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

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Abstract

The transcriptional coactivator SRC-3 plays a key role in enhancing prostate cancer cell proliferation. Although SRC-3 is highly expressed in advanced prostate cancer, its role in castration-resistant prostate cancer (CRPC) driven by PTEN mutation is unknown. We documented elevated SRC-3 in human CRPC and in PTEN-negative human prostate cancer. 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 with 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 noncastrated 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. Cancer Res; 73(13); 1–12. ©2013 AACR.

Introduction

Prostate cancer 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 prostate cancer. Despite transient regression, most tumors recur and become hormone-refractory with the androgen-independent phenotype termed "castration-resistant prostate cancer" (CRPC). As 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). Although 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 that 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). In addition, SRC-3 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 prostate cancer, increased SRC-3 expression is associated with a more aggressive phenotype and worse prognosis (14, 15). In addition, studies in prostate cancer cell lines like LNCaP and PC-3 have highlighted the relevance of SRC-3 in promoting prostate cancer proliferation through insulin-like growth factor (IGF)–Akt signaling pathway (16). Moreover, SRC-3 promotes cell migration and invasion in prostate cancer.
cancer by coactivating transcriptional activity of AP-1 and PEA3, transcription factors that upregulate targets including matrix metalloproteinases (MMP; ref. 17). Our group showed that global knockout of SRC-3 in transgenic adenocarcinoma of the mouse prostate (TRAMP) 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 prostate cancer. In addition, 70% of late-stage prostate cancer show altered PTEN–phosphoinositide 3-kinase (PI3K)–AKT pathway (7). The mouse model harboring prostate-specific deletion of Pten faithfully mimics human prostate cancer, recapitulating well-defined stages of hyperplasia, prostatic intraepithelial neoplasia (PIN), and invasive carcinoma (20). Because SRC-3 plays a key role in prostate cancer 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 prostate cancer 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.

Materials and Methods

Generation of Pb-Cre;Pten+/f, SRC-3–f/f and Pb-Cre;Pten+/f mutant mice

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

Castration

Twelve-week-old male mice were anesthetized by intraperitoneal (i.p.) injection of Avertin (2.5% in saline, 15 μL per gram body weight). Mice were castrated via a scrotal incision as previously 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 after castration. The lower urogenital tracts were dissected out in a Petri dish containing PBS. Prostate tissues were isolated and each lobe was separated and weighed. Prostate lobes from 1 side were fixed in 4% paraformaldehyde at 4°C overnight, dehydrated, processed, paraffin-embedded, and then sectioned in 5-μm thickness for hematoxylin and eosin (H&E) staining, Trichrome staining, and immunohistochemistry (IHC). Lobes from the other side were frozen in liquid nitrogen and stored at −80°C for RNA and protein analyses.

Histology and IHC

Tissue sections were stained with H&E and Trichrome as described previously (18, 23). For IHC, antigen retrieval was conducted (in a microwave oven) using 10 mmol/L sodium citrate, pH 6. 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 (2126, Cell Signaling Technology), AR (sc-816, Santa Cruz Biotecnology), Ki-67 (550609, BD Biosciences), p63 (sc-8431, Santa Cruz Biotechnology), Troma-1 (K8; DSHB), E-cadherin (610182, BD Biosciences), smooth muscle α-actin (SMA; M0851, DAKO), CD31 (ab28364, Abcam), phospho-S6 (#5364, Cell Signaling Technology), cytokeitin 5 (K5; PBB160P, Covance), and Vimentin (ab8978, Abcam). Secondary antibodies were obtained from Vector Labs and diluted 1:400. The signal was enhanced using the Vectastain ABC system and visualized with a DAB kit. The slides were counterstained with Harris Modified Hematoxylin. For double fluorescence staining, Tyramide Signal Amplification kit (Life technologies) was used according to the manufacturer's instructions.

RNA isolation and quantitative reverse transcription PCR analysis

Total RNA was prepared from frozen prostate tissues using the TRizol reagent (Life technologies), cDNA was generated using Tetro reverse transcriptase kit and quantitative reverse transcription PCR (qRT-PCR) analysis was conducted using the TaqMan system (Roche).

Immunoblotting

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

Cell culture

PC-3 cells at passage 25 were obtained from the American Type Culture Collection, used for experiment at passage 77, and validated by STR DNA fingerprinting with an AmpFISTR Identification kit (Applied Biosystems) in the University of
Texas MD Anderson Cancer Center Characterized Cell Line Core. PC-3 cells were cultured in Dulbecco's Modified Eagle Medium (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). Nontargeting siRNAs (siGENOME SMARTpool control) were 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 prostate cancer (24). The gene expression data were log-transformed, median-centered per array, and the SD was normalized to 1 per array. The database results were analyzed using GraphPad Prism Software. Statistical analysis comparisons were done with the Student t test for tissue weight and gene expression.

Results

SRC-3 expression correlates negatively with PTEN expression and prostate-specific antigen recurrence-free survival in patients with prostate cancer

We downloaded 2 sets of expression data published by Tomlins and colleagues and Chandran and colleagues (25, 26) from the Oncomine database (24) and compared SRC-3 expression between primary prostate tumors and CRPC samples. In both datasets, SRC-3 mRNA expression is significantly higher in CRPC tumors versus primary tumors (Fig. 1A and B), suggesting SRC-3 may play an important role...
in prostate cancer progression. Statistical analysis of data from Tomlins and colleagues also showed SRC-3 expression is negatively associated with PTEN expression (Fig. 1C). Analysis of another Oncomine dataset published by Grasso and colleagues (27) further showed 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 2 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 (Spearman ρ = −0.156; P = 0.013) in the same prostate cancer cohort. Furthermore, we found patients with high SRC-3, and no PTEN protein had a significantly higher risk of prostate-specific antigen (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 patients with prostate cancer.

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

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

To evaluate the contribution of SRC-3 to prostate tumorigenesis induced by Pten loss, we generated PB-Cre;Ptenf/f mice (designated as PtenCKO hereafter) and PB-Cre;Ptenf/f;SRC-3f/f (designated as Pten3CKO hereafter) mice by interbreeding PB-Cre, Ptenf/f, and SRC-3f/f mouse lines. In prostate of 12-week-old PtenCKO mice, SRC-3 protein was absent in virtually all LECs but retained in some basal cells (Fig. 2A). SRC-3 mRNA was reduced by approximately 50% compared with that in PtenCKO prostate (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 prostate 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 prostate at 18 weeks (Fig. 2D).

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

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 to ensure that tumors would develop from the high PIN stage in an androgen-depleted environment. Previous reports showed that Pten CKO prostate tumors initially responded to androgen ablation, as indicated by increased cell death and reduced size. However, after prolonged castration, tumors regrew and developed into CRPC (20). In the present 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 Trichrome, which stains collagen blue and fibrin pink. We found significantly increased blue staining in tumors from castrated versus noncastrated 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, whereas staining in the noncastrated tumor is a dense, well-defined monolayer boundary that surrounds PECs (Fig. 4C). In addition, IHC for angiogenesis marker CD31 identified significantly more endothelial cells in tumors from castrated versus noncastrated 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 conducted IHC for epithelial differentiation markers such as AR, p63, and E-cadherin. AR is more widely expressed in tumor cells of noncastrated mice, and 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 noncastrated tumors, but total numbers of E-cadherin(+) cells are not significantly different between 2 groups (Fig. 4D). These results indicate that prostate tumors from castrated mice are less differentiated than tumors from age-matched noncastrated 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 (Supplementary Fig. S2). IHC 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 (Supplementary Fig. S2). In keeping with the similar tumor size at this stage, indices of Ki-67 proliferation were comparable in PtenCKO and Pten3CKO prostate tumors (Supplementary Fig. S2). Given these results showing only subtle impact of SRC-3 deletion on prostate tumor growth and histology, we decided to carry out surgical castration on both PtenCKO and Pten3CKO mice at 12 weeks of age, a timepoint before 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 postcastration), 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 noncastrated mice, exhibiting well-differentiated glandular...
structures with a clear basement membrane boundary (Fig. 5B). Importantly, Ki-67 IHC also showed significantly fewer proliferating cells in Pten3CKO versus PtenCKO tumors under castration conditions. Statistical analysis showed that the Ki-67 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 coexpress SRC-3. On the contrary, in castrated Pten3CKO tumors, the number of p63(+) cells was significantly lower, and no colocalization 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, whereas none

Figure 3. Deletion of SRC-3 decreases tumor proliferation and changes cellular composition of the tumor. A, Ki-67 IHC (brown color) on prostate tumor sections of 18-week-old PtenCKO and Pten3CKO mice. Scale bar, 50 μm. B, quantitative analysis of Ki-67(+) cells (IHC is shown in A) and p63(+) cells (IHC is shown in C) in PtenCKO and Pten3CKO prostate tumors (n = 6 per group). The percentage of Ki-67(+) 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 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 percentage of 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 t test.
were present in Pten3CKO tumors (Fig. 5D). E-cadherin, an epithelial marker, was higher in Pten3CKO than PtenCKO tumors, whereas immunostaining for CD31, which indicates the extent of angiogenesis, showed more blood vessels in PtenCKO versus Pten3CKO tumors (Fig. 5D). Taken together, these results show that specific deletion of SRC-3 inhibits reactive stromal infiltration and tumor cell dedifferentiation 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 upregulating 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. IHC showed 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 (Supplementary Fig. S3). Immunoblotting conducted on whole-tumor lysates confirmed this finding (Fig. 6B). pRPS6 is phosphorylated by S6 kinase 1 (S6K1), a protein with both cytosolic (p70) and nuclear (p85) isoforms generated from alternative usage of 2 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 prostate cancer 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 knockdown 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 prostate cancer, we downloaded a set of data with clear readouts of both SRC-3 and S6K1 mRNA expression in prostate tumors from Oncomine.
by Taylor and colleagues (7). Analysis of this dataset indicated SRC-3 expression positively correlated with \textit{S6K1} mRNA expression in human prostate tumors (\(P < 0.0001\); Fig. 6E). These results support the notion that SRC-3 promotes \textit{S6K1} expression in prostate cancer, although it is currently unclear whether \textit{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 show SRC-3 is overexpressed in CRPC and enhances the Akt–mTOR pathway in prostate cancer, while previous reports indicate SRC-3 coactivates AR-mediated transcription in cultured prostate cancer cell lines under androgen-depleted conditions (31). In order to investigate the impact of SRC-3 on AR signaling \textit{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 2 tumor types (Supplementary Fig. S4A). We then evaluated a group of differentiation genes including \textit{probasin}, \textit{Nkx3.1}, \textit{FKBP5}, \textit{Ets2}, and \textit{Igfbp-3} (30). Among AR-activated genes, the expression levels of \textit{probasin} and \textit{Nkx3.1} were similar in the 2 tumor types (Supplementary Fig. S4B), whereas the expression of \textit{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 \textit{Ets2} and \textit{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, \textit{TK1} expression was significantly upregulated in Pten3CKO versus PtenCKO tumors, while \textit{MMP10} and \textit{cyclin E2} expression levels were significantly lower in Pten3CKO versus PtenCKO tumors in castrated mice (Fig. 7C). These results show 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 prostate cancer responds initially to androgen deprivation therapy but eventually recurs as hormone-refractory
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CRPC. As CRPC is highly metastatic and incurable, understanding its molecular mechanism is crucial for designing effective therapies for advanced prostate cancer. The SRC family members coactivate nuclear receptors such as estrogen receptor and AR (1, 6, 7) and other transcription factors such as E2F1, AP-1, and PEA3 to drive cancer growth and progression (1, 6, 34). Studying SRC coactivators in CRPC may, therefore, identify molecular targets for treating both androgen-dependent prostate cancer and CRPC.

SRC-3 expression is elevated in clinical prostate cancer samples, correlating positively with Gleason score and negatively with prognosis (14, 15, 35). Here, we show elevated SRC-3 expression in human CRPC and prostate cancer 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 show 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 histopathologic progression of tumors. Taken together, these results suggest oncogenic potential of SRC-3 is both intrinsic to tumor cells and dependent on nontumor 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 levels of androgens 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 on the basis of 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 knock-out 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 dedifferentiation. 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 showing 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 qRT-PCR 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 is a potential therapeutic target for preventing CRPC development in the context of antiandrogen therapy.

The activation of the PI3K–Akt–mTOR pathway is critical for cancer cell survival, proliferation, and metastasis. Previous
studies reported that SRC-3 activates the PI3K–Akt pathway through upregulating of IGF-I-signaling components including IGF-1, IGF-1R, 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 hyperactivation of the 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 prostate cancer samples. Accordingly, p85S6K1 protein and RPS6 phosphorylation are significantly reduced in SRC–3-depleted mouse and human prostate cancer 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 the 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 PSA expression, suggesting SRC-3 acts as an AR coactivator in these cells (data not shown; ref. 31). Previous in vitro studies also showed SRC-3 and other SRC family members are authentic AR coactivators in cell lines (42–44). Intriguingly, our 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 noncastrated mice (data not shown). Third, knockdown 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 knockdown 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 E2s, Lgfbp3, 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 physiologic 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 because these 2 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 because FKbp5 inhibits Akt activity through stabilizing Phlp3 phosphatase (46–48). In summary, SRC-3 expression is elevated in CRPC and negatively correlated with Pten expression and recurrence-free survival of prostate cancer 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 antiandrogen or anti-AR therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


8. Buras S, Southey MC, Venter DJ. Overexpression of the steroid receptor coactivator AIB1 in breast cancer correlates with the absence...
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