Transient Sox9 Expression Facilitates Resistance to Androgen Targeted Therapy in Prostate Cancer

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Running Title: Transient Sox9 Facilitates Prostate Cancer Progression

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Abstract

**Purpose:** Metastatic prostate cancer (PCa) patients are increasingly presenting with treatment-resistant, androgen receptor-Negative/Low (AR\textsuperscript{\text{Low}}) tumors, with or without neuroendocrine characteristics, in processes attributed to tumor cell plasticity. This plasticity has been modeled by Rb1/p53 knockdown/knockout and is accompanied by overexpression of the pluripotency factor, Sox2. Here we explore the role of the developmental transcription factor Sox9 in the process of PCa therapy response and tumor progression.

**Experimental Design:** Unique PCa cell models that capture AR\textsuperscript{\text{Low}} stem cell-like intermediates were analyzed for features of plasticity and the functional role of Sox9. Human PCa xenografts and tissue microarrays were evaluated for temporal alterations in Sox9 expression. The role of NF-κB pathway activity in Sox9 overexpression was explored.

**Results:** PCa stem cell-like intermediates have reduced Rb1 and p53 protein expression and overexpress Sox2 as well as Sox9. Sox9 was required for spheroid growth, and overexpression increased invasiveness and neural features of PCa cells. Sox9 was transiently upregulated in castration-induced progression of PCa xenografts and was specifically overexpressed in neoadjuvant hormone therapy (NHT) treated patient tumors. High Sox9 expression in NHT-treated patients predicts biochemical recurrence. Finally, we link Sox9 induction to NF-κB dimer activation in PCa cells.

**Conclusions:** Developmentally reprogrammed PCa cell models recapitulate features of clinically-advanced prostate tumors including downregulated Rb1/p53 and
overexpression of Sox2 with Sox9. Sox9 is a marker of a transitional state that identifies PCa cells under the stress of therapeutic assault and facilitates progression to therapy resistance. Its expression may index the relative activity of the NF-κB pathway.

Translational Relevance

The introduction of stringent androgen receptor pathway blockers into the treatment paradigm for metastatic prostate cancer patients is increasing the occurrence of aggressive tumors lacking a prostate-like differentiation. Employing our unique prostate cancer cell models of therapy-induced dedifferentiation, we identified Sox9 as an effector of a transitional state with aggressive features that serves as an early marker of progression. As such, Sox9 might provide a novel therapeutic target to suppress the development of certain types of castration-resistant prostate cancer.
Introduction

Prostate cancer (PCa) is a disease that develops and progresses under the influence of the androgen receptor (AR) protein (1,2). For this reason, patients with metastatic disease are invariably treated with therapies that suppress AR function (3). Androgen deprivation therapy (ADT) is currently the preferred front-line approach (4). But ADT-treated patients typically progress to castration resistant prostate cancer (CRPC) that continues to grow and spread despite castrate-levels of serum testosterone (5,6). Potent anti-androgens (enzalutamide or abiraterone) are then used as secondary agents, before or after taxanes (7). Eventually however, as with ADT, most metastatic CRPC patients treated with anti-androgens will continue to progress to recurrent disease, which foreshadows lethal outcomes (8,9).

Resistance to primary hormonal therapy is mainly driven by adaptions of the androgen receptor (AR) signaling pathway in tumor cells (4). Mutations, gene amplification and/or expression of truncated splice variants of AR, any of which can override the constraints of androgen targeted therapy (ATT), contribute to this phenotype (10-12). But the introduction of stringent anti-androgens is increasing the occurrence of AR-/low tumors, with or without neuroendocrine (NE) characteristics (13). These latter tumor types are refractory to hormonal therapies and are thought to represent a resistance driven by cellular “lineage plasticity” (14,15). This refers to an ability of cancer cells to change their phenotypic characteristics as an adaption to adverse microenvironments or therapeutics. The most striking example of this plasticity is the generation of NE-like tumors from prostate-like adenocarcinomas. Differentiated prostate features are lost while mature NE features are gained in a process typically
described as a transdifferentiation (16-18). There is extensive interest in defining the mechanistic pathway leading to this conversion. Putative NE transdifferentiation (NETD) driver events may involve gains in expression (N-myc, AURKA, Sox11, PEG-10) or loss of REST expression, and these are thought to directly guide PCa cells from the prostate-like state to the NE-like state (19-22). These observations, however, never address the reasons that the putative driver genes are differentially expressed. Others have established the importance of a plasticity phenotype driven primarily by loss of the tumor suppressor functions of Rb1 and p53 (15,20,23). This is often accompanied by increased expression of Sox2, a transcription factor best known for its role in the maintenance of self-renewal and pluripotency of embryonic stem cells (24).

The upregulation of Sox2 and its role in establishing the plastic state implies that plasticity might recapitulate a developmental process involving a transition from a poorly differentiated, plastic state to a more highly differentiated state. Based on observations of diverse cell phenotypes in a culture of transdifferentiated PCa cells, we reasoned that transdifferentiation may first require a de-differentiation to an intermediate stem cell-like state that then re-differentiates to alternate cell lineages (25). As the spectrum of differentiated states represented mature cell lineages generated by neural crest stem cells (26,27), we tested a means to convert, capture and maintain PCa cells in this stem-like state by culture in a modified neural crest stem transition media (STM). STM-mediated culture allowed us to convert differentiated AR+ PCa cells, en-masse, to a metastable stem-like phenotype over the course of 14 days, and propagate and maintain them as such. We termed this technique Developmental Reprogramming for the clonogenic, invasive, tumorigenic and primitive neural crest stem-like phenotype it
presented. Moreover, we showed that cells in this reprogrammed state could subsequently be re-differentiated to several distinct neural-/neural crest-(N-/NC-) derived cell lineages by growth in cell-specific differentiation mediums. Similarly, we could return the stem-like cells back to an AR$^+$ prostate-like state by growth in standard culture medium with serum and/or androgens. Based upon our results, we proposed that NEtD requires a de-differentiation to a metastable N/NC stem-like state that then differentiates to a NE-like cell.

Here we report further studies using our developmental reprogramming model, including demonstrating that the transition to the plastic state in our cell models involves a loss of Rb1 and p53 function. Moreover, we now show that reprogramming is accompanied by upregulation of another Sox gene family member, Sox9, which may, at least partially, confer the N-/NC-like characteristics of this reprogrammed state. Sox9 has already been described as an effector of aggressive cancers, including PCa (28-31), and has a prominent role during both prostate and NC development. Further, Sox9 was consistently upregulated in stem-like reprogrammed PCa cells, and was part of a common gene signature that was enriched in tumors from patients with aggressive clinical behaviors. In this current study, clinical and patient derived xenografts (PDXs) demonstrated transient overexpression of Sox9 in response to ATT, while Sox9 perturbations in our models resulted in abrogation of aggressive features. Finally, in both our stem-like reprogramming cell models and in vivo PDX models, we show evidence that Sox9 induction may be mediated by increased NF-$\kappa$B signaling, which has been previously associated with PCa progression and acquired castration resistance (32-34).
Materials and Methods

Cell Culture
LNCaP, VCaP, 22Rv1 (ATCC, Manassas, VA) and LAPC4 cell line (obtained from Dr. Charles Sawyer), were maintained in phenol red-free ATCC-recommended media with 10% fetal bovine serum (FBS). HEK-293FT cells (ATCC, Manassas, VA) were maintained in DMEM with 5% FBS media. Stem-Like models were established as previously described (25). Cell line identifies were validated by short tandem repeat profiling and interspecies contamination test (IDEXX). Cells were screened weekly for mycoplasma with MycoAlert detection kit (Lonza). See supplementary materials and methods for extensive cell culture details.

Lentiviral Transduction
The pLKO.1-shCTRL, pLKO-RB1-shRNA63, pLKO.1-shp53, pLKO.1-GFP-shRNA or pLKO.1-sh-hSOX9 (Addgene) were combined with pCMV-dR8.91 (packaging plasmid) and pCMV-VSV-G (envelope plasmid), as described previously (49).

Plasmid Transfection
Plasmid transfection with Lipofectamine3000 reagents (Invitrogen) was conducted per manufacturers instructions for 48 hours. The pcDNA3-1(C)-SOX9OE vector was a kind gift from the laboratory of Steven Balk. The pGL2-NF-κB Luciferase reporter was a kind gift from the laboratory of Albert Baldwin. The pcDNA3-1(C)-Empty and pCMV-eGFP control plasmids were purchased from Addgene.
Patient Derived Xenografts

The raw data from the LTL331 longitudinal microarray samples (GSE59986) were normalised to pre-castrate sample expression levels and displayed as fold-changes over time. The LTL313B RNA-Seq samples (PRJEB9660), were converted from log2 normalised to fold changes, compared to pre-castrate sample expression levels, and displayed as fold-changes compared to pre-castrate expression levels.

Immunoblotting

Cells were harvested in 1% NP40 lysis buffer supplemented with a protease and phosphatase inhibitor cocktail. Samples were run on pre-cast 4-15% gradient gels (BioRad), and fast-transferred to PVDF membranes using the TransBlot Turbo system (BioRad). See supplementary materials and methods for antibodies and the titers employed.

Quantitative real-time PCR

RNA was extracted from cell lines using an RNeasy Mini kit (Qiagen) before reverse transcription with Maxima kit (Thermo Scientific) according to the manufacturer’s instructions. Subsequent qPCR was performed using the ABI ViiA7 with SYBR Green detection (Applied Biosystems). See supplementary materials and methods for primer sequences.

Luciferase Assays
Luciferase assays were conducted with the Dual Luciferase Kit (Promega) employing manufacturers protocols. Briefly, PCa cells were transiently co-transfected with pGL2-NF-κB Luciferase reporter and pCMV-eGFP for 48 hours, treated for 72 hours with STM, cell lysates were prepared in 1X Passive Lysis Buffer, incubated for three freeze/thaw cycles of 15 minutes each. Detection was performed using Infinite M200 plate reader (Tecan Group Ltd., Männedorf, Switzerland). Values were normalized to relative GFP intensity.

**Flow Cytometry**

Cells were detached by treatment with Accutase (Gibco), fixed in ethanol, re-suspended in propidium iodide (PI) staining solution (50 µg/mL PI, 0.1 mg/mL RNase A, and 0.05% Triton X-100) and analysed on a FACSCanto™ II (BD Biosciences) as described previously (25).

**Immunohistochemistry**

Immunohistochemical staining of patient tumor sections was completed on a Ventana autostainer model Discover XT (Ventana Medical System, Tuscan, Arizona). Representative cores were manually identified by Dr. Ladan Fazli, Pathologist. All biomarkers were scored as previously described (50). See supplementary materials and methods for detailed IHC protocols and NHT cohort data.

**Proliferation, Neurite and Sphere Formation Assays**
Cell proliferation, neurite and sphere formation assays were conducted using IncuCyte (Essen) and CyQuant (Invitrogen) system’s as previously described (25). See supplementary materials and methods for detailed protocols.

Invasion Assay
The invasive abilities of cell models was assessed using Matrigel-coated 24-well plate inserts (Corning® BioCoat™ Matrigel® Invasion, Corning, NY) according to manufacturer’s instructions. Briefly, 20,000 cells were seeded in the top chamber of a Matrigel-coated 24-well plate inserts in serum-free medium. 10% FBS was added to the lower chamber as a chemo-attractant. After 20 hours, cells were fixed and stained with DAPI, the filter was fluorescently imaged and cells remaining on the filter counted using ImageJ software.

Gene Set Enrichment Analysis (GSEA)
The GenePattern server (http://www.genepattern.org/), and default parameters, were employed to identify significantly-enriched gene signatures in reprogrammed PCa cells (p<0.05, fold change>1.5). The gene sets used for enrichment were obtained from the “Curated” and “Hallmark” MSigDB collections.

Gene Expression Analysis and Correlation with cohorts of PCa patients
Processed signal from GSE66850 was analysed for genes which were up/down-regulated with a fold-change≥2 and p-value≤0.05 in reprogrammed LNCaP-SL, LAPC4-SL and VCaP-SL models compared to parental controls, producing a list of 59 genes, constituting a “Stem-Like Signature”. Patient samples
from the Vancouver Prostate Centre (VPC), cumulatively totalling 76 specimens, were collected and RNA-sequencing analysis pipeline and algorithms were processed as previously described (39). However, in the present study, we updated this pipeline to use the Hg38 human genome build and Ensembl GRCh38.86 gene tracks.

**Statistical Analysis**

Unpaired t-test were employed to calculate two-tailed P-values to estimate statistical significance of differences between two treatment groups. One-way or two-way ANOVA and Bonferroni correction were applied to assess differences between multiple groups. Statistically significant P values, indicated by asterisks, are as follows: *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. Data were analyzed by GraphPad Prism software. The programming language R v3.0 was used for unsupervised hierarchical clustering (UHC). Unsupervised Hierarchal Clustering (UHC) was performed with the 59-gene stem-like signature on samples from the VPC cohort using the h.clust package with Pearson correlation for distance and complete linkage. The clustering and heatmaps generated were built using the heatmap.3 function. Prior clustering, samples expression values were log2 transformed, and then standardized/scaled using a Z-score.

**Results**

Based on reports that cellular plasticity of PCa is driven by loss of Rb1 and p53 (15,23), we analyzed parental and reprogrammed PCa cells (LNCaP, VCaP, LAPC4) for
expression of these tumor suppressors. Immunoblotting of lysates from parental (P) and reprogrammed stem-like (SL) models demonstrated reduced expression of both Rb1 and p53 protein in the stem-like state as compared to the parental state (Fig. 1A). This loss of Rb1/p53 protein was not accompanied by reduced expression of \textit{RB1} or \textit{TP53} mRNAs (Fig. 1B). A cycloheximide-chase experiment showed that Rb1 protein was degraded at a faster rate in STM-cultured LNCaP and LAPC4 cells than in FBS-cultured cells (Supplementary Fig. S1A,B). While, VCaP and LAPC4 harbour p53 mutations, and LNCaP does not, the consistent regulation of Rb1/p53 suggests reprogrammed cells require loss of tumor suppressors to promote stem-like fates. Loss of Rb1/p53 in the reprogrammed cells was accompanied by upregulated expression of Sox2 mRNA (Fig. 1C), although we were unable to detect Sox2 protein by immunoblotting. However, we found that mRNA encoding another SRY-Related HMG Box protein, SOX9, was also up-regulated in all of the reprogrammed cell lines (Fig. 1C). Here we were able to detect greatly enhanced expression of Sox9 protein in reprogrammed PCa cells on immunoblots (Fig. 1D). Sox9 migrates as a doublet on immunoblots, with the lower band representing unphosphorylated Sox9, and the upper band representing the phosphorylated Sox9, with enhanced transcriptional activity (35).

As Rb1 and p53 loss alone have been reported to increase expression of stem cell transcription factor Sox2, we hypothesized that the loss of Rb1 and p53 may also alter expression of Sox9. Employing a stable short-hairpin for \textit{RB1}, we found that knockdown of \textit{RB1} in LAPC4, but not LNCaP cells, markedly increased expression of Sox9 (Supplementary Fig. S1C). LAPC4 cells harbour a deactivation mutation in the \textit{TP53} gene, while LNCaP cells do not (36), which may explain the discrepancy in Sox9
protein overexpression in response to RB1 knockdown between LAPC4 and LNCaP cells. Employing a TP53 knockdown shRNA vector, we were able to demonstrate Sox9 mRNA overexpression in the context of TP53 knockdown, with or without RB1 knockdown, in LNCaP cells, concomitant with SOX2 overexpression (Fig. 1E,F).

LNCaP-shP53 cells not only overexpressed Sox9 mRNA and protein, but also a putative Sox9 target gene, COL2A1. At an mRNA & protein level, loss of P53 seems more consequential than RB1 for Sox9 expression in LNCaP. Functionally, TP53 knockdown significantly increased the proliferation of LNCaP cells in both androgen-containing (FBS) and androgen-deprived (CSS) containing medium (Fig. 1G), consistent with previous reports (37). The STM formulation contains both FGF and EGF, typically employed in other stem cell culture media, but also requires a neural supplement that is key to developmental reprogramming of PCa cells from a prostate-like (AR+/p53High/Rb1High/Sox9Low) state, to a stem-like (AR-/p53Low/Rb1Low/Sox9High) state. While shP53 cells were not able to phenocopy STM cultured cells in media lacking the neural supplement (containing only the FGF/EGF factors), LNCaP-shP53 cells appeared to demonstrate enhanced growth compared to LNCaP-shCTRL in FGF/EGF (Supplementary Fig. S1D), although the difference was not significant in terms of spheroid area after 14 days (Fig. 1H). Interestingly, shP53 cells produced significantly larger spheroids than shCTRL cells in STM (Fig. 1H), suggesting that the loss of p53 function coordinates with developmental reprogramming phenotypes.

The role of Sox9 in developmental reprogramming was further examined by SOX9 knockdown using shRNA in LNCaP and LAPC4 cells (Supplementary Fig. S2A). Knockdown of SOX9 significantly reduced sphere forming potential during the 14-day
developmental reprogramming protocol (Fig. 2A,B). Interestingly, in LAPC4 cells which
can proliferate under androgen-deprived conditions, shSOX9 resulted in the abrogation
of proliferation in androgen-free CSS media, while only reducing the proliferation of cells
cultured in androgen-containing FBS media (Fig. 2C). Expression of Rb1 and p53 are
reduced by culture in CSS (Supplemental Fig. S2B), suggesting that the inhibitory
effects in proliferation by Sox9 knockdown are dependent on RB1 and/or p53 loss. To
confirm that Sox9 overexpression plays a significant role in the aggressive features of
therapeutically challenged PCa cells, we sought to express exogenous SOX9. Both
LNCaP and LAPC4 cells demonstrated significant overexpression of Sox9 protein with
SOX9 vector transfection (31) (Supplemental Fig. S2C). In the context of exogenous
SOX9, LNCaP cells demonstrated overexpression of NC genes like NRCAM, BRN3A,
ASCL1, and NANOG, as well as genes associated with NEtD like SOX2, NCAM1, SYP
and NSE (Fig. 2D). Putative Sox9 target genes COL2A1 and COL11A2 served as
positive controls of Sox9 activity, and both were upregulated in LNCaP-SOX9 cells.
Notably, we previously reported that the NC and NEtD genes described above are
highly upregulated in developmentally reprogrammed PCa cells (25), while a panel of
NC and NEtD genes are reduced after knockdown of SOX9 in LNCaP cells (data not
shown). Unexpectedly, overexpression of SOX9 alone failed to increase sphere
formation in LNCaP or LAPC4 cells without the presence of the complete STM
formulation (Supplementary Fig. S2D,E). Further, results of sphere formation
calculations under STM culture conditions was not consistent between LNCaP and
LAPC4 cells. Similarly, SOX9 overexpression alone did not alter androgen independent
growth in LNCaP or LAPC4 cells (Supplementary Fig. S2F). Nevertheless, in 24 hour
Matrigel invasion assays, *SOX9* overexpression was able to significantly increase *in vitro* invasiveness of both LNCaP and LAPC4 cells (Fig. 2E). Furthermore, *SOX9* overexpression increased both the length and number of branch points in neurites formed by LNCaP cells under androgen deprived conditions (Fig. 2F,G, Supplementary Fig. S2G,H,I). This suggests that Sox9 enhances complex, neural-like features of ATT-treated PCa cells. Finally, we found that both *RB1* and *TP53* knockdown recapitulated this phenotype, as both shRB1 and shP53 cells also demonstrated enhanced neurite length and branching under androgen-deprived conditions (Supplementary Fig. S2G,H).

**Transient upregulation of SOX9 in cell and PDX models, and in patient tumors following ATT**

Culture in STM increased Sox9 expression in a time dependent manner for LNCaP and LAPC4 cells (Fig. 3A). In contrast, acute androgen-deprivation in both LNCaP and LAPC4 cells in CSS medium resulted in a transient upregulation of Sox9 that gradually returned to control levels (Fig. 3B). Extending our results beyond *in vitro* models, we employed microarray and RNA-Seq datasets from PDX models (20,38). Examination of longitudinal microarray samples in intact mice bearing LTL331 PDXs indicated that *SOX9* expression spikes post-castration and remained elevated up to recurrence in this *in vivo* NEPC recurrence model (Fig. 3C). *SOX9* expression peaked at over 3 times the pre-castration levels, and remained elevated up to 21 days post-castration, while *PSA* (*KLK3*) expression levels plummeted after castration, and remained extremely low even after recurrence. Similarly, the LTL313B PDX model, that
recurs as AR+ CRPC rather than AR− NEPC, showed the same transient upregulation of SOX9 post-castration, that declined upon recurrence (Fig. 3D).

Global gene expression profiling of three reprogrammed cell lines (LNCaP, VCaP and LAPC4) showed an overlapping set of 59 genes consistently altered in stem-like cells compared to their parental counterparts (fold-change≥2, P≤0.05), and containing several N/NC stem cell genes including ALCAM (CD166) and SOX9 (Supplementary Table 1). To determine if these genes were clinically relevant, we validated the gene sets expression in a published sequenced cohort profiled at the Vancouver Prostate Centre (VPC) (39). Using unsupervised hierarchical clustering, a distinct separation between clades of untreated and neoadjuvant hormone therapy (NHT) treated samples was observed (Supplementary Fig. S3A). This suggests an association of these 59 genes to NHT status. Of interest, within the untreated samples two predominate clusters were observed, where one cluster contained the bulk of intermediate/high risk disease patients (Gleason>7). This suggests a gene set derived from stem-like PCa cells may be associated with therapy response and PCa staging.

Clinical validation of enhanced Sox9 expression in response to ATT comes from analysis of patient-derived tissue microarrays (TMAs). Immunohistochemical (IHC) staining of a TMA containing untreated (n=38), NHT treated (n=23), CRPC (n=40) and NEPC-like (n=41) samples with Sox9 antibody revealed that Sox9 expression was significantly elevated in NHT specimens compared to untreated, CRPC and NEPC-like patient tissues (Fig. 4A,B). These results mirror longitudinal microarray and RNA-Seq data in PDX models showing transient overexpression of Sox9 up to recurrence (Fig. 3C,D). Unlike Sox9, Sox2 expression increased through NHT and was highest in CRPC
and NEPC samples, where it reached significance (Fig. 4A,B). As anticipated, markers of NEPC, Synaptophysin and NCaM, were highest in NEPC-like samples, and PSA was highest in untreated and CRPC samples, while proliferation marker Ki67 was highest in both CRPC and NEPC (Fig. 4A,B).

To expand on our preliminary findings in patient tissues we conducted Sox9 IHC on larger TMAs containing samples from 273 patients at varying time points before and after NHT and from CRPC. Here again, the staining of Sox9 was significantly elevated in NHT samples compared to untreated or CRPC (Fig. 5A,B). Furthermore, Sox9 expression was able to predict biochemical recurrence in this patient cohort (Fig. 5C), despite no correlation between PSA secretion and Sox9 expression at sampling (data not shown), suggesting that the actions of Sox9 may be independent of AR. Notably, Ki67 staining of TMA samples indicated significantly enhanced proliferation in CRPC, but not NHT tissues, and a significantly negative correlation between Sox9 and Ki67 in CRPC samples (Fig. 5D, Supplementary Fig. S4A). This mirrors results from the smaller TMA, and may explain the lack of enhanced proliferative potential in exogenous SOX9-expressing cell lines (Supplementary Fig. S2E).

Sox9 is regulated by NF-κB signalling during development and in malignant cells (40,41). In PCa models, NF-κB pathway activation was demonstrated by increased luciferase reporter assays in LNCaP, LAPC4 and VCaP cells under acute STM treatment compared to FBS-cultured controls (Fig. 5E). Expression of mRNA from NF-κB pathway members and downstream targets was also increased when comparing parental and stem-like LNCaP, VCaP, LAPC4 and even 22Rv1 cells (Fig. 5F). Further, Gene Set Enrichment Analysis (GSEA) indicated that a core NF-κB pathway signature
was highly enriched in LNCaP-SL and VCaP-SL cells compared to parental cells (Supplementary Fig. S4B), while Ingenuity Pathway Analysis (IPA) returns NF-κB Signaling as one of the most enriched pathways in LNCaP-SL, VCaP-SL and LAPC4-SL cells (Supplementary Fig. S4C). Notably, the top hit in IPA is Dendritic Cell Maturation, which is a process that requires NF-κB signalling and high levels of NF-κB proteins for both survival and differentiation of activated dendritic cells, as well as dendritic cell-mediated T cell induction (42,43). Analysis of the transcriptomes of PDX models post-castration showed a transient increase in the expression of NF-κB transcription factors and NF-κB target genes that mirrors the transient increase seen in SOX9 expression after castration (Supplementary Fig. S4D,E). Finally, transcriptomic data from genetically engineered mouse models showed that expression of NF-κB transcription factors and NF-κB target genes mirrors that of SOX9, with TP53 loss alone producing the greatest induction of SOX9 expression (Supplementary Fig. S4F).

In addition to the broad activation of the NF-κB transcriptome, protein analysis indicates that both the canonical NF-κB pathway, characterized by p65 (RelA) phosphorylation, and the non-canonical pathway, characterized by p100 cleavage to p52, are activated in reprogrammed stem-like cells (Fig. 6A,B). Strikingly, IHC analysis of p65 in untreated, NHT treated and CRPC patient samples demonstrated that this canonical NF-κB subunit is highly up-regulated in response to NHT treatment in a time dependent manner (Supplementary Figure S5A). This enhanced expression is lost in tissues from recurrent patients, mimicking transient Sox9 upregulation post-NHT. As proof of principle, we examined whether suppression of NF-κB (using the proteasome inhibitor Bortezomib as a tool compound) could affect Sox9 expression in our cell
models. Bortezomib prevents the activation of both the classical and non-classical NF-κB pathways, which both rely on proteasomal cleavage for signal transduction (34). Bortezomib treatment was found to suppress Sox9 expression under androgen-deprived conditions (CSS or STM) in LNCaP and LAPC4 cells (Fig. 6C, Supplementary Fig. S5B). Notably, dose response experiments indicated that cells under androgen-deprived conditions appear sensitized to Bortezomib treatment, with significantly lower doses necessary for 50% growth inhibition (Fig. 6D, Supplementary Fig. S5C,D). Cell cycle analysis indicated that the VCaP model, the cell line with highest basal expression of Sox9 protein, appears most sensitive to Bortezomib treatment based on the population of Sub-G0 cells (Supplementary Fig. S5E), while LNCaP and LAPC4 cells both show increased S-Phase populations in response to Bortezomib treatment (Fig. 6E, Supplementary Fig. S5F). As Bortezomib-mediated proteasomal inhibition has broad effects across the cell, we elected to test a second NF-κB targeting strategy by employing the diterpenoid epoxide, Triptolide, shown to prevent phosphorylation of p65 in therapeutically challenged cancer models (44). The use of increasing doses of Triptolide alone was also shown to decrease Sox9 protein expression under androgen-deprived CSS culture conditions in LNCaP and LAPC4 cells (Supplementary Fig. S5G). Collectively, this suggests that NF-κB mediated induction of Sox9 in response to ATT may facilitate the de-differentiation of AR⁺/Sox9\textsuperscript{Low} prostate adenocarcinomas to AR\textsuperscript{Low}/Sox9\textsuperscript{High} NC stem-like cells, promoting therapy resistance and disease progression (Fig. 6F).
Discussion

The introduction of potent anti-androgens into the armamentarium for CRPC has created a vexing clinical dilemma where patients progress to AR\(^{-/\text{Low}}\) disease that may have characteristics of aggressive neuroendocrine cancer (13). This transdifferentiation is a striking example of tumor cell plasticity driving therapeutic resistance. Others have suggested that this plasticity is a direct consequence of simple gain/loss of certain gene functions (19,20,22), whereas we presented evidence from cultured PCa cell models of a transition through an intermediate stem cell-like state that might ultimately underlie transdifferentiation to NE-like cancer or even transition to AR\(^{+}\), but resistant adenocarcinoma (25). Our characterization of this stem-like state showed that it shared many characteristics with NC stem cells, including a gene signature, expression of common cell surface markers, multipotency, and functional features like high tumorigenicity and enhanced migration and invasion, \textit{in vitro} and \textit{in vivo} (25). We showed here that our conditional stem-like cell models demonstrate significant loss of p53 and Rb1 protein expression, accompanied by an upregulation of pluripotency factor Sox2, molecular features previously associated with transition to NEPC by others (15,23). Our findings then support the idea that we are observing a plastic state transition that was previously described using gene knockdown/knockout approaches (15,23). The reduced expression of tumor suppressors in our cell models appears to be consequence of reduced protein stability and not a loss of mRNA expression. As the appearance of NEPC in treated patients is usually accompanied by genomic alterations of p53 and Rb1 (45), loss of tumor suppressor function by non-genomic factors may explain the transient nature of our cell plasticity models.
We also previously described how a commonly altered gene set, derived from microarray analysis of PCa stem-like cells, was able to discriminate PCa patients that experience adverse events including biochemical recurrence, metastasis and PCa-specific mortality (25). Here, we showed that a gene set of consistently altered genes in stem-like cells is also associated with NHT treated PCa. Both these gene signatures included SOX9, and here we confirmed that Sox9, like Sox2, was upregulated in the transition to a stem-like state. Moreover, knockdown of RB1/TP53 was sufficient to drive expression of Sox9, demonstrating that the plastic state established by the loss of these tumor suppressor proteins includes overexpression of Sox9. Sox9, like Sox2, is a member of the extended SRY-Related HMG-box containing gene family of transcription factors that regulates pluripotency and differentiation (26). Though best known for its role in chondrogenesis and skeletal development in adult tissues (40), Sox9 is also functionally required during dissemination of NC progenitors in early embryonic development (27) and for normal prostate development (46). In tumors, Sox9 has been described as a facilitator of invasiveness in lung and prostate cancer, and a factor in acquired endocrine resistance of breast cancer (28-30).

We reasoned that idiopathic activation of the Sox9 protein in our reprogramming medium may play a significant role in the NC stem cell-like phenotype observed. Indeed, knockdown of Sox9 significantly reduced spheroid formation during the 14-day STM culture protocol in LNCaP and LAPC4 cells. Further, knockdown of SOX9 abrogated endogenous androgen-independent proliferation in LAPC4 cells, suggesting that it might be a contributing factor to the development of CRPC. While transient SOX9 overexpression alone was insufficient to drive spheroid formation and growth in PCa
cells, this is not surprising since this behavior is likely to require concerted action with Sox2, tumor suppressor loss, and other developmental regulatory proteins to reproduce the stem cell-like state. Nevertheless, transient SOX9 overexpression was sufficient to induce a more neural phenotype on PCa cells, identified by upregulation of NC genes and NE markers, and increased neurite formation. Furthermore, exogenous SOX9 expression conferred a more invasive phenotype on LNCaP and LAPC4 cells, recapitulating its role in dissemination of NC stem cells during embryogenesis (27). This suggests that Sox9 may be key in the enhanced invasiveness of developmentally reprogrammed cells (25).

While growth of PCa cells in our androgen-free STM medium maintains high Sox9 expression, re-differentiation of the stem-like cells to prostate-like cells in androgen-containing growth medium reduced Sox9 expression to the levels found in parental cells (data not shown). We had proposed that this stem-like state is transient, and our findings of a transient upregulation of Sox9 in in vitro models (initiated by growth in androgen-depleted medium) as well as in the in vivo LTL331 and LTL331B PDX models (initiated by castration of the host) further supports this concept. Finally, observations of significantly elevated Sox9 in tumors from patients receiving NHT just prior to prostatectomy, but not in untreated or CRPC specimens, also lend credence to the idea that the stem cell-like state (with high Sox9 expression) is transiently induced by ATT, preceding the development of CRPC (Fig. 6E). It is of further interest that while Sox2 was also upregulated in NHT-treated prostate tumors, it remained elevated in CRPC. This divergence upon transition to CRPC suggests that Sox2 may remain important for the resistant phenotype whereas Sox9 is only a transitional factor.
Sox9 overexpression vectors did not produce enhanced proliferation in LNCaP or LAPC4 cells, under either FBS or CSS conditions (Supp. Fig. S2E). Others have correlated Ki67 (a proliferation marker) with Sox9 expression in genetically engineered mouse PCa models (28,47). Here in patient models, we see that this correlation could not be validated. In fact, in CRPC Sox9 and Ki67 expression was negatively correlated. This suggests that previous reports linking Sox9 expression to enhanced proliferation are not backed by our in vitro findings or patient data. These findings, however, are consistent with the role of Sox9 as a facilitator of invasion, but not proliferation in lung cancer cells (30).

Mechanistically, Sox9 induction during developmental reprogramming may be a consequence of elevated NF-κB signaling. The NF-κB pathway is a known regulator of Sox9 in both normal and malignant cells (40,41,48), and here we found that expression of both the classical (phospho-p65) and non-classical (p52) effectors of NF-κB signaling are induced by growth in STM, and that NF-κB reporter activity was likewise induced. NF-κB signalling is notoriously difficult to inhibit, therefore we exploited an established NF-κB pathway inhibitor strategy, with the proteasome inhibitor Bortezomib. We were able to confirm that pharmacological targeting of NF-κB not only reduced induction of Sox9 expression in STM-reprogramming but also in androgen deprived PCa cells. We also explored a more targeted method to suppress NF-κB, employing the diterpenoid epoxide, Triptolide, an inhibitor of p65 phosphorylation to similar effect as Bortezomib. Notably, androgen-deprived PCa cells appeared to be especially sensitized to NF-κB pathway targeting tool compounds like Bortezomib.
Collectively, our data indicate that PCa cells under the pressures of ATT may exploit plasticity-related pathways to transiently de-differentiate into NC stem-like cells, allowing for re-differentiation into a therapy-resistant and aggressive CRPC. This transitory stem-like cell may rely on abnormal expression of NC master regulator and prostate development gene, Sox9, as evidenced by *in vitro*, *in vivo* and clinical data. Inhibition of SOX9 expression ameliorated aggressive functional features associated with therapy resistance, including spheroid formation and androgen-independent proliferation, while SOX9 overexpression induced a N/NC-like transcriptomic profile, enhanced neural features, and promoted *in vitro* invasiveness. Finally, targeting developmental reprogramming by inhibiting upstream regulators of Sox9 expression successfully prevented Sox9 induction and hindered androgen-independent proliferation of PCa cells.

**Figure Legends**

**Figure 1:** Loss of Rb1 and p53, and gain of Sox9 overexpression may be responsible for establishment of the developmental reprogramming.

A, Western blot of LAPC4, VCaP and LNCaP parental and stem-like cells, demonstrating Rb1 and p53 protein loss during developmental reprogramming. B, The mRNA signal for both RB1 and TP53 was not altered by developmental reprogramming, despite protein loss. C, Both SOX2 and SOX9 mRNA is up-regulated in reprogrammed (SL) PCa cells compared to parental cells, as measured by RT-qPCR. Bars represent Mean ± standard deviation. D, Western Blot analyses of parental and reprogrammed stem-like (SL) PCa cells showed consistent up-regulation of Sox9 protein. Note that
Sox9 often migrates as a doublet on immunoblots (35). E, SOX9 and Sox9 target mRNA is up-regulated in RB1 and P53 knockdown LNCaP cells, as measured by RT-qPCR. Bars represent relative expressions compared to shCTRL cells. Mean ± standard deviation. F, Western Blot analyses of LNCaP shRB1, shP53 and shRB1/shP53 cells showed consistent up-regulation of Sox9 protein. G-H, LNCaP cells demonstrate enhanced androgen independent proliferation (G) and sphere formation (H) in P53 knockdown cells compared to controls. Mean ± standard deviation.

**Figure 2: Sox9 perturbation abrogate functional features of developmental reprogramming, while over-expression promotes invasiveness in PCa cells.**

A-B, LNCaP and LAPC4 cells stably expressing short-hairpin targeting SOX9 display significantly reduced sphere formation in IncuCyte area calculations. Mean ± standard error. C, LAPC4-shSOX9 cells demonstrated significantly reduced proliferation compared to control cells by IncuCyte confluence calculations. Androgen-independent proliferation (CSS) is most adversely effected compared to culture in androgen containing media (FBS). Mean ± standard error. D, Overexpression of SOX9 leads to concomitant increase in the mRNA of neural crest, neuroendocrine PCa and putative Sox9 targets in LNCaP cells. Bars represent relative expressions compared to empty vector control cells. Mean ± standard deviation. E-G, Overexpression of SOX9 increased *in vitro* invasiveness of LNCaP and LAPC4 cells in Matrigel invasion assays (E), and complex neural features like neurite formation and branching in IncuCyte NeuroTrack assays (F,G). Mean ± standard deviation.
Figure 3: Sox9 expression is upregulated in response to androgen deprivation in in vitro and in vivo models.

A-B, Western Blot analyses of STM (A) and CSS (B) treatment time courses indicates consistent up-regulation of Sox9 protein early during developmental reprogramming (STM) and transient upregulation of Sox9 under androgen deprivation (CSS) in LNCaP and LAPC4 cells. C, Longitudinal microarray assessment of PDX samples with NEPC recurrence shows early spike in SOX9 post-castration. D, Longitudinal microarray assessment of PDX samples with CRPC recurrence shows early spike in SOX9 post-castration.

Figure 4: Analysis of patient tissues confirm transient Sox9 upregulation in neoadjuvant hormone therapy treated patients.

A, Immunohistochemistry of untreated, NHT treated, CRPC and NEPC-like patient tissues (n=142) indicate Sox9 IHC score spike post-NHT, that returns to basal levels in CRPC and NEPC tissues, unique in comparison to other markers of disease progression like Sox2, Synaptophysin, NCaM, PSA and Ki67. Each column represents one patient. B, Mean scoring of marker expression in untreated, NHT treated, CRPC and NEPC-like patient TMAs. Mean ± standard error.

Figure 5: Analysis of neoadjuvant hormone therapy treated patient tissues validate transient Sox9 upregulation.

A, Immunohistochemistry of Sox9 and Ki67 expression in untreated, NHT treated, and CRPC patient tissues (n=273) indicate transient increase in Sox9 expression in NHT
treated samples, not correlated to Ki67 staining. **B,** Mean scoring of Sox9 expression in untreated, NHT treated, and CRPC patient TMA. Mean ± standard error. **C,** Sox9 expression predicts PSA recurrence in PCa patients. **D,** Mean scoring of Ki67 expression in untreated, NHT treated, and CRPC patient TMA. Mean ± standard error. **E,** Luciferase reporter assays indicate significant NF-κB transcriptional activity in response to acute STM exposure in LNCaP, VCaP and LAPC4 cells. Mean ± standard deviation. **F,** Developmentally reprogrammed LNCaP, VCaP, LAPC4 and 22Rv1 stem-like cells demonstrate enhanced expression of NF-κB pathway members and putative target genes. Bars represent relative expressions compared to parental control cells. Mean ± standard error.

**Figure 6: Targeting the NF-κB signalling axis hinders Sox9 expression and androgen-independent proliferation of PCa cells.**

**A,** Western blot analysis indicates consistent activating cleavage of NF-κB transcriptional regulator p100 to p52 in LNCaP, LAPC4, and VCaP stem-like cells compared to parental controls. Representative western blot analysis (left), and p52/p100 ratio based on optical density of western blot bands (right). **B,** Western blot indicates consistent activating phosphorylation of NF-κB transcriptional regulator p65 in LNCaP, LAPC4, and 22Rv1 stem-like cells compared to parental controls. **C,** Western blot analysis indicates that increasing doses of Bortezomib, reduces expression of Sox9 under androgen deprived (CSS and STM) conditions. **D,** Representative Bortezomib dose response proliferation assays indicate that LNCaP cells in CSS are sensitized to Bortezomib treatment. **E,** Cell cycle analysis with Bortezomib treatments indicate a dose
dependent increase in Sub-G0 populations in LNCaP cells cultured in FBS or CSS, with concomitant rise in S-Phase population. F, Graphical abstract of therapy-induced, transient Sox9 expression in PCa cells, leading to disease progression.

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References


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Figure 1

A. Western blot analysis of Rb1, p53, and GAPDH in LNCaP-shP53 and LNCaP-shRb1.

B. mRNA expression levels of TP53 and RB1 in different cell lines: LNCaP-P, LNCaP-SL, VCaP-P, VCaP-SL, LNCaP-shP53, and LNCaP-shRb1. The mRNA fold change is compared to the LNCaP-P control.

C. mRNA expression levels of SOX2 and SOX9 in different cell lines: LNCaP-shCTRL, LNCaP-shP53, LNCaP-shRb1, and LNCaP-shP53-Rb1. The mRNA fold change is compared to the LNCaP-shCTRL control.

D. Western blot analysis of Sox9 and GAPDH in LNCaP-shP53 and LNCaP-shRb1.

E. mRNA expression levels of RB1, TP53, SOX2, SOX9, and COL2A1 in different cell lines: LNCaP-shCTRL, LNCaP-shP53, LNCaP-shRb1, and LNCaP-shP53-Rb1. The mRNA fold change is compared to the LNCaP-shCTRL control.

F. Western blot analysis of Rb1, Sox9, p53, and Vinculin in shCTRL, shP53, shRb1, and shRb1/P53 groups.

G. Confluence percentage over time in different conditions: shCTRL + FBS, shCTRL + CSS, shP53 + FBS, and shP53 + CSS. The confluence is compared to the shCTRL + FBS control.

H. Relative spheroid area over time in different conditions: shCTRL + FGF/EGF, shCTRL + STM, shP53 + FGF/EGF, and shP53 + STM. The relative spheroid area is compared to the shCTRL + FGF/EGF control.
Figure 2

**A**

![Graph showing Relative Spheroid Area over Day](image)

**B**

![Graph showing Relative Spheroid Area over Day](image)

**C**

![Graph showing Confluence Fold Change over Hours](image)

**D**

![Bar graph showing mRNA Fold Change](image)

**E**

![Histogram showing Cells on Filter](image)

**F**

![Graph showing Neurite Length (mm/mm²) over Hours](image)

**G**

![Graph showing Neurite Branch Points (1/mm²) over Hours](image)
Figure 3

A

B

C

D

LTL331 (NEPC, AR+)

- SOX9
- KLK3

LTL313B (CRPC, AR+)

- SOX9
- KLK3

Fold Change Relative to Time Zero

Fold Change Relative to Time Zero

Pre-Cx Post-Cx Recurrent

Research.
**Figure 5**

A. Sox9 and Ki67 immunohistochemistry (IHC) images for untreated, NHT-treated, and CRPC conditions.

B. Sox9 IHC score in untreated, NHT-treated, and CRPC conditions.

C. PSA recurrence-free survival graph showing different Sox9 expression levels (Low vs. Med/High).

D. Ki67 immunoreactivity graph comparing untreated, NHT-treated, and CRPC conditions.

E. Relative luminescence intensity for LNCaP, VCaP, and LAPC4 cells treated with FBS and STM.

F. mRNA fold change for NFKB1, NFKB2, RELA, RELB, IL8, and FN1 in LNCaP-SL, VCaP-SL, LAPC4-SL, and 22RV1-SL conditions.
Figure 6

A. Western blot analysis showing the expression of p100, p52, Vinc, p65, and P-p65 proteins in different cell lines.

B. Graph showing the relative protein signal for parental vs. stem-like p100 cleavage.

C. Table comparing gene expression levels in LNCaP cells under different conditions.

D. Graph illustrating the fold change in gene expression under various conditions.

E. Bar graph showing the percentage of cells in different phases of the cell cycle under different conditions.

F. Diagram depicting the transformation of normal prostate cells to prostate adenocarcinoma, followed by targeted therapy and neural crest stem-like intermediate, leading to castrate resistant prostate cancer.

Research.

Clinical Cancer Research

Transient Sox9 Expression Facilitates Progression to Castrate Resistant Prostate Cancer

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