

Androgen Deprivation of the Prohormone Convertase-310 Human Prostate Cancer Model System Induces Neuroendocrine Differentiation¹

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ABSTRACT

Neuroendocrine (NE) cells are androgen-independent cells and secrete growth-modulating neuropeptides via a regulated secretory pathway (RSP). We studied NE differentiation after androgen withdrawal in the androgen-dependent prostate cancer xenograft PC-310. Expression patterns of chromogranin A, secretogranin III, and prohormone convertase-1 were analyzed at both protein and mRNA level to mark the kinetics of NE differentiation both *in vivo* and *in vitro*. PC-310 tumor-bearing nude mice were killed at 0, 2, 5, 7, 14, and 21 days postcastration. PC-310C cultures initiated from collagenase-treated tumor tissue could be maintained up to four passages, and androgen-deprivation experiments were performed similarly. PC-310 tumor volumes decreased by 50% in 10 days postcastration. Proliferative activity and prostate-specific antigen (PSA) serum levels decreased to zero postcastration, whereas PSA levels in PC-310C culture media first decreased and subsequently increased after 5 days. *In vivo*, androgen receptor (AR) expression decreased initially but returned to control level from 5 days postcastration on. CgA, secretogranin III, and secretogranin V expression increased *in vivo* from 5 days postcastration on. Subsequently, prohormone convertase-1 and peptidyl α -amidating monooxygenase as well as the vascular endothelial growth factor were expressed from 7 days postcastration on, and, finally, growth factors such as gastrin-releasing peptide and serotonin were expressed in a small part of the NE cells 21 days postcastration. The PC-310 tumors did not show colocalization of the AR on the NE cells in the tumor residues after 21 days.

As in the PC-310 xenograft, NE differentiation was induced and AR expression relapsed after prolonged androgen suppression in PC-310C. For PC-310C cells, this relapse was associated with the secretion of PSA. PC-310C is the first culture of human prostatic cancer cells having the NE phenotype. The PC-310 model system is a potential androgen-dependent model for studying the role of NE cells in the progression of clinical prostate cancer. Androgen deprivation of NE-differentiated prostate cancer may induce the formation of both NE- and AR-positive dormant tumor residues, capable of actively producing NE growth factors via a RSP, possibly leading to hormone refractory disease.

INTRODUCTION

NE³ differentiated cells form an androgen-independent subpopulation of the prostatic glandular cells (1). NE cells are considered to be nonproliferating cells that do not express the AR (2) and, therefore, are assumed to be unaffected by androgen deprivation. NE cells have been found in nearly all clinical prostatic adenocarcinomas in different frequencies (3–9).

Prostatic NE cells may regulate homeostasis and secretion of prostatic fluid, either actively or passively. NE cells can be identified by immunohistochemistry with specific antibodies against secreted products, for example, 5-HT (10), GRP, and secretion-associated proteins such as CgA (11–14), which is the commonly used marker for NE differentiation. NE cells show a heterogeneous cytokeratin expression pattern inasmuch as there are basal, luminal, and intermediate NE cell types (10, 15, 16). They are often found near Bcl-2 positive cells in prostate cancer (17, 18). Xue *et al.* (10) showed no coexpression of 5-HT with the anti-apoptotic oncogene *Bcl-2*. Likewise, we previously showed that CgA-positive prostate cancer cells did not coexpress Bcl-2 (19) and that there was a proliferation-independent induction of NE differentiation after androgen withdrawal. This induction did not allow progression of tumor growth of non-NE tumor cells after prolonged androgen suppression. Other studies revealed that androgen deprivation in clinical prostate cancer induced NE differentiation (20) or showed correlation of NE differentiation with progressive behavior (21). Most of the studies on the prognostic value of CgA for NE differentiation did not show a correlation between CgA-expressing NE cells and poor prognosis or progression of prostate cancer (4–9).

There are more markers of the NE phenotype next to CgA because NE-differentiated cells have an activated RSP (22) next to the lysosomal and an exocrine constitutive pathway. Along the RSP, pathway secretion and processing of bioactive neuropeptides and hormones such as insulin and glucagon in the pancreas (23, 24) are regulated. The RSP consists of a sequence of processes linked from the transcription/translation of various factors to the final secretion of neuropeptides at the plasma membrane from secretory granules (25). Different markers can be identified, such as the granular markers SgIII and 7B2 (26–28), carboxy peptidase E and the processing enzymes prohormone convertase 1 and 2 (PC1 and PC2). The enzyme PAM (29, 30) is expressed in, or in the near vicinity of, NE cells. Evaluation of these markers of the RSP is ongoing in *Xenopus laevis* and human lung and prostate cancer (31, 32).

The role of NE cells in the progression of prostate cancer toward androgen-independent growth is still unclear. Unfortunately, there are not many representative prostate cancer models with NE differentiation. Several groups have recently been developing prostate tumor models that involve both *in vitro* cell lines (33) and *in vivo* xenografts (34–36). The presently available prostate cancer cell lines lack the potency to differentiate into NE cells. NE differentiation was studied in the panel of *in vivo* human prostate cancer xenograft models developed at our institution. Some did not express the NE phenotype at all, whereas some lost their NE phenotype after a few passages in nude mice (37). It seemed that in the androgen-dependent PC-295 and PC-310 models, part of the cells constitutively has the NE phenotype. These two models are very suitable for studying NE differentiation in prostate cancer and the role that NE cells may play in the progression of prostate cancer. In both the PC-295 and PC-310 models, androgen deprivation induces increased numbers of NE cells. The kinetics of NE differentiation have been intensively studied in the PC-295 model (19). In this completely androgen-dependent model, the tumor rapidly regressed, and, like the non-NE cells, the NE cells also died.

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³ The abbreviations used are: NE, neuroendocrine; RSP, regulated secretory pathway; AR, androgen receptor; PAM, peptidyl α -amidating monooxygenase; CgA, chromogranin A; SgIII, secretogranin III; 7B2, secretogranin V; GRP, gastrin-releasing protein; 5-HT, serotonin; VIP, vasoactive intestinal peptide; VEGF, vascular endothelial growth factor; P/S, penicillin and streptomycin; PI, propidium iodine; RT-PCR, reverse transcription PCR; β 2-MG, β 2 microglobulin.

The PC-310 xenograft does not regress completely after androgen withdrawal. Therefore, we considered this tumor model more suitable for studying the process of NE differentiation of androgen-dependent cells to NE cells and the consecutive maturation of these NE cells in the long-term. We first characterized the kinetics of NE differentiation after androgen depletion in the PC-310 model by using CgA, MIB-1, AR, and PSA as cellular markers, and we analyzed the expression of SgIII, PC-1, PAM, and 7B2 as the different markers of the RSP at both mRNA and protein levels. Furthermore, the expression of different growth factors such as 5-HT, GRP, VIP, and VEGF was studied after androgen suppression. Among the limited number of available *in vitro* cell lines of human prostate cancer, none showed the NE phenotype at our institution (37). Attempts were made to set up *in vitro* cultures of the PC-310 xenograft, and the effect of androgen deprivation was evaluated as to whether the *in vitro* PC-310 culture had the potency of NE differentiation and behaved similarly to the xenograft *in vivo* in an androgen-depleted environment.

MATERIALS AND METHODS

PC-310 Xenograft Model. The nude mouse human prostate cancer xenograft model PC-310 was established from a primary prostatic tumor after radical prostatectomy of a previously untreated patient (36). The tumors grow with a doubling time of 16 days and a lag phase of 2–3 months. The model represents a strictly androgen-dependent and histologically moderately differentiated tumor, organized in solid sheets and microacini. In short, PC-310 tumors were implanted s.c. at both shoulders of intact NMRI male nude mice (Harlan, Horst, the Netherlands). Optimal growth conditions were reached by supplementation of PC-310 transplanted mice with testosterone implants, as described previously (38). Subcutaneously growing tumors developed within 2 months and were grown to a volume of 2000 mm³. Tumor volume was followed weekly by two perpendicular diameter measurements (D_1 and D_2) after which the volume (V) was calculated from the formula:

$$V = \frac{\Pi}{6} (D_1 \times D_2)^{3/2}$$

Castration Experiment with the PC-310 Human Prostate Cancer Xenograft Model. A castration experiment was performed with testosterone-supplemented PC-310 bearing male NMRI mice (Harlan) at the Erasmus Center for Animal Research (project 102.98.02). Androgen withdrawal was performed by castrating the mice under hypnorm anesthesia (Janssen Pharmaceuticals, Oxford, United Kingdom) and by removing the silastic testosterone implant. Four mice per time point were killed at 0, 2, 5, 7, 14, and 21 days after castration.

The mice were killed after blood samples were taken for determining serum PSA levels. The tumors were removed and either fixed in 4% buffered formalin and paraffin-embedded for immunohistochemical analysis or snap-frozen in liquid nitrogen and stored at -80°C for biochemical analysis. The paraffin-embedded material was processed routinely for H&E staining.

***In Vitro* Initiation of Primary Cultures from PC-310 Xenograft Tissue.** Initiation of primary cultures from xenograft material was performed according to Limon *et al.* (39). Tumors were mechanically disaggregated after washing the tumor tissue in RPMI 1640 and P/S (Life Technologies, Inc., Breda, the Netherlands), and necrotic tissue and blood clots were removed. The tissue was minced into 1- to 2-mm³ pieces, which were put into RPMI 1640 and P/S. After sedimentation for 10–15 min the supernatant was separated from the pellet. The supernatant was centrifuged at $175 \times g$ for 10 min, and the resulting pellet was resuspended in 3 ml of Swedish culture medium, *i.e.*, in DMEM F12, supplemented with various growth factors: 2% FCS, 10^{-10} M R1881 (a synthetic androgen), and P/S as described previously (33). Medium (10 ml) containing collagenase A (final concentration, 250 units/ml) was added to the pellet obtained after sedimentation, and this suspension was incubated at 37°C for 2–3 h. The cell suspension was transferred into centrifuge tubes, RPMI 1640 + P/S was added, and the cells were centrifuged at $175 \times g$ for 10 min. After sedimentation of the resuspended pellet for 10–15 min, the ensuing supernatant was centrifuged, resuspended in medium, and transferred

into culture flasks. Subsequent supernatant cultures were established. The PC-310 xenograft was initially grown as a mixed culture of mouse fibroblasts and PC-310C human epithelial cells. After passaging of the cultures and subsequent treatment with Amphotericin B, which could effectively kill mouse fibroblasts, pure cultures of PC-310C cells were obtained.

Androgen Deprivation of PC-310C Cultures. Series of confluent flasks with PC-310C were rinsed with PBS and subsequently cultured in medium with dextran charcoal-treated (*i.e.*, androgen-depleted) FCS for 0, 2, 5, 7, 14, and 21 days. At each time point, culture medium was sampled, and cells were harvested and frozen at -80°C for RNA and protein extraction. Likewise, small Petri dishes were plated with PC-310C cells ($n = 2$), and cells were grown in medium containing DCC-treated FCS. At the different time points, the level of proliferation was assessed in terms of the percentage of cells with mitotic figures, and the level of apoptosis was assessed in terms of the percentage of cells containing apoptotic bodies, which was determined by a quantitative immunofluorescence microscopy of Hoechst 33342 and PI staining of the PC-310C cultures.

The PC-310C cells were also grown on glass slides under various conditions and were used for immunohistochemical staining for NE-associated proteins.

Immunohistochemistry. To identify the fraction of cells expressing the NE phenotype, paraffin-embedded tissue sections of the PC-310 xenografts were stained with antibodies against: (a) CgA [monoclonal clone LK2H10 (ICN Pharmaceuticals, Aurora, OH) or polyclonal rabbit antihuman CgA (DAKO/ITK Diagnostics, Uithoorn, the Netherlands)]; (b) SgIII (rabbit polyclonal antibody, provided by the Department of Animal Physiology, University of Nijmegen; Ref. 40); and (c) PC1 and PC2 (Alexis Biochemicals, 10P's, Breda, the Netherlands). For identification of the proliferative capacity, tissue sections were stained with the antibody against the proliferation-associated Ki-67 antigen (MIB-1, Immunotech, Marseilles, France). In addition, apoptotic cells were identified by counting the apoptotic bodies in the H&E staining of the tissues. Other antibodies used were directed against the AR (clone F39.4, kindly provided by Dr. A. O. Brinkmann, Erasmus University, Rotterdam) as follows: (a) the cytoplasmic Bcl-2 antigen (clone 124, Dako); (b) PAM (monoclonal α -284, from Dr. A. M. Treston, National Cancer Institute, Bethesda, MD); and (c) the growth factor VEGF (rabbit polyclonal, DAKO). Antibodies used against the neuropeptides were as follows: (a) GRP [rabbit polyclonal antihuman GRP (DAKO) and anti-GRP monoclonal antibody 2A11 (kindly provided by Dr. F. Cuttitta, National Cancer Institute)]; (b) 5-HT (rabbit polyclonal antibody, DAKO); (c) VIP (rabbit polyclonal antibody, DAKO); and (d) calcitonin (rabbit polyclonal antibody, DAKO).

Paraffin-embedded xenograft tissues were cut at $4\text{-}\mu\text{m}$ sections for single immunostaining and at $2\text{-}\mu\text{m}$ for double immunostaining. The sections were mounted on 3-amino-propyl-triethoxysilane (Sigma Chemicals Co, St. Louis)-coated glass slides and were incubated overnight at 60°C . The slides were deparaffinized, and endogenous peroxidase activity was blocked with 3.3% H_2O_2 in methanol for 10 min. The following steps were also performed for the PC-310C glass slides after a 10-min fixation in 4% formaldehyde. Antigen retrieval was performed in 10 mmol/L citrate buffer (pH 6.0) in a microwave oven at 700 W for 15 min (41). The slides were allowed to cool down to room temperature and were then put into the sequenza immunostaining system (Shandon, Uncorn, United Kingdom) and rinsed with PBS. All of the slides were preincubated with normal goat serum (DAKO) diluted 1:10 in PBS for 15 min. The primary antibody was incubated at the appropriate concentration for 2 h at room temperature or overnight at 4°C . The secondary antibody was incubated for 30 min, being either horseradish-peroxidase conjugated goatmouse or goatrabbit (1:50), or biotinylated goat antimouse and goat antirabbit (1:400) for monoclonal and polyclonal antibodies (all DAKO), respectively. In the case of biotinylated goat antimouse and goat antirabbit, a horseradish-peroxidase:streptavidin-biotin complex (DAKO) diluted 1:1:200 in PBS, prepared at least 30 min before use, was incubated for a subsequent 30 min. Between the subsequent steps, the slides were rinsed four times with PBS. The bound horseradish-peroxidase was visualized in 10 min with diaminobenzidine (DAB, Fluka, Neu-Ulm, Germany) in PBS containing 0.075% H_2O_2 as substrate. Slides were rinsed extensively in tap water and, finally, were counterstained in Mayer's hematoxylin, dehydrated through a series of alcohols, and embedded in malinol.

To assess the properties of the NE cells, double staining of CgA with MIB-1, AR, or Bcl2 was performed on the PC-310 xenograft tumors. The double-staining procedure consisted of two consecutive stainings with two

primary antibodies, the first always being a horseradish-peroxidase-related stable amino-ethyl-carbazole (AEC) complex; the second staining was performed with an alkaline phosphatase-conjugated goat antimouse secondary antibody, which was colored with Fast Blue substrate. In between both stainings, the slides were rinsed with PBS for 1 h and boiled again in a microwave oven in 10 mmol/L citrate buffer (pH 6.0) for 10 min. As negative control, PBS replaced the primary antibody in all of the stainings. Radical prostatectomies, containing normal prostatic tissue were used as positive control for CgA, SgIII, PC1, PC2, AR, GRP, 5-HT, VEGF, Bcl2, and MIB-1 expression.

For MIB-1, CgA, SgIII and Bcl-2, the number of immunoreactive cells was determined by quantitative counts of all of the visible cells in tumor squares at $\times 310$, from which the number of positive cells per mm^2 was calculated. The percentage of positive cells was determined in ≥ 10 squares. For AR, VEGF, GRP, 5-HT, PC1, and PC2 the level of immunostaining was assessed semi-quantitatively.

Western Blot Analysis. We further confirmed the expression of CgA, SgIII, Bcl-2, 7B2, and AR in our castration series of the PC-310 tumor by Western blotting. As positive controls, we used material of human pheochromocytoma for CgA, of rat pituitary for SgIII, and of the human *in vitro* cell line LNCaP for AR expression. The procedure of protein extraction has been described previously (37). Frozen tissues of the PC-310 xenografts were crushed in a liquid-nitrogen-chilled metal cylinder. The tissue homogenates or PC-310C cell culture pellets were transferred into a lysis buffer [10 mM TRIS (pH 7.4), 150 mM NaCl (Sigma), 1% Triton X-100 (Merck, Germany), 1% deoxycholate (Sigma), 0.1 SDS (Life Technologies, Inc.), 5 mM EDTA (Merck), and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 50 mg/L leupeptin, 1 mM benzamide, and 1 mg/L pepstatin—all from Sigma). After centrifugation of the mixture at 100,000 rpm at 4°C for 10 min, the protein content of the supernatants was measured by the Bradford method (Bio-Rad protein assay, Munich, Germany).

Each sample (20 μg) was transferred to a SDS polyacrylamide gel, and gel electrophoresis was performed with prestained markers as size standards (Novex, San Diego, CA). The gels were blotted to a 0.45- μm cellulose nitrate membrane (Schleicher & Schuell, Dassel, Germany). The immunoblot was blocked with PBS (pH 7.7) containing 0.1% Tween 20 (Sigma) and 5% dry milk for 1 h. The CgA, 7B2 (Mon 102/144, kindly provided by Prof. Dr. W. M. van der Ven, Leuven, Belgium), Bcl-2, AR, or SgIII antibodies were added in their optimal concentration and incubated overnight on an orbital shaker at 4°C. After rinsing four times for 15 min with PBS, incubation for 1 h was performed with the secondary horseradish-peroxidase-conjugated antibodies and goat antimouse for mouse monoclonal antibodies and goat antirabbit for rabbit polyclonal antibodies. Subsequently, a short incubation with a mixture of 10 ml of luminal and 100 μl of oxidizing agent (BM chemiluminescence kit, Boehringer Mannheim GmbH, Mannheim, Germany) followed, after washing for four times for 15 min with PBS. Excess reagent was removed, and antibodies were visualized by exposure of the blots to an X-ray film.

RT-PCR. RNA was isolated by using the single-step RNeasy method (Campro, the Netherlands; Ref. 42). Frozen tissue (100 mg) or cell culture pellets were homogenized in 1 ml of RNeasy. Chloroform (0.1 ml) was added, and the mixture was vortexed for 15 s followed by 5 min incubation on ice. The homogenate was then centrifuged at 4°C at $12,000 \times g$ for 15 min. The upper water phase containing the RNA was removed and mixed with an equal volume of isopropanol. This mixture was then kept at 4°C for 15 min and, after that, was centrifuged at 4°C at $12,000 \times g$ for 15 min. The supernatant was removed, and the RNA pellet was washed twice with 75% ethanol by vortexing and centrifugation at 4°C at $12,000 \times g$. The pellet was then dried and resuspended in sterile H_2O . The concentration was determined at $A_{260\text{ nm}}$, and solutions of 1 $\mu\text{g}/\mu\text{l}$ were prepared for further use in RT-PCR or Northern blot analysis. The quality of the isolated RNA was checked by determining the 260:280 ratio and by formaldehyde gel-electrophoresis to check the ribosomal (28S and 18S) bands.

RT-PCR was performed for CgA, SgIII, PAM, PC1 and 2, and $\beta 2$ -MG with a standard protocol. Reverse transcriptase reaction was performed with a mastermix containing 5 mM MgCl_2 , PCR buffer, 10 mM dNTPs, RNase inhibitor (10 units), reverse transcriptase (25 units), 2.5 mM random hexamer primers, and 0.5 μg RNA in a total volume of 10 μl covered with 50 μl of mineral oil. The mastermix was then processed at 42°C for 60 min followed by 15-min incubation at 99°C, and the reaction was stopped at 4°C for 5 min. The two negative controls, water and -RT in the reverse transcriptase reaction

were negative in each RT-PCR protocol. The cDNA mix that was formed was then used totally with the mastermix of the PCR protocol. In this protocol, the mastermix contained reaction buffer, Supertaq polymerase (HT Biotechnology Ltd., Cambridge, United Kingdom; 1 unit), 15 μM sense and antisense primer in a total volume of 40 μl . All of the samples were first denatured at 94°C for 10 min, and then amplification was performed for 35 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C and a final extension at 72°C for 10 min. The PCR product was checked on a 1% agarose gel and, if necessary, was followed by Southern blotting. The internal control of human $\beta 2$ -MG clearly showed the equal amounts of mRNA that were used for each time point.

Northern Blot Analysis. Northern blot analysis was performed by running a formaldehyde gel in MOPS buffer containing 20 μg of each sample including the controls. The RNA content of the gel was consequently blotted overnight onto a hybond- N^+ filter in $10\times$ SSC ($10\times$ SSC: 1.5 M sodium chloride and 0.15 M sodium citrate). The filter was checked for RNA and ribosomal bands were marked. After rinsing the filter in $2\times$ SSC, the blot is cross-linked in a GS gene linker UV chamber (Bio-RAD, Munich, Germany) and ready for (pre)hybridization with different probes against CgA, SgIII, PAM, and $\beta 2$ -MG as control.

RESULTS

Androgen withdrawal in the PC-310 xenograft showed a decrease in volume directly after castration. The PC-310 tumor doubling time was 16 days before castration. After castration, the PC-310 tumors had regressed with 50% within 9 days. Between 14 and 21 days postcastration tumor volume was maintained at about 30–40% of the initial tumors. (Fig. 1A). The decline in tumor volume was associated by a rapid decrease of proliferating, MIB-1 expressing, cells from 20% in the controls to zero at 7 days postcastration (Fig. 1A). Apoptotic counts increased after 2, 5, and 7 days postcastration and returned to control levels after 14 and 21 days (data not shown). Serum PSA levels rapidly dropped below detection levels postcastration (Fig. 1B). The expression of the M_r 110,000–112,000 AR protein decreased directly after castration, but a markedly increased expression was observed on Western blot after 5–7 days postcastration (Fig. 2A), which was confirmed by immunohistochemistry.

Expression of the NE marker CgA increased rapidly after castration, up to an approximately 40-fold increase after 14 days of androgen withdrawal (Fig. 1A). It is clear that the changes in NE differentiation cannot be explained by a selection of NE cells as a 40-fold increase in the number of NE cells was observed while tumor volumes were still 30–40% of the controls at 14 and 21 days postcastration. On Western blot, the increased expression of CgA at M_r 78,000 and 68,000 could clearly be visualized as could also the increased processing of the protein into proteins of M_r 49,000, 31,000, and 18,000 after 14 and 21 days of androgen withdrawal (Fig. 2B). Immunohistochemical double-labeling studies revealed that approximately 50% of the epithelial tumor cells expressed the AR (CgA⁻, AR⁺), and the other 50% were NE cells (CgA⁺, AR⁻) after 21 days of androgen withdrawal. The AR-positive cells and NE cells were found as a mixed population of cells dispersed over the whole tumor tissue.

The induction of NE differentiation was further confirmed by increased mRNA levels for NE markers as found for CgA in Northern blot analysis (Fig. 3, A and B). RNA of this same series of tissues was reverse transcribed, and the resulting cDNA was used in subsequent PCR reactions analyzing the expression of CgA and other markers of NE differentiation. Expression of markers of the RSP was found to be increased after androgen deprivation of PC-310 *in vivo*. Induction of SgIII and 7B2 were found to be comparable to the increased CgA expression at 5 or 7 days after androgen withdrawal (Fig. 4). Temporal induction of mRNA expression of PC1 and PC2 was found at day 5 and 7, respectively. PAM-mRNA expression was temporarily increased at day 7 postcastration, when the expression of all of the three PAM splice variants (29) was increased. Increased expression of

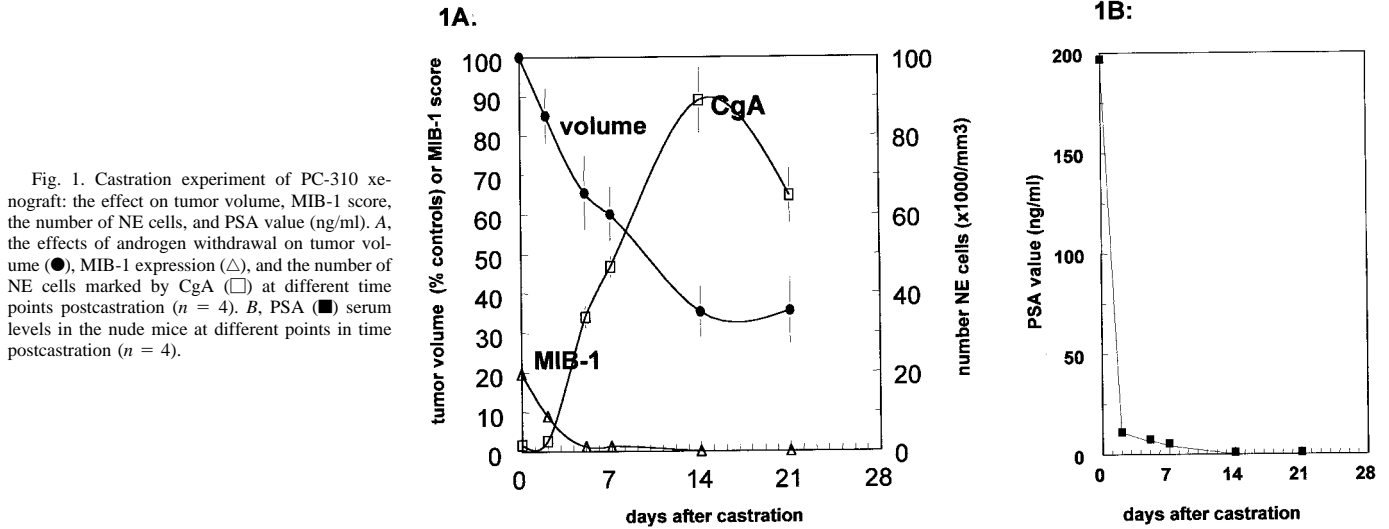


Fig. 1. Castration experiment of PC-310 xenograft: the effect on tumor volume, MIB-1 score, the number of NE cells, and PSA value (ng/ml). A, the effects of androgen withdrawal on tumor volume (●), MIB-1 expression (△), and the number of NE cells marked by CgA (□) at different time points postcastration ($n = 4$). B, PSA (■) serum levels in the nude mice at different points in time postcastration ($n = 4$).

the RSP markers was confirmed by immunohistochemistry and Western blotting (Table 1). From this table, it is clear that the expression of SgIII and 7B2 is already apparent at day 5 and 7 postcastration, whereas expression of the processing enzymes PC1, PC2, and PAM in the NE cells starts only around day 7 and becomes more apparent at day 14 and 21 postcastration. The anti-apoptotic protein Bcl-2 was expressed at low levels in the androgen-supplemented mice but decreased at both mRNA and protein level after androgen withdrawal (result not shown). Furthermore, expression of the VEGF was clearly increased at 14 and 21 days postcastration. Interestingly, GRP and 5-HT expression could also be clearly detected as a relatively late event of NE differentiation from 14 days postcastration forward.

Expression of vasoactive intestinal peptide or calcitonin was not found during the 21-day period studied.

In vitro, androgen depletion of PC-310C cultures led to increased numbers of apoptotic cells and a decreased level of proliferation as determined by Hoechst 33342 and PI staining (Table 2). The total amount of apoptotic cells was counted as the increased number of Hoechst 33342 or Hoechst 33342/PI staining nuclei containing fragmented DNA and/or apoptotic bodies, whereas the number of blue, Hoechst 33342-stained, mitotic figures decreased in time after androgen suppression. PSA levels of the culture medium initially decreased, but, with the refreshing of the culture medium once a week, the PSA levels increased in time. Some proliferating cells were still present after 21 days of androgen withdrawal, and PSA levels in the medium were high. AR expression initially decreased to a limited extent after androgen withdrawal, but, after 5 days of androgen depletion, an increased level of the M_r 110,000–112,000 AR protein was found.

Similar to the *in vivo*-grown PC-310 tumor, in PC-310C cultures, expression of the NE markers CgA and SgIII increased after 14 days of androgen withdrawal both at the mRNA (Figs. 3B and 5) and protein levels (Fig. 6B). The mRNA levels of PC1 and PC2 did not increase, and a slight increase in the levels of PAM-mRNA was found (Fig. 5).

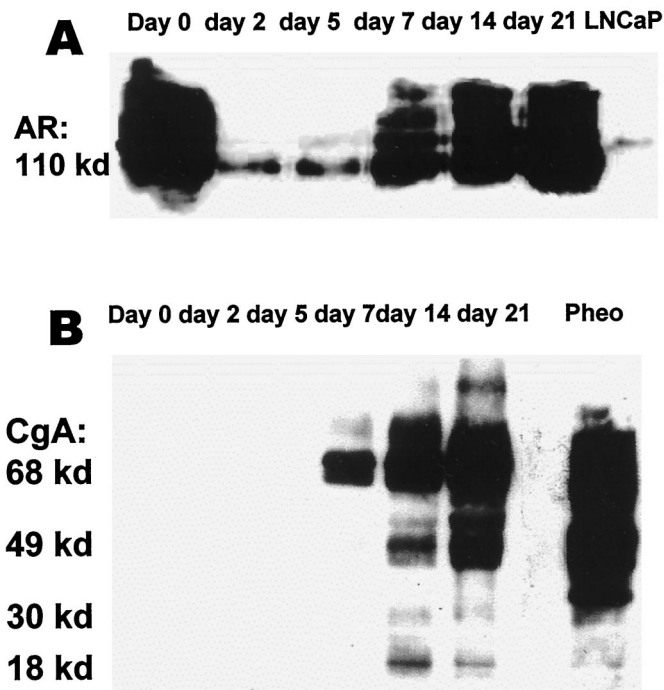


Fig. 2. Expression of the AR and CgA after castration in the PC-310 xenograft model. Both panels show the time points T_0 , T_2 , T_5 , T_7 , T_{14} , and T_{21} after androgen withdrawal. In A, the signal of AR expression is found at M_r 110,000–112,000, which first rapidly decreased and was subsequently reexpressed in the PC-310 model. The increased CgA signal can be found between M_r 68,000 and 78,000 in B, which is the highest band in the control pheochromocytoma (*Pheo*). After 7 days clear processing of the protein is shown by smaller fragments at M_r of $\pm 49,000$, $\pm 30,000$, and $18,000$.

DISCUSSION

This study of the human androgen-dependent PC-310 prostatic carcinoma xenograft model aims at a comparison of the kinetics of inducible NE differentiation caused by androgen depletion both *in vivo* and *in vitro*. The PC-310 xenograft model was established from the primary tumor of a previously untreated prostatic tumor and behaves as an androgen-dependent clinical tumor. The growth and regression profiles of the PC-310 xenograft in the presently described castration experiments was comparable to studies performed earlier (36). Hormonal suppression of the model induced an initial increase of apoptosis, decreased proliferation, and down-regulation of the AR. This was paralleled by a rapid and prominent decrease of serum PSA levels, demonstrating the androgen-dependent character of the PC-310 model, as was previously demonstrated for the PC-295 model (19). As a consequence, the PC-310 tumors regressed, and, after 21 days of androgen withdrawal, only 30–40% of the initial tumor volumes were left. The rapid loss of AR expression in the PC-310 tumors was comparable to that observed previously in the androgen-dependent human PC-82 xenograft after castration (43). However, after 5 days of

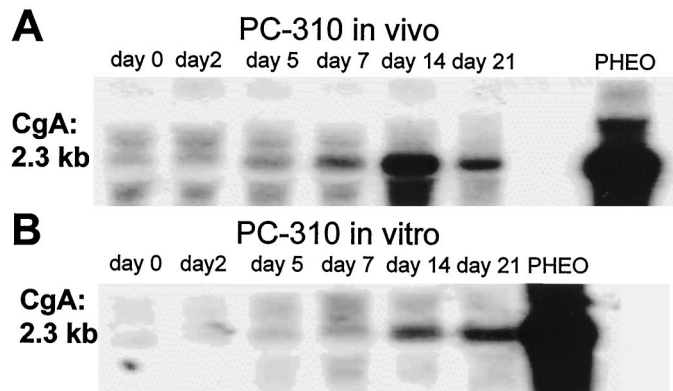


Fig. 3. mRNA expression of CgA on Northern blot at 2.3 kb in the PC-310 xenograft model (A) and in the *in vitro* cell culture PC-310C (B) after androgen withdrawal.

androgen depletion, the expression level of the AR returned to above normal. Constitutive high AR expression has been found in transurethral resection specimens of clinical hormone refractory tumors (44), but lower expression of the AR was noted during regression of hormonally treated prostate cancer (45). Similarly, AR-positive prostatic cancer cell lines, such as the LNCaP, continue to express the AR after hormone depletion (46, 47).

NE differentiation was clearly induced at 5 days after androgen withdrawal as shown by expression of CgA. At that time point, only a few proliferating non-NE cells were left. Analysis for CgA at both RNA and protein level demonstrated the increased expression of the CgA in time from a low basal-expression level in the controls to a maximum at 21 days postcastration. Despite intertumoral differences in basal CgA expression levels at the moment of castration, increased CgA expression was observed at 5, 7, 14, and 21 days postcastration by immunohistochemistry, which was confirmed by Western blot analysis, RT-PCR, and Northern blot analysis. By comparing the Western blots for CgA and AR postcastration, it was extrapolated that, from day 7 postcastration onward, there were both NE cells present as well as cells expressing the AR. However, immunohistochemical double staining for AR and CgA confirmed that CgA and AR did not colocalize. Thus postcastration, the NE cells represent the androgen-independent, nonproliferating part of the prostatic tumor, and the rest of the PC-310 tumor cells express the AR after an initial down-regulation. These latter cells that are still androgen-sensitive could possibly become androgen-independent cells also, after long-term androgen depletion.

From the immunostainings for CgA and MIB-1, it was seen that the proliferation at 7 days postcastration was near zero, whereas the numbers of NE-differentiated tumor cells still increased. It can be concluded that the greater part of the NE cells was in the G_0 phase of the cell cycle, which is in agreement with our previous study of the PC-295 model (19) as well as with other studies on NE differentiation and proliferation in prostate cancer (15, 16). In clinical prostate cancer specimens, Bonkhoff *et al.* (16) found that AR-negative NE cells expressed cytokeratins 18 and 5, and that these cells were found in the proximity of proliferating cells. In the PC-310 experimental tumor model, during a period of 21 days postcastration NE cells were found scattered over the tumors and were not associated with proliferative activity of the surrounding non-NE cells.

The kinetics of NE differentiation, after androgen withdrawal was clearly demonstrated by the time-related induction of other secretogranins and processing enzymes belonging to the RSP (25) next to the observed induction of CgA. RT-PCR analysis of the RSP markers clearly showed the time-dependent induction of 7B2, SgIII, PC1, PC2, and PAM in the PC-310 NE cells. The kinetics of NE differentiation

and the maturation of secretory granules in prostatic NE cells was also shown by immunohistochemistry in the PC-310 model. Increased expression at protein level of SgIII by immunohistochemistry and 7B2 by Western blotting was detected from 5 to 7 days postcastration onward, whereas induction of PC1, PC2, and PAM occurred later, *i.e.*, at day 14 and 21 postcastration during apparent maturation. These results indicate that the NE cells in the PC-310 model possess an active RSP and also demonstrate the time-dependent maturation of the secretory granules as shown by the increased expression of the PC1 and the increased processing of CgA coinciding at 14 days postcastration (Fig. 2B). Clearly, the M_r 78,000 and 68,000 CgA protein (37) is processed by PC1 to form products and processed proteins ranging from M_r ~49,000 to ~31,000 and ~18,000 as was shown previously by others (48–50).

PAM is most likely expressed in NE cells because the protein activates neuropeptides by amidation mainly in the secretory granules of the RSP, but expression in non-NE cells has also been described (51). The increase of PAM in the PC-310 model after castration coincides with the time-related induction of expression of RSP-associated proteins. We found that PAM is expressed both in NE cells and in the neighboring non-NE cells. The immunohistochemical data indicate increased PAM expression with a granular NE-like staining pattern 14 days postcastration and a low cytoplasmic membranous staining in the non-NE cells (data not shown).

VEGF, GRP, and 5-HT, but not VIP and calcitonin were expressed postcastration only in the PC-310 model. The expression of VEGF colocalized in the NE cells, and the expression was induced shortly in time after the expression of CgA and SgIII. Expression of GRP and 5-HT was only found in a small part of the NE cells relatively late, at 14 and 21 days postcastration. This indicates that possibly other neuropeptides and growth factors for endothelial cells can be induced

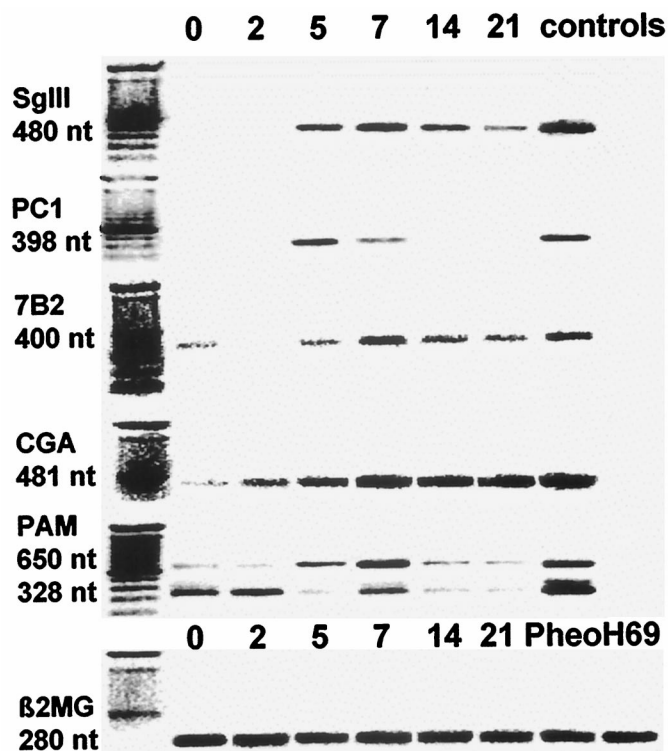


Fig. 4. NE differentiation and RSP. RT-PCR performed for SG3 (*SgIII*), PC1, 7B2, CgA, PAM, and β 2-MG expression in PC-310 xenograft after castration. H69 was used to check PC1, 7B2, and PAM analysis and Pheo was used for SgIII and CgA. The products of the human specific primer sets are indicated as the length in nucleotides (nt) of the mRNA that is amplified.

Table 1 Expression^a of NE markers, neuropeptides, and growth factors in PC-310 *in vivo* after androgen suppression

CgA, SgIII, PC1, PAM, GRP, VEGF, and 5-HT were all determined by IHC, whereas 7B2 was determined from Western blot.

Time (days)	CgA	SgIII (Sg3)	7B2	PC1	PAM	GRP	VEGF	5-HT
Control	+	±	±	-	-	-	-	-
2	+	±	±	-	-	-	-	-
5	++	+	+	-	-	-	-	-
7	+++	++	++	±	+	±	+	-
14	++++	+++	+++	++	++	+	++	±
21	+++++	++++	+++	++++	+++	+	++++	++

^a Expression was scored as follows: -, no expression; ±, sporadic expression; +, focal expression; ++, few cells positive per optical field; +++, clustered cells positive per optical field; +++++, more clusters of positive cells per optical field; ++++++, 50% of tumor positive. The expression of 7B2 was determined from weak expression (±) to high expression (++++).

Table 2 Effects of androgen withdrawal on PC-310C *in vitro*: proliferation, apoptosis, and culture medium PSA levels after androgen withdrawal

PSA ($n = 2$) was determined by quantitative ELISA at the Department of Clinical Chemistry, Academica Hospital Dijkzigt, Rotterdam. The percentages of apoptotic and mitotic cells were determined by scoring the individual and total number of cells in at least 10 optical microscopic fields ($\times 165$) per time-point and mean values and SDs were calculated from this.

Time (days)	Apoptosis (%) (total)	Mitotic cells (%)	PSA medium levels (ng/ml)
Control	2.1 ± 0.5	2.8 ± 1.3	278 ± 89
2	5.7 ± 1.3	2.3 ± 0.9	610 ± 140
5	7.8 ± 1.5	0.9 ± 0.9	943 ± 178
7	10.0 ± 2.7	2.1 ± 1.6	948 ± 195
14	6.3 ± 2.3	1.2 ± 0.6	186 ± 90
21	5.9 ± 1.6	0.4 ± 0.6	505 ± 159

postcastration in the PC-310 model. Apparently, the PC-310 tumors respond to castration by induced VEGF, GRP, or 5-HT expression that may induce proliferation of endothelial cells or non-NE cells. Induction of VEGF may be a consequence of NE differentiation, but, alternatively, VEGF expression can be induced by hypoxia (52), which is a common condition observed in prostatic tumors (53). Possibly, increased hypoxic stress after androgen withdrawal induced VEGF expression in the NE-differentiated cells.

Previously, we suggested that the NE cells and their lack of AR expression may play a key role in the survival of prostate cancer cells after androgen withdrawal, whereas Bcl-2 protects the remaining androgen-independent cells from undergoing apoptosis (19). Here we show another unexpected behavior of androgen-dependent prostatic tumor cells, being the drop in AR expression shortly after androgen withdrawal followed by a relapse of expression after 5 days postcastration. Thus, part of the initially androgen-dependent PC-310 cells

differentiated into NE cells after androgen withdrawal, whereas the remaining carcinoma cells are surviving as androgen-sensitive tumor cells by constitutively expressing the AR in an androgen-deprived environment. In contradiction to what has been reported about the LuCap 23 model (35) and to our previous observations in the PC-295 model (19), here we showed an alternative route of overcoming cell death after androgen deprivation by both a strong induction of NE differentiation and a transient down-regulation of the AR, without inducing Bcl-2 expression.

The establishment of *in vitro* cultures from PC-310 epithelial cells in a specialized culture medium has enabled us to study the *in vitro* effect of androgen deprivation on the kinetics of NE differentiation in PC-310C and to compare it with the behavior of PC-310 *in vivo*. To our knowledge, PC-310C is the first human cell culture of a primary untreated prostatic adenocarcinoma-containing cells with the NE phenotype and that can be induced to differentiate into NE, *i.e.*, CgA and SgIII, positive cells after androgen depletion. The *in vitro* model PC-310C resembles the *in vivo* model for most parameters such as the decrease in proliferation, the induction of apoptosis, the regulation of AR expression, and the less strong but significant induction of CgA mRNA expression. The main difference with the *in vivo* situation was the observation that, after an initial decrease of PSA *in vitro*, the PSA levels increased again to control level.

The time-related induction of PC1 and PAM expression after androgen withdrawal indicates that the NE cells do have the potency to actively secrete growth factors such as neuropeptides from secretory granules (25, 40). This statement is fortified by the relatively late induction of GRP and 5-HT expression during granular maturation. Likewise, the induced expression of VEGF may lead to the growth of new blood vessels, which may play a decisive role in the progression

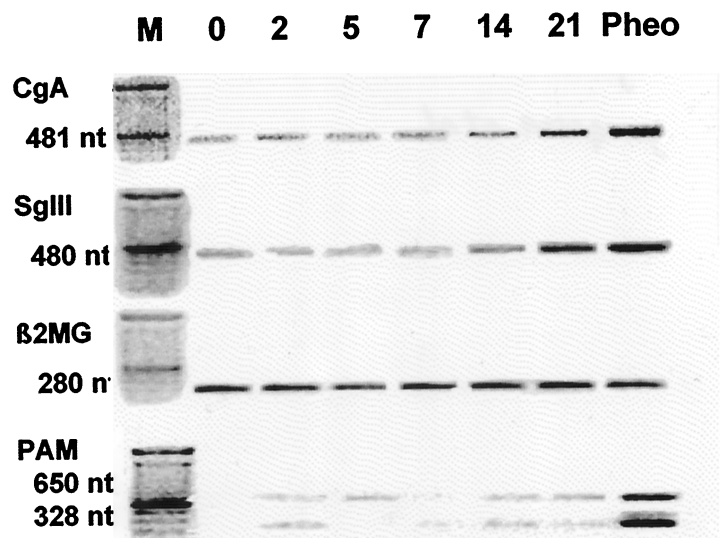


Fig. 5. Expression of CgA, SG3, PAM, and β 2-MG in PC-310C. The products of the primer sets are indicated as the length in nucleotides (nt) of the mRNA that is specifically amplified. Pheochromocytoma was used as a control for CgA and Sg3 expression, and H69 was used for PAM expression.

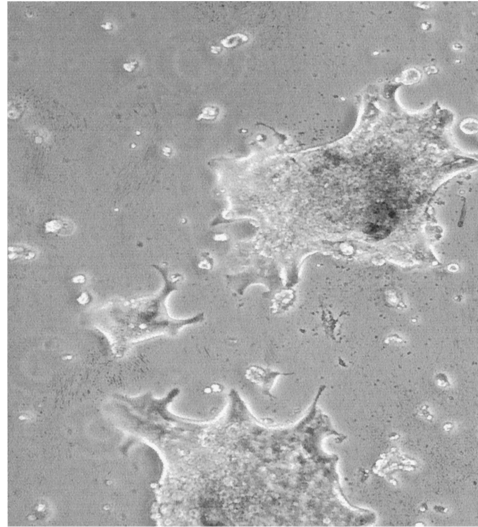
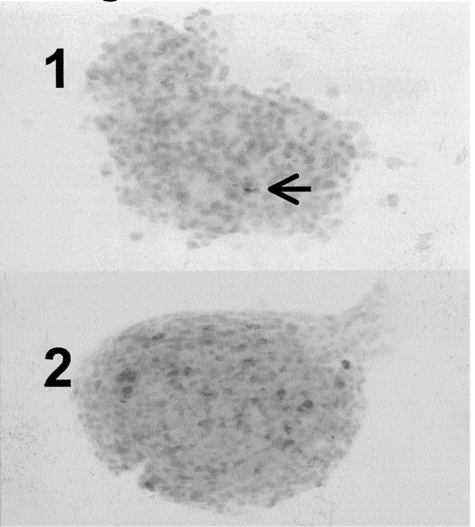
A: PC-310C**B: CgA in PC-310C**

Fig. 6. NE differentiation *in vitro* in PC-310C cultures after androgen withdrawal. A, overview PC-310C after five passages in culture. $\times 40$. B, CgA immunohistochemistry in PC-310C cultures. 1, androgen supplemented; arrow, one CgA positive cell. 2, NE differentiation 14 days postcastration. $\times 310$.

of dormant prostate cancer to hormone independence. After 21 days of androgen withdrawal, a tumor residue is formed in which, 50% of the growth-arrested tumor cells expresses the AR without any colocalization with NE markers. During prolonged androgen deprivation, the NE cells may play a role in the maintenance of the dormant G_0 -arrested non-NE PC-310 tumor cells. However, these NE cells may also induce the androgen-independent growth of these dormant non-NE cells by producing growth-activating neuropeptides. Furthermore, we consider the possibility of androgen-independent activation of the AR, for instance by neuropeptides such as GRP and insulin-like growth factor 1 (54) or cytokines such as interleukin 6, which cannot be excluded (55) in the PC-310 model. The finding of this kind of dormant prostate cancer residues is unique to our knowledge, and additional studies on the PC-310 model will probably teach us more about the development of androgen-independent progression in prostate cancer.

The PC-310 model system is a unique model for additional studies on the induction of NE differentiation and AR modulation by androgen withdrawal both *in vivo* and *in vitro*—the role of the RSP in the progression of prostate cancer in particular—because the role in the progression of prostate cancer is still unclear (20, 21). In addition, androgen-independent growth or prostate cancer progression is currently under investigation in a long-term androgen withdrawal experiment of the PC-310 *in vivo* model. Induction of NE differentiation, the maturation of NE cells, and temporal inhibition of AR protein expression in prostate cancer may also be the clinical behavior of hormonally treated prostate cancer patients who relapse after an initial 1–2 years of remission of the disease. The only cells in the PC-310 tumor left after 21 days are androgen-independent CgA-expressing NE cells and AR-expressing androgen-sensitive cells, which neither proliferate nor undergo apoptosis. Additional studies on the PC-310 model system will yield information as to whether NE cells play a role in the maintenance of dormant tumor residues and induce progression to hormonal independence by initiating growth induction of AR-positive non-NE cells, for example, by different growth factors or the induction of genetic changes.

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