Neuroendocrine Transdifferentiation in Human Prostate Cancer Cells: An Integrated Approach

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Abstract

Prostate cancer is highly sensitive to hormone therapy because androgens are essential for prostate cancer cell growth. However, with the nearly invariable progression of this disease to androgen independence, endocrine therapy ultimately fails to control prostate cancer in most patients. Androgen-independent acquisition may involve neuroendocrine transdifferentiation, but there is little knowledge about this process, which is presently controversial. In this study, we investigated this question in a novel model of human androgen-dependent LNCaP cells cultured for long periods in hormone-deprived conditions. Strikingly, characterization of the neuroendocrine phenotype by transcriptomic, metabonomic, and other statistically integrated analyses showed how hormone-deprived LNCaP cells could transdifferentiate to a nonmalignant neuroendocrine phenotype. Notably, conditioned media from neuroendocrine-like cells affected LNCaP cell proliferation. Predictive in silico models illustrated how after an initial period, when LNCaP cell survival was compromised by an arising population of neuroendocrine-like cells, a sudden trend reversal occurred in which the neuroendocrine-like cells functioned to sustain the remaining androgen-dependent LNCaP cells. Our findings provide direct biologic and molecular support for the concept that neuroendocrine transdifferentiation in prostate cancer cell populations influences the progression to androgen independence. Cancer Res; 75(15): 1–12. ©2015 AACR.

Major Findings

Our integrated approach merging mathematical modeling with experimental data examines androgen-dependent prostate cancer response to long-term/sustained hormone-deprived conditions and the role of neuroendocrine in the progression to hormone-refractory status. Using the tools of applied mathematics, we demonstrated that the nonmalignant neuroendocrine phenotype, achievable under permanent androgen deprivation conditions, over time paradoxically contributes to the sustainment of undetectable androgen-dependent malignant cells.

Introduction

Neuroendocrine cells are highly specialized neuron-like cells with peculiar secretory functions, which are widely scattered throughout the human body including non-neuroendocrine glands like prostate. In normal prostatic parenchyma, neuroendocrine cells are part of a diffuse system that contributes to the homeostasis of the surrounding epithelial population. The neuroendocrine system acts through its secreted products such as calcitonin, parathyroid hormone-related protein (PTHrP), chromogranins (CgA, CgB), neuron-specific enolase (NSE), neurotensin, serotonin, bombesin, and somatostatin. These peptide hormones and biogenic amines can either be released into the bloodstream or act locally by paracrine or autocrine signaling in an androgen-independent way. Neuroendocrine cells and the associated neuropeptides play also a crucial role in sustaining both growth and progression of many, if not all, conventional prostate adenocarcinomas with a wide preclinical and clinical evidence of a poor prognosis correlation. However, the nature and the origin of neuroendocrine cells in prostate tumor lesions and their underlying molecular mechanisms are still controversial. Most likely, this is due to the complex heterogeneity and the multifaceted way in which neuroendocrine cells are linked to tumor progression. The ability of neuroendocrine cell to induce an early onset of a hormone-refractory status is very intriguing and clinically relevant. The transition from hormone-sensitive to hormone-insensitive status is one of the most critical issues in prostate cancer research as the conventional primary androgen deprivation therapy is only transiently successful. Over a period of 16 to 18 months, the tumor progresses to a hormone-independent status also known as castrate-resistant prostate cancer (CRPC). One emerging aspect of CRPC is that the androgen receptor signaling remains persistent. On the basis of the overall survival advantages, the U.S. FDA recently approved the “second” hormone therapy when patients develop CRPC. The mechanisms that upregulate intracellular androgens and/or androgen receptor, leading to ongoing androgen receptor–directed cancer growth despite a castrate level of serum androgens are not understood yet. It is widely believed that transdifferentiation from

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Quick Guide to Equations and Assumptions

Here, we present a nonlinear system of delay differential equations describing the interaction between malignant LNCaP and nonmalignant transdifferentiated neuroendocrine-like cell populations. The system in first approximation mimics experiments on cells growing in a Petri dish in androgen-deprived conditions and builds mainly off previous works by Adimy and colleagues (1, 2), adapting their model for cell cycle in hematopoiesis to our case. Furthermore, the model acknowledges previous works on prostate cancer development in single patients (3, 4), where most of the models consider two populations of cancer cells: one population androgen-dependent and the other population androgen-independent (3). These studies describe in vivo conditions, and both androgen-dependent and androgen-independent cells are able to proliferate. In our model, we also consider two cell populations, one androgen-sensitive (LNCaP) and the other androgen-insensitive (neuroendocrine-like), with sizes at time $t$ represented by $L(t)$ and $N(t)$, respectively. We assume that neuroendocrine-like cells are postmitotic and are produced through transdifferentiation by the first population. In addition, we consider that LNCaP cells go through asymmetrical cell division, generating both undifferentiated and differentiated daughter cells. Finally, the model describes the dynamics of androgen concentration by referring to the percentage of charcoal-stripped serum introduced in the medium $[A(t)]$. As the serum was the only exogenous source of androgen in the experiments, and as there is a direct proportionality between serum and androgen content, from now on we refer in an equivalent way to charcoal-stripped serum and androgen levels. The following system represents the dynamics of the three introduced quantities:

$$\frac{dA(t)}{dt} = -\varphi A(t) + \kappa N(t)$$  \hspace{1cm} (A.1)

$$\frac{dL(t)}{dt} = -\delta L(t) - k_2 a(A(t))L(t) - \beta(L(t), A(t))L(t) + 2(1 - k_2 a(A(t - \tau_1)))e^{-\delta \tau_1} \beta(L(t - \tau_1), A(t - \tau_1))L(t - \tau_1)$$ \hspace{1cm} (A.2)

$$\frac{dN(t)}{dt} = -\mu N(t) + 2k_2 a(A(t - \tau_1))e^{-\delta \tau_1} \beta(L(t - \tau_1), A(t - \tau_1))L(t - \tau_1) + k_3 e^{-\delta \tau_1} a(A(t - \tau_2))L(t - \tau_2)$$ \hspace{1cm} (A.3)

LNCaP cells have a constant per capita mortality rate $\delta$ and can differentiate into neuroendocrine-like cells with an androgen-dependent rate $k_2 a(A)$, where $k_2$ represents the differentiation efficiency. Experiments proved that LNCaP transdifferentiation only occurs for low androgen levels. To capture this evidence, we represented the dependence of differentiation on the androgen with the Ricker function:

$$a(A) = raAe^{-\alpha A}$$ \hspace{1cm} (B)

which is a standard choice for bell-shaped patterns skewed to the right. The positive constants $r$ and $a$, respectively, define the slope with which the differentiation rate increases and the inverse of the differentiation rate maximum point (1/a). Experimental evidences suggested that the differentiation induced by androgen deprivation is delayed. We therefore assumed that differentiating cells need a time $\tau_2$ to perform all processes necessary to fully transdifferentiate in neuroendocrine-like cells. The equation for transdifferentiating cells can be written as

$$\frac{dT(t)}{dt} = -\delta T(t) + k_2 a(A(t))L(t) - e^{-\delta \tau_2} k_3 a(A(t - \tau_2))L(t - \tau_2)$$ \hspace{1cm} (A.4)

where $T(t)$ is the population size at time $t$. From Eq. A.4, we can write

$$T(t) = \int_{t-\tau_2}^{t} e^{-\delta(s-\delta)}a(A(s))L(s)ds.$$

We also assumed that the LNCaP cells population is divided into proliferating and quiescent/mature cells. LNCaP cells need a time $\tau_1$ to perform all processes required for cell division by mitosis, for example, $\tau_1$ is the duration of cell cycle. The proliferating cells mortality rate was $\gamma$ and, by the end of the mitotic phase, each cell had divided into two cells, which either were LNCaP entering in the quiescent phase or differentiated neuroendocrine-like cells as previously reported (1). The equation for proliferating cells can be written as

$$\frac{dP(t)}{dt} = -\gamma P(t) + \beta(L(t), A(t))L(t) - e^{-\gamma \tau_1} \beta(L(t - \tau_1), A(t - \tau_1))L(t - \tau_1)$$ \hspace{1cm} (A.5)
where \( P(t) \) is the population size at time \( t \). From Eq. A.5, it follows that the proliferating cell population can be explicitly calculated as

\[
P(t) = \int_{t_{-t}} e^{-\beta(L(s), A(s)) A(s)} ds.
\]

The asymmetric cell division occurs at low androgen levels as described by Eq. B, with \( k_o \) representing its efficiency. The rate at which resting cells enter the proliferating phase depends both on level of androgen in the medium and on cell density. We assumed that a high level of androgen aids proliferation of LNCaP cells (5), whereas a high cell density inhibits it. The model describing the resting-to-proliferative phase rate \( \beta(L, A) \) consists of a continuous function that is zero in the absence of androgen in the medium \( \beta(L, 0) = 0 \), increases as the androgen concentration increases, and decreases as the LNCaP cell concentration increases (1, 2):

\[
\beta(L, A) = \beta_0 \frac{\theta^p}{\theta^p + L^n} \frac{A}{b + A}.
\]

On the right hand side of Eq. C, the first term represents the inhibition of the mitotic reentry rate \( \beta \) from \( L(t) \) into \( P(t) \) due to cell concentration and is described by a Hill function. The two positive constants \( \theta \) and \( n \) have similar roles to the Hill coefficients, and together they define the response to cell concentration changes. The second term is a Michaelis–Menten function, where \( b \) describes the androgen level at which \( \beta \) is half. Finally, the positive constant \( \beta_0 \) represents the maximum rate of cell movement from the resting phase into proliferation, which is achieved in the absence of the inhibition caused by low androgen levels or high cell concentrations.

The differentiated cells are postmitotic and die with a mortality rate \( \mu \). Androgen is assumed homogeneously distributed in the whole medium, and we do not differentiate between the intracellular and extracellular one. The exponent \( \psi \) controls the decay of androgen concentration in the medium, and neuroendocrine-like cells secrete A-like factors with a constant rate \( k \).

Other authors such as Morken and colleagues (3) and Portz and colleagues (4) assumed that proliferation of prostate cancer cells is androgen-dependent, but none of the existing models considered cell differentiation and its dependence on the androgen content, nor the possibility that differentiated cells could play an active role in sustaining the tumor in androgen-deprived conditions. We could therefore consider these as the major novelties of this model.

Materials and Methods

Cell culture and neuroendocrine transdifferentiation

The human prostate carcinoma cell line LNCaP (clone FG6; CRL-1740; passage number 10–40) was obtained from ATCC in 2013. Morphology check by microscope and cell growth curves was performed routinely. Cells were cultured in RPMI medium supplemented with 10% heat-inactivated FBS (Gibco-Invitrogen) according to the manufacturer’s instructions in 37°C in a 5% CO2-enriched humidified air atmosphere. In experiments assessing LNCaP neuroendocrine transdifferentiation protocol, cells were seeded at 4 × 10^5 cells per 100-mm dishes and left for 24 hours in regular media containing 10% heat-inactivated FBS before switching to various differentiation media (RPMI medium supplemented with different percentages of dextran-coated charcoal-stripped FBS, dcc-str, FBS; Sigma). Cells were maintained in those conditions until they started elongating their shape and activating a neuron-like morphology characterized by a progressive and sustained expression of neuroendocrine markers up to 14 days. For the parameterization of the mathematical model, we differentiated LNCaP in 1% dcc-str FBS (n = 4) and counted cells (days 3, 6, 10, and 14) either with Burker chamber or with Millipore’s Scepter automated handheld cell counter.

NMR and PCR data integration

\(^1\)H-NMR and PCR data were pretreated with centering and unit variance scaling, respectively. Then, we scaled the corresponding matrices to an equal total sum of squares to avoid the dominance of any of them. We integrated datasets by Orthogonal Projections to Latent Structures (O2PLS) to implement a bilinear statistical
model and reveal jointed variation. The influence of the original variables on the OPLS model was interpreted by inspection of the predictive regression coefficients, which are related to how each variable influences the model for prediction of the response variables. We derived O2PLS models from both polar and lipophilic NMR data in correlation with PCR data. To identify the subset of most responsible metabolites and transcripts characterizing the transdifferentiation process, variables were selected using a combination of VIP (variable importance in projection) value >1 and correlation loadings $p[q corr] >0.8$ for the polar model. We also generated a correlation map with hierarchical clustering by combining transcript values and selected polar metabolite buckets where we considered Euclidian distance for the metrics and WARD method for clustering criterion.

Pathway analysis
Pathway topology and biomarker analysis on selected and more representative metabolites in class separation were applied to the pathway topology search tool in Metaboanalyst 2.0 (17, 18). We calculated the centrality through the Pathway Impact, a combination of the centrality and pathway enrichment results. Metabolites were selected by evaluating both VIP values $>1$ in class discrimination and correlation values $| p[q corr] | > 0.8$. Homo sapiens pathway library was chosen and analyzed using the Fisher exact test for overrepresentation and relative-betweenness centrality for pathway topology analysis.

Formulation of the mathematical model
The mathematical model introduced in the Quick Guide was used to describe laboratory experiments on the interaction between the human prostate cancer cells LNCaP and the transdifferentiated neuroendocrine-like cell population. Most of the mathematical models for prostate cancer analyze the effects of continuous (19) or intermittent (20–25) androgen deprivation treatments on cancer progression. Prostatic tumor cells are usually divided in androgen-dependent and androgen-independent, with androgen-dependent transforming in androgen-independent in a reversible or irreversible way (25). In some models, the mutation rate is considered directly dependent on the androgen concentration (4, 19, 21, 25), whereas in others, the switch rate depends on a cell quota of bound androgen receptor (3, 4). All these studies investigate the well-known mechanism of androgen-independent relapse and do not consider cell transdifferentiation as such. Also, differently from the mathematical approach followed in other types of tumors, for example, colonic crypt and colorectal cancer (26–28), in the case of prostate cancer the well-established theory of cancer stem cells, which assumes an asymmetrical cell division (29), has not been considered in the representation of tumor growth (1, 30). In our model (A1–A3) according with the cancer stem cell theory, we assumed that LNCaP mitosis leads to the formation of both undifferentiated and differentiated cells. The differentiation, which is driven by environmental factors (i.e., prolonged hormone deplanation), is considered irreversible and differentiated cells apoptotic, as our experiments did not show any reversibility or proliferation of neuroendocrine-like cells. The model also represents androgen dynamics. The experiments were run considering different percentages of charcoal-stripped serum; however, given the direct proportionality between the serum and its androgen content in the model, we directly refer to androgen levels. Processes such as proliferation and transdifferentiation depend on the androgen level.

Parameterization of the mathematical model
The model was parameterized from data on the basis of the experiments described above, and with values and ranges taken from the suitable literature when experimental data were not able to provide the required information. We took from the literature values for LNCaP and neuroendocrine-like cell death rates $\delta$ and $\mu$, for the androgen degradation rate $\psi$ (3), and for the Hill parameter $n$ (5). To parameterize the Ricker function, representing the relationship between androgen levels and transdifferentiation (Eq. B), we designed an experiment that would provide the maximum differentiation rate at different androgen levels. The nonlinear regression of these data provided the following parameter values $r = 3 \pm 2$ day$^{-1}$ and $a = 1.3 \pm 0.3$ (Supplementary Fig. S1). The proliferation parameters $\rho_0 = 1.8 \pm 0.2$ day$^{-1}$ and $\theta = 0.9 \pm 0.1$ cells were estimated from empirical data of LNCaP grown in regular growth medium.

According to the manufacturer, the value for LNCaP cell-cycle duration $\tau_1$ was set equal to 1.43 day, whereas the value of the delay in the differentiation process $\tau_2 = 7$ days was estimated from the progressive expression of neurotensin over time (Fig. 1). The value of the Michaelis–Menten half-saturation constant $b = 0.2$ was based on the observation that even at very low levels of androgen LNCaP cells are still able to proliferate (Fig. 2). Finally, the fit of 14-day experiments of LNCaP growth in androgen-deprived conditions provided values for the differentiation efficiency $k_1 = 0.6 \pm 0.2$, and the asymmetrical division parameter $k_2 = 0.5 \pm 0.2$. Values for the parameter $x$ were considered in the range of $0.003$ to $0.03$ day$^{-1}$. Numerical simulations and parameterization were performed with MATLAB R2014a.

Results
Acquisition of neuroendocrine phenotype with antiproliferative action on parental LNCaP cells
To investigate the role of neuroendocrine cells during androgen-independent acquisition, we cultured androgen-dependent LNCaP cells in long-term hormone-deprived conditions. Different culture conditions (from 0% to 5% dcc-srt FBS) were tested. Morphologic and transcriptional data analyzed all over the 14-day experimental period indicated the best differentiation medium for the study. Low-androgen content rather than complete androgen depletion appeared to be crucial, as the 1% rate was an exclusive condition for the occurrence of this cellular phenomenon. Only when hormone content was kept at 1% dcc-str FBS, cells started elongating their shape and showing a neuronal-like morphology. From day 1 to day 3, cells retained the typical LNCaP morphology, a spindle shape with occasional pseudopodium-like extensions. Starting from days 8 to 10, cells presented an elongation of their shape that resolved (day 14) in the development of long-branched neuritic-like processes with small cell bodies (Fig. 1A–H). The elongating shapes were characterized by a progressive and sustained expression of neuroendocrine markers up to 14 days (i.e., NSE, neurotensin; Fig. 1I and J). To investigate paracrine interactions between the two cellular phenotypes, we determined the effect of neuroendocrine conditioned media on LNCaP cell growth by means of $[^{3}H]$-thymidine incorporation. LNCaP cells were exposed (48 hours) to various conditioned media collected during neuroendocrine transdifferentiation at different time points (see scheme in Fig. 2). DNA synthesis
of LNCaP cells was significantly \( P < 0.001 \) affected by the complete deprivation of serum (nonserum medium), whereas nonconditioned differentiation medium showed no per se effect when compared with regular growth medium (Fig. 2A). Already after 3 days, we observed that conditioned differentiation medium significantly \( P < 0.01 \) inhibited \([H]-\) thymidine incorporation of LNCaP cells (Fig. 2B).

**NMR- and PCR-based prostate cancer discriminates classes revealing a nonmalignant phenotype**

NMR and PCR analyses were performed in parallel on LNCaP (day 0) and neuroendocrine-like cells (day 14). Conspicuous differences were found in their metabolic profiles. In particular, some hydrophilic signals, commonly identified as prostate cancer biomarkers (31), such as creatine and phosphocreatine (Cr + PCr), glycine (Gly), and alanine (Ala), were mostly prominent in LNCaP compared with neuroendocrine-like samples (Fig. 3A). Also, the hydrophobic fraction denoted a different and phenotype-based distribution of total cholesterol content (Fig. 3B). Free cholesterol was more pronounced in neuroendocrine cells, whereas the form prevailing in LNCaP cells was the esterified one (Fig. 3C and D), recently related to prostate cancer aggressiveness (32). Differences were also found in the relative quantification of selected gene expression. The applied prolonged hormone-deprived conditions led to a significant upregulation of common neuroendocrine secretory...
products (i.e., NSE, neurotensin, and CgA). While other hormone-related targets (i.e., estrogen receptor GPER and androgen-regulated channel TRPM8) were upregulated in neuroendocrine-like cells, the expression of the prostatic tumor biomarker α-methyl-acyl-CoA racemase (AMACR) was strongly reduced in respect to LNCaP cells (Fig. 4). Also, mRNA levels of a common indicator of prostate cancer [i.e., prostate-specific antigen (PSA)] were significantly mislaid in neuroendocrine transdifferentiated cells when compared with parental malignant cells. The transdifferentiation process (Fig. 4) did not affect gene expression of androgen receptor. Both, NMR-based metabolic profiles and PCR-based analysis of relevant genes indicated that androgen deprivation drove the development to a less-malignant neuroendocrine phenotype. Accordingly, principal component analysis applied both to NMR and to PCR data smoothly discriminated androgen-dependent LNCaP cells from their relative transdifferentiated neuroendocrine-like cells. The complete separation between classes was achieved with an unsupervised analysis (Supplementary Fig. S2).

Data integration and joint systematic variation suggests the use of healthy neuroendocrine biomarkers as putative indicators of androgen-independent prostate cancer

Once we established a clear discrimination between the two phenotypes, we explored the shared variance between NMR and PCR values via O2PLS. In this way, we pointed out those variations that consistently and concurrently occurred during the transdifferentiation process (Fig. 5A). The joint predictive structure showed that 95% of the variation in the polar NMR block (66% for lipid dataset) correlates with 96% of the variation in the transcript block (72% for lipid dataset), which is a substantial overlap. This approach produced a functional model for interchangeable predictions between NMR and PCR data. Class discrimination was achieved by considering the shared variation between transcripts and metabolites. We found a close linking between the two biologic information sets (see regression coefficients in Supplementary Fig. S3) via O2PLS analysis that displays sample scores and variable loadings in a joint predictive model (leave-one-out classification approach showed that only 3 of 10 samples were outliers). This model allows projecting the transcripts and metabolites on a common two-dimensional plane (Fig. 5A), where the positions describe the cell phenotype and the proximity between transcripts and metabolites relates with the correspondence of their changes. The analysis revealed that both PSA and AMACR, highly expressed in LNCaP cells, positively correlated with Cr+PCr, Gln, Pro, and Ala. These transcripts also positively correlated with fatty acids and phospholipidic compound (PC and PE) in LNCaP cells. We found minor positive correlations for choline and ethanolamine moieties (PE and GPC) and
nucleoside derivatives (ribose and UDPG). Moreover, all the genes upregulated in neuroendocrine-like population (i.e., TRPM8, CgA, and GPER) correlated with Gln, myo-inositol (myo-Ins), and citrate (Cit). We observed different correlations for the 2 genes associated with the neuroendocrine phenotype. Individually, NSE appeared correlated to PE/PC, Gln, and GSH, whereas neurotensin was mostly influenced by myo-Ins and acetate (Ace).

Interestingly, neuroendocrine-like cell population appeared mainly characterized by the joint variation of neurotensin and GPER with free cholesterol. We used a correlation matrix-based hierarchical clustering (CMBHHC) for visualization purposes. The results of hierarchical clustering were then visualized as a tree structure plot (dendogram), which clearly classified the two cell populations based on transcripts and metabolites correlation coefficients (Fig. 5B). As a final point, we applied Metabolite Set Enrichment Analysis (MSEA) to draw biologic inferences about selected metabolites and to identify their relevance in significant metabolic pathways. The applied selection showed that at least 30 major metabolic pathways were involved (Supplementary Table S1). Among these, alanine/aspartate/glutamate ($P = 1.81 \times 10^{-4}$, impact = 0.52); glutamine/glutamate ($P = 1.22 \times 10^{-3}$, impact = 0.35); glutathione ($P = 1.45 \times 10^{-2}$, impact = 0.24); glycine/serine/threonine ($P = 1.44 \times 10^{-3}$, impact = 0.19); and arginine/proline ($P = 3.95 \times 10^{-4}$, impact = 0.13) metabolisms emerged. Considering both Holm $P$ values and the False Discovery Rate (FDR) correction, we focused on alanine/aspartate/glutamate metabolism (Holm $P = 1.39 \times 10^{-2}$, FDR = $3.62 \times 10^{-3}$), which appeared to be the most affected between cell populations, with glutamine/glutamate metabolism (Holm $P = 9.17 \times 10^{-2}$, FDR = $1.63 \times 10^{-2}$) to a minor extent (Fig. 6).

Figure 3.
NMR-based metabolome analysis. A, representative $^1$H-NMR spectra of LNCaP and neuroendocrine (NE) cells polar extracts. B, representative $^1$C-NMR spectra of LNCaP and neuroendocrine cells lipid extracts. C and D, bucket variations relative to free cholesterol (C) and esterified cholesterol (D) content in LNCaP and neuroendocrine lipophilic fractions. Bin values were normalized to the total spectral area intensity. Ac, acetate; Al, alanine; Leu, leucine; Chol, free cholesterol; Chol E, esterified cholesterol; Cr/PCr, creatine/phosphocreatine; Gln, glutamine; Glu, glutamate; Lac, lactate; Gly, glycine; ML, myo-inositol; Pro, proline; SFA, saturated fatty acid; Tau, taurine; Val, valine.
Mathematical modeling of neuroendocrine transdifferentiation supports a self-sustaining mechanism for LNCaP cells

On the basis of the experimental evidence, we developed a mathematical model of LNCaP cells dynamics and transdifferentiation. We assumed a mechanism of androgen production by neuroendocrine-like cells and lack of androgen sources external to the system (see Eqs. A.1–A.3). Two types of analyses were applied, one to describe the cell growth dynamics (descriptive) and the other to forecast future behaviors (predictive). The descriptive analysis well represented all the outcomes of the in vitro experiments. The obtained growth curve resembled the increase in cell numbers (from $3 \times 10^5$ cells at day 0 to $2.5 \times 10^6$ cells at day 16) measured as described above (Fig. 7A). Figure 7B shows the gradual acquisition of a neuroendocrine phenotype over time as we obtained from both morphologic and transcriptomic analyses. The predictive analysis allowed a comparison between the outcome of the model and the results of a continuous androgen deprivation therapy. Keeping the same parameter values tuned on data from the 14-day experiment (Supplementary Table S2), we ran a simulation for 400 days (Fig. 7C and D). After a first period of approximately 150 days, during which LNCaP cells almost appeared extinguished, whereas neuroendocrine-like cells were nearly constant in number, the system behavior suddenly changed and neuroendocrine-like cell population density started increasing followed by an increase of LNCaP cells (Fig. 7D). Both populations reached a steady state after further 150 days. The simulations showed how LNCaP cells self-sustain by enhancing a concentration of neuroendocrine-like cells sufficient to produce enough A-like factor to allow androgen-dependent cell proliferation (Fig. 7C).

Discussion

Despite the advances in early detection due to a widespread use of PSA-based screening, a high rate of patients still present locally advanced prostate cancer when diagnosed. Furthermore, most of the patients who successfully go through endocrine therapy are often at high risk of recurrence. This unfavorable prognosis is due to the progression of primarily androgen-independent cancer cells that escape hormonal ablation. Neuroendocrine transdifferentiation is one of the hallmarks of this phenomenon; nonetheless, the clinical significance of the coexistence of neuroendocrine phenotype in the context of classic prostate cancer is still controversial. The approach we proposed in this study is based on the use of “omics” sciences and mathematics to investigate how neuroendocrine transdifferentiation contributes to tumor progression and
hormone therapy failure. A growing body of literature describes neuroendocrine transdifferentiation from LNCaP cells as a result of short-term (72 hours) androgen withdrawal, or a dramatic increase of c-AMP levels, or a cytokine-based induction. Those cells were described as cancerous terminally transdifferentiated neuroendocrine cells lacking of androgen receptor and PSA expression. Herein, differently from previously described \textit{in vitro} protocols (33, 34), we cultured androgen-dependent LNCaP cells in a long-term (up to 14 days) hormone-deprived conditions. Such conditions led to a possibly closer "human-like" model of androgen resistance. Unexpectedly, metabolic profiles revealed that LNCaP cells transdifferentiated into a nonmalignant neuroendocrine phenotype, resembling a clinically positive response to primary androgen deprivation therapy. Experiments also showed that some environmental factors (i.e., minimal androgen content rather than its depletion) are crucial for this particular transdifferentiation process. Neuroendocrine conditioned media (collected at various days of the transdifferentiation process) affected LNCaP cell proliferation. However, further investigations are needed to clarify if the reduction of LNCaP cells is only due the androgen-depleted conditions or if there is a concurrent neuroendocrine-mediated anti-mitogenic effect.

Metabolome analysis described the dynamic changes in the pattern of malignancy observed during the considered experimental period. LNCaP cells (day0) were characterized by Ala, Glu, Cr, and Gly (the precursor of sarcosine) previously described as prostate cancer metabolic biomarkers (35–37). In agreement with the well-known Warburg effect (38), these cells showed an increased rate of energetic expenditure, as the relevance of metabolic-related nucleosides (i.e., AMP, NADP, NAD, ATP, UDP) indicates. Prostate cancer generally presents a high glucose oxidation and low levels of \textit{myo}-inositol (39, 40), so the increase in glutathione (in its reduced form, GSH), Gln, and \textit{myo}-inositol signals observed in neuroendocrine-like cells at day 14 is a further confirmation of their nonmalignant phenotype. Neuroendocrine cells exhibited high levels of cholesterol, the precursor of the entire androgen synthesis cascade. Very recently, it has been reported that an aberrant accumulation of its esterified form is related to prostate cancer aggressiveness (32). However, the abundance of cholesterol in day 14 cells was mostly due to its free and not esterified form, which instead resulted more pronounced in LNCaP day0 cells. Moreover, enriched pathway analysis showed that the alanine/aspartate/glutamate metabolism was the most profoundly affected during transdifferentiation. In particular, we found that androgen deprivation produced a downregulation of L-Asp in neuroendocrine-like cells. This finding is in agreement with the reported evidence that in prostate epithelial cells, L-Asp is an important source for citrate synthesis via oxaloacetate through testosterone positively regulated L-Asp transporter (41). The two cell populations under study showed distinct transcripts related to their phenotypes (upregulation of PSA and AMACR in day 0 cells
and upregulation of NSE, neurotensin, and CgA in day 14 cells, respectively). Furthermore, under low serum condition, nonmalignant neuroendocrine-like cells exhibited a higher content of hormone-related targets (GPER and TRPM8) that could be related with the induction of androgen independence in adjacent malignant phenotype. TRPM8, in fact, is positively regulated by androgens, and we showed how neuroendocrine cells under persistent low levels of hormones can increase the production of free cholesterol, precursor of testosterone. We did not observe a parallel reduction of AR expression in transdifferentiated neuroendocrine day 14 cells. This can be explained assuming that the abundance of free cholesterol found in neuroendocrine cells sustains the expression of AR even in low androgen conditions.

We applied a descriptive in silico analysis to represent the transdifferentiation process and to further investigate the possible role of nonmalignant camouflaged neuroendocrine cells on potential prostate cancer relapse. The mathematical model we developed combined all information provided by experiments and statistical analysis, and the numerical simulations well represented the outcomes of the 14-day experiments. Predictive analyses provided a possible explanation to the reason why tumorigenic LNCaP cells differentiate into nonmalignant neuroendocrine-like cells. In fact, long-term simulations showed a peculiar ability of neuroendocrine cells in sustaining the system. Numerical results also provided a possible reason why most patients, but not all, develop androgen-resistant prostate cancer, as the simulated outcome depended on the initial size of LNCaP cell population and on an eventual minimum size for the neuroendocrine-like cell population. The key assumption for the model behavior is that neuroendocrine-like cells have a putative role in hormonally treated cancers by releasing paracrine factors that promote residual prostate cancer cell growth and progression (neuroendocrine-based feeding support).

Given the in silico results, we can assert that malignant androgen-dependent LNCaP cells react to hormone deprivation by favoring the establishment of a nonmalignant neuroendocrine cell population. We can consider these neuroendocrine cells as ‘hidden’ androgen-resistant clones coexisting with scattered malignant cells. This apparent positive response to early hormone-based therapy leads to the increase of neuroendocrine components, which, over time, show their “evil-side” by thwarting the recovery of malignant LNCaP proliferation through a paracrine mechanism.

In conclusion, we produced an original in vitro model to investigate the pathophysiology of neuroendocrine cells in hormone-refractory transition of prostate cancer. The nonmalignant phenotype achieved in our model represents an intriguing link between neuroendocrine cell differentiation and the occurrence of hormone-refractory prostate cancer status. These androgen-independent cells are able to recover the proliferation index of surrounding non-neuroendocrine phenotype cancer cells by the secretion of neuroendocrine products through a paracrine mechanism.

Figure 6.
A, topology-based pathway analysis showing metabolic networks potentially affected during neuroendocrine (NE) transdifferentiation. The most impacted metabolic pathways are specified by the volume and the color of the spheres (yellow, least relevant; red, most relevant) according to their statistical relevance $P$ and impact value. B, metabolic scheme reporting alanine, aspartate, and glutamate pathway potentially affected during the neuroendocrine transdifferentiation process.
mechanism. The predictive forecasts of the mathematical model support the notion that, also in a clinical setting, treatment-related neuroendocrine cells generate tardive inductive stimuli on quiescent/undetectable tumor cells. The statistical analyses provided a link between transcripts and metabolites that were highly co-responsible for class distinction. All the found correlations are important for the future development of new diagnostic tools for androgen-independent prostate cancer. Translated into a clinical setting this bidirectional model could be used for predictions in both directions between NMR and PCR data matrices, offering new possibilities of monitoring the response of patients with prostate cancer to treatments. Further in vivo analyses are required (i) to validate new putative biomarker candidates during the follow-up of treated prostate cancer and (ii) to elucidate the feasibility of this complex functional network between epithelial PSA secretory cells and "dual face" neuroendocrine cells. The understanding of the biologic duality of these neuroendocrine cells, with a nonmalignant phenotype that "sneakily" sustains hormone-dependent tumor cells, will be clinically relevant in the management of advanced, relapsing, and castration-resistant prostate cancer as well as in the development of new strategies for targeted therapies and/or diagnostic biomarkers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Figure 7.
Mathematical simulations of cell behavior over time. A, total cell number plotted against experimental counting. B, numerical simulation of LNCaP transdifferentiation into neuroendocrine cells, as reported from the experimental outcome. C, predictive simulation of androgen-like factor over a time interval of 400 days. D, predictive simulation of LNCaP/neuroendocrine cell system over a time interval of 400 days.


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