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Running title: PCDH-PC/AR cross-talk in driving NE differentiation

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Abbreviations: ADT, androgen deprivation therapy; AR, androgen receptor; CRPC, castration resistant prostate cancer; DHT, dihydrotestosterone; NE, neuroendocrine; PCDH-PC, protocadherin-PC

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Abstract

Castration-resistant prostate cancers (CRPC) that relapse after androgen deprivation therapies are responsible for the majority of mortalities from prostate cancer. While mechanisms enabling recurrent activity of androgen receptor (AR) are certainly involved in the development of CRPC, there may be factors that contribute to the process including acquired neuroendocrine cell-like behaviors working through alternate (non-AR) cell signaling systems or AR-dependent mechanisms. In this study, we explore the potential relationship between the AR axis and a novel putative marker of neuroendocrine (NE) differentiation, the human male Protocadherin-PC (PCDH-PC) in vitro and in human situations. We found evidence for a NE transdifferentiation process and PCDH-PC expression as an early-onset adaptive mechanism following androgen deprivation therapy, and elucidate AR as a key regulator of PCDH-PC expression. PCDH-PC overexpression, in turn, attenuates the ligand-dependent activity of the AR, enabling certain prostate tumor clones to assume a more NE phenotype and promoting their survival under diverse stress conditions. Acquisition of a NE phenotype by prostate cancer cells positively correlated with resistance to cytotoxic agents including docetaxel, a taxane chemotherapy approved for the treatment of patients with metastatic CRPC. Furthermore, knockdown of PCDH-PC in cells that have undergone a NE transdifferentiation partially sensitized cells to docetaxel. Together, these results reveal a reciprocal regulation between the AR axis and PCDH-PC signals, observed both *in vitro* and *in vivo,* with potential implications in coordinating NE transdifferentiation processes and progression of prostate cancer towards hormonal and chemo-resistance.

INTRODUCTION

PCa (PCa) is the most commonly diagnosed malignancy amongst men in Western nations [1]. It is well recognized that androgens working through androgen receptor, so termed the androgen receptor (**AR**), play a key role in PCa disease initiation and progression [2], and are known to stimulate the PCa cell growth and diminish their rate of apoptosis. This is the basis for the use of androgen deprivation therapy (**ADT**) in the form of medical or surgical castration as standard front line therapy for patient with advanced disease [3]. Despite the fact that ADT has been proven to extend life span in accordance with its effect of limiting the growth of “androgen sensitive” PCa cells and inducing cell death of “androgen-dependent” PCa cells, one important aspect of PCa is that the majority of cases eventually develop resistance to ADT and the emergence of castration resistant disease (CRPC) emerges. Although there are a number of approved and promising therapies for metastatic CRPC, including taxane chemotherapies (ie. docetaxel, cabazitaxel) and potent AR targeted agents (ie. abiraterone, MDV3100) [4], all patients develop resistance and as such, metastatic CRPC accounts for most PCa-related deaths.

A key mechanism involved in progression of PCa from a hormone sensitive to castration resistant state includes acquisition of molecular alterations of the androgen/AR axis, such that PCa cells retain active AR even in the setting of castrate levels of circulating testosterone [5]. However, an alternative mechanism that dominates in some cases of CRPC involves transformation towards an androgen-independent state, in which certain PCa cells offset their sensitivity to androgens by altering their

apoptotic pathways such that active Androgen/AR signaling is no longer mandatory for their survival. These androgen-independent cell populations may either arise from progenitor or neuroendocrine- (NE-) like cells in the primary prostate tumor or from prostate adenocarcinoma cells that transdifferentiate to NE-like cells. It has been more than a decade since the concept first emerged from *in vitro* studies suggesting the latter, that under certain circumstances, including hormonal manipulation, PCa cells have the potential to transdifferentiate to acquire NE characteristics [6-10]. Despite evidence of up-regulated NE differentiation in patients receiving ADT [11, 12], the origin of NE cells in the prostate remains uncertain. Moreover, the relative lack of knowledge regarding the chain of events and the mechanistic paradigm underlying the trans-differentiation process supports the need for further investigations.

We previously reported that overexpression of protocadherin-PC (PCDH-PC also referred to as *PCDH11Y*), a gene primarily identified for its anti-apoptotic properties that encodes from the Y-chromosome at Yp11.2 [13, 14], can drive NE transdifferentiation in LNCaP [15], a cell line originally established from a lymph node metastatic lesion of human PCa characterized by its androgen-dependent growth [16]. Here, by exploring the potential relationship between the Androgen-AR axis and PCDH-PC, we investigated the possibility that PCa progression toward androgen independence is indeed characterized by a putative subpopulation of cancer cells that undergo a NE transdifferentiation. We also explore the extent to which the emergence of these populations is influenced by current therapies for advanced CRPC.

MATERIALS and METHODS

Cell culture and chemicals

The human PCa cell lines LNCaP and 22Rv1 were obtained from ATCC, authenticated at this site and maintained in recommended medium. For androgen-reduced conditions, cells were cultured in phenol red–free RPMI supplemented with 10% dextran charcoal-stripped FBS (CS-FBS). The LNCaP-PCDH-PC cells were previously described [17]. Steroids and chemotherapeutic agents were obtained from Sigma-Aldrich. Bicalutamide was obtained from LKT Laboratories.

Human prostate tissue samples.

The prostate samples have been collected as part of an IRB approved protocol at Henri Mondor Hospital. Specimens consisted of FFPE tissues from Hormone-naive PCa (HNPC; n=222), neoadjuvant hormone therapy treated (HTPC; n=32) obtained from radical prostatectomy specimens, CRPC specimens (n=60) of which 54 were collected at the time of the transurethral resection of the prostate for obstructive CRPC, and 6 isolated from rapid autopsy specimens with metastatic lesions. The study also included a few specimens derived from normal prostates of young donors.

Immunohistochemistry and immunofluorescence.

Paraffin-embedded tissues were sectioned at 5 µm thickness, deparaffinized, and endogenous peroxidase activity was inactivated in a solution containing 3% hydrogen peroxide (H_2O_2) for 10 minutes. Sections were then cleared in running water followed by phosphate-buffered saline. Antigen unmasking was performed by heat-retrieval with citrate buffer, pH 6 (Dako). The primary antibodies used are listed in Table W2.

Antibodies purified from HB 0337 SSA hybridoma and raised against PCDH-PC are available upon request to Pr F. Vacherot (vacherot@u-pec.fr). Biotin-labelled antibodies (Jackson ImmunoResearch) were used as secondary antibodies. Antigen-antibody reactions were revealed using the streptavidin method with diaminobenzidine (DAB) as substrate. All slides were read by a genitourinary pathologist (YA) and the intensity of staining was scored as null (0), weak (1), moderate (2) and strong (3). In this analysis, a case was considered positive only when the score was 2 or more in at least 10% of cancer cells, whereas cases with less than 10% staining, or scored below 2 were considered as negative. For dual immunofluorescence staining, samples were processed as above but using as secondary antibodies, anti–mouse Alexa Fluor 488 (Life technologies) and biotinylated anti-rabbit antibodies (Jackson ImmunoResearch) with subsequent incubation with Streptavidin-Fluoprobes 647H (Interchim). Slides were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and inspected by confocal microscopy.

Transient transfection and luciferase Reporter Assays

Transient transfection assays and measures of luciferase and beta-gal activities were performed as previously described [15] with minor modifications. The PSA-61-Luc plasmid was described previously [18] and used as reporter of AR activity. Briefly, Cells (6×10^5 per well) were plated in 24-well plate and cotransfected the next day using Lipofectamine 2000 (Life technologies) mixed with up to 400ng of PCDH-PC, vector or empty pcDNA3 along with 500ng of a PSA-61-luc and 50ng of a Lac-Z luciferase plasmid as a transfection control; so that all wells received ~1µg of DNA. On the next day, cells were treated with DHT for 24h after which cell lysates were prepared and

processed for luciferase activity and β-Gal activity using the Luciferase Reporter Assay and β-Gal Reporter Gene Assay Kits (Roche Diagnostics), respectively. Measures have been performed using Wallac victor^3 1420 Multilabel counter (Perkin-Elmer, Courtaboeuf, France).

PCDH-PC knockdown

All siRNAs were from Thermo Scientifics. Knockdown of PCDH-PC in 22Rv1 cells was performed using ON-TARGET *plus* SMART pool Human PCDH11Y (L-013624, Thermo Scientifics) 100nmol/L of ON-TARGET *plus* Non-Targeting Pool (D-001810) or siRNAs against PCDH-PC were transfected in 22Rv1 cells as indicated using lipofectamine 2000. Knockdown of PCDH-PC in LNCaP-NE like cells was carried out using Accell SMARTpool - Human PCDH11Y (E-013624). Accell Non-targeting Pool D-001910 as well as Accell Green Non-targeting siRNA were also used. LNCaP-NE-like cells were incubated in Accell siRNA Delivery Media mixed with either 1 µmol/L of Non-Targeting siRNAs or siRNAs against PCDH-PC according to the manufacturer's instructions. On the next day, media was changed and cells were subsequently cultured in the indicated medium.

Cell growth and Cell viability and. Cell growth was monitored by cell counting and the population doubling time (DT) estimated (in hours) by using the formula: doubling time = h*ln(2)/ln(C2/C1); C1 and C2 are the cell concentrations at the beginning and the end of the chosen period of time. Cell viability was assessed by the MTT assay [19] or WST-1 assay (Roche Diagnostics) as described previously [20].

Western blot analysis. Protein lysates were prepared and processed as described previously [21].

cDNA synthesis and real-time PCR. RNA was extracted using the TRIzol reagent (Life technologies), subjected to DNase treatment (DNA-free kit; Applied Biosystems) according to the manufacturer's instructions. 1μg of total RNA was then reverse transcribed using SuperScript II (Life technologies). Quantitative PCR was performed using SYBR Green dye on a StepOnePlus Real-Time PCR System (Applied Biosystems). Unless indicated, the amount of each target gene relative to the housekeeping genes *RPLP0* or *HMBS* was determined for each sample using $2^{-\Delta\Delta CT}$ method. Primer sequences are provided in Table W3.

Statistical analysis: For qualitative data, χ2 test and Fisher's exact test were applied. For *in vitro* studies, comparisons between groups were performed using the Student's t test. All statistical tests used a two-tailed $\alpha = 0.05$ level of significance and were performed using GraphPad Prism (GraphPad Software, Inc.).

RESULTS

Phenotypic changes in the PCa cell line LNCaP upon androgen depletion.

LNCaP cells are commonly used *in vitro* to model the response to ADT of PCa in patients following hormone manipulation [22]. Thus, we first searched for perturbation in *PCDH-PC* expression and various markers in LNCaP cells maintained in androgen-depleted medium for an extended period. This included known androgen-upregulated gene products *KLK3* (PSA) and *KLK2*, previously described androgen-repressed genes, the Neuron Specific Enolase (NSE)[6], *TUBB3* (Neuronal class III β-tubulin)[7] and the hedgehog ligand *SHH* [23], as well as various genes assumed to be critical in PCa progression comprising Bcl-2, Akt, TP53, *MYC* and AR [5, 24]. Western Blot and qRT-PCR analyses showed that when cells are switched to androgen-deficient medium, NSE and *TUBB3*, two prominent markers of neuroendocrine differentiation, are induced along with *PCDH-PC* which shows a peak expression (~125 fold increase) at 2 weeks (Figures 1*A*-*B* and W1*A*). *SHH* was also augmented (Figure W1*B*). This period was associated with a decreased of cell growth accompanied by the emergence of neurite-like outgrowths from the cells (Figure 1C). We likewise observed a down-regulation of PSA and *KLK2* levels, two AR target genes, during the first weeks of androgen depletion, as expected. We also noted some increase in phosphorylated-AKT, and a decrease in expression of p53 and *MYC* (Figures 1*A*-*B* and W1*A*). Intriguingly, *PCDH-PC* expression was found to be gradually decreased with time in conjunction with reappearance of an epithelial-like morphology and a loss of neurite outgrowth (Figure 1*C*). After 3 months of culturing in androgen-depleted medium, PSA and *KLK2* were again detected suggestive

of AR activity (Figures 1*A-B* and W1*A*). This was concomitant with the down-modulation of *PCDH-PC*, NSE, *TUBB3* and increased expression of active phosphorylated-AKT, p53 and *MYC*. Together, these observations further qualified *PCDH-PC* as a novel *in vitro* marker of NE differentiation in PCa cells and indicate that its expression may fluctuate in concordance with AR activity. After more than 11 months of culturing, the obtained LNCaP derivative grows perfectly in androgen-depleted media, and express significant levels of AR and PSA. The growth rate was comparable to cultures of parental LNCaP cells grown in normal media (Figure W1*C*). For subsequent studies, these cells will be referred to LNCaP-Androgen-Independent (LNCaP-AI).

The androgen-Androgen Receptor axis regulates *PCDH-PC* expression

We then sought to determine the extent to which the androgen-AR axis regulates *PCDH-PC* expression. LNCaP were treated during 24 hours with increasing concentrations of the androgen dihydrotestosterone (DHT), and *KLK3* (PSA) and *PCDH-PC* mRNA levels were measured by qRT-PCR. The increased level of *KLK3*, an AR targeted gene, was used as a positive control of the AR activity in the presence of DHT. In DHT-treated cells, we observed a 4-fold reduction in *PCDH-PC* mRNA levels in conjunction with increased *KLK3* expression (Figure 2*A*). The temporal effects of androgen were further tested in an experiment where the cells were maintained in androgen-depleted media for 72h and then DHT was added back for 6h, 12h, 24h. In such conditions, inhibition of PCDH-PC expression was detectable as early as 6h following DHT supplementation suggesting that the androgen-AR axis directly mediates PCDH-PC expression (Figure 2*B*).

Moreover, PCDH-PC expression was similarly reduced when cells were chronically exposed to androgens (Figure *W2A*), oestrogen or progesterone which are two alternative ligands of mutated AR in this line [25]. We then asked whether a functional AR is required to mediate the repressive effect of androgens on *PCDH-PC* expression. LNCaP cells were incubated in the presence of the antiandrogen, bicalutamide [26]. A 10 day treatment resulted in augmenting by 7 fold *PCDH-PC* expression (Figure 2*C*) while expectedly reducing *KLK3* expression. Changes in cell morphology were also visible upon the treatment (Figure *W2B)*. We next applied bicalutamide treatment to the LNCaP-AI derivative. We observed a dose-dependent relative decrease in *KLK3* and *KLK2* expression compared to non-treated cells with a concurrent increase in *PCDH-PC* expression (Figure 2*D*). To ascertain our assumption that *PCDH-PC* is repressed by AR activity we next treated the LNCaP-AI cells with docetaxel. docetaxel is the standard of care first line chemotherapy for men with metastatic CRPC. In PCa cells, recent studies showed that short-term treatment with docetaxel impeded AR activity [27]. Here, we exposed LNCaP-AI cells to 2,5 nM docetaxel for a prolonged period, and examined expression of *PCDH-PC* and NE markers over time. After 15 days, we found that the cell populations surviving this chronic exposure to docetaxel had greater levels of NE markers *NSE* (~2-4 fold increase), *TUBB3* (~2-5 fold increase) and *PCDH-PC* (~25-125 fold increase) compared to untreated cells (Figure 2*E*). The morphology of the cells also changed substantially with the formation of neurite outgrowths (Figure 2*F*). This data suggests that NE-like cancers cells likely emerged via trans-differentiation following the chronic exposure to docetaxel.

PCDH-PC is a negative mediator of ligand-dependent AR transcriptional activity

We earlier found that transient overexpression of *PCDH-PC,* under certain circumstances, can perturb AR protein stability in LNCaP cells through a complex mechanism that involves AKT activation and increase proteasomal activity towards AR [28]. However, the potential links between AR activity, *PCDH-PC* expression, and phenotypic changes in LNCaP cells have not been investigated. Here we tested the possibility that *PCDH-PC* expression could disrupt androgen signaling. We transiently overexpressed *PCDH-PC* using cultures of LNCaP cells. Increased expression of *PCDH-PC* was verified by qRT-PCR (Figure W3*A*), Western blot analysis showed a marked downregulation of PSA in PCDH-PC-transfected cells, while expectedly increased NSE and phospho-AKT levels (Figure 3*A*). There was also significant enrichment for inactivated phospho-GSK-3β (Ser9). The AR level was not perturbed suggesting that *PCDH-PC* expression disrupted androgen signaling by inhibition of AR activity in our conditions. To further explore this inhibitory effect, we performed luciferase reporter assays on these latter cells following transfection of incremental amounts of the *PCDH-PC* expression construct. These analyses demonstrated a dose-dependent decrease of the PSA promoter transactivation (Figure 3*B*). We then investigated long-term effects of *PCDH-PC* expression by analyzing PSA expression in LNCaP derivatives stably-transfected with *PCDH-PC.* In normal culture conditions, these cells showed more neurites and a minor decrease in cell growth as compared to control cells (Figure 3*C*). PCDH-PC mRNA and protein levels in LNCaP-pcDNA3 and LNCaP-PCDH-PC are depicted in Figures W3*B* and W3*C*. Stable transfectants exhibited reduced AR activity compared to vector transfected-LNCaP cells (Figure 3*D*). These cells have enhanced levels of endogenous NSE, phospho-AKT and phospho-GSK-3β, comparable AR

expression, but lower levels of PSA protein as compared the vector-transfected, or LNCaP-AI cells (Figure 3*E*). Interestingly, inhibition of PI3K/AKT signal using the PI3K inhibitor LY294002 compromised NE features in these cells (Figure 3*F*). We next investigated whether knockdown of PCDH-PC could affect the AR activity in the 22Rv1 prostate cancer cells [29], which endogenously express *PCDH-PC*. 22Rv1 cells are androgen-independent given that they can grow in the absence of androgens. However, they remain AR dependent expressing several AR target genes including *KLK3* and *KLK2*. When 22Rv1 cells were maintained in the presence of androgens, ablation of *PCDH-PC* with *PCDH-PC* targeted siRNAs did not significantly affect *KLK3* expression (Figure 3*G*). By contrast, this led to *KLK2* levels that were approximately 12-fold higher. It was earlier demonstrated that 22Rv1 is androgen responsive for *KLK2* but weakly for *KLK3* expression [30]. We confirmed this information in an experiment where cells were exposed 10 nM DHT for 24h (Figure 3*H*). Thus, we conceived that *PCDH-PC* is a potential repressor of ligand-dependent AR activity in this line. To pursue this possibility, we transiently transfected 22Rv1 cells with a PCDH-PC expression construct or control vector and measured *KLK2* and *KLK3* in either control (ethanol) or DHT-treated cells. Overexpression of *PCDH-PC* resulted in a significant decrease in *KLK2* expression as compared to minor changes for *KLK3* (Figure 3*I*) and the effect was perceived only in the presence of DHT. Together, these results strongly suggest that PCDH-PC overexpression inhibits ligand-dependent activity of AR in PCa cells, with no or marginal effects on its ligand-independent activity.

PCDH-PC expression during PCa progression

By immunohistochemistry, we then explored the distribution of PCDH-PC protein in normal and pathological specimens. In tissues derived from normal prostate, luminal epithelial cells were consistently found to be negative for PCDH-PC and pronounced expression of this protein was observed in lonely cells scattered within the epithelium (Figure 4*A*, i). Occasionally, a faint staining was detected in the basal cell layer (Figure W4). A series of hormone naïve PCa (HNPC) specimens was examined using Tissue Microarrays. This analysis revealed moderate to high expression of PCDH-PC in at most 11% (25 out of 222) of evaluable cases (Table 1). There was no significant correlation with clinico-pathological data (Table W1). Evaluation of PCDH-PC expression in CRPC samples indicated a much higher proportion of positive cases (that is, 61%, 33 of 54 CRPC) (Figure 4*A,* ii; Table 1). It is noteworthy that, PCDH-PC protein was also detectable in cancer cells of metastatic CRPC lesions present in the brain and the lymph nodes of patients (Figure 4*A*, iii-iv). Despite only 6 cases were analyzed, this suggested that deregulated expression of PCDH-PC in CRPC disease is not restricted to recurrent lesions localized to the prostate.

We then evaluated a series of prostatectomy specimens of PCa obtained from patients treated for 3 to 6 months with neoadjuvant hormone therapy (HTPC). Of the 32 cases of HTPC evaluated, 14 (43,7%) were recorded as positive for PCDH-PC (Table 1). Especially, intense expression was consistently detected in clusters comprising of 5 to 100 cells (Figure 4*A*, v). For the overall HTPC group, PCDH-PC was found to be significantly higher when compared with the HNPC group as evaluated by Fisher's exact test ($p<0.0001$). To test further the hypothesis that ADT is causative for increased expression of PCDH-PC in these specimens, we examined the hormone-naïve tissues

of these patients by examining their initial prostatic biopsies. Matched biospsy specimens were available in 7 cases. In 6 of these index cases, we found no evidence of PCDH-PC expression after analyzing cancer foci of several biopsy specimens (Figure 4*A*, vi) and one other case showed strong positivity for PCDH-PC but in dispersed isolated cells rather than in clusters. These results demonstrate that high PCDH-PC expression is rare in men with still hormonally-untreated PCa, but substantially increases in response to hormonal manipulation.

PCDH-PC expression associates with neuroendocrine features in human prostate tissues.

Given the apparent link between PCDH-PC and NE features *in vitro*, we explored the value of PCDH-PC as novel candidate marker for NE trans-differentiation in human PCa specimens. Examination of the hormone-treated samples for CgA and PSA expressions consistently revealed that cancer cells expressing PCDH-PC are present in tumor foci showing a large majority of CgA expressing cells, but with reduced expression of PSA (Figure 4*B-C*; Figure 5*A*; Figure W5*A*). Dual immunofluorescence procedure also revealed that in these tumor areas, not all cells exhibited the same NE characteristics such that varied levels of NE markers were observed in the cells (Figure 5*B*). In adjacent benign epithelia, we detected a few isolated cells staining positive for both CgA and PCDH-PC likely representing non-malignant NE cells (Figure W5*B*).

On further analysis of cancer foci positive for PCDH-PC, we found positivity for the AR as well as for NSE and synaptophysin, two established NE marker, but we consistently failed to detect staining for CD56 (NCAM1) (Figure 5*C*), another NE marker.

Of note, cancer areas within the different tissues analyzed (PCDH-PC positive and negative) were consistently negative for the Ki67 antigen (not shown). Moreover, PCDH-PC expressing cells were negative for the basal cytokeratins 5/6 and p63, but positive for α-Methylacyl-CoA racemase (AMACR) (Figure 5*C*), a highly specific marker of PCa epithelia thus supporting a PCa origin [31].

Collectively, these observations strongly suggest PCDH-PC as a novel early marker for transition from epithelial to neuroendocrine phenotype in PCa treated by ADT. Intriguingly however, at the castration state of prostate adenocarcinoma, the relationship between PCDH-PC expression and NE (as assessed by CgA staining) appeared to be lost, and although PCDH-PC immunostaining of PCa cells sometimes coincides with staining for NE markers such as NSE (Figure W5*C*), in many cases the PCDH-PC positive contingents examined did not show coincidental staining (not shown).

NE-like prostate cancer cells are resistant to chemotherapeutic agents

Several pieces of evidence suggest that PCa NE-like cells are resistant to multiple therapeutics agents [32, 33]. Here, we assessed further the chemoresistance spectrum of LNCaP NE-like. After culturing LNCaP cells for 15 days in androgen-depleted medium, the cells exhibit a NE-like phenotype, and reduced growth (Figure 6*A*) concomitant with a loss of their epithelial characteristics. Sensitivity with respect to diverse agents was evaluated 96h after treatment of LNCaP-NE-like, LNCaP or LNCaP-AI cells. Treatments included two taxanes, docetaxel and paclitaxel as well as TPA and Camptothecin, two well-known inducers of apoptosis in LNCaP cells [34, 35]. At the

indicated doses, LNCaP-NE-like cells were overwhelmingly resistant to these drugs compared to LNCaP or LNCaP-AI cells (Figure 6*B*). LNCaP-NE-like cells also showed enhanced resistance to various cytotoxic agents commonly used in management of various malignancies (Figure W6*A*). We next wanted to gauge the dependence of LNCaP-NE-like cells with respect to PCDH-PC expression for their viability. To this end, LNCaP-NE-like cells were treated for 24h with Accell Green Non-Targeting siRNAs used to control effective uptake of the siRNAs (Figure W6*B*), pools of Accell non-targeting siRNAs, or Accell siRNAs raised against PCDH-PC transcripts, then cultured for 8 days in hormone-deprived medium supplemented or not with docetaxel (10 nmol/L). PCDH-PC silencing was found to be efficient in these conditions (Figure 6*C*). In the presence of docetaxel, LNCaP-NE cells that had been pre-incubated with the PCDH-PC siRNAs showed a significant decrease in cell viability (relative to cells exposed to NT siRNA in the presence or absence of docetaxel) while in the absence of docetaxel, PCDH-PC siRNA treatment had limited effect (Figure 6*D*). Moreover, the effect was not seen when similar treatments were applied to the chemosensitive PC3 prostate cancer lineage (Figure 6*E*) which lack *PCDH-PC*, or LNCaP-AI which expresses low amounts of *PCDH-PC* (Figure 6*F*). Subsequent analyses showed that attenuating PCDH-PC expression similarly sensitized LNCaP-NE-like cells to TPA and Camptothecin (Figure W6*C-D*). These data argue for a chemoprotective role for PCDH-PC in LNCaP-NE-like.

DISCUSSION

The androgen-AR axis remains active in the vast majority of CRPC. However, as prostate tumors develop resistance to treatment, NE differentiation has been proposed as a mechanism for hormonal escape or AR independence [4, 10-12, 36-38]. Yet, the impact of NE differentiation on the clinical outcome, the mechanisms by which NE differentiation emerges after ADT, and the consequence of targeting these cell populations remains uncertain. The current study significantly expands our understanding of NE differentiation in PCa and qualifies PCDH-PC, as surrogate marker for human PCa cell subpopulations experiencing NE transdifferentiation under hormonal treatment.

With respect to progression towards a castration resistant phenotype, results obtained from LNCaP cultures grown in androgen-reduced medium support a model in which AR function is attenuated in a first phase following ADT, concomitantly with the acquisition of neuroendocrine features by PCa cells. *In situ*, we found evidence that high PCDH-PC expression, also parallels CgA and other NE markers in clusters of tumor cells from neo-adjuvant hormonally-treated PCa. The fact that normal NE cells are considered as post-mitotic [39], coupled with data showing that the proliferating rate of prostate cancer cells is relatively low in primary prostate tumors [40] strongly suggests that NE–like clusters revealed in this study originated from the NE transdifferentiation of pre-existing epithelial-looking PCa cells. Thus, we propose that in clinical setting, overexpression of PCDH-PC and concomitant induction of NE transdifferentiation by a fraction of PCa cells in early response to hormonal treatment reflects one route for PCa cells to adapt and survive in a low androgen environment.

In a second step, AR may be reactivated [5, 41, 42] to promote proliferation in conjunction with partial, or total loss of NE features along with reappearance of significant amounts of PSA as observed in LNCaP-AI cells. Further studies are warranted to decrypt the mechanisms involved in reactivation of AR in these cells.

Enigmatically, the relationship between PCDH-PC and NE differentiation was not evident in CRPC specimens. This could reflect the multifaceted role of PCDH-PC in the more advanced stages of PCa with functions that may occur independently of NE differentiation. Alternatively, this could be indicative of various subtypes of NE differentiation (from well-differentiated to poorly differentiated) in tumors with varied proliferative activity and expressing various levels of NE markers [43, 44]. In that respect, it will be important to examine the role of PCDH-PC in the setting of small cell carcinoma of prostate, a rare poorly differentiated neuroendocrine PCa associated with poor prognosis and poor response to therapies [45]. It is also tempting to speculate that AR plays a crucial role in this potential molecular switch as AR is consistently implicated in the growth of castrate resistant tumors [41, 46]. We have shown here that PCDH-PC expression inhibits AR activity. But this inhibition appeared to be incomplete in the sense that it's likely restricted to the ligand-dependent activity of AR. Although we already know that PI3K/AKT activity may be an important mediator of this effect, the precise mechanism through which PCDH-PC regulates the ligand-dependent AR activity has yet to be fully determined.

If confirmed, this regulation could also indicate that among castrate resistant tumors, those overexpressing PCDH-PC might progress to the favor of tumor clones dependent on a ligand-independent activity of AR [46-48].

Our experimental data consistently revealed that androgen exposure inhibits PCDH-PC expression in LNCaP cells, although it is unlikely that androgens completely switch off PCDH-PC expression. Likewise, the contribution of other recurrent alterations found in PCa, such as *TMPRSS2-ERG* gene fusion or loss of PTEN, known to perturb AR signalling, should be considered [49, 50].

Another interesting observation is that the NE status of LNCaP cells correlates with resistance to a wide range of chemotherapeutic agents including docetaxel, the current standard for metastatic CRPC. One could suggest that those resistances are likely linked to the reduced growth rate of LNCaP-NE-like cells. Indeed, from a clinical perspective, the observation that NE transdifferentiation could confer a multidrug resistant phenotype allowing a cell to remain arrested until it can reacquire the ability to proliferate, could make that process a formidable tumor promoter at any stage of PCa progression. Interestingly, by targeting NE-like PCa cells using RNA interference against PCDH-PC it was possible to sensitize cells to chemo-hormonal treatment. Together with prior work identifying PCDH-PC as an anti-apoptotic factor in PCa cells [13], this qualifies PCDH-PC as a general survival factor in PCa cells and provides a biological rationale for further assessment of targeting malignant NE-like cells.

Although not emphasized here, in neo-adjuvant hormonally-treated tumors, we found many instances with NE-like PCa (PCDH-PC^{+}, CgA^{+}, PSA^{-}) cells adjacent to malignant epithelial-like (PCDH-PC^{-}, CgA^{-}, PSA^{+}) cells thus continuing to use the androgen-AR axis despite ADT (Figure 4). Clearly the manifestation of these mixed populations, gives reason to further examine whether these phenotypically distinct cell populations may

cooperate to promote transition towards castration resistance [8, 51], which would either help support or refute a rationale of treating both adenocarcinoma and neuroendocrine components.

In summary, our study provides support for the likelihood of transdifferentiation model of PCa cells to explain the emergence of neuroendocrine differentiation in human PCa following ADT. We substantiate PCDH-PC, a human male specific protocadherin, as a critical factor in this process that appears to be regulated by cross-modulation between PCDH-PC and AR. Along this line, our data revealed novel paradigms linking the AR axis and NE transdifferentiation in PCa cells with apparent implications for the emergence of chemohormonal resistance.

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Table 1: PCDH-PC expression before and after androgen deprivation therapy

Prostate carcinoma		PCDH-PC negative No of samples (%)	PCDH-PC positive No of samples (%)
Hormone Naïve PCa (HNPC)		197 (88.8)	25 (11.2)
Hormone Therapy Treated PCa (HTPC)		18 (56.3)	14 (43.7)
Castration Resistant PCa (CRPC)		21 (38.9)	33 (61.1)
Pearson's chi- square test:			p<0.0001
Fisher's exact test:	HNPC/HTPC		p<0.0001
	HTPC/CRPC		p = 0.178
	HNPC/CRPC		p<0.0001

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LEGENDS

Figure 1 Phenotypic changes in LNCaP cells upon long term androgen deprivation. At Day 0, monolayer cultures of LNCaP cells were grown in 10% CS-FBS containing medium. (A) qRT-PCR analysis for mRNA expression *of PCDH-PC*, *TUBB3*, *KLK2* and *MYC*. (B) Western blot analysis for indicated proteins. Beta-actin is used as loading control. Densitometry of some WB bands is provided in Supplementary Figure S1A. (C) Morphology of cultured LNCaP cells maintained in medium containing 10%FBS (day 0) or 10% CS-FBS containing medium for 15, 30 or 345 days. Photomicrographs are taken at x 10 objective magnification under inverted light microscopy.

Figure 2 Androgenic regulation of PCDH-PC gene. (A) Cultures of LNCaP cells were grown 24 hrs in 10% CS-FBS media supplemented or not with incremental doses of DHT. RT-PCR analysis for *PCDH-PC* (Left) and *KLK3* (right) levels in DHT-treated over vehicle treated. (B) LNCaP cells were grown for 72h in 5% CS-FBS media then refreshed with media supplemented with 100nM DHT, and *PCDH-PC as well as KLK3* levels inspected as the indicated time. (C) LNCaP cells were grown in 10% FBS in the presence or absence of bicalutamide 10 μmol/L for 10 days and mRNA levels for *PCDH-PC* and *KLK3* examined. (D) Histograms showing normalized levels of *KLK2* (left), *KLK3* (middle), *PCDH-PC* (right) from LNCaP-AI cultures treated with bicalutamide for 8 days. (E) Time course expression of *NSE* (left)*, TUBB3* (middle) *and PCDH-PC* (right) in LNCaP-AI cells cultivated at 2,5 nmol/L docetaxel. Bars, means ± SEM of two independent experiments done in triplicate. (F) Morphology of LNCaP-AI cells

maintained in medium containing 10% CS-FBS (left panel); supplemented with docetaxel for 15 days (middle), or 30 days (right); Scale bar, 200 μm.

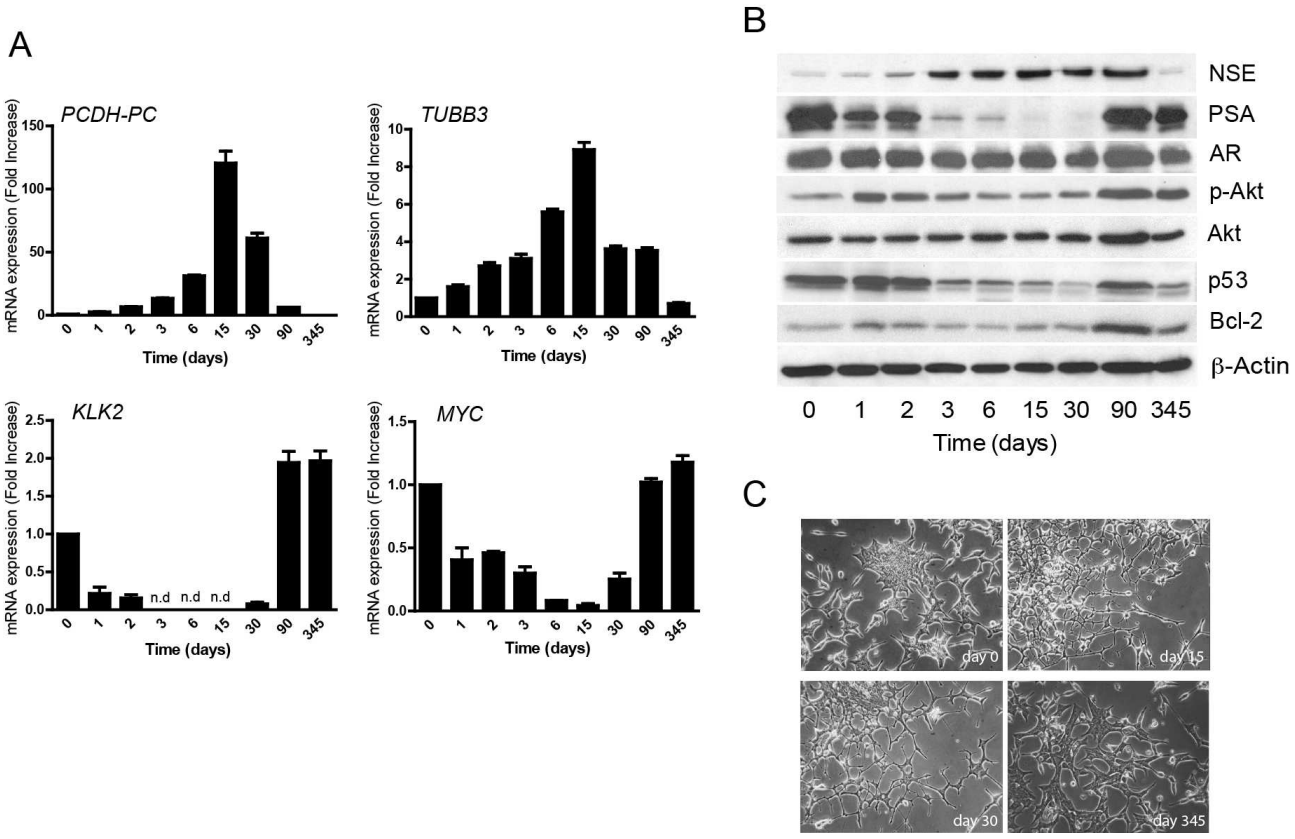
Figure 3 *PCDH-PC* expression reduces ligand-bound AR activity. (A) Western blot analysis 48h following transient transfection of *PCDH-PC* cDNA or the control vector. (B) PSA promoter activity was assessed in transfected-LNCaP cells by measuring luciferase activity 24h after DHT treatment in cellular extracts normalized to β-galactosidase activity. (C) Stably transfectants of vector- and PCDH-PC-transfected LNCaP (LNCaP-PCDH-PC) were examined for differences in cell morphology and cell growth (doubling time; DT), and PSA promoter activity (D) as in (B). (D) Western blot made against proteins from LNCaP-pcDNA3, LNCaP-AI and LNCaP-PCDH-PC cells showing reduced PSA and increased levels of NSE in the LNCaP-PCDH-PC cells. (E) LNCaP-PCDH-PC cells were treated for 3 days with either the PI3K inhibitor LY294002 (10 μmol/L) or vehicle (DMSO). A western blot was performed and probed as above. (F) 22Rv1 cells transfected either with siRNAs raised against PCDH-PC mRNA or non-targeting siRNA were analyzed for mRNA expression of *PCDH-PC*, *KLK3* and *KLK2*. Down-regulation of *PCDH-PC* is accompanied by elevation of *KLK2* mRNA but had minor effects of *KLK3*. (G) 22Rv1 cells were treated with vehicle (EtOH) or DHT (10 nmol/L) for 24h and endogenous levels of *KLK3* and *KLK2* were examined. (H) 22Rv1 cells pre-transfected with PCDH-PC plasmid were treated with vehicle (EtOH) or DHT (10 nmol/L) for 24h, and *PCDH-PC*, *KLK3*, *KLK2* levels were compared by qPCR.

Figure 4 (A) Expression of PCDH-PC in human prostatic tissues. anti-PCDH-PC identifies single normal cells in the prostatic epithelium of a healthy subject (i), in PCa cells in prostate tissue of CRPC (ii), in brain containing PCa metastases (iii) and in a lymph node metastase (iv) of CRPC. (v) Positive PCDH-PC-staining in cancer cells of a section of the surgical piece from a patient who had received 3 to 6 months of neoadjuvant ADT. (vi) Representative biopsy core from the same patient before neoajduvant ADT showing negativity for PCDH-PC. (B-C) Expression of PCDH-PC correlates with neuroendocrine characteristics in human PCa. Representative consecutive sections stained with antibodies to PCDH-PC, CgA, PSA of primary PCa from a patient treated by neoadjuvant ADT. Immunohistochemical stains reveal mixed populations of cancer cells suggesting a common origin

Figure 5 (A) Immunohistochemical analysis further validating the inverse correlation between protocadherin-PC/CgA stainings and PSA expression in tumor foci of a hormonally-treated case. (B) Dual-immunofluorescence in the previous index case identifies cancer cells co-expressing PCDH-PC and CgA. The cells can express varied levels of the two proteins. (C) A positive PCDH-PC cancer focus was analyzed for expression of Synaptophysin (SYN), Neuron Specifc Enolase (NSE), N-CAM (CD56), Androgen receptor (AR), basal cytokeratins 5/6, AMACR and p63. Note the areas positive for NSE and CD56 (arrows) but negative for the other markers representing non-tumoral nerves present in the prostate tissue.

Figure 6 Acquired NE phenotype correlates with chemoresistance in LNCaP cells. (A) LNCaP, LNCaP-NE like and LNCaP-AI derivatives were examined for differences in cell cell growth. (B) Viability assay of LNCaP (white bars), LNCaP-NE-like (gray bars), LNCaP-AI (black bars) at 96 hours after treatment with docetaxel, paclitaxel, Camptothecin, or phorbol ester (TPA) relative to untreated cells. (C) verification of efficient *PCDH-PC* knockdown by qRT-PCR in LNCaP-NE-like pre-treated 24h with either Accell Non-Targeting or PCDH11Y siRNAs, and then maintained in the presence or absence of docetaxel for 48h. (D) Cell viability as assessed by WST-1 assay using siRNAs treated LNCaP-NE-like cells alone or subsequently treated with docetaxel for 8 days. (E) As in (C) except using PC3 cells and 96h docetaxel treatment. (F) As in D except using LNCaP-AI cells. Bars, means ± SEM of quintuplets from one experiment representative of three independent experiments.

FIGURE 1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TUBB3
KLK2
MYC
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Time (days)
n.d
B
NSE
PSA
AR
p-Akt
Akt
p53
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β-Actin
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Time (days)
C
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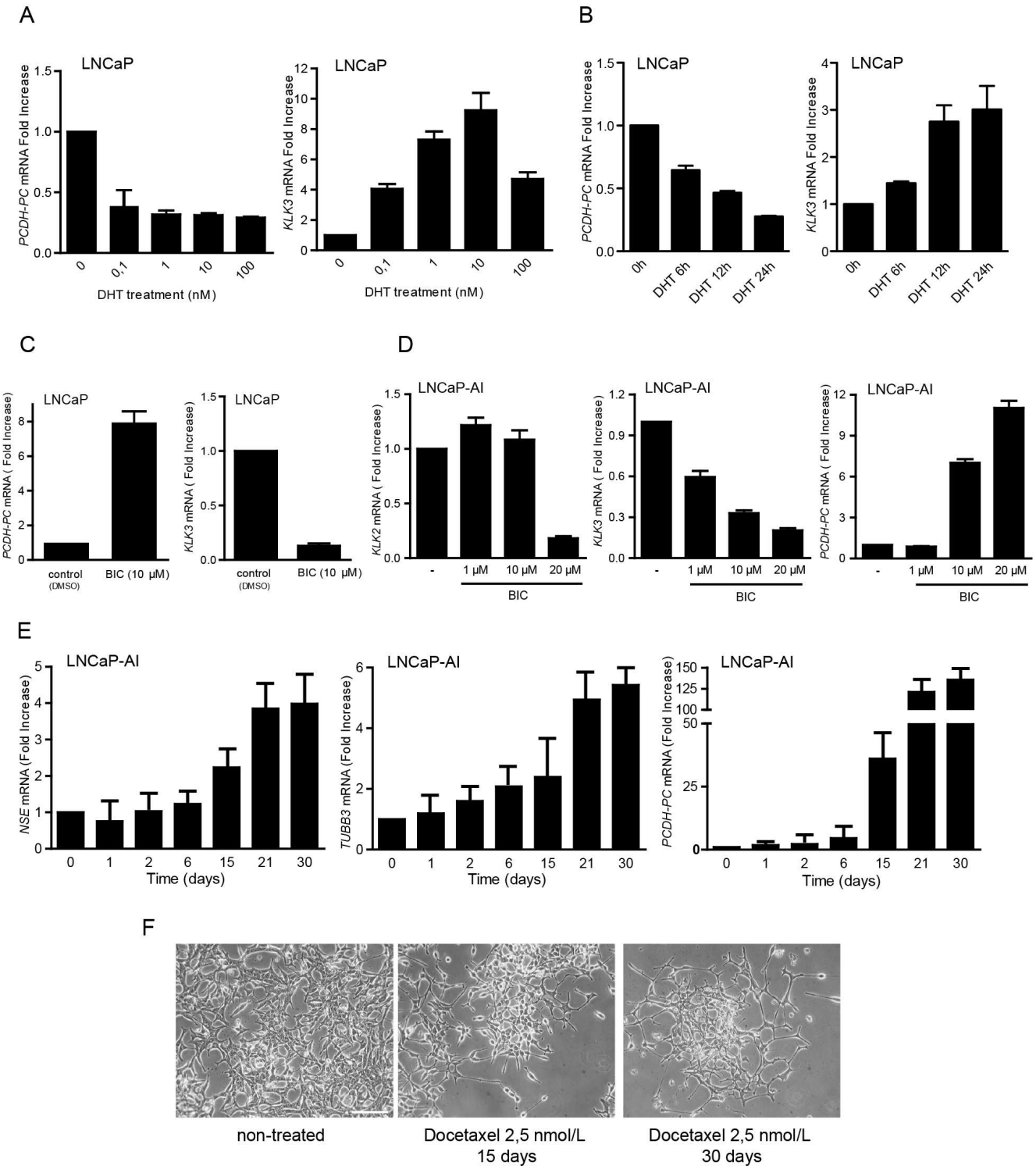
day 15
day 30
day 345

FIGURE 2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LNCaP
PCDH-PC mRNA Fold Increase
DHT treatment (nM)
LNCaP
KLK3 mRNA Fold Increase
DHT treatment (nM)
B
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PCDH-PC mRNA Fold Increase
0h
DHT 6h
DHT 12h
DHT 24h
LNCaP
KLK3 mRNA Fold Increase
C
LNCaP
PCDH-PC mRNA (Fold Increase)
control (DMSO)
BIC (10 µM)
LNCaP
KLK3 mRNA (Fold Increase)
D
LNCaP-AI
KLK2 mRNA (Fold Increase)
LNCaP-AI
KLK3 mRNA (Fold Increase)
LNCaP-AI
PCDH-PC mRNA (Fold Increase)
1 µM
10 µM
20 µM
BIC
E
LNCaP-AI
NSE mRNA (Fold Increase)
Time (days)
LNCaP-AI
TUBB3 mRNA (Fold Increase)
LNCaP-AI
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F
non-treated
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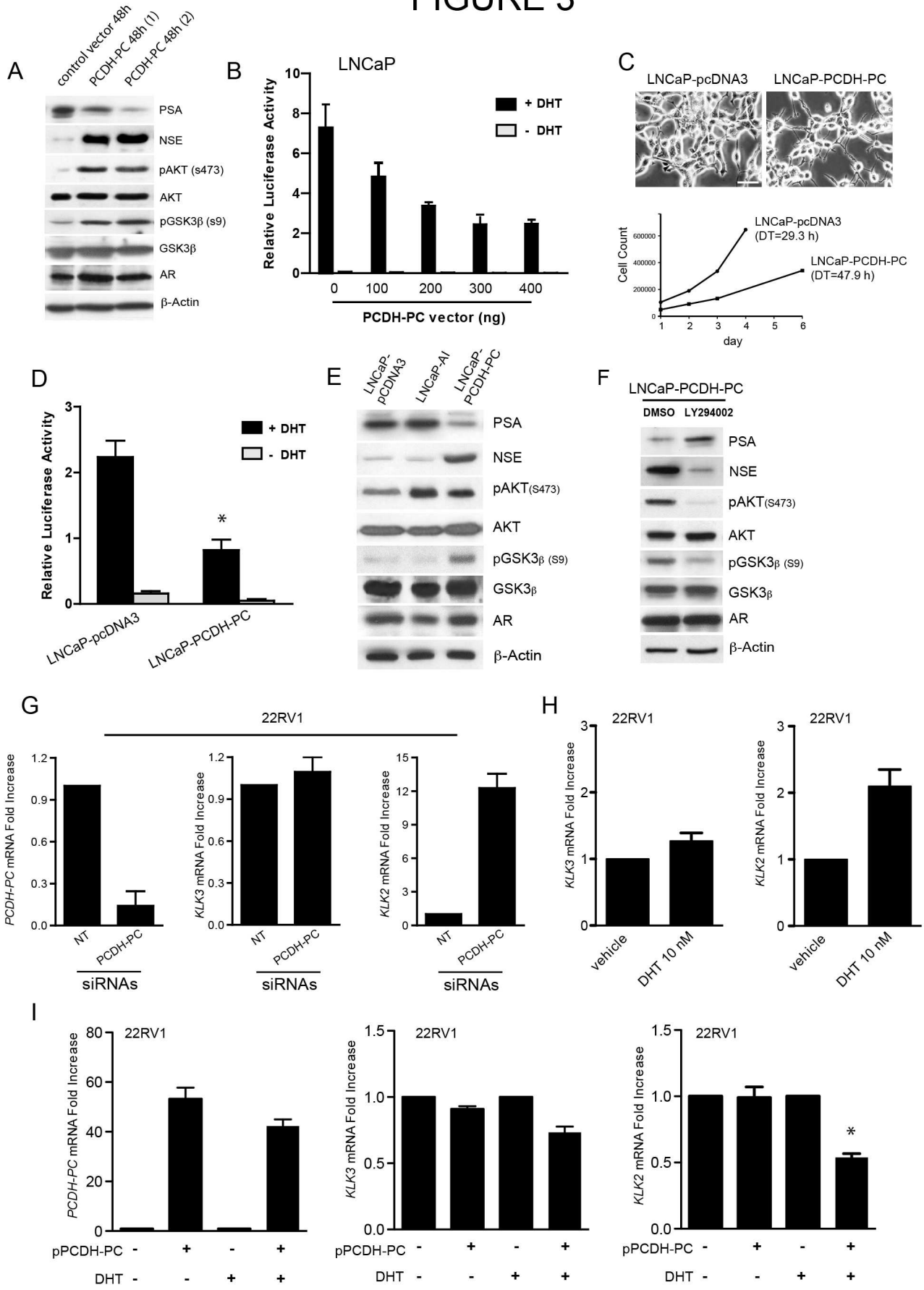
Docetaxel 2,5 nmol/L 30 days

769

FIGURE 3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NSE
pAKT (s473)
AKT
pGSK3β (s9)
GSK3β
AR
β-Actin
B
LNCaP
Relative Luciferase Activity
+ DHT
- DHT
PCDH-PC vector (ng)
C
LNCaP-pcDNA3
LNCaP-PCDH-PC
Cell Count
LNCaP-pcDNA3 (DT=29.3 h)
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day
D
Relative Luciferase Activity
+ DHT
- DHT
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LNCaP-PCDH-PC
E
LNCaP-pcDNA3
LNCaP-AI
LNCaP-PCDH-PC
PSA
NSE
pAKT(S473)
AKT
pGSK3β (S9)
GSK3β
AR
β-Actin
F
LNCaP-PCDH-PC
DMSO
LY294002
PSA
NSE
pAKT(S473)
AKT
pGSK3β (S9)
GSK3β
AR
β-Actin
G
22RV1
PCDH-PC mRNA Fold Increase
KLK3 mRNA Fold Increase
KLK2 mRNA Fold Increase
NT
PCDH-PC
siRNAs
H
22RV1
KLK3 mRNA Fold Increase
KLK2 mRNA Fold Increase
vehicle
DHT 10 nM
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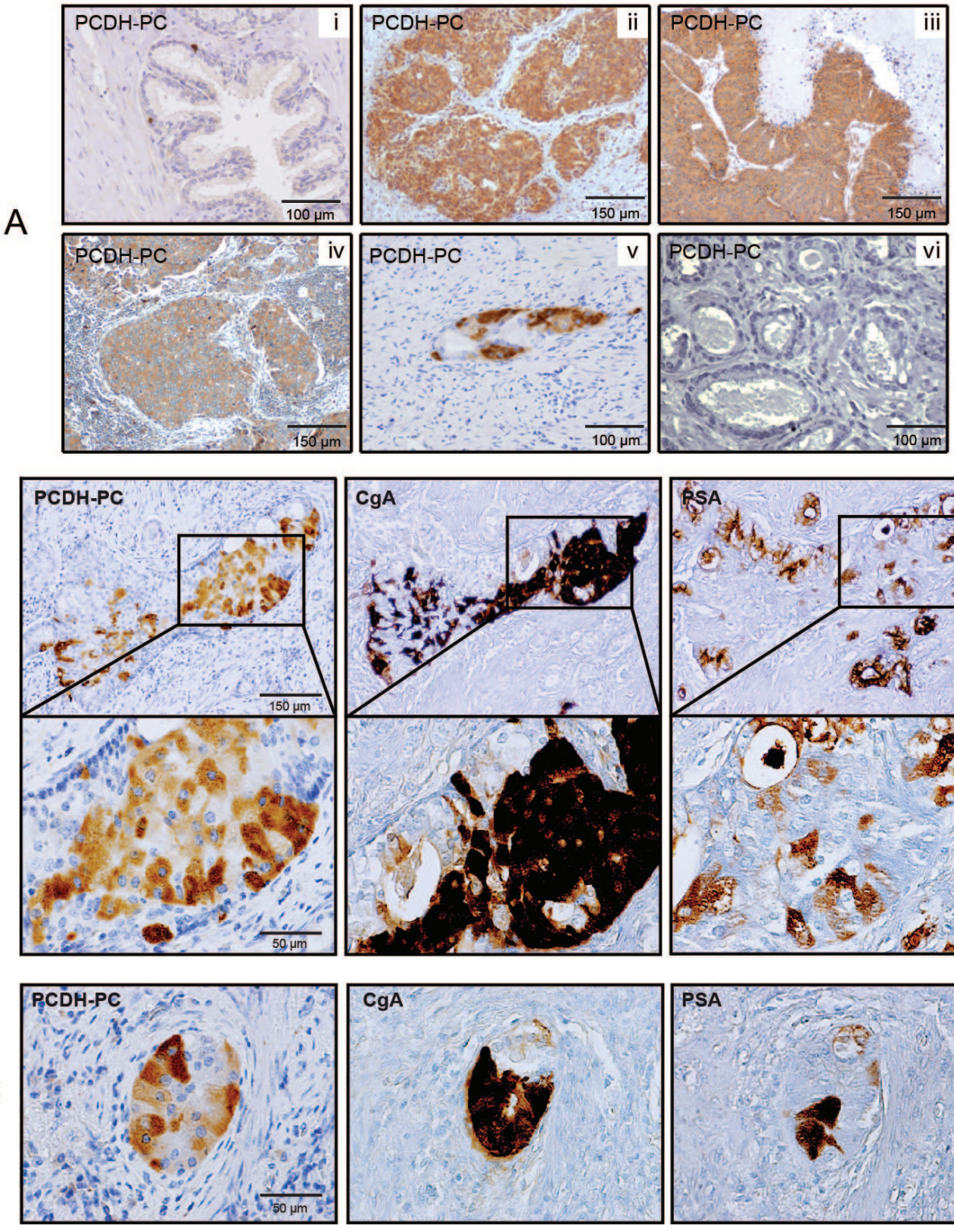
KLK3 mRNA Fold Increase
KLK2 mRNA Fold Increase
pPCDH-PC
DHT

770

FIGURE 4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PCDH-PC
i
100 µm
PCDH-PC
ii
150 µm
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iii
150 µm
PCDH-PC
iv
150 µm
PCDH-PC
v
100 µm
PCDH-PC
vi
100 µm
B
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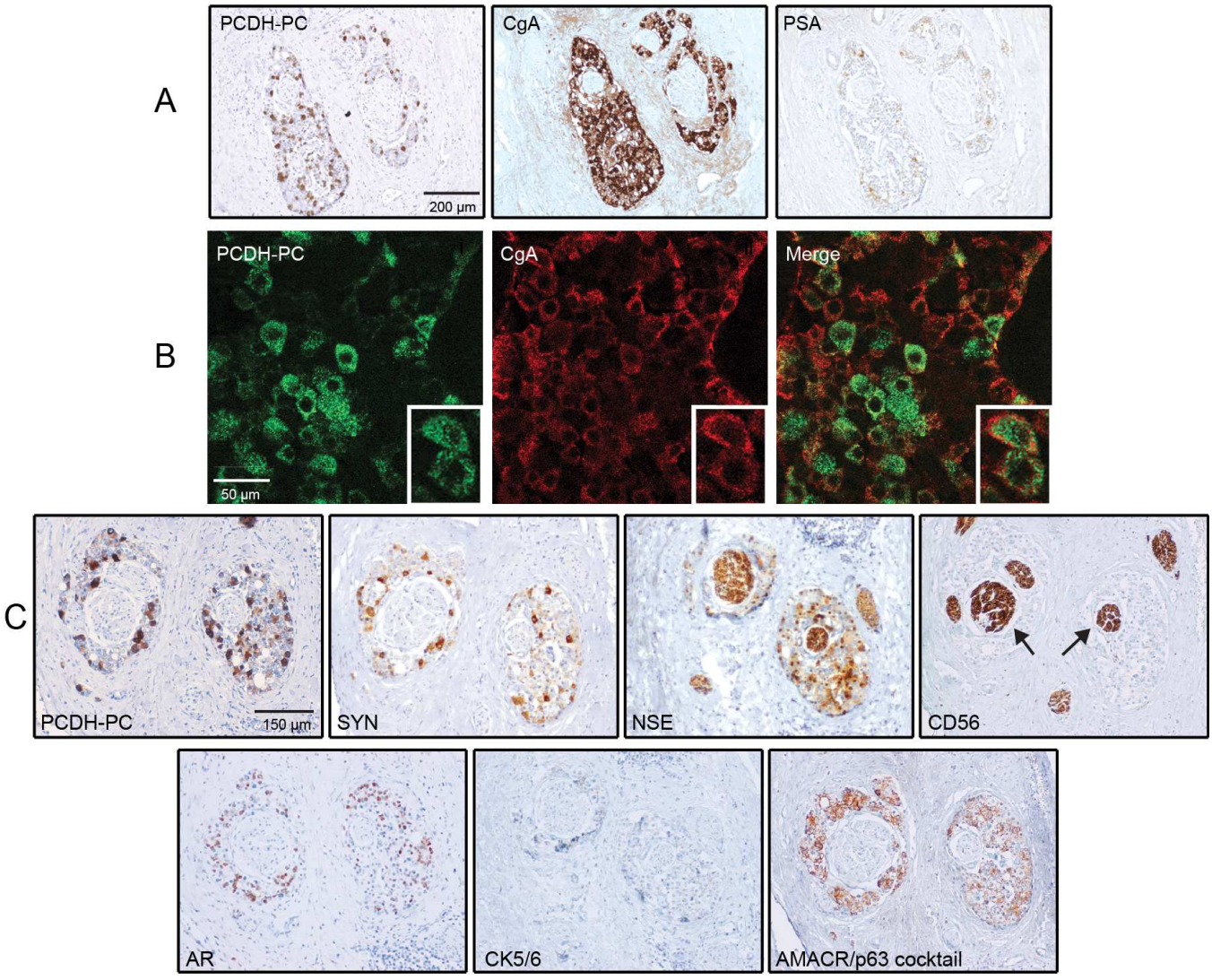
150 µm
50 µm
CgA
PSA
C
PCDH-PC
50 µm
CgA
PSA

771

FIGURE 5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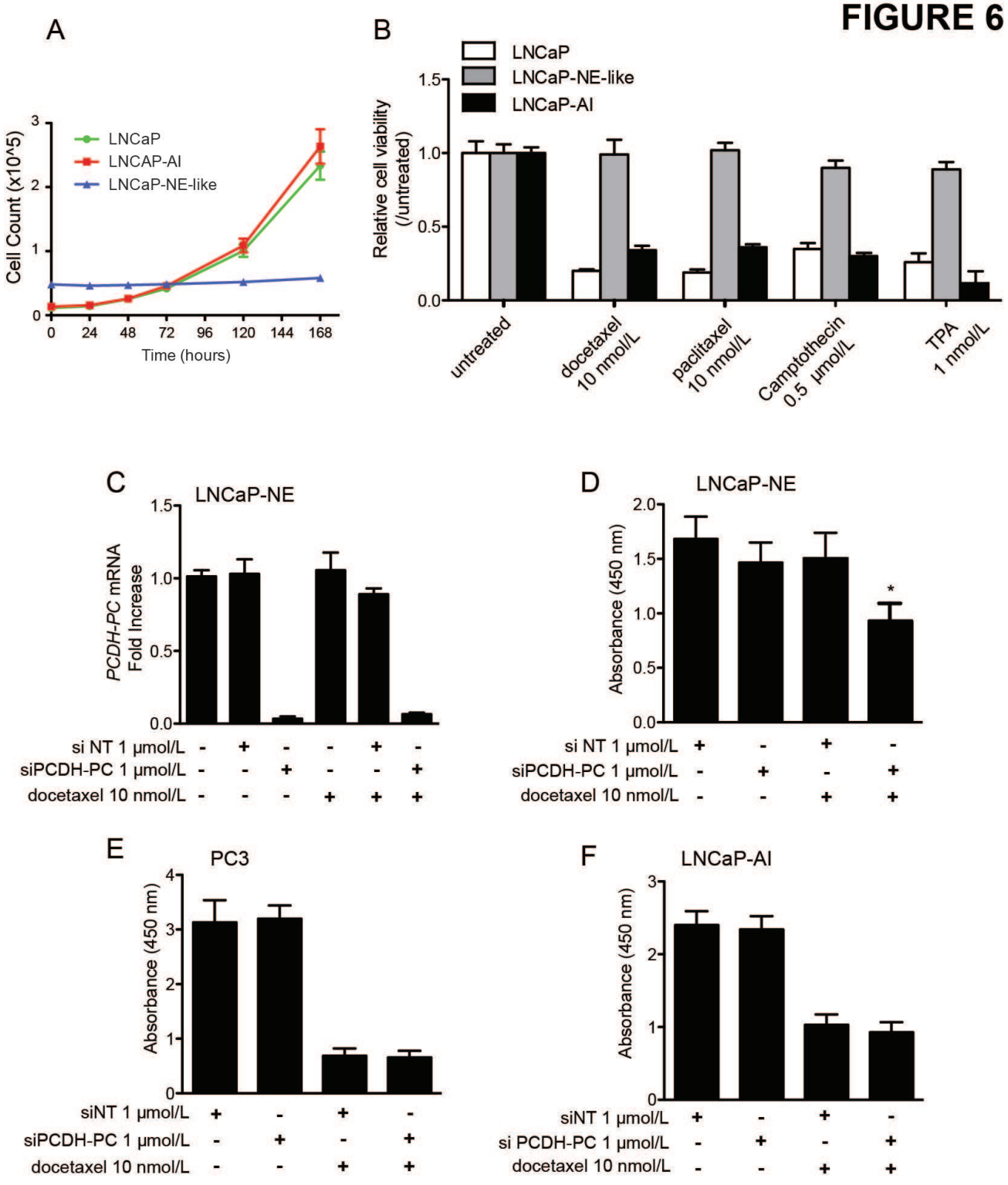
PCDH-PC
200 µm
CgA
PSA
B
PCDH-PC
50 µm
CgA
Merge
C
PCDH-PC
150 µm
SYN
NSE
CD56
AR
CK5/6
AMACR/p63 cocktail

FIGURE 6


A
Cell Count (x10^5)
LNCaP
LNCAP-AI
LNCaP-NE-like
Time (hours)
B
LNCaP
LNCaP-NE-like
LNCaP-AI
Relative cell viability (/untreated)
untreated
docetaxel 10 nmol/L
paclitaxel 10 nmol/L
Camptothecin 0.5 µmol/L
TPA 1 nmol/L
C
LNCaP-NE
PCDH-PC mRNA Fold Increase
si NT 1 µmol/L
siPCDH-PC 1 µmol/L
docetaxel 10 nmol/L
D
LNCaP-NE
Absorbance (450 nm)
si NT 1 µmol/L
siPCDH-PC 1 µmol/L
docetaxel 10 nmol/L
E
PC3
Absorbance (450 nm)
siNT 1 µmol/L
siPCDH-PC 1 µmol/L
docetaxel 10 nmol/L
F
LNCaP-AI
Absorbance (450 nm)
siNT 1 µmol/L
si PCDH-PC 1 µmol/L
docetaxel 10 nmol/L