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Mode of Growth Determines Differential Expression of Prostasomes in Cultures of Prostate Cancer Cell Lines and Opens for Studies of Prostasome Gene Expression

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Abstract

The exocrine secretion of the acinar gland cells in the human prostate consists of, among other components, a serous secretion and prostasomes. The prostasomes are functionally associated with both reproduction and prostate cancer development and are capable to raise autoantibodies at various pathologies. Therefore, we are trying to characterize prostasome antigens by analysing prostasome-producing cell lines of prostate cancers with the cDNA microarray technique. To obtain one state with synthesis of prostasomes and another state without synthesis, we checked whether the prostasome differentiation was influenced by the mode of growing the cells, that is, whether the cells had been growing on a solid support or on a flexible one.

We studied the expression of prostasomes in the cell lines PC3, DU145 and LNCaP. We grew the cells with the following methods: Monocellular layers on microbeads, multicellular spheroids, single cells in suspension cultures, and xenotransplants in nude rats. The presence of prostasomes was examined by ELISA, immunocytochemistry or electron microscopy.

The results showed that growing the cells on microbeads (solid support) produced a differentiation of prostasomes, while growing the cells in multicellular spheroids (flexible support) did not. Thus it should be possible to apply cDNA microarray analyses for characterizing the genes which are active at the cellular expression of prostasomes and then deduce the prostasome antigens.

Introduction

The exocrine secretion of the acinar gland cells in the human prostate consists of, among other components, a serous secretion and small bodies (prostasomes) both of which are located within storage vesicles in the supranuclear parts of the cells. The prostasomes are complex structures and exert several abilities, which are associated with both reproduction and prostate cancer development (1). This has made them interesting in our research on immunocontraception (2) and prostate cancer metastases (3). As a further step in these studies, we have work in progress to find ways to map the prostasome genes.

The prostasome gene expression can be deduced by, among other methods, cDNA microarray analyses. In this case, two states of growth of the prostate cells have to be compared: one state with synthesis of prostasomes and another state

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without synthesis. As yet, though, no culturing technique of prostate cancer cell lines for achieving this has been reported.

Various cellular differentiation parameters of cultured cells, however, can be influenced by the mode of growing the cells. For instance, the expression of differentiation markers in cultured cells of the choriocarcinoma cell-line BoWo was influenced by the type of support for the growing cells (4), that is, whether the cells had been growing on a solid support or on a flexible one. Now, we know that the cells of prostate cancer lines do produce prostasomes when growing as monolayers on the solid support of Petri dishes (5) and that these prostasomes have properties similar to those of seminal prostasomes (6, 7). Therefore, we have checked whether also the synthesis of prostasomes is dependent upon the type of cell support during culturing, that is, whether the prostasome differentiation could be influenced by the mode of growing the cell lines.

We studied the expression of prostasomes in the cell lines PC3, DU145 and LNCaP. We grew the cells with the following methods: 1. Monocellular layers on microbeads. 2. Multicellular spheroids. 3. Single cells in suspension cultures. 4. Xenotransplants in nude rats. The presence of prostasomes was examined by ELISA, immunocytochemistry or by electron microscopy. The use of electron microscopy was required due to the small size of the prostasomes.

Materials and Methods

Cell lines

The human prostate carcinoma cell lines PC3, DU145 and LNCaP were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in RPMI 1140 cell culture medium containing 5 µg/mL phenol red and supplemented with 10% heat-inactivated fetal calf serum, 1% glutamine, 100 µg/mL penicillin and 100 ug/mL streptomycin.

Growth on solid support

Growth on microbeads

The cells were grown on Cytodex beads 1, 2 and 3 (Pharmacia Fine Chemicals, Uppsala , Sweden) in culture dishes (100 mm) of tissue culture plastic (Falcon, Becton Dickinson) at 37°C in humidified atmosphere of 5% CO_2 in air using the liquid-overlay technique (see later). Cell carpets were obtained after about a week. The beads were saved by centrifugation. The cells were released by trypsination, washed twice in PBS and frozen at –70°C.

Frozen prostate cancer cells were thawed and pooled, and the suspension of disintegrated cells was centrifuged at 1500 x g for 30 min. The supernatant obtained was subjected to a second centrifugation at 6000 x g for 20 min. The new supernatant containing prostasomes was ultracentrifuged at 100,000 x g for 2 h. The pelleted material was suspended in isotonic Tris-HCl buffer, pH7.6, and run through a

Sephadex G200 column (Amersham Biotech., Uppsala, Sweden) at 6 mL/h, and 2 mL fractions were collected. The isotonic Tris-HCl buffer was the eluant, and the eluate was read at 260 and 280 nm. The fractions with initially elevated UV absorbances and with a positive reaction on the anti-prostasome mAb 78 were pooled and ultracentrifuged at 100,000 x g for 2 h. Isotonic Tris-HCl buffer was added to the pellet, and the resulting mixture was regarded as suspensions of prostasomes.

For enzyme-linked immunosorbent assay (ELISA), plates were coated with the prepared prostasomes diluted in 100 mM $NaHCO_3$, pH 9.5 (coating buffer), for 2 h at 37º C. The plates were blocked overnight at 4º C with coating buffer containing 3% BSA, then washed 3 times with 200 µL PBS-0.05% Tween 20 (PBS-T) and incubated with either a monoclonal mouse anti-prostasome antibody or a polyclonal hen anti-prostasome antibody, both biotin conjugated, for 2 h at 37º C. After 3 washings with 200 µL of PBS-T, the plates were incubated with Streptavidin-alkaline phosphatase conjugate (1:500 in PBS) at 37º C for 1 h and then washed 3 times with 200 µL of PBS-T. Finally, 200 µL of alkaline phosphatase substrate solution (1mg/mL p-nitrophenyl phosphate in 1M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8) were added and the plates were incubated for 25 min at room temperature in the dark. The reaction was stopped by 50 µL of 5M NaOH. Absorbance was measured at 405 nm in an ELISA reader system (Spectra Max 250, Molecular Devices, Sunnyvale, CA, USA).

Growth on flexible support

Multicellular spheroids

Liquid-overlay technique was used to obtain the spheroids (8–10). In short, tissue culture dishes were coated with 0.5 mL of 1% agarose (Sigma, St Louis, USA) that was boiled in sterile RPMI-1640 without serum. Cells of PC3 or DU145 were plated in RPMI-1640 supplemented with 10% fetal calf serum and 10 mM HEPES (Sigma). Since the cells could not attach onto the agarose layer of the culture dishes, the cells adhered onto each other and formed cell-clumps, that is, multicellular spheroids. The spheroids were fed every 3 days. The size of the cell spheroids increased over time and reached, after about two weeks, a diameter of approximately 1 mm. By then, the cell spheroids were fixed in formalin or Bouin´s solution for 2 h. The specimens were embedded without delay according to the conventional techniques for immunocytochemistry or electron microscopy.

Single-cell suspension cultures

Single cells were noted in the culture medium of Petri dishes. These cells were embedded in paraffin, using small tubes, for conventional immunostaining with the DAB technique.

Xenotransplants

Cells from monolayer cultures of PC3, DU145 or LNCaP in Petri dishes, were scraped from the tissue culture dishes, washed free of growth medium, and injected

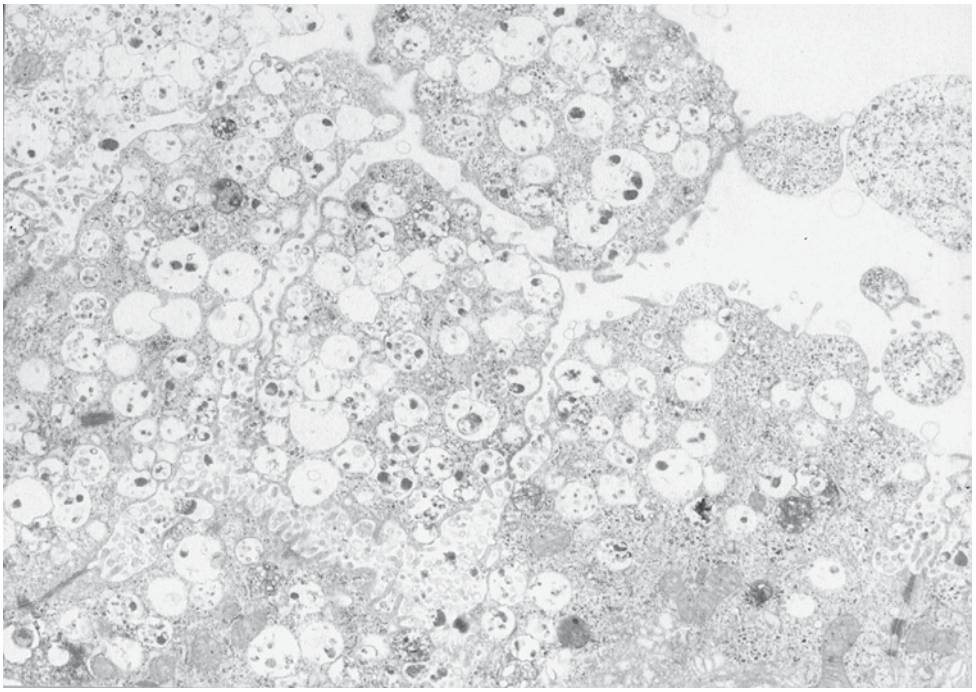

Figure 1. Apical parts of normal prostate epithelial cells. The cytoplasm is filled with numerous storage vesicles containing small vesicles and dark bodies, i.e. prostasomes. Mag. 9000 X.

s.c. into the flank region of nude rats. When the tumours were palpable, they were dissected free after various times to obtain tumours of various sizes. The specimens were processed for immunostaining or electron microscopy.

Immunocytochemistry

The following protocol, rinsings excluded, was used for immunostaining.

1. Blocking with 3% BSA and 1% normal horse serum in PBS for 30 min
2. Primary antibody, being undiluted mouse monoclonal IgG1 mAb78 supernatant for 60 min
3. Secondary antibody, being biotinylated horse anti-mouse IgG, diluted 1:200 (Sigma) for 60 min
4. Alkaline phosphatase complex (Dako Pat) for 30 min
5. Substrate Vector Red or DAB for appropriate times

Positive controls were sections of human prostate gland and negative controls were obtained by omitting the primary antibody.

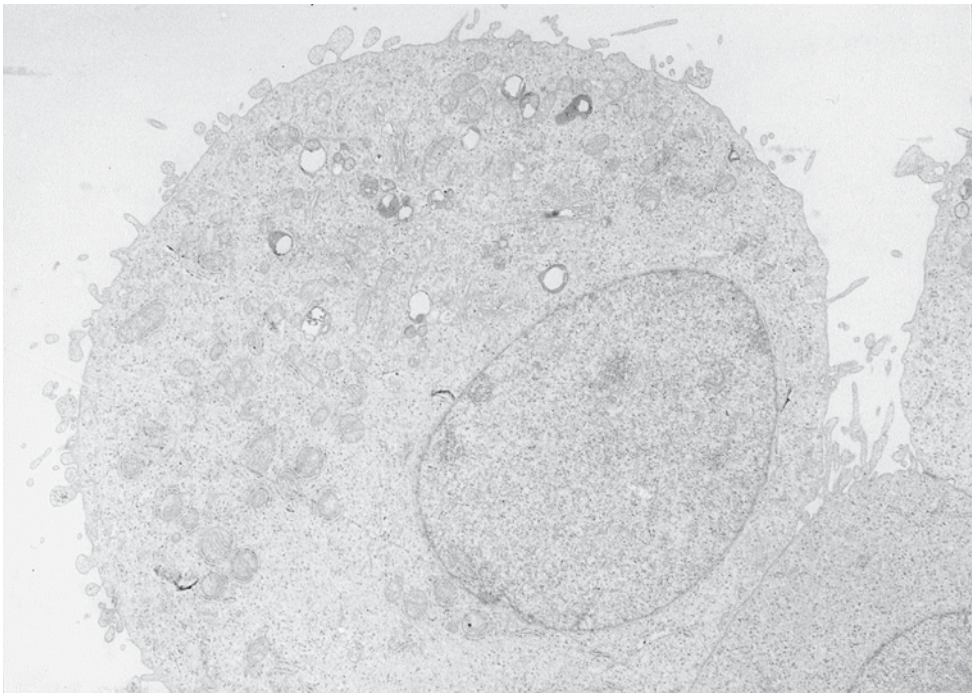

Figure 2. A cell from the prostate cancer cell line PC3 growing at the periphery of a multicellular spheroid. The vacuoles are remnants of dissolved lipid granules, and no storage vesicles containing prostasomes are visible. Mag. 6000 X.

Results

Ultrastructure of normal prostate cells

Electron microscopy of normal prostate epithelium shows that the apical parts of the cytoplasm are filled with numerous storage vesicles containing small, dark bodies, i.e. prostasomes (Fig. 1).

Growth in Petri dishes

The general morphology of the growing monolayers were similar to earlier reports (11–13). At confluence, the cultures showed a continuous carpet of cells, but eventually small grape-like groups of cells appeared on the monolayers.

Trypsinized monolayers of PC3, DU145 and LNCaP cells, prepared as cytospin specimens and immunostained with monoclonal anti-prostasome antibody, demonstrated that most cells expressed prostasomes and secretion but that scattered cells or small groups of cells did not show any prostasomes.

Growth on microbeads

PC3 cells grown on three types of Cytodex beads were tested by ELISA for the presence of secretion. Binding of anti-prostasome antibody was demonstrated in

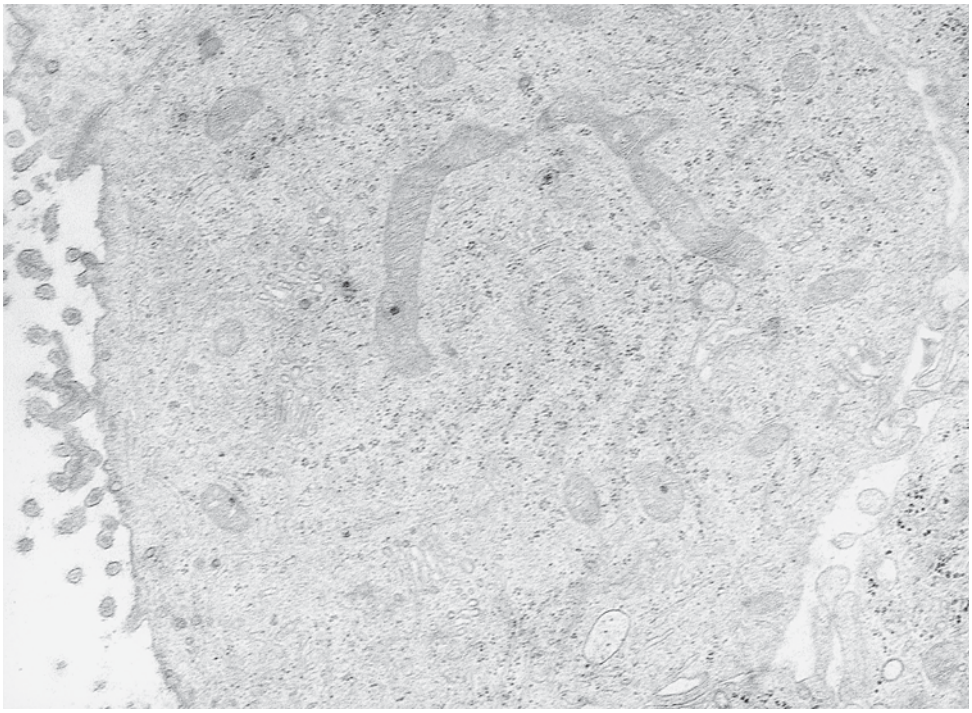

Figure 3. Part of the cytoplasm of a cell from the cancer cell line DU145 growing in the periphery of a multicellular spheroid. The surface of the spheroid is observed to the left, and an intercellular space to a neighbouring cell is noticed at the right side of the picture. The cytoplasm contains the ordinary organelles and inclusions but no storage vesicles with prostasomes. Mag. 13,000 X.

preparations from cells grown on Cytodex 2 and 3, with Cytodex 3 having the highest absorbance value (1.45) compared to Cytodex 2 (0.38). Preparations from cells grown on Cytodex 1 beads did not show any significant increase in absorbance value compared to negative controls.

Multicellular spheroids

Electron microscopy of the spheroid-like structures demonstrated that none of the cell lines of PC3 (Fig. 2), DU145 (Fig. 3) or LNCaP cells contained storage vesicles with prostasomes.

Suspension cultures of single cells

Immunostaining of paraffin-embedded cells demonstrated that some cells contained secretion, while others did not.

Xenotransplants

Most of the mice inoculated subcutaneously with cells of the prostate cancer lines PC3, DU145 or LNCaP developed tumours. The tumours consisted of lobular

groups of cells surrounded by strands of host connective tissue, which in addition formed a capsule around the tumour. No storage vesicles containing prostasomes were observed in the cells of the transplanted cell lines.

Discussion

The apical parts of the normal prostate epithelium contain numerous storage vesicles with secretion and prostasomes. Also cell lines of prostate cancers, grown on the solid support of Petri dishes, are known to produce prostasomes (5). In the present experiments, we grew the prostate cell lines on the solid support of Sephadex microbeads and found that the cells also then were expressing prostasomes. However, when the cells were growing in multicellular spheroids or as xenographs and thus being offered only the flexible support of their cell neighbours, we could not demonstrate any prostasomes. Thus, the prostasome differentiation of the cancer cell lines PC3, DU145 and LNCaP is influenced by the mode of growth of the cells.

Cells growing in culture dishes, prepared by the cytospin technique and then immunostained contained secretion and prostasomes, although some single cells did not (5). We assume that these cells had been squeezed-out from confluent monolayers and by loosing the solid support of the Petri dish, the cells ceased to make prostasomes. Consequently, studies of paraffin-embedded single cells from the cells suspended in the culture medium showed that some cells contained secretion while others did not. Thus, there is a gradual slowing down of the secretory activity, when the cells loose their attachment to a solid support and begin living as free-floating cells or as aggregated cells in cell spheroids.

Xenotransplants of cancer cell lines grown in immune-deficient mice or rats are often considered as counter-parts to poorly differentiated metastases of prostate cancers (14,15). However, poorly differentiated prostate cancer cells in metastases contain cells which still have the ability to produce prostasomes (16, 17), but our xenotransplants did not demonstrate any prostasomes-containing cells. Thus, the influence of the mode of growth on the expression of the differentiation markers studied has to be considered when planning experiments involving prostate cancer cell lines.

Differentially expressed genes related to prostate cancer have been studied in prostate cancer cell lines with various aims (for references, see 18). For instance, by comparing two LNCaP cell lines, one androgen-dependent and the other androgen-independent, the gene expression changes during prostate cancer progresssion could be evaluated (19, 20).

Our results disclose that the genes coding for the prostasome synthesis are differentially expressed due to their mode of growth, that is, prostasomes are expressed by cells growing on solid supports but not on flexible supports. Thus, for exemple, by comparing a cancer cell line grown on microbeads (solid support) with the same cell line grown as multicellular spheroids (flexible support), it should be possible

to study the expression of prostasome genes and co-expressed genes by cDNA microarray screening.

Additional methods to deduce the prostasome gene expression are also available, for instance, mass spectrometry and screening of cDNA libraries. Each of these techniques has its drawback and advantage. Thus, mass spectrometry requires preparation of the prostasomes with the risk of loosing some prostasome components during the procedures. Screening of cDNA libraries can be time consuming, but the method defines the genes, which correspond to existing monoclonal anti-prostasome antibodies. cDNA microarray studies are based on whole cells containing unaffected prostasomes and can offer a broad list of genes active at the cellular expression of prostasomes.

Since we have shown that prostasomes can raise autoantibodies both at immunological infertility and in patients with metastasizing prostate cancer, well-characterized prostasome antigens would be a valuable tool in our current research. It seems that cDNA microarray analyses of prostasome genes followed by protein mappings (www.proteinatlas.org) offer a way to obtain specific prostasome polypeptides which can be used, for instance, in studies of anti-prostasome autoantibodies.

Acknowledgements

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References

1. Ronquist G, Nilsson O (2004) The Janus-faced nature of prostasomes: Their pluripotency favours the normal reproductive process and malignant prostate growth. Prostate Cancer Prostatic Dis 7: 21–31.
2. Carlsson, L., Larsson, A., Ronquist, G. and Nilsson, B.O. Prostasome antigens as targets for sperm agglutinating antibodies demonstrated by 1-D gel electrophoresis and immunoblottings. Int J Androl 27:360–367, 2004.
3. Nilsson BO, Carlsson L, Larsson A, Ronquist G (2001) Autoantibodies to prostasomes as new markers for prostate cancer. Upsala J Med Sci 105: 43–50.
4. Hohn H-P, Grummer R, Bosserhoff S, Graf-Lignau S, Reuss B, Bäcker C, Denker H-W (1996) The role of matrix contact and of cell-cell interactions in chorioncarcinoma cell differentation. Eur J Cell Biol 69: 76–85.
5. Nilsson BO, Lennartsson L, Carlsson L, Nilsson S, Ronquist G (1999) Expression of prostasome-like granules by the prostate cancer cell lines PC3, DU145 and LnCaP grown in monolayer. Upsala J Med Sci 104: 199–206.
6. Wang J, Lundquist M, Carlsson L, Nilsson O, Lundquist Ö, Ronquist G (2000) Prostasome-like granules from the PC3 prostate cancer cell line increase the motility of washed human spermatozoa and adhere to the sperm. Eur J Obstet Gynecol Reprod Biol 96: 88–97.
7. Carlsson L, Nilsson O, Larsson A, Stridsberg M, Sahlén G, Ronquist G (2003) Characteristics of human prostasomes isolated from three different sources. Prostate 54: 322–330.
8. Donaldson JT, Tucker JA, Keane TE, Walther PJ, Webb KS (1990) Characterization of a new model of human prostatic cancer: The multicellular tumor spheroid. Int J Cancer 46: 238–244.
9. Ballangrud M, YangWH, Dnistrian A, Lampen NM, Sgouros G (1999) Growth and characterization of LNCaP prostate cancer cell spheroids. Clin Cancer Res 5: Suppl., 3171s–3176s.

10. Santini MT, Rainaldi G (1999) Three-dimensional spheroid model in tumor biology. Pathobiol 67: 148–157.
11. Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF, Jones LW (1979) Establishment and characterization of a human prostatic carcinoma cell line (PC3). Invest Urol 17: 16–23.
12. Stone KR, Mickey DD, Wunderli H, Mickey GH, Paulson DF (1978) Isolation of a human prostate carcinoma cell line (DU145) . Int J Cancer 21: 274–81.
13. Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, Mirand EA, Murphy GP (1983) LNCaP model of human prostates carcinoma. Cancer Res 43: 1809–18.
14. Fizazi K, Navone NM (2005) Modèles précliniques de cancer de la prostate. Bull Cancer 92: 129–41.
15. Kinoshita Y, Kuratsukuri K, Newman N, Rovito PM, Kaumaya PT, Wang CY, Haas GP (2005) Targeting epitopes in prostate-specific membrane antigen for antibody therapy of prostate cancer. Prostate Cancer Prostatic Dis 8: 359–63.
16. Nilsson BO, Egevad L, Jin M, Ronquist G, Busch C (1999) Distribution of prostasomes in neoplastic epithelial prostate cells. Prostate 35: 36–40.
17. Sahlén G, Ahlander A, Frost A, Ronquist G, Norlén BJ, Nilsson BO (2004) Prostasomes are secreted from poorly differentiated cells of prostate cancer metastases. Prostate 61: 291–297.
18. Calvo A, Gonzalez-Moreno O, Yoon CY, Huh JI, Desai K, Nguyen QT, Green JE (2005) Prostate cancer and the genomic revolution: Advances using microarray analyses. Mutat Res 576: 66–79.
19. Vaarala MH, Porvari K, Kyllönen A, Vihko P (2000) Differentially expressed genes in two LNCaP prostate cancer cell lines reflecting changes during prostate cancer progression.Lab Invest 80: 1259–1268.
20. Zhao H, Kim Y, Lapoint J, Tibshirani R, Pollack JR, Brooks JD (2005) Genome-wide characterization of gene expression variations and DNA copy number changes in prostate cancer cell lines. Prostate 63: 187–197.

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