

Investigating the role of opiorphin genes in prostate cancer and the possible genetic mechanisms by which they modulate tumor growth and androgen-sensitivity

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Research article

Keywords: Prostate Cancer, opiorphin, ProL1, hSMR3A, hSMR3B, xenograft, hypoxia, androgen-sensitivity.

Posted Date: October 9th, 2019

DOI: <https://doi.org/10.21203/rs.2.15829/v1>

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Abstract

Background The opiorphin family of genes (represented in humans by *ProL1*, *hSMR3A* and *hSMR3B*) encode peptides which act as potent neutral endopeptidase (NEP) inhibitors. Because modulated NEP activity is associated with cancer development, several years ago a review postulated that dysregulated opiorphin expression may be involved in oncogenesis. This has recently been supported by reports associating changes in expression of opiorphin-encoding genes with breast and oropharyngeal cancer. Based on these reports, we initiated the studies described here to determine if opiorphin-encoding genes play a role in prostate cancer (PrCa). **Methods** We screened publicly available data bases for evidence of an association between PrCa and opiorphin gene expression and then confirmed these findings using PrCa tissue arrays. Cell-lines representing early (LNCaP) and late stage (PC3) PrCa were engineered to overexpress *ProL1* and the growth of xenografted tumors from these lines compared to parent cell lines in male and female nude mice. The changes in global gene expression caused by overexpression of *ProL1* in these cell lines was determined by RNA-Seq to identify possible genetic mechanisms related to growth and progression of PrCa. **Results** Two datasets available on GEO showed an association between upregulation of opiorphin-encoding genes and PrCa, which was confirmed using tissue arrays. Xenografted tumors derived from PC3 engineered to overexpress *ProL1* had an initial growth advantage over parent cell lines whilst tumors derived from LNCaP cells overexpressing *ProL1* had impaired growth in female mice. Global gene expression analysis suggested that overexpression of *ProL1* causes modulated expression of genes involved in signaling, angiogenesis and steroid response pathways. These pathways might be involved in overcoming the hypoxic barrier of the growing tumor and the development of androgen insensitivity. **Conclusions** This is the first report to show an association between upregulation of opiorphin-encoding genes and PrCa and that their overexpression in PrCa cell-lines modulates tumor growth in mouse xenograft models. Overexpression of opiorphin-encoding genes modulates genetic pathways that previous reports have associated with PrCa growth, suggesting possible genetic mechanisms for tumor development.

Background

Prostate cancer (PrCa) is the most prevalent of all cancers in men in the United States and the second most common cause of all cancer deaths. Despite high long-term survival rates with localized PrCa, metastatic PrCa remains largely incurable, even after intensive multimodal therapy (1). 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. Based on recent reports that dysregulated expression of opiorphin-encoding genes are associated with some cancers we initiated the present studies to determine if they might also play a role in PrCa.

In humans, the opiorphin family genes are represented by *ProL1*, *hSMR3A* and *hSMR3B*, which are highly homologous genes that have neighboring genomic locations on chromosome 4 (cytogenetic location: 4q13.3) and have coregulated expression. Of the family of opiorphin-encoding genes, *ProL1* has been the most studied in the determination of their molecular and biochemical functions. The genes encode pre-

proteins that are post-translationally processed to generate a bioactive pentapeptide, opiorphin, which acts as a potent endogenous neutral endopeptidase (NEP) inhibitor (2). Because changes in NEP activity are associated with cancer development, a review article published in 2015 suggested that because opiorphin is an NEP inhibitor, it may play a role in cancer. This has been supported by several recent reports associating dysregulated expression opiorphin-encoding genes with cancer. Upregulated *ProL 1* expression was observed in invasive multifocal/multicentric compared to unifocal breast cancer (3) and increased *hSMR3A* expression was associated with unfavorable survival outcomes following surgery for oropharyngeal squamous cell carcinoma (4, 5). With the exception of a brief conference report from our laboratory (6), there are no published reports associating dysregulated expression of opiorphin-encoding genes with PrCa.

In addition to their activity as NEP inhibitors, members of opiorphin-encoding gene family have also been shown to play a role in the hypoxic response. For example, the rat homologue of *ProL 1*, *vcsa1*, is upregulated in corporal smooth muscle cells in response to hypoxia, where it acts as a “master regulator” of the hypoxic response. Since the metabolic demands of growing tumors creates a growth limiting hypoxic microenvironment(7-9) this might be expected to result in upregulation of *ProL 1* which may then play a role in PrCa by overcoming the limits in tumor size imposed by the hypoxic barrier. Given reports suggesting hypoxia may be a driver of malignant progression (10), *ProL 1* might also be a factor in development of castration-resistant/androgen-insensitive PrCa.

In the present report we demonstrate that upregulated expression of opiorphin-encoding genes are associated with human PrCa and confirmed that *ProL 1* overexpression modulates the growth of mouse xenografted PrCa tumors. Global gene expression analysis on the effects of overexpressing *ProL 1* in PrCa cell-lines suggested possible genetic mechanisms by which *ProL 1* modulates tumor growth and androgen-sensitivity consistent with its known biochemical and functional activities as an NEP inhibitor and master regulator of the hypoxic response.

Methods

Identification of upregulated *ProL 1* 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 (11) 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 6 of these data sets allowed both gene expression comparison between benign and cancerous prostate cancer tissue and included the opiorphin-encoding genes *ProL 1*, *hSMR3A* or *hSMR3B*. These 6 datasets were analyzed using the NCBI online data analysis tool (GEO2R) to compare expression of *ProL 1* (referred to as *OPRN* in this data set), *hSMR3A* and *hSMR3B* between cancerous and non-cancerous prostate tissue.

In order to confirm the upregulation of *ProL 1* in PrCa, a cDNA array from normal and cancerous prostate tissue was purchased from OriGene (HPRT103; TissueScan™ cDNA Arrays; Rockville, MD, USA). This

array has 48 samples covering 9-normal and 18-Stage II, 19-III and 2-IV PrCa. Quantitative RT-PCR analysis of the *ProL1* transcripts was performed as described below.

Cell-lines

PC3 (NCI-PBCF-CRL1435 (PC-3)/ATCC®CRL-1435™) and LNCaP clone FGC (NCI-PBCF-CRL1740 (LNCaP Clone FGC)/ATCC® CRL-1740™; hereafter termed LNCaP) prostate cancer cell-lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD), and maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Carlsbad, CA) with 10% Fetal Calf Serum (Thermo Fisher Scientific, Waltham, MA), supplemented with 100 U/ml penicillin G and 100 ng/ml streptomycin (Invitrogen). All cell-lines were passaged at 2–3 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 Mycoplasma testing 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 Technologies, Rockville, MD) according to manufacturers' protocol. The transduced cell-lines were designated PC3-ProL1+ and LNCaP-ProL1+. RNA seq data indicated *ProL1* was overexpressed 5 995-fold in PC3-ProL1+ (see Supplemental Table 1) and 28 560-fold in LNCaP-ProL1+ (Supplemental Table 2) compared to their respective parent cell-lines.

Mice Xenograft studies

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. Athymic nude (homozygous, nu/nu aged 8-12 weeks) male and female mice (Charles River Laboratories, Wilmigton, MA) were housed under standard pathogen-free conditions at a maximum of four mice per cage. Tumours 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, Teterboro, NJ) and HBSS using a 27-gauge needle. For PC3 and PC3-ProL1+ cell-lines, 1.5×10^6 cells, and for LNCaP and LNCaP-ProL1+ cell-lines, 2.5×10^6 cells were injected. The numbers of animals in each group are described in the Figure legends. Tumors were measured twice a week using Vernier Calipers and volume (mm^3) calculated by length \times width \times height \times 0.52. When tumors reached a diameter > 1 cm mice were euthanized through inhalation of CO_2 to effect.

RNA Isolation

To prepare total RNA from *in vitro* experimental samples to be used for quantitative-RT-PCR or RNA-seq analysis was isolated with the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) following the manufacturers' 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). Real-time PCR was performed by the Applied Biosystems 7300 Real Time PCR System (Thermo Fisher) using the standard cycling mode recommended by the PowerUp SYBR protocol (50 °C for 2 minutes, 95 °C for 2 minutes, and then 40 cycles of 95 °C for 15 seconds along with 60 °C for 1 minute). Commercially available 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 was analyzed using the $\Delta\Delta C_t$ method, with gene expression normalized to 60S ribosomal protein L19 (RPL19) and expressed as the average fold-change relative to controls.

RNA-sequencing (RNA-seq)

Prior to RNA-seq, RNA samples were treated with RNase-free DNase I and then quantified using RiboGreen (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity number (RIN) was assessed with an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) to ensure each sample had a RIN ≥ 8 . RNA libraries were prepared using the Illumina TruSeq Stranded Total RNA kit, with Ribosomal depletion (Illumina, San Diego, CA, USA). Each library was sequenced using the Illumina HiSeq2500 platform and generated 125 nt paired-end (PE) reads at either 30 million or 60 million reads per sample. RNA-seq analysis of gene expression for each cell-line was performed in triplicate (each sample prepared separately). [Raw sequencing data sets will be deposited in the Sequence Read Archive of NCBI (<http://www.ncbi.nlm.nih.gov/sra>) upon acceptance of this publication with accession number included here]

Bioinformatics analysis

The reads were aligned with STAR (version 2.4.0c) (12) and genes annotated in Gencode v18 were quantified with featureCounts (v1.4.3-p1)(13). Normalization and differential expression analyses were performed with the Bioconductor package DESeq2 (14). Differentially expressed gene (DEG's) were assessed using a two-sided t-test and fold-change on log-transformed expression values. All samples were median centered. Cut-off criteria for DEG was >1 log₂-fold or <-1 log₂-fold change in gene expression with a p-value <0.01 .

Gene ontology (GO) annotation analysis of differentially expressed genes (DEG) was performed using online analysis tools available from the database for annotation, visualization and integrated discovery (DAVID, vers 6.8, *Homo sapien* GOTERM_GO_Direct database (15, 16)), the Gene Ontology Consortium (GOC, *Homo sapien*, biological function database (17, 18)) and the Kyoto Encyclopedia of Genes and Genomes database (KEGG, (19-21)).

Statistical analyses

Statistical analyses were performed using either Microsoft Excel (Microsoft, Seattle, WA) or Prism 8.2. (GraphPad Software, Inc., La Jolla, CA). To determine statistical significance of two group comparisons, unpaired, two-tailed t-tests were performed, and results reported in Tables and Figures. Error bars represent standard error of mean or standard deviation (as described in figure legends).

Results

ProL1 is overexpressed in prostate cancer

Of the 6 data sets available data sets on the GEO database that were suitable for our analysis, 2 demonstrated a significant association between PrCa and opiorphin-encoding genes (Table 1).

DataSet GSE55945 was generated from a study aimed at identifying novel biomarkers and immunotherapy targets for PrCa (22) and contained 8 normal (benign) prostate tissue samples and 13 prostate cancer tissues (sub-divided into ERG fusion positive (N=6) and ERG fusion negative (N=7) PrCa). As shown in Table 1, comparing the 8 normal prostate tissue samples and 13 prostate cancer tissues and using a p-value of <0.05 to define significance, PrCa was associated with overexpression of both *ProL1* and *hSMR3A*. When the group of prostate cancer tissues was sub-divided into ERG fusion positive and ERG fusion negative PrCa, there was a greater significance and fold-increase of *ProL1* expression in ERG fusion positive PrCa tissue compared to non-cancerous prostate tissue (2.25-fold, p-value 9.7×10^{-4}) than when the same analysis was performed with ERG fusion negative PrCa tissue (2.16-fold, p-value 1.3×10^{-2}).

Dataset GSE3325 (23) was generated in a study to identify the genetic signatures of metastatic progression and contained 6 samples from normal (benign) prostate tissue samples and 13 PrCa tissues (sub-divided into localized (N=6) and metastatic (N=7) PrCa). As shown in Table 1, PrCa was associated with overexpression of *hSMR3B*, with a trend for greater overexpression with progression from localized to metastatic disease, from 5-fold (p-value 0.0035) to 9.32-fold, (p-value 0.00038), respectively.

Given that *ProL1* has been the most intensively studied member of the opiorphin gene family, we focused on this gene as representative of the opiorphin family. Figure 1A shows the relative expression levels of *ProL1* determined in a tissue cDNA array containing human non-cancerous and cancerous (at different stages) prostate tissue. This data, shown in Figure 1B, is consistent with the microarray analysis and demonstrates that *ProL1* is overexpressed in PrCa tissue, with a trend for higher levels of expression as the cancer stage increased.

Xenografted tumors derived from PC3 cell show time-dependent increase in the expression of *ProL1* and *VEGFA*

Expression levels of two markers of the hypoxic response, *ProL1* and *VEGFA*, were compared between the PC3 cell-line (isolated from castration-resistant/androgen-insensitive PrCa) prior to injection and in tumors derived from this cell-line at 2- and 4-weeks post-injection into nude male mice. As shown in Figure 2, the PC3-derived tumors showed a progressive and significant increase in both *ProL1* and *VEGFA* expression, such that the 4-week old tumors had approximately a 20-fold increase in *ProL1* expression, and approximately a 3.2-fold increase in *VEGFA* expression, compared to the levels of these genes in the parent PC3 cell-line.

Xenografted tumors derived from a PC3 cell-line engineered to over-express *ProL1* (PC3-ProL1+) initially exhibit accelerated growth compared to its parent cell-line in male nude mice

In order to determine if 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 can be seen in Figure 3, in male nude mice at early stages of tumor detection (at 10- and 13- days post-implantation), there was a significantly greater rate of tumor growth in mice implanted with PC3-ProL1+ compared to PC3. However, at later time-points (from 17-days onwards) in the male mice, and at any time-point in female nude mice, there was no significant size difference in the tumors derived from PC3-ProL1+ or parent PC3 cells (data not shown).

Overexpression of *ProL1* in PC3 cells modulates expression of genes associated with the hypoxic response

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 PC3 and PC3-*ProL1*+ cell-lines. This analysis identified 1 698 DEG's between the two cell lines (Supplemental Table 1) with *ProL1* being the most DEG (5 995-fold, p-value 2.82×10^{-26}). To identify biological functions that may be regulated through overexpression of *ProL1* in PC3 cells, the list of DEG's (minus *ProL1*) was submitted to the DAVID, GOC and KEGG databases, which identified 1 250, 1 366 and 480 unique genes within each database, respectively, which were then used for ontological analysis. The most significant, broadly defined, functional ontologic groups with over-representation of DEG's were involved in morphogenesis and signal transduction (Supplemental Tables 3 and 4). For example, there was over-representation of DEG's in the ontological groups "anatomical structure development/morphogenesis" (GOC: GO:0048856 and GO:0009653, p-value 2.05×10^{-22} and 2.21×10^{-20} , respectively, and more specifically "prostate gland morphogenesis" GOC: GO:0060512, p-value 6.39×10^{-4}), "extracellular matrix organization" (DAVID: GO:0030198, 1.54×10^{-9}), "signaling" (GOC: GO:0023052, p-value 3.35×10^{-14}) and "signal transduction" (DAVID: GO:0007165, p-value 4.29×10^{-5}). In Table 2 we present the more defined ontologic groups where there was significant over-representation of DEG's. Both DAVID and GOC analysis identified DEG's in ontological groups involved in vascularization (such as genes involved in angiogenesis, blood vessel development, organ growth and morphogenesis) and also groups that function in hypoxia and direct modulation of blood flow (such as genes involved in over-coming hypoxia, vasodilation, blood vessel diameter and regulation of smooth muscle contraction). Analysis of the list of DEG's using the KEGG database identified the ontologic group "pathways in cancer" as having the greatest number of DEG's (41 genes, p-value 8.96×10^{-3}) (Supplemental Table 5). DEG's 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 over-express *ProL1* (LNCaP-*ProL1*+) have gender-specific modulated growth compared to its parent cell-line.

The PC3 cell-line used in the preceding experiments was isolated from advanced stage, castration-resistant/androgen-independent PrCa. In order to determine if upregulated *ProL1* expression might also play a role in earlier stages of PrCa growth, we compared the growth of xenografted tumors in nude male and female mice derived from LNCaP cells (isolated from androgen-sensitive PrCa) engineered to over-express *ProL1* (LNCaP-*ProL1*+) with tumors derived from its parent cell-line (LNCaP). Similar to previous studies (24), xenografted tumors derived from LNCaP are more rapidly established in male compared to female nude mice (the LNCaP tumor size at 4-weeks post-implantation is significantly greater in male compared to female mice, Figure 4A). We also observed (as previously reported (24)) that once LNCaP tumors are established their growth rate is not significantly different between male and female mice

(Figure 4A). However, overexpression of *ProL1* in LNCaP cells significantly modulated these gender specific growth characteristics. LNCaP-*ProL1*+ derived tumors have a significantly impaired growth rate in female compared to male mice (Figure 4B). Although there was no significant difference in growth rate of tumors derived from LNCaP-*ProL1*+ or LNCaP in male mice (Supplemental Figure 1A), there was significantly impaired growth of tumors derived from LNCaP-*ProL1*+ compared to tumors derived from LNCaP in female mice (Supplemental Figure 1B). Therefore, the observed gender specific differences in xenografted tumor growth caused by overexpression of *ProL1* is primarily mediated by impairing LNCaP tumor growth in female mice, rather than conferring a growth advantage in male mice. This contrasts to our observations in PC3 cells, where overexpression of *ProL1* conferred (at least during the early time-points following transplant) a growth advantage to xenografted tumors in male mice, but had no effect on growth of tumors in female mice (Figure 3).

Overexpression of *ProL1* in LNCaP cells modulates expression of genes associated with steroid metabolism and 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 the LNCaP-*ProL1*+ and its parent cell-line, LNCaP identifying 1 110 DEG's (Supplemental Table 2). Of these 1 100 DEG's, 209 were in common with PC3 when *ProL1* was overexpressed (Supplemental Table 2). *ProL1* was the most DEG (28 560-fold, p-value 24.36×10^{-36}). To identify biological functions that may be regulated through overexpression of *ProL1* in LNCaP cells, the list of DEG's (minus *ProL1*) was submitted to the DAVID, GOC and KEGG databases, identifying 665, 812 and 287 unique genes within each database, respectively, which were then used for ontological analysis. Although the analysis identified fewer ontologic groups with significant overrepresentation of DEG's caused by *ProL1* overexpression in LNCaP compared to PC3 cells, many these ontologic groups were the same, or served similar functions (Supplemental Tables 6-8). In common with PC3, *ProL1* overexpression regulated gene expression in ontological groups involved in morphogenesis and signal transduction (Supplemental Tables 6 and 7). For example, there was overrepresentation of genes in the ontological groups "anatomical structure development/morphogenesis" (GOC: GO:0048856/GO:0009653, p-value 2.76×10^{-8} and 2.09×10^{-8} , respectively), "cell-cell signaling" (GOC: GO:0007267, p-value 1.46×10^{-10} and DAVID: GO 0007267, p-value 6.00×10^{-3}) and "signal transduction" (GO:0007165, p-value 2.66×10^{-4}). DEG's were also overrepresented in more defined ontological groups potentially 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 DEG's in biochemical pathways using the KEGG database, also identified similar effects caused by overexpression of *ProL1* in PC3 and LNCaP cells (Supplemental Table 8). For example, overexpressing *ProL1* regulated genes that were significantly over-represented in the ontologic groups "pathways in cancer" (p-value 5.97×10^{-2}), 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-value 3.16×10^{-197}) and a decrease in expression of the estrogen receptor (ESR1, 2.7-fold reduced, p-value 3.38×10^{-49}), in LNCaP neither of these genes were changed in expression (Supplemental Table 1 and 2). In contrast, overexpression of *ProL1* in LNCaP reduced expression of the progesterone receptor gene (PGR, 2.17-fold reduced, p-value 4.41×10^{-3}), whereas PGR expression was not changed in PC3 cells.

Discussion

This is the first report associating elevated expression levels of opiorphin-encoding genes with the development of human PrCa. Our data suggests that expression levels of the opiorphin-encoding genes correlate with PrCa progression. As shown in Table 1 there is a much greater significance of elevated *ProL1* expression in ERG-fusion positive PrCa (which is generally considered to represent a more advanced stage PrCa (25)) compared to ERG-fusion negative PrCa (Table 1) and a trend for higher levels of *ProL1* with cancer stage (Figure 1D). Also shown in Table 1 there was over-expression of the *ProL1* homologue, *hSMR3B*, in localized PrCa compared to non-cancerous prostate tissue (5-fold, p-value 0.0035), with greater over-expression in metastatic PrCa compared to non-cancerous prostate tissue (9.32-fold, p-value 0.00038). Although other DataSets did not show significant differences in expression of opiorphin encoding genes in cancerous compared to non-cancerous prostate tissue samples, this may be a result of differences in sample preparation. It has been shown that many PrCa DataSets have significant stromal contamination which has a tendency to mask epithelial gene expression across PrCa profiling studies (26).

We also demonstrate that there is significantly modulated growth of xenografted tumors derived that PrCa cell-lines engineered to overexpress *ProL1* compared to their parent cell-lines. In PC3 cells (isolated from castration-resistant/ androgen-insensitive PrCa) overexpression of *ProL1* increases the growth rate of xenografted tumors in nude male mice at early time-points post-transplantation (Figure 3), whereas in LNCaP cells (isolated from androgen-sensitive PrCa) overexpression of *ProL1* impairs the growth of xenografted tumors in nude female mice (Figure 4). As discussed in more detail below, comparing global gene expression in cell-lines overexpressing *ProL1* with their parent cell-lines suggests possible genetic mechanisms by which *ProL1* overexpression might modulate the growth rate of PrCa tumors.

In order to gain insight into the possible genetic mechanisms by which upregulated *ProL1* modulates PrCa growth, we compared global gene expression between PrCa cell-lines engineered to over-express *ProL1* and their parent cell-lines. Overexpression of *ProL1* resulted in relatively large numbers of genes being identified as significantly differentially expressed in both PC3 and LNCaP cell-lines (1 698 and 1 110, respectively). There was both a lower total number and average fold-change in DEG's when *ProL1* was overexpressed in LNCaP compared to PC3. For example, the average fold-change of the top 250 most upregulated DEG's in PC3 was 11.2-fold and in LNCaP, 3.49-fold (p-value $< 1 \times 10^{-5}$). The differences in numbers and fold-change caused by *ProL1* overexpression in the two cell-lines may arise because of a

greater intrinsic chromosome instability reported for PC3 relative to LNCaP, resulting in increased copy numbers of genes in PC3 (27). This would result in a proportionately greater change in the expression levels of genes regulated by *ProL1* in PC3 compared to LNCaP as well as increasing the number of the total number of significant DEG's.

Ontologic analysis of the genes regulated by *ProL1* in PC3 cells demonstrated that it primarily affects genetic pathways involved in morphogenesis and signalling (Supplemental Tables 3-5, and Table 2). Since *ProL1* encodes opiorphin, a potent endogenous NEP inhibitor, its overexpression would be expected to inhibit the break-down of signal peptides bound to their receptors, effectively increasing their half-lives. Therefore, the genes changed in expression within the ontologic groups related to signalling likely represent compensatory genetic pathways to dampen signalling pathways. In a previous study on rat corporal smooth muscle cell-lines, knock-down of *vcsa1* (a rat *ProL1* homologue, encoding an opiorphin homologue that functions as a potent NEP inhibitor) resulted in modulated expression of several genes in the G-protein coupled receptor (GPCR) family (28). This effect was proposed to be a compensatory mechanism to increased NEP activity, leading to shorter half-lives of signal peptides bound to their GPCR's.

The regulation of genes involved in morphogenesis by *ProL1* may represent a genetic mechanism for the initial growth advantage in male mice of xenografted tumors derived from PC3-*ProL1*+ compared to PC3. By priming genetic pathways involved in the reorganization of tissues that increase local blood supply, tumors may more rapidly overcome the hypoxic barrier in the microenvironment generated through the metabolic demands associated with PrCa growth. Within the broad functional groups related to morphology there was significant over-representation of *ProL1*-regulated genes in more specific ontologic groups involved in angiogenesis, blood vessel development and vascularization. In addition, *ProL1* overexpression also regulated genes in ontologic groups that might directly regulate blood flow to the tumor, such as vasodilation, blood vessel diameter and smooth muscle contraction. Evidence from previous studies has demonstrated that opiorphin-encoding genes act as "master regulators" of the hypoxic response in rat corporal smooth muscle cells (29, 30), regulating the expression of genes involved in angiogenesis and vascular smooth muscle tone. The data presented here would suggest that in PrCa cells, the expression of genes known to be involved in the hypoxic response are also regulated by *ProL1*.

ProL1 overexpression in PC3 cells had a significant effect on expression of genes in the PI3K-Akt signaling pathway (Table 2). The PI3K-Akt signaling pathway is considered one of the most commonly dysregulated pathways in PrCa. Approximately 40% of early stage, and 70–100% of advanced stage PrCa, exhibit dysregulation of this pathway, suggesting PI3K-Akt dysregulation is associated with PrCa progression (31-33). Furthermore, there is a direct connection between the PI3K-Akt pathway and androgen receptor (AR) signaling, which is also dysregulated with progression and development of castration resistant PrCa(31, 34). In PC3-*ProL1*+ cells we observed not only changes in the expression of genes in the PI3K-Akt pathway, but also a significant upregulation of the AR (7.46-fold, p-value 3.16×10^{-197} , Supplemental 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 MAPK pathway, which was identified as having significant over-representation of DEG's with *ProL1* overexpression.

Similar to PC3, when *ProL1* was overexpressed in LNCaP cells it also modulated expression of genes significantly over-represented in ontologic groups related to morphogenesis and signaling (Supplemental Tables 6-8 and Table 3). As discussed above, compared to PC3, overexpression of *ProL1* in LNCaP resulted in lower-fold and fewer total genes with changed expression in these ontological groups. Although expression of genes involved in morphogenesis might be expected to confer growth advantages to the developing tumor (as described above for PC3), no significant growth advantage was observed for xenografted tumors derived from LNCaP-*ProL1*+ compared to LNCaP cells in male mice. The differences in the growth of tumors derived from LNCaP compared to PC3 cell-lines overexpressing *ProL1* may result from the proportionately greater fold-change in gene expression in PC3 cells, such that in PC3 DEG's that impact tumor growth reach a threshold level for fold-change in expression that is not attained in LNCaP. Alternatively, given that *ProL1* overexpression results in significantly greater numbers of DEG's in PC3 compared to LNCaP, *ProL1* may regulate expression of specific genetic pathways related to tumor growth in PC3, but not LNCaP cell-lines.

ProL1 overexpression in both LNCaP and PC3 cells resulted in overrepresentation of DEG's involved in steroid metabolism, with the specific genes in these pathways having similar fold-changes and significance in the two cell lines (in contrast to the difference in fold-change and significance when the entire list of DEG's was compared, as discussed above, Supplemental Table 1 and 2). However, because overexpression of *ProL1* caused similar changes in genetic pathways of steroid metabolism in LNCaP and PC3 cell-lines, but only in the case of LNCaP did overexpression of *ProL1* impair tumor growth in female mice, the changes in steroid metabolism were considered unlikely to represent a genetic mechanism for the impaired growth rate. Overexpression of *ProL1* did however have significant and differential effects on the expression of androgen, estrogen and progesterone receptors in LNCaP and PC3 cells (Supplemental Tables 1 and 2). For example, overexpression of *ProL1* in LNCaP reduced expression of the progesterone receptor gene, but had no effect on its expression in PC3 cells. Activation of PGR is a positive modulator of cell division, and its detection correlates with PrCa progression, with 54% of castration-resistant cases having >20% PGR positive cells in tumors(35, 36). Reduced expression of *PGR* in LNCaP-*ProL1* cells would therefore be expected to have a negative effect on tumor growth in female mice.

Our global gene expression analysis on the effects of overexpressing *ProL1* in PrCa cell-lines suggest possible genetic mechanisms by which *ProL1* modulates tumor growth and androgen-sensitivity, consistent with its known biochemical and functional activities as an NEP inhibitor and master regulator of the hypoxic response. These mechanisms are summarized in Figure 5. The hypoxic barrier to growth developed when tumors reach about 2mm³ would be expected to upregulate *ProL1* expression. As shown in Figure 2 xenografted tumors derived from PC3 cell-line do indeed demonstrate progressive upregulation of *ProL1* expression. As a master regulator of the hypoxic response, overexpression of

ProL1 would then be expected to activate genes involved in the vascularization and regulation of blood supply to the tumor. Our global gene expression studies on the effect of *ProL1* overexpression in PrCa cell-lines confirm significant overrepresentation of genes in ontological groups with these functions (Table 2 and 3), which would allow tumors to overcome the hypoxic barrier and allow continued PrCa growth. In addition, overexpression of *ProL1* in response to hypoxia may drive malignant progression of PrCa(10, 37) since overexpression of *ProL1* was also shown to activate genes involved in steroid metabolism and androgen response which are pathways known to be associated with the progression of tumors to castration resistance/androgen insensitivity.

The studies reported here have focused on *ProL1*, which represents the best characterized of the opiorphin gene family. Since both *hSMR3A* and *hSMR3B* also encode the opiorphin pentapeptide, at least some of the effects of modulated opiorphin gene expression would be expected to be shared in common with *ProL1*. However, other bioactive peptides are known to be generated by processing of the protein product of *ProL1*, *hSMR3A* and *hSMR3B*, and which might also play roles in the genetic mechanisms by which they modulates tumor growth. As with the other studies associating opiorphin-encoding genes with other forms of cancer, our studies are limited to the transcriptional level, since at present there are no useful antibodies for determine expression of opiorphin or other proteins encoded by the genes.

Conclusions

In conclusion, this is the first report to provide evidence for a role of opiorphin-encoding genes in PrCa. We demonstrate significantly higher expression levels of *ProL1* in cancerous, compared to non-cancerous, prostate tissue and that overexpression of *ProL1* in PrCa cell-lines modulates tumor growth in mouse xenograft models. We have demonstrated that overexpression of *ProL1* modulates genetic pathways that previous reports have associated with PrCa growth and progression and therefore may represent possible genetic mechanisms for the effect of *ProL1* on tumor development. However, additional research will be needed to establish that the genetic pathways modulated by *ProL1* are actually responsible for changes in tumor growth, and that the DEG's caused by overexpression of *ProL1* in cultured PrCa cells are similar to those in xenografted-tumors.

Abbreviations

PrCa, Prostate Cancer; NEP, neutral endopeptidase; GEO, Gene Expression Omnibus; GO, gene ontology; DEG, differentially expressed genes; DAVID, database for annotation, visualization and integrated discovery; GOC, Gene Ontology Consortium; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Declarations

- **Ethics approval and consent to participate**
- 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.

-
- **Consent for publication**
- Not applicable.
-
- **Availability of data and materials**

· [Raw sequencing data sets will be deposited in the Sequence Read Archive of NCBI (<http://www.ncbi.nlm.nih.gov/sra>) upon acceptance of this publication with accession number included here].

· **Competing interests**

· The authors declare that they have no competing interests.

· **Funding**

· Funding for these studies were provided by NIH R01 DK107807 (PI: Kelvin P. Davies).

• **Authors' contributions**

- "AM conducted, analyzed and interpreted data in manuscript and contributed to writing the manuscript. AP conducted, analyzed and interpreted data in manuscript and contributed to writing the manuscript. LW AP conducted and analyzed data in manuscript. KPD conceived of the study, analyzed and interpreted data in manuscript and was the major contributor to writing the manuscript. All authors read and approved the final manuscript.

• **Acknowledgements**

- We thank Mark Schoenberg, MD, for his initial involvement in discussions in the study conception.

• **Authors' information (optional)**

- None.

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Tables

Table 1. The publicly available datasets GSE55945 and GSE3325 were analyzed using the GEO2R online tool to compare expression of opiorphin encoding genes (*ProL1*, *hSMR3A* and *hSMR3B*) between cancerous and non-cancerous prostate tissue samples.

Comparison	Gene	p-value	logFC	Fold Change
Dataset GSE55945				
All PrCa data compared to non-cancerous prostate tissue.	ProL1(OPRN)	0.0013	1.14	2.20
	hSMR3A	0.0098	0.52	1.43
ERG fusion positive PrCa data compared to non-cancerous prostate tissue.	ProL1(OPRN)	0.00097	1.17	2.25
	hSMR3A	0.026	0.38	1.30
ERG fusion negative PrCa data compared to non-cancerous prostate tissue.	ProL1(OPRN)	0.013	1.11	2.16
	SMR3A	0.012	0.64	1.56
Dataset GSE3325				
All PrCa data compared to non-cancerous prostate tissue.	hSMR3B	0.000083	2.74	6.68
Localized PrCa data compared to non-cancerous prostate tissue.	hSMR3B	0.0035	2.3	4.92
Metastatic PrCa data compared to non-cancerous prostate tissue.	hSMR3B	0.00039	3.22	9.32

Table 2: Ontologic groups containing overrepresented differentially expressed genes (DEG's) when *ProL1* is overexpressed in PC3 cells.

Biological process/ molecular pathway	GO/hsa identifier	Analysis	Ref List (GOC:20996) (DAVID:16792) (KEGG:6879)	<i>ProL1</i> - overexpression list	Fold- enrichment.	P-value
Angiogenesis	0001525	GOC	315	56	2.65	9.33x10 ⁻¹⁰
Blood vessel development	0001568	GOC	486	74	2.27	1.53x10 ⁻⁹
Blood vessel morphogenesis	0048514	GOC	404	68	2.51	1.37x10 ⁻¹⁰
Blood circulation	0008015	GOC	386	63	2.44	2.55x10 ⁻⁹
Circulatory system process	0003013	GOC	394	77	2.18	3.9x10 ⁻⁹
Angiogenesis	0001525	DAVID	223	38	2.47	5.46x10 ⁻¹¹
Regulation of blood vessel diameter	0097746	GOC	130	31	2.66	1.25x10 ⁻⁵
Regulation of blood vessel size	0050880	GOC	137	25	2.73	2.71x10 ⁻⁵
Positive regulation of animal organ morphogenesis	0110110	GOC	86	23	2.99	7.79x10 ⁻⁵
Vasculogenesis	0001570	DAVID	56	12	3.10	1.35x10 ⁻³
Positive regulation of PI3K signaling	0014068	GOC	85	15	2.64	1.4x10 ⁻³
Regulation of blood pressure	0045776	DAVID	30	13	2.90	1.47x10 ⁻³
Positive regulation of steroid metabolic process	0045940	GOC	28	8	4.27	1.47x10 ⁻³
Response to hypoxia	0001666	DAVID	172	22	1.85	7.7x10 ⁻³
PI3K-Akt signaling pathway	04151	KEGG	345	36	1.50	1.4x10 ⁻²
VEGF signaling pathway	04370	KEGG	61	10	2.35	2.4x10 ⁻²
MAPK signaling pathway	04010	KEGG	253	26	1.47	4.67x10 ⁻²
Vasodilation	0042311	DAVID	14	4	4.13	6.74x10 ⁻²

Table 3: Ontologic groups containing overrepresented differentially expressed genes (DEG's) when *ProL1* is overexpressed in LNCaP cells.

Biological process/ molecular pathway	GO/hsa identifier	Analysis	Ref List (GOC:20996) (DAVID:16792) (KEGG:6879)	<i>ProL1</i> - overexpression list	Fold- enrichment.	P-value
Animal organ morphogenesis	0009887	GOC	930	68	1.79	1.16x10 ⁻⁵
Smooth muscle contraction	0006939	GOC	51	10	4.8	1.20x10 ⁻⁴
Circulatory system development	0072359	GOC	832	59	1.7	1.60x10 ⁻⁴
Blood circulation	0008015	GOC	386	33	2.09	1.72x10 ⁻⁴
Steroid hormone biosynthesis	00140	KEGG	58	8	3.31	9.75x10 ⁻³
Angiogenesis	0001525	DAVID	223	16	1.81	3.19x10 ⁻²
Positive regulation of MAPK cascade	0043410	DAVID	81	8	2.49	4.10x10 ⁻²
Positive regulation of blood pressure	0045777	DAVID	20	4	5.05	4.25x10 ⁻²

Figures

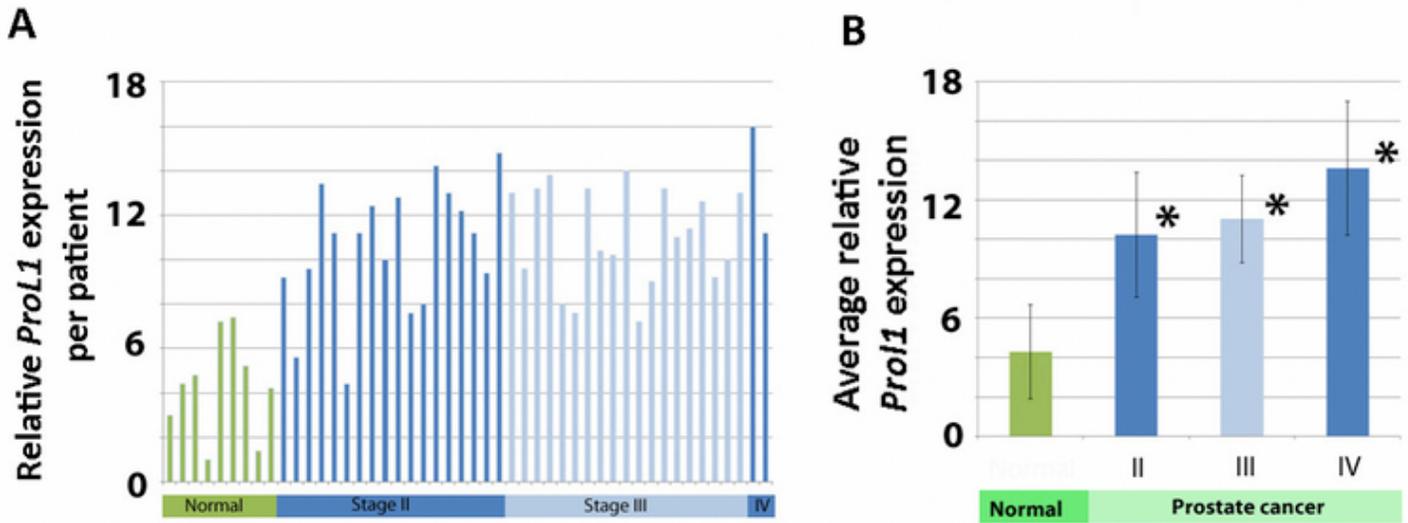


Figure 1

ProL1, the gene encoding opioidin is upregulated in PrCa lines. A, Quantitative RT-PCR was used to determine relative expression levels of ProL1 in a tissue RNA array with sample from control and different stages of PrCa. B, The data for individual samples was 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 levels of ProL1 in PrCa tissue, with a trend for higher levels of expression as the cancer stage increased. Error bars = Std. Dev., *= p-value <0.05.

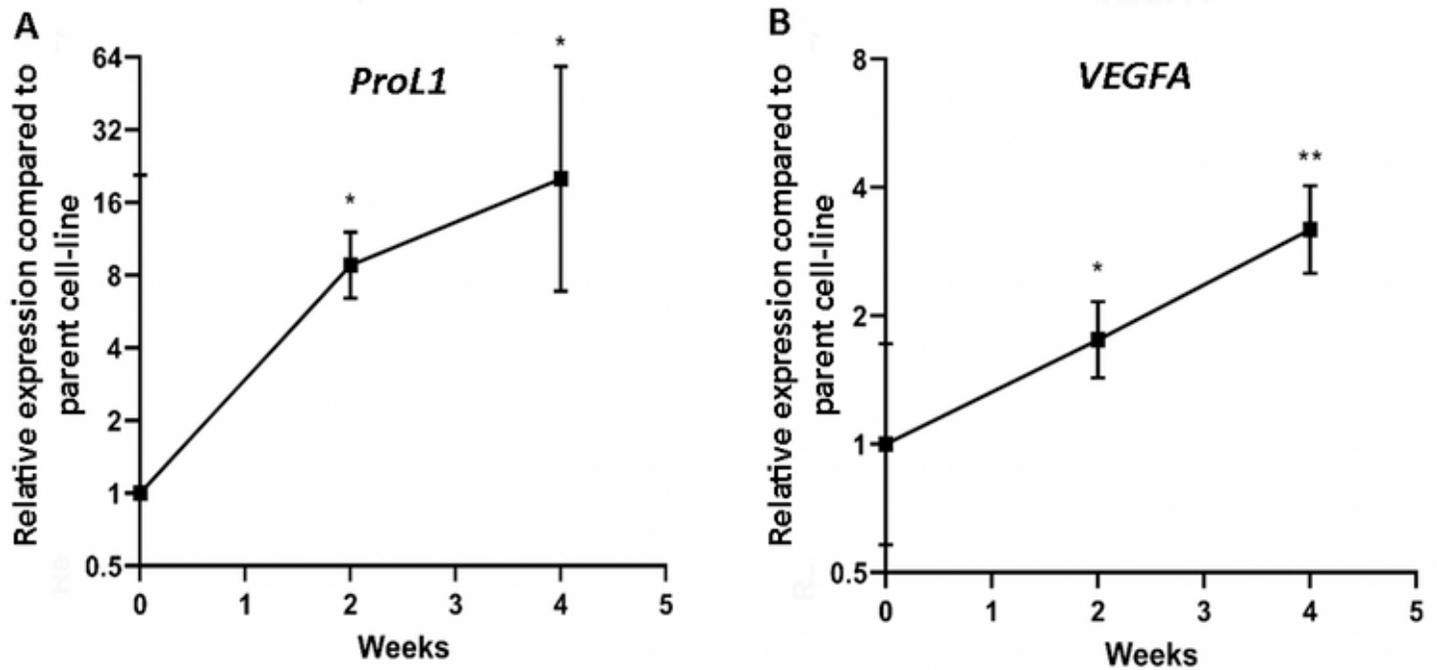


Figure 2

Athymic nude male mice were injected with a prostate cancer cell-line (PC3). At 2-weeks and 4-weeks post implantation animals were euthanized, tumors excised, and RNA extracted to perform qt-RT-PCR for the

opiorphin encoding gene, A) ProL1 and B) VEGFA as a marker of hypoxia (b). The expression of genes is represented as fold-change compared to the starting population of the parent PC3 cell-line (N=5, error bars =95% Confidence Interval, *= p-value <0.05, **= p-value <0.01).

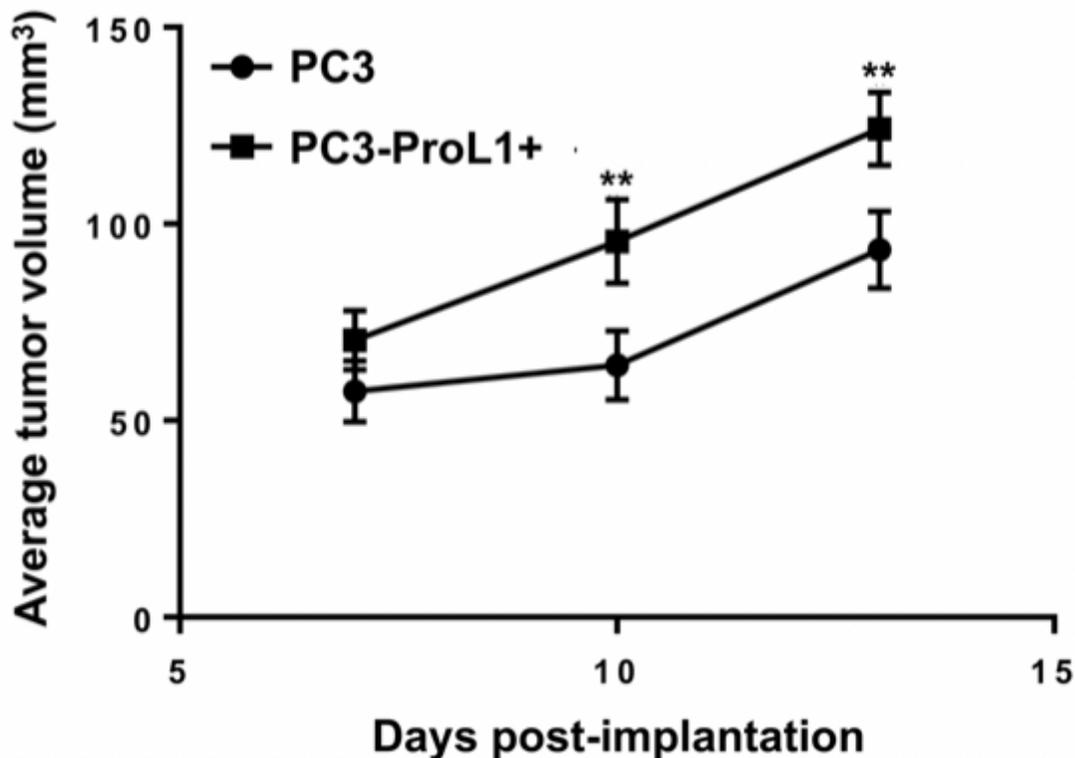


Figure 3

PC3 cells engineered to overexpress ProL1 have initially faster tumor growth than the parent cell line. PC3 and PC3-ProL1+ cell lines were injected into male nude mice and tumor size determined by standard caliper measurements (N=10). The average tumor volume is shown for each time point. Error bars = Std. error *= p-value <0.05.

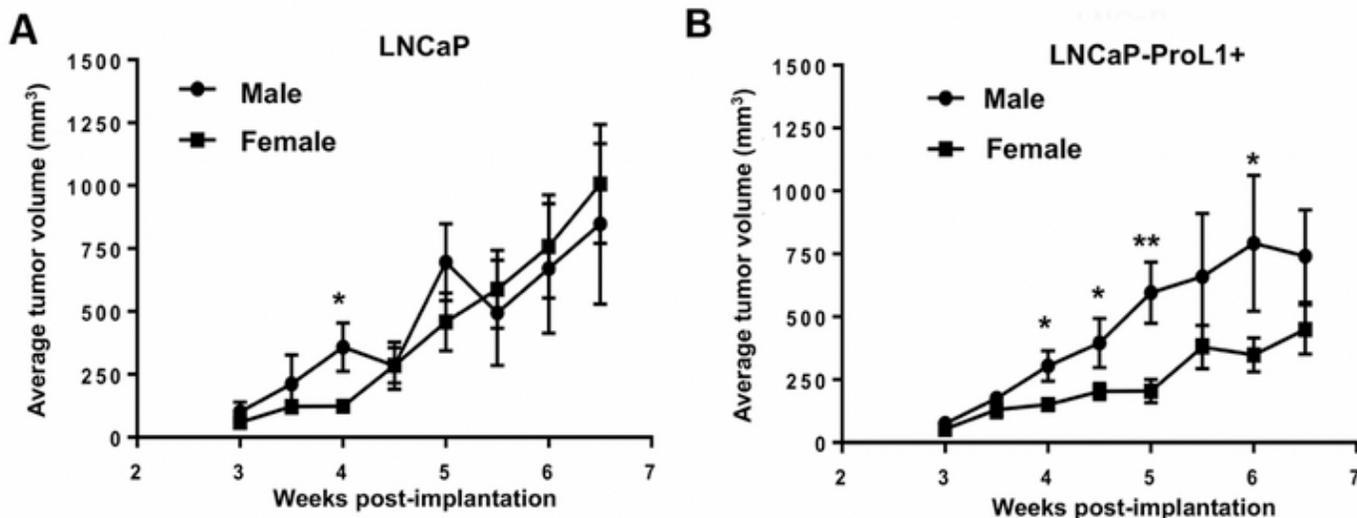


Figure 4

Growth of xenografted tumors derived from LNCaP-ProL1+ or LNCaP cell lines in male or female mice. LNCaP and LNCaP-ProL1+ cell-lines were injected into male and female nude mice and tumor size determined by standard caliper measurements (N=10-14). The average tumor volume is shown for each time-point. Error bars = Std. Error. *p-value <0.1, **p-value<0.05. A: Growth of LNCaP derived tumors in male compared to female mice. B: Growth of LNCaP-ProL1+ derived tumors in male compared to female mice.

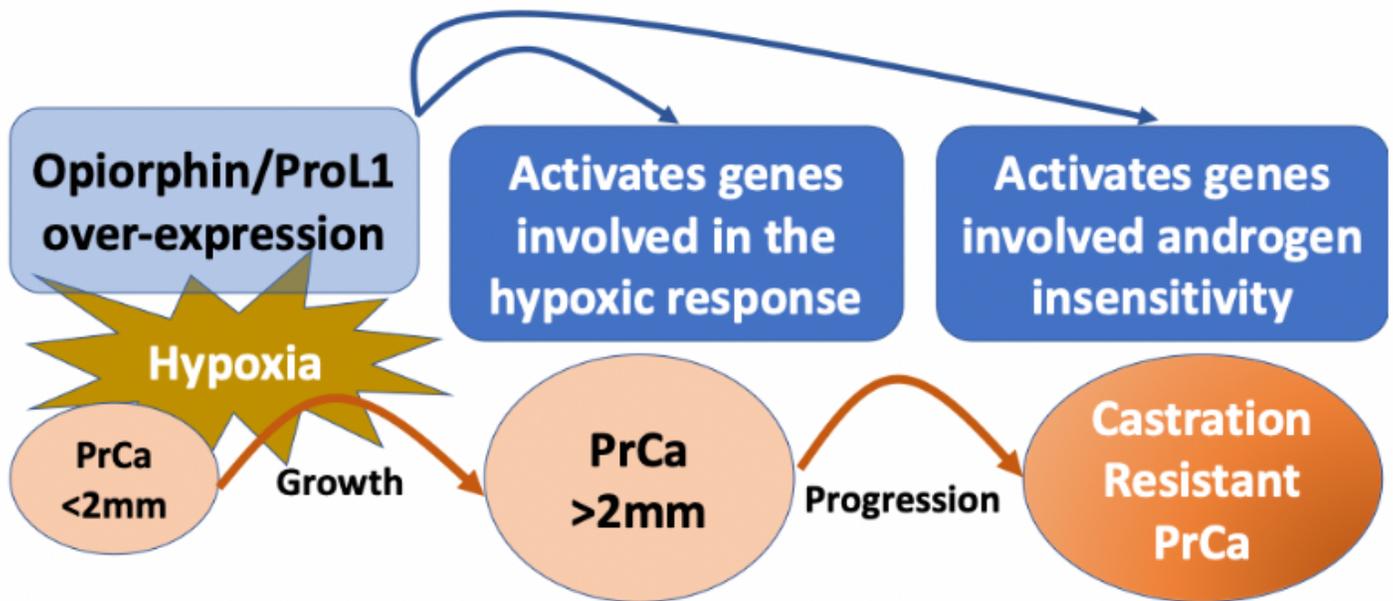


Figure 5

In our recent publication we demonstrated that in corporal smooth muscle cells opiorphin is a master regulator of the hypoxic response. Therefore, we hypothesize that as the tumor grows above 1-2mm hypoxia first causes up-regulation of opiorphin, which in turn activates expression of genes involved in the hypoxic response and the development of androgen insensitive/ castration resistant PrCa.

Supplementary Files

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