Effect of progranulin (PGRN) on the proliferation and senescence of cervical cancer cells

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ABSTRACT. We investigated the effect of progranulin (PGRN) expression on the proliferation and senescence of cervical cancer cells. PGRN small interfering RNA (siRNA) was introduced into the SiHa and HeLa cell lines of human cervical carcinoma using liposome-mediated transfection. The expression levels of PGRN in each cell line after transfection of PGRN siRNA were detected by reverse transcription-polymerase chain reaction (RT-PCR). Senescence in the cell lines was detected using the β-galactosidase-staining test, and proliferation was detected by clone formation. The RT-PCR assay showed that the expression of PGRN in all of the cell lines transfected with PGRN siRNA markedly decreased. In the clone-forming test, compared with the control group, the colony-forming ability in all cell lines decreased significantly after transfection with PGRN siRNA. The β-galactosidase-staining experiments showed that the phenomenon of cell aging in the PGRN interference group was more obvious than in the control group. After the cervical cancer cells had been transfected with PGRN siRNA, cell senescence was accelerated and clone-forming ability was markedly reduced. This suggests that PGRN can promote the proliferation of the cervical cancer cell line; proliferation of cervical

cancer cells is achieved by inhibiting their senescence.

Key words: PGRN; Cell senescence; Cell proliferation; Cervical cancer

INTRODUCTION

Research shows that progranulin (PGRN) is a novel growth factor that plays an important role in embryonic development, wound repair, inflammation modulation, and tumor pathology (Gijselinck et al., 2008). The expression of PGRN is higher in rapidly proliferating tissues and organs, such as skin tissue, the gastrointestinal system, and mammary epithelial tissue (Cui et al., 2006). In addition, PGRN is highly expressed in human tumors such as ovarian, breast, liver, prostate, and bladder cancers (Khanna et al., 2011; Ren et al., 2011; Lin et al., 2012; Niemelä et al., 2012). Studies conducted *in vivo* and *in vitro* have also shown that PGRN is closely related to the characteristics of proliferation, differentiation, invasion, and drug resistance of tumors (He and Bateman, 1999). Currently, the expression status and the clinical significance of PGRN in clinical specimens of cervical carcinoma are rarely reported. Whether the multifunctional growth factor of PGRN has an effect on the development of cervical cancer is still unclear. Our previous clinical trials showed that the expression of PGRN in cervical cancer tissue was significantly higher than in normal cervical tissue. According to this phenomenon, we conducted a preliminarily study to investigate the effect of PGRN on the senescence of cervical cancer cells.

MATERIAL AND METHODS

Materials

1) SiHa and HeLa cell lines of cervical cancer were donated by Wang Hongyan, who is an associate professor of the Department of Microbiology of Weifang Medical University.

2) The PGRN small interfering RNA (siRNA) interference sequence was synthesized by Shanghai GenePharma Co. Ltd., Shanghai, China; it was prepared using 20 μM RNase-free water and preserved at -20°C. The sequence of PGRN siRNA was S1: 5'-CCUGAUAGUCAGUUCGAAU -3'; S2: 5'-AUUCGAACUGACUAUCAGG-3'.

3) The cells were transfected with liposomes; we used Lipofectamine™ 2000 reagent and a TRIzol kit, which were purchased from Invitrogen.

4) A β-galactosidase *in situ* staining kit was purchased from the Beyotime Technology Co., Ltd., Shanghai, China.

5) Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 medium, and Opti-MEM medium were purchased from Gibco, USA.

6) The reverse transcription (RT) kit was purchased from Fermentas.

Methods

RNA interference experiment

SiHa and HeLa cells were cultured in 10% fetal bovine serum and DMEM in a conventional culture incubator at 37°C and 5% CO_2. The cells were passaged with 0.25% trypsin digestion every 2 days.

The SiHa and HeLa cells were spread on a 6-hole plate at a concentration of 1 × 10^5/hole, placed in a cell culture incubator, and cultured for 24 h. The pSUPER-si-PGRN (si-) and the control comprising airborne pSUPER (con) were transfected into the SiHa and HeLa cell lines for

each group in three holes, and culture was continued for 72 h, followed by extraction of RNA and collection of the cells. The expression of PGRN mRNA was detected by polymerase chain reaction (PCR) after RT synthesis of complementary DNA (cDNA).

β-galactosidase-staining method

Cells were spread on the 6-hole plate and transfected with PGRN siRNA for 96 h, then stained with β-galactosidase liquid along with the control. The medium was drained from the six-hole plate, which was flushed with phosphate-buffered saline (PBS), and the β-galactosidase-staining solution was fixed *in situ* for 10 min at room temperature. Cells were washed three times for 3 min each time with PBS after removing the fixed liquid. After removing the PBS, adding 1 mL staining liquid to each hole, and incubating at 37°C overnight, the cells were investigated under an optical microscope.

Clone-forming test

The transfected cells of the experimental and control groups were digested and counted after culture for 72 h. The two groups of cells were inoculated on the six-hole plate (300 per hole). The plate was incubated at 37°C in 5% CO_2 for 10-14 days. The culture medium was discarded and the cells were flushed with PBS, fixed with methanol for 5 min, washed with PBS, stained with Giemsa dye for 10 min, and flushed with PBS. The cloning groups with more than 50 cells were counted.

Data analysis

Data were analyzed by single-factor analysis of variance using the SPSS 19.0 software, and the results are reported as means ± SD. The analysis of data between different groups was conducted using the Student *t*-test, and $P < 0.05$ was considered to be statistically significant.

RESULTS

PGRN protein levels in the SiHa and HeLa cells decreased after interference by PGRN mRNA

Using actin as a reference, we used RT-PCR to detect the expression of PGRN mRNA in SiHa and HeLa cell lines after transfection with PGRN siRNA. As shown in Figure 1, the expression of PGRN mRNA was significantly lower than in the control group after the SiHa and HeLa cell lines had been transfected with PGRN siRNA, which indicates the successful silencing of the PGRN gene.

Senescence of cancer cells was obvious after interference by PGRN mRNA

As shown in Figure 2A-D, when the PGRN interference was expressed in the SiHa and HeLa cell lines, there were more cells stained blue by β-galactosidase, revealing the phenomenon of aging. When the SiHa cell line was transfected with the interference sequence of PGRN, the color ratio was 19.03 ± 1.36%, while the color ratio in the control group was 10.01 ± 4.80% ($P = 0.035$). In the HeLa cell line, a similar trend of staining was observed. After transfection with the PNRN interference sequence, the color ratio was 15.03 ± 1.22%, while the color ratio in the control group was 7.71 ± 2.14% ($P = 0.029$). There was a significant difference between the interference and control groups according to variance analysis. The colored cells increased after PGRN interference, indicating that intracellular senescence-associated-β-galactosidase activity increased.

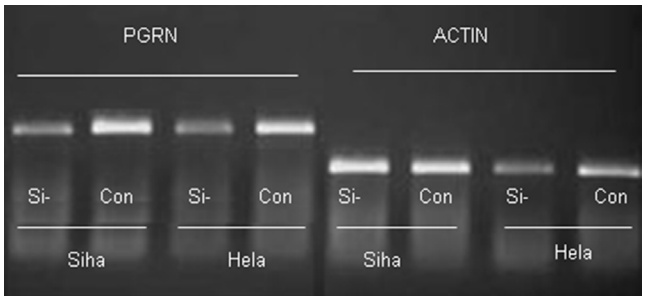

Figure 1. PGRN mRNA interference in the SiHa and HeLa cell lines.

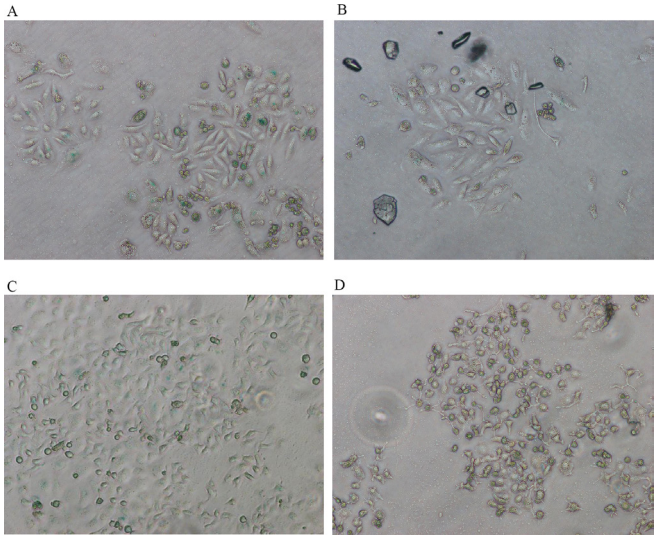

Figure 2. β-galactosidase enzyme dyeing. **A.** SiHa cell line after PGRN mRNA interference. **B.** SiHa cell line control. **C.** HeLa cell line after PGRN mRNA interference. **D.** HeLa cell line control. (A-D) 100X magnification.

Figure 3 illustrates the increase in senescent cells following interference. Figure 4A-D shows that the interference of PGRN can significantly inhibit the proliferation of SiHa and HeLa cell lines. In the SiHa cell line, the cell clone number was 136.77 ± 19.12 colonies/plate in the interference group, while the average cell clone number in the control group was 206.57 ± 12.15 colonies/plate (P = 0.03); the cell clone number formed in the interference groups was around 33% less than in the control group. In the HeLa cell experimental group, we observed similar results: the cell clone number in the interference group was 151.67 ± 22.12 colonies/plate, whereas in the control group, the average cell clone was 236.67 ± 26.08 colonies/plate (P = 0.01); the cell clone number formed was approximately 36% less in the interference groups than in the control group. Comparing with the control group, the number of SiHa and HeLa cells in the interference group all showed statistical significances. In addition, the formed cell clone number in the interference group was also smaller, which suggests that PGRN promotes the proliferation and growth of SiHa and HeLa cells (see Figure 5).

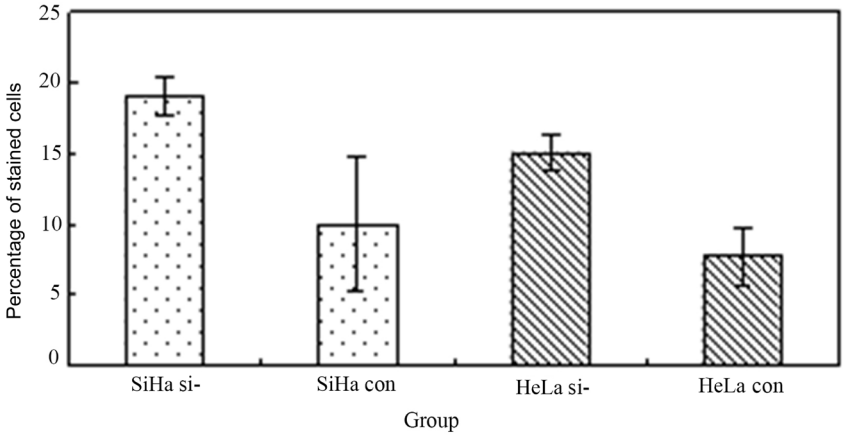

Figure 3. SiHa and HeLa cell line-dyeing experiments.

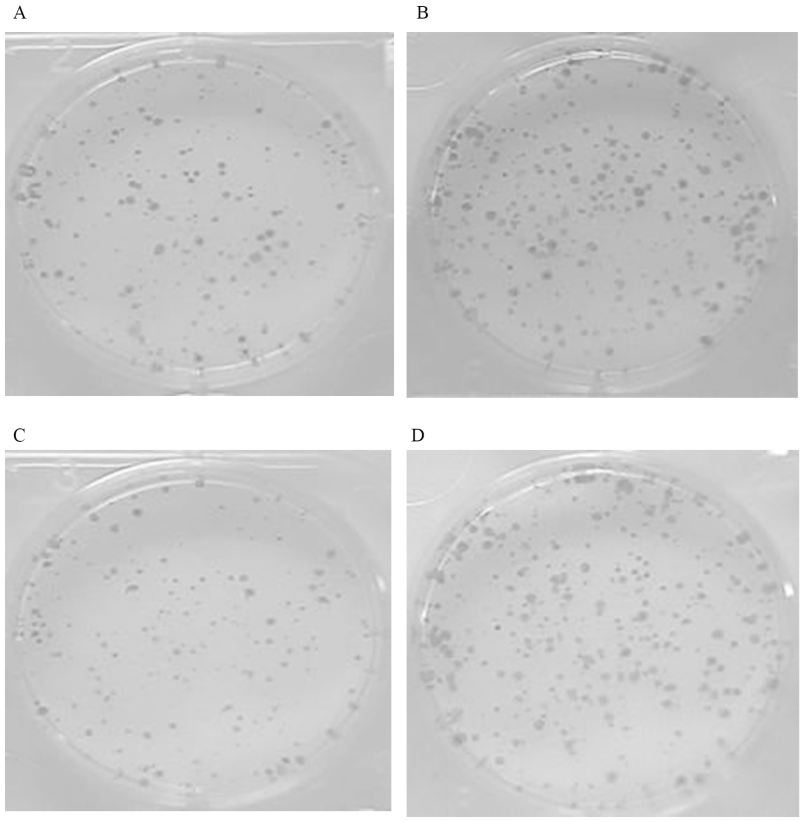

Figure 4. Cell survival by colony formation assay. **A.** SiHa group after PGRN mRNA interference. **B.** SiHa control group. **C.** HeLa group after PGRN mRNA interference. **D.** HeLa control group.

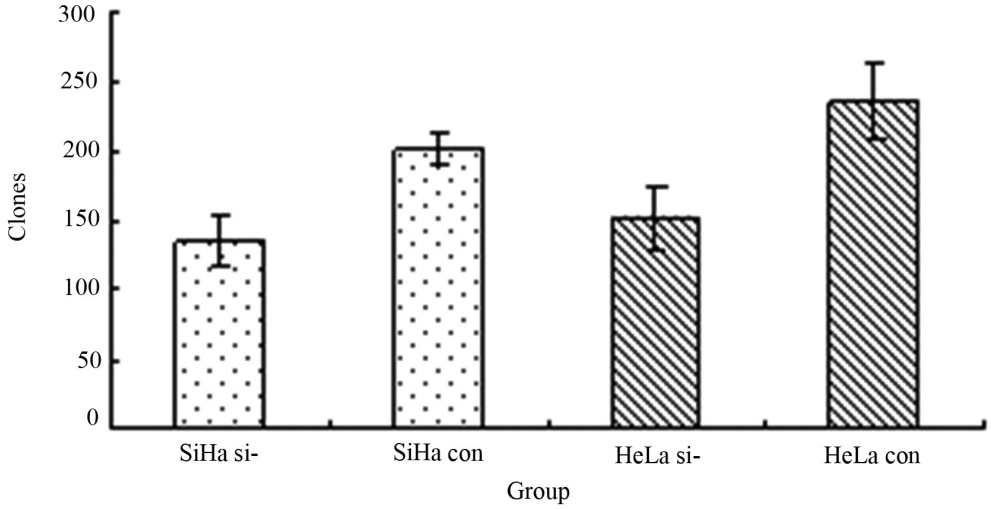

Figure 5. Cell survival in the SiHa and HeLa cell lines by clone formation assay.

DISCUSSION

Cervical cancer is the second most common malignant tumor in women worldwide after breast cancer (Greer et al., 2010). In recent years, there has been a trend towards a higher incidence of cervical cancer in younger women (Waggoner, 2003). The mechanisms by which cervical cancer acts, and the search for effective biomarkers and drug targets are matters of the highest priority.

PGRN belongs to a novel family of growth factors that are secreted glycoproteins. It is the largest member of the granule protein growth factor family and is composed of 593 amino acids (Bhandari and Bateman, 1992; Hrabal et al., 1996). Recent studies have proven that PGRN is expressed to some extent in most organs of the body (He and Bateman, 1999; Khanna et al., 2011; Lin et al., 2012), but the content of PGRN increases significantly in a variety of cancer tissues (Khanna et al., 2011; Ren et al., 2011; Lin et al., 2012; Niemelä et al., 2012). Our previous experiment detected that the expression of PGRN in cervical cancer was significantly higher than in normal cervical tissue, indicating that PGRN can be a potential molecular marker for the diagnosis and evaluation of cervical cancer. Therefore, it is important to investigate the effect of PGRN expression on the incidence and development of cervical cancer.

There are generally two ways by which growth factors promote the formation of tumors: one is by the promotion of tumor cell proliferation, and the other is the stimulation of the non-transformed mesenchymal cells that participate in angiogenesis (Aaronson, 1991). Based on the analysis of the expression of the PGRN gene in multiple-tumor samples and the study of PGRN in the tumor model, researchers believe that the main mechanism by which PGRN is involved in the formation of tumors is the promotion of the proliferation of tumor cells. When the PGRN gene is overexpressed, the cell mitotic rate, independent growth ability, and tumorigenicity ability in nude mice are improved. However, the normally expressed PGRN mRNA in the cells is combined with antisense primer, which may affect the proliferation of cells (He and Bateman, 1999). In the present

study, the aging phenomenon of cervical cancer cells was enhanced significantly after inhibition of the PGRN gene, the clone-forming ability was weakened, the clone formation of cell clusters was reduced, and the number decreased. It has been suggested that the decreased expression of the endogenous PGRN gene may promote senescence in cancer cells, which inhibits the growth of cervical cancer cells and shortens their life. This is corroborated by the previous research.

Studies have shown that cellular senescence is the third anti-cancer mechanism after cellular DNA repair and apoptosis. Cell senescence is irreversible periodic stagnation, which may end the indefinite proliferation of cancer cells. Therefore, cell senescence has become a new hot topic of research in the treatment of cancer because it can inhibit the occurrence and development of cancer. In 2005, it was confirmed for the first time, from animal models and human body specimens, that cellular senescence can inhibit the malignancy of tumors and prolong survival time in an animal model (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Michaloglou et al., 2005), suggesting that cellular senescence plays an important role in the genesis and development of tumors. Increasing evidence suggests that the induction of cell senescence can also enhance the efficacy of chemotherapy drugs and other therapies (Schmitt et al., 2002; Tai and Chung, 2007; Wu et al., 2007; Xue et al., 2007).

Therefore, cell senescence is closely related to the occurrence, development, and treatment of tumors (Collado et al., 2007; Hornsby, 2007). The balance between cell proliferation and cell senescence determines whether malignant tumors form. The induction of tumor cell senescence has broad application prospects for the inhibition of cancer. The results suggested that the inhibition of PGRN expression in cervical cancer cells has an anti-tumor function, which may provide strong evidence at the cellular level to provide a therapeutic target strategy for PGRN in cervical carcinoma. Our results were also confirmed by Lu et al. (2014) and Zhang et al. (2014). The mechanism by which the PGRN gene allows cervical cancer cells to escape from normal cell senescence is still unclear. Therefore, gene therapy with PGRN as the target for cervical cancer requires further investigation.

Conflicts of interest

The authors declare no conflict of interest.

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