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1 **Cross-modulation between the androgen receptor axis and**
2 **Protocadherin-PC in mediating neuroendocrine transdifferentiation**
3 **and therapeutic resistance of prostate cancer**

4
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8
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18
19 **Running title:** PCDH-PC/AR cross-talk in driving NE differentiation

20 **Keywords:** prostate cancer; protocadherin-pc; androgen receptor; neuroendocrine transdifferentiation; therapy.

21
22 **Abbreviations:** ADT, androgen deprivation therapy; AR, androgen receptor; CRPC, castration resistant prostate
23 cancer; DHT, dihydrotestosterone; NE, neuroendocrine; PCDH-PC, protocadherin-PC

24
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29
30

Abstract

31

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

53

54

INTRODUCTION

55

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

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

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

88

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

MATERIALS and METHODS

100 **Cell culture and chemicals**

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

107

108 **Human prostate tissue samples.**

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

116

117 **Immunohistochemistry and immunofluorescence.**

118 Paraffin-embedded tissues were sectioned at 5 μ m thickness, deparaffinized, and
119 endogenous peroxidase activity was inactivated in a solution containing 3% hydrogen
120 peroxide (H₂O₂) for 10 minutes. Sections were then cleared in running water followed by
121 phosphate-buffered saline. Antigen unmasking was performed by heat-retrieval with
122 citrate buffer, pH 6 (Dako). The primary antibodies used are listed in Table W2.

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

137

138 **Transient transfection and luciferase Reporter Assays**

139 Transient transfection assays and measures of luciferase and beta-gal activities were
140 performed as previously described [15] with minor modifications. The PSA-61-Luc
141 plasmid was described previously [18] and used as reporter of AR activity. Briefly, Cells
142 (6×10^5 per well) were plated in 24-well plate and cotransfected the next day using
143 Lipofectamine 2000 (Life technologies) mixed with up to 400ng of PCDH-PC, vector or
144 empty pcDNA3 along with 500ng of a PSA-61-luc and 50ng of a Lac-Z luciferase
145 plasmid as a transfection control; so that all wells received $\sim 1\mu\text{g}$ of DNA. On the next
146 day, cells were treated with DHT for 24h after which cell lysates were prepared and

147 processed for luciferase activity and β -Gal activity using the Luciferase Reporter Assay
148 and β -Gal Reporter Gene Assay Kits (Roche Diagnostics), respectively. Measures have
149 been performed using Wallac victor³ 1420 Multilabel counter (Perkin-Elmer,
150 Courtaboeuf, France).

151

152 **PCDH-PC knockdown**

153 All siRNAs were from Thermo Scientific. Knockdown of PCDH-PC in 22Rv1 cells was
154 performed using ON-TARGET *plus* SMART pool Human PCDH11Y (L-013624, Thermo
155 Scientific) 100nmol/L of ON-TARGET *plus* Non-Targeting Pool (D-001810) or siRNAs
156 against PCDH-PC were transfected in 22Rv1 cells as indicated using lipofectamine
157 2000. Knockdown of PCDH-PC in LNCaP-NE like cells was carried out using Accell
158 SMARTpool - Human PCDH11Y (E-013624). Accell Non-targeting Pool D-001910 as
159 well as Accell Green Non-targeting siRNA were also used. LNCaP-NE-like cells were
160 incubated in Accell siRNA Delivery Media mixed with either 1 μ mol/L of Non-Targeting
161 siRNAs or siRNAs against PCDH-PC according to the manufacturer's instructions. On
162 the next day, media was changed and cells were subsequently cultured in the indicated
163 medium.

164

165 **Cell growth and Cell viability and.** Cell growth was monitored by cell counting and the
166 population doubling time (DT) estimated (in hours) by using the formula: doubling time =
167 $h \cdot \ln(2) / \ln(C2/C1)$; C1 and C2 are the cell concentrations at the beginning and the end of
168 the chosen period of time. Cell viability was assessed by the MTT assay [19] or WST-1
169 assay (Roche Diagnostics) as described previously [20].

170

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

173
174 **cDNA synthesis and real-time PCR.** RNA was extracted using the TRIzol reagent (Life
175 technologies), subjected to DNase treatment (DNA-free kit; Applied Biosystems)
176 according to the manufacturer's instructions. 1µg of total RNA was then reverse
177 transcribed using SuperScript II (Life technologies). Quantitative PCR was performed
178 using SYBR Green dye on a StepOnePlus Real-Time PCR System (Applied
179 Biosystems). Unless indicated, the amount of each target gene relative to the
180 housekeeping genes *RPLP0* or *HMBS* was determined for each sample using $2^{-\Delta\Delta CT}$
181 method. Primer sequences are provided in Table W3.

182
183 **Statistical analysis:** For qualitative data, χ^2 test and Fisher's exact test were applied.
184 For *in vitro* studies, comparisons between groups were performed using the Student's t
185 test. All statistical tests used a two-tailed $\alpha = 0.05$ level of significance and were
186 performed using GraphPad Prism (GraphPad Software, Inc.).

187

188

189

RESULTS

190

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

192 LNCaP cells are commonly used *in vitro* to model the response to ADT of PCa in
193 patients following hormone manipulation [22]. Thus, we first searched for perturbation in
194 *PCDH-PC* expression and various markers in LNCaP cells maintained in androgen-
195 depleted medium for an extended period. This included known androgen-upregulated
196 gene products *KLK3* (PSA) and *KLK2*, previously described androgen-repressed genes,
197 the Neuron Specific Enolase (NSE)[6], *TUBB3* (Neuronal class III β -tubulin)[7] and the
198 hedgehog ligand *SHH* [23], as well as various genes assumed to be critical in PCa
199 progression comprising Bcl-2, Akt, TP53, *MYC* and AR [5, 24]. Western Blot and qRT-
200 PCR analyses showed that when cells are switched to androgen-deficient medium, NSE
201 and *TUBB3*, two prominent markers of neuroendocrine differentiation, are induced along
202 with *PCDH-PC* which shows a peak expression (~125 fold increase) at 2 weeks (Figures
203 1A-B and W1A). *SHH* was also augmented (Figure W1B). This period was associated
204 with a decreased of cell growth accompanied by the emergence of neurite-like
205 outgrowths from the cells (Figure 1C). We likewise observed a down-regulation of PSA
206 and *KLK2* levels, two AR target genes, during the first weeks of androgen depletion, as
207 expected. We also noted some increase in phosphorylated-AKT, and a decrease in
208 expression of p53 and *MYC* (Figures 1A-B and W1A). Intriguingly, *PCDH-PC* expression
209 was found to be gradually decreased with time in conjunction with reappearance of an
210 epithelial-like morphology and a loss of neurite outgrowth (Figure 1C). After 3 months of
211 culturing in androgen-depleted medium, PSA and *KLK2* were again detected suggestive

212 of AR activity (Figures 1A-B and W1A). This was concomitant with the down-modulation
213 of *PCDH-PC*, *NSE*, *TUBB3* and increased expression of active phosphorylated-AKT,
214 p53 and *MYC*. Together, these observations further qualified *PCDH-PC* as a novel *in*
215 *vitro* marker of NE differentiation in PCa cells and indicate that its expression may
216 fluctuate in concordance with AR activity. After more than 11 months of culturing, the
217 obtained LNCaP derivative grows perfectly in androgen-depleted media, and express
218 significant levels of AR and PSA. The growth rate was comparable to cultures of
219 parental LNCaP cells grown in normal media (Figure W1C). For subsequent studies,
220 these cells will be referred to LNCaP-Androgen-Independent (LNCaP-AI).

221

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

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

235 Moreover, PCDH-PC expression was similarly reduced when cells were chronically
236 exposed to androgens (Figure W2A), oestrogen or progesterone which are two
237 alternative ligands of mutated AR in this line [25]. We then asked whether a functional
238 AR is required to mediate the repressive effect of androgens on *PCDH-PC* expression.
239 LNCaP cells were incubated in the presence of the antiandrogen, bicalutamide [26]. A
240 10 day treatment resulted in augmenting by 7 fold *PCDH-PC* expression (Figure 2C)
241 while expectedly reducing *KLK3* expression. Changes in cell morphology were also
242 visible upon the treatment (Figure W2B). We next applied bicalutamide treatment to the
243 LNCaP-AI derivative. We observed a dose-dependent relative decrease in *KLK3* and
244 *KLK2* expression compared to non-treated cells with a concurrent increase in *PCDH-PC*
245 expression (Figure 2D). To ascertain our assumption that *PCDH-PC* is repressed by AR
246 activity we next treated the LNCaP-AI cells with docetaxel. docetaxel is the standard of
247 care first line chemotherapy for men with metastatic CRPC. In PCa cells, recent studies
248 showed that short-term treatment with docetaxel impeded AR activity [27]. Here, we
249 exposed LNCaP-AI cells to 2,5 nM docetaxel for a prolonged period, and examined
250 expression of *PCDH-PC* and NE markers over time. After 15 days, we found that the cell
251 populations surviving this chronic exposure to docetaxel had greater levels of NE
252 markers *NSE* (~2-4 fold increase), *TUBB3* (~2-5 fold increase) and *PCDH-PC* (~25-125
253 fold increase) compared to untreated cells (Figure 2E). The morphology of the cells also
254 changed substantially with the formation of neurite outgrowths (Figure 2F). This data
255 suggests that NE-like cancers cells likely emerged via trans-differentiation following the
256 chronic exposure to docetaxel.

257

258

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

260 We earlier found that transient overexpression of *PCDH-PC*, under certain
261 circumstances, can perturb AR protein stability in LNCaP cells through a complex
262 mechanism that involves AKT activation and increase proteasomal activity towards AR
263 [28]. However, the potential links between AR activity, *PCDH-PC* expression, and
264 phenotypic changes in LNCaP cells have not been investigated. Here we tested the
265 possibility that *PCDH-PC* expression could disrupt androgen signaling. We transiently
266 overexpressed *PCDH-PC* using cultures of LNCaP cells. Increased expression of
267 *PCDH-PC* was verified by qRT-PCR (Figure W3A), Western blot analysis showed a
268 marked downregulation of PSA in PCDH-PC-transfected cells, while expectedly
269 increased NSE and phospho-AKT levels (Figure 3A). There was also significant
270 enrichment for inactivated phospho-GSK-3 β (Ser9). The AR level was not perturbed
271 suggesting that *PCDH-PC* expression disrupted androgen signaling by inhibition of AR
272 activity in our conditions. To further explore this inhibitory effect, we performed luciferase
273 reporter assays on these latter cells following transfection of incremental amounts of the
274 *PCDH-PC* expression construct. These analyses demonstrated a dose-dependent
275 decrease of the PSA promoter transactivation (Figure 3B). We then investigated long-
276 term effects of *PCDH-PC* expression by analyzing PSA expression in LNCaP derivatives
277 stably-transfected with *PCDH-PC*. In normal culture conditions, these cells showed more
278 neurites and a minor decrease in cell growth as compared to control cells (Figure 3C).
279 PCDH-PC mRNA and protein levels in LNCaP-pcDNA3 and LNCaP-PCDH-PC are
280 depicted in Figures W3B and W3C. Stable transfectants exhibited reduced AR activity
281 compared to vector transfected-LNCaP cells (Figure 3D). These cells have enhanced
282 levels of endogenous NSE, phospho-AKT and phospho-GSK-3 β , comparable AR

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

304

305

306

307 **PCDH-PC expression during PCa progression**

308 By immunohistochemistry, we then explored the distribution of PCDH-PC protein in
309 normal and pathological specimens. In tissues derived from normal prostate, luminal
310 epithelial cells were consistently found to be negative for PCDH-PC and pronounced
311 expression of this protein was observed in lonely cells scattered within the epithelium
312 (Figure 4A, i). Occasionally, a faint staining was detected in the basal cell layer (Figure
313 W4). A series of hormone naïve PCa (HNPC) specimens was examined using Tissue
314 Microarrays. This analysis revealed moderate to high expression of PCDH-PC in at most
315 11% (25 out of 222) of evaluable cases (Table 1). There was no significant correlation
316 with clinico-pathological data (Table W1). Evaluation of PCDH-PC expression in CRPC
317 samples indicated a much higher proportion of positive cases (that is, 61%, 33 of 54
318 CRPC) (Figure 4A, ii; Table 1). It is noteworthy that, PCDH-PC protein was also
319 detectable in cancer cells of metastatic CRPC lesions present in the brain and the lymph
320 nodes of patients (Figure 4A, iii-iv). Despite only 6 cases were analyzed, this suggested
321 that deregulated expression of PCDH-PC in CRPC disease is not restricted to recurrent
322 lesions localized to the prostate.

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

331 of these patients by examining their initial prostatic biopsies. Matched biopsy
332 specimens were available in 7 cases. In 6 of these index cases, we found no evidence
333 of PCDH-PC expression after analyzing cancer foci of several biopsy specimens (Figure
334 4A, vi) and one other case showed strong positivity for PCDH-PC but in dispersed
335 isolated cells rather than in clusters. These results demonstrate that high PCDH-PC
336 expression is rare in men with still hormonally-untreated PCa, but substantially
337 increases in response to hormonal manipulation.

338
339 **PCDH-PC expression associates with neuroendocrine features in human prostate**
340 **tissues.**

341 Given the apparent link between PCDH-PC and NE features *in vitro*, we explored
342 the value of PCDH-PC as novel candidate marker for NE trans-differentiation in human
343 PCa specimens. Examination of the hormone-treated samples for CgA and PSA
344 expressions consistently revealed that cancer cells expressing PCDH-PC are present in
345 tumor foci showing a large majority of CgA expressing cells, but with reduced
346 expression of PSA (Figure 4B-C; Figure 5A; Figure W5A). Dual immunofluorescence
347 procedure also revealed that in these tumor areas, not all cells exhibited the same NE
348 characteristics such that varied levels of NE markers were observed in the cells (Figure
349 5B). In adjacent benign epithelia, we detected a few isolated cells staining positive for
350 both CgA and PCDH-PC likely representing non-malignant NE cells (Figure W5B).

351
352 On further analysis of cancer foci positive for PCDH-PC, we found positivity for the
353 AR as well as for NSE and synaptophysin, two established NE marker, but we
354 consistently failed to detect staining for CD56 (NCAM1) (Figure 5C), another NE marker.

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

360 Collectively, these observations strongly suggest PCDH-PC as a novel early
361 marker for transition from epithelial to neuroendocrine phenotype in PCa treated by
362 ADT. Intriguingly however, at the castration state of prostate adenocarcinoma, the
363 relationship between PCDH-PC expression and NE (as assessed by CgA staining)
364 appeared to be lost, and although PCDH-PC immunostaining of PCa cells sometimes
365 coincides with staining for NE markers such as NSE (Figure W5C), in many cases the
366 PCDH-PC positive contingents examined did not show coincidental staining (not
367 shown).

368

369 **NE-like prostate cancer cells are resistant to chemotherapeutic agents**

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

378 indicated doses, LNCaP-NE-like cells were overwhelmingly resistant to these drugs
379 compared to LNCaP or LNCaP-AI cells (Figure 6B). LNCaP-NE-like cells also showed
380 enhanced resistance to various cytotoxic agents commonly used in management of
381 various malignancies (Figure W6A). We next wanted to gauge the dependence of
382 LNCaP-NE-like cells with respect to PCDH-PC expression for their viability. To this end,
383 LNCaP-NE-like cells were treated for 24h with Accell Green Non-Targeting siRNAs used
384 to control effective uptake of the siRNAs (Figure W6B), pools of Accell non-targeting
385 siRNAs, or Accell siRNAs raised against PCDH-PC transcripts, then cultured for 8 days
386 in hormone-deprived medium supplemented or not with docetaxel (10 nmol/L). PCDH-
387 PC silencing was found to be efficient in these conditions (Figure 6C). In the presence of
388 docetaxel, LNCaP-NE cells that had been pre-incubated with the PCDH-PC siRNAs
389 showed a significant decrease in cell viability (relative to cells exposed to NT siRNA in
390 the presence or absence of docetaxel) while in the absence of docetaxel, PCDH-PC
391 siRNA treatment had limited effect (Figure 6D). Moreover, the effect was not seen when
392 similar treatments were applied to the chemosensitive PC3 prostate cancer lineage
393 (Figure 6E) which lack *PCDH-PC*, or LNCaP-AI which expresses low amounts of *PCDH-*
394 *PC* (Figure 6F). Subsequent analyses showed that attenuating PCDH-PC expression
395 similarly sensitized LNCaP-NE-like cells to TPA and Camptothecin (Figure W6C-D).
396 These data argue for a chemoprotective role for PCDH-PC in LNCaP-NE-like.

397

398

DISCUSSION

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

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

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

425
426 Enigmatically, the relationship between PCDH-PC and NE differentiation was not
427 evident in CRPC specimens. This could reflect the multifaceted role of PCDH-PC in the
428 more advanced stages of PCa with functions that may occur independently of NE
429 differentiation. Alternatively, this could be indicative of various subtypes of NE
430 differentiation (from well-differentiated to poorly differentiated) in tumors with varied
431 proliferative activity and expressing various levels of NE markers [43, 44]. In that
432 respect, it will be important to examine the role of PCDH-PC in the setting of small cell
433 carcinoma of prostate, a rare poorly differentiated neuroendocrine PCa associated with
434 poor prognosis and poor response to therapies [45]. It is also tempting to speculate that
435 AR plays a crucial role in this potential molecular switch as AR is consistently implicated
436 in the growth of castrate resistant tumors [41, 46]. We have shown here that PCDH-PC
437 expression inhibits AR activity. But this inhibition appeared to be incomplete in the sense
438 that it's likely restricted to the ligand-dependent activity of AR. Although we already
439 know that PI3K/AKT activity may be an important mediator of this effect, the precise
440 mechanism through which PCDH-PC regulates the ligand-dependent AR activity has yet
441 to be fully determined.

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

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

450
451 Another interesting observation is that the NE status of LNCaP cells correlates with
452 resistance to a wide range of chemotherapeutic agents including docetaxel, the current
453 standard for metastatic CRPC. One could suggest that those resistances are likely
454 linked to the reduced growth rate of LNCaP-NE-like cells. Indeed, from a clinical
455 perspective, the observation that NE transdifferentiation could confer a multidrug
456 resistant phenotype allowing a cell to remain arrested until it can reacquire the ability to
457 proliferate, could make that process a formidable tumor promoter at any stage of PCa
458 progression. Interestingly, by targeting NE-like PCa cells using RNA interference against
459 PCDH-PC it was possible to sensitize cells to chemo-hormonal treatment. Together with
460 prior work identifying PCDH-PC as an anti-apoptotic factor in PCa cells [13], this
461 qualifies PCDH-PC as a general survival factor in PCa cells and provides a biological
462 rationale for further assessment of targeting malignant NE-like cells.

463
464 Although not emphasized here, in neo-adjuvant hormonally-treated tumors, we found
465 many instances with NE-like PCa (PCDH-PC⁺, CgA⁺, PSA⁻) cells adjacent to malignant
466 epithelial-like (PCDH-PC⁻, CgA⁻, PSA⁺) cells thus continuing to use the androgen-AR
467 axis despite ADT (Figure 4). Clearly the manifestation of these mixed populations, gives
468 reason to further examine whether these phenotypically distinct cell populations may

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

472
473 In summary, our study provides support for the likelihood of transdifferentiation model of
474 PCa cells to explain the emergence of neuroendocrine differentiation in human PCa
475 following ADT. We substantiate PCDH-PC, a human male specific protocadherin, as a
476 critical factor in this process that appears to be regulated by cross-modulation between
477 PCDH-PC and AR. Along this line, our data revealed novel paradigms linking the AR
478 axis and NE transdifferentiation in PCa cells with apparent implications for the
479 emergence of chemohormonal resistance.

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483
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491

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

Prostate carcinoma	PCDH-PC negative	PCDH-PC positive
	No of samples (%)	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)
<i>Pearson's chi-square test:</i>		p<0.0001
<i>Fisher's exact test:</i>	HNPC/HTPC	p<0.0001
	HTPC/CRPC	p = 0.178
	HNPC/CRPC	p<0.0001

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LEGENDS

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

688

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

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

703

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

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

736

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

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33

747 **Figure 6** Acquired NE phenotype correlates with chemoresistance in LNCaP cells. (A)
748 LNCaP, LNCaP-NE like and LNCaP-AI derivatives were examined for differences in cell
749 cell growth. (B) Viability assay of LNCaP (white bars), LNCaP-NE-like (gray bars),
750 LNCaP-AI (black bars) at 96 hours after treatment with docetaxel, paclitaxel,
751 Camptothecin, or phorbol ester (TPA) relative to untreated cells. (C) verification of
752 efficient *PCDH-PC* knockdown by qRT-PCR in LNCaP-NE-like pre-treated 24h with
753 either Accell Non-Targeting or PCDH11Y siRNAs, and then maintained in the presence
754 or absence of docetaxel for 48h. (D) Cell viability as assessed by WST-1 assay using
755 siRNAs treated LNCaP-NE-like cells alone or subsequently treated with docetaxel for 8
756 days. (E) As in (C) except using PC3 cells and 96h docetaxel treatment. (F) As in D
757 except using LNCaP-AI cells. Bars, means \pm SEM of quintuplets from one experiment
758 representative of three independent experiments.
759

FIGURE 1

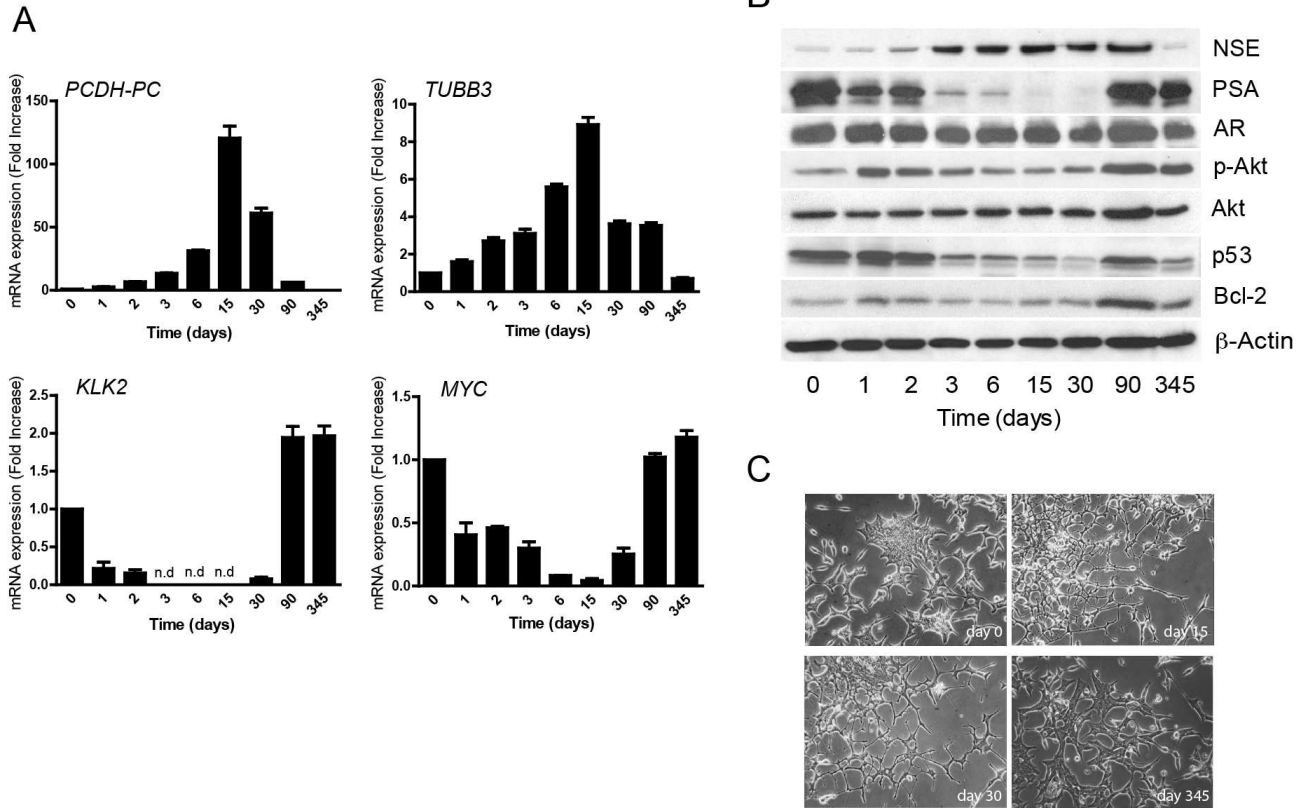


FIGURE 2

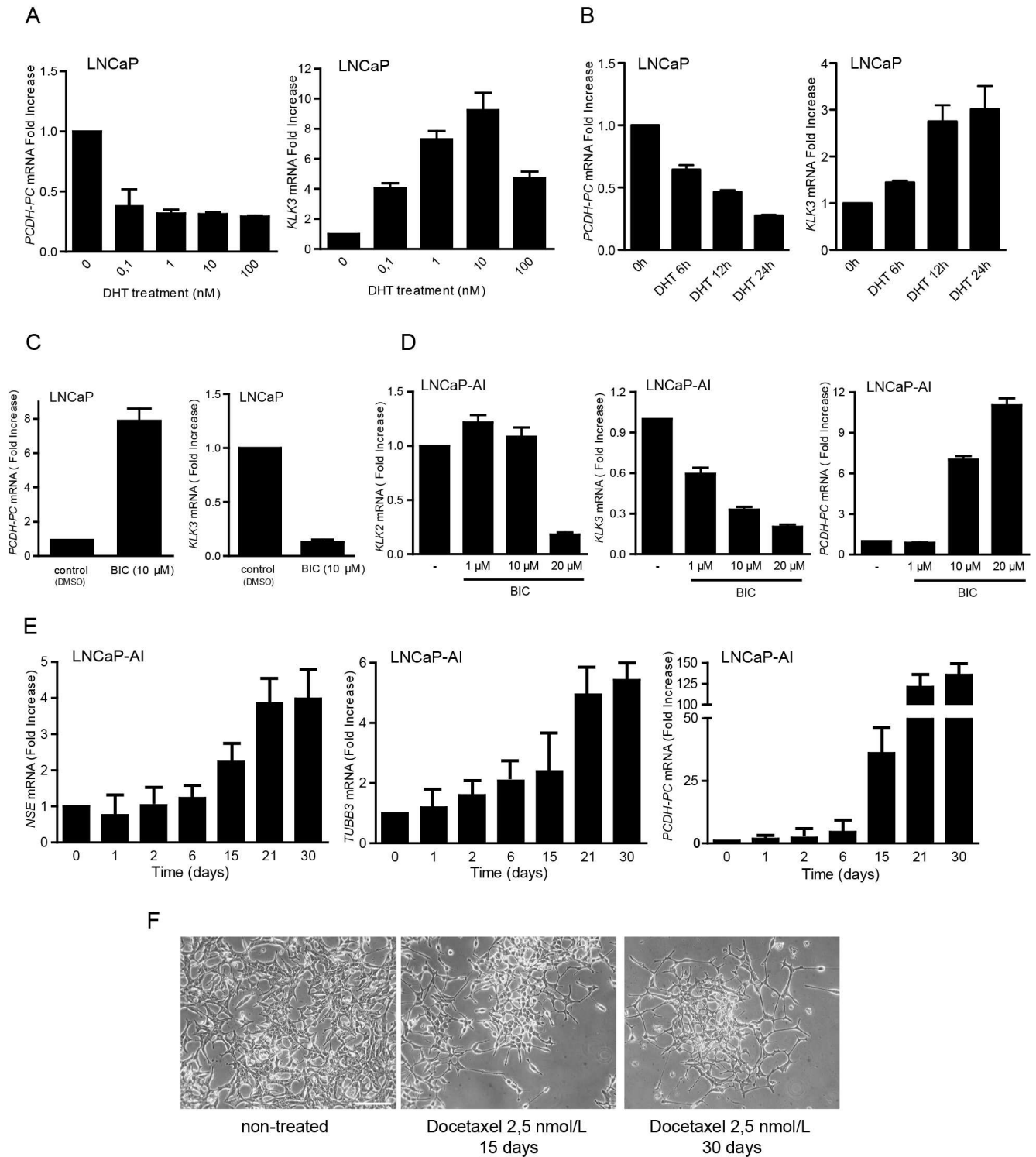


FIGURE 3

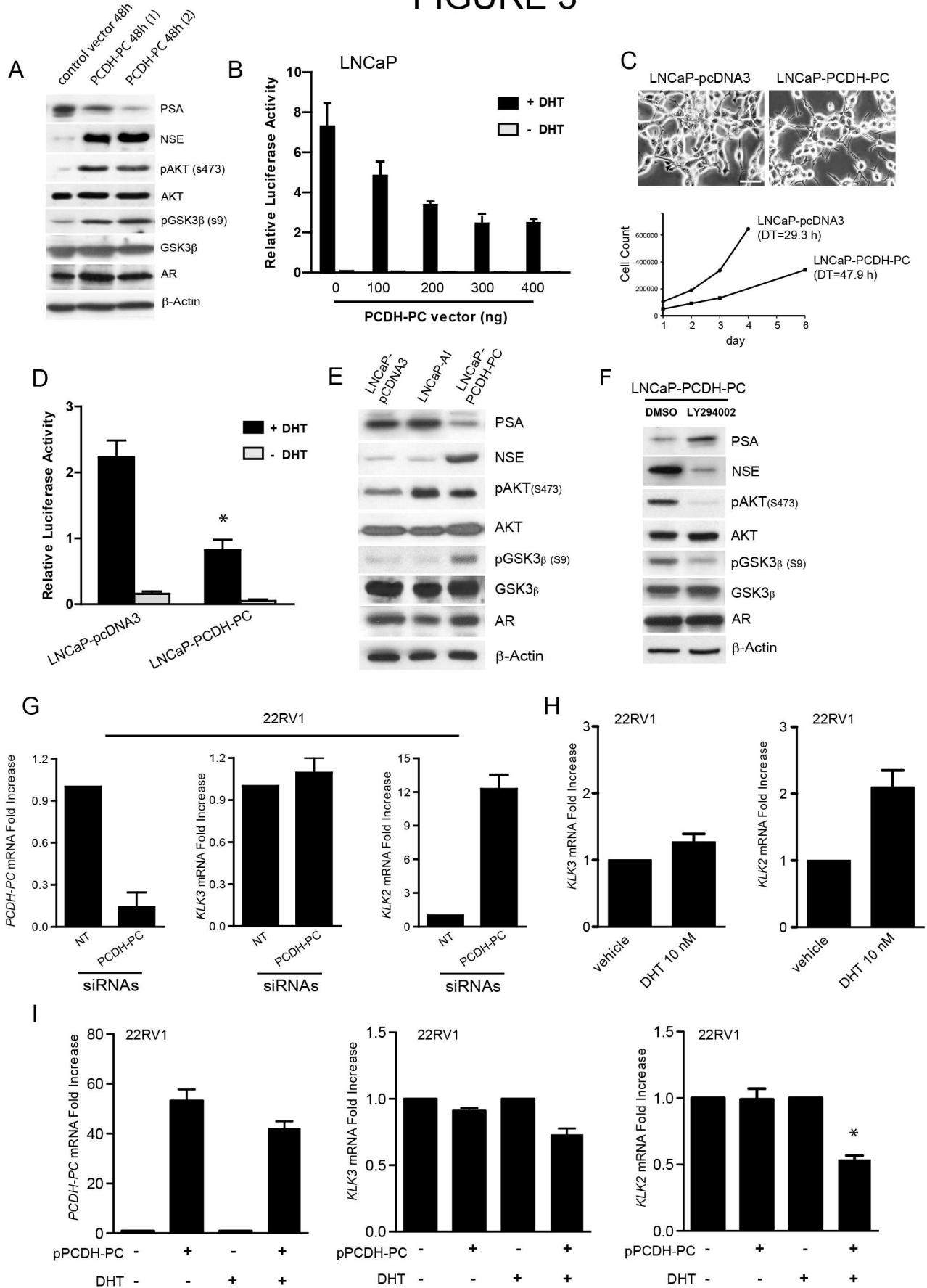
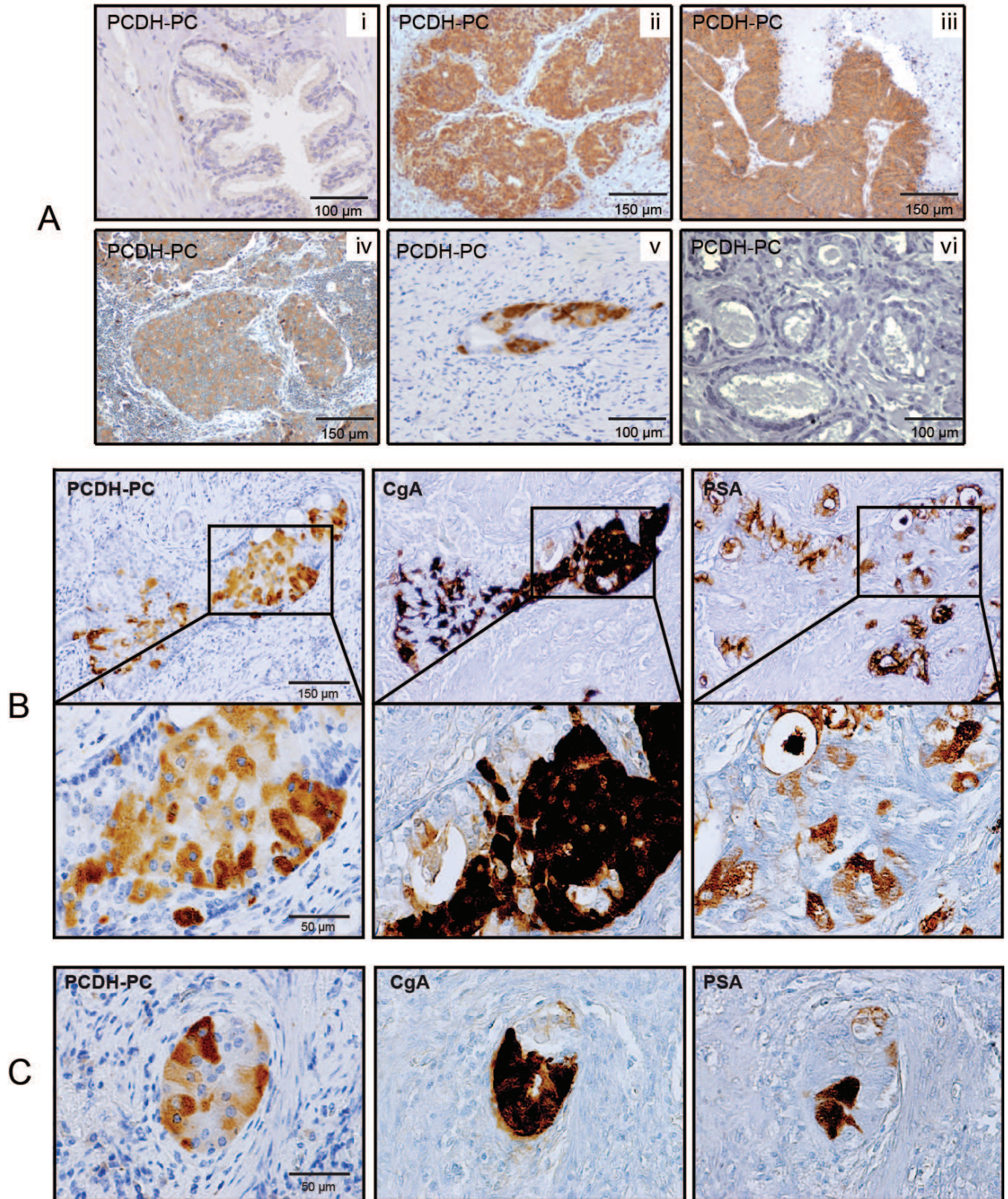


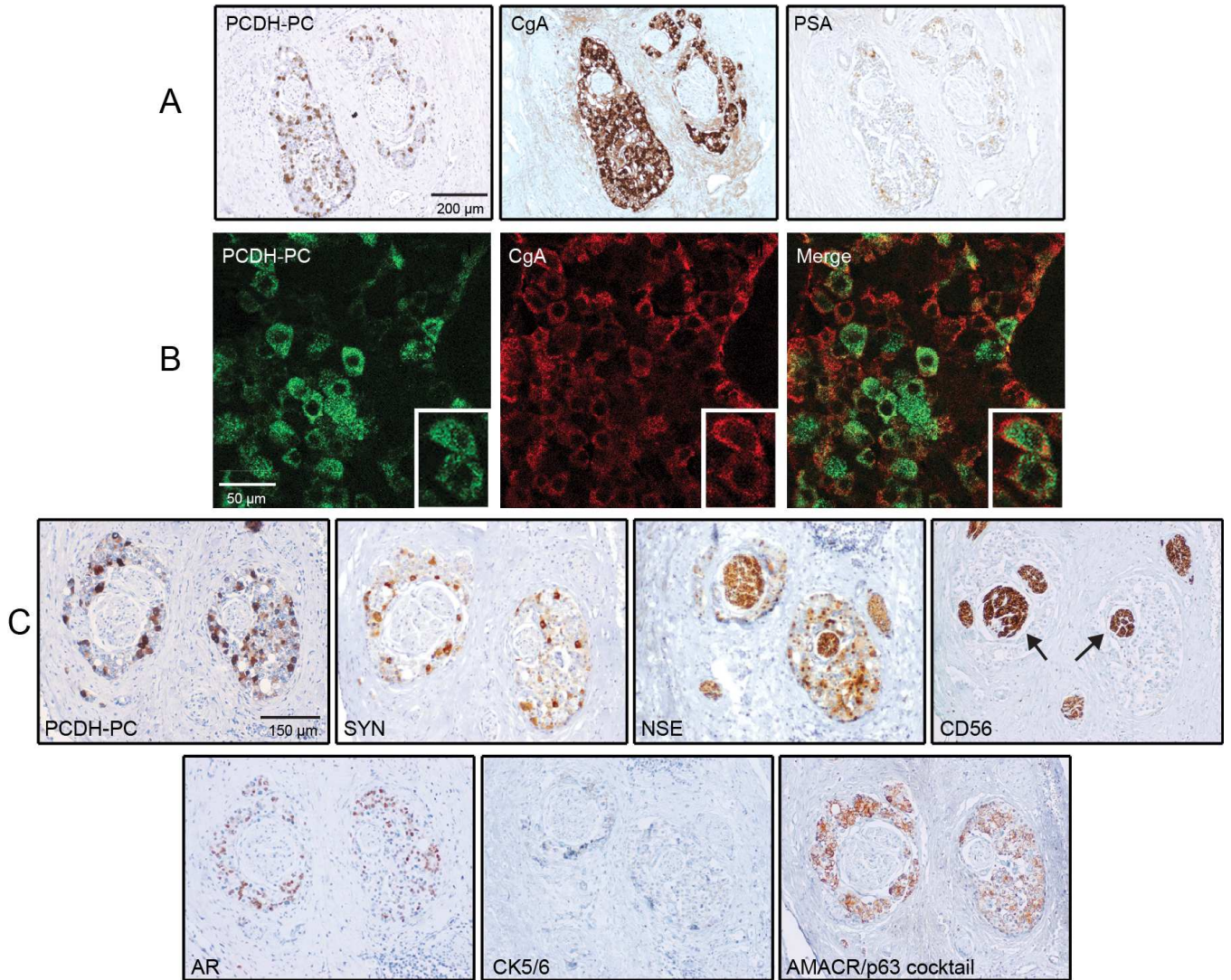
FIGURE 4



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FIGURE 5



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FIGURE 6

