Growth inhibition of human salivary gland tumor cells by introduction of progesterone (Pg) receptor and Pg treatment

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Abstract

Cancer of the salivary gland is one of the common cancers in the head and the neck regions. This type of cancer develops in the minor and the major salivary glands, and it sometimes metastasizes to other organs, particularly the lung. Morphologic mimicry and similarity in the expression of steroid hormone receptors between salivary gland tumors (SGTs) and breast tumors are well-known phenomena and are occasionally debated in the field of surgical pathology. Progesterone (Pg), one of the female sex steroid hormone, is intimately involved in the development of the mammary gland. Further, it is believed that Pg plays a role in breast cancer progression. However, little is known regarding its role in SGTs. In this study, we used ACCM, a human adenoid cystic carcinoma cell line established from the salivary gland, in order to clarify the role of the Pg receptor (PR) on cell proliferation. No effect of Pg on cell proliferation was observed in the PR-deficient aggressive ACCM cells. However, after introducing PR into the ACCM cells, Pg markedly inhibited the proliferative activity of the cells. This inhibitory effect on cell proliferation was accompanied by p21 upregulation, and Id1 and c-myc downregulation. Moreover, Pg-treated PR transfectants showed significant morphological change; they appeared more flattened and spread out when compared with the ethanol-treated control cells. Our results provided significant insights into the mechanism of suppression of the proliferative property of the cells via the function of PR, and suggested that PR reintroduction therapy might be a viable method of inhibiting human SGT progression.

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Introduction

In cancer cells, several genetic alterations are responsible for giving the cells the ability for proliferation, migration, and metastasis. These genetic alterations should be elucidated in order to understand the mechanisms of cancer progression, to attain the diagnosis, and to develop a cancer treatment. In the case of the reproductive organs, hormonal stimulation is also critically involved in carcinogenesis. The sex steroid hormones, estrogen and progesterone (Pg), play an important role in normal mammary gland development, and it is believed that breast cancer progression is influenced by these hormones and their receptors (Knight et al. 1980, Kester et al. 1987, Clarke & Sutherland 1990, Horwitz 1993). Morphologic mimicry between salivary gland tumors (SGTs) and breast tumors is a well-known phenomenon in the field of surgical pathology. Human SGT has been reported to have some similarity with mammary gland tumor with regard to its histology and steroid hormone receptor status (Wick et al. 1998). Moreover, SGTs occur more frequently in females (Lima et al. 2005, Toida et al. 2005), and the adenoid cystic carcinomas (ACCs) of the salivary glands show remarkable similarity to the ACCs of the breasts (Pia-Foschini et al. 2003). Some studies have shown the possibility of the involvement of steroid hormone receptor in SGT progression (Ozono et al. 1992, Shick et al. 1995, Teymoortash et al. 2001, Toida et al. 2005). Therefore, we hypothesized that some hormonal regulations are involved in their pathogenesis.
The reported 5-year survival rates of the patients with salivary ACC were not low; however, the 10- to 20-year survival rates were extremely poor (Jones et al. 1997, Spiro 1997, Lopes et al. 1998) because of the gradual tumor progression in the primary and metastatic sites. Malignant SGTs are highly aggressive neoplasms that invade adjacent tissues and metastasize to distant organs at an early stage of the disease (Lopes et al. 1998, Lima et al. 2005). Particularly, it is extremely difficult to salvage the patient in the case of recurrent ACC, because of the ineffectiveness of radiotherapy and chemotherapy or the limitation in performing wide surgical resection due to cosmetic and anatomic reasons (Marandas et al. 1990, Takagi et al. 2001). Therefore, a new treatment modality for SGTs needs to be introduced. We have already reported that in human aggressive breast cancer cells, introduction of Pg receptor (PR) after treatment with Pg is sufficient to reduce the malignant phenotypes (Sumida et al. 2004). Therefore, we hypothesize that the same event in terms of Pg–PR system is observed in SGTs. The loss of PR may induce the expression of tumorigenic proteins, whereas the reintroduction of PR may function to suppress human SGT progression. We used the PR-deficient human ACC cell line to clarify the role of Pg and its receptor on the aggressive phenotype, and reintroduced PR into this cell line. In this study, we demonstrate the possibility for treating ACC with Pg-based hormonal therapy as a completely novel approach for controlling SGT.

Materials and methods

Cell culture

ACCM, a human cell line derived from an ACC of the salivary gland (Guan et al. 1997), was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Science (Shanghai, China). Cells of the human breast cancer cell lines (T47D, MDA-MB231) were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). They were cultured in phenol red-containing RPMI-1640 (from the University of California, San Francisco) that was supplemented with 1, 5, or 10% charcoal-stripped fetal bovine serum (FBS) and insulin (5 μg/ml; Sigma Chemical Co.) at 37 °C in the presence of 5% CO₂. FBS was omitted in the experiments in which serum-free medium was used. ACCM cells lack PR and estrogen receptor (ER), therefore, the estrogenic effect of phenol red is considered to be disregarded in this study.

Introduction of PR into the ACCM cells

Vectors hPR1 and hPR2 contain human PR cDNA coding for isoforms, PR-B and PR-A respectively in pSG5 plasmid (generously donated by Prof. P Chambon, Institute of Genetics and Molecular and Cellular Biology, Strasbourg, France; Kastner et al. 1990). Vector pBK-CMV (Stratagene, La Jolla, CA, USA) that contains the neomycin-resistant gene was cotransfected with hPR1 and hPR2 into the ACCM cells using LipofectAMINE PLUS Reagent (Life Technologies Inc). Neomycin-resistant clones were selected in the medium containing G418. The cells that were cotransfected with empty pSG5 and pBK-CMV were used as the control cells. We isolated 32 clones containing a control clone and both PR isoforms. After determining the protein content of PR-A/PR-B by western blotting, we selected five populations of ACCM cells that expressed various levels of PR.

Chemicals

Pg was obtained from Sigma Chemical Co. Cells were treated with Pg stock solution in ethanol. Most of the experiment was performed in 10 ml culture medium under the concentration of 10 nM Pg, therefore, in these cases, the stock solution of 10 μM Pg in ethanol was used. This gave a final concentration of ethanol of 0.1% in the medium. Treatment controls received 0.1% ethanol only. For thymidine incorporation assays, the cells were treated with ethanol or Pg once daily, and for other assays, this treatment was repeated twice daily.

Western blot analysis

The cells were lysed in 2× Laemmli buffer (Maniatis et al. 1989) and stored at −70 °C. The protein concentration was determined using the DC (detergent compatible) protein assay kit (Bio-Rad). The samples (30 μg) were separated by SDS-PAGE and were transferred onto a PVDF (poly vinylidenedifluoride) membrane (Hybond P, Amersham) by standard methods (Maniatis et al. 1989). The membrane was blocked for 1 h at room temperature with TBST (20 mM Tris base, 137 mM NaCl, 3.8 mM HCl, and 0.1% Tween 20) containing 10% nonfat milk. Next, it was incubated for 1 h with a rabbit polyclonal antibody specific for Id-1 (Z-8; Santa Cruz Biotechnology), MDA-MB231), PR-A or PR-B (C-20; Santa Cruz Biotechnology), p21 (187; Santa Cruz Biotechnology), c-myc (9E10; Santa Cruz Biotechnology), or actin (C4; Chemicon). The membrane was washed and incubated with secondary antibody
(goat anti-rabbit or anti-mouse IgG-horseradish peroxidase; Santa Cruz Biotechnology), washed again, and then developed for enhanced chemiluminescence using the Amersham ECL or ECL-plus kit, according to the manufacturer’s instructions.

**Immunofluorescence microscopy**

Cells were grown on glass cover slips in six-well plates and treated with Pg or ethanol for 48 h. After rinsing with PBS, the cells were fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 for 10 min. This was followed by incubation with 2% normal horse serum in PBS for 1 h to block non-specific binding. All the subsequent incubations with antibody were carried out in PBS containing 2% normal horse serum. Antibody to rhodamine phalloidin (R-415; Molecular Probes) and vinculin (V9131-2ML; Sigma) were incubated with the cells overnight at 4°C, followed by incubation with FITC-conjugated goat anti-mouse IgG at room temperature for 1 h. After washing in PBS, the cover slips were mounted on slides with fluorescence mounting media. Stained cells were viewed and photographed using the confocal laser scanning microscope.

**Flowcytometric analysis**

The distribution of the cell cycle was measured by flowcytometric analysis. Samples for flowcytometric analysis were prepared from the cultured cells using a Cycle TESTTM PLUS DNA reagent kit (Becton Dickinson Immunocytometry Systems, Ventura, CA, USA). Flowcytometric analysis was performed in a FACScalibur flowcytometer equipped with a Cell QUEST software program (Becton Dickincon & Co., Mountain View, CA, USA). Analysis was carried out on $2 \times 10^4$ nuclei per sample using ACCM-PRCL1 cells.

**[3H]thymidine incorporation assay**

The cells were cultured on cover slips in 1% serum, and either ethanol or Pg was added at 24 and 48 h before harvesting. [3H]thymidine (10 $\mu$Ci/ml; 60–80 Ci/mmole; Amersham) was added to the cells during the last 16 h, and the cells were fixed with 1:1 methanol/acetone (v/v). The cells were treated with either ethanol or Pg for total 48 h. The cell nuclei were stained with DAPI diluted to 1:10 000 in PBS. [3H]thymidine labeling was developed as described previously (Desprez et al. 1998). The percentage of labeled nuclei was calculated by comparing the number of [3H]thymidine-labeled nuclei with the number of DAPI-stained nuclei in a given field using light and fluorescence microscopy.

**PCR-based telomerase assay**

Telomeric repeat amplification protocol assay was performed as described by Kim et al. (1994), with minor modifications. The protein was extracted from the cells that were cultured in the medium with 10% serum for 72 h with either ethanol or Pg. We analyzed 0.6 $\mu$g protein extracts. An internal control was used for the semiquantitative estimation of telomerase activity levels and identification of false-negative tumor samples containing Taq polymerase inhibitors. A 15 $\mu$l aliquot of the PCR products was electrophoresed on a 12% non-denatured polyacrylamide mini gel. The gel was stained with SYBR Green 1 nucleic acid gel stain (diluted to 1:10 000; FMC Bioproducts, Rockland, ME, USA) for 30 min. The stained gel was observed with an u.v. transilluminator.

**Reverse transcriptase PCR analysis**

For c-DNA synthesis, we used 3 $\mu$g of the total RNA from the cells, which were cultured in medium with 10% serum for 72 h with either ethanol or Pg. The primer design for the telomerase catalytic subunit, hTERT (human telomerase reverse transcriptase), and PCR conditions have been described previously (Sumida et al. 1999). GAPDH mRNA served as an internal control for the reaction. A 10 $\mu$l aliquot of the PCR products was electrophoresed on a 1% agarose gel with ethidium bromide. The expression of hTERT-mRNA relative to the housekeeping gene GAPDH was defined as the respective expression level.

**Matrigel invasion assay**

Invasion assays were performed in modified Boyden chambers with 8 mm pore filter inserts for 24-well plates (Becton Dickinson Labware). Filters were coated with 12 $\mu$l ice-cold Matrigel (7.5 mg/ml protein; Becton Dickinson Labware). Cells ($5 \times 10^4$ per well), which were pretreated with ethanol or Pg for 72 h, were added to the upper chamber in 200 $\mu$l of the appropriate medium containing 5 $\mu$g/ml insulin. The lower chamber was filled with 300 $\mu$l NIH-3T3 cell-conditioned medium. After a 16 h incubation, cells were fixed with 2.5% glutaraldehyde in PBS and were stained with 0.5% toluidine blue in 2% Na2CO3. Cells that remained in the Matrigel or attached to the upper side of the filter were removed with cotton tips. Cells on the lower side of the filter were counted using light microscopy. Assays were performed in triplicate and the results were averaged.
Results

PR introduction into the ACCM cells

As described in Materials and methods, parental ACCM cells were transfected with both isoforms of the human PR (PR-A and PR-B). Using western blot analysis, we screened all the clones that were isolated from the transfected cells. We selected five cell populations from the many clones that expressed various levels of PR-A and PR-B (Fig. 1A). ACCM-PRCL5 also has a very unclear faint band of PR-B. As controls, we used the T47D cells, which express both isoforms of the human PR, and the MDA-MB231 control cells, which express none. The lower band observed in T47D and ACCM-PRCL1 is the N-terminally truncated form.

Morphological change in the PR-transfected ACCM cells after Pg treatment

Following the Pg treatment, the PR transfectants showed drastic morphological change: the transfectants appeared more flattened and spread out when compared with the ACCM control cells (Fig. 1B, panel B and E). The morphological changes began at ~8 h after treatment, stopped occurring 16–24 h after treatment; the changes continued to occur as long as Pg was added. The PR transfectants treated with the vehicle alone did not show any morphological change (panel D). Moreover, there were notable increase in focal contacts and stress fibers in PR transfectants with Pg treatment (panel F) compared with ACCM control cells (panel C).

Figure 1 (A) The expression of progesterone receptor (PR) isoforms in T47D, MDA-MB231, control ACCM, and PR-transfected ACCM clones. The proteins were extracted and PR was analyzed by western blotting, as described in Materials and methods. PR was detected using a polyclonal antibody against human PR-A and PR-B. The position of the two isoforms of PR is indicated. Actin was used as a control for protein integrity and quantitation. (B) Morphological changes of the PR-transfected cells due to the presence of progesterone (Pg). The cells cultured in 10% serum were photographed after a 48 h treatment with either ethanol (Et) or Pg. Panels A and B, ACCM-pSG5CL1 cells, which are ACCM cells transfected with pSG5 empty vector; D and E, ACCM-PRCL1 cells, which are cells that express both PR-A and PR-B; C and F, ACCM-pSG5CL1 and ACCM-PRCL1 cells respectively which are co-stained with actin filaments (red) and vinculin (green).
Pg reduces the proliferation of PR-transfected ACCM cells

To investigate the relationship between the PR isoform expression and the proliferative activity of these cells, we determined the proliferation rate by thymidine incorporation assay. A significant reduction in the proliferative activity of the transfectants was observed after Pg treatment (Fig. 2A). The percentage of labeled nuclei reduced significantly in the PR transfectants (Fig. 2B).

This Pg-induced inhibitory effect was dose dependent. The effect of various concentrations of Pg on thymidine incorporation is shown in Fig. 3A. Pg had no effect on the cells transfected with control empty vector. On the other hand, all clones showed significant reduction in the proliferative activity after 48 h of treatment in a dose-dependent manner. Figure 3B illustrates the effect of Pg in time course. We can observe the inhibitory effect of Pg after 24 h of treatment; it reaches a plateau after 48 h. This inhibitory effect continued to occur as long as Pg was added.

However, the PR transfected cells cultured on poly(2-hydroxyethyl methacrylate) did not show significant difference in proliferation between vehicle treatment and Pg treatment (data not shown). Therefore, we considered that the inhibition of proliferation needs the change of the cell shape.

Effect of Pg on cell cycle kinetics

The growth-inhibitory effect of Pg in the PR-transfected ACCM cells was associated with dose-dependent reductions in the percentage of the S-phase cells along with an increase in the G0–G1 phase cells (Fig. 4A).

Pg inhibits cell cycle by the downregulation of Id-1 and c-myc proteins and the upregulation of p21

The inhibitory effect of Pg on the proliferative activity of the ACCM cells transfected with both isoforms of PR was investigated by extracting the cell protein at different time points and by western blot analysis. As shown in Fig. 4B, the expression of Id-1 and c-myc was downregulated in three different clones after Pg treatment. Conversely, p21 expression level was upregulated after treatment. This upregulation was observed within 24 h after starting the treatment; it continued to occur as long as Pg was added. Thus, the expression of these cell cycle-associated proteins were reasonable and almost the same as we expected. Id-1 and c-myc were usually upregulated in the phase of the cell proliferation; on the other hand, p21 can retard cell cycle. We also determined the telomerase activity and hTERT-mRNA expression as another proliferative marker. There was no significant change after 72 h of Pg treatment (Fig. 4C).

Pg reduces invasive property

To investigate the effects of Pg on invasive properties, cells were plated in Matrigel invasion chambers. After
72 h pre-treatment with Pg and ACCM-PR cells that contain both PR isoforms, the invasive capacity of the cells was markedly inhibited (Fig. 5). There was no Pg effect on invasiveness in cells transfected with the control empty vector.

ACCM cells regain their proliferative activity and morphology after the withdrawal of Pg

Next, we determined that the Pg-induced inhibitory effect is reversible after the withdrawal of Pg. After 48 h of Pg treatment, the PR-transfected ACCM cells showed significant reduction in the proliferative activity, as shown in Fig. 2A. However, after the cells were cultured in a medium without Pg, we observed the restoration of the proliferative activity. After 48 h of Pg withdrawal from the culture, the proliferative activity was restored to approximately the same level as that of the empty vector-transfected control cells (Fig. 6A). Moreover, as shown in Fig. 1B, following the Pg treatment, PR transfectants showed drastic morphological changes. However, after the withdrawal of Pg, the original round morphology was also restored (Fig. 6B).

Discussion

The Pg–PR system plays an important role in various gynecologic malignant tumors (Kauppila et al., 1982, McGuire & Clark, 1985, Munstedt et al., 2000). In patients with breast cancer, the level of these steroid hormone receptors is a strong prognostic factor and has been used in clinical management as an indicator of endocrine responsiveness (Knight et al., 1980, Horwitz, 1993). Many advanced breast cancers are negative for PR and ERs, and hence fail to respond to endocrine therapy (Osborne et al., 1980, Young et al., 1980). Moreover, it has been recently reported that the lung cancer also expresses PR, and the PR status is associated with better clinical outcome of the patients (Ishibashi et al., 2005). Although progestins are
Currently used for treating advanced breast cancer (Sitruk-Ware et al. 1999), their effect on the malignant phenotype remains controversial. Depending on the tissue type, Pg is classified as a proliferative or a differentiative hormone (Graham & Clarke 1997, Groshong et al. 1997). PR is a member of the nuclear receptor superfamily, which specifically regulates the expression of target genes in response to hormonal stimulus. In most rodents and humans, PR exists in two isoforms, PR-A and PR-B. These isoforms are produced from a single gene by translation initiation at two distinct start codons under the control of separate promoters (Wen et al. 1994). The T47D cell line has high levels of PRs and ERs (Lin et al. 2000a); therefore, this cell line is widely used as a model system to study the effects of sex steroid hormone on human breast cancer cells. A poorly differentiated breast cancer cell line, MDA-MB231, does not contain PR and possesses only small amounts of endogenous ER-b isoform (Tong et al. 2002), this characteristic, therefore, makes the cell line a good model for hormone-independent aggressive breast cancer. On the other hand, only limited human SGT cell lines are available, and no good model to study the Pg–PR system is established. Thus, in the present study, PR-deficient aggressive ACC cells were used, and some cell populations with PR expression were obtained from the parental cells. We have already reported that in human aggressive breast cancer cells without PR, reintroduction of PR after the Pg treatment is sufficient to reduce the malignant phenotypes (Sumida et al. 2004). Hence, we hypothesize that PR also plays an important role in SGTs. This is because some investigators have reported that SGTs often express PR (Ozono et al. 1992, Shick et al. 1995, Wick et al. 1998, Teymoortash et al. 2001), and determined if it would be possible to reduce the aggressive phenotypes of SGTs following the introduction of PR in the ACCM cells after Pg treatment.

The cells used in this study were derived from human ACC, which is one of the most common malignant tumors of the major and minor salivary glands (Szanto et al. 1984). This tumor has a tendency to grow gradually; however, compared with other

Figure 5 The indicated cells were treated with Pg for 72 h and then assayed in Matrigel invasion as described in Materials and methods. Bars represent the method percentage in three independent assays with standard error. The percentage of all the cells treated with ethanol was defined as 100%.

Figure 6 Reversion assay of the effect of progesterone (Pg) on ACCM-PRCL1 cells. (A) The results of the proliferation assay after reverse treatment. The cells were cultured in a medium with 5% serum and treated with Pg for 48 h. Furthermore, the cells were cultured in a medium with 5% serum and treated with only solvent for 48 h. [3H]thymidine was added during the last 16 h. The percentage of labeled nuclei of all the cells treated with control solvent was normalized as 100%. (B) Morphological changes of the PR-transfected cells when the cells were first treated with Pg and then cultured in a medium without Pg for 48 h. Panels A and B, ACCM-pSG5CL1 cells; C and D, ACCM-PRCL1 cells, which express both PR-A and PR-B.
malignant SGTs its long-term survival rate is extremely low (Spiro et al. 1974). Although ACC shows various histological findings, distinguishing its invasive nature is difficult. In fact, ACC is reported to be resistant to chemotherapy, and radiotherapy has enabled better control of the lesion focus in only some patients (Marandas et al. 1990, Takagi et al. 2001). To date, surgical resection is considered to be the best primary treatment for patients with ACC. However, it occasionally becomes difficult to perform this surgery with sufficient surgical margins because there are several cosmetic and functional limitations. Therefore, we need to introduce a new treatment modality for patients with ACC.

First, we obtained cell populations with various levels of PR expression from PR-deficient ACCM cells; Pg treatment affected these cells in various ways. Significant reduction in cell proliferation was observed after Pg treatment. This effect was observed even at extremely low concentrations of Pg and was dose dependent. In time course studies, the inhibitory effect of Pg began after 24 h of treatment. To investigate further details, the PR-transfected cells were analyzed by flowcytometry. The growth inhibitory effect of PR-transfected cell lines was associated with the reduction in the S-phase cells along with an increase in the G0–G1 phase cells. Furthermore, we noted that Pg-induced growth inhibition was accompanied with a reduction in the cell cycle-associated protein. Pg-induced growth inhibition was accompanied by p21 upregulation, which generally plays a critical role in regulating cell growth inhibition, and by c-myc and Id1 expression, which usually accelerates cell growth. These changes in the ACCM cells after Pg treatment have also been observed in human aggressive breast cancer cells (Sumida et al. 2004); therefore, we believe that the same mechanisms with regard to cell proliferation must exist in both salivary and breast cancer cells. Some investigators have indicated a relationship between Pg–PR and human telomerase activity and telomerase reverse transcriptase expression, which are also considered the proliferative marker (Wang et al. 2000, Lebeau et al. 2002). However, in this study, no significant change with regard to the telomerase activity was observed between the cells treated with Pg and the control cells. Therefore, telomerase may not be involved in Pg signal transduction in human ACC cells. Based on these observations, we can conclude that Pg acts as a suppressor of cell proliferation via PR by regulating some proteins that are associated with the proliferation of the ACC cells.

Drastic morphologic changes were observed after Pg treatment. Further, identical changes were observed in the PR-introduced breast cancer cell lines (Sumida et al. 2004), and these morphologic changes were induced by the formation of new stress fibers called focal adhesion assemblies (Lin et al. 2000b). We believe that these morphologic changes may explain the decrease in the malignant potential of these cells. Therefore, we are now examining these morphological changes in greater detail. Actually, the PR transfected cells cultured on poly(2-hydroxyethyl methacrylate) did not show the difference in proliferation between vehicle treatment and Pg treatment (data not shown). Therefore, we considered that the inhibition of proliferation needs the change of the cell shape. On the other hand, the effect of the cell shape change on invasion is still unclear. Lin et al. (2001) reported that Pg could inhibit the invasiveness of PR-transfected MDA-MB231 cells because of the downregulation of urokinase plasminogen activator and upregulation of tissue-type plasminogen activator. We also observed the decrease in the invasiveness of PR-transfected MDA-MB231 cells by Matrigel invasion assay (Sumida et al. 2004). In this study, we have already confirmed the decrease in the invasiveness of PR-transfected ACCM cells after Pg treatment by Matrigel invasion assay.

It is known that female sex steroid hormones play an important role in the growth and development of gynecologic cancers. Anti-estrogenic Tamoxifen is presently used as a well-accepted drug for the treatment of receptor-positive breast cancer (Rove 1989, Osborne & Fuqua 1994). In contrast, there is no other effective second-line therapy besides surgery for human malignant SGTs. Some new strategies for the treatment of SGTs were proposed. For example, it was reported that differentiation therapy (Sato 1993), adoptive immunotherapy (Ueta et al. 1998), and gene therapy (Zhang et al. 2001) might be a new aspect for treatment of SGTs. Thus, in this paper, we have demonstrated Pg-based hormonal therapy as a completely new therapeutic possibility for SGTs. Further, we believe that considerably low doses of Pg or even physiological circulating level of Pg may effectively inhibit cancer growth because of a high inhibitory potential of Pg in these PR-transfected cells.

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