Role of opiorphin genes in prostate cancer growth and progression

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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.

First draft submitted: 23 December 2020; Accepted for publication: 4 February 2021; Published online: 17 February 2021

Keywords: androgen sensitivity • hypoxia • opiorphin • PROL1 • prostate cancer • SMR3A • SMR3B • xenograft

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].
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 O2] enriched by 5% CO2 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 × width × height × 0.52. In all experimental groups, when tumors reached a diameter >1 cm the mice were euthanized through inhalation of CO₂. 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. #PCRPH18637A; PROL1, cat. #PPH10360A; and VEGFA, cat. #PPH00251C). Quantitative RT-PCR was performed in triplicate for each sample. Data were analyzed using the ΔΔCt 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
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**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.

* * < 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 x 10^{-10} |
| Blood vessel development                                | 0001568           | GOC      | 486                                             | 74                        | 2.27           | 1.53 x 10^{-9}  |
| Blood vessel morphogenesis                              | 0048514           | GOC      | 404                                             | 68                        | 2.51           | 1.37 x 10^{-10} |
| Blood circulation                                      | 0008015           | GOC      | 386                                             | 63                        | 2.44           | 2.55 x 10^{-9}  |
| Circulatory system process                              | 0003013           | GOC      | 394                                             | 77                        | 2.18           | 3.9 x 10^{-6}   |
| Angiogenesis                                           | 0001525           | DAVID    | 223                                             | 38                        | 2.47           | 5.46 x 10^{-11} |
| Regulation of blood vessel diameter                     | 0097746           | GOC      | 130                                             | 31                        | 2.66           | 1.25 x 10^{-5}  |
| Regulation of blood vessel size                         | 0050880           | GOC      | 137                                             | 25                        | 2.73           | 2.71 x 10^{-5}  |
| Positive regulation of animal organ morphogenesis       | 0110110           | GOC      | 86                                              | 23                        | 2.99           | 7.79 x 10^{-5}  |
| Vascularogenesis                                        | 0001570           | DAVID    | 56                                              | 12                        | 3.10           | 1.35 x 10^{-3}  |
| Positive regulation of PI3K signaling                   | 0014068           | GOC      | 85                                              | 15                        | 2.64           | 1.4 x 10^{-3}   |
| Regulation of blood pressure                            | 0045776           | DAVID    | 30                                              | 13                        | 2.90           | 1.47 x 10^{-3}  |
| Positive regulation of steroid metabolic process        | 0045940           | GOC      | 28                                              | 8                         | 4.27           | 1.47 x 10^{-3}  |
| Response to hypoxia                                     | 0001666           | DAVID    | 172                                             | 22                        | 1.85           | 7.7 x 10^{-3}   |
| PI3K-Akt signaling pathway                              | 04151             | KEGG     | 345                                             | 36                        | 1.50           | 1.4 x 10^{-2}   |
| VEGF signaling pathway                                  | 04370             | KEGG     | 61                                              | 10                        | 2.35           | 2.4 x 10^{-2}   |
| MAPK signaling pathway                                  | 04010             | KEGG     | 253                                             | 26                        | 1.47           | 4.67 x 10^{-2}  |
| Vasodilation                                           | 0042311           | DAVID    | 14                                              | 4                         | 4.13           | 6.74 x 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 x 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 x 10^{-3}; 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 x 10^{-197}) and a decrease in expression of the estrogen receptor (ESR1, 2.7-fold reduced; p = 3.38 x 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 x 10^{-5}), whereas PGR expression was not changed in PC3 cells.
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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:5879) | 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
Role of opiorphin genes in prostate cancer growth and progression

Research Article

Activates genes involved in androgen insensitivity

Activates genes involved in the hypoxic response

Opiorphin/ProL1 over-expression

Hypoxia

Growth

PrCa <2 mm

PrCa >2 mm

Progression

Castration resistant PrCa

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.

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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; \( p = 3.16 \times 10^{-197} \); 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 mm³ 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

Author contributions

A Mukherjee and A Park conducted experiments, analyzed and interpreted data and contributed to writing the manuscript. L Wang conducted experiments and analyzed data. K Davies conceived of the study, analyzed and interpreted data, and was the major contributor to writing the manuscript.

Acknowledgments

The authors wish to thank M Schoenberg for his initial involvement in discussions about the study conception.

Funding & competing interests disclosure

Funding for these studies were provided by NIH R01 DK107807 and DK109314 (PI: K Davies). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.
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.

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