# SRC-3 Is Required for Prostate Cancer Cell Proliferation and Survival

Hai-Jun Zhou,[1] Jun Yan,[1] Weiping Luo,[5] Gustavo Ayala,[2,4] Sue-Hwa Lin,[5] Halime Erdem,[2] Michael Ittmann,[2,4] Sophia Y. Tsai,[1,3] and Ming-Jer Tsai[1,3,4]

Departments of [1]Molecular and Cellular Biology and [2]Pathology, [3]Program of Development, and [4]Baylor Prostate Cancer Specialized Programs of Research Excellence, Baylor College of Medicine; and [5]Department of Molecular Pathology, University of Texas, M.D. Anderson Cancer Center, Houston, Texas

## Abstract

Prostate cancer is the most common cancer in men in America. Currently, steroid receptor coactivators have been proposed to mediate the development and progression of prostate cancer, at times in a steroid-independent manner. Steroid receptor coactivator-3 (SRC-3, p/CIP, AIB1, ACTR, RAC3, and TRAM-1) is a member of the p160 family of coactivators for nuclear hormone receptors including the androgen receptor. SRC-3 is frequently amplified or overexpressed in a number of cancers. However, the role of SRC-3 in cancer cell proliferation and survival is still poorly understood. In this study, we show that SRC-3 is overexpressed in prostate cancer patients and its overexpression correlates with prostate cancer proliferation and is inversely correlated with apoptosis. Consistent with patient data, we have observed that reduction of SRC-3 expression by small interfering RNA decreases proliferation, delays the $G_1$-S transition, and increases cell apoptosis of different prostate cancer cell lines. Furthermore, with decreased SRC-3 expression, proliferating cell nuclear antigen and Bcl-2 expression, as well as bromodeoxyuridine incorporation in prostate cancer cells are reduced. Finally, knockdown of SRC-3 with inducible short hairpin RNA expression in prostate cancer cells decreased tumor growth in nude mice. Taken together, these findings indicate that SRC-3 is an important regulator of prostate cancer proliferation and survival. (Cancer Res 2005; 65(17): 7976-83)

## Introduction

Current studies have shown that steroid receptors, especially androgen receptor (AR), play pivotal roles in all stages of prostate carcinogenesis (1, 2). Because the activity of steroid receptors is potentiated by a variety of coactivators (3, 4), it is reasonable to believe that these proteins may also be involved in prostate carcinogenesis. Indeed, recent studies have found that some steroid receptor coactivator mRNAs are overexpressed in prostate cancer tissues and cell lines (5); however, the underlying mechanisms of how these coactivators may be involved in prostate cancer initiation and/or progression are not well understood.

Steroid receptor coactivator-3 (SRC-3; p/CIP/AIB1/ACTR/RAC3/TRAM-1) is a member of the p160 SRC family, which interacts with steroid receptors in a ligand-dependent manner and enhances their transactivation (6–10). SRC-3 has a histone acetyltransferase activity and works together with CBP, p300, PCAF, and CARM1 to mediate chromatin conformation changes and the transcriptional process of steroid receptors (11, 12). However, further analysis indicates that SRC-3 plays a more global role in its ability to activate transcription factors other than steroid receptors (10). Ablation of SRC-3 in mice results in growth retardation and more global phenotypes than ablation of its other family members, SRC-1 or SRC-2 (13–16), suggesting the functional importance of SRC-3 in cell growth and proliferation in many tissues.

The link between SRC-3 and cancer is first suggested by its overexpression and amplification in breast and ovarian cancers (6, 17). Subsequently, other groups have shown that SRC-3 is also overexpressed in other steroid targeted tumors, such as endometrial carcinoma (18) and prostate cancer (19). Further investigations of breast cancer have revealed that SRC-3 is involved in the regulation of estrogen-dependent cyclin D1 transcription (20) and that SRC-3 can promote breast cancer growth under hormone administration (21). Furthermore, androgen also directs the recruitment of SRC-3 onto prostate-specific antigen (PSA) promoter (22). The physical interaction between nuclear receptors and SRC-3 has been proposed to be important in the tumorigenesis.

However, current data strongly suggested hormone-independent roles of SRC-3 in the development of cancer. First, SRC-3 overexpression is not restricted in cancer of steroid-targeted tissues. Recent studies have shown SRC-3 is also overexpressed in steroid hormone-independent gastric cancer (23) and pancreatic cancer cell lines (24), which suggest SRC-3 can contribute to tumorigenesis through transcription factors other than steroid hormone receptors. Indeed, SRC-3/AIB1 deficiency has been shown to suppress v-Ha-ras-induced breast cancer initiation and progression (25) in an ovarian hormone-independent manner. SRC-3 has also been shown to interact with E2F1 and promote both hormone-dependent and -independent breast cancer cell proliferation (26). Furthermore, our previous work has shown that SRC-3 increases cell size of AR-negative prostate cancer cells in a cell autonomous manner (27). All these results indicate that SRC-3/AIB1 is important for progression of a variety of cancers through factors other than steroid receptors.

In this study, we show that SRC-3 is overexpressed in prostate cancer patients and overexpression of SRC-3 correlates well with the prostate cancer proliferation and cell survival. Knocking down of SRC-3 in prostate cancer cells leads to decreased cell proliferation, inhibition of cell cycle progression, and increased apoptosis. Most importantly, down-regulation of SRC-3 protein in prostate cancer cell lines results in decreased tumor growth in nude mice. Therefore, our data support the importance of SRC-3 in prostate cancer and raises the possibility of using SRC-3/AIB1 as an indicator for prostate cancer treatment.

## Materials and Methods

**Prostate cancer tissue microarrays and immunohistochemistry.** The tissue microarray used to study SRC-3 expression in 480 clinically localized prostate cancers has been described previously (28). Patients received no adjuvant therapy such as radiation or hormonal therapy. Other patient characteristics were as described previously (29). Immunohistochemistry was done as described previously (28). Antigen retrieval was done in 10 mmol/L citrate buffer (pH 9.0) for 20 minutes in a rice cooker. After blocking endogenous peroxidase, sections were incubated with anti-SRC-3 mouse monoclonal antibody (BD Transduction Laboratories, San Jose, CA) at 1.25 µg/mL at 4°C overnight and staining visualized using the Dako Envision Plus (DAKO, Carpinteria, CA) with diaminobenzidine substrate followed by counterstaining with hematoxylin. Slides were scanned using a Bliss automated slide scanner system to produce high-resolution digital images. The proliferation rate and phosphor-Akt (p-Akt) index of prostate cancer cells in human tissues was determined using the immunoperoxidase method with antibodies against Ki-67 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and p-Akt (Ser$^{473}$; Cell Signaling Technology, Beverly, MA), respectively. Briefly, the slides were deparaffinized and rehydrated in graded ethanol, and antigens were retrieved using steam in 10 mmol/l citrate buffer (pH 6.0) for 30 minutes. The slides were subsequently blocked with blocking protein (DAKO) for 10 minutes and incubated with the primary antibody at a dilution of 1:50 for 1 hour at room temperature. The secondary biotinylated antibody was applied for 30 minutes followed by 30 minutes of incubation with streptavidin peroxidase (DAKO LSAB+HRP kit). After rinsing, slides were visualized by diaminobenzidine chromogen solution (DAKO) and counterstained with routine hematoxylin. Positive staining of Ki-67 was confined to the nucleus. The proliferation index was defined as the ratio of Ki-67-positive cancer cells to total cancer cells in the highest positive stain fields (at least 2,000 cells), using a microscopic grid at 400× magnification. p-Akt (Ser$^{473}$) index was defined with the same method.

***In situ* labeling of apoptotic bodies.** The detection of DNA fragmentation was determined *in situ* by the terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) technique as described previously (30). Apoptotic bodies were counted under a light microscope (×400) equipped with an ocular grid (10,000 m$^2$). The area with highest positive stain was selected for counting. The number of apoptotic bodies was determined in a total of ∼2,000 prostate cancer cells, normalized to 100 cells and defined as apoptotic index. At least 10 representative areas without necrosis were selected. Positively staining cells or apoptotic bodies located in the stroma and lumen were excluded because these apoptotic cells or apoptotic bodies might have originated from other cell types.

**Cell culture.** LNCaP and DU145 cells were maintained in RPMI (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS, Invitrogen). PC3 cells were maintained in DMEM (Invitrogen) with 10% FBS. NIH3T3 cells were maintained in DMEM (Invitrogen) with 10% FCS (Invitrogen). All these cell lines were from American Type Culture Collection (Manassas, VA).

**Small interfering RNA transfection.** SRC-3 small interfering RNA (siRNA; ref. 27) and nonspecific siRNA were obtained from Dharmacon Research, Inc. (Dallas, TX). siRNAs were transfected with LipofectAMINE 2000 (Invitrogen) into PC3 and DU145 cells at 30 nmol/L and into LNCaP cells at 60 nmol/L following manufacturer's protocol. For NIH3T3 cells, mouse SRC-3 Smart pool siRNA and control were from Dharmacon Research and used at 40 nmol/L for final concentration.

**Short hairpin RNA stable cell line selection.** The DNA oligonucleotides, encoding short hairpin RNA (shRNA) targeting the human SRC-3 (shSRC-3), were synthesized, annealed, and cloned into *Bgl*II/*Hin*dIII sites of the RNA polymerase III–based expression vector pTER (ref. 31; kindly provided by Dr. Hans Clevers at Hubrecht Laboratory, Centre for Biomedical Genetics, Utrecht, the Netherlands). The TetR expression plasmid (pcDNA6/TR) was transfected into PC3 cells with FuGene6 transfection reagent (Roche, Indianapolis, IN) and cells were selected with blasticidin (10 µg/mL) for 2 weeks. TetR clone was then transfected with pTER-shSRC-3 and selected by zeocin (100 µg/mL). Zeocin-resistant clones were tested for their ability to down-regulate SRC-3 after doxycyclin induction by Western blotting.

**Cell proliferation and bromodeoxyuridine incorporation.** Cell proliferation was measured every 2 days by cell counting in triplicate. For bromodeoxyuridine (BrdUrd) incorporation, PC3 cells were transfected with siRNA for 24 hours and starved for another 24 hours without serum. Cells were then stimulated with 10% FBS containing medium and pulse labeled for 20 minutes with 10 µmol/L BrdUrd (Sigma, St. Louis, MO). After harvesting and fixing with 70% ethanol, nuclei were isolated and incubated with anti-BrdUrd antibody (Sigma) and with FITC conjugated secondary antibody (Jackson ImmunoResearch Lab, West Grove, PA). Nuclei were then stained with propidium iodide and subjected to fluorescence-activated cell sorting (FACS) analysis.

**Immunoblot analysis.** Cell lysates were prepared as described previously (27) and cell extracts with equal amounts of protein were analyzed by immunoblotting. The SRC-3 (AIB1) antibody (BD Transduction Laboratories) was used at 1:2,500 dilutions. Antibodies for proliferating cell nuclear antigen (PCNA, 1:1,000), Bcl-2 (1:1,000), and β-actin (1:5,000) were from Sigma and antibodies for cleaved caspase-3 (1:1,000) and cleaved caspase-7 (1:1,000) were from Cell Signaling Technology.

**Immunohistochemistry of cell lines.** PC3 cells were seeded onto coverslip and transfected at 70% confluence. Two days later, cells were fixed and incubated with anti-SRC-3 (BD Transduction Laboratories) and anti-cleaved caspase-3 (Cell Signaling Technology) antibodies. The signal was visualized by FITC-conjugated and Texas red–conjugated secondary antibodies (Jackson ImmunoResearch Lab). Ten representative areas in each group were imaged. Cleaved caspase-3 positive cancer cells to total cancer cells in the same fields were counted at 100× magnification and the numbers were normalized by 1,000 cells.

**Cell cycle analysis.** For cell cycle analysis, 2 days after siRNA transfection, cells were incubated in medium containing aphidocolin (10 µg/mL) for 30 hours. Cells were harvested at 0, 6, and 10 hours after replacing the medium with fresh medium containing 10% FBS, stained with propidium iodide, and subjected to FACS analysis.

**Apoptosis.** Sub-$G_0$-$G_1$ phase cell was detected by FACS analysis. Briefly, 3 days after siRNA transfection, cells were harvested and fixed with 70% ethanol. After stained with propidium iodide, cell cycle distribution was analyzed.

**Reverse transcription-PCR.** Total RNAs were isolated 48 hours after siRNA transfection using Trizol (Invitrogen) reagent following manufacturer's instruction. First-strand cDNA was reverse transcribed with 2 µg of total RNA by Superscript III first-strand synthesis system (Invitrogen). The final volume was 20 µL. cDNA (2 µL) was subject to PCR with 35 cycles. *Bcl2*- and β-actin-specific primers were as described previously (32).

**Tumorigenesis assay.** Male, 4- to 5-week-old nude mice were inoculated with $1 \times 10^6$ PC-3 cells stably transfected with inducible shRNA expression plasmids or parental PC-3 cells. Harvested cells were resuspended in 0.1 mL of PBS and injected s.c. into the flanks of mice. Each clone was injected into six mice and each mouse was inoculated with cells at four different sites. In the experiments with and without doxycyclin treatment, the animals were randomized into two groups (three animals each). Immediately after cell inoculation, mice were injected i.p. with 100 µg of doxycyclin for the doxycyclin treatment group. Afterwards, animals were fed with drinking water containing 5% sucrose in the presence or absence of 200 µg of doxycyclin per mL (Sigma). Drinking water was changed every other day for the period of study. The sizes of the tumors were monitored weekly from day 14 of cell inoculation. The volume of tumor was calculated as volume = (length × width × height) × 0.5.

## Results

**SRC-3 is overexpressed in prostate cancer patients.** To evaluate the expression of SRC-3 in prostate cancer, we did immunohistochemistry on tissue microarray of clinically localized prostate cancers using an anti-SRC-3 antibody. These arrays contain 0.6-mm tissue cores from cancers in triplicate as well as nonneoplastic peripheral zone tissue cores (also in triplicate) from patients undergoing radical prostatectomy. Stained slides were digitized and staining scored separately for both extent of nuclear

and cytoplasmic staining (scale of 0-3) and intensity of such staining (scale of 0-3). A nuclear and cytoplasmic staining index was calculated from the average extent of staining score for the three cores multiplied by the average staining intensity score so that the staining index ranged from 0 (no staining) to 9 (extensive, strong staining). Staining was present almost exclusively in basal cells, luminal epithelial cells, and cancer cells but not in the stroma. In most cases, staining, if present, was only nuclear, but in 50 of the cancer tissues staining was present in the cytoplasm. Such cytoplasmic staining was usually weak, although in 13 cases it was moderate to strong. In all but four cases, the presence of cytoplasmic staining was associated with absence of nuclear staining. The presence of cytoplasmic staining did not seem an independent predictor of tumor aggressiveness (data not shown) so all further analysis was based on nuclear staining. Interestingly, the normal epithelium had variable nuclear staining indices, ranging from 0 to 9. The three cores from each patient were highly correlated with each other so that this variability seemed to reflect differences between patients rather than heterogeneity within each specimen. Examples of the variability in the staining of normal tissues are shown in Fig. 1*A-B*. The staining indices of 480 cancers were determined. The cancers also had quite variable staining indices (0-9) and again this seemed to represent variation between patients rather than heterogeneity within a given tumor. Examples of tumor staining are shown in Fig. 1*C-D*. Approximately half of prostate cancers had intermediate to intense nuclear staining of anti-SRC-3 antibody (index 2-9), whereas only a third of normal prostate epithelial cells had a similar level of staining. Overall, there was a statistically significant increase in SRC-3 staining index in the cancer ($P < 0.001$, *t* test).

**SRC-3 overexpression correlates with prostate-specific antigen recurrence and prostate cancer cell proliferation and survival.** PSA recurrence following radical prostatectomy is an
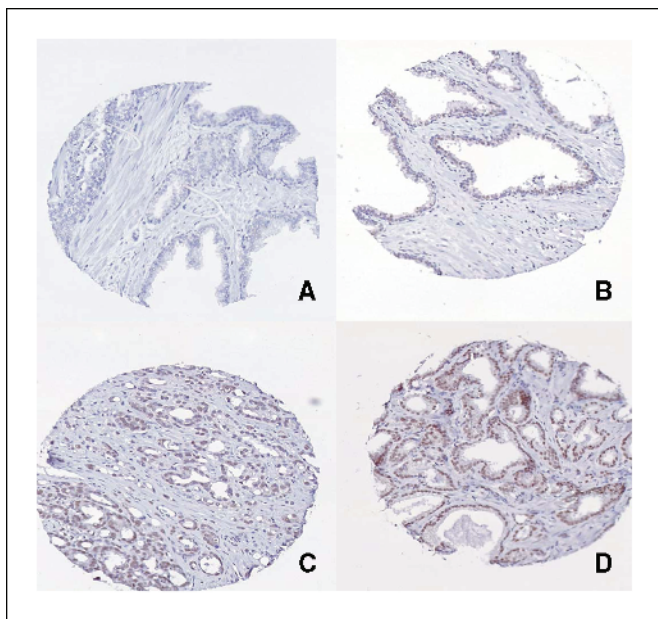


**Figure 1.** Elevated SRC-3 expression in prostate cancer. Expression of SRC-3 protein in normal prostate (*A-B*) and prostate cancer (*C-D*) was determined by immunohistochemistry of tissue microarrays. *A,* normal prostate peripheral zone tissue with no expression of SRC-3. *B,* normal prostate with intermediate expression of SRC-3 in basal and luminal epithelial cells. *C,* prostate cancer with intermediate SRC-3 expression. *D,* prostate cancers with strong nuclear SRC-3 expression.
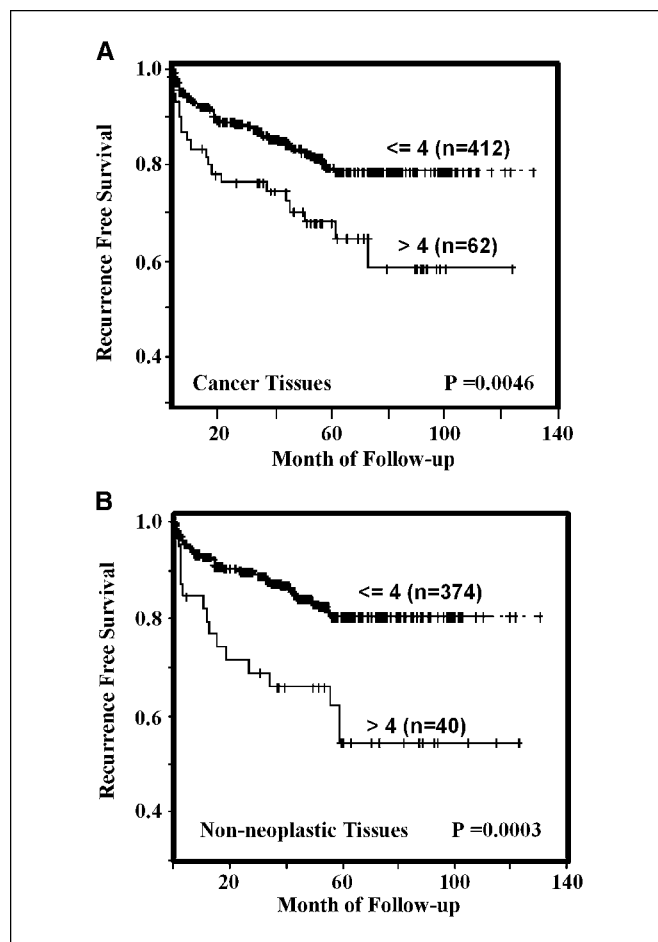


**Figure 2.** Clinical correlation of increased nuclear SRC-3 expression. Kaplan-Meier analysis of PSA recurrence-free survival following radical prostatectomy for men with SRC-3 nuclear staining index of >4 or ≤4. The number of patients (*n*) and *P* values were shown in the graphs. *A,* cancer tissues; *B,* nonneoplastic tissues.

indicator of clinically more aggressive prostate cancer and is associated with disease progression and metastasis in a subset of patients with PSA recurrence (33). When the time to PSA recurrence was compared, both cancer and nonneoplastic tissues with strong SRC3 expression (index >4) were compared with those with lower SRC-3 expression (index ≤4) by the Kaplan-Meier method (Fig. 2). There was a statistically significant increase risk of PSA recurrence for men with SRC-3 staining index of ≥4. These data suggest patients with higher SRC-3 expression underwent prostate cancer recurrence sooner. Therefore, SRC-3 is an indicator for prostate cancer recurrence.

Prostate cancers with strong SRC-3 expression (index >4) had a significantly higher rate of proliferation, as assessed by Ki-67 immunohistochemistry (Fig. 3*A*), and a decreased apoptotic index, as determined by TUNEL assay (Fig. 3*B*). Moreover, strong SRC-3 expression (index >4) significantly correlated with p-Akt expression (Fig. 3*C*). These differences were statistically significant by Mann-Whitney rank sum test ($P = 0.0035$ for Ki67, $P = 0.0002$ for TUNEL, and $P < 0.0001$ for p-Akt). Analysis of Spearman's correlations revealed a significant positive correlation of SRC-3 staining intensity with proliferation ($P < 0.00001$, $\rho = 0.201$), and a negative correlation with apoptosis ($P = 0.0001$, $\rho = -0.197$). Thus, in clinically localized cancers, increased SRC-3 expression was
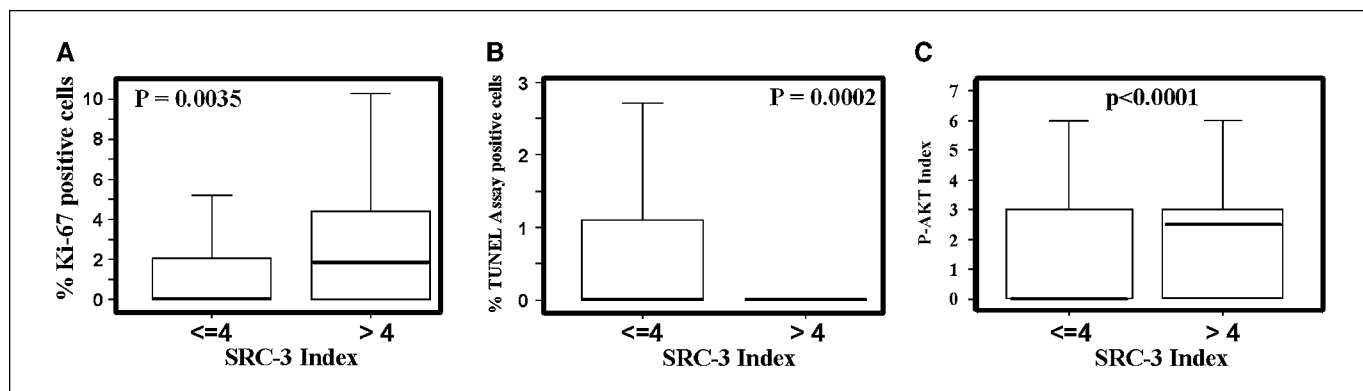
**Figure 3.** Biological correlation of increased nuclear SRC-3 expression with proliferation and survival in prostate cancer. Comparison of (*A*) Ki67 staining index (% nuclei positive for Ki67 by immunohistochemistry), (*B*) TUNEL index (% nuclei positive by TUNEL assay), and (*C*) p-Akt index in men with SRC-3 nuclear staining index of >4 or ≤4. *P* values were shown in the graphs.

associated biologically with increased proliferation and decreased apoptosis. This is the first direct evidence that indicates SRC-3 expression associates with both prostate cancer cell proliferation and survival. The correlation between SRC-3 and p-Akt also validates our previous finding that SRC-3 up-regulated p-Akt in prostate cancer cells (27).

**Down-regulation of SRC-3 expression with specific small interfering RNA significantly decreases cell proliferation in prostate cancer cells but not in noncancer cells.** To investigate the role of SRC-3 in the proliferation of prostate cancer cells, we designed a specific siRNA against SRC-3 (34) to down-regulate its expression in PC3 cells. As shown in Fig. 4*A*, SRC-3 protein was dramatically knocked down after 3 days of siRNA treatment, compared with the untreated or nonspecific siRNA-treated cells. The cell growth curve was then determined by counting cells every 2 days after siRNA treatment. Significantly, down-regulation of SRC-3 expression in PC3 cells resulted in the decrease of cell proliferation (Fig. 4*B*). By the sixth day after siRNA treatment, cell number decreased >30% for SRC-3 siRNA-treated cells, whereas cells treated with nonspecific siRNA grew as well as cells with no siRNA treatment, indicating SRC-3 is important for PC3 cell proliferation.

To further investigate the role of SRC-3 in proliferation, we first determined the effect of SRC-3 down-regulation on DNA synthesis by BrdUrd incorporation. BrdUrd incorporation was significantly decreased in specific siRNA-treated cells with decreased SRC-3 expression in comparison with the control cells (Fig. 4*C*). Furthermore, PCNA, a proliferation marker, was down-regulated as well (Fig. 4*A*). Therefore, the decreased proliferation is one reason for the cell number decrease when SRC-3 was knocked down. To substantiate this notion, we tested if down-regulation of SRC-3 could affect cell cycle progression. After siRNA treatment, cells were synchronized in $G_1$ phase with a reversible cell cycle inhibitor, aphidocolin, and the percentage of the S phase of the cell cycle was measured by flow cytometry after release of aphidocolin inhibition. As shown in Fig. 4*D*, cells with down-regulated SRC-3 expression entered the S phase from $G_1$ phase significantly slower than the control groups. Taken together, these several lines of evidence support our conclusion that SRC-3 promotes prostate cancer cell proliferation and cell cycle progression.

Finally, because PC3 cells are AR- and PTEN-negative prostate cancer cells, we extended our study to LNCaP (AR positive/PTEN negative) and DU145 (AR negative/PTEN positive) cells which have

different AR and PTEN status. As expected, with the effective knocking down of SRC-3 expression by SRC-3-specific siRNA, the proliferation rate of LNCaP (Fig. 5*A*) and DU145 (Fig. 5*B*) cells decreased as well. These data suggested that SRC-3 functioned in prostate cancer cells regardless of AR or PTEN status. Therefore, our findings indicate that SRC-3 is important for a wide spectrum of prostate cancer cell lines, which implies its wide usage in the clinical investigations.

Importantly, in a parallel experiment, we knocked down the expression of SRC-3 in NIH3T3 cells and monitored the cell proliferation. As shown in Fig. 5*C*, with efficient SRC-3 knockdown, there was just a slight decrease of cell proliferation. This result indicates that SRC-3 plays more important roles in cancer cells than noncancer cells. We speculate that this is because SRC-3 is overexpressed in cancer cells but not in NIH3T3 cells. Indeed, as
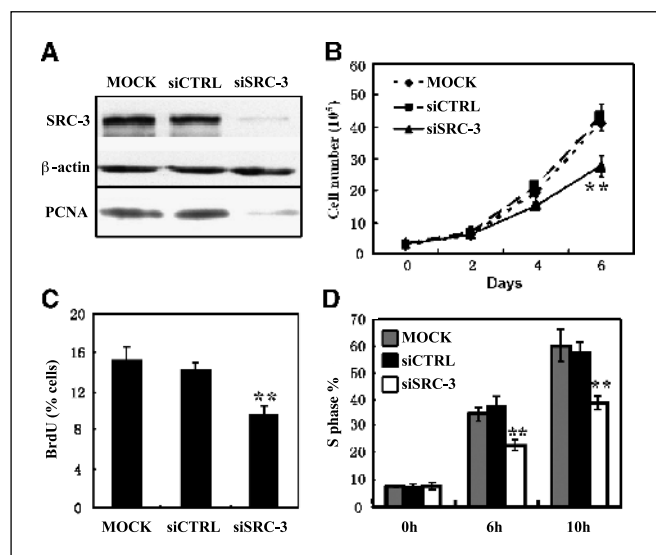


**Figure 4.** Down-regulation of SRC-3 expression decreases prostate cancer cell proliferation. *A,* Western blot analysis showed the decreased expression of SRC-3 and PCNA after SRC-3 siRNA treatment. *B,* PC3 cells were transfected with siRNA and cell number was counted every 2 days after transfection in triplicate. *C,* BrdUrd incorporation assay was done as described in Materials and Methods in triplicate. *D,* $G_1$-S transition assay was determined by FACS analysis as described in Materials and Methods. *Mock,* no siRNA but transfection reagent; *siCTRL,* nonspecific siRNA control; *siSRC-3,* SRC-3-specific siRNA. **, *P* < 0.01, significant difference with *t* test.
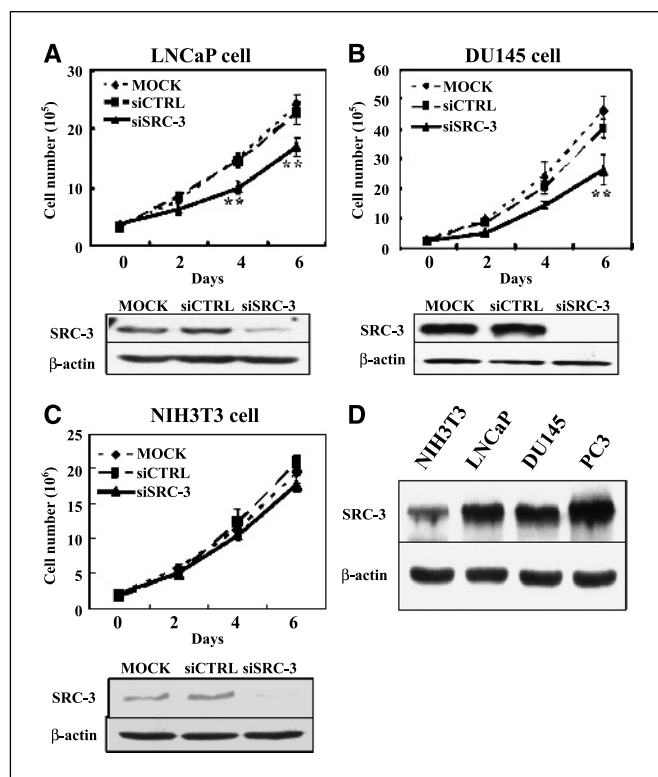
**Figure 5.** Cell proliferation decreases after down-regulation of SRC-3 expression in LNCaP and DU145 cells but not in NIH3T3 cells. Prostate cancer cell lines LNCaP cells (*A*) and DU145 cells (*B*) and noncancer cell line NIH3T3 cells (*C*) were transfected with siRNA to SRC-3 and cell number was counted every 2 days after transfection. Western blot showed decreased expression of SRC-3. *Mock,* with transfection reagent but no siRNA; *siCTRL,* nonspecific siRNA control; *siSRC-3,* SRC-3-specific siRNA. **, *P* < 0.01, significant difference with *t* test. *D,* SRC-3 expression levels in NIH3T3, LNCaP, DU145, and PC3 cells were measured by Western blot.

shown in Fig. 5*D,* the expression level of SRC-3 is much higher in LNCaP, DU145, and PC3 cells than in NIH3T3 cells. This result is consistent with our clinical data that SRC-3 was expressed highly in epithelium and cancer cells but not in stroma cells.

**SRC-3 sustains prostate cancer cell survival through Bcl-2.** Decrease in cell number could be a consequence of either reduced proliferation or increased cell death, or both. To characterize whether SRC-3 could function as a survival factor in prostate cancer cells, we did Western blotting to assess if players involved in apoptotic pathway could be activated when we efficiently knocked down SRC-3 level. As shown in Fig. 6*A,* cleaved caspase-3 and cleaved caspase-7 were clearly detected when SRC-3 was knocked down. In addition, immunohistochemistry analysis illustrated a 4- to 5-fold increase of cleaved caspase-3-positive cells when SRC-3 was knocked down in comparison with the control cells (Fig. 6*B*). These data indicate prostate cancer cells are prone to undergo apoptosis in the absence of SRC-3.

To strengthen the above notion, we carried out another apoptosis assay using flow cytometry to determine cell death. LNCaP and PC3 cells transfected with siRNA were harvested 3 days later and stained with propidium iodide. The sub-$G_0$-$G_1$ population of cells was then measured by FACS analysis. As shown in Fig. 6*C-D,* the sub-$G_0$-$G_1$ dead cells increased 2- or 3-fold compared with the untreated or nonspecific siRNA-treated cells, suggesting apoptosis did increase when SRC-3 was down-regulated. As a contrast in NIH3T3 cells, same experiment was done; however, we

could not observe a significant increase of apoptosis when SRC-3 was knocked down (Fig. 6*E*). Consistent with the clinical data shown earlier, the above results strongly indicate that SRC-3 has a survival function in prostate cancer cells and apoptosis increases when SRC-3 is decreased or absent.

Our previous work has shown that SRC-3 could up-regulate Akt expression in prostate cancer cells (27), which can be one reason why SRC-3 protected prostate cancer cells from apoptosis. However, other mechanisms, such as SRC-3-regulating cell survival genes, may also contribute to this phenotype. Therefore, we examined the expression of the important cell survival gene, *bcl-2,* in prostate cancer cells when SRC-3 was down-regulated. Prostate cancer cells were transfected with siRNA; cell lysates and RNA were extracted and Western blotting and reverse transcription-PCR (RT-PCR) were done. Interestingly, our data in Fig. 7*A* showed that Bcl-2 protein level decreased when expression of SRC-3 was reduced. Furthermore, RT-PCR showed clearly that level of *bcl-2* transcript decreased when SRC-3 was knocked down (Fig. 7*B*). A bar graph representing the average measurement of three experimental data sets was also presented. The results indicated a significant decrease of Bcl-2 protein and mRNA levels with the down-regulation of SRC-3. These data suggest that SRC-3 functions to promote cell growth and survival through multiple mechanisms.
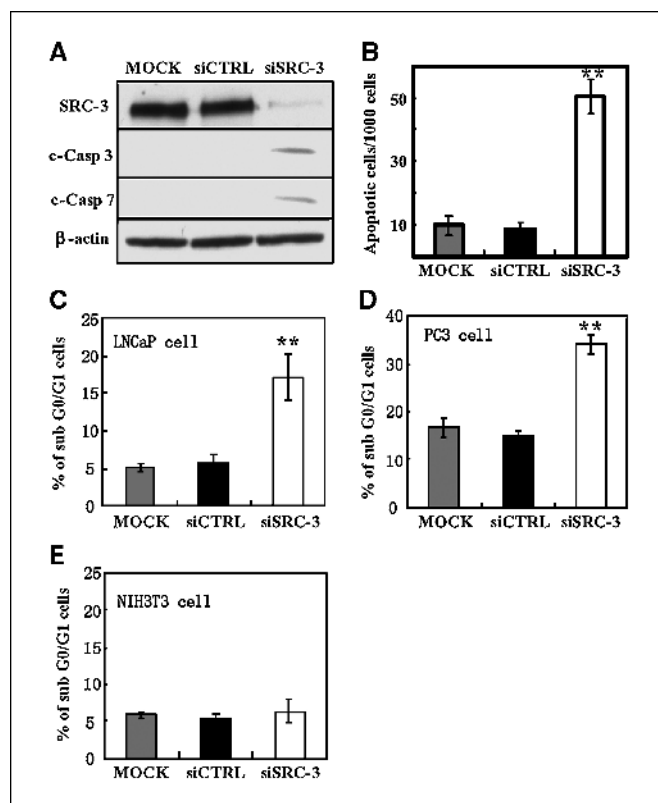


**Figure 6.** Knocking down of SRC-3 expression induces apoptosis in prostate cancer cells. *A,* prostate cancer cells were transfected with siRNA, 3 days later, cells including floating cells were harvested for Western blot analysis to detect apoptosis with cleaved caspase-3 and cleaved caspase-7 expression. *B,* PC3 cells were seeded onto coverslips and transfected with siRNA. Cleaved caspase-3 staining cells were counted and normalized as indicated in Materials and Methods. *C-D,* prostate cancer cells were transfected with siRNA. Three days later, cells including floating cells were harvested for flow cytometric analysis as described in Materials and Methods. All experiments were done in triplicate. *Mock,* no siRNA but with transfection reagent; *siCTRL,* nonspecific siRNA control; *siSRC-3,* SRC-3-specific siRNA. **, *P* < 0.01, significant difference with *t* test.

**SRC-3 promotes tumorigenesis in nude mice.** Recent reports illustrated that *in vivo* expressed shRNA could work as siRNA in mammalian cells for a lengthy period of time. To study the effect of down-regulation of SRC-3 on tumor growth, we tried to establish stable PC3 cells constitutively expressing SRC-3 shRNA. However, we were not able to do so presumably due to cellular requirements of SRC-3 for proliferation and survival. Therefore, we used a tetracycline-inducible expression system (31). Three clones were selected and treated with doxycycline; Western blot analysis was done to detect the efficiency of SRC-3 knock down. As shown in Fig. 8*A*, SRC-3 is effectively knocked down in the presence of doxy-cycline in clones 3 and 8, indicating efficient shRNA expression.

We then used these two cell lines together to determine their proliferation ability before and after induction of shRNA to SRC-3. As shown in Fig. 8*B-D*, after doxycycline induction, control cell line did not show significant proliferation difference; however, for clones 3 and 8, cell proliferation went down significantly after induction of the shRNA to down-regulate SRC-3 expression. These results confirmed our observation of siRNA transfection experiments.

To determine whether down-expression of SRC-3 would decrease tumorigenesis *in vivo*, we injected both inducible shRNA clones and their parental control cells s.c. into nude mice to induce tumors and followed the tumor growth in the presence or absence of doxycy-cline for 5 weeks. During this period of time, all mice in each group survived. As shown in Fig. 8*E-G*, animals in which SRC-3 was down-regulated by supplementing their drinking water with doxycycline developed significantly smaller tumors as compared with controls. In agreement with the Western blotting assay, clone 3 cells harboring the most efficient SRC-3 knockdown (Fig. 8*A*) showed the most significant difference after doxycycline administration, with a 70% decrease in tumor volume. Clone 8, with a bit higher residual SRC-3 expression showed a less (35-50%) reduction of tumor growth. In contrast, there was no significant difference in the parental control cells before and after doxycycline treatment. These data indicate clearly that SRC-3 is a tumor-promoting factor *in vivo*.

## Discussion

Although the expression profile of SRC-3 in prostate cancer has been investigated primarily by other groups (5, 19), the small number of the patient cases and the detection method in their studies have not provided extensive insight of SRC-3 protein expression pattern in prostate cancer. After analyzing 480 clinical prostate cancer patients by immunohistochemistry, we have shown in this study that prostate cancer patients express increased level of nuclear SRC-3 in comparison with matched normal prostate tissue (Fig. 1). Clinically, patients with high SRC-3 expression underwent recurrence sooner (Fig. 2). Consistent with this clinical behavior, prostate cancers with increased nuclear SRC-3 staining displayed both increased proliferation and decreased apoptosis in this large-scale analysis (Fig. 3). These clinical observations suggested SRC-3 could play proliferation and survival roles in prostate cancer cells. The parallel cell culture study at same time confirmed this notion.

SRC-3 was first isolated as a steroid receptor coactivator; however, its role in tumorigenesis seems not limited to its action in stimulation of steroid hormone receptors. Recent results have indicated that SRC-3 acts through cell cycle regulator E2F1 and promotes breast cancer cell proliferation regardless of ER status (26). Furthermore, breast carcinogenesis is delayed independent of ovarian hormones in SRC-3 null mice (25). Finally, although the interaction between SRC-3 and AR has been shown by several groups (22, 35), our results in this study have shown that SRC-3 knockdown impaired the proliferation of both AR-positive and -negative prostate cancer cells (Figs. 4 and 5). These results suggest that an androgen-independent mechanism exist in the process of prostate cancer cell proliferation in response to SRC-3. However, we cannot exclude the importance of the interaction between SRC-3 and AR in the AR-dependent cancer cells. Interestingly, we found that SRC-3 played more important roles in cancer cells versus noncancer cells (Figs. 4-6). However, we have to emphasize that our studies should not be taken to mean that requirement of SRC-3 is prostate cancer specific. Actually, many data suggest and we believe that SRC-3 is required for other cancer cells as well.

The function of SRC-3 in prostate cancer is strikingly correlated with growth signal pathways. We have shown that overexpression of SRC-3 in prostate cancer cells induced AKT expression and phosphorylation (27). Moreover, it has been shown that SRC-3 is a phospho-protein which can be regulated by different protein kinases, such as mitogen-activated protein kinase (36) and IκB kinase (37). Wu et al. recently identified six phosphorylation sites in SRC-3 protein, and the phosphorylation of these sites mediated multiple steroid-dependent and -independent transcription activ-ities and oncogenic transformations (38). These studies have suggested that SRC-3 is likely to be involved in multiple signaling pathways during the development of cancer. In breast cancer, overexpression of SRC-3 correlated with the expression of the HER-2/*neu* oncogene (39, 40) and overexpression of HER-2/*neu* induced SRC-3 phosphorylation (41). This interplay between SRC-3 and HER-2/*neu* may contribute to the tamoxifen resistance in breast cancer patients. Through these crosstalks, SRC-3 may synergize with various signal pathways to reinforce its biological functions involved in prostate cancer cell survival, proliferation, and tumorigenesis.

Our investigation also sheds light on how SRC-3 maintains cell survival. Our data have indicated that expression of SRC-3 correlates inversely with apoptosis index in prostate cancer tissue. Consistent with this observation, down-regulation of SRC-3 increases apoptosis of prostate cancer cells *in vitro*. Our previous data have shown SRC-3 up-regulated AKT pathway (27), which can at least account for one of the mechanisms explaining the consequence of SRC-3 knockdown. More interestingly in this study,
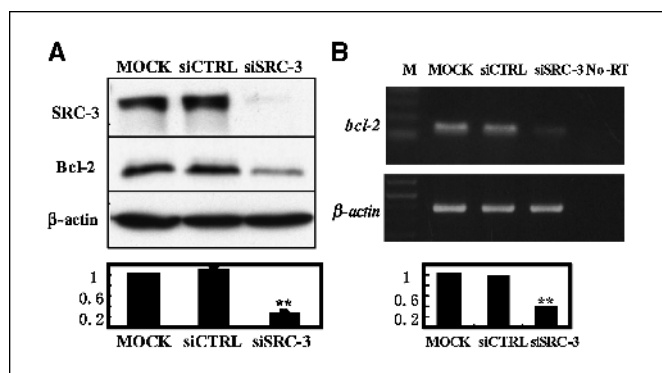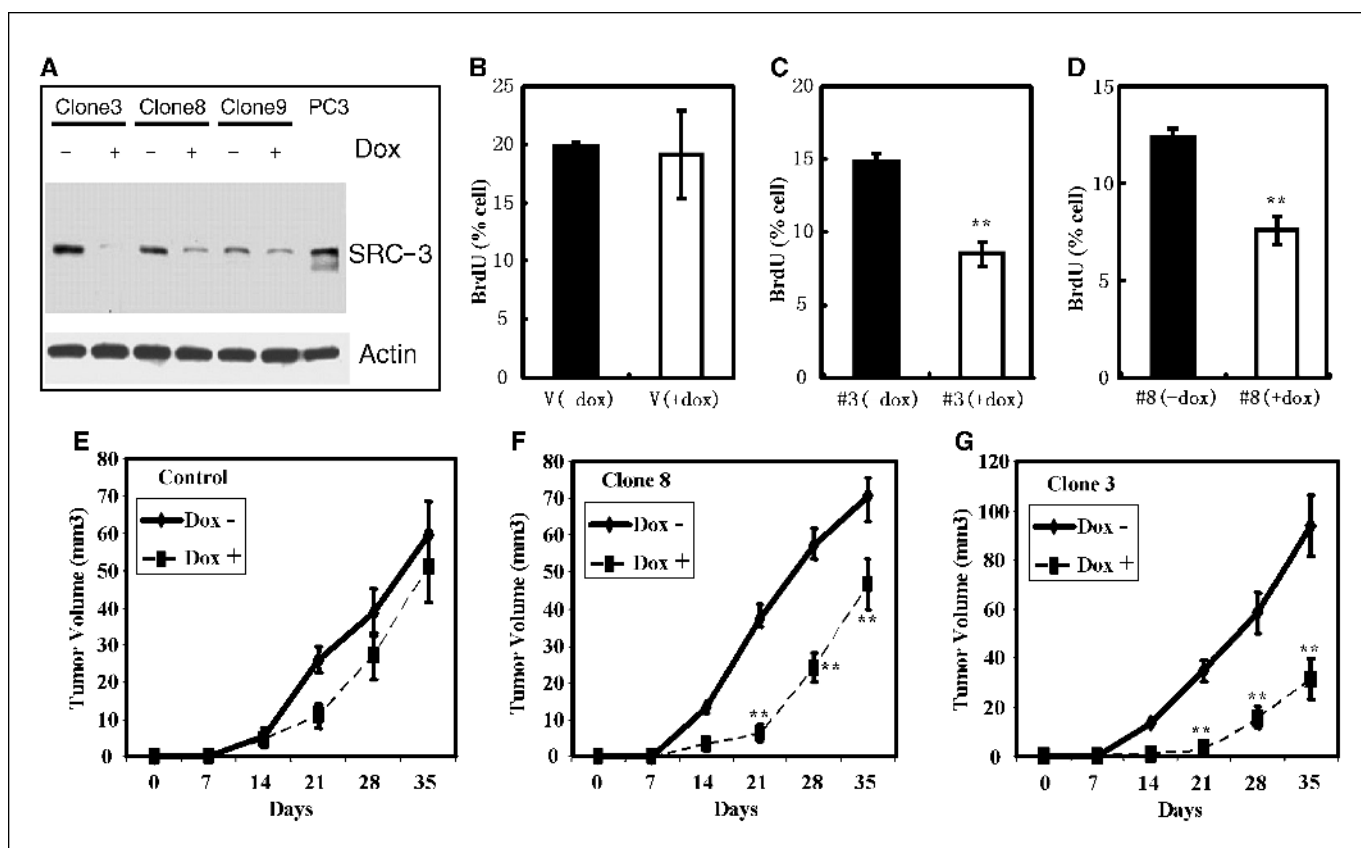


**Figure 7.** Down-regulation of SRC-3 expression decreases Bcl-2 expression. *A*, after 3 days of SRC-3 siRNA transfection, cells were harvested and lysates were analyzed by SDS-PAGE and immunoblotted with anti-Bcl-2 antibody. *B*, PC3 cells were transfected with siRNA to SRC-3 for 3 days and total RNAs were extracted and RT-PCR was done with *bcl-2*–specific primers. β-Actin transcripts were analyzed as control. Triplicate experiment were done and the decreased expression of Bcl-2/*bcl-2* was illustrated as arbitrary unites normalized by β-actin. *M*, DNA ladder; *Mock*, no siRNA but transfection reagent; *siCTRL*, nonspecific siRNA control; *siSRC-3*, SRC-3-specific siRNA; *No-RT*, No-RT control. **, *P* < 0.01, significant difference with *t* test.

**Figure 8.** Down-regulation of SRC-3 expression in stable PC-3 cell lines decreases prostate cancer cell proliferation and tumor growth in nude mice. *A,* cell extracts from cells treated with (+) or without (−) doxycyclin (*dox*, 2 μg/mL, 72 hours) were separated by SDS-PAGE and blotted and probed with anti-SRC-3 and anti-actin antibody. Actin was used as a loading control. Parental cell clone (*B*) and clones 3 (*C*) and 8 (*D*) were used to determine their proliferation rate before (−*dox*) and after (+*dox*) induction of shRNA to SRC-3 by BrdUrd incorporation assay. *E-G,* doxycycline-regulated expression of SRC-3 inhibits the tumorigenesis of PC-3 prostate cancer cells in nude mice. PC-3 stably transfected with shRNA expression plasmids or parental PC-3 cells were harvested and injected s.c. into the flank region of male nude mice. The mice were fed with water with (+) or without (−) 200 μg of doxycycline per mL. The change in tumor volume over a 35-day period is shown in the graph. **, $P < 0.01$, significant difference with $t$ test.

we detected the decreased expression of Bcl-2 after SRC-3 knockdown, which extended the function of SRC-3 into the regulation of the antiapoptosis genes. Because SRC-3 is involved in the nuclear factor-κB pathway (37) that regulates bcl-2 expression, SRC-3 may serve as a mediator in signal pathways to confer its survival functions. Considering the importance of Bcl-2 in prostate cancer survival and progression (42), SRC-3-regulated Bcl-2 expression may serve as a novel pathway to contribute to the androgen-independent progression in prostate cancer.

In summary, consistent with overexpression study in transgenic mice (43), our analysis of clinical specimens and *in vitro* studies indicate that SRC-3 overexpression in prostate cancer cells also results in both increased proliferation and decreased apoptosis. Knockdown of SRC-3 has led to decreased tumor growth in nude

mice. Our studies illustrate that SRC-3 is an important indicator in prostate cancer diagnosis and prognosis, and disruption of functions of SRC-3 or its target genes and pathways may have potential therapeutic advantages in prostate or other cancer treatment.

## Acknowledgments

## References

1. Abate-Shen C, Shen MM. Molecular genetics of prostate cancer. Genes Dev 2000;14:2410–34.
2. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. Nat Rev Cancer 2001;1:34–45.
3. McKenna NJ, Lanz RB, O'Malley BW. Nuclear receptor coregulators: cellular and molecular biology. Endocr Rev 1999;20:321–44.
4. McKenna NJ, O'Malley BW. Combinatorial control of gene expression by nuclear receptors and coregulators. Cell 2002;108:465–74.
5. Linja MJ, Porkka KP, Kang Z, et al. Expression of androgen receptor coregulators in prostate cancer. Clin Cancer Res 2004;10:1032–40.
6. Anzick SL, Kononen J, Walker RL, et al. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science 1997;277:965–8.
7. Chen H, Lin RJ, Schiltz RL, et al. Nuclear receptor coactivator ACTR is a novel histone acetyltransferase

and forms a multimeric activation complex with P/CAF and CBP/p300. Cell 1997;90:569–80.
8. Li H, Gomes PJ, Chen JD. RAC3, a steroid/nuclear receptor-associated coactivator that is related to SRC-1 and TIF2. Proc Natl Acad Sci U S A 1997;94:8479–84.
9. Takeshita A, Cardona GR, Koibuchi N, Suen CS, Chin WW. TRAM-1, a novel 160-kDa thyroid hormone receptor activator molecule, exhibits distinct properties from steroid receptor coactivator-1. J Biol Chem 1997;272:27629–34.

10. Torchia J, Rose DW, Inostroza J, et al. The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. Nature 1997;387:677–84.

11. Chen D, Ma H, Hong H, et al. Regulation of transcription by a protein methyltransferase. Science 1999;284:2174–7.

12. Teyssier C, Chen D, Stallcup MR. Requirement for multiple domains of the protein arginine methyltransferase CARM1 in its transcriptional coactivator function. J Biol Chem 2002;277:46066–72.

13. Wang Z, Rose DW, Hermanson O, et al. Regulation of somatic growth by the p160 coactivator p/CIP. Proc Natl Acad Sci U S A 2000;97:13549–54.

14. Xu J, Liao L, Ning G, Yoshida-Komiya H, Deng C, O'Malley BW. The steroid receptor coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty, female reproductive function, and mammary gland development. Proc Natl Acad Sci U S A 2000;97:6379–84.

15. Gehin M, Mark M, Dennefeld C, Dierich A, Gronemeyer H, Chambon P. The function of TIF2/GRIP1 in mouse reproduction is distinct from those of SRC-1 and p/CIP. Mol Cell Biol 2002;22:5923–37.

16. Xu J, Qiu Y, DeMayo FJ, Tsai SY, Tsai MJ, O'Malley BW. Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. Science 1998;279:1922–5.

17. List HJ, Reiter R, Singh B, Wellstein A, Riegel AT. Expression of the nuclear coactivator AIB1 in normal and malignant breast tissue. Breast Cancer Res Treat 2001;68:21–8.

18. Glaeser M, Floetotto T, Hanstein B, Beckmann MW, Niederacher D. Gene amplification and expression of the steroid receptor coactivator SRC3 (AIB1) in sporadic breast and endometrial carcinomas. Horm Metab Res 2001;33:121–6.

19. Gnanapragasam VJ, Leung HY, Pulimood AS, Neal DE, Robson CN. Expression of RAC 3, a steroid hormone receptor co-activator in prostate cancer. Br J Cancer 2001;85:1928–36.

20. Planas-Silva MD, Shang Y, Donaher JL, Brown M, Weinberg RA. AIB1 enhances estrogen-dependent induction of cyclin D1 expression. Cancer Res 2001;61:3858–62.

21. List HJ, Lauritsen KJ, Reiter R, Powers C, Wellstein A, Riegel AT. Ribozyme targeting demonstrates that the nuclear receptor coactivator AIB1 is a rate-limiting factor for estrogen-dependent growth of human MCF-7 breast cancer cells. J Biol Chem 2001;276:23763–8.

22. Louie MC, Yang HQ, Ma AH, et al. Androgen-induced recruitment of RNA polymerase II to a nuclear receptor-p160 coactivator complex. Proc Natl Acad Sci U S A 2003;100:2226–30.

23. Sakakura C, Hagiwara A, Yasuoka R, et al. Amplification and over-expression of the AIB1 nuclear receptor co-activator gene in primary gastric cancers. Int J Cancer 2000;89:217–23.

24. Ghadimi BM, Schrock E, Walker RL, et al. Specific chromosomal aberrations and amplification of the AIB1 nuclear receptor coactivator gene in pancreatic carcinomas. Am J Pathol 1999;154:525–36.

25. Kuang SQ, Liao L, Zhang H, Lee AV, O'Malley BW, Xu J. AIB1/SRC-3 deficiency affects insulin-like growth factor I signaling pathway and suppresses v-Ha-ras-induced breast cancer initiation and progression in mice. Cancer Res 2004;64:1875–85.

26. Louie MC, Zou JX, Rabinovich A, Chen HW. ACTR/AIB1 functions as an E2F1 coactivator to promote breast cancer cell proliferation and antiestrogen resistance. Mol Cell Biol 2004;24:5157–71.

27. Zhou G, Hashimoto Y, Kwak I, Tsai SY, Tsai MJ. Role of the steroid receptor coactivator SRC-3 in cell growth. Mol Cell Biol 2003;23:7742–55.

28. Kwabi-Addo B, Wang J, Erdem H, et al. Expression of Sprouty1, an inhibitor of fibroblast growth factor signal transduction, is decreased in human prostate cancer. Cancer Res 2004;64:4728–35.

29. Ayala G, Wang D, Wulf G, et al. The prolyl isomerase Pin1 is a novel prognostic marker in human prostate cancer. Cancer Res 2003;63:6244–51.

30. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992;119:493–501.

31. van de Wetering M, Oving I, Muncan V, et al. Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. EMBO Rep 2003;4:609–15.

32. Metcalfe AD, Hunter HR, Bloor DJ, et al. Expression of 11 members of the BCL-2 family of apoptosis regulatory molecules during human preimplantation embryo development and fragmentation. Mol Reprod Dev 2004;68:35–50.

33. Swindle PW, Kattan MW, Scardino PT. Markers and meaning of primary treatment failure. Urol Clin North Am 2003;30:377–401.

34. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001;411:494–8.

35. Tan JA, Hall SH, Petrusz P, French FS. Thyroid receptor activator molecule, TRAM-1, is an androgen receptor coactivator. Endocrinology 2000;141:3440–50.

36. Font de Mora J, Brown M. AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. Mol Cell Biol 2000;20:5041–7.

37. Wu RC, Qin J, Hashimoto Y, et al. Regulation of SRC-3 (pCIP/ACTR/AIB-1/RAC-3/TRAM-1) coactivator activity by I κ B kinase. Mol Cell Biol 2002;22:3549–61.

38. Wu RC, Qin J, Yi P, et al. Selective phosphorylations of the SRC-3/AIB1 coactivator integrate genomic responses to multiple cellular signaling pathways. Mol Cell 2004;15:937–49.

39. Bouras T, Southey MC, Venter DJ. Overexpression of the steroid receptor coactivator AIB1 in breast cancer correlates with the absence of estrogen and progesterone receptors and positivity for p53 and HER2/neu. Cancer Res 2001;61:903–7.

40. Osborne CK, Bardou V, Hopp TA, et al. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/*neu* in tamoxifen resistance in breast cancer. J Natl Cancer Inst 2003;95:353–61.

41. Shou J, Massarweh S, Osborne CK, et al. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/*neu* cross-talk in ER/HER2-positive breast cancer. J Natl Cancer Inst 2004;96:926–35.

42. Catz SD, Johnson JL. BCL-2 in prostate cancer: a minireview. Apoptosis 2003;8:29–37.

43. Torres-Arzayus MI, De Mora JF, Yuan J, et al. High tumor incidence and activation of the PI3K/AKT pathway in transgenic mice define AIB1 as an oncogene. Cancer Cell 2004;6:263–74.