA are then incorporated into a nuclease complex, the RNA-inducing silencing complex, which targets and cleaves mRNA containing a sequence identical to that of the RNAi (27). In the last few years, RNAi has developed into a powerful approach for the targeted silencing of gene expression, and has provided researchers with a formidable method of cancer gene therapy (28). Furthermore, the specificity and potency of siRNA in cell culture and animal studies suggest that it may be a robust and effective therapeutic agent.

In the present study, we chemically synthesized siRNA targeting the *hPRLR* gene and constructed a eukaryotic expression plasmid (p*Silencer* 2.0) with the U6 promoter driving expression of RNA composed of 2 identical 19-nucleotide sequence motifs in an inverted orientation separated by a 9-bp spacer to form hairpin dsRNA capable of mediating *hPRLR* inhibition. After transfection into T-47D breast cancer cells, we measured the inhibitory effects had on hPRLR mRNA and protein expression by real-time quantitative RT-PCR and Western blot anaysis, and found that the siRNA-hPRLR-1/p*Silencer*-hPRLR had a pronounced effect on hPRLR silencing as compared to the control groups. This efficacy is perhaps related to the precise position of the selected 19-nucleotide motif, as targeting different regions of *hPRLR* mRNA can produce varying silencing effects. In this study, we reduced the expression of hPRLR mRNA and protein in T-47D cells using a specific siRNA, observed cell growth inhibition by MTT, and detected an evident block in the G1/G0 phase of the cell cycle distribution by flow cytometry. This research lays the foundation for further investigation of the anti-breast tumor activity of silencing PRLR, perhaps leading to future therapeutic interventions that exploit this effect.

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