

# Knockdown of *p57* gene inhibits breast cancer cell proliferation

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**Abstract.** The aim of the study was to investigate possible effects of *p57* on the growth of the human MCF-7 and rat SHZ-88 breast cancer cell lines. Specific oligonucleotide sequences containing small hairpin structure were inserted into a small interfering RNA (siRNA) expression vector. The human MCF-7 and rat SHZ-88 breast cancer cell lines were transfected with recombinant plasmids. The *p57* gene expression was blocked in the human MCF-7 breast and rat SHZ-88 breast cancer cells, using chemically modified siRNA. The *p57* expression level was evaluated using quantitative polymerase chain reaction (qPCR) and western blot analysis. Immunofluorescence was conducted to detect *p57* expression in the breast cancer cells. Tetrazolium blue (MTT) method was employed to detect the effect of *p57* inhibition on the proliferation of the MCF-7 and SHZ-88 cell lines. Cell proliferation in the experimental group was significantly reduced. Immunofluorescence assay results showed *p57* siRNA effectively inhibited the *p57* level in the MCF-7 and SHZ-88 cells. RT-PCR results showed that 48 h after transfection, the *p57* mRNA level in the transfected group was significantly lower compared with the control group. In conclusion, *p57* effectively inhibited the proliferation of breast cancer after stable interference.

## Introduction

Breast cancer ranks second in female cancer mortality rate, and the incidence rate in developing countries is on the increase (1). Cell cycle-dependent kinase inhibitor *p57* is a tumor suppressor gene coding for a multifunctional protein involved in cell proliferation regulation, apoptosis, cell differentiation,

tumor invasion, metastasis and angiogenesis (2-4). Previous findings showed that in a variety of human tumor cells, *p57* was downregulated, suggesting that low levels of *p57* increased the risk of malignant tumor development. Thus, *p57* downregulation is important in the pathogenesis of human ovarian cancer, gastric cancer, colorectal cancer and lymphoma (5,6).

There are reports suggesting that *p57* downregulation is linked to the onset and development of breast cancer (7). In the present study, we investigated the effects of *p57* on two breast cancer cell lines. The results however did not show any effects of knocked down *p57* gene on breast cancer cell proliferation.

## Materials and methods

**Materials.** Human HMCF-7 and rat SHZ-88 breast cancer cell lines were purchased from the Shanghai Institute of Cell Research, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in media containing 10% fetal bovine serum RPMI-1640 (Gibco BRL, Gaithersburg, MD, USA), with 5% CO<sub>2</sub> at 37°C in a humid environment.

**Main reagent.** Lipofectamine® 2000 and TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA); AMV First Strand cDNA Synthesis kit (Toyobo Co., Ltd., Osaka, Japan); and anti-*p57* antibody (rb-1527) (Lab Vision/neomarkers, San Diego, CA, USA) were used in the present study.

**Small interfering RNA (siRNA) design synthesis.** *p57* mRNA sequences in human and rat were obtained from NCBI, and siRNAs were designed based on mRNA sequences using siRNA online design software and structure 4.4 (8-10). In order to enhance the stability of siRNA, the sense chain was modified by fluorine (chemical was produced by the Shanghai Jima Company, Shanghai, China).

## Experimental method

**Transfection.** We used Lipofectamine® 2000 for transfection. Transfection conditions were optimized using the negative control siRNA with universal fluorescent labeling. Cells in the logarithmic growth phase were collected and cell density was adjusted. The cells were inoculated into a 12-well plate containing RPMI-1640 culture medium with 10% serum without antibiotics. Lipofectamine® 2000 reagent was diluted in culture medium without serum and antibiotics. At 24 h later,

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the previous culture medium was discarded and replaced with diluted Lipofectamine® 2000 reagent and siRNA. They were mixed for 5 min and after 20 min at room temperature they were incubated for 6 h and then observed under a fluorescent microscope (IX70; Olympus, Tokyo, Japan).

*Cell proliferation was detected by CCK-8 method.* Normal and transfected human MCF-7 and rat SHZ-88 breast cancer cells were inoculated into a 96-well plate ( $5 \times 10^3$  cells/well). Cell proliferation was measured at 1, 2, 3 and 4 days after culture. CCK-8 (10  $\mu$ l) reagent was added to each well followed by 2 h incubation at 37°C, and absorbance was measured at 450 nm. The absorbance value represented cell proliferation.

*Immunofluorescence.* MCF-7 and SHZ-88 breast cancer cells were inoculated into 12-well plates with coverslips ( $1 \times 10^5$ /ml, 1 ml per well). After 48 h incubation, the cells were washed with PBS twice, and fixed with 4% formaldehyde at room temperature for 10 min. Then, 0.2% Triton X-100 was added and left at room temperature for 10 min. BSA (2%) was added, followed by 30 min incubation. Anti-p57 antibody (dilution, 1:50) was added and the sample was incubated at 4°C overnight. After incubation, the cells were washed three times with PBS, 5 min each time, and secondary anti FITC-IgG (dilution, 1:100) was added at room temperature followed by 1 h incubation in the dark. The samples were then examined under a fluorescent microscope.

*RT-polymerase chain reaction (PCR) detection.* Total RNA was extracted and the concentration was measured using a UV spectrophotometer (Hitachi, Ltd., Tokyo, Japan) and the integrity of RNA was examined by electrophoresis (A260/A280 ratio was 1.8-2). cDNA FastQuant was used for cDNA synthesis. The reaction conditions were: 95°C for 3 min and 42°C for 15 min, and then kept on ice. Primer sequences are presented in Table I.

*Western blot analysis.* Lysis solution (including protease inhibitor) was added and the cells were ground (on ice). The sample was centrifuged at 12,000 x g for 20 min at 4°C and supernatant was collected. Protein content in the supernatant was quantified and the sample was electrophoresed (10% SDS-PAGE) and transferred to a PVDF membrane. The membrane was incubated with skimmed milk (10%) for 1 h and rabbit monoclonal p57 antibody (dilution, 1:1,000; cat. no. ab75974; Abcam, Cambridge, MA, USA) was added and the membrane was incubated at 4°C overnight. The membrane was washed with TTBS followed by adding secondary goat anti-rabbit (HRP) IgG antibody (dilution, 1:2,000; cat. no. ab6721; Abcam) and incubating at room temperature for 1 h. The membrane was washed with TTBS and color-substrate solution was added.

*Statistical analysis.* SPSS 17.0 software (IBM SPSS, Armonk, NY, USA) was used for statistical analysis. The Kaplan-Meier method was used for single factor analysis of the relevant data in survival analysis. Log-rank test was employed for comparisons of significant difference. The Cox proportional hazard model was used to study the effects of various clinical and pathological factors on the recurrence time.  $P < 0.05$  was considered to indicate a statistically significant difference.

Table I. RT-polymerase chain reaction primer sequences of p57 mRNA of MCF-7 and SHZ-88 cells.

Gene name	Primer sequence
MCF-7	5'-3' CTGATCTCCGATTTCCTTCGC 3'-5' TCTTTGGGCTCTAAATTGG
SHZ-88	5'-3' TCTCCTGTCTGTGTGCCTACC 3'-5' CAGGTCAACTGCCTACACAGAGC

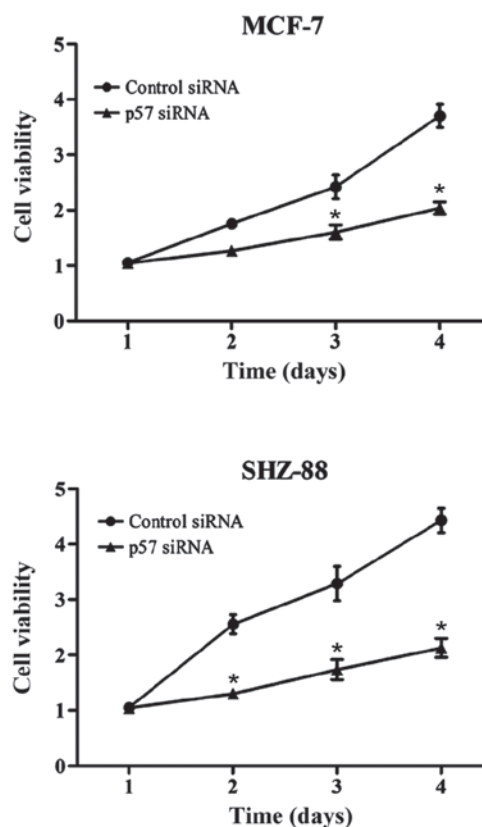


Figure 1. CCK-8 method to detect the proliferation activity of MCF-7 and SHZ-88 breast cancer cells after transfection of small interfering RNA (siRNA) compared with the blank control group. \* $P < 0.05$ .

## Results

*Cell proliferation after p57 interference.* Cell proliferation in the experimental group was significantly reduced. In the MCF-7 cells, absorbance value in the experimental group and the negative control group were  $2.67 \pm 0.09$  and  $3.87 \pm 0.12$ , respectively. In the SHZ-88 cells these values were  $2.06 \pm 0.07$  and  $4.37 \pm 0.12$  in the experimental and negative control groups, respectively. Differences were statistically significant ( $P < 0.05$ ; Fig. 1).

*Immunofluorescent assay.* siRNA effectively inhibited the p57 expression in MCF-7 and SHZ-88 cells. Compared with the control group, p57 protein levels were significantly lower in the siRNA-transfected cells. Immunofluorescent assay showed that p57 was expressed in the MCF-7 and SHZ-88 cells, 48 h after transfection (Fig. 2).

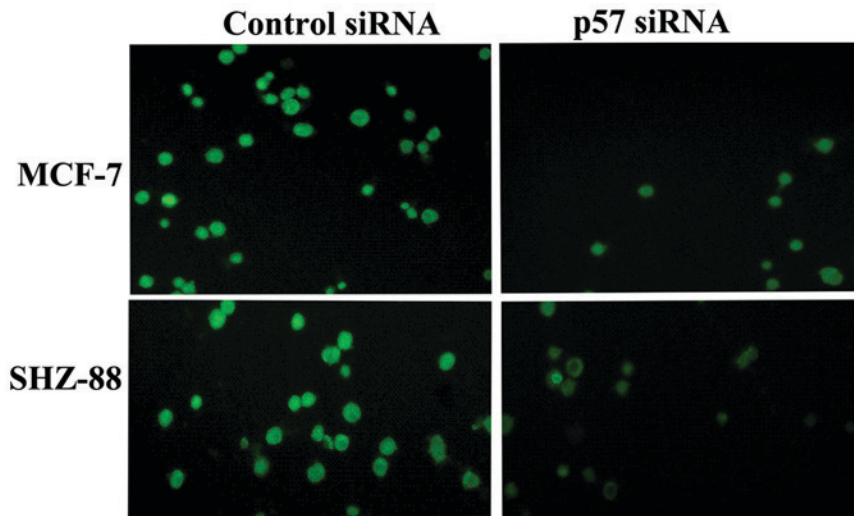


Figure 2. p57 expression in the MCF-7 and SHZ-88 cells ( $P < 0.01$ ). siRNA, small interfering RNA.

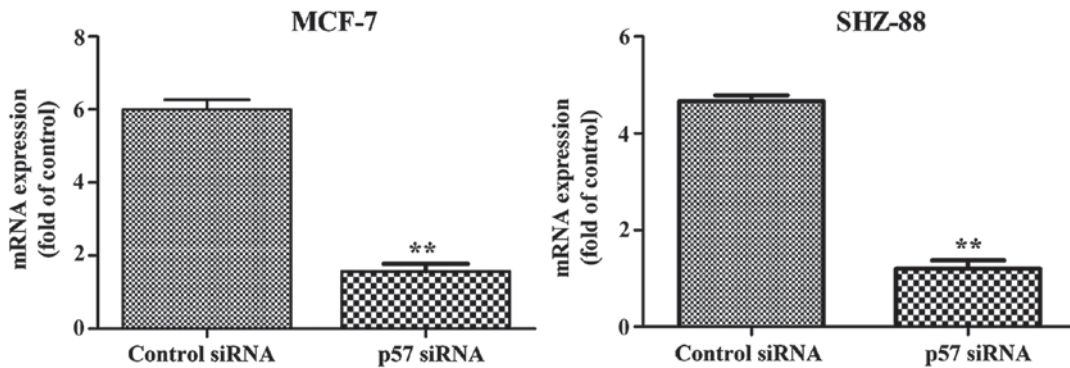


Figure 3. p57 expression in MCF-7 and SHZ-88 cells ( $^{**}P < 0.01$ ). siRNA, small interfering RNA.

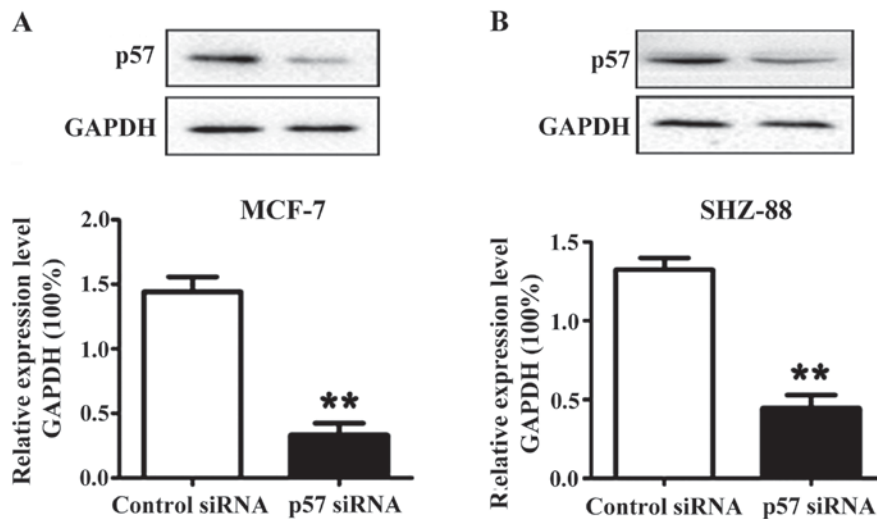


Figure 4. p57 protein expression in the (A) MCF-7 and (B) SHZ-88 cells ( $^{**}P < 0.01$ ). siRNA, small interfering RNA.

*Effect of p57 siRNA on p57 transcription and translation.* RT-PCR results showed that 48 h after transfection, the mRNA level in the transfected group was significantly lower compared with the control group (Fig. 3). This suggested that the design of our siRNA was successful and siRNA was able

to effectively inhibit the *p57* gene transcription in the MCF-7 and SHZ-88 cells. The results obtained from western blotting showed that the level of p57 protein was significantly lower in the transfected cells, which suggested that the design of our siRNA was successful (Fig. 4).

## Discussion

Breast cancer is one of the most common malignant tumors in women. In China, the incidence of this type of cancer ranks first. According to GLOBOCAN 2008 statistics, new cases of breast cancer accounted for 23% of all tumors in 2011, and accounted for 14% of all cancer deaths (11-14). Tumor formation is associated with the out-of-control cell cycle regulation (15). The cell cycle control mechanism includes cyclins, cyclin-dependent protein kinases (CDKs) and cyclin-dependent kinase inhibitor (CKI). Any disturbance in the mechanisms that control these factors can potentially lead to excessive cell proliferation, reduction of apoptosis and tumor formation. p57 is a CKI that belongs to the Cip/Kip family, which includes p21, p27 and p57. p57 can regulate cell cycle progression and is involved in the regulation of transcription, apoptosis, differentiation, development, and migration. p57 is a tumor suppressor that can regulate cyclins, CDKs and cyclin-CDK complexes in the G1-S transition, modulating DNA replication (16). Therefore, absence of p57 may result in an unusual increase in the number of cells in S phase (16). It has been reported that P57 protein is downregulated in colon cancer (17), gastric cancer (18) and human brain glioma (19,20).

In the present study, we blocked the expression of p57 in human and rat breast cancer cell lines (MCF-7 and SHZ-88) by transfecting cells using Lipofectamine<sup>®</sup> 2000 and specific p57 siRNAs. The results showed that p57 siRNA successfully knocked down the expression of p57. We demonstrated that the construction of an appropriate p57 siRNA was directly linked to p57 silencing efficiency. Thus, the knockdown of p57 cells may be useful in the field of tumor therapy.

## Competing interests

The authors declare that they have no competing interests.

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