Notch Signaling Modulates Hypoxia-Induced Neuroendocrine Differentiation of Human Prostate Cancer Cells

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Abstract

Prostate carcinoma is among the most common causes of cancer-related death in men, representing 15% of all male malignancies in developed countries. Neuroendocrine differentiation (NED) has been associated with tumor progression, poor prognosis, and with the androgen-independent status. Currently, no successful therapy exists for advanced, castration-resistant disease. Because hypoxia has been linked to prostate cancer progression and unfavorable outcome, we sought to determine whether hypoxia would impact the degree of neuroendocrine differentiation of prostate cancer cells in vitro.

Results: Exposure of LNCaP cells to low oxygen tension induced a neuroendocrine phenotype, associated with an increased expression of the transcription factor neurogenin3 and neuroendocrine markers, such as neuron-specific enolase, chromogranin A, and β3-tubulin. Moreover, hypoxia triggered a significant decrease of Notch 1 and Notch 2 mRNA and protein expression, with subsequent downregulation of Notch-mediated signaling, as shown by reduced levels of the Notch target genes, Hes1 and Hey1. NED was promoted by attenuation of Hes1 transcription, as cells expressing a dominant-negative form of Hes1 displayed increased levels of neuroendocrine markers under normoxic conditions. Although hypoxia downregulated Notch 1 and Notch 2 mRNA transcription and receptor activation also in the androgen-independent cell lines, PC-3 and Du145, it did not change the extent of NED in these cultures, suggesting that androgen sensitivity may be required for transdifferentiation to occur.

Conclusions: Hypoxia induces NED of LNCaP cells in vitro, which seems to be driven by the inhibition of Notch signaling with subsequent downregulation of Hes1 transcription. Mol Cancer Res; 10(2); 230–8. ©2011 AACR.

Introduction

Carcinoma of the prostate is the second leading cause of cancer-related death in men, representing 15% of all male malignancies in developed countries (1). Although the incidence of prostate cancer varies according to race, about 85% of patients are diagnosed after the age of 65 years (2).

However, little is known about the molecular mechanisms that underlie its development and progression. Neuroendocrine differentiation (NED) of prostate cancer leads to a worst prognosis and is associated with a lack of response to androgen deprivation therapy, which is the only effective treatment for advanced metastatic disease. Indeed, it has been suggested that neuroendocrine-differentiated prostate cancer cells present within the tumor mass sustain proliferation, invasion, and metastasis through the production and release of peptide hormones (3). Moreover, NED has been associated with chemotherapy resistance (4).

Neuroendocrine cells exist in normal adult prostate, where they regulate growth, differentiation, and secretory properties of the gland. Malignant neuroendocrine cells have been identified in all human prostate cancer tissues, with a prevalence that varies among different studies (5, 6). This may be due, at least in part, to inconsistency in techniques used for the identification of the most widely used markers of prostate NED, neuron-specific enolase (NSE), and chromogranin A (CGA; ref. 7).

How prostate cancer shifts toward the neuroendocrine phenotype is still debated. Androgen deprivation therapy seems to favor the appearance of neuroendocrine cells

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through the activation of PI3K-AKT-mTOR intracellular signaling pathway (8).

In a tumor mass, oxygen tension is constantly changing, following changes in microvascular supply, with periods of acute and chronic hypoxic conditions (9). Hypoxia is involved in cancer progression and has been linked to modulation of Notch signaling in solid tumors (10, 11). Notch is an evolutionary conserved receptor/ligand system that mediates several biologic processes: cell fate specification, differentiation, proliferation, apoptosis, migration, and angiogenesis (12, 13). Dysregulation of Notch signal occurs in several types of tumors (14–16), including prostate cancer (17). Notch signaling is also required for normal prostate development (18, 19). When Notch receptor interacts with its ligands, it undergoes a series of proteolytic cleavages that result in the production of an intracellular domain (NICD), which translocates to the nucleus where it binds to the transcription factor CBF1 (13). The formation of the CBF1-NICD complex leads to the recruitment of the nuclear protein MAML1 that functions as a transcriptional activator of CBF1-dependent genes, such as transcriptional repressors belonging to the Hes and Hey families (20).

This study was aimed at investigating whether hypoxia influences the degree of NED of prostate cancer cells, in vitro, and whether neuroendocrine transdifferentiation is supported by Notch signaling.

Materials and Methods

Antibodies and chemicals

Mouse monoclonal antibody against β3-tubulin (TUJ-1; 1:2,500) and rabbit polyclonal antibody against human Notch 1 (sc-6014-R; 1:1,000) were obtained from Santa Cruz Biotechnology Inc. (DBA); mouse monoclonal antibody against HIF-1α (1:1,000) was obtained from Novus Biologicals (DBA); mouse monoclonal antibody against human NSE (1:1,500) was obtained from Dako Cytomation; rabbit monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1,000), mouse monoclonal antibody against HA epitope tag (1:5,000) was purchased from Invitrogen SRL. Mouse monoclonal antibody against V5 epitope tag (1:5,000) was purchased from Invitrogen SRL. Horseradish peroxidase (HRP)-conjugated secondary anti-mouse and anti-rabbit antibodies were purchased from GE Healthcare Italia and Pierce Biotechnology Inc. (Euroclone), respectively. All other reagents were obtained from Sigma-Aldrich S.r.l. The γ-secretase inhibitor DAPT (20 mmol/L stock solution) was dissolved in dimethyl sulfoxide. Dihydrotestosterone (DHT) was dissolved in ethanol.

Cell cultures

LNCaP (androgen-dependent human prostate cancer cell line), PC-3 and Du145 (androgen-independent human prostate cancer cell lines) were obtained from American Type Culture Collection, maintained in liquid nitrogen, and used within few weeks after thawing. Cells were grown in RPMI-1640 (LNCaP) or Dulbecco’s Modified Eagle’s Medium (DMEM; PC-3 and Du145; Euroclone), supplemented with 10% (vol/vol) FBS (Euroclone), 1-glutamine (Euroclone), and 1% (vol/vol) antibiotic/antimycotic solution (Gibco, Invitrogen S.r.l.). Hypoxia was achieved by maintaining the cells at 2% oxygen, in a CO2 incubator (Forma Series II, Thermo Scientific) with oxygen sensor control, and with CO2 and N2 gas regulators, for up to 14 days. The cells were split every 4 days.

Cell growth analysis

To assess cell growth under normoxia and hypoxia, cultures were plated at 40,000 cells per well, in a 6-well tissue culture plate. At day 3 and 5 after plating, cells were detached by trypsin and counted using a hemocytometer. Four wells were counted for each point. Growth response to DHT stimulation was studied by plating 80,000 cells per well, in a 6-well tissue culture plate. Forty-eight hours later, culture medium was changed with medium containing 1% FBS and 4% charcoal-stripped FBS, and the cells were put in normoxic or hypoxic environment. After 5 days, cells were stimulated with DHT (10 nmol/L); 7 days after treatment, cells were detached by trypsin and counted using a hemocytometer. Four wells were counted for each point. Student t test was used for statistical analysis.

Phase contrast microscopy

Phase contrast photographs of the cells, under normoxic and hypoxic conditions were taken with a phase contrast Zeiss Axiovert 25 inverted microscope, equipped with an AxioCam MR camera and Axiovision software.

Immunocytochemistry

Mouse monoclonal antibody against NSE (prediluted) was obtained from CellMarque (Ventana). The immunocytochemical analysis was carried out by Benchmark XT Ventana System. Cells, grown on poly-l-lysine–treated glass slides, were rinsed with PBS and fixed in 50% ethanol. Immunostaining was carried out using the HRP Multimer System and specific mouse monoclonal antibodies; the 3,3’-diaminobenzidine (DAB, Ventana) was used as a substrate chromogen solution for the development of the peroxidase activity (UltraView Universal DAB Detection Kit, Ventana).

RNA extraction and quantitative real-time RT-PCR

Total RNA was isolated using the RNEasy Mini Kit (Qiagen), following the manufacturer’s instructions. During RNA purification, the sample was treated with RNase-free DNase I (Qiagen) to eliminate any genomic DNA contamination. The concentration of total RNA was determined spectrophotometrically with Nanodrop ND-1000 (National Instruments Corporation). Total RNA (1–2 μg) was reverse transcribed into cDNA by using TaqMan reverse transcription reagents, with random hexamers (Applied Biosystem Inc.). The profile of the reverse transcription reaction was 10 minutes at 25°C, 30 minutes at 48°C, and 5 minutes at 95°C. Each reverse transcription was carried out in triplicate. Expression of Notch (N1), N2, N3, N4, Jagged

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(J1, J2, Delta-like (Dll)-1, Dll-3, Dll-4, Hes1, Hey1, androgen receptor, neurogenin3 (Ngn3), and CGA mRNA was determined by quantitative real-time PCR (qPCR) carried out using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems), according to the manufacturer’s instructions. All PCR amplifications were carried out using MicroAmp optical 96-well reaction plate with TaqMan Fast Universal PCR Master Mix and with TaqMan Gene Expression Assay (Applied Biosystems).

Adenoviral transduction

Dominant-negative Hes1 (dnHes1:HA; ref. 21), constitutively active Notch 1 (caN1:V5) consisting of Notch 1 intracellular domain (22), and LacZ (control gene), cloned in a pAdlox adenoviral construct, were used to transduce LNCaP cells. Recombinant adeno viruses were produced, purified, and titrated as described (23). Briefly, CRE8 cells were transfected with SfiI-digested pAdlox-derived constructs and infected with the ψ85 virus. Lysates were prepared 4 days after infection. Viruses were passed twice through CRE8 cells and purified from the second passage using a caesium density gradient. The viruses were quantified by optical density at 260 nm, and the bioactivity was determined by the plaque-forming unit assay. Adenoviral transduction was carried out in serum-free DMEM with approximately 10^5 viral particles per cell in the presence of poly-L-lysine hydrobromide (Sigma-Aldrich), for 2 hours at 37°C. Then the adenovirus-containing medium was removed and replaced with serum-containing medium.

Western blot analysis

Cells grown under normoxic and hypoxic conditions were scraped in cold PBS, centrifuged and resuspended in cold lysis buffer (10 mmol/L Tris-HCl, pH 7.4, 25 mmol/L MgCl2, 1% Triton X-100, 1 mmol/L dithiothreitol, 0.1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mmol/L Na3VO4). Lysates were obtained by sonication on ice, followed by centrifugation to collect supernatants. After measurement of total protein content (Coomassie Plus-Bradford Assay kit, Pierce), equal amounts of cell lysates (50 μg) were resolved by 10% (w/v) SDS-PAGE, transferred to a nitrocellulose membrane (Hybond C, Amersham Pharmacia Biotech) and immunoblotted using primary antibodies. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) assay (Amersham Pharmacia Biotech), following manufacturer’s instructions. Densitometric analysis was conducted using GAPDH immunoreactive bands for normalization. Each experiment was carried out at least 3 times.

Results

LNCaP cells exposed to hypoxia adopt a neuroendocrine phenotype

LNCaP cells were exposed to 2% oxygen (hypoxia) for up to 14 days. Similar to other reports (24), we found that transcription of HIF-1α mRNA was constitutive in LNCaP and was nearly unchanged after 24 hours of exposure to hypoxia (data not shown). However, the expression of HIF-1α protein was enhanced under reduced oxygen tension, indicating stabilization of the protein (ref. 25; Fig. 1A). Cells grown at 2% oxygen for at least 3 days changed their morphology and assumed a neuronal-like phenotype, with the appearance of long dendritic-like processes in numerous cells, suggesting NED. The phenotypic change was more striking after 7 days of hypoxia (Fig. 1B) and still evident at 14 days. To confirm that the change in phenotype was due to NED, we analyzed the expression of 2 neuroendocrine markers: NSE (Fig. 1C, D, and F) and class III beta (B3)-tubulin (refs. 26, 27; Fig. 1E and G); both markers were significantly upregulated after 7 days of exposure to hypoxia. Because Ngn3, a proneurogenic bHLH transcription factor, was shown to be expressed in the neuroendocrine prostate cancer mouse model, 12T-10 (28), we analyzed the expression of Ngn3 by qPCR, under normoxic and hypoxic conditions. As shown in Fig. 1H, Ngn3 transcription was significantly upregulated by hypoxia. Neither PC-3 nor Du145 cells showed any change in morphology suggestive of NED when grown under hypoxic conditions. According to this observation, the level of expression of NED markers did not increase when the cells were exposed to reduced oxygen tension (data not shown).

Hyoxia modulates the expression of Notch receptors and ligands

Because Notch signaling has a key role in cell fate and differentiation, and hypoxia has been shown to modulate Notch signaling in some tumors (10), we sought to determine whether Notch was involved in the hypoxia-mediated NED of LNCaP cells. Notch1 to 4 (N1-4), Jagged 1 (J1) and J2, Delta-like (Dll)-1, 3 and 4 mRNA levels were assessed by qPCR, under normoxic and hypoxic conditions. N4 and Dll-3 were undetectable under both conditions. N1 and N2 mRNA were abundant under normoxia and decreased significantly under hypoxia (Fig. 2A). N3 mRNA was less represented and slightly decreased under hypoxic conditions (Supplementary Fig. S1A). We also determined the expression of N1 and N2 proteins by Western blotting. As shown in Fig. 2C, compared with normoxia (lane 1), the expression of the transmembrane (TM) form of N1 was undetectable and TM N2 was significantly reduced under hypoxia (lane 2). The decrease of N1 and N2 receptor proteins was not modified by treatment of the cells with γ-secretase inhibitor DAPT (lane 3), indicating that it was related to reduced protein translation, rather than receptor activation. The decreased expression of Notch receptors was maintained up to 14 days (data not shown). J1 and J2 were the main Notch ligands expressed in LNCaP; J1, J2, Dll-1, and Dll-4 mRNA levels decreased significantly under hypoxic conditions (Fig. 2B). Similarly to Notch receptors, hypoxia-induced modification of ligand expression was still maintained after 14 days of exposure to hypoxic conditions (data not shown). To determine whether the modulation of Notch receptor/ligand system under hypoxia was a common feature of prostate cancer cells, we examined PC-3 and Du145 for the transcription levels of Notch1–4 and their ligands, under...
normoxic and hypoxic conditions: PC-3 and Du145 expressed abundant levels of N1, N2 and N3; J1 and J2 were the main ligands (Supplementary Fig. S1B). N4 andDll-3 were undetectable. Under hypoxia, N1 and N2 were significantly downregulated in both cell lines (Supplementary Fig. S2A). With the exception of J2 in PC-3 cells, ligands were also significantly downregulated under hypoxic conditions (Supplementary Fig. S2B).

Hypoxia modulates Notch-mediated signaling

We next studied Notch signaling under normoxic and hypoxic conditions and found that the transcription of Notch target genes, Hes1 and Hey1, was significantly downregulated when LNCaP cells were exposed to 2% oxygen, in comparison with normoxia, indicating that Notch-mediated signaling was turned down. Cells treated with the γ-secretase inhibitor DAPT were used as controls for inhibition of Notch activation (Fig. 2D). As in LNCaP cells, Hes1 and Hey1 expression was significantly downregulated when PC-3 and Du145 were grown at 2% oxygen (Supplementary Fig. S3).

Notch signaling modulates the extent of NED in LNCaP cells

Because Notch signaling was downregulated under hypoxia, and because in the developing neuroblast, Notch signaling and transcription of Hes genes are repressed (29), we sought to determine whether hypoxia-mediated NED was supported by the downregulation of Notch signal. To do so, we transduced LNCaP cells with a dominant-negative form of Hes1 (dnHes1; ref. 21). Cells transduced with LacZ were used as controls. Ninety-six hours after infection, the cells were analyzed for the proneurogenic transcription factor Ngn3 and CGA mRNA expression, using qPCR (Fig. 3A and B), and for NSE and β3-tubulin protein expression, by Western blotting (Fig. 3C). Cells

**Figure 1.** A, Western blotting for the detection of HIF-1α in LNCaP cells exposed to normoxia (N) or hypoxia (H) for 24 hours. Detection of GAPDH was used for normalization of protein loading. B and C, LNCaP cells exposed to hypoxia for 7 days, compared with cells exposed to normoxia, assessed by phase contrast microscopy (B) and by immunoperoxidase staining (brown) for the detection of NSE (C). D and E, representative immunoblot for the expression of NSE (D) and β3-tubulin (E) in LNCaP cells exposed to N and H for 7 days. Detection of GAPDH was used for normalization of protein loading. F and G, densitometric analysis of 3 independent experiments for the detection of NSE (F) and β3-tubulin (G); values are expressed as mean ± SE. H, neurogenin (Ngn)3 mRNA expression in LNCaP cells exposed to normoxia and hypoxia, at different time points, assessed by real-time qPCR.
expressing dnHes1 upregulated Ngn3, CGA, NSE, and β3-tubulin, compared with cells infected with the LacZ construct. These data suggest that hypoxia-induced NED may be facilitated by the attenuation of Notch receptor activity, through the downregulation of Hes1 transcription. To confirm that the extent of neuroendocrine features correlated to the level of Notch receptor activation, we next transduced LNCaP cells with a construct expressing a constitutively active form of Notch 1 (caN1; ref. 22). Cells transduced with LacZ were used as controls. Ninety-six hours after infection, we analyzed the level of neuroendocrine markers: GCA by qPCR (Fig. 3D), and NSE and β3-tubulin by Western blotting (Fig. 3E). In cells virally transduced with caN1, the expression of all 3 neuroendocrine markers was significantly reduced.

**Hypoxia modulates the expression and function of androgen receptor**

Because it has been reported that malignant prostatic neuroendocrine cells are androgen independent (30), we analyzed the growth of the cells and the expression of androgen receptor mRNA by qPCR, under normoxic and hypoxic conditions. As shown in Fig. 4A, cell growth was not inhibited under hypoxia but rather enhanced. However, transcription of androgen receptor significantly decreased when LNCaP cells were exposed to reduced oxygen tension (Fig. 4B). Moreover, stimulation of the cells with 5α-DHT for 7 days was able to sustain cell growth under normoxia but not under hypoxic conditions (Fig. 4C), indicating that at 2% oxygen cells were unresponsive to exogenous hormone stimulation. To test whether there was a correlation between the expression of androgen receptor and the level of Notch signal, we analyzed cells transduced with caN1 and dnHes1 for androgen receptor mRNA expression, by qPCR. Cells transduced with LacZ were used as controls. As reported in Fig. 4D, caN1 potentiated, whereas dnHes1 attenuated the transcription of the receptor. These data suggest that downregulation of Notch signal may contribute to the downregulation of androgen receptor when the cells are grown under reduced oxygen tension.

**Discussion**

Progression of prostate cancer to a hormone-independent state is associated with resistance to androgen deprivation therapy and poor prognosis. A growing body of evidence suggests that NED plays a role in the development of androgen independency. Moreover, a recent report showed that focal NED in prostate cancer is a powerful independent predictor of outcome (31). NED can range from the presence of scattered clusters of neuronal-like cells within the adenocarcinoma tumor mass, to a small cell carcinoma or carcinoid of the prostate, which is composed of 100% neuroendocrine cells (32, 33). A recent report showed that a xenograft of neuroendocrine-differentiated prostate cancer cells implanted into a castrated mouse enabled the growth of a xenograft of androgen-dependent tumor cells implanted into the opposing flank. This result suggests that neuroendocrine-differentiated cells, within the tumor mass, may support the growth of androgen-dependent cancer cells, even in the absence of androgenic stimulation (3), likely favoring the selection of hormone-independent clones. For
this reason, understanding the molecular mechanisms that underlie neuroendocrine transdifferentiation should be of great interest.

Like many solid tumors, hypoxia develops in prostate cancerous tissues and has a role in tumor development and progression (34). We showed that exposure of LNCaP cells to hypoxia induced NED. In a tumor mass, changes in microvascular supply lead to the development of acute and chronic hypoxic conditions (9). Pathologic hypoxia has been established under 5% oxygen; however, compromised blood flow in specific areas may result in oxygen tension less than 1% (35). We chose to grow the cells at 2% oxygen to be able to study the “long term,” chronic effect of oxygen reduction. Exposure of LNCaP cells to hypoxia induced a profound modification of Notch receptor–ligand expression profile.

Notch signaling is central in cell fate specification, in embryonic and adult tissues (12, 13). Notch-mediated signal has been linked increasingly to carcinogenesis; however, its role in cancer is highly context dependent. In lung and breast cancer, hypoxia seems to increase Notch signaling (10, 11); in breast cancer cells, hypoxia upregulates the expression of Notch receptors and ligands, as well as of Notch target genes, Hes1 and Hey1 (11). In LNCaP cells, low oxygen tension produced the opposite effect on Notch expression and activity, suggesting a further tissue-specific regulation of the Notch system. Hypoxia downregulated N1 and N2 mRNA transcription also in the androgen-independent cell lines, PC-3 and Du145, although it did not change the extent of neuroendocrine transdifferentiation in these models. It is possible that androgen sensitivity may be required for transdifferentiation to occur, as shown by a number of studies that pointed out how NED progresses more rapidly if androgen deprivation is more intense (36, 37).

Moreover, as suggested by Marchiani and colleagues (38), there is variability in the pathways leading to NED among different cell lines, in vitro, which may mirror tumor variability of prostate cancer cells, in vivo.

Notch is a major regulator of neuronal development, through the well-known mechanism of lateral inhibition: in a cell differentiating toward the neuronal lineage, Notch is not activated and, consequently, transcription of Hes genes is repressed; the cell expresses the transcription factor Ngn, which leads to the expression of Notch ligands. The expression of ligands on the developing neuroblast activates Notch receptors on adjacent cells, leading to the transcription of Hes genes that function as repressors of neuronal differentiation (29). This mechanism prevents the cells surrounding the newly formed neuroblast to differentiate toward the neuronal lineage as well. Hes1 has been shown to be expressed by almost all undifferentiated cells, and it is crucial for suppressing

Figure 3. A–C, LNCaP cells virally transduced with LacZ or with a dominant-negative form of Hes1 (dnHes1), in frame with an HA-tag, were analyzed for Ngn3 and CGA mRNA expression 4 days after transduction, by real-time qPCR (A and B) and for NSE and β3-tubulin protein expression, by Western blotting (C). Detection of HA-tag was used to determine the expression of the transduced dnHes1 construct. Detection of GAPDH was used for normalization of protein loading.
and Nkx2.2, associated with a loss of Hes1 transcript (28). The downregulation of Hes1 allowing neuronal associated differentiation of neural stem cells. Interestingly, Hes1-deficient mice exhibit premature differentiation and severe defects in the brain, eye, and pancreas (29). Recently, several reports have suggested the involvement of Notch signaling in pancreatic exocrine and endocrine cell fate, through a mechanism similar to lateral inhibition: in the developing pancreas, a cell committed to the endocrine lineage expresses Ngn3, forcing neighboring cells to adopt a nonendocrine phenotype. The existence of a cross-talk between Ngn and Hes transcription factors in the developing pancreas has been shown by several lines of evidence: overexpression of Ngn3 or disruption of Hes1 gene results in an increased number of endocrine progenitor cells, with depletion of precursors bearing the capacity to differentiate into exocrine pancreas (39, 40). It has been shown that Hes1 is able to halt Ngn3 expression by binding to several silencer sites near the transcription initiation site of the gene (39).

We report that under hypoxic conditions, LNCaP cells downregulate Notch signal and Hes1 mRNA transcription and upregulate the expression of Ngn3 and the extent of neuroendocrine features. Because a dominant-negative Hes1 construct is also able to increase the level of Ngn3 transcription and NED, we propose that the loss of Notch signaling may determine NED in a manner similar to what has been described during pancreas development, with downregulation of Hes1 allowing neuronal associated proteins to be synthesized. In the previous study, immunohistochemical analysis of 12T-10 transgenic neuroendocrine prostate cancer mouse model showed the presence of proneural transcription factors, such as Foxa2, Ngn3, and Nkx2.2, associated with a loss of Hes1 transcript (28). Moreover, in 80 samples of human prostate cancers, grouped according to the level of CGA expression, human achaete-scute homolog 1 (hASH1), which is negatively regulated by Notch signal, was expressed and colocalized with CGA, in neuroendocrine-differentiated samples, and its expression correlated positively to the extension of neuroendocrine features (41). In addition, during the preparation of this article, an article was published reporting that the formation of neuroendocrine prostate tumors in the TRAMP mouse model is regulated by HIF-1α availability. The authors also showed that HIF-1α cooperates with the proneural transcription factor Foxa2 to initiate the transcriptional program required for the neuroendocrine phenotype to develop (42). Our data confirm that hypoxia can trigger the appearance of NED in prostate cancer cells and suggest a further level of complexity, with modulation of Notch signaling as an additional contributor. Interestingly, using osteoblastic skeletal prostate metastatic cancer cells, Zayzafoon and colleagues showed that expression of N1 and Hes1 was increased, when compared with the primary tumor, indicating that Notch signaling was activated and likely participated in the acquisition of osteoblastic properties (43). Therefore, modulation of Notch signaling seems to be crucial for prostate cancer progression by determining cancer cell differentiation and controlling the acquisition of specific phenotypes.

Several reports have indicated that malignant prostatic neuroendocrine cells are androgen receptor negative (30). When LNCaP cells were grown under hypoxic conditions, androgen receptor mRNA transcription was significantly downregulated, although not completely absent. We also...
found that the level of androgen receptor transcription correlated with the level of Notch signal. Therefore, we propose that the attenuation of Notch-mediated signal, detected under hypoxia, promotes not only NED but also a reduction of androgen receptor expression.

Some authors have reported an increased sensitivity of prostate cancer cells to hormone stimulation, under hypoxia (44). When we assessed cell growth in our experimental system, we found that LNCaP cells exposed to hypoxia were able to grow even faster than cells exposed to normoxia. At the same time, at 2% oxygen, the cells did not respond to androgen stimulation. These results are in accordance with Suzuki and colleagues that reported that DHT-dependent growth and ARE-mediated transcriptional activity of LNCaP cells were depressed under reduced oxygen tension (45). Although this behavior seems to suggest the acquisition of an androgen-independent phenotype, when we passaged the cells with charcoal-stripped serum, which is considered a model of androgen deprivation, their growth was hampered equally under normoxia and hypoxia (data not shown). One possible explanation is that charcoal-stripped serum is deprived not only of androgens but also of other mitogens that may be necessary to sustain the growth of prostate cancer cells, under hypoxia, in our experimental system. Further studies will be necessary to establish whether chronic hypoxia favors the appearance of an androgen-independent phenotype and to elucidate which mitogenic stimuli sustain the growth of the cells under reduced oxygen tension. One likely candidate is the prototype member of the fibroblast growth factor family, FGF-1. Indeed FGF-1 is a well-known mitogenic stimulus for prostate cancer cells (46) and its secretion has been linked to a downregulation of N1 receptor activity (47).

Several lines of evidence indicate that Notch signaling may have a facilitating role in the progression of prostate cancer; indeed, J1 is highly expressed in metastatic tumors and in clinically localized tumors with higher risk of recurrence (48). Moreover, downregulation of N1 and J1 inhibits cancer cell growth, migration, and invasion (49, 50). For all these reasons, strategies aimed at inhibiting Notch pathway have been proposed as alternative anti-cancer therapies for metastatic prostate cancer, refractory to endocrine therapy, whose treatment is currently based on docetaxel (51). However, on the basis of our results, great caution should be exerted before proposing such strategy as single therapy in patients with advanced prostate cancer, as it may favor the appearance of a neuroendocrine-mediated, hormone-independent phenotype with self-sustained proliferative capacity and aggressive clinical behavior. At the same time, characterizing the downregulation of Notch signal as a mechanism involved in NED may open new perspectives for treatment of hormone-refractory tumors. Indeed, while somatostatin analogues have been used with quite elusive results in prostate cancers characterized by neuroendocrine features (52), Notch signaling may be regarded as a novel molecular target to prevent/revert neuroendocrine transdifferentiation.

In conclusion, hypoxia induces downregulation of Notch-mediated signal, sustaining NED of prostate cancer cells in vitro. Further studies are needed to understand whether the modulation of Notch, in combination with strategies aimed at inhibiting signaling pathways responsible for the self-sustained proliferative capacity (53), may slow down the progression of prostate cancer toward a neuroendocrine-mediated, androgen-independent phenotype.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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In Memoriam

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</table>