Multipathways for transdifferentiation of human prostate cancer cells into neuroendocrine-like phenotype

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Abstract

The neuroendocrine (NE) cell is a minor cell population in normal human prostate glands. The number of NE cells is increased in advanced hormone-refractory prostate carcinomas (PCA). The mechanism of increased NE cell population in these advanced tumors is poorly understood. We examined molecular mechanisms which may be involved in the regulation of the transdifferentiation process of human PCA cells leading to a NE phenotype. We compared PCA cell lines LNCaP and PC-3 in the following medium conditions: steroid-reduced (SR), interleukin-6 (IL-6)-supplemented, or dibutyrate cAMP (db-cAMP)-supplemented. We found that androgen-responsive C-33 LNCaP cells responded to all treatments, having a neuronal-like morphology. In contrast, C-81 LNCaP cells, having a decreased androgen responsiveness, had a less pronounced effect although followed a similar trend. Androgen-unresponsive PC-3 cells showed little change in their morphology. Grown in the SR condition, the level of neuron-specific enolase (NSE), a marker of neuronal cells, was upregulated in C-33 LNCaP cells, while to a lesser degree in the presence of IL-6. In the presence of db-cAMP, the NSE level in C-33 cells was decreased, lower than that in control cells. An opposite effect was observed for C-81 LNCaP cells. Nevertheless, the NSE level was only elevated in db-cAMP-treated PC-3 cells, but no change was found in PC-3 cells grown in the SR- or IL-6-supplemented medium. Thus, a similar gross phenotypic change may correlate with differential molecular expressions. We also analyzed the expression of protein tyrosine phosphatase \( \alpha \) (RPTP\( \alpha \)) since it plays a critical role in normal neuronal differentiation and signaling. Our results showed that the expression of RPTP\( \alpha \) correlates with the NE phenotypic change of LNCaP cells in the SR condition. In summary, our data clearly show that the molecular process by which cultured human prostate cancer cells undergo a transdifferentiation process to a NE cell-like phenotype is accompanied by differential expressions of different markers, and a gross NE cell-like phenotype can occur by exposing PCA cells to different pharmacological agents. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Prostate cancer is the most common malignancy in
men, and the second leading cause of cancer-related mortality in US males [49]. Although androgen ablation is the most effective therapy for patients with advanced prostate cancer, progression of cancer cells to androgen independence evidently occurs and remains the main obstacle to improve the survival and quality of life [21]. The tumor progression to androgen-refractory stage is a complex process that may be involving both clonal selection and adaptive mechanisms in heterogeneous tumors composed of cells that respond differently to androgen ablation. The present research is focused on understanding the molecular mechanisms that allow human prostate cancer cells to acquire androgen independence.

The cells in human prostate gland include basal epithelial, secretory epithelial, and neuroendocrine (NE) cells, in addition to stromal cells [7,23]. The NE or endocrine–paracrine cells, although only representing a minor cell population in normal prostate glands, are known to produce and secrete some potent neuro-hormones, including bombesin/gastrin, calcitonin, and parathyroid hormone-related peptide, that likely regulate the activity and homeostasis of the adjacent basal and/or secretory epithelial cells [31]. NE cells may represent one potential pathway of differentiation of prostatic stem cells. Recently, results of several clinical studies suggested that NE cells may also have a role in the progression of prostate cancer cells to the hormone-independent state [22,24,25]. Histological studies reveal that NE cells are significantly more abundant in cancerous tissues than in non-cancerous tissues [32]. In fact, the overall abundance of NE cells is increased in proportion to the progression of prostate cancer from the early stage to the advanced, hormone-resistant state [61]. Results from analyses on sera from hormone-dependent and clinically advanced, hormone-refractory prostate cancer patients show an increased level of chromogranin A [1,37] and neuron-specific enolase (NSE) [58], markers for NE cells, in advanced stage patients. These observations have led to the hypothesis that NE cells function to provide paracrine stimulatory factors in an androgen-deprived condition, leading to an accelerated growth and progression of surrounding prostate cancer cells toward an androgen-independent state. This hypothesis is supported by the observation of an increased proliferation index of tumor cells in the close proximity to the NE cell foci [9]. Thus, NE cells can apparently modulate the proliferation of adjacent epithelial cells including cancerous and non-cancerous cells.

Although the origin of the NE cell population in prostate cancer tissues remains uncertain, a number of recent studies has shown that prostate cancer cells themselves have the ability to ‘transdifferentiate’ (or convert) into cells with a NE-like phenotype in culture. This phenomenon can occur when cultured prostate cancer cells are exposed to pharmacological agents that increase the intracellular level of cyclic AMP (cAMP) [4,16], in the presence of interleukin-6 (IL-6) [47,51], or in a steroid-reduced medium [10,55]. These treatments induce a morphological change in cell phenotype, resembling cultured neuronal cells with narrow cell bodies and elongated processes. However, no systematic study has been performed to analyze the susceptibility of each cell line to different treatments that relate to clinical phenomena. The molecular mechanism of transdifferentiation also remains to be further delineated.

Protein tyrosine phosphorylation plays a critical role in regulating various cellular events, including cell differentiation and proliferation. Protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) function dynamically to maintain cellular homeostasis. Unbalanced tyrosine phosphorylation can alter signaling for cell growth and differentiation, leading to neoplastic diseases or cell growth arrest. Protein tyrosine phosphatase \( \alpha \) (RPTP\( \alpha \)), also known as leukocyte common antigen-related phosphatase (LRP), is a transmembrane member of the PTP family [39,40,54]. It has been demonstrated that RPTP\( \alpha \) can be involved in GRB2-mediated signaling [19,57], and participate in activation of mitogen-activated protein kinases (MAPKs) and c-Jun transcription [66]. Additionally, RPTP\( \alpha \) can regulate the specific activity of Src kinase by dephosphorylating its carboxyl-terminal tyrosine [20,67]. Interestingly, RPTP\( \alpha \) has also been determined to play a critical role in normal neuronal differentiation [60,62]. Thus, RPTP\( \alpha \) is involved in multisignaling pathways. We have reported that RPTP\( \alpha \) mRNA is expressed in normal and cancerous prostate cells [64]. However, its functional role in those cells remains to be clarified.

It has been established that modification of nucleosome structure is an important regulatory mecha-
nism of gene expression. For example, butyrate is a non-competitive inhibitor of histone deacetylase, and thus provokes a hyperacetylation level of core histones [44]. The hyperacetylation of histones by butyrate induces a more open state of chromatin, which leads to the activation of expression of numerous genes and cellular differentiation [12], similar to that by trichostatin A (TSA), a potent specific inhibitor of histone deacetylase [63]. Butyrate treatment can also increase the phosphorylation of MAPK, i.e., ERK1/2 [27], and induce differentiation in many cell types [15,29,53]. Interestingly, some transcriptional effects by butyrate can be abolished by inhibitors of protein phosphatases [17]. Thus, the molecular mechanism of butyrate in cellular differentiation involving phosphorylation signaling remains to be delineated.

In an effort to understand how NE cells progressively emerge in prostate malignancies and their relationship to hormone-refractory prostate carcinomas, we studied the pharmacological effect on transdifferentiation of two human prostate cancer cell lines, LNCaP and PC-3. Although LNCaP parental cells, i.e. C-33 LNCaP cells, have been studied with regard to their growth patterns in response to various culture conditions, less is known about transdifferentiation of PC-3 cells nor is their transdifferentiation related to prostate cancer progression. We also examined the transdifferentiation process of C-81 LNCaP cells, a subline of LNCaP cells which lack androgen responsiveness of growth stimulation, despite the expression of a functional AR. We investigated the biochemical parameters of transdifferentiation, and examined the effect of butyrate on transdifferentiation of LNCaP cells. Our results suggest that different transdifferentiation processes are involved in the accumulation of NE cells in advanced hormone-refractory human prostate cancers.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS), cell culture medium RPMI 1640, gentamicin, horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulins (IgGs) were purchased from Life Technologies (Grand Island, NY). Charcoal/Dextran-treated, certified FBS with testosterone concentration below 3 ng/dl was from HyClone (Logan, UT). Protein molecular weight standard markers, acrylamide, and the protein assay kit were obtained from Bio-Rad (Hercules, CA). Sodium butyrate, trichostatin A, dibutyrate cAMP (db-cAMP), and β-actin-specific antibody were from Sigma (St. Louis, MO). IL-6 and NSE-specific antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). RPTPα-specific antibody was obtained from Transduction Laboratories (San Diego, CA). Gastrin (GS), chromogranin A (ChA), and neurtensine (NT)-specific antibody and ABC Staining System were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were obtained as described in previous publications [30,42].

2.2. Methods

2.2.1. Cell culture

Commercially available human prostate carcinoma cell lines, LNCaP and PC-3, were originally obtained from the American Type Culture Collection, and routinely maintained in phenol red (PR)-containing RPMI 1640 medium supplemented with 5% FBS, 1% glutamine, and 0.5% gentamicin, as described in previous publications [30,42]. LNCaP cells are androgen-responsive and express PSA, while PC-3 cells are androgen-insensitive and lack the expression of PSA [38,42]. For experiments, LNCaP cells that had passage numbers less than 33 were designated as C-33, passage numbers between 80 and 120 as C-81, as described in our previous publication [42]. Despite the expression of functional AR in C-81 cells, the degree of androgen-stimulating cell growth is decreased. Furthermore, the growth rate of C-81 cells is more rapidly than that of C-33 cells with a doubling time half that of C-33 cells [42]. For experiments, cells were routinely seeded in PR-containing RPMI 1640 medium supplemented with 5% FBS for 2 days prior to any treatment. The total cell number was counted using a Coulter Counter Z1 model [42,64].

2.2.2. Effect of steroid-depleted condition on prostate cancer cells

For these experiments, LNCaP and PC-3 cells
were plated in the regular medium at a cell density of 0.5×10⁶ per T-25 flask for PC-3 and C-81 LNCaP cells, and 1×10⁶ cells per T-25 flask for C-33 LNCaP cells, and allowed to attach for 48 h. Cells were then fed with a steroid-reduced (SR) medium, i.e., PR-free RPMI 1640 medium containing heat-inactivated 5% charcoal-stripped fetal bovine serum (CS-FBS) and gentamicin, for 5 days as described previously [64].

2.2.3. IL-6 and db-cAMP-induced neuroendocrine differentiation

Cells were seeded in a density of 0.5×10⁶ cells per T-25 flask for PC-3 and C-81 LNCaP cells, and 1×10⁶ cells per T-25 flask for C-33 LNCaP cells in RPMI 1640 medium containing 5% FBS for 2 days, then 24 h in a SR medium, and followed by treatment with 50 ng/ml IL-6 or 1 mM db-cAMP for 3 days as described previously [4,51].

2.2.4. Sodium butyrate effect

A cell density of 1×10⁶ cells per T-25 flask for C-33 LNCaP cells and 0.5×10⁶ cells per T-25 flask for C-81 cells were plated in RPMI 1640 medium supplemented with 5% FBS for 2 days. Cells were then fed with a SR medium with or without sodium butyrate as described previously [64]. After 72 h, cells were harvested for Western blot analyses.

2.2.5. Trichostatin A effect

A cell density of 1×10⁶ cells per T-75 flask for C-33 LNCaP cells was plated and maintained in RPMI 1640 medium supplemented with 5% FBS for 2 days. Cells were then fed with a SR medium with or without TSA. After 48 h, cells were harvested for Western blot analysis.

2.2.6. Immunocytochemical analysis

For immunocytochemical analysis, C-33 LNCaP cells were plated at 2×10⁴ cells per well in four-well glass slides (Nalge Nunc). Immunostaining was performed after treatments with a modified avidin–biotin complex technique [35]. Cells were washed briefly with PBS, fixed in −10°C methanol for 5 min and air-dried. The nonspecific immunoglobulin binding was blocked by incubation with 1.5% normal donkey serum (Santa Cruz Biotechnology) for GS, NT, ChA antibodies or goat serum (Santa Cruz Biotechnology) for NSE antibody for 1 h. The primary antibodies were applied to cells and incubated for 30 min at room temperature. The slides were then incubated with biotinylated IgG secondary antibody (goat anti-mouse or donkey anti-goat depending on the primary antibody host; Santa Cruz Biotechnology) in PBS with 1.5% normal blocking serum at room temperature for 30 min. Avidin–biotin complexes were made according to the manufacturer’s instructions, applied to the section, and incubated at room temperature for 30 min. Immunopositivity was visualized with the chromogen diaminobenzidine (0.025%) in substrate buffer and 0.1% hydrogen peroxide. Immunostaining with the negative controls (no primary antibody) was proceeded as described for the primary antibody.

2.2.7. Western blotting of cellular protein and medium

Cells were harvested and lysed as described previously [46]. Briefly, cells were scraped, pelleted and rinsed with ice-cold HEPES-buffered saline, pH 7.0, then lysed in an ice-cold cell lysis buffer containing a battery of protease and phosphatase inhibitors. The cellular lysates were spun, the supernatants were recovered and the protein concentration was determined using the Bio-Rad protein assay kit. The medium was harvested and spun at 4°C for 10 min to remove any cell debris before usage. For immunoblotting, an aliquot of total lysate protein (30 μg) or 15 μl of culture medium in a 2×SDS-PAGE sample buffer (1:1, v/v) was electrophoresed and transferred to nitrocellulose filters. Filters were incubated with appropriate primary antibody in Tris-buffered saline, pH 7.5, containing 0.1% Tween 20 (TBST) and 5% skim milk for 3 h, subsequently with secondary antibody in TBST buffer for 1 h. The protein bands were visualized by an ECL detection system. For reprobing, filters were agitated with the stripping buffer for 30 min at 50°C [42,45]. After two washes with TBST buffer, the filters were reacted with specific antibodies and the signal was detected by an ECL method.

2.3. Data analysis

The intensities of hybridization bands were semi-quantified by densitometric analyses of autoradiograms with different exposure time periods utilizing
3. Results

3.1. Effect of steroid-depleted medium, IL-6 and db-cAMP on transdifferentiation of prostate cancer cells

Since several pharmacological reagents have been reported individually to induce transdifferentiation of LNCaP or PC-3 human prostate cancer cells to a NE-like phenotype, we examined if the same reagent can have a similar effect on these two prostate cancer cells. C-81 LNCaP cells which exhibit androgen-unresponsive cell growth despite the expression of functional AR were also included for analysis. Due to the androgenic activity of PR [41], all cells were maintained in a SR condition, i.e., PR-free, steroid-reduced medium, in the presence or absence of IL-6, or db-cAMP. Control cells were maintained in the routine growth condition, i.e. PR-positive medium containing 5% FBS.

In the SR condition alone or with the addition of db-cAMP or IL-6, PC-3 cells showed little change in their morphology in comparison with control cells grown in the PR-positive medium containing 5% FBS (data not shown and [4]). C-33 LNCaP control cells had an epithelial morphology (Fig. 1A), and tended to grow in clusters with a somewhat acinar appearance (data not shown). In contrast, in the SR
condition, C-33 LNCaP cells had a neuronal appearance characterized by multipolar cells with small cell bodies and long, irregular processes (Fig. 1B). Similar morphologic effects were seen for these cells grown in the IL-6-containing medium although less pronounced (Fig. 1C), while db-cAMP had a more potent effect on cellular morphology (Fig. 1D). In comparison, C-81 LNCaP cells followed a similar trend to C-33 LNCaP cells in the phenotype alteration although the effect was less pronounced (data not shown).

To determine whether acquisition of the NE-like phenotype of C-33 LNCaP cells affects the expression of distinct NE cell markers, immunocytochemical stainings with antibodies against NSE, ChA, GS, and NT were performed. As shown in Fig. 2, LNCaP cells expressed elevated levels of ChA and NT in SR conditions or in the presence of IL-6 although the
degree of elevation is low. These data showed that LNCaP cells exhibit the ability to express ChA and NT prior to induced transdifferentiation by exogenous stimuli. In contrast, the immunoreactivity with NSE and GS antibodies was found to be highly increased in cells maintained in the SR condition and in the presence of IL-6. Interestingly, while db-cAMP had a potent effect on cellular morphology (Fig. 1D), it did not significantly affect the levels of NSE, ChA, GS, and NT. Nevertheless, the immunostaining clearly showed increasing NE characteristics of C-33 LNCaP cells in SR conditions including manifestation of the cytoplasmic NE granules in ChA staining and the cytosolic staining of NSE. Thus, different growth conditions including pharmacological reagents can induce NE-like phenotypic changes in prostate cancer cells, while they may have differential effects on the expression of biomarkers. Based on these data, we used NSE as the major marker in our further studies to monitor the transdifferentiation process of LNCaP cells into NE-like cells.

Western blot analyses were performed to semi-quantify the expression of NSE in cells grown in different conditions. As shown in Fig. 3 in C-33 LNCaP cells grown in the SR condition, the NSE level was elevated by approx. 3-fold, while to a lesser degree with an addition of IL-6, as observed in Fig. 2. Unexpectedly, in the presence of db-cAMP, the expression of NSE was decreased, even lower than that in control cells grown in the regular growth condition. An opposite effect was observed in C-81 LNCaP cells in which the NSE level was decreased in the SR condition, while elevated in db-cAMP-supplemented condition (Fig. 3). The level of NSE was not significantly altered by adding IL-6 to those cells. Interestingly, as shown in Fig. 3, PC-3 control cells expressed a high basal level of NSE in comparison with LNCaP cells. The NSE level was only elevated in the db-cAMP-treated cells, but did not change in the SR medium or IL-6-supplemented medium. Thus, despite a similar effect on the morphology of C-33 and C-81 LNCaP cells by the steroid-reduced condition and the db-cAMP supplement, these growth conditions have an opposite effect on the NSE expression. Thus, differential molecular expressions can correlate with a similar gross phenotypic change.

PC-3 cells are poorly differentiated prostate cancer cells, and do not express any known biomarkers including PSA. Similar to the observations by Bang et al. [4], we found that PC-3 cells only exhibited a minor change in their phenotype and a marginal elevation of NSE level upon db-cAMP treatment. We therefore focused our efforts on characterizing biomarkers during transdifferentiation utilizing genetically lineaged C-33 and C-81 LNCaP cells since they are derived from the same cell origin, and may represent different stages of tumor progression regarding androgen responsiveness [42], but not PC-3 cells. We utilized these two cells as our model system for further studies.

3.2. Effect of steroid-depleted medium, IL-6, and db-cAMP on PSA and AR expression in LNCaP cells

To determine molecular changes that are associated with NE differentiation of prostate cells, we
analyzed the expression of PSA and AR in LNCaP cells grown in different growth conditions. As shown in Fig. 4A, in the SR medium in the presence or absence of IL-6, the cellular level of PSA was downregulated in both C-33 and C-81 cells, although a lesser effect was obtained in C-33 cells. Db-cAMP treatments resulted in an increase in the intracellular level of PSA in both LNCaP cells (Fig. 4A). These data were consistent with the secreted level of PSA in

Fig. 4. Effect of the steroid-depleted condition, IL-6, or db-cAMP on PSA and AR expression in LNCaP cells. C-33 and C-81 LNCaP cells were plated and treated with 50 ng/ml IL-6 (IL-6) or 1 mM db-cAMP (db-cAMP) as described in Fig. 3. The same set of samples was used for Western blot analyses to detect the expression level of PSA (A) and AR (B). 30 μg total lysate proteins and 25 μl medium were used for Western blot analyses on PSA level in LNCaP cell lysates and in their conditioned medium. The ratio of PSA expression in cell lysates was similar to that in conditioned medium from LNCaP cells. Similar results were obtained from three sets of independent experiments.

Fig. 5. Western blot analyses of RPTPα expression in LNCaP cells. Cells were cultured at growth conditions in a PR-positive medium supplemented with 5% FBS (C), PR-negative medium supplemented with 5% SR-FBS (SR), PR-negative medium supplemented with 5% SR-FBS and 50 ng/ml IL-6 (IL-6), or 5% SR-FBS and 1 mM db-cAMP (db-cAMP). 30 μg total lysate proteins were electrophoresed and blotted on a nitrocellulose membrane, and the membrane was subsequently hybridized with anti-RPTPα antibody. It should be noted that a prolonged exposure showed a low level of RPTPα expression in C-33 cells grown in the IL-6-containing medium (data not shown). A portion of membrane was dissected for Coomassie blue staining to show a similar amount of loaded protein in each lane (data not shown). Similar results were obtained from three sets of independent experiments.
In the SR condition, the level of AR was elevated in C-33 LNCaP cells, while markedly decreased in C-81 cells (Fig. 4B). IL-6 also caused an increase of AR in C-33 cells, but a decrease in C-81 cells (Fig. 4B). Db-cAMP exhibited a negative effect on the AR level in C-81 cells, but not in C-33 cells. Thus, PSA, an epithelial cell differentiation marker, is regulated differently from NSE, a NE cell marker.

3.3. Expression of RPTPα in LNCaP cells during neuroendocrine transdifferentiation

It has been shown that RPTPα plays a critical role in neuronal differentiation and signaling [60,62]. We investigated if RPTPα may also be involved in prostate NE differentiation. As shown in Fig. 5, in cells cultured in a SR medium, the level of RPTPα expression was increased in both LNCaP cells, correlating with their morphological changes. However, in cells grown in the presence of IL-6 or db-cAMP, the expression of RPTPα was markedly decreased (Fig. 5). Thus, in the androgen-depleted condition, the expression of RPTPα correlates with phenotypic changes of LNCaP cells, independent of their androgen responsiveness. Furthermore, the expression of RPTPα in these two cells has the same response in different growth conditions.
3.4. Sodium butyrate effect on NSE and RPTPα expression in LNCaP cells

To further examine the correlation of NSE and RPTPα expression with NE differentiation of prostate carcinoma cells, sodium butyrate (NaBu) was utilized to induce cell differentiation [2,33]. NaBu induced a NE-diifferentiation phenotype (Fig. 6A), similar to the IL-6 effect (Fig. 1C) that correlated with a diminished cell growth (Fig. 6B). Interestingly, NaBu treatment resulted in an elevated level of NSE and RPTPα protein in both LNCaP cells although it had a lesser effect with different dose-response patterns in C-81 cells than in C-33 cells (Fig. 6C). Thus, the treatment of LNCaP cells with NaBu leads to a marked increase of NSE and RPTPα levels, which correlates with a NE-like phenotype of LNCaP cells, independent of androgen responsiveness of growth regulation.

3.5. Trichostatin A effect on NSE and RPTPα expression in C-33 LNCaP cells

NaBu exhibits several biological effects including that it can function as an inhibitor of histone deacetylases (HDAC) and cause a hyperacetylation of core histones. To investigate if histone hyperacetylation has the same effect on the expression of NSE and RPTPα during NE differentiation by NaBu, C-33 cells were treated with trichostatin A (TSA), a specific HDAC inhibitor. As shown in Fig. 7, TSA induced NSE expression in a dose-dependent manner, while it inhibited the expression level of RPTPα. The results thus indicated that the activation of RPTPα expression by NaBu is mediated by the activation of cellular pathways other than hyperacetylation of core histones.

4. Discussion

Clinical results indicate that NE cells may play a critical role in androgen-refractory growth of prostate cancer cells [9]. While the origin of these NE cells is not clearly defined in clinical studies, recent experimental studies have shown that prostate cancer cells can directly give rise to cells with a NE-like phenotype. This process is referred to as transdifferentiation [4,51,55]. Although different prostate cancer cells can be transdifferentiated into a similar NE cell-like phenotype by different treatments [4,13,26,51,55], there is no direct comparison among those treatments in different cells. Thus, it is not clear if different cells can follow the same pathway of transdifferentiation. The experimental work in this study is to investigate molecular mechanisms of transdifferentiation in different prostate cancer cells including different LNCaP cells. LNCaP cells are partially differentiated prostate cancer cells and express PSA, a prostate epithelium-specific differentiation marker. In different LNCaP cells, despite a similar expression level of functional AR and PSA, the responsiveness of androgen stimulation of cell proliferation is altered, imitating prostate tumor progression [42]. Thus, different LNCaP cells represent an interesting, unique cell model in studying the transdifferentiation of prostate cancer cells during tumor progression.

In our experiments, the end product of prolonged maintenance of C-33 LNCaP cells in the SR condition is a cell that resembles the phenotype of NE cells much more than the morphology of prostate epithelial cells. By growth in this condition, the altered phenotype of NE transdifferentiated C-33 LNCaP cells is dramatically different from that of control cells with differentiated characteristics of prostate epithelial cells (Fig. 1A vs. B). We found that andro-
gen-responsive C-33 LNCaP cells grown in steroid-reduced condition express higher levels of different markers of NE cells including NSE, NT, ChA and GS than control cells (Figs. 2 and 3). Thus our results clearly showed that C-33 LNCaP cells can undergo transdifferentiation into a cell type with strong characteristics of NE cells, suggesting the possibility that such an event might underlie the apparent accumulation of NE cells in hormone-refractory tumors. This phenomenon may also reflect clinical observations that, during androgen-ablation therapy, the androgen-responsive prostate cancer cells can transdifferentiate into NE-like cells, and the population of NE cells is thus increased.

In archival specimens of normal, hyperplastic and neoplastic human prostate, NE cells lack AR and are considered to be androgen independent [8,36]. In contrast to NE cells which are histologically considered nonproliferative [23] and represent a terminally differentiated cell population [6], we found that NE-like cells derived from LNCaP cells still express AR (Fig. 4B) and could proliferate in steroid-restricted conditions. Furthermore, a recent report shows the reversibility of the NE phenotype of LNCaP cells [16], suggesting that the acquisition of NE characteristics may be an epigenetic phenomenon that can occur gradually, depending on the phenotypic potential of a cell and the influence of its environment. These findings together indicate that there are apparent differences between NE-like cells and NE cells of the prostate.

Interestingly, in C-81 cells grown in steroid-reduced condition, the NSE level was not increased, while RPTPα had an increased expression as in C-33 cells (Fig. 3 vs. Fig. 5). C-81 LNCaP cells are an androgen-unresponsive subline of LNCaP cells and express PSA (Fig. 4A and [42]). Thus, these cells exhibit different biochemical properties from parental C-33 LNCaP cells. Although these cells express a functional AR (Fig. 4B), their growth is androgen-unresponsive [42] and exhibits a rapid growth rate in steroid-restricted condition, similar to androgen-independent PC-3 cells. In this condition, RPTPα expression, but not NSE, was increased in steroid-restricted medium as parental C-33 cells. This could be due to the fact that RPTPα is a different marker from NSE, and involved in different cellular functions in neuronal differentiation. RPTPα may be involved in the early stage of differentiation, while NSE is a differentiated marker. The regulations of their expressions are therefore via different molecular mechanisms. The detailed molecular mechanism is under investigation.

It has been reported that the IL-6 level in circulation is elevated in a subset of patients with advanced prostate carcinomas who have failed androgen-ablation therapy [59]. Upregulation of IL-6 in these patients may be due to the negative regulation of the IL-6 gene by androgenic hormones [5] since the expression of IL-6 receptor is increased after withdrawal of androgens [43]. These findings together point to the importance of cross-talks between IL-6 and androgen signaling pathways in prostatic carcinomas [45,50]. We have observed that IL-6-treated LNCaP cells acquire a neuron-like morphology (Fig. 1C). To determine whether these IL-6-treated LNCaP cells exhibit some characteristics of NE cells, we analyzed the expression of NSE (Figs. 2 and 3). We also compared these cells with poorly differentiated androgen-unresponsive PC-3 cells which express a high basal level of NSE (Fig. 3). IL-6 has no effect on the NSE level in PC-3 cells, correlating with a limited morphological change of these cells. However, IL-6 has an intriguing effect in LNCaP cells. In androgen responsive C-33 cells, although the NSE level is higher in IL-6-treated cells than in control cells grown in the regular growth condition, the NSE level is decreased, lower than in the SR alone. On the contrary, in C-81 cells, the NSE level is higher in the IL-6-containing medium than in the SR alone, indicating IL-6 counteracts the effect by the SR medium. The results clearly show that the intracellular signaling of IL-6 is different in androgen-responsive and -unresponsive prostate cancer cells. One possibility is that different signal transduction pathways are activated in those cells, due to differences in the expression of signaling molecules. For example, it has been reported that PC-3 cells may not express functional STAT3 [56], a transducer of IL-6 signaling. Of course, there are other explanations. Further studies are required to determine the IL-6 signaling in prostate cancer cells.

It has been shown that cAMP can reverse the transformed phenotype of some cancer cells, possibly by restoring the normal regulatory pathway [3]. The critical genes regulated by cAMP in prostate carci-
noma cells may include genes that induce NE differentiation as well as downregulate cell cycle progression. Our data show that an elevation of cAMP induces transdifferentiation primarily in androgen-unresponsive C-81 LNCaP and PC-3 cells, indicated by NSE expression and morphology change.

Our data thus reveal that androgen-unresponsive human prostate cancer cells respond differently from androgen-responsive cells to pharmacological treatments. This phenomenon thus resembles the heterogeneity of prostate cancer cells. Furthermore, although cAMP induces a dramatic change in the phenotype of C-33 LNCaP cells (Fig. 1D), cAMP caused a decrease of NSE in those cells (Figs. 2 and 3). The differential responses of NSE expression in different prostate cells is also similar to the response of c-myc gene expression to protein kinase C (PKC) action on those same cells [34]. Thus, a similar change in gross phenotype may accompany differential gene expressions.

As described above, IL-6 and db-cAMP have differential effects on different prostate cancer cells. In prostate cancer cells, results of several studies have shown that IL-6 and db-cAMP can stimulate or inhibit cell growth, depending on the targeted cells. It has been proposed that IL-6 and db-cAMP may share the same MAPK signaling pathway through a cross-talk with ErbB2 which activates the MAPK pathway in LNCaP cells, while it inhibits it in PC-3 cells [11,50,56]. Taken together all these findings indicate that PC-3 may respond differently from LNCaP cells in the presence of different pharmacological agents. The detailed molecular mechanisms remain to be clarified.

To further examine the regulatory mechanism of transdifferentiation of human prostate cells, we utilized different LNCaP cells, a genetically lineageed cell model system. PSA is expressed only in differentiated secretory prostatic epithelial cells, and serves as a marker of prostate differentiation [14]. Both LNCaP cells express a high level of PSA (Fig. 4A and [42]). In our studies, IL-6 induces NE-like morphology changes and has an inhibition of PSA expression in both LNCaP cells. Contrarily, db-cAMP upregulates the PSA level. It has been shown that human AR can be activated through an alternate signaling pathway involving db-cAMP in a ligand-independent manner in prostate cancer cells [18,48]. The activation of AR by db-cAMP can thus enhance the specific activity of AR, but not its protein level, which increases PSA expression.

The transmembrane nature of the receptor-like PTPs indicates that these PTPases can be involved in transducing extracellular signals. RPTPα has a high level of expression in brain [28,54], and can associate with contactin, a neuronal cell adhesion molecule, through the extracellular region [65]. Several lines of evidence have demonstrated that RPTPα plays a critical role in normal neuronal differentiation [20,60,67]. We have shown that in the steroid-reduced condition, RPTPα expression in both LNCaP cells is upregulated (Fig. 5), correlating with the phenotype change (Fig. 1B). To understand the possible functional role of RPTPα in prostate NE transdifferentiation, NaBu is utilized since this reagent can induce partial differentiation of PC-3 cells [33]. This reagent is currently under clinical consideration as a potential agent for the management of advance prostate cancer [52]. However, the molecular mechanism in this process is not completely known. In this study, NSE and RPTPα are used as markers to investigate the signaling pathway by NaBu in cell differentiation. Our data clearly show that NaBu treatment results in morphological changes and elevated NSE levels (Fig. 6), and thus resembles a transdifferentiation pathway of LNCaP cells. NaBu treatment results in an upregulation of RPTPα expression in C-33 and C-81 LNCaP cells (Fig. 6C). Since TSA is a specific inhibitor of histone deacetylases [63], it is a useful tool to study the role of histone acetylation in the regulation of NSE and RPTPα expression during NE differentiation by butyrate. Unexpectedly, TSA induces a dose-dependent activation of NSE, while it inhibits RPTPα expression in C-33 LNCaP cells (Fig. 7). Taken collectively the data suggest that NaBu activation of NSE and RPTPα expression is mediated by different signaling pathways although both NaBu and TSA have a similar effect on histone acetylation. For the first time, our data show that RPTPα may act as a molecular mediator in the signaling pathway initiated by NaBu although the link between sodium butyrate and RPTPα remains to be identified. Since cell growth and differentiation can be regulated by protein phosphorylation and dephosphorylation, RPTPα may play an important role in the modulation of gene...
expression for transdifferentiation of prostate carcinoma cells during androgen-ablation therapy, and may also serve as an additional marker for this process.

Results of clinical studies show that the NE cell population is increased in hormone-refractory carcinomas, although the origin of those NE cells is not known. Based on our results, we propose that there are multipathways for the transdifferentiation of prostate cancer cells to NE-like cells. As shown in Fig. 8, in normal prostate gland, NE cells are apparently derived from prostate stem cells. Nevertheless, hormonal-based prostate cancer therapies have the potential of altering androgen-responsive prostate cancer cells to NE-like cells in a manner that might accelerate the development of more aggressive prostate tumors. For androgen-unresponsive cells, an additional pharmacological effect is apparently required to facilitate this transdifferentiation process. Taken together, our data suggest that the transdifferentiation process is responsible for the apparent accumulation of NE cells in hormone-refractory tumors during relapse progression.

In conclusion, the molecular process by which cultured human prostate cancer cells undergo a transdifferentiation process to a NE cell-like phenotype is accompanied by changes in the expression of different protein markers. The NE cell-like phenotype can occur by exposing prostate cancer cells to different pharmacological agents. Furthermore, different molecular pathways can be involved in transdifferentiation of different prostate cancer cells, indicating the heterogeneity of prostate cancer. Thus, controlling these signaling events may provide us with alternative therapeutic targets to block tumor progression. Further studies are required to determine the functional role of NE cells in the pathogenesis of prostate carcinoma.

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