# Steroid Receptor Coactivator-3 and Activator Protein-1 Coordinately Regulate the Transcription of Components of the Insulin-Like Growth Factor/AKT Signaling Pathway

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## Abstract

**Steroid receptor coactivator (SRC)-3, also called amplified in breast cancer 1, is a member of the p160 nuclear receptor coactivator family involved in transcriptional regulation of target genes. SRC-3 is frequently amplified and/or overexpressed in hormone-sensitive and hormone-insensitive tumors. We reported previously that SRC-3 stimulated prostate cell growth in a hormone-independent manner through activation of AKT signaling pathway. However, the underlying mechanism remains undefined. Here, we exploited the mifepristone-induced SRC-3 LNCaP prostate cancer cell line generated in our laboratory to identify SRC-3-regulated genes by oligonucleotide microarray analysis. We found that SRC-3 up-regulates the expression of multiple genes in the insulin-like growth factor (IGF)/AKT signaling pathway that are involved in cell proliferation and survival. In contrast, knockdown of SRC-3 in PC3 (androgen receptor negative) prostate cancer cells and MCF-7 breast cancer cells reduces their expression. Similarly, in prostate glands of SRC-3 null mice, expressions of these components in the IGF/AKT signal pathway are also reduced. Chromatin immunoprecipitation assay revealed that SRC-3 was directly recruited to the promoters of these genes, indicating that they are direct targets of SRC-3. Interestingly, we showed that recruitment of SRC-3 to two target promoters, IRS-2 and IGF-I, requires transcription factor activator protein-1 (AP-1). Taken together, our results clearly show that SRC-3 and AP-1 can coordinately regulate the transcription of multiple components in the IGF/AKT pathway to ensure ligand-independent cell proliferation and survival of cancer cells.** (Cancer Res 2006; 66(22): 11039-46)

## Introduction

Steroid receptor coactivator (SRC)-3 was first identified as coactivator of nuclear receptors (1–4). It also was independently isolated from a chromosomal region often amplified in breast cancer patients and thus named amplified in breast cancer 1 (AIB1; ref. 5). SRC-3 is a member of the p160 SRC family, including SRC-1 and SRC-2/TIF2/GRIP1 (for review, see ref. 6). Similar to other p160 coactivators, SRC-3 functions as a cofactor that associates with hormone-bound nuclear receptor through the conserved LXXLL interaction domains in the nuclear receptor. SRC-3 recruited to the promoter/enhancer of target genes by nuclear receptors leads to further recruit other chromatin modification factors, such as acetyltransferases (CBP and p300), histone acetyltransferases, and methyltransferases (CARM1 and PRMT1) to mediate chromatin remodeling and enhance receptor-dependent transcription (6).

SRC-3$^{-/-}$ null mice displayed many developmental defects, including growth retardation and reduction in body size, suggesting that SRC-3 might play a proliferative role *in vivo* (7–9). The link between SRC-3 and cancer was first established in breast cancer. Amplification and overexpression of SRC-3 was detected in 5% to 10% and 30% to 60% of breast cancer biopsies, respectively (5, 10). Furthermore, SRC-3 was shown to be overexpressed in other hormone-dependent tumors, such as prostate and ovarian cancers (11–13). SRC-3 transgenic mice developed a high incidence of spontaneous tumors in mammary glands, pituitary, and uterus, whereas SRC-3 deficiency can reduce the incidence of breast cancer induced by H-ras or carcinogen, 7,12-dimethylbenz(*a*) anthracene (14–16). These results suggest that SRC-3 is a bona fide oncogene.

Recently, SRC-3 overexpression was found to occur also in estrogen receptor (ER)-α-negative and progesterone receptor-negative breast cancer specimens (17). In SRC-3 transgenic mouse model, there is ~16.7% (8 of 48) of mammary gland adenocarcinomas that are ER-α negative (14). The ablation of SRC-3 has been shown to suppress v-Ha-ras-induced breast cancer initiation and progression in an ovarian hormone-independent manner (15). Furthermore, cumulative clinical evidences revealed that SRC-3 is highly associated with a variety of cancer not known to be hormone dependent, such as gastric, liver, pancreatic, and colon cancers (18–21). Consistent with these observations, our previous study indicated that overexpression of SRC-3 can induce hormone-independent growth of LNCaP prostate cancer cell line, whereas depletion of SRC-3 in androgen receptor (AR)–negative PC3 and DU145 prostate cancer cells reduced cell proliferation and induced apoptosis (12, 22). Collectively, the available data suggest that SRC-3 has nuclear receptor-independent functions, which may also contribute to the carcinogenesis.

Insulin-like growth factor (IGF)/AKT signaling is implicated in cell growth and frequently found to be activated in cancer patients (23, 24). IGF-I and IGF-II are expressed in the normal tissues and promote mitogenesis and survival of normal epithelial cells and cancer cells *in vitro* (23). Furthermore, IGF-I or IGF-II transgenic mice promote mammary tumorigenesis (25–27). These IGF signalings stimulate the mitogenic, transforming, differentiating, and antiapoptotic actions through IGF-I receptor (IGF-IR). Activated IGF-IR in turn mediates its functions through activation of intracellular substrates, such as insulin receptor substrate (IRS)-1 and IRS-2 (28, 29). Recently, it was shown that IRS-1 and IRS-2 are

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doi:10.1158/0008-5472.CAN-06-2442

essential intermediates in the activation of phosphatidylinositol 3-kinase (PI3K). PI3K consists of a p85 regulatory subunit containing two SH2 domains and one SH3 domain as well as a p110 catalytic subunit that phosphorylates inositol lipids specifically at the D-3 position of the inositol ring (30). AKT is a downstream target of PI3K. AKT binds to the lipid products of PI3K and is then activated through the phosphorylation of $Thr^{308}$ and $Ser^{473}$ by the PI3K-dependent kinases PDK1 and PDK2 (24). Activation of IGF/AKT signaling in cancer cells can stimulate cell proliferation and increase the threshold for cell apoptosis.

In this study, we now show that SRC-3 coordinately and directly regulate the transcription of many IGF/AKT signaling components. Additional experiments reveal that the recruitment of SRC-3 to the promoters of IRS-2 and IGF-I requires activator protein-1 (AP-1). Our data suggest that SRC-3 may induce hormone-independent cell growth via coactivation of AP-1.

## Materials and Methods

**Tissue culture.** The LNCaP human prostate cancer cell line with inducible expression of SRC-3, generated in our laboratory (22), was maintained in the RPMI 1640 supplemented with 10% dialysed FCS, 50 μg/mL penicillin-streptomycin, 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate (Invitrogen Corp., Carlsbad, CA). MCF-7 and HeLa cells were maintained in the DMEM and supplemented as above. PC3 cells were maintained in the DMEM/F-12 and supplemented as above. All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO2.

**Mice.** The targeted SRC-3 mutant mice with a mixed 129SvEv and C57BL/6J background were described previously (7). Genotyping was done by using specific primers for SRC-3 as described previously (7). Littermates were used in the comparison.

**Microarray analysis.** Induced SRC-3 LNCaP cells were treated with $10^{-8}$ mol/L mifepristone (Invitrogen) or vehicle for 8 and 24 hours, respectively. Total RNA was isolated from three independent experiments using the RNeasy Total RNA Isolation Midi kit (Qiagen Ltd., Crawley, United Kingdom). The cDNA reverse transcription from 20 μg total RNA and fluorescent labeling reactions were carried out using cyanine 5-labeled nucleotides for the control cDNA and cyanine 3-labeled nucleotides for experimental samples. Arrays were hybridized as described previously (31) for 4 hours at 50°C against a microarray chip carrying oligonucleotides representing >21,000 human genes and transcripts obtained from the Gene Array Facility of the Prostate Centre at Vancouver General Hospital.[5] After hybridization and posthybridization washes, slide was scanned immediately in Axon 4000A dual channel scanner (Axon Instruments, Foster City, CA) and the data were analyzed using GenePix version 4.01 software package (Axon). Data with low signal intensity, high background, and high variability were eliminated. The control RNA, Universal Human Reference RNA, composed of 10 different cell lines for broad gene coverage on human microarrays, was purchased from Stratagene (La Jolla, CA).

The data obtained from the GenePix were further analyzed by loading in to GeneSpring software (version7.0; Agilent Technologies, Palo Alto, CA). Array-specific data normalization was then done using the LOWESS, "locally-weighted regression and smoothing scatter plots," procedure. The fold difference of the samples over the reference of these genes were calculated and grouped according to their differentially expressed values.

**Semiquantitative reverse transcription-PCR analysis.** Total RNA was isolated using the Trizol reagent (Invitrogen) according to the instructions of the manufacturer. Reverse transcription was carried out with 3 μg RNA using a SuperScriptase II First-Strand synthesis system (Invitrogen) according to the instructions of the manufacturer. Specific primers used for reverse transcription-PCR (RT-PCR) are listed below. Human SRC-3, 5′-cgtcctccatataacccagc-3′ and 5′-tcataggttccattcttccg-3′; human β-actin, 5′-atcatgtttgagaccttcaa-3′ and 5′-catctcttgctcgaagtcca-3′; human IGF-I, 5′-catgcccaagacccagaag-3′ and 5′-cagcccgagttgtgtagaa-3′; human IGF-II, 5′-gcgctggcagaggagtgt-3′ and 5′-ggggtatctggggaagttg-3′; human IRS-1, 5′-ggcggcgtggatttcaga-3′ and 5′-tcgtggtggccccttagca-3′; human IRS-2, 5′-gtgcacccgtacctatgga-3′ and 5′-ggcgtttgcaatgtgaagta-3′; human PIK3CA, 5′-aaggccgaaagggtgctaaa-3′ and 5′-catctgggctacttcatctc-3′; human AKT1, 5′-atggcgctgagattgtgtc-3′ and 5′-atctgggccgtgaactcct-3′; human Bcl2, 5′-gccctcctgccctccttc-3′ and 5′-gcaattagcccccgtgacc-3′; human PDK1, 5′-tacctgccggctatgtcg-3′ and 5′-ccgggtggctctggtatc-3′; human IGFR1α, 5′-cggccgcagacacctacaa-3′ and 5′-ggacagcgacgggcagagc-3′; mouse β-actin, 5′-cctgaaccctaaggccaaccg-3′ and 5′-gctcatagctcttctccaggg-3′; mouse IGF-I, 5′-gaagtgcaggaaacaagac-3′ and 5′-ctacgtgggaagaggtgaa-3′; mouse IGF-II, 5′-gtggcatcgtggaagagt-3′ and 5′-ggggtgggtaaggagaaa-3′; mouse IRS-1, 5′-ggcaggggaggacttgag-3′ and 5′-ctgggtggagggttgttg-3′; mouse IRS-2, 5′-gtagcggcggagaacgag-3′ and 5′-acgacccggacgactgaca-3′; mouse PIK3CA, 5′-tggagccacagacactact-3′ and 5′-ggccaaacctctgactgac-3′; mouse AKT1, 5′-ccacgctacttcctcctc-3′ and 5′-tgcccttgcccagtagttt-3′; mouse Bcl2, 5′-ctcgtcgctaccgtcgtga-3′ and 5′-acttgtggcccaggtatgc-3′; mouse PDK1, 5′-gtcgggttgctacacatt-3′ and 5′-ttccccatcacctaacaga-3′; and mouse IGF-IR, 5′-acatccggagaggcaataa-3′ and 5′-ttcggaggcaggtctacat-3′.

**Immunoblotting and immunohistochemistry.** Total protein (40 μg) was separated by 8% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane overnight at 4°C. Western blotting analysis was done with specific antibodies against SRC-3 (BD Transduction Laboratories, Lincoln Park, NJ), IRS-1, IRS-2 (Upstate Biotechnology, Lake Placid, NY), PIK3CA, AKT1, Bcl2 (Cell Signaling Technology, Beverly, MA), or β-actin (Sigma, St. Louis, MO) according to the instructions of manufacturer. After the primary antibody incubation, the membrane was washed thrice for 5 minutes each with PBST and then incubated with a horseradish peroxidase–conjugated secondary antibody (Amersham, Piscataway, NJ) at a dilution of 1:3,000. The fluorescent signal was detected using the enhanced chemiluminescent detection system (Amersham) according to the instructions of the manufacturer.

Ventral prostate gland from 1-month-old wild-type (WT) or SRC-3 null mice were dissected immediately and fixed in 4% paraformaldehyde overnight at 4°C, washed with PBS, dehydrated, embedded in paraffin, and sectioned at 7-μm thick. Primary antibodies were used at the following dilutions: anti-IGF-I, 1:50 (Chemicon, Temecula, CA); anti-IGF-II, 1:80 (Chemicon); anti-IRS-1, 1:50 (Cell Signaling); anti-IRS-2, 1:50 (Cell Signaling); and anti-PIK3CA, 1:50 (Cell Signaling). Primary incubations were done overnight at 4°C (tissues). Primary antibodies were detected by immunoperoxidase using a Vectastain avidin-biotin complex method kit (Vector Laboratories, Burlingame, CA).

**Small interfering RNA treatment and transient transfection.** For the small interfering RNA (siRNA) experiments, MCF-7 and PC3 cells were seeded the night before transfection at such a density that cells reach about 60% to 70% confluence by the time of transfection. siSRC-3 SMART pool (40 nmol/L; Dharmacon, Lafayette, CO) was used for transfection using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. Transfected cells were continued in culture for 2 to 3 days before harvesting for further analyses. The efficiency of the siRNA knockdown was determined by Western analysis using corresponding specific antibodies.

Transient transfections of DNA were done using LipofectAMINE 2000 according to the manufacturer's instructions. Most of the transient transfection experiments were done with triplicates and repeated at least thrice.

**Chromatin immunoprecipitation.** Chromatin immunoprecipitation (ChIP) analyses were done as described previously (32). The crude chromatin solution was diluted and incubated overnight at 4°C with specific antibodies against SRC-3, c-Fos (Santa Cruz Biotechnology, Santa Cruz, CA). The protein-bound DNA precipitated from the ChIP assay was purified with the QIAquick PCR purification kit (Qiagen) and eluted in 50 μL elution buffer. Primers used for each gene are listed below. IGF-I, 5′-ttgtcaccatgcccaaaaaa-3′ and 5′-ttgcgcaggctctatctgc-3′; IGF-II, 5′-gccgcctcctcttcatctac-3′ and 5′-cgggcgcccagctcggtttg-3′; IRS-1, 5′-agctggcaccatccttgtt-3′ and 5′-cttgcccatccgtgtgac-3′; IRS-2, 5′-gccggcatccacaacaa-3′ and 5′-ctaagaagagcaaaacaaca-3′; primer 1 sets of PIK3CA, 5′-gttgagggcagggagtgaa-3′

[5] http://www.prostatecentre.com/facilities.php?pageID=26.

and 5′-gaggctatgggaagaatgaa-3′; primer 2 sets of PIK3CA, 5′-gccttttgatatactcttaca-3′ and 5′-tctgactgaaatgaatctaca-3′; primer 1 sets of AKT1, 5′-ggggcgctgtggtttagg-3′ and 5′-atggccccgtttgctctc-3′; and primer 2 sets of AKT1, 5′-ccaggtggccacttcttga-3′ and 5′-tgccgcctgcctttaccat-3′.

## Results

**Overexpression of SRC-3 in LNCaP prostate cancer cells up-regulates genes involved in the IGF/AKT signaling pathway.** To identify the potential target genes of SRC-3 during oncogenesis, we used mifepristone-induced system to overexpress SRC-3 in an LNCaP prostate cancer cell line, which we have described previously (22). Semiquantitative RT-PCR revealed that SRC-3 was induced on treatment with mifepristone for 4 hours and the induction reached a maximun with 8 hours of treatment and remained at high levels for 8 to 24 hours (Fig. 1*A*). RNA harvested from LNCaP cells treated with mifepristone or vehicle for 8 and 24 hours was analyzed using oligonucleotide microarrays that represent 21,000 transcripts. Each time point was done in triplicate. We found that 1,081 genes were up-regulated and 719 genes were down-regulated at 8 hours of induction, whereas 504 genes were up-regulated and 379 genes were down-regulated at 24 hours of induction by 1.44-fold change (Fig. 1*B*). Among these statistically significant altered genes, we found up-regulation and down-regulation of several genes related to cell proliferation, apoptosis, tumor cell invasion, and metastasis. Interestingly, some SRC-3 up-regulated genes are involved in cell cycle progression, such as *cyclin D2, cyclin E2*, and *cdc25A*, and some in nuclear factor-κB (NF-κB) signaling, such as *RelA, Birc2, Birc3*, and *Gadd45β* (Fig. 1*C*). Most importantly, our microarray data revealed that many components in the IGF/AKT signaling showed significant induction during 8 to 24 hours of treatment of mifepristone. Those genes include *IGF-I, IGF-II, IRS-1, IRS-2, PIK3CA*, and *AKT1* (Fig. 1*C*).

**Validation of microarray data by semiquantitative RT-PCR and Western blotting analysis.** To confirm the induction of expressions of genes involved in IGF/AKT signaling, we conducted semiquantitative RT-PCR analysis. Our results revealed that seven genes (*IRS-1, IRS-2, IGF-I, IGF-II, PIK3CA, AKT1*, and *Bcl2*) involved in the IGF/AKT signaling were induced after 4 hours of mifepristone induction of SRC-3 levels. The induction reached a peak at 8 hours and remained high after 24 hours (Fig. 2*B*). To confirm that altered SRC-3 gene expression was reflected also at the protein level, we investigated SRC-3 expression by Western blot analysis (Fig. 2*C*). Mifepristone induced SRC-3 protein by 2.7-fold at 24 hours. In addition, IRS-2, PIK3CA, and AKT1 proteins were induced. We also examined the IRS-1 protein level but could not measure the signal. Several reports have indicated that no IRS-1 protein was detected in LNCaP cells (33, 34). The reason for our inability to detect IRS-1 protein even when its mRNA was induced is not clear. We also examined AKT2 and AKT3 mRNA level. The results showed that AKT2 level was not changed during SRC-3 induction and AKT3 was not detected in LNCaP cells (data not shown), which is consistent with previous report (35). In any event, the data collectively suggest that SRC-3 coordinately regulates the expression of many components of the IGF/AKT signaling.

**Depletion of endogenous SRC-3 reduces the expression of the IGF/AKT signaling components.** To further confirm our microarray data, we used RNA interference to knock down endogenous SRC-3 in two cancer cell lines. These are PC3 human prostate cancer cell line (without AR expression) and MCF-7 human breast cancer cell line, which have been found to express a large amount of SRC-3 (5, 12). Semiquantitative RT-PCR showed clearly that SRC-3 was reduced dramatically after 72 hours of exposure to SRC-3 siRNA in both cell lines (Fig. 3). In addition, we observed that Bcl2 expression was reduced as well. This is consistent with our earlier observation that knockdown of SRC-3 resulted in the reduction of Bcl2 expression (12). Parallel to the reduction of SRC-3, mRNA levels for IGF-I, IGF-II, IRS-1, IRS-2, and PIK3CA were all decreased. In PC3 cells, AKT1 and IRS-1 were dramatically reduced, whereas AKT1 showed only slight reduction in MCF-7 cells (Fig. 3). Moreover, we found that two other genes in this IGF/AKT signaling are reduced in SRC-3 knocked down cells. These are *IGF-IR* and *PDK1*. Similar data were also obtained in LNCaP prostate cell line

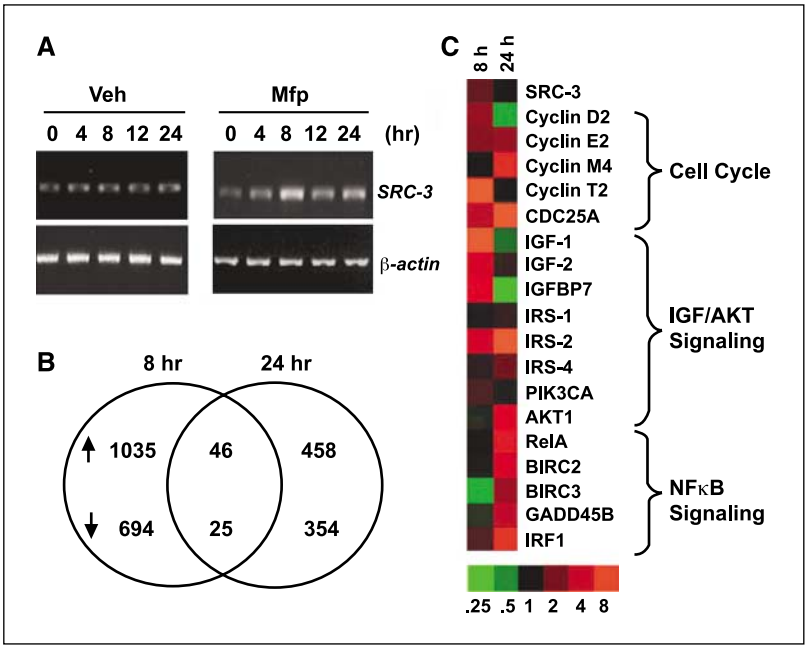


**Figure 1.** SRC-3 induces multiple genes involved in cell cycle, IGF/AKT signaling, and NF-κB signaling. *A*, total RNA was isolated after mifepristone (*Mfp*; $10^{-8}$ mol/L) treatment at indicated time points. Semiquantitative RT-PCR was done to confirm the induction of SRC-3 in LNCaP-SRC-3 stable cell line. *B*, Venn diagrams shows the numbers of significant changed genes at 8 and 24 hours of mifepristone treatment compared with vehicle treatment groups. ↑, up-regulated genes; ↓, down-regulated genes. *C*, illustration of genes induced by SRC-3 in LNCaP-SRC-3 stable cells represented in the oligonucleotide microarray. Genes are listed in rows. The ratio of gene expression levels in the mifepristone-treated group to the vehicle-treated group is color coded. The differential expression fold is indicated in the color key.

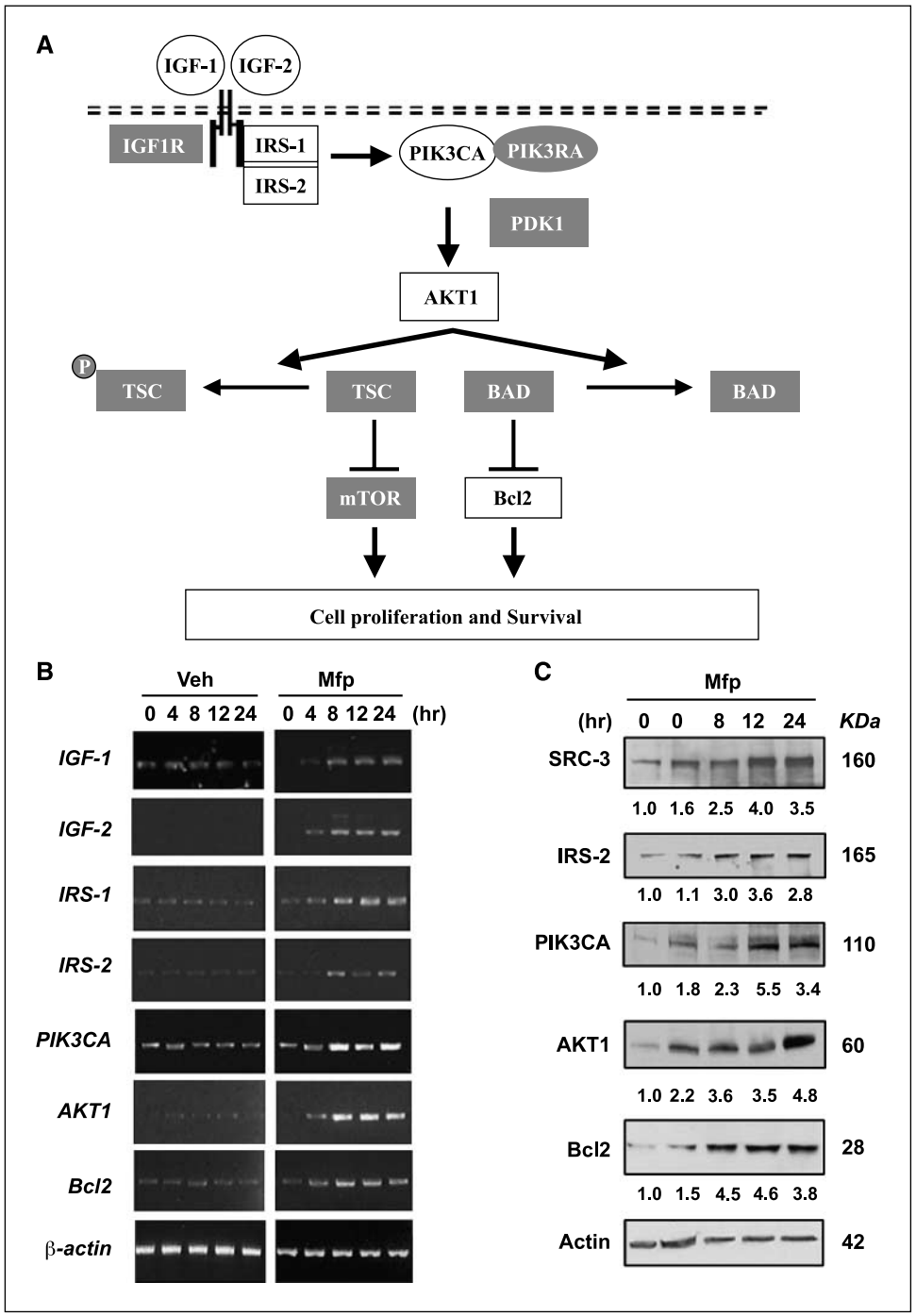


**Figure 2.** Up-regulation of IGF/AKT signaling components by SRC-3 in LNCaP prostate cancer cell line. *A,* diagram of IGF/AKT signaling pathway. According to microarray data, the genes shown induction by SRC-3 are in empty circles or box. *B,* treatment with mifepristone ($10^{-8}$ mol/L) induces IGF-I, IGF-II, IRS-1, IRS-2, PIK3CA, and AKT1 in LNCaP-SRC-3 stable cell lines showed by semiquantitative RT-PCR assay. Vehicle (*Veh*) treatment did not induce these genes expression. *C,* Western blot analysis showed the induced expression of SRC-3, IRS-2, PIK3CA, and AKT1 during 24 hours of mifepristone treatment.

treated with SRC-3 siRNA (data not shown). Overall, these results are consistent with our overexpression data showing that SRC-3 indeed regulates the expression of many components of the IGF/AKT pathway in different cancer cell lines.

**Reduction of the expression of the IGF/AKT signaling components in the prostate gland of SRC-3 knockout mice.** As shown above, *in vitro* ectopic expression and knockdown of SRC-3 in cell culture models have shown that these IGF/AKT signaling components are tightly regulated by SRC-3 expression. Next, we asked whether this regulation also occurs *in vivo* in a SRC-3 null mouse model. For this purpose, we collected prostate glands from SRC-$3^{+/+}$, SRC-$3^{+/-}$, and SRC-$3^{-/-}$ mice and isolated RNA for semiquantitative RT-PCR analysis (Fig. 4*A*). The data showed that these IGF/AKT signaling components, except PDK1, are reduced in SRC-$3^{-/-}$ mice, whereas there was no significant difference between SRC-$3^{+/+}$ and SRC-$3^{+/-}$ mice.

To further confirm our RT-PCR results, we did immunohistochemistry on the prostate glands of 1-month-old SRC-3 WT and knockout mice. Three parts of prostate glands (ventral, dorsolateral, and anterior prostates) of SRC-3 null mice are all smaller than its WT littermate. We found IGF-I, IGF-II, IRS-1, IRS-2, and PIK3CA expressed mainly in luminal epithelial cells of ventral prostate

gland of 1-month-old WT mice, whereas some signals were also detected from stromal cells and basal epithelial cells. In comparison with WT ventral prostate gland, the prostate gland from SRC-3 null mice displayed reduction of staining intensity, which is consistent with our findings by semiquantitative RT-PCR assay (Fig. 4*B*).

Therefore, both our *in vitro* and *in vivo* results indicate that SRC-3 coordinately regulates components of the IGF/AKT signaling pathway.

**Recruitment of SRC-3 onto the promoters of the IGF/AKT signaling components.** Because we found that these IGF/AKT signaling components are tightly regulated by SRC-3 at the mRNA expression level, we sought to determine whether SRC-3 is directly involved in transcriptional control of the expression of these IGF/AKT signaling components by examining whether SRC-3 is recruited to their promoter. To address this question, we used the ChIP method. We designed primer sets localized within 2 kb upstream of transcription start sites of these six genes. Representative ChIP results are shown in Fig. 5. After induction of SRC-3 in LNCaP prostate cancer cell line for 12 hours, SRC-3 was observed to occupy the promoters of IGF-I, IGF-II, IRS-1, IRS-2, PIK3CA, AKT1, and Bcl2, which indicate that these genes are direct transcriptional targets of SRC-3. We further show that the association is site specific: primer 1 sets derived from human PIK3CA and AKT1 promoters displayed strong occupancy by SRC-3, respectively, whereas primer 2 sets from other regions of the promoters of these two genes did not detect SRC-3 binding activity.

To reconfirm the result above derived from SRC-3-overexpressed cell line, we carried out ChIP assay with endogenous SRC-3 in PC3 prostate cancer cell line. Similar results were obtained in these PC3 cells, indicating that they are direct SRC-3 target genes (Fig. 5).

**SRC-3 coactivates AP-1 to induce IRS-2 transcription.** To understand how SRC-3 is recruited to the promoters of its target genes, we examined IRS-2 promoter, which responds to SRC-3. Approximately 2.1 kb of a genomic DNA fragment containing the exon 1 of IRS-2 was isolated and subcloned into pGL3-basic plasmid, as described previously (36). Serial deletions of the IRS-2

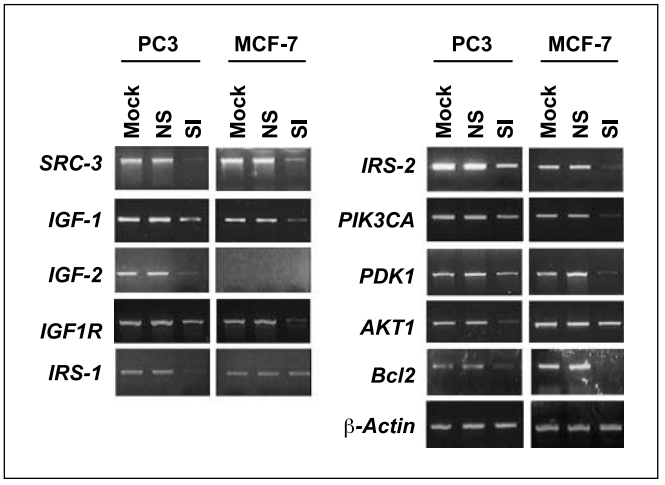


**Figure 3.** Depletion of endogenous SRC-3 decreases the IGF/AKT signaling components in PC3 human prostate cancer cell line and MCF-7 human breast cancer cell line. PC3 and MCF-7 cells were transfected with siRNA to SRC-3 for 3 days and total RNAs were extracted and semiquantitative RT-PCR was conducted with different primer sets. β-Actin transcripts were served as control. *Mock,* no siRNA but transfection reagent; *NS,* nonspecific siRNA control; *SI,* SRC-3-specific siRNA.

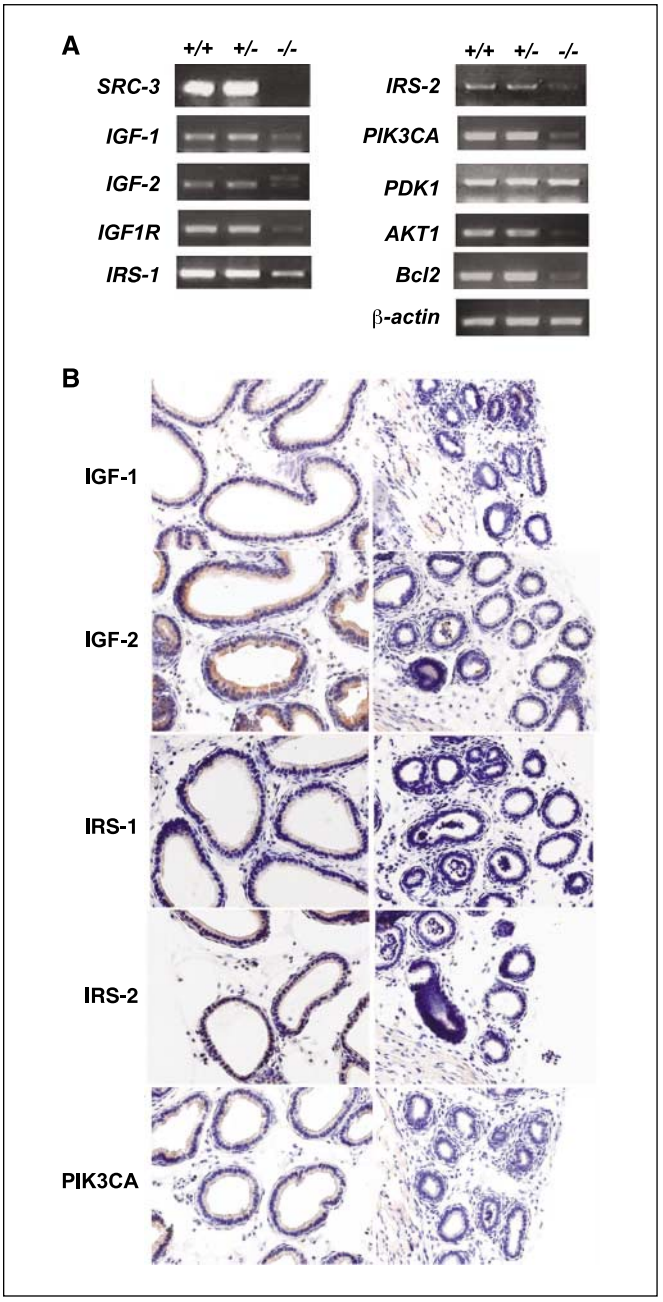


**Figure 4.** IGF/AKT signaling components are reduced in the prostate glands of SRC-3 null mice. *A,* total RNAs were harvested from prostate glands of 25-day SRC-$3^{+/+}$, SRC-$3^{+/-}$, and SRC-$3^{-/-}$ male mice. Semiquantitative RT-PCR was conducted with different primer sets. β-Actin transcripts were served as control. *B,* immunohistochemical staining of paraffin section of the ventral prostate gland from 1-month-old SRC-3 WT and SRC-3 null littermate mice with antibodies against IGF-I, IGF-II, IRS-1, IRS-2, and PIK3CA.

promoter and a promoterless control vector, pGL3, were transfected with SRC-3 expression plasmid into HeLa cells. As shown in Fig. 6*A*, pGL3-IRS-2 promoter can be regulated by SRC-3 (~8-fold) and the major response element for SRC-3 is located between −320 and −42. Next, we analyzed its sequence with the MatInspector and identified a conserved putative AP-1 binding site located around −42 (Fig. 6*B*). Because it was reported that SRCs could coactivate c-Fos, a subunit of the AP-1 complex (37), we examined the effects of AP-1 and SRC-3 on an IRS-2 promoter. AP-1 and

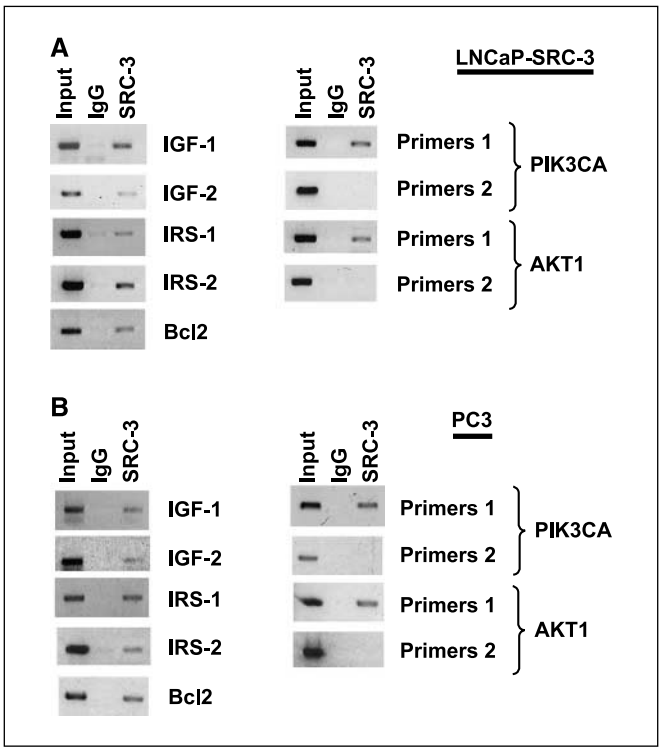


**Figure 5.** SRC-3 is recruited to the promoters of IGF/AKT signaling components. *A,* ChIP analysis of SRC-3 occupancy on IGF/AKT signaling components in LNCaP-SRC-3 stable cells. Cells were harvested 12 hours after mifepristone treatment. *B,* ChIP analysis of endogenous SRC-3 recruitment to the promoter of IGF/AKT signaling components in PC3 prostate cancer cell line. The ChIP assay was done using α-SRC-3 antibody. Normal IgG was used as negative control.

SRC-3 were transfected alone or combined with pGL3-IRS-2 (−320) into HeLa cells. Transfection of SRC-3 enhanced luciferase activity, whereas transfection of AP-1 also stimulated IRS-2 promoter activity. More importantly, cotransfection of SRC-3 and AP-1 synergistically induced the IRS-2 promoter activity, indicating that AP-1 may recruit SRC-3 to the IRS-2 promoter and together they regulate its expression.

**AP-1 is required for the recruitment of SRC-3 to the IRS-2 and IGF-I promoter.** To further confirm the direct regulation of IRS-2 by AP-1 and SRC-3, we knocked down endogenous SRC-3 and c-Fos in PC3 prostate cancer cells. As shown in Fig. 7*A* and as expected from the earlier data, knockdown of SRC-3 expression reduced the IRS-2 expression. Importantly, depletion of cellular c-Fos also reduced the level of IRS-2 mRNA expression. Similarly, we detected reduction of IGF-I expression in PC3 cells when either SRC-3 or c-Fos was depleted by siRNA. In SRC-3-LNCaP cells, c-Fos, together with SRC-3, was recruited to the IRS-2 and IGF-I promoter (Fig. 7*B*). Moreover, because SRC-3 and c-Fos were recruited to the same region, we asked whether SRC-3 is recruited by AP-1 to these promoters. For this reason, we knocked down the expression of c-Fos by siRNA in PC3 cells and asked whether SRC-3 recruitment to these promoters is affected. To this end, we carried out ChIP assay using SRC-3 antibody to precipitate chromatin DNA and used the same primer set to do PCR. Our results showed that depletion of c-Fos reduced or eliminated the occupancy of SRC-3 to the promoter of IRS-2 and IGF-I, respectively (Fig. 7*C*). Taken together, our result clearly showed that AP-1 is required for the recruitment of SRC-3 to the IRS-2 and IGF-I promoter.

## Discussion

We and others have found that SRC-3 overexpression can enhance AKT signaling, eventually stimulating cell growth and proliferation and suppressing apoptosis (14, 22). In the present study, we showed by microarray analysis that induction of expression of SRC-3 in LNCaP human prostate cancer cells altered expression of numerous genes. Among them, multiple IGF/AKT signaling components were up-regulated at mRNA and protein levels. These genes include *IGF-I, IGF-II, IRS-1, IRS-2, PIK3CA, AKT1*, and *Bcl2*. Moreover, depletion of SRC-3 in two cancer cell lines (PC3 and MCF-7) or ablation of SRC-3 in mouse prostate gland caused a decrease in their expression. Most importantly, we showed by ChIP assays that the coordinate regulation of these genes by SRC-3 is at the transcription level and it occurs via direct recruitment of SRC-3 to the promoters of those genes. The recruitment of SRC-3 to the promoters of *IRS-2* and *IGF-I* genes required the presence of c-Fos, a subunit of the AP-1 complex, suggesting that coactivation of AP-1

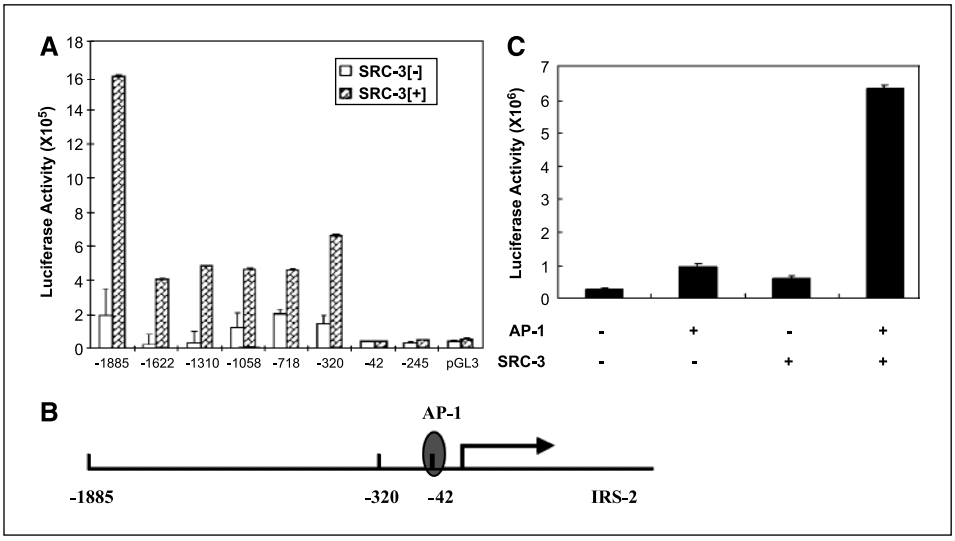


**Figure 6.** SRC-3 coactivates AP-1 and stimulate IRS-2 promoter activity. *A,* HeLa cells were cotransfected with the indicated reporter plasmids, expression constructs for SRC-3, or empty vector. Luciferase activities were normalized with protein mass. *B,* the putative AP-1 binding site was identified with MatInspector program (*gray circle*). *C,* SRC-3 and/or c-Fos/c-Jun were cotransfected with the pGL3-IRS-2 (−320) reporter in the HeLa cells. A synergistic induction of IRS-2 promoter activity was detected in SRC-3 and c-Fos/c-Jun group.

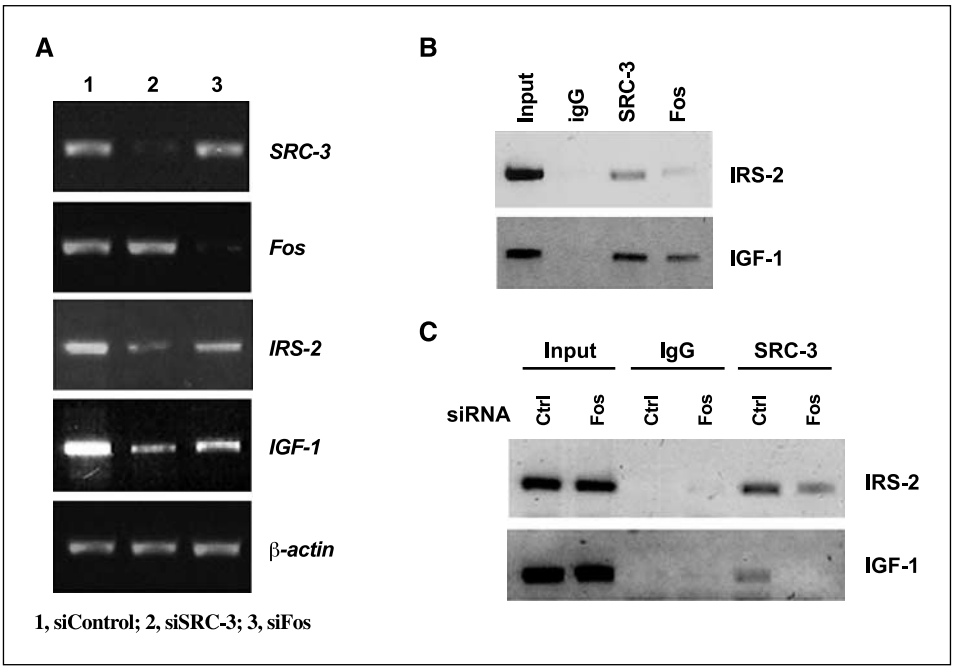


**Figure 7.** c-Fos is required for the recruitment of SRC-3 to IRS-2 and IGF-I promoter. *A,* depletion of SRC-3 or c-Fos decreases the IRS-2 and IGF-I expression in PC3 cells by semiquantitative RT-PCR. *B,* recruitments of c-Fos, as well as SRC-3, to IRS-2 and IGF-I promoters. LNCaP-SRC-3 cells were harvested 12 hours after mifepristone treatment. The ChIP assay was done using α-SRC-3 antibody. Normal IgG was used as negative control. *C,* endogenous c-Fos was knocked down in PC3 cells by siRNA method. The ChIP assay was done using α-SRC-3 antibody. Normal IgG was used as negative control.

by SRC-3 may promote carcinogenesis. Our data indicated that SRC-3 can activate IGF/AKT signaling pathway through coordinate induction of the transcription of multiple major components in this signaling pathway and, consequently, through the enhancement of the phosphorylation cascade in the PI3K/AKT signaling pathway to promote cell growth and inhibit apoptosis.

The IGF/AKT signaling pathway has important roles in regulating cellular proliferation and apoptosis (23). Alteration of IGF/AKT signaling is frequently detected in a variety of cancer types. Higher circulating IGF-I levels have been correlated with increased risk for several common cancers (38). IGF-I or IGF-II transgenic mice showed a high frequency of mammary tumors (25–27). In addition, deregulation of IRS-1, IRS-2, PIK3CA, and/or AKT1 have been detected in a wide variety of malignancies, including prostate cancer, and are thought to play a central role in cancer progression (24, 39–41). Primary epithelial cell cultures from virgin WT and SRC-3 transgenic mammary glands revealed that IGF/AKT signaling was activated by SRC-3 through phosphorylation of IGF-IR, AKT1, and their downstream effectors, such as glycogen synthase kinase-3, mammalian target of rapamycin, and P70S6K (14). Our previous data indicated that signaling downstream of IGF/AKT was affected by overexpression of SRC-3 in LNCaP cells via induction and phosphorylation of AKT1 (22). Here, we found that SRC-3 could coordinately regulate many components of upstream and downstream of this signaling pathway. The activation of many important components of the IGF/AKT signaling at both transcription and phosphorylation levels should lead to tumor progression by ensuring the efficient downstream signaling to enhance cell growth and reduce apoptosis. In agreement with this notion, a previous study showed that SRC-3 null mouse embryonic fibroblasts were resistant to IGF-I-induced proliferation (42). Furthermore, in a breast cancer cell line, IGF-I-induced survival of cells in suspension and changes in gene expression were reported to be dependent on AIB1 (43).

SRC-3 has been implicated in hormone-independent and hormone-dependent carcinogenesis (41). Recently, two studies showed that SRC-3 can interact and coactivate E2F1 to induce cell cycle–related genes, such as cyclin A, cyclin E, and cyclin-dependent kinase 2, independent of ER (44, 45). In our study, we showed that the recruitment of SRC-3 to the promoters of its target genes, IRS-2 and IGF-I, requires c-Fos, a subunit of AP-1 complex. Because AR is not expressed in PC3 prostate cancer cells, SRC-3 coactivates AP-1 in an AR-independent manner. Moreover, our previous study also revealed that SRC-3 could modulate AKT1 activity in a steroid-independent manner (22). Hence, our data suggested that SRC-3 could induce hormone-independent cancer cell growth via coactivation of AP-1.

AP-1 is implicated in diverse cellular processes, including cell proliferation and transformation (46). c-Fos and c-Jun are correlated with mammary carcinomas and androgen-independent prostate cancer (47, 48). The expression level of c-Fos is critical for MCF-7 breast cancer cell growth (49). Therefore, the coactivation of AP-1 by SRC-3 can contribute to carcinogenesis.

SRC-3, initially identified as nuclear receptor coactivator, has a broad spectrum of interacting proteins, including E2F1, AP-1, and NF-κB (37, 44, 50). Amplified and/or overexpressed in cancer cells, SRC-3 can coactivate and integrate multiple signaling pathways involved in cell proliferation and survival. In hormone-dependent cancer cells, SRC-3 can coactivate nuclear receptors (ER or AR) and induce target genes, such as cyclin D1, to accelerate cell cycle progression (44). But in hormone-independent stage and nonsteroid target tumors, higher expression of SRC-3 was also detected, suggesting that SRC-3 still can coordinately regulate gene expression to ensure hormone-independent cell proliferation and survival.

Taken together, available data suggests that, similar to a bacterial operon, SRC-3 coordinately and directly regulates the transcription activation of many important transcription factors that together control cell proliferation and survival by up-regulating cell cycle progression proteins and components of the IGF/AKT signaling through AP-1. Our data thus explain why SRC-3 is such a potent oncogene, which can bypass the feedback control of individual

pathways to ensure tumor growth when it is overexpressed. As such, it represents an important target in cancer therapy due to its central role in these important growth pathways.

## Acknowledgments

Received 7/5/2006; revised 8/23/2006; accepted 9/18/2006.

**Grant support:** NIH grant DK62821, National Cancer Institute Baylor Prostate Cancer Specialized Programs of Research Excellence program grant P50CA058204 (M-J. Tsai and M. Ittmann), and Nuclear Receptor Signaling Atlas grant DK62434 (M-J. Tsai and S.Y. Tsai).

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We thank Dr. Hashiramoto for providing the pGL3-IRS-2 promoter plasmids, Dr. Bert W. O'Malley for comments on our article, Microarray facility of Baylor, and Michael E. DeBakey Veterans Affairs Medical Center for the use of the facilities.

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# Steroid Receptor Coactivator-3 and Activator Protein-1 Coordinately Regulate the Transcription of Components of the Insulin-Like Growth Factor/AKT Signaling Pathway

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*Cancer Res* 2006;66:11039-11046.