Role of opiorphin genes in prostate cancer growth and progression

Amarnath Mukherjee[1], Augene Park[1], Li Wang[1] & Kelvin P Davies*,[1,2]
[1]Department of Urology, Albert Einstein College of Medicine/Montefiore Medical Center, NY 10461, USA
[2]Department of Physiology & Biophysics, Albert Einstein College of Medicine/Montefiore Medical Center, NY 10461, USA
*Author for correspondence: kelvin.davies@einsteinmed.org

Background: We describe the first studies investigating a role for opiorphin genes (*PROL1*, *SMR3A* and *SMR3B*) in prostate cancer (PrCa). **Materials & methods:** Databases and PrCa tissue arrays were screened for opiorphin expression. Xenografted tumor growth of human PrCa cells overexpressing *PROL1* was compared with controls in nude mice. Modulated gene expression by overexpression of *PROL1* was determined by RNA sequencing. **Results:** PrCa is associated with overexpression of opiorphin genes. Xenografted androgen-sensitive PrCa cells overexpressing *PROL1* developed into tumors in castrated male mice (in contrast to parental cells). *PROL1* overexpression modulates expression of genes in angiogenesis, steroid and hypoxic response pathways. **Conclusions:** Opiorphins promote the development of androgen-insensitive PrCa and activate pathways that potentially overcome the hypoxic barrier generated during tumor growth.

Worldwide, prostate cancer (PrCa) is the second most prevalent cancer in men, accounting for 15.5% of all male cancers in 2018 [1]. Although long-term survival rates with localized PrCa are high, metastatic PrCa remains largely incurable, resulting in 3.8% of all cancer-related deaths in men [2,3]. Identifying novel molecular markers of PrCa and their involvement in genetic mechanisms leading to tumor growth and progression may lead to new therapeutic strategies to prevent and treat PrCa.

There is a growing body of evidence that dysregulated expression of opiorphin-encoding genes (*PROL1*, *SMR3A* and *SMR3B*) is associated with the development of cancer. This was first suggested in a meta-analysis reported in 2008, where rank aggregation identified *PROL1* as one of the top 50 genes with changed expression levels in common between different cancer types [4]. Since this report, modulated expression of opiorphin-encoding genes has been associated with invasive breast cancer [5,6], head and neck adenoid cystic carcinoma [7,8] and oropharyngeal squamous cell carcinoma [9,10]. However, with the exception of a brief conference report from our laboratory [11], there are no published reports associating dysregulated expression of opiorphin-encoding genes with PrCa.

The biochemical activities and involvement of opiorphins in benign disease have been well studied and suggest possible mechanisms by which they may be involved in cancer development and progression. Posttranslational processing of the full-length opiorphin proteins generates peptides which act as potent endogenous neutral endopeptidase (NEP) inhibitors. A review from 2015 suggested that because modulated NEP activity is often associated with cancer development, opiorphins may play a role in cancer [12]. Opiorphin has also been demonstrated to act as master regulator of the hypoxic response, activating pathways that are associated with increased blood flow and angiogenesis [13]. Given that the metabolic demands of growing tumors create a growth-limiting hypoxic microenvironment [14–16], upregulated opiorphin expression might be involved in overcoming this hypoxic barrier. In addition, because hypoxia is a driver of malignant progression [17], the involvement of opiorphin genes in overcoming the hypoxic barrier may also be a factor in the development of castration-resistant/androgen-insensitive PrCa. Initial published studies have suggested an association between opiorphin and steroid response pathways in head and neck squamous cell carcinoma, where radioresistant tumors have elevated levels of both *SMR3A* and *ER2* [8].

Future Medicine

The aim of the present study was to investigate whether upregulated expression of opiorphin genes is associated with human PrCa and to test the hypothesis that *PROL1* overexpression modulates the growth and androgen sensitivity of mouse xenografted PrCa tumors. Global gene expression analysis was performed on PrCa cell lines overexpressing *PROL1* to determine whether the genetic mechanisms by which *PROL1* modulates tumor growth and androgen sensitivity might involve the known functional activities of opiorphin as an NEP inhibitor and regulator of the hypoxic response.

Materials & methods
Identification of upregulated *PROL1* expression in prostate cancer
An initial screen was conducted of publicly available gene expression datasets on the Gene Expression Omnibus (GEO) profiles database at NCBI [18] to determine if there was prior but unrecognized evidence of an association between modulated expression of opiorphin-encoding genes and PrCa. Using the search term 'prostate cancer' identified 79 datasets. However, only six of these datasets (GSE55945 [19], GSE45016 [20,21], GSE26910 [22], GSE6919 [23,24], GSE3325 [25] and GSE25136 [26]) both allowed gene expression comparison between benign and cancerous prostate cancer tissue and included the opiorphin-encoding genes *PROL1*, *SMR3A* or *SMR3B*. These six datasets were analyzed using the NCBI online data analysis tool (GEO2R) to compare expression of *PROL1* (referred to as *OPRPN* in this dataset), *SMR3A* and *SMR3B* between cancerous and noncancerous prostate tissue. A change in expression of a gene of interest was considered significant if it resulted in a log2-fold change of >0.56 or <-0.56 (i.e., a 1.5-fold change) with a p-value < 0.05 compared with the control group. To provide context on how this analysis might also provide evidence of an association between PrCa and genes previously identified as markers of PrCa development and progression [27], we also screened the same six datasets to identify whether PrCa was associated with changed expression of *AR*, *BRCA1*, *BRCA2*, *CDKN1B*, *CHEK2*, *CYP1B1*, *ELAC2*, *TP53*, *PTEN* or *RAS*.

In order to confirm the upregulation of *PROL1* in PrCa, a cDNA array from normal and cancerous prostate tissue was purchased from OriGene (HPRT103; TissueScan™ cDNA Arrays; Origene, MD, USA). This array has 48 cDNA samples covering nine normal, 18 stage II, 19 stage III and two stage IV tumors. Quantitative (real-time)-PCR analysis of the *PROL1* transcripts was performed as described below.

Cell lines
PrCa cell line PC3 (NCI-PBCF-CRL-1435/ATCC®CRL-1435™) and lymph node prostate carcinoma clone FGC (NCI-PBCF-CRL1740/ATCC® CRL-1740™, hereafter termed LNCaP) were obtained from the American Type Culture Collection (ATCC, MD, USA) and maintained in Roswell Park Memorial Institute 1640 medium (Invitrogen, CA, USA) with 10% fetal calf serum (Thermo Fisher Scientific, MA, USA), supplemented with 100 U/ml penicillin G and 100 ng/ml streptomycin (Invitrogen). Cells were maintained under standard conditions (37°C, atmospheric air [21% volume fraction of O_2] enriched by 5% CO_2 and humidity provided by spontaneous water evaporation). All cell lines were passaged at 2- to 3-day intervals on reaching 70% confluency, using a 0.25% trypsin–EDTA solution (Thermo Fisher Scientific). Cell morphology and viability were monitored by microscopic observation and regular testing for mycoplasma was performed (Universal Mycoplasma Detection Kit; ATCC).

Generation of *PROL1*-overexpressing cell lines
PC3 and LNCaP cells (as described above) were transduced with commercially available human *PROL1* lentiviral particles (Origene) according to the manufacturer's protocol. The transduced cell lines were designated PC3-ProL1+ and LNCaP-ProL1+, and overexpression of *PROL1* was confirmed by quantitative RT-PCR. RNA sequencing data indicated that *PROL1* was overexpressed 5995-fold in PC3-ProL1+ (Supplementary Table 1) and 28,560-fold in LNCaP-ProL1+ (Supplementary Table 2) compared with their respective parent cell lines. The doubling time of PC3-ProL1+ and LNCaP-ProL1+ cell lines in culture was not significantly different from that of their parent cell lines.

Mice xenograft studies
Athymic nude (homozygous nu/nu, aged 8–12 weeks) male, female and castrated male mice (Charles River Laboratories, MA, USA) were housed under standard pathogen-free conditions at a maximum of five mice per cage. Tumors were established by subcutaneous injection on the upper left flank of nude mice randomly ascribed to groups that received PC3, PC3-ProL1+, LNCaP or LNCaP-ProL1+ cells in a 1:1 mixture of Matrigel (Corning

Life Science, NJ, USA) and Hanks' balanced salt solution using a 27-gauge needle. For PC3 and PC3-ProL1+ cell lines, 1.5×10^6 cells (based on [28]), and for LNCaP and LNCaP-ProL1+ cell lines, 2.5×10^6 cells (based on [29]) were injected. The numbers of animals in each group are detailed in the figure legends. Tumors were measured twice a week using Vernier calipers and their volume (mm^3) calculated by length $\times$ width $\times$ height $\times$ 0.52. In all experimental groups, when tumors reached a diameter >1 cm the mice were euthanized through inhalation of CO_2. For the determination of molecular markers of hypoxia (*PROL1* and *VEGF*), xenografted PC3 tumors were excised 14 days after implantation (the earliest time point at which tumors could be reliably identified and surgically removed while avoiding potential contamination from surrounding tissue) and at the termination of the xenograft studies.

RNA isolation

For quantitative RT-PCR or RNA sequencing analysis from *in vitro* experimental samples (PC3, PC3-ProL1+, LNCaP and LNCaP-ProL+ cell lines grown in culture), RNA was isolated with the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. To prepare total RNA from xenografted tumors for quantitative RT-PCR analysis, tumors were first flash-frozen in liquid nitrogen and then ground to a homogenate using a pestle and mortar. RNA was isolated from 30 mg of the homogenate using a combination of the TRIzol™ (Invitrogen) method and the RNeasy Plus Mini Kit (Qiagen).

Quantitative RT-PCR

Generation of cDNA was achieved using the SuperScript III First-Strand Synthesis System (Invitrogen) using 1 µg total RNA as the starting material. For PCR analysis, samples were prepared in a 25-µl reaction mix containing the PowerUp SYBR Green PCR Master Mix (Thermo Fisher Scientific). Rt-PCR was performed by the Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific) using the standard cycling mode recommended by the PowerUp SYBR protocol (50°C for 2 min, 95°C for 2 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min). Primers were obtained from Qiagen (*RPL19*, cat. #PCRPPH18637A; *PROL1*, cat. #PPH10360A; and *VEGFA*, cat. #PPH00251C). Quantitative RT-PCR was performed in triplicate for each sample. Data were analyzed using the $\Delta\Delta Ct$ method, with gene expression normalized to 60S ribosomal protein L19 (RPL19) and expressed as the average fold-change relative to controls.

RNA sequencing

Prior to RNA sequencing, RNA samples were treated with RNase-free DNase I and then quantified using RiboGreen (Thermo Fisher Scientific). RNA integrity number was assessed with an Agilent 2100 Bioanalyzer (Agilent, CA, USA) to ensure each sample had an RNA integrity number ≥ 8. RNA libraries were prepared using the Illumina TruSeq Stranded Total RNA kit, with ribosomal depletion (Illumina, CA, USA). Each library was sequenced using the Illumina HiSeq2500 platform and generated 125-nt paired-end reads at either 30 million or 60 million reads per sample. RNA sequencing analysis of gene expression was performed in triplicate for each cell line (each sample prepared separately). Raw sequencing datasets have been deposited in the Sequence Read Archive of NCBI (http://www.ncbi.nlm.nih.gov/sra) with accession number GSE156223.

Bioinformatics analysis

The reads were aligned with STAR (v. 2.4.0c) [30] and genes annotated in Gencode version 18 were quantified with featureCounts (v. 1.4.3-p1) [31]. Normalization and differential expression analyses were performed with the Bioconductor package DESeq2 [32]. Differentially expressed genes were assessed using a two-sided *t*-test and fold-change on log-transformed expression values. All samples were median centered. Cutoff criteria for differentially expressed genes were >1 log2-fold change or <-1 log2-fold change in gene expression, with a p-value < 0.01.

Gene ontology annotation analysis of differentially expressed genes was performed using online analysis tools available from the database for annotation, visualization and integrated discovery (DAVID, v. 6.8, *Homo sapiens* GOTERM_GO_Direct database [33,34]), the Gene Ontology Consortium (GOC, *Homo sapiens*, biological function database [35,36]) and the Kyoto Encyclopedia of Genes and Genomes database (KEGG [37–39]).

Table 1. The publicly available datasets GSE55945 and GSE3325 were analyzed using the GEO2R online tool to compare expression of opiorphin-encoding genes (*PROL1, SMR3A and SMR3B*) between cancerous and noncancerous prostate tissue samples.

Comparison	Gene	p-value	logFC	Fold change
Dataset GSE55945				
All PrCa data compared with noncancerous prostate tissue	*PROL1 (OPRN)*	0.0013	1.14	2.20
	SMR3A	0.0098	0.52	1.43
ERG fusion-positive PrCa data compared with noncancerous prostate tissue	*PROL1 (OPRN)*	0.00097	1.17	2.25
	SMR3A	0.026	0.38	1.30
ERG fusion-negative PrCa data compared with noncancerous prostate tissue	*PROL1 (OPRN)*	0.013	1.11	2.16
	SMR3A	0.012	0.64	1.56
Dataset GSE3325				
All PrCa data compared with noncancerous prostate tissue	*SMR3B*	0.000083	2.74	6.68
Localized PrCa data compared with noncancerous prostate tissue	*SMR3B*	0.0035	2.3	4.92
Metastatic PrCa data compared with noncancerous prostate tissue	*SMR3B*	0.00039	3.22	9.32

logFC: Log2-fold change; PrCa: Prostate cancer.

Statistical analyses

Statistical analyses were performed using either Microsoft Excel (Microsoft, WA, USA) or GraphPad Prism 8.2 (GraphPad Software, Inc., CA, USA). To determine the statistical significance of two-group comparisons, unpaired two-tailed *t* tests were performed.

Results

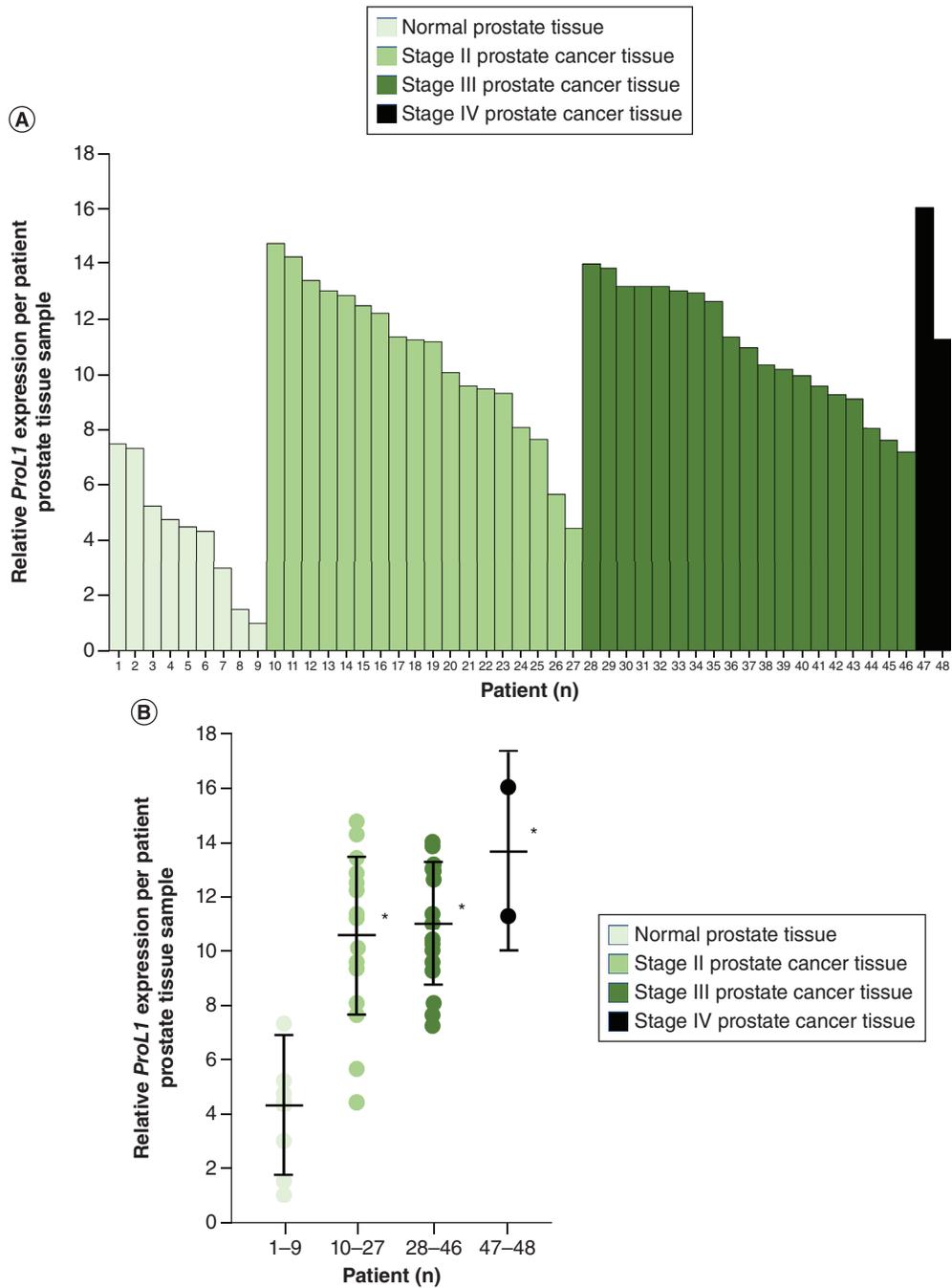### *PROL1* is overexpressed in prostate cancer

Of the six datasets in the GEO database that were suitable for our analysis, two demonstrated a significant association between PrCa and overexpression of opiorphin-encoding genes (Table 1 & Supplementary Table 3). This was comparable to the number of datasets in which genes with well-defined associations with PrCa were overexpressed; although *BRCA1* was overexpressed in four of the six datasets, the other genes (*PTEN*, *CYP1B1*, *AR*, *BRCA2*, *CDKN1B*, *CHEK2*, *ELAC2*, *RAS* and *TP53*) were also overexpressed in only two (Supplementary Table 3).

Analysis of dataset GSE55945 [19] demonstrated that PrCa was associated with overexpression of the opiorphin-encoding genes *PROL1* and *SMR3A* (Table 1). When the group of PrCa tissues in this dataset was subdivided into *ERG* fusion-positive and *ERG* fusion-negative PrCa, *ERG* fusion-positive PrCa was associated with a greater fold increase in *PROL1* expression and significance (2.25-fold; p = 9.7 × 10⁻⁴) than *ERG* fusion-negative PrCa (2.16-fold; p = 1.3 × 10⁻²). Similarly, dataset GSE3325 [25] demonstrated that PrCa was associated with overexpression of the opiorphin-encoding gene *SMR3B*, with a trend for greater overexpression in metastatic (9.32-fold; p = 0.00038) compared with localized PrCa (4.92-fold; p = 0.0035).

Given that *PROL1* has been the most intensely studied member of the opiorphin gene family, we focused on this gene as representative of the opiorphin family. Figure 1A & B shows the relative expression levels of *PROL1* determined in a tissue cDNA array containing human noncancerous and cancerous (at different stages) prostate tissue. The data from the tissue array demonstrate that *PROL1* is overexpressed in PrCa tissue (at a similar level of overexpression as observed in the dataset analysis), with a trend for higher levels of expression as the cancer stage increases. Overall, these analyses suggest that PrCa is associated with overexpression of *PROL1*.

Xenografted tumors derived from the PC3 cell line show a tumor environment-dependent & time-dependent increase in expression of *PROL1* & *VEGFA*
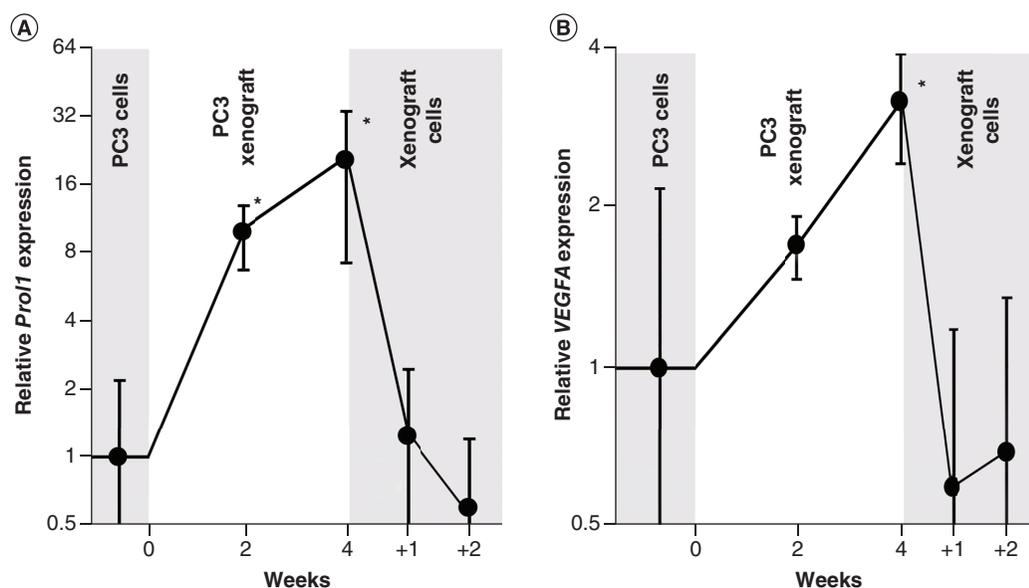
Xenografted tumors derived from the PC3 cell line (isolated from castration-resistant/androgen-insensitive PrCa) demonstrated a progressive and significant increase in both *PROL1* and *VEGFA* expression relative to cells kept under normoxic culture conditions (Figure 2A & B). After 4 weeks, tumors had approximately a 20-fold increase in *PROL1* expression and approximately a 3.2-fold increase in *VEGFA* expression, compared with the levels of these genes in the parent PC3 cell line. In cells isolated from these tumors and cultured under normoxic conditions, the expression of both *PROL1* and *VEGFA* returned to levels that were not significantly different from those of the

Figure 1. **PROL1, the gene encoding opiorphin, is upregulated in prostate cancer lines. (A)** Quantitative real-time PCR was used to determine relative expression levels of *PROL1* in a tissue cDNA microarray with samples from control tissue and different stages of PrCa. **(B)** The data for individual samples were normalized to the sample with the lowest level of *PROL1* expression in the tissue array. Columns represent the average *PROL1* expression level for each grade of cancer. There was significantly higher expression of *PROL1* in PrCa tissue, with a trend for higher levels of expression as the cancer stage increased.
*p < 0.05; error bars = standard deviation.
PrCa: Prostate cancer.

Figure 2.   Relative expression of *PROL1* and *VEGFA* in the PC3 PrCa cell line before xenografting into nude male mice, in the xenografted tumors and in cells isolated from the tumors and kept under normoxic cell culture conditions. At 2 and 4 weeks post-xenograft, animals were euthanized and tumors excised, and the tumor tissue was used to isolate cells for cell culture. RNA was extracted from cells and tissues for RT-PCR for the *PROL1* and *VEGFA* genes, with expression normalized to the housekeeping gene *RPL19*. The relative change in expression of **(A)** *PROL1* and **(B)** *VEGFA* is represented as fold change compared with the starting population of the parent PC3 cell line. *p < 0.05; n = 5; error bars = 95% CI.

parent PC3 cells. Overall, these data suggest that the hypoxic tumor microenvironment results in overexpression of *PROL1* and *VEGFA* in the PC3 tumor xenograft.

Xenografted tumors derived from a PC3 cell line engineered to overexpress *PROL1* (PC3-ProL1+) initially exhibit accelerated growth compared with the parent cell line in male nude mice

In order to determine whether overexpression of *PROL1* might directly affect PrCa tumor growth, we compared the growth of xenografted tumors in male and female nude mice derived from a PC3 cell line genetically engineered to overexpress *PROL1* (PC3-ProL1+) with its parent cell line (PC3). As shown in Figure 3A, at early time points (10 and 13 days after implantation) there was a significantly greater rate of tumor growth in male mice implanted with PC3-ProL1+ compared with PC3. However, at later time points (from 17 days onwards) tumor growth in both male and female mice was not significantly different between tumors derived from PC3-ProL1+ and the parental PC3 cells (Figure 3B & C).

Overexpression of *PROL1* in PC3 cells modulates expression of genes associated with the hypoxia & steroid response pathways

In order to identify possible genetic mechanisms by which upregulated *PROL1* expression in PC3 cells might modulate tumor growth, we compared global gene expression between the PC3 and PC3-ProL1+ cells grown in culture. This analysis identified 1698 differentially expressed genes between the two cell lines (Supplementary Table 1), with *PROL1* being the most differentially expressed gene (5995-fold; p = 2.82 × 10^{-26}). To identify biological functions that may be regulated through overexpression of *PROL1* in PC3 cells, the list of differentially expressed genes (minus *PROL1*) was submitted to the DAVID, GOC and KEGG databases, which identified 1250, 1366 and 480 unique genes, respectively; these were then used for ontological analysis (Supplementary Tables 4–6). Table 2 shows ontological groups where there was significant overrepresentation of differentially expressed genes. Both DAVID and GOC analysis identified differentially expressed genes in ontological groups involved in vascularization (such as genes involved in angiogenesis, blood vessel development, organ growth and morphogenesis) as well as groups that function in hypoxia and direct modulation of blood flow (such as genes
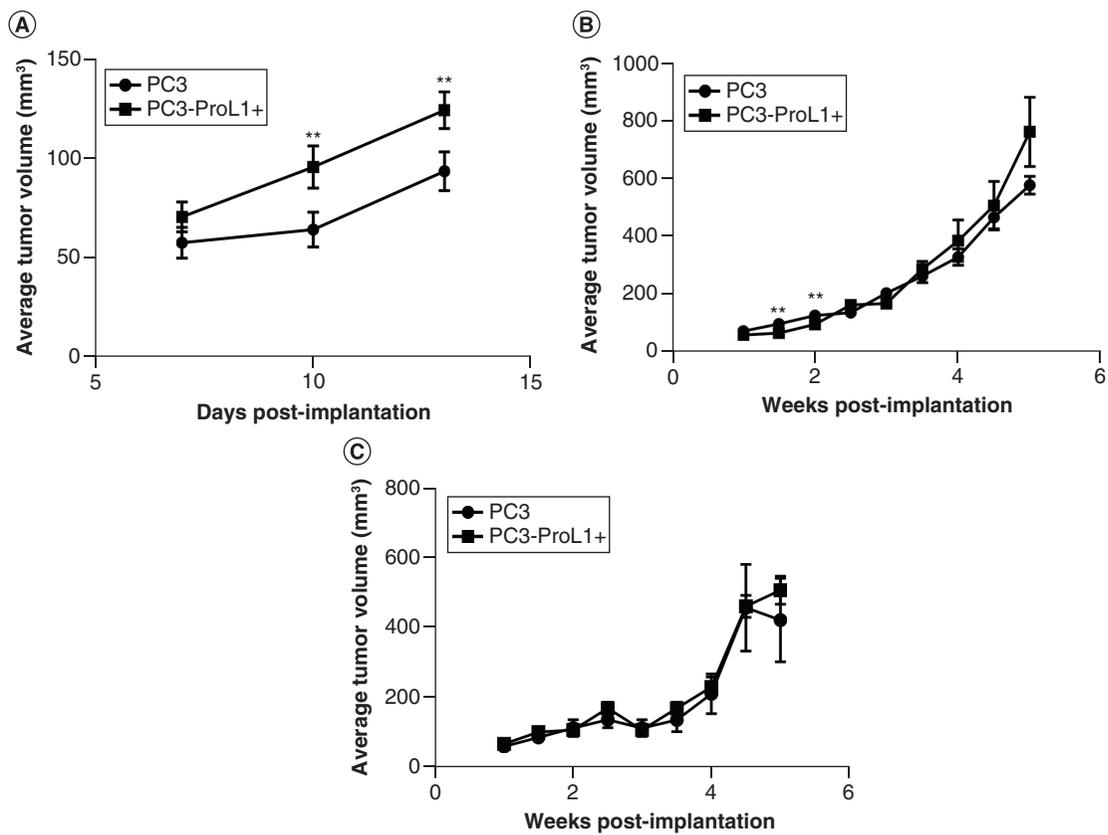
Figure 3. PC3 cells engineered to overexpress *PROL1* have initially faster tumor growth in male mice compared with the parent cell line.
PC3 and PC3-ProL1+ cell lines were injected into male and female nude mice and tumor size determined by standard caliper measurements (n = 10 per group). All mice developed tumors. The average tumor volume is shown for **(A)** male mice at early time points, **(B)** all time points for male mice and **(C)** all time points for female mice.
*p < 0.05; error bars = standard error.

involved in overcoming hypoxia, vasodilation, blood vessel diameter and regulation of smooth muscle contraction). Analysis of the list of differentially expressed genes using the KEGG database identified the ontologic group 'pathways in cancer' as having the greatest number of differentially expressed genes (41 genes; p = 8.96 × 10^{-3}) (Supplementary Table 6). Differentially expressed genes were also identified with significant overrepresentation in specific biochemical pathways such as the P13K-Akt, VEGF and MAPK signaling pathways and steroid metabolic pathways (Table 2).

Xenografted tumors derived from LNCaP cells engineered to overexpress *PROL1* (LNCaP-ProL1+) have gender-specific modulated growth compared with the parent cell line

We expanded the studies described above to determine whether upregulated *PROL1* expression might also play a role in tumor development in an androgen-sensitive cell line (LNCaP). As shown in Figure 4, although overexpression of *PROL1* in LNCaP cells did not affect tumor growth in intact male mice (Figure 4A), it significantly modulated tumor growth in both castrated male and female mice (Figure 4B & C). As expected for an androgen-sensitive cell line, no tumors developed when LNCaP cells were xenografted into castrated male mice (Figure 4B). However, remarkably, xenografted LNCaP-ProL1+ cells were able to develop tumors in castrated male mice. As seen in previous studies [40], xenografted tumors derived from LNCaP did develop tumors in female mice, albeit at a delayed time for detection compared with male nude mice (Figure 4C). However, once LNCaP tumors are established, their growth rate is not significantly different between male and female mice. Interestingly, in the female mice there was significantly impaired growth of tumors derived from LNCaP-ProL1+ compared with tumors derived from LNCaP (Figure 4C). Overall, these observations suggest that overexpressing *PROL1* in the

Table 2. Ontological groups containing overrepresented differentially expressed genes when *PROL1* is overexpressed in PC3 cells.

| Biological process/molecular pathway	GO/hsa identifier	Analysis	Ref list (GOC: 20996) (DAVID: 16792) (KEGG: 6879)	*PROL1* overexpression list	Fold enrichment	p-value
Angiogenesis	0001525	GOC	315	56	2.65	9.33×10^{-10}
Blood vessel development	0001568	GOC	486	74	2.27	1.53×10^{-9}
Blood vessel morphogenesis	0048514	GOC	404	68	2.51	1.37×10^{-10}
Blood circulation	0008015	GOC	386	63	2.44	2.55×10^{-9}
Circulatory system process	0003013	GOC	394	77	2.18	3.9×10^{-9}
Angiogenesis	0001525	DAVID	223	38	2.47	5.46×10^{-11}
Regulation of blood vessel diameter	0097746	GOC	130	31	2.66	1.25×10^{-5}
Regulation of blood vessel size	0050880	GOC	137	25	2.73	2.71×10^{-5}
Positive regulation of animal organ morphogenesis	0110110	GOC	86	23	2.99	7.79×10^{-5}
Vasculogenesis	0001570	DAVID	56	12	3.10	1.35×10^{-3}
Positive regulation of PI3K signaling	0014068	GOC	85	15	2.64	1.4×10^{-3}
Regulation of blood pressure	0045776	DAVID	30	13	2.90	1.47×10^{-3}
Positive regulation of steroid metabolic process	0045940	GOC	28	8	4.27	1.47×10^{-3}
Response to hypoxia	0001666	DAVID	172	22	1.85	7.7×10^{-3}
PI3K-Akt signaling pathway	04151	KEGG	345	36	1.50	1.4×10^{-2}
VEGF signaling pathway	04370	KEGG	61	10	2.35	2.4×10^{-2}
MAPK signaling pathway	04010	KEGG	253	26	1.47	4.67×10^{-2}
Vasodilation	0042311	DAVID	14	4	4.13	6.74×10^{-2}

DAVID: Database for Annotation, Visualization and Integrated Discovery; GO: Gene ontology; GOC: Gene Ontology Consortium; KEGG: Kyoto Encyclopedia of Genes and Genomes.

androgen-sensitive LNCaP cell line modulates the hormonal sensitivity of xenografted tumors toward a more androgen-insensitive phenotype.

Overexpression of *PROL1* in LNCaP cells modulates the expression of genes associated with steroid metabolism & the androgen response

In order to determine possible mechanisms for the difference in xenografted LNCaP tumor growth when *PROL1* is overexpressed, we compared global gene expression between LNCaP-ProL1+ and LNCaP (its parent cell line) growing in culture, identifying 1110 differentially expressed genes (Supplementary Table 2). *PROL1* was the most differentially expressed gene (28,560-fold; p = 24.36×10^{-36}). To identify biological functions that may be regulated through overexpression of *PROL1* in LNCaP cells, the list of differentially expressed genes (minus *PROL1*) was submitted to the DAVID, GOC and KEGG databases, which identified 665, 812 and 287 unique genes, respectively; these were then used for ontological analysis (Supplementary Tables 7–9). There was considerable overlap in the ontological groups with overrepresentation of differentially expressed genes when *PROL1* was overexpressed in either LNCaP or PC3 (Table 3). These included ontological groups involved in vascularization (angiogenesis, circulatory system development and organ morphogenesis) and regulation of blood flow (smooth muscle contraction and blood pressure) (Table 3). Analysis for overrepresentation of differentially expressed genes in biochemical pathways using the KEGG database also identified 'pathways in cancer' as a significant ontological group (p = 5.97×10^{-2}; Supplementary Table 9) as well as steroid metabolism and the regulation of MAPK signaling (Table 3).

However, there were differences in the regulation of specific genes related to the androgen response between LNCaP and PC3. Unlike PC3, where overexpression of *PROL1* caused a significant increase in expression of the androgen receptor gene (*AR*, 7.46-fold; p = 3.16×10^{-197}) and a decrease in expression of the estrogen receptor (*ESR1*, 2.7-fold reduced; p = 3.38×10^{-49}), in LNCaP neither of these genes changed in expression (Supplementary Tables 1 & 2). In contrast, overexpression of *PROL1* in LNCaP reduced expression of the progesterone receptor gene (*PGR*, 2.17-fold reduced; p = 4.41×10^{-3}), whereas *PGR* expression was not changed in PC3 cells.
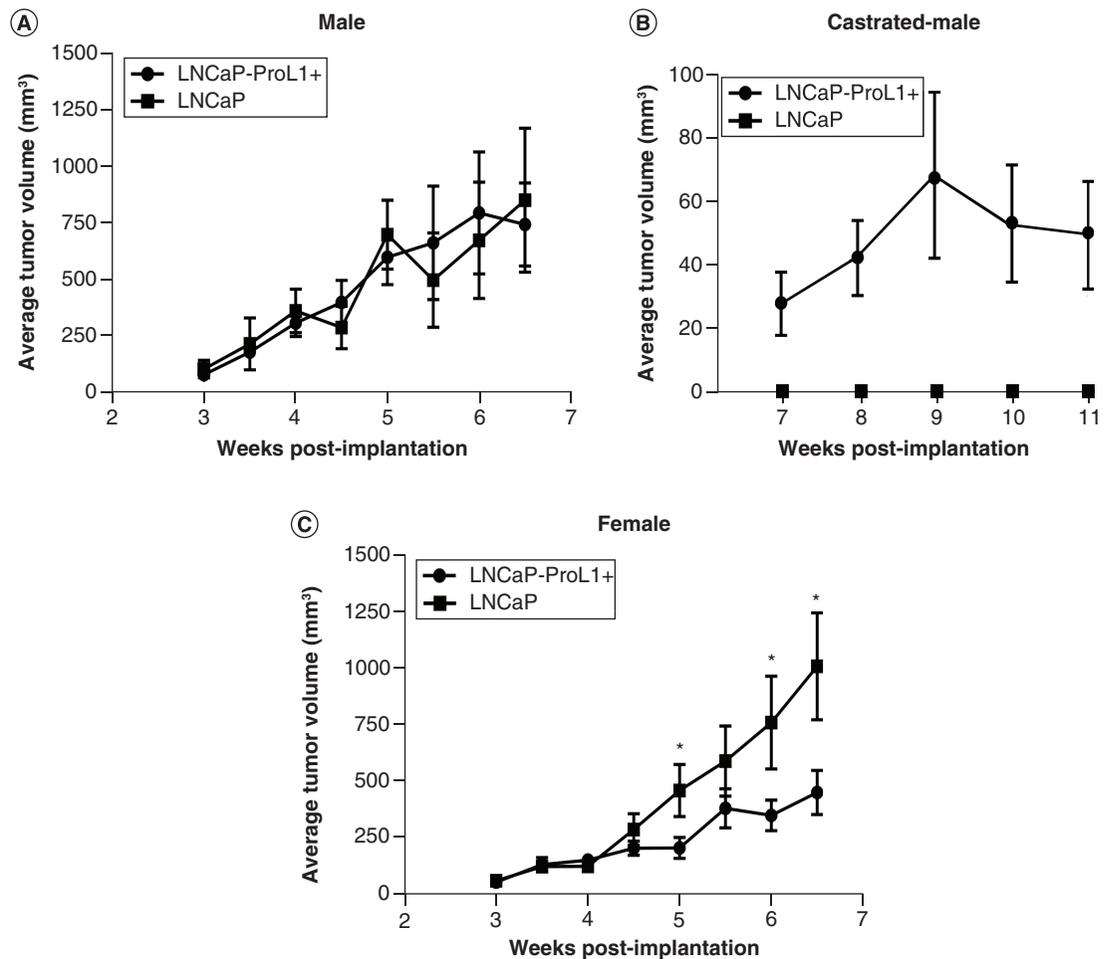
Figure 4. Growth of xenografted tumors derived from LNCaP-ProL1+ or LNCaP cell lines in male, castrated male or female mice. LNCaP and LNCaP-ProL1+ cell lines were injected into male, castrated male and female nude mice and tumor size determined by standard caliper measurements. The average tumor volume is shown for each time point. **(A)** Growth of tumors derived from LNCaP or LNCaP-ProL1 cell lines in male mice (n = 10 per group). All mice developed tumors. **(B)** Growth of tumors derived from LNCaP or LNCaP-ProL1+ cell lines in castrated male mice (n = 5 per group). Two of five mice developed tumors in the LNCaP-ProL1+ group and none of five developed tumors in the LNCaP group. **(C)** Growth of tumors derived from LNCaP or LNCaP-ProL1+ cell lines in female mice (n = 10 per group). All mice developed tumors.
*p < 0.1; **p < 0.05; error bars = standard error.

Discussion

This is the first report associating elevated expression levels of opiorphin-encoding genes with the development of human PrCa. An initial screen of publicly available databases identified an association between expression levels of the opiorphin-encoding genes and PrCa. There was a much greater significance of elevated *PROL1* expression in *ERG* fusion-positive PrCa (generally considered to represent a more advanced stage PrCa [41]) compared with *ERG* fusion-negative PrCa (Table 1). There was overexpression of the *PROL1* homologue *SMR3B* in localized PrCa compared with noncancerous prostate tissue (fivefold; p = 0.0035), with greater overexpression in metastatic PrCa compared with noncancerous prostate tissue (9.32-fold; p = 0.00038). Although only two of the six datasets publicly available on GEO associated an upregulation of opiorphin genes with cancer development, a similar search for an association with several genes widely accepted to be involved in PrCa revealed associations in similar numbers of datasets (Supplementary Table 3). In a meta-analysis of 20 microarray datasets using a rank aggregation approach to identify changes in gene expression in common between different cancer types, the maximum number of lists that any gene appeared in was three [4]. In that study, of the aggregated list of top-50 genes, 36 had been previously

Table 3. Ontological groups containing overrepresented differentially expressed genes when *PROL1* is overexpressed in LNCaP cells.

Biological process/ molecular pathway	GO/hsa identifier	Analysis	Ref list (GOC:20996) (DAVID:16792) (KEGG:6879)	*PROL1*-overexpression list	Fold enrichment	p-value
Animal organ morphogenesis	0009887	GOC	930	68	1.79	1.16×10^{-5}
Smooth muscle contraction	0006939	GOC	51	10	4.8	1.20×10^{-4}
Circulatory system development	0072359	GOC	832	59	1.7	1.60×10^{-4}
Blood circulation	0008015	GOC	386	33	2.09	1.72×10^{-4}
Steroid hormone biosynthesis	00140	KEGG	58	8	3.31	9.75×10^{-3}
Angiogenesis	0001525	DAVID	223	16	1.81	3.19×10^{-2}
Positive regulation of MAPK cascade	0043410	DAVID	81	8	2.49	4.10×10^{-2}
Positive regulation of blood pressure	0045777	DAVID	20	4	5.05	4.25×10^{-2}

DAVID: Database for Annotation, Visualization and Integrated Discovery; GO: Gene ontology; GOC: Gene Ontology Consortium; KEGG: Kyoto Encyclopedia of Genes and Genomes.

implicated in cancer (and often in multiple cancers) and the authors suggested that the other 14 genes 'may contain some novel cancer genes that may deserve further scrutiny in the future'. It is interesting to note that *PROL1*, the gene encoding opiorphin, was one of these 14 genes.

The apparent low-level representation in datasets of the differentially expressed opiorphin genes, as well as other markers of PrCa, may represent the highly heterogeneous nature of samples used in gene expression studies [42]. Not only is PrCa in general highly heterogeneous in nature, but even in the same patient there are multiple distinct cancer foci at the time of primary diagnosis. It has also been shown that many PrCa datasets have significant stromal contamination, which has a tendency to mask epithelial gene expression across PrCa profiling studies [43]. Subsequently, PrCa tissue arrays confirmed that *PROL1* is significantly upregulated in PrCa, with a trend for higher levels of overexpression with stage (Figure 1).

The gene products of opiorphin are posttranslationally processed to peptides that are released into the bloodstream; therefore the association between upregulated opiorphin gene expression and PrCa suggests that determining the levels of opiorphin peptides in the blood may be useful as a prognostic marker. Several studies have already demonstrated the measurement of opiorphin levels in blood, saliva and seminal fluid and showed that opiorphin levels could be prognostic markers of disease (e.g., burning mouth syndrome [44], dental pain [45] and male factor infertility [46]).

Our data (presented in Figure 2) suggest that overexpression of opiorphin genes is a response to the tumor microenvironment. Because our prior published studies have demonstrated that opiorphins are upregulated in smooth muscle cells in response to hypoxia [13], and that the metabolic demands of developing tumors create a hypoxic microenvironment [14–16], our hypothesis is that it is the hypoxic environment of the developing tumor that results in opiorphin overexpression.

We also demonstrate that engineered constitutive overexpression of *PROL1* modulates the growth of xenografted tumors. The effect on tumor growth caused by overexpression of *PROL1* is highly pronounced in LNCaP cells (isolated from castration-sensitive/androgen-sensitive PrCa) (Figure 4). Remarkably, in contrast to the parent cells, xenografted LNCaP-ProL1+ cells were able to develop tumors in castrated male mice, and in female mice overexpression of *PROL1* significantly impaired growth of tumors. In PC3 cells (a castration-resistant/androgen-insensitive PrCa cell line), although overexpression of *PROL1* increases the growth rate of xenografted tumors in nude male mice at early time points there is no significant difference at later time points (Figure 3). These experiments provide strong evidence that *PROL1* overexpression modulates the androgen sensitivity of tumors, such that the xenografted tumor exhibits a more androgen-insensitive phenotype.

Global analysis of the changes in gene expression caused by overexpression of *PROL1* in PC3 and LNCaP cells supports a role for *PROL1* in the modulation of genetic pathways involved in both overcoming hypoxia and the development of androgen insensitivity. Opiorphins have previously been shown to be directly involved in the regulation of blood flow to tissues through their modulation of smooth muscle tone [47–49], thus their upregulation in tumors may directly contribute to overcoming the hypoxic barrier that develops in the growing tumor. In addition, overexpression of *PROL1* in both PC3 and LNCaP cells modulated the expression of genes involved
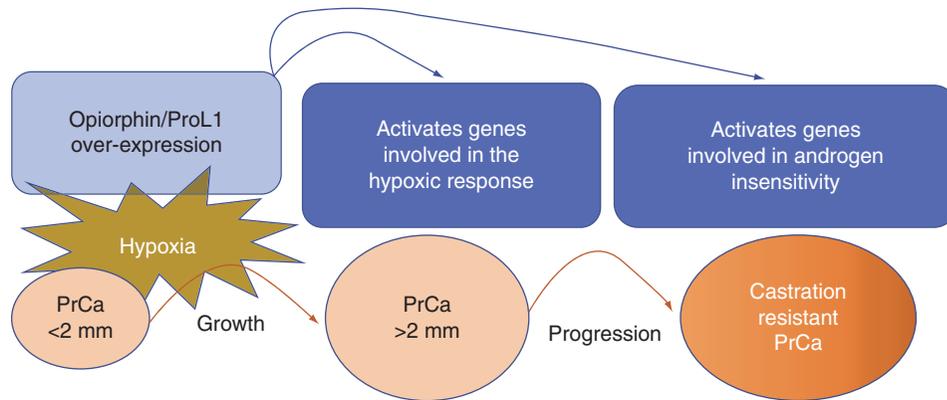
Figure 5.   Proposed mechanisms by which overexpression of opiorphin-encoding genes are involved in prostate cancer growth and progression. In our recent publication we demonstrated that in corporal smooth muscle cells opiorphin is a master regulator of the hypoxic response [13]. Therefore we hypothesize that as the tumor grows above 1–2 mm. hypoxia first causes upregulation of opiorphin, which in turn activates expression of genes involved in the hypoxic response and the development of androgen-insensitive/castration-resistant prostate cancer. PrCa: Prostate cancer.

in angiogenesis and morphogenesis (Tables 2 & 3); the activation of these pathways would likely be involved in vascularization of the tumor and thereby also contribute to overcoming hypoxia.

Overexpression of *PROL1* also affected genes involved in steroid metabolism and response pathways in both LNCaP and PC3 (Tables 2 & 3), which could potentially contribute to the modulation of androgen sensitivity. For example, overexpression of *PROL1* in LNCaP reduced expression of the progesterone receptor gene, *PGR*. Activation of *PGR* is a positive modulator of cell division [50,51] and the reduced expression of *PGR* in LNCaP-ProL1 cells might therefore explain the negative effect on tumor growth in female mice. In PC3-ProL1+ cells we observed a significant upregulation of the AR (7.46-fold; p = 3.16 × 10^{-197}; Supplementary Table 1). Increased activity of the AR could subsequently lead to the activation of secondary messengers involved in modulating the activity of other signaling pathways such as the PI3K-Akt and MAPK pathways, which had significant overrepresentation of differentially expressed genes in PC3-ProL1+ cells. The PI3K-Akt signaling pathway is considered one of the most commonly dysregulated pathways in PrCa, with approximately 40% of early stage and 70–100% of advanced-stage PrCa exhibiting dysregulation of this pathway, suggesting that PI3K-Akt dysregulation is associated with PrCa progression [52,53].

Overall, our data associate the development of PrCa with overexpression of opiorphin-encoding genes and suggest genetic pathways by which they contribute to tumor growth and the development of androgen insensitivity. These mechanisms are summarized in Figure 5. The hypoxic barrier to growth developed when tumors reach about 2 mm^3 would be expected to upregulate opiorphin gene expression. As a master regulator of the hypoxic response, overexpression of opiorphin would both directly relax vascular smooth muscle cells neighboring the tumor and activate pathways involved in the vascularization and regulation of blood supply to the tumor, allowing the tumor to overcome the hypoxic barrier and promoting PrCa growth. In addition, overexpression of *PROL1* in response to hypoxia may activate genes involved in steroid metabolism and androgen response, modulating the androgen sensitivity of the tumor and potentially driving the development of castration resistance/androgen insensitivity.

Conclusion

In conclusion, this is the first report associating upregulated opiorphin gene expression and PrCa. Overexpression of *PROL1* in PrCa cell lines modulates tumor growth and, in the androgen-sensitive LNCaP cell line, xenografted tumors exhibit a more androgen-insensitive phenotype. Global analysis of the changes in gene expression caused by overexpression of *PROL1* in PC3 and LNCaP cells supports that it has a role in the modulation of genetic pathways involved in both overcoming hypoxia and the development of androgen insensitivity. Therefore opiorphins may play a role in PrCa development by activating pathways that overcome the hypoxic environment of the developing tumor and modulate its androgen sensitivity. Strategies that target opiorphin expression may represent a novel means of preventing the development and progression of PrCa.

Future perspective

The protein products of the opiorphin genes are processed and released from cells and can be detected in blood. Therefore the development of tests measuring levels of opiorphin in the blood could be used diagnostically for determining the stage and androgen sensitivity of PrCa. Strategies that target opiorphin gene expression, such as siRNA, may be used as anti-PrCa therapeutics that prevent the development and progression of PrCa.

Summary points

- There is a growing body of evidence that opiorphins (represented in humans by *PROL1*, *SMR3A* and *SMR3B*) are associated with cancer development. This is the first report associating upregulated opiorphin gene expression with prostate cancer (PrCa).
- When *PROL1* is overexpressed in androgen-sensitive PrCa cell lines, they can develop tumors when xenografted into castrated male mice, unlike the parent cell lines; therefore overexpression of *PROL1* plays a role in the development of castration-resistant PrCa.
- Global gene analysis demonstrated that *PROL1* overexpression in PrCa cells results in modulated expression of genes involved in steroid response pathways, supporting a mechanism by which overexpression of *PROL1* might lead to the development of androgen insensitivity.
- Previous studies have suggested that opiorphins are master regulators of the hypoxic response in smooth muscle cells. Data presented here suggest a similar role in mediating the hypoxic response in PrCa; as tumors grow, the expression of *PROL1* correlates with the expression of other markers of the hypoxic response (e.g., *VEGFA*), and global gene analysis demonstrated that *PROL1* overexpression in PrCa cells results in modulated expression of genes involved in the hypoxic response.
- Once tumors reach about 2 mm^3 they generate a hypoxic barrier, preventing further growth; as a master regulator of the hypoxic response, overexpression of opiorphin regulates genes involved in vascularization and regulation of blood supply to the tumor, allowing tumors to overcome the hypoxic barrier.
- Future experiments planned include the development of PrCa cell lines in which *PROL1* is silenced, which will be used to support the present studies identifying pathways modulated through *PROL1* expression and confirm its involvement in tumor growth in animal models following xenograft.
- Although changes in ontological groups of genes with differential expression at the mRNA level have a robust correlation with ontological groups of proteins with differential expression, changes in mRNA expression levels of a specific gene identified by global gene analysis often correlate poorly with changes in the level of protein expressed from that gene. In future studies, particularly where global gene expression analysis is used to identify potential therapeutic targets for PrCa that are downstream of *PROL1*, confirmation of the effect of *PROL1* expression on the protein levels resulting from specific genes will be important.
- Overall, our data suggest a role for opiorphins in PrCa that involves the activation of pathways that allow growing tumors to overcome the hypoxic barrier and the development of androgen insensitivity/castration resistance.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/fon-2020-1299

Ethical conduct of research

All animal studies were conducted in accordance with the Animal (Scientific Procedures) Act 1986 and approved by the Institutional Animal Care and Use Committee (IACUC) of Albert Einstein College of Medicine.

Availability of data & materials

Raw sequencing datasets have been deposited in the Sequence Read Archive of NCBI (http://www.ncbi.nlm.nih.gov/sra) with accession number GSE156223.

Open access

This work is licensed under the Attribution-NonCommercial-NoDerivatives 4.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-nd/4.0/

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* 68(6), 394–424 (2018).

2. Wang G, Zhao D, Spring DJ, Depinho RA. Genetics and biology of prostate cancer. *Genes Dev.* 32(17–18), 1105–1140 (2018).

3. Rawla P. Epidemiology of prostate cancer. *World J. Oncol.* 10(2), 63–89 (2019).

4. Pihur V, Datta S, Datta S. Finding common genes in multiple cancer types through meta-analysis of microarray experiments: a rank aggregation approach. *Genomics* 92(6), 400–403 (2008).

5. Lang Z, Wu Y, Pan X, Qu G, Zhang T. Study of differential gene expression between invasive multifocal/multicentric and unifocal breast cancer. *J. BUON* 23(1), 134–142 (2018).

6. Lv X, He M, Zhao Y *et al.* Identification of potential key genes and pathways predicting pathogenesis and prognosis for triple-negative breast cancer. *Cancer Cell Int.* 19, 172 (2019).

7. Thierauf J, Veit JA, Grunow J *et al.* Expression of submaxillary gland androgen-regulated protein 3A (SMR3A) in adenoid cystic carcinoma of the head and neck. *Anticancer Res.* 36(2), 611–615 (2016).

8. Grunow J, Rong C, Hischmann J *et al.* Regulation of submaxillary gland androgen-regulated protein 3A via estrogen receptor 2 in radioresistant head and neck squamous cell carcinoma cells. *J. Exp. Clin. Cancer Res.* 36(1), 25 (2017).

9. Koffler J, Holzinger D, Sanhueza GA *et al.* Submaxillary gland androgen-regulated protein 3A expression is an unfavorable risk factor for the survival of oropharyngeal squamous cell carcinoma patients after surgery. *Eur. Arch. Otorhinolaryngol.* 270(4), 1493–1500 (2013).

10. Wu Q, Cao R, Chen J, Xie X. Screening and identification of biomarkers associated with clinicopathological parameters and prognosis in oral squamous cell carcinoma. *Exp. Ther. Med.* 18(5), 3579–3587 (2019).

11. Mukherjee A, Wang L, Schoenberg M, Davies K. Upregulation of opiorphin is associated with prostate cancer; a potential novel therapeutic target. *Urology* 197(4), e1167–e1168 (2017).

12. Mizerska-Dudka M, Kandefer-Szerszeń M. Opioids, neutral endopeptidase, its inhibitors and cancer: is there a relationship among them? *Arch. Immunol. Ther. Exp. (Warsz.)* 63(3), 197–205 (2015).

13. Fu S, Tar MT, Melman A, Davies KP. Opiorphin is a master regulator of the hypoxic response in corporal smooth muscle cells. *FASEB J.* 28(8), 3633–3644 (2014).

14. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 407(6801), 249–257 (2000).

15. Fox WD, Higgins B, Maiese KM *et al.* Antibody to vascular endothelial growth factor slows growth of an androgen-independent xenograft model of prostate cancer. *Clin. Cancer Res.* 8(10), 3226–3231 (2002).

16. Chung LW, Baseman A, Assikis V, Zhau HE. Molecular insights into prostate cancer progression: the missing link of tumor microenvironment. *J. Urol.* 173(1), 10–20 (2005).

17. Rudolfsson SH, Bergh A. Hypoxia drives prostate tumour progression and impairs the effectiveness of therapy, but can also promote cell death and serve as a therapeutic target. *Expert Opin. Ther. Targets* 13(2), 219–225 (2009).

18. Barrett T, Wilhite SE, Ledoux P *et al.* NCBI GEO: archive for functional genomics data sets – update. *Nucleic Acids Res.* 41(Database issue), D991–995 (2013).

19. Arredouani MS, Lu B, Bhasin M *et al.* Identification of the transcription factor single-minded homologue 2 as a potential biomarker and immunotherapy target in prostate cancer. *Clin. Cancer Res.* 15(18), 5794–5802 (2009).

20. Tamura K, Furihata M, Satake H *et al.* SHISA2 enhances the aggressive phenotype in prostate cancer through the regulation of WNT5A expression. *Oncol. Lett.* 14(6), 6650–6658 (2017).

21. Satake H, Tamura K, Furihata M *et al.* The ubiquitin-like molecule interferon-stimulated gene 15 is overexpressed in human prostate cancer. *Oncol. Rep.* 23(1), 11–16 (2010).

22. Planche A, Bacac M, Provero P *et al.* Identification of prognostic molecular features in the reactive stroma of human breast and prostate cancer. *PLoS ONE* 6(5), e18640 (2011).

23. Chandran UR, Ma C, Dhir R *et al.* Gene expression profiles of prostate cancer reveal involvement of multiple molecular pathways in the metastatic process. *BMC Cancer* 7, 64 (2007).

24. Yu YP, Landsittel D, Jing L *et al.* Gene expression alterations in prostate cancer predicting tumor aggression and preceding development of malignancy. *J. Clin. Oncol.* 22(14), 2790–2799 (2004).

25. Varambally S, Yu J, Laxman B *et al.* Integrative genomic and proteomic analysis of prostate cancer reveals signatures of metastatic progression. *Cancer Cell* 8(5), 393–406 (2005).

26. Sun Y, Goodison S. Optimizing molecular signatures for predicting prostate cancer recurrence. *Prostate* 69(10), 1119–1127 (2009).

27. Fu S, Davies KP. Opiorphin-dependent upregulation of CD73 (a key enzyme in the adenosine signaling pathway) in corporal smooth muscle cells exposed to hypoxic conditions and in corporal tissue in pre-priapic sickle cell mice. *Int. J. Impot. Res.* 27(4), 140–145 (2015).

28. Li Y, Rizvi SM, Ranson M, Allen BJ. 213Bi-PAI2 conjugate selectively induces apoptosis in PC3 metastatic prostate cancer cell line and shows anti-cancer activity in a xenograft animal model. *Br. J. Cancer* 86(7), 1197–1203 (2002).

29. Zheng X, Cui XX, Khor TO *et al.* Inhibitory effect of a γ-tocopherol-rich mixture of tocopherols on the formation and growth of LNCaP prostate tumors in immunodeficient mice. *Cancers (Basel)* 3(4), 3762–3772 (2011).

30. Dobin A, Davis CA, Schlesinger F *et al.* STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29(1), 15–21 (2013).

31. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30(7), 923–930 (2014).

32. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15(12), 550 (2014).

33. Huang Da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* 4(1), 44–57 (2009).

34. Huang Da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* 37(1), 1–13 (2009).

35. Ashburner M, Ball CA, Blake JA *et al.* Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat. Genet.* 25(1), 25–29 (2000).

36. The Gene Ontology Consortium. The Gene Ontology Resource: 20 years and still GOing strong. *Nucleic Acids Res.* 47(D1), D330–D338 (2019).

37. Kanehisa M, Furumichi M, Tanabe M, Sato Y, Morishima K. KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res.* 45(D1), D353–D361 (2017).

38. Kanehisa M, Sato Y, Furumichi M, Morishima K, Tanabe M. New approach for understanding genome variations in KEGG. *Nucleic Acids Res.* 47(D1), D590–D595 (2019).

39. Yamamoto S, Teng W, Kakiuchi T, Tsukada H. Disturbance of cerebral blood flow autoregulation in hypertension is attributable to ischaemia following subarachnoid haemorrhage in rats: a PET study. *Acta Neurochir (Wien)* 141(11), 1213–1219 (1999).

40. Horoszewicz JS, Leong SS, Kawinski E *et al.* LNCaP model of human prostatic carcinoma. *Cancer Res.* 43(4), 1809–1818 (1983).

41. Song C, Chen H. Predictive significance of *TMRPSS2-ERG* fusion in prostate cancer: a meta-analysis. *Cancer Cell Int.* 18, 177 (2018).

42. Carm KT, Hoff AM, Bakken AC *et al.* Interfocal heterogeneity challenges the clinical usefulness of molecular classification of primary prostate cancer. *Sci. Rep.* 9(1), 13579 (2019).

43. Tomlins SA, Mehra R, Rhodes DR *et al.* Integrative molecular concept modeling of prostate cancer progression. *Nat. Genet.* 39(1), 41–51 (2007).

44. Boucher Y, Braud A, Dufour E *et al.* Opiorphin levels in fluids of burning mouth syndrome patients: a case–control study. *Clin. Oral Investig.* 21(7), 2157–2164 (2017).

45. Ozdogan MS, Gungormus M, Ince Yusufoglu S, Ertem SY, Sonmez C, Orhan M. Salivary opiorphin in dental pain: a potential biomarker for dental disease. *Arch. Oral Biol.* 99, 15–21 (2019).

46. Bosler JS, Davies KP, Neal-Perry GS. Peptides in seminal fluid and their role in infertility: a potential role for opiorphin inhibition of neutral endopeptidase activity as a clinically relevant modulator of sperm motility: a review. *Reprod. Sci.* 21(11), 1334–1340 (2014).

47. Calenda G, Tong Y, Kanika ND *et al.* Reversal of diabetic vasculopathy in a rat model of Type 1 diabetes by opiorphin-related peptides. *Am. J. Physiol. Heart Circ. Physiol.* 301(4), H1353–H1359 (2011).

48. Tong Y, Tar M, Melman A, Davies K. The opiorphin gene (*ProL1*) and its homologues function in erectile physiology. *BJU Int.* 102(6), 736–740 (2008).

49. Davies KP, Tar M, Rougeot C, Melman A. Sialorphin (the mature peptide product of Vcsa1) relaxes corporal smooth muscle tissue and increases erectile function in the ageing rat. *BJU Int.* 99(2), 431–435 (2007).

50. Bonkhoff H. Estrogen receptor signaling in prostate cancer: implications for carcinogenesis and tumor progression. *Prostate* 78(1), 2–10 (2018).

51. Bonkhoff H, Fixemer T, Hunsicker I, Remberger K. Progesterone receptor expression in human prostate cancer: correlation with tumor progression. *Prostate* 48(4), 285–291 (2001).

52. Carver BS, Chapinski C, Wongvipat J *et al.* Reciprocal feedback regulation of PI3K and androgen receptor signaling in PTEN-deficient prostate cancer. *Cancer Cell* 19(5), 575–586 (2011).

53. Edlind MP, Hsieh AC. PI3K-AKT-mTOR signaling in prostate cancer progression and androgen deprivation therapy resistance. *Asian J. Androl.* 16(3), 378–386 (2014).

54. Shankavaram UT, Reinhold WC, Nishizuka S *et al.* Transcript and protein expression profiles of the NCI-60 cancer cell panel: an integromic microarray study. *Mol. Cancer Ther.* 6(3), 820–832 (2007).

55. Koussounadis A, Langdon SP, Um IH, Harrison DJ, Smith VA. Relationship between differentially expressed mRNA and mRNA-protein correlations in a xenograft model system. *Sci. Rep.* 5, 10775 (2015).