The Steroid Receptor Coactivator-3 Is Required for the Development of Castration-resistant Prostate Cancer

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Running title: SRC-3 in CRPC Development

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ABSTRACT

The transcriptional coactivator SRC-3 plays a key role to enhance prostate cancer (CaP) cell proliferation. Although SRC-3 is highly expressed in advanced CaP, its role in castration resistant CaP (CRPC) driven by PTEN mutation is unknown. We documented elevated SRC-3 in human CRPC and in PTEN-negative human CaP. Patients with high SRC-3 and undetectable PTEN exhibited decreased recurrence-free survival. To explore the causal relationship in these observations, we generated mice in which both Pten and SRC-3 were inactivated in prostate epithelial cells (Pten3CKO mice), comparing them to mice in which only Pten was inactivated in these cells (PtenCKO mice). SRC-3 deletion impaired cellular proliferation and reduced tumor size. Notably, while castration of PtenCKO control mice increased the aggressiveness of prostate tumors relative to non-castrated counterparts, deletion of SRC-3 in Pten3CKO mice reversed all these changes. In support of this finding, castrated Pten3CKO mice also exhibited decreased levels of phospho-Akt, S6 kinase (RPS6KB1) and phosphorylated S6 protein (RPS6), all of which mediate cell growth and proliferation. Moreover, these tumors appeared to be more differentiated as evidenced by higher levels of Fkbp5, an AR-responsive gene that inhibits Akt signaling. Lastly, these tumors also displayed lower levels of certain androgen-repressed genes such as cyclin E2 and MMP10. Together, our results show that SRC-3 drives CRPC formation and offer preclinical proof of concept for a transcriptional coactivator as a therapeutic target to abrogate CRPC progression.
INTRODUCTION

Prostate cancer (CaP) is the most commonly diagnosed cancer and the leading cause of cancer death in American men. Virtually all mortality results from advanced tumors that invade adjacent organs and metastasize to distant sites. Androgen ablation therapy is the first-line treatment for advanced CaP. Despite transient regression, most tumors recur and become hormone-refractory with androgen-independent phenotype termed “castration resistant CaP” (CRPC). Since the molecular basis of CRPC remains unknown, a mechanistic understanding of its formation will be crucial for designing novel CRPC therapies.

Steroid Receptor Coactivator-3 (SRC-3, AIB1, ACTR or NCOA3) is a member of the p160 SRC family (1). Members of this family, which also includes SRC-1 (NCOA1) and SRC-2 (TIF2, GRIP1 or NCOA2), mediate transcriptional functions of nuclear receptors and other transcription factors like E2F1, PEA3 and AP-1 (2-5). While SRC-1 amplification in human cancer is rare, amplification of the SRC-2 or SRC-3 gene in human cancers is frequent (1, 6, 7). In breast cancer, SRC-3 is an established oncogene. Clinical studies have revealed SRC-3 amplification significantly correlates with tumor size. SRC-3 is overexpressed in 35% of ductal carcinoma in situ (DCIS), 31% of invasive tumors and 38% of metastatic disease (1, 8). In mice, knockout of SRC-3 decreases somatic growth and mammary gland development (9, 10) and delays v-Ha-ras-induced breast cancer initiation and progression (11). Conversely, SRC-3 overexpression results in spontaneous mouse mammary tumors, as well as tumors in other organs (12). SRC-3 also promotes other hormone-related cancers such as endometrial carcinoma and ovarian cancer, in addition to hormone-independent esophageal squamous cell carcinoma, gastric...
cancer, colorectal carcinoma and pancreatic cancer (13).

In CaP, increased SRC-3 expression is associated with a more aggressive phenotype and worse prognosis (14, 15). In addition, studies in CaP cell lines like LNCaP and PC-3 have highlighted the relevance of SRC-3 in promoting CaP proliferation through IGF-Akt signaling pathway (16). SRC-3 also promotes CaP cell migration and invasion by coactivating transcriptional activity of AP-1 and PEA3, transcription factors that upregulate targets including MMPs (17). Our group has demonstrated that global knockout of SRC-3 in TRAMP (transgenic adenocarcinoma of the mouse prostate) mice arrested prostate tumor progression at the well-differentiated stage (18). One caveat of this model lies in the fact that global SRC-3 knockout causes somatic growth retardation accompanied by significant decrease in IGF-1 level (9, 19). This makes it difficult to distinguish the cell-autonomous role of SRC-3 from possible impact of systemic factors such as IGF-1. In addition, the function of SRC-3 in CRPC has not been investigated.

The PTEN tumor suppressor gene is mutated in 50% of human CaP. In addition, 70% of late stage CaP show altered PTEN/PI3K/AKT pathway (7). The mouse model harboring prostate-specific deletion of Pten faithfully mimics human CaP, recapitulating well-defined stages of hyperplasia, prostatic intraepithelial neoplasia (PIN) and invasive carcinoma (20). Since SRC-3 plays a key role in CaP but has not been investigated in the setting of PTEN deletion and/or castration, we aim to characterize the expression and molecular interaction relationships between SRC-3 and PTEN in human and mouse CaP, while also defining the role of SRC-3 in CRPC development. We report here that SRC-3 expression is elevated in human CRPC and negatively correlated with PTEN expression and recurrence-free survival. Specific knockout of SRC-3 in Pten deletion-induced prostate tumors effectively prevents tumors from
developing into CRPC.
METHODS

Generation of Pb-Cre;\textit{Pten}^{\textit{f/f}};\textit{SRC-3}^{\textit{f/f}} and Pb-Cre;\textit{Pten}^{\textit{f/f}} mutant mice

The \textit{SRC-3} conditional line (\textit{SRC-3}^{\textit{f/f}}) was previously generated in our lab (21). \textit{Pten}^{\textit{f/f}} mice were reported previously (20) and obtained from Jackson Laboratory. ARR2PBi-Cre (Pb-Cre) mice were described previously (22). \textit{SRC-3}^{\textit{f/f}} mice were first crossed with \textit{Pten}^{\textit{f/f}} mice to generate \textit{SRC-3}^{\textit{f/+}} and \textit{Pten}^{\textit{f/+}}. The F1 mice were then either inbred to generate \textit{Pten}^{\textit{f/f}} and \textit{SRC-3}^{\textit{f/+}} or crossed with Pb-Cre mice to obtain Pb-Cre;\textit{Pten}^{\textit{f/+}};\textit{SRC-3}^{\textit{f/+}} mice. The F2 mice are crossed to generate experimental Pb-Cre;\textit{Pten}^{\textit{f/f}};\textit{SRC-3}^{\textit{f/f}} and control Pb-Cre;\textit{Pten}^{\textit{f/f}};\textit{SRC-3}^{\textit{f/+}} mice. All animal protocols were approved by the Animal Care and Use Committee of Baylor College of Medicine.

Castration

Twelve-week-old male mice were anesthetized by i.p. injection of Avertin (2.5% in saline, 15 \textmu l/g body weight). Mice were castrated via a scrotal incision as described (18).

Tissue analysis

Prostate tumors in experimental and control mice were analyzed at 9, 12, 18 and 24 weeks of age. Prostate tumors in mice for the androgen deprivation experiment were examined 6 weeks post-castration. The lower urogenital tracts were dissected out in a Petri dish containing phosphate-buffered saline (PBS). Prostate tissues were isolated and each lobe was separated and weighed. Prostate lobes from one side were fixed in 4% paraformaldehyde at 4\textdegree C overnight, dehydrated, processed, paraffin-embedded, and sectioned in 5-\textmu m thickness for H&E staining,
Trichrome staining and immunohistochemistry. Lobes from the other side were frozen in liquid nitrogen and stored at -80°C for RNA and protein analyses.

**Histology and immunohistochemistry**

Tissue sections were stained with H&E and Trichrome as described previously (18, 23). For immunohistochemistry, antigen retrieval was performed (in a microwave oven) using 10 mM sodium citrate, pH6. The sections were then rinsed with PBS and blocked with either 10% normal serum or M.O.M (Vector Laboratories) kit for 1 hour at room temperature. Sections were incubated with primary antibody overnight at 4°C. Primary antibodies were against SRC-3 (Cell Signaling, #2126), AR (Santa Cruz, sc-816), Ki67 (BD Biosciences, 550609), p63 (Santa Cruz, sc-8431), Troma-1 (K8) (DSHB), E-Cadherin (BD Biosciences, 610182), smooth muscle α-actin (SMA) (DAKO, M0851), CD31 (abcam, ab28364), phospho-S6 (Cell Signaling, #5364), cytokeratin 5 (K5) (Covance, PRB160P) and Vimentin (abcam, ab8978). Secondary antibodies were obtained from Vector labs and diluted 1:400. The signal was enhanced using the Vectastain ABC system and visualized with DAB kit. The slides were counterstained with Harris Modified Hematoxylin. For double fluorescence staining, Tyramide Signal Amplification kit (Life technologies) was used per manufacturer’s instructions.

**RNA isolation and qRT-PCR analysis**

Total RNA was prepared from frozen prostate tissues using the TRIzol reagent (Life technologies). cDNA was generated using Eurogen reverse transcriptase kit and real time quantitative RT-PCR (qRT-PCR) analysis was performed using the TaqMan system (Roche).
Immunoblotting

Dissected mouse prostate samples were snap-frozen in liquid nitrogen. Protein was isolated in RIPA buffer (150 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris-HCl; pH 8.0). Twenty μg of each protein sample was loaded per lane in a polyacrylamide gel. Primary antibodies for immunoblotting were against phospho-Akt (Cell Signaling, #4060), Akt (Cell Signaling, #9272), SRC-3 (Cell Signaling, #2126), phospho-S6 ribosomal protein (Cell Signaling, #5364), S6 Ribosomal protein (Cell Signaling, #2217), p70 S6 Kinase (Cell Signaling, #2708) and β-actin (Sigma, A1978).

Cell culture

PC-3 cells at passage 25 were obtained from American Type Culture Collection (Manassas, VA), used for experiment at passage 77, and validated by STR DNA fingerprinting with an AmpFISTR Identification kit (Applied Biosystem) in the University of Texas MD Anderson Cancer Center Characterized Cell Line Core. PC-3 cells were cultured in DMEM/F12 medium containing 10% FBS. SRC-3 knockdown was achieved by transfection with siGENOME SMARTpool siRNAs targeting hSRC-3 (Dharmacon), using Lipofectamine 2000 (Invitrogen). Non-targeting siRNAs (siGENOME SMARTpool control) was used as the control.

Databases and statistical analysis

We used Cancer Microarray datasets in Oncomine database to study the expression profiles of SRC-3, PTEN and S6K1 in human CaP (24). The gene expression data were log transformed, median centered per array and the standard deviation was normalized to one per array. The database results were analyzed using GraphPad Prism Software (San Diego, USA).
Statistical analysis comparisons were done with Student’s t-test for tissue weight and gene expression.

RESULTS

SRC-3 expression correlates negatively with PTEN expression and prostate-specific antigen (PSA) recurrence-free survival in CaP patients

We downloaded two sets of expression data published by Tomlins et al. and Chandra et al. (25, 26) from Oncomine database (24) and compared SRC-3 expression between primary prostate tumors and CRPC samples. In both data sets, SRC-3 mRNA expression is significantly higher in CRPC tumors versus primary tumors (Fig. 1A, B), suggesting SRC-3 may play an important role in CaP progression. Statistical analysis of Tomlins’s data also showed SRC-3 expression is negatively associated with PTEN expression (Fig. 1C). Analysis of another Oncomine data set published by Grasso et al. (27) further demonstrated an elevated SRC-3 mRNA in prostate tumors with PTEN deletion (Fig. 1D). At the protein level, SRC-3 and PTEN had been examined by IHC in a set of tissue microarrays from 480 radical prostatectomy specimens in Dr. Ittmann’s laboratory, although data on these two proteins were reported separately (15, 28). Here, analysis of these protein expression data revealed a significant negative correlation between higher SRC-3 expression (index > 4) and PTEN levels (rho = -0.156; p = 0.013; Spearman’s) in the same CaP cohort. Furthermore, we found patients with high SRC-3 and no PTEN protein had a significantly higher risk of PSA recurrence versus patients with low SRC-3 and any PTEN levels (p<0.0001). Patients with high SRC-3 and detectable PTEN proteins were not significantly different from either group, possibly due to small sample size.
(number of events = 7), however it appeared more similar to the low risk group with low SRC-3 and any PTEN levels (p=0.2850) rather than the high risk group with high SRC-3 and no PTEN (p=0.0703) (Fig. 1E). These results indicate SRC-3 expression positively correlates with CRPC development and negatively correlates with PTEN expression and PSA recurrence-free survival of CaP patients.

**Conditional knockout of SRC-3 in PECs does not affect prostate morphogenesis but inhibits Pten loss-induced prostate tumor growth**

To define the specific role of SRC-3 in PECs, we first generated $SRC-3^{ff}$ and PB-Cre;$SRC-3^{ff}$ mice. Immunohistochemistry detected SRC-3 protein in PECs of wild type and $SRC-3^{ff}$ mice, while it did not detect SRC-3 in luminal epithelial cells (LECs) of PB-Cre;$SRC-3^{ff}$ mice when examined at 8 weeks of age (Fig. S1A). This indicated SRC-3 was specifically and efficiently deleted in PECs of PB-Cre;$SRC-3^{ff}$ mice. In agreement with nearly normal prostate development in $SRC-3$ null mice (18), conditional knockout of $SRC-3$ in PECs did not significantly change prostate morphogenesis (Fig. S1B).

To evaluate the contribution of SRC-3 to prostate tumorigenesis induced by Pten loss, we generated PB-Cre;$Pten^{ff}$ (designated as PtenCKO hereafter) and PB-Cre;$Pten^{ff},SRC-3^{ff}$ (designated as Pten3CKO hereafter) mice by interbreeding PB-Cre, $Pten^{ff}$ and $SRC-3^{ff}$ mouse lines. In prostates of 12-week-old Pten3CKO mice, SRC-3 protein was absent in virtually all LECs but retained in some basal cells (Fig. 2A). $SRC-3$ mRNA was reduced about 50% versus PtenCKO prostates (Fig. 2B). This is consistent with LEC-specific $SRC-3$ deletion, with $SRC-3$ retention in the basal and stromal compartments. We assessed the impact of $SRC-3$ ablation on tumor growth by comparing weights of Pten3CKO and PtenCKO prostates harvested at 6, 9, 12,
18 and 24 weeks of age (Fig. 2C). Indeed, prostate weight was lower by more than 50% in Pten3CKO versus PtenCKO mice at 18 and 24 weeks. Tumor growth in the anterior prostate (AP) lobes principally accounted for this difference in tumor size (Fig. 2D). In contrast, AP tumor histology was not markedly different between groups, with the exception of an increase in large cystic spaces seen in PtenCKO prostates at 18 week (Fig. 2D).

To understand the cause of the tumor size difference, we quantified cellular proliferation and apoptosis. Ki67 immunohistochemistry detected nearly twice as many proliferating cells in 18-week PtenCKO versus Pten3CKO tumors, translating into proliferation indices of 9% and 5%, respectively (Fig. 3A, B). However, no difference existed between the numbers of apoptotic cells in these groups (data not shown). Immunohistochemistry for cell type-specific biomarkers revealed that Pten3CKO tumors had a dramatic increase in the percentage of p63(+) basal cells versus PtenCKO tumors (Fig. 3B, C). This correlated with an increased ratio of the cytokeratin 5 (K5)(+) basal cell number to the K8(+) tumor cell number, which was mainly caused by the decreased K8(+) tumor cell number in Pten3CKO mice (Fig. 3C, D). No change was observed in AR distribution (Fig. 3C, D). In sum, SRC-3 knockout yields smaller tumors with lower proliferation index and a specific decrement in K8-positive luminal tumor cells. This is coupled with relative expansion of the basal cell compartment.

Castration increases cellular proliferation, stromal reactivity and tumor cell dedifferentiation in Pten loss-induced mouse CaP

We evaluated tumor progression and determined that epithelial hyperplasia, low PIN, high PIN and cancer developed in PtenCKO mice at 6, 9, 12 and 18 weeks of age, respectively (Fig. 4A). Therefore, in order to establish a CRPC model we surgically castrated PtenCKO mice
at 12 weeks of age so that tumors would develop from the high PIN stage in an androgen-depleted environment. Previous reports demonstrate that PtenCKO prostate tumors initially respond to androgen ablation, as indicated by increased cell death and reduced size. However, after prolonged castration, tumors re-grow and develop into CRPC (20). In the current study, we analyzed tumors at 18 weeks of age. We found that prolonged castration indeed increased cellular proliferation, while also promoting stromal infiltration and disorganized PEC morphology (Fig. 4B). Invasive adenocarcinoma increases reactive stroma by inducing a desmoplastic response in which collagen is deposited in the surrounding stromal tissue (23). Therefore, we measured this response using Mason’s Trichrome, which stains collagen blue and fibrin pink. We found significantly increased blue staining in tumors from castrated versus non-castrated mice (Fig. 4C). Another marker for reactive stroma is SMA. Loss of the dense SMA layer usually corresponds with tumor invasiveness. Here, we found SMA distribution in castrated tumors is less dense and exhibits a disorganized multilayer pattern, while staining in the non-castrated tumor is a dense, well-defined monolayer boundary that surrounds PECs (Fig. 4C). In addition, immunohistochemistry for angiogenesis marker CD31 identified significantly more endothelial cells in tumors from castrated versus non-castrated mice (Fig. 4C). These results suggest castration of PtenCKO mice increases cellular proliferation and aggressiveness of prostate tumors.

To further characterize the castration-induced phenotype, we performed immunohistochemistry for epithelial differentiation markers such as AR, p63 and E-cadherin. AR is more widely expressed in tumor cells of non-castrated mice, also stained more intensely in these cells versus those of castrated mice. Conversely, the number of p63(+) basal cells is higher in tumors of castrated mice. E-cadherin expression is lower in individual cells in castrated versus
non-castrated tumors, but total numbers of E-cadherin(+) cells are not significantly different between two groups (Fig. 4D). These results indicate that prostate tumors from castrated mice are less differentiated than tumors from age-matched non-castrated mice. These results, together with the increased proliferation and reactive stroma, indicate that the Pten loss-induced prostate tumors undergo structural remodeling and molecular reprogramming in response to androgen ablation.

**Deletion of SRC-3 significantly reduces tumor size and cellular proliferation in CRPC**

As shown in Fig. 2C, prostate tumor weights were not significantly different in PtenCKO and Pten3CKO mice before 12 weeks of age. At 12 weeks, these tumors were also similar in histology, consisting only of PIN lesions (Fig. S2). Immunohistochemistry confirmed SRC-3 expression in tumor, luminal epithelial and basal cells in PtenCKO mice and the efficient SRC-3 knockout in tumor cells and LECs in Pten3CKO mice (Fig. S2). In keeping with the similar tumor size at this stage, Ki67 proliferation indices were comparable in PtenCKO and Pten3CKO prostate tumors (Fig. S2). Given these results showing only subtle impact of SRC-3 deletion on prostate tumor growth and histology, we decided to perform surgical castration on both PtenCKO and Pten3CKO mice at 12 weeks of age, a time point prior to the development of invasive adenocarcinoma, to assess the role of SRC-3 in CRPC.

When prostate and tumor samples were analyzed at 18 weeks of age (6 weeks post-castration), we found that tumors from Pten3CKO mice were much smaller than those from PtenCKO mice. Pten3CKO prostates were only 30% the weight of PtenCKO prostates (Fig. 5A). SRC-3 protein was highly expressed in tumor cells of castrated PtenCKO mice but absent in all tumor cells and LECs of castrated Pten3CKO mice. PtenCKO tumors had substantial stromal
infiltration with tumor cells aggressively invading stromal tissue. On the contrary, Pten3CKO tumors retained histology characteristic of non-castrated mice, exhibiting well-differentiated glandular structures with a clear basement membrane boundary (Fig. 5B). Importantly, Ki67 immunohistochemistry also demonstrated significantly fewer proliferating cells in Pten3CKO versus PtenCKO tumors under castration conditions. Statistical analysis showed Ki67 proliferation index of Pten3CKO tumors was reduced 75% versus that detected in PtenCKO tumors (Fig. 5B). In conclusion, deletion of SRC-3 drastically reduced tumor size and prevented transition from a benign to an aggressive tumor during development of CRPC.

**Deletion of SRC-3 reverses castration-induced changes in tumor cell type and stromal reactivity**

To further characterize the role of SRC-3 in CRPC development, we compared cellular components, epithelial differentiation markers and stromal reactivity in prostate tumors from castrated PtenCKO and Pten3CKO mice. Castration caused an increase in the number of p63(+) cells inside both the basal cell layer and the tumor mass in PtenCKO prostates. Double staining of p63 and SRC-3 in castrated PtenCKO tumors revealed clusters of p63(+) cells, the majority of which co-express SRC-3. On the contrary, in castrated Pten3CKO tumors, the number of p63(+) cells was significantly lower, and no co-localization with remnant SRC-3(+) cells was visible (Fig. 5C). AR staining was more intense and distributed among more cells in Pten3CKO versus PtenCKO tumors, indicating cancer cells in Pten3CKO animals were more differentiated (Fig. 5C). Vimentin/SMA double staining is a measure of reactive stroma (23). Both vimentin and SMA immunoreactivities were increased, while their distribution patterns were more disorganized in PtenCKO tumors versus Pten3CKO tumors. In Pten3CKO tumors, few
vimentin(+) cells were detected and SMA immunostaining signal showed a monolayer surrounding the ductal glands (Fig. 5C). Trichrome staining also revealed significant collagen deposit in PtenCKO tumors, while none were present in Pten3CKO tumors (Fig. 5D). E-cadherin, an epithelial marker, was higher in Pten3CKO than PtenCKO tumors, while immunostaining for CD31, which indicates the extent of angiogenesis, demonstrated more blood vessels in PtenCKO versus Pten3CKO tumors (Fig. 5D). Taken together, these results demonstrate that specific deletion of SRC-3 inhibits reactive stromal infiltration and tumor cell de-differentiation in castrated mice.

**SRC-3 deletion downregulates Akt-mTOR signaling in CRPC tumors**

We next aimed to elucidate the molecular mechanism by which SRC-3 promotes cell proliferation, tumor growth and CRPC progression. It is known that SRC-3 enhances Akt activation by up-regulating multiple components of the IGF-I-signaling pathway in breast and prostate cancers (11, 16). Indeed, tumors from castrated Pten3CKO mice had significantly reduced phosphorylated Akt versus tumors from castrated PtenCKO mice (Fig. 6A), suggesting SRC-3 is also required for Pten loss-induced Akt activation in the setting of castration. Phosphorylated ribosomal protein S6 (pRPS6) is an indicator of Akt-mTOR pathway activity. Immunohistochemistry demonstrated that pRPS6 was widely distributed in the prostatic epithelial and tumor cells of PtenCKO mice but reduced more than 7-fold in the same cell populations of Pten3CKO mice (Fig. S3). Immunoblotting performed on whole-tumor lysates confirmed this finding (Fig. 6B). RPS6 is phosphorylated by S6 kinase 1 (S6K1), a protein with both cytosolic (p70) and nuclear (p85) isoforms generated from alternative usage of two ATG codons within the same mRNA (29). We found that, while the level of cytosolic p70S6K1 was
unchanged between PtenCKO and Pten3CKO tumors, the level of nuclear p85S6K1 was significantly lower in Pten3CKO tumors (Fig. 6B). In cultured AR-negative PC-3 CaP cells, SRC-3 knockdown also reduced phospho-Akt, pRPS6, as well as p70S6K1 and p85S6K1 kinases (Fig. 6C). These results indicate that in CRPC, SRC-3 knockout inhibits the Akt-mTOR pathway, potentially at multiple steps.

We next assessed S6K1 at the mRNA level in vivo. S6K1 mRNA was reduced 40% in Pten3CKO tumors versus PtenCKO tumors from castrated mice (Fig. 6D). To evaluate the relationship between SRC-3 and S6K1 expression in human CaP, we downloaded a set of data with clear readouts of both SRC-3 and S6K1 mRNA expression in prostate tumors from Oncomine by Taylor et al (7). Analysis of this data set indicated SRC-3 expression positively correlated with S6K1 mRNA expression in human prostate tumors (p<0.0001) (Fig. 6E). These results support the notion that SRC-3 promotes S6K1 expression in CaP, although it is currently unclear whether S6K1 is a direct or indirect SRC-3-regulated gene.

**Targeting SRC-3 differentially affects the expression of a subgroup of AR-regulated genes promoting differentiation in the mouse CRPC**

Increased Akt-mTOR pathway activity characteristic of Pten ablation-driven tumorigenesis inhibits the androgen-signaling pathway (30). Our data demonstrate SRC-3 is overexpressed in CRPC and enhances the Akt-mTOR pathway in CaP, while previous reports indicate SRC-3 coactivates AR-mediated transcription in cultured CaP cell lines under androgen-depleted conditions (31). In order to investigate the impact of SRC-3 on AR signaling in vivo, we compared androgen signaling in PtenCKO and Pten3CKO prostate tumors from castrated mice at 18 weeks of age. AR protein levels were comparable between the two tumor types (Fig. S4A).
We then evaluated a group of differentiation genes including *probasin*, *Nkx3.1*, *FKBP5*, *Ets2* and *Igfbp-3* (30). Among AR-activated genes, the expression levels of *probasin* and *Nkx3.1* were similar in the two tumor types (Fig. S4B), while the expression of *Fkbp5*, a specific inhibitor of Akt signaling, was significantly increased in Pten3CKO versus PtenCKO tumors from castrated mice (Fig. 7A). AR-repressed genes such as *Ets2* and *Igfbp-3* were significantly downregulated in Pten3CKO versus PtenCKO tumors from castrated mice at 18 weeks (Fig. 7B). Furthermore, a broader panel of AR-repressed genes that were previously reported to be overexpressed in human CRPC (32) was evaluated and the results were summarized in Fig. 7C. Among these genes, *TK1* expression was significantly upregulated in Pten3CKO versus PtenCKO tumors, while *MMP10* and *cyclin E2* expression levels were significantly lower in Pten3CKO versus PtenCKO tumors in castrated mice (Fig. 7C). These results demonstrate that SRC-3 knockout causes an increase in expression of specific AR-activated genes while yielding either increases or decreases in certain AR-repressed genes.

**DISCUSSION**

Advanced CaP responds initially to androgen deprivation therapy but eventually recurs as hormone-refractory CRPC. Since CRPC is highly metastatic and incurable, understanding its molecular mechanism is crucial for designing effective therapies for advanced CaP. The SRC family members coactivate nuclear receptors such as estrogen receptor and AR (1, 6, 7) and other transcription factors like E2F1, AP-1 and PEA3 to drive cancer growth and progression (1-3, 16, 17, 33, 34). Studying SRC coactivators in CRPC may therefore identify molecular targets for treating both androgen-dependent CaP and CRPC.

SRC-3 expression is elevated in clinical CaP samples, correlating positively with Gleason
score and negatively with prognosis (14, 15, 35). Here, we demonstrate elevated SRC-3 expression in human CRPC and CaP with PTEN deletion and its negative correlation with PTEN expression. Moreover, we show patients with tumors characterized by high SRC-3 and no PTEN expression have worse recurrence-free survival rates than patients with detectable PTEN and either low or high SRC-3 expression. These findings suggest SRC-3 can cooperate with PTEN loss to promote CRPC development, resulting in poor recurrence-free survival.

We previously reported global knockout of murine SRC-3 caused arrest of SV40 T/t-induced prostate tumor progression at well-differentiated stages (18). Here, we demonstrate prostate epithelium-specific knockout of SRC-3 in Pten loss-induced tumors does not significantly inhibit prostate tumor growth at early stages (before 12 weeks of age), but significantly reduces cell proliferation and tumor growth after 18 weeks of age, without affecting histopathological progression of tumors. Taken together, these results suggest oncogenic potential of SRC-3 is both intrinsic to tumor cells and dependent on non-tumor cells (perhaps via paracrine or endocrine growth signaling). As such, SRC-3 may well be a viable drug target in multiple cell types.

Androgen depletion causes marked acceleration of prostate tumorigenesis in Nkx3.1+/−;Pten+/− mutant mice (36). Prostate tumors exposed to low-level androgen share similar gene expression profiles with androgen-independent prostate tumors, suggesting androgen ablation may promote prostate tumorigenesis by selecting for molecular events that promote more aggressive, hormone-refractory tumors (36). In this study, we also investigated the effect of androgen deprivation on CRPC development in PtenCKO mice and found androgen deprivation makes Pten loss-induced prostate tumors more aggressive. Specifically, castration significantly increased cell proliferation and stromal reactivity, while making tumors less differentiated based
on epithelial markers. These findings indicate changes in both the tumor and microenvironment, suggesting an androgen depletion-induced interaction between tumor cells and stroma. Remarkably, specific knockout of SRC-3 in tumor epithelial cells of castrated Pten3CKO mice abolished virtually all changes induced by androgen deprivation. SRC-3 deletion inhibited tumor cell proliferation and tumor growth, suppressed stromal reactivity, reduced tumor cell invasiveness and, finally, prevented Pten3CKO tumor cells from undergoing de-differentiation. The cellular and molecular events responsible for these effects resulted from epithelial knockout of SRC-3. Tumor phenotype as a whole likely results from intrinsic effects of SRC-3 ablation in these cells and reciprocal signaling between SRC-3-deficient epithelial cells and an SRC-3-replete stroma. In assessing the intrinsic role of SRC-3 in tumor epithelial cell proliferation, we took note of previous studies demonstrating the ability of SRC-3 to upregulate c-Myc and cyclin D1 expression and facilitate HER2 and EGFR activation in breast cancer cells, as well as enhance AKT activation in breast and prostate cancer cells (11, 16, 37-39). On the contrary, the possible role of SRC-3 in epithelial-stromal interaction has not been investigated. As for reciprocal interaction between the tumor and stroma, several growth factors and cytokines including TGF-beta1, CTGF, IL-6, and FGF1/2/7/9/10/17 and FGFR1 have been implicated in induction of stromal reactivity (40, 41). Despite this, our qPCR measurements could not identify any significant corresponding expression changes of these paracrine factors between tumors of castrated Pten3CKO and PtenCKO mice (data not shown). Therefore, it is currently unclear how SRC-3 in the epithelial tumor cells stimulates stromal reactivity and whether reactive stroma reciprocally promotes CRPC development in the setting of castration. Further assessment of the important but complex mechanisms underlying these processes will warrant future study. In all, these findings indicate SRC-3 is required for CRPC development in Pten loss-induced prostate
tumors, suggesting SRC-3 as a potential therapeutic target for preventing CRPC development in the context of anti-androgen therapy.

The activation of PI3K-Akt-mTOR pathway is critical for cancer cell survival, proliferation and metastasis. Previous studies reported that SRC-3 activates PI3K/Akt pathway through upregulating of IGF-I-signaling components including IGF-I, IGF-IR, IRS-1 and IRS-2 (11, 12, 16). The inability of the current study to find altered IGF1R and IRS-1/2 expression was probably due to Pten loss-induced hyper activation of PI3K-Akt pathway (data not shown). Nonetheless, we discovered decreased Akt activity and S6K1 expression in Pten3CKO versus PtenCKO tumors in castrated mice, as well as a positive correlation between SRC-3 and S6K1 expression in clinical CaP samples. Accordingly, p85S6K1 protein and RPS6 phosphorylation are significantly reduced in SRC-3-depleted mouse and human CaP cells. Although it is currently unclear why only p85S6K1 but not p70S6K1 protein is reduced in Pten3CKO tumors in castrated mice, these results suggest SRC-3 can upregulate Akt-mTOR pathway by promoting Akt activation and upregulating S6K1 expression.

In androgen-sensitive LNCaP and insensitive C4-2 cells, knockdown of SRC-3 reduces AR-activated 

\[ FKBP5 \]

and \n
\[ PSA \]

expression, suggesting SRC-3 acts as an AR coactivator in these cells [data not shown and reference (31)]. Previous \textit{in vitro} studies also showed SRC-3 and other SRC family members are authentic AR coactivators in cell lines (42-44). Intriguingly, our \textit{in vivo} data do not provide a clear role of SRC-3 in AR function in the mouse prostate. First, prostate morphogenesis and growth in SRC-3 null mice are essentially normal, and androgen-induced prostate regeneration in castrated SRC-3 null mice is also unaffected (18). Second, expression of AR target genes is largely unchanged in mouse prostates with epithelial SRC-3 knockout as well as in Pten3CKO tumors of non-castrated mice (data not shown). Third, knockout of SRC-3 in
Pten3CKO tumors of castrated mice has no influence on most AR-regulated genes (Fig. S4B and Fig. 7C) and does not show unidirectional effects on either AR-activated or AR-repressed genes. SRC-3 knockout can even be seen to upregulate the expression of AR-activated *Fkbp5* gene and the AR-repressed *TK1* gene while downregulating the expression of AR-repressed genes such as Ets2, Igfbp3, MMP10 and cyclin E2. The overall effect of these gene expression changes is to promote differentiation and suppress proliferation and extracellular matrix remodeling. Taken together, our results are unable to support a straightforward role of SRC-3 in AR function in the prostate epithelial and tumor cells in mice. Under physiological conditions, SRC-1 and SRC-2 may compensate SRC-3 deficiency for supporting AR transcriptional activity. It is also possible that Pten loss- and SRC-3 knockout-induced Akt/mTOR/S6K1 activation may modify the interactive features between AR and SRC-3 and their specificity and activity on different target gene promoters, resulting in differential effects on the expression of AR-activated and AR-repressed genes. In addition, SRC-3 knockout decreases the PI3K/Akt/mTOR signaling pathway, which may partially reduce the inhibitory effect of this pathway on AR signaling pathway since these two pathways are reciprocally inhibited (30, 45). Conversely, the increased *Fkbp5* expression may be partially responsible for SRC-3 knockout-induced inhibition of Akt-mTOR signaling since Fkbp5 inhibits Akt activity through stabilizing Phlpp phosphatase (46-48).

In summary, SRC-3 expression is elevated in CRPC and negatively correlated with PTEN expression and recurrence-free survival of CaP patients. SRC-3 is required for CRPC development induced by Pten deficiency in castrated mice. SRC-3 may promote CRPC principally through enhancing Akt activity and S6K1 expression. Therefore, inhibiting SRC-3 is a promising future strategy for abrogating CRPC development resulting from anti-androgen or anti-AR therapy.
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FIGURE LEGENDS

Fig. 1. SRC-3 expression is elevated in human hormone refractory prostate cancer (HRPC) and negatively correlated with PTEN expression and recurrence-free survival.  

A. and B. SRC-3 expression in human HRPCs and primary prostate tumors. The mRNA expression data sets by Chandra et al. (Panel A) and by Tomlins et al. (Panel B) were downloaded from Oncomine. Data sets in each panel were from the same study source and were statistically analyzed by Mann-Whitney U test.  

C. Pearson’s correlation analysis of the data set by Tomlins et al. (n=59) revealed a negative correlation between SRC-3 and PTEN expression in human prostate tumors.  

D. The relative expression levels of SRC-3 mRNA in human prostate tumors with PTEN wild type and PTEN deletion. The expression data sets were from Oncomine (Grasso et al. 2012).  

E. Patients with prostate tumors expressing high SRC-3 and no PTEN exhibit poor recurrence-free survival. Based on immunostaining scores, patients were divided into high SRC-3 with no PTEN (n=17), high SRC-3 with detectable PTEN (n=21) and low SRC-3 with any levels of PTEN (n=214). The data sets were analyzed using Kaplan-Meier Estimator for PSA (biochemical) recurrence (serum PSA>0.2 ng/ml) following radical prostectomy. The p values are indicated in the text.

Fig. 2. Specific knockout of SRC-3 in PECs reduced Pten deletion-induced prostate tumor growth.  

A. Immunohistochemistry of SRC-3 (brown) on the prostate sections with PIN lesions prepared from 12-week-old PtenCKO and Pten3CKO mice. Note the negative SRC-3 immunostaining in the luminal epithelial and tumor cell compartments. Scale bar, 50 µm.  

B.
Relative SRC-3 mRNA levels in PtenCKO and Pten3CKO prostates with tumors. Total RNA samples (n=8 for each group) were used in real time RT-PCR measurements. The mRNA level was normalized to β-actin mRNA level in the same sample. *, p<0.05 by Student’s t-test. 

C. SRC-3 knockout significantly reduced prostate weight at 18 and 24 weeks of age. The relative prostate weight was obtained by normalizing to body weight. Ten mice were used for each age stage for each genotype group. *, p<0.05 and **, p<0.01 by Student’s t-test. 

D. Gross picture showing the size difference between PtenCKO and Pten3CKO tumors at 18 weeks and H&E stained sections comparing the histology between the two groups. AP, DP, LP and VP, anterior, dorsal, lateral and ventral prostates; scale bar, 100 µm.

**Fig. 3. Deletion of SRC-3 decreases tumor proliferation and changes cellular composition of the tumor.** A. Ki67 immunohistochemistry (brown color) on prostate tumor sections of 18-week-old PtenCKO and Pten3CKO mice. Scale bar, 50 µm. 

B. Quantitative analysis of Ki67(+) cells (IHC is shown in Panel A) and p63(+) cells (IHC is shown in Panel C) in PtenCKO and Pten3CKO prostate tumors (n=6 per group). The percentage of Ki67(+) and p63(+) cells were calculated by dividing the number of positive cells by the number of total cells per visual field. *, P <0.05 and **, p < 0.01 by Student’s t-test. 

C. Immunostaining of p63 basal cell marker (brown), K8 LEC marker (green) and K5 basal and precursor cell marker (red) as well as AR LEC marker (brown) on prostate tumor sections of 18-week-old PtenCKO and Pten3CKO mice. Scale bar, 50 µm. 

D. Quantitative analysis of percent K8(+)-, K5(+) and AR(+) -staining areas to total areas on prostate tumor sections prepared from 18-week-old PtenCKO and Pten3CKO mice (n=6). The respective areas were measured using the NIH ImageJ software. *, p<0.05 by Student’s t-test.
Fig. 4. Castration increases tumor aggressiveness and cellular proliferation in PtenCKO mice. A. In PtenCKO mice, Pten deletion started at 2 weeks of age, which induced prostate tumor initiation and progression as indicated. PtenCKO mice were castrated at 12 weeks of age and analyzed at 18 weeks of age. B. Analysis of proliferating cells by Ki67 IHC and assessment of tumor histology by H&E staining in prostate tumors of non-castrated and castrated PtenCKO mice. The percentages of Ki67(+) cells in each group and the statistical analysis results are indicated. C. Trichrome staining of collagen (blue) and IHC of SMA and CD31 (brown) for assessment of reactive stroma in prostate tumors of non-castrated and castrated PtenCKO mice. For quantitative analysis indicated below each panel, stained areas and total areas were measured in 9 independent samples using NIH ImageJ software for statistical analysis. D. Immunohistochemistry of AR, p63 and E-Cadherin in prostate tumors of non-castrated and castrated PtenCKO mice. The decreased AR, increased p63 and decreased E-cadherin staining in tumors of castrated PtenCKO mice signify a de-differentiated phenotype. Quantitative analyses of the percentages of AR(+) cells, p63(+) cells and E-cadherin(+) areas in tumors of non-castrated and castrated PtenCKO mice (n=9 per group) are indicated. All scale bars in panels B–D, 100 µm.

Fig. 5. Deletion of SRC-3 significantly reduces tumor size and cellular proliferation in CRPC. A. PtenCKO and Pten3CKO mice were castrated at 12 weeks of age and their prostates were imaged at 18 weeks of age. Their anterior prostate weights were measured and normalized to their body weights. *, p<0.05 by Student’s t-test, n=8 per group. B. SRC-3 and Ki67 IHC (brown). SRC-3 was present in the prostate tumor cells of castrated PtenCKO mice, but absent in
the tumor cells of castrated Pten3CKO mice. The percentage of Ki67(+) cells was significantly higher in prostate tumors of castrated PtenCKO mice (n=6) versus castrated Pten3CKO mice (n=6). *, p<0.05 by Student’s t-test. Scale bar, 50 µm.  

C and D. Deletion of SRC-3 reversed castration-induced changes in tumor cell type and stromal reactivity. Many SRC-3(+) cells (green) including tumor cells and non-tumor basal and stromal cells were observed in PtenCKO tumors, but less number of SRC-3(+) non-tumor basal and stromal cells were observed in Pten3CKO tumors. Many more p63(+) cells (red) were observed in PtenCKO tumors versus Pten3CKO tumors, and many of the p63(+) cells in PtenCKO tumors co-expressed SRC-3 as evidenced by yellow color after merging the green and red colors in the same cells (upper panels in C). AR immunostaining (brown) was increased in Pten3CKO tumors versus PtenCKO tumors (medial panels in C). SMA (red) was detected in the stromal layer surrounding the PIN lesions of Pten3CKO mice, while SMA(+) stromal cells are disorganized in PtenCKO tumors. Many vimentin(+) cells (Green) were detected in PtenCKO tumors but not in Pten3CKO tumors (lower panels in C). Trichrome-stained collagen (blue) was abundant in PtenCKO tumors but little in Pten3CKO tumors (upper panels in D). E-cadherin immunostaining (brown) was higher in Pten3CKO tumors versus PtenCKO tumors (medial panels in D). The number of CD31(+) endothelial cells (arrows) were decreased in Pten3CKO tumors versus PtenCKO tumors (lower panels in D). All indicated quantitative results for immunostaining signals were calculated from positively stained areas versus total areas measured by ImageJ software. Scale bars in C and D, 100 µm.

Fig. 6. SRC-3 deletion downregulates Akt-mTOR signaling in CRPC tumors. A. Western blot analysis of phospho-Akt and total Akt levels in prostate tumors of castrated PtenCKO and
Pten3CKO mice. B. Western blot analysis of indicated protein levels in prostate tumors of castrated PtenCKO and Pten3CKO mice. C. Western blot analysis of indicated protein levels in PC-3 human prostate cancer cells transfected with non-targeting control siRNA or SRC-3-knockdown siRNAs. Note the significant declines of p85S6K1 and pRPS6 in SRC-3-knockdown cells. D. The significant decrease in the S6K1 mRNA expression in Pten3CKO versus PtenCKO tumors from castrated mice. *, p<0.05 by Student’s t-test, n=6 per group. E. SRC-3 expression positively correlates with S6K1 expression in human CaP samples (n=185). Data was downloaded from Oncomine database as indicated.

**Fig. 7. Targeting SRC-3 in CRPC tumors altered some of the AR target gene expression.** A. The expression of the AR-activated gene *Fkbp5* in prostate tumors of castrated Pten3CKO mice (n=6) is significantly higher than that in prostate tumors of castrated PtenCKO mice (n=6). B. The expression of AR-suppressed genes *Ets2* and *Igfbp3* in prostate tumors of castrated Pten3CKO mice (n=6) is significantly lower than that in prostate tumors of castrated PtenCKO mice (n=6). C. A broader panel of AR-repressed genes was measured in the tumors of castrated PtenCKO and Pten3CKO mice (n=6). In all panels, the mRNA levels were measured by real time RT-PCR. *, p<0.05 and **, p<0.01 by Student’s t-test.
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Fig. 1

A. **P < 0.0001**

B. *P = 0.0216*

C. \( P < 0.05, r = -0.285 \)

D. **P = 0.0005**

E. Recurrence-free Survival

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Relative SRC-3 Expression

Relative PTEN Expression

PTEN Status

Wild Type (n=68)

Deletion (n=26)

Low SRC3 / Any PTEN

High SRC3 / Detectable PTEN

High SRC3 / No PTEN
Fig. 2

A

PtenCKO

Pten3CKO

B

C

Relative Prostate Tumor Weight

Weeks of Age

D

PtenCKO

Pten3CKO

DP

LP

VP

AP

Fig. 2
Fig. 3
A Pten deletion hyperplasia Low PIN high PIN cancer
0 2 6 9 12 18 (wk) +/- Castration Analysis

B Non-castrated Castrated
Ki67
7.85%±2.43%; n=9 20.71%±3.64%; n=9
(p < 0.01, t-test)

H&E

C Non-castrated Castrated
Trichrome
5.53%±0.45%; n=9 17.66%±1.23%; n=9
(p < 0.0005, t-test)

SMA
14.04%±1.30%; n=9 24.58%±1.33%; n=9
(p < 0.0001, t-test)

CD31
0.27%±0.06%; n=9 1.71%±0.51%; n=9
(p < 0.05, t-test)

D Non-castrated Castrated
AR
19.67%±3.13%; n=9 6.33%±1.12%; n=9
(p < 0.005, t-test)

p63
12.13%±1.34%; n=9 25.24%±1.39%; n=9
(p < 0.00001, t-test)

E-Cad
27.08%±3.86%; n=9 22.21%±3.39%; n=9
(p > 0.05, t-test)

Fig. 4
Fig. 6
Fig. 7
The Steroid Receptor Coactivator-3 Is Required for the Development of Castration-resistant Prostate Cancer


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