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Androgen-induced Long Noncoding RNA (lncRNA) *SOCS2-AS1* Promotes Cell Growth and Inhibits Apoptosis in Prostate Cancer Cells*

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Long noncoding RNAs (lncRNA) have been associated with the development of cancer. However, the interplay between lncRNAs and androgen receptor (AR) signaling in prostate cancer is still unclear. Here, we identified lncRNAs induced by androgen in AR-positive prostate cancer cells, where induction was abolished by AR knockdown as well as an anti-androgen, bicalutamide. By combining these data, we identified an androgen-regulated lncRNA, suppressor of cytokine signaling 2-antisense transcript 1 (*SOCS2-AS1*), the expression of which was higher in castration-resistant prostate cancer model cells, *i.e.* long-term androgen-deprived (LTAD) cells, than in parental androgen-dependent LNCaP cells. *SOCS2-AS1* promoted castration-resistant and androgen-dependent cell growth. We found that *SOCS2-AS1* knockdown up-regulated genes related to the apoptosis pathway, including tumor necrosis factor superfamily 10 (*TNFSF10*), and sensitized prostate cancer cells to docetaxel treatment. Moreover, we also demonstrated that *SOCS2-AS1* promotes androgen signaling by modulating the epigenetic control for AR target genes including *TNFSF10*. These findings suggest that *SOCS2-AS1* plays an important role in the development of castration-resistant prostate cancer by repressing apoptosis.

Recent advances in sequencing technologies have greatly enhanced our understanding of the human transcriptome, revealing that more than 90% of the human genome is actively transcribed, although only a minority of it is translated into proteins (1, 2). Most of the noncoding transcripts of more than 200 nt are known as long noncoding RNAs (lncRNAs)[3] (3). Although the number of known human lncRNAs is still evolving, some of them have been functionally characterized and experimentally validated.

Androgen receptor (AR) and its downstream signaling have a crucial role in the development and progression of both localized and advanced metastatic prostate cancer (4–6). High-risk localized prostate cancer is treated with androgen deprivation therapies in addition to surgery and radiotherapy (7–9). However, many prostate cancers inevitably escape from androgen dependence leading to castration-resistant prostate cancer (CRPC) (10). Past studies have revealed that elevated AR expression (4), activation of AR transcription (11), and development of AR variants (12) are observed in the progression to CRPC, suggesting the importance of identifying AR downstream signals and new molecular mechanisms for AR activation to improve the treatment of CRPC.

Some lncRNAs studied in prostate cancer, act in a highly prostate-specific manner (13–18). In prostate cancer, the first prominent lncRNA, *PCA3*, was initially described as a novel biomarker of disease (19) and subsequently defined as a promising urine test for this disease (20). Similarly, the lncRNA *PCGEM1* has been implicated in prostate cancer as a regulator of apoptosis (21). *PCAT-1* was characterized as a novel prostate-specific lncRNA, regulator of cell proliferation, and target of the polycomb repressive complex 2 (PRC2) (15). *HOTAIR* RNA binds to AR protein to block the interaction with the E3 ubiquitin ligase MDM2, thereby preventing protein degradation and AR activation (22).

In a previous study, we analyzed global AR transcriptional network by mapping genome-wide transcriptional start sites regulated by androgen and AR binding sites (ARBS). This integrative genomic study revealed comprehensive AR-regulated transcripts from intergenic or AS regions of genes in prostate cancer cells (23). In addition, we investigated the functional roles of these novel androgen-responsive long noncoding

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[3] The abbreviations used are: lncRNA, long noncoding RNA; AR, androgen receptor; CRPC, castration-resistant prostate cancer; ARBS, AR binding site; RIP, RNA immunoprecipitation; RNA-seq, RNA sequencing; LTAD, long-term androgen-deprived; DHT, 5α-dihydrotestosterone; siAR, siRNA-targeting androgen receptor; siNC, negative control siRNA; GO, gene ontology; DMSO, dimethyl sulfoxide; PARP, poly(ADP-ribose) polymerase; qPCR, quantitative PCR; qRT-PCR, quantitative RT-PCR; MTS, methanethiosulfonate.

RNAs, such as a lncRNA located at the AS region of the C-terminal-binding protein 1 *(CTBP1)* gene, *CTBP1-AS*, revealing an oncogenic role of androgen-regulated lncRNAs in prostate cancer progression (24).

In the present study, to explore more androgen-dependent lncRNAs that have roles in prostate cancer progression, we performed high-throughput sequencing analysis in androgen-treated prostate cancer cell lines. Among the androgen-induced lncRNAs in LNCaP and VCaP cells, we focused on suppressor of cytokine signaling 2-antisense transcript 1 (*SOCS2-AS1*), the expression of which was higher in long-term androgen-deprived (LTAD) and VCaP-LTAD cells, which are castration-resistant prostate cancer cells derived from LNCaP and VCaP cells (GenBank^{TM} accession number NR_038263). *SOCS2-AS1* promoted cell growth and migration and repressed several genes related to the apoptosis pathway, including *TNFSF10*, suggesting that androgen-induced *SOCS2-AS1* would play an important role in the progression of prostate cancer.

Results

Identification of Androgen-induced lncRNAs by Directional RNA Sequencing—To investigate hormone-regulated lncRNAs in prostate cancer, we performed directional RNA sequencing (RNA-seq) and identified lncRNAs induced by androgen in prostate cancer cell lines. For lncRNA analysis, we used two databases, GENCODE V19 (25) and NONCODE v4. AR-positive prostate cancer cell lines, LNCaP and VCaP, and their corresponding castration-resistant cell lines, LTAD and VCaP-LTAD (24), were treated with vehicle (ethanol) or 10 nM 5α-dihydrotestosterone (DHT). In addition, LNCaP and VCaP cells were also treated with DHT plus bicalutamide or with 10 nM siRNA-targeting AR (siAR). After 24 h, total RNAs were extracted, and then RNA-seq analysis was performed. Bioinformatic analysis identified lncRNAs that were up-regulated more than 1.5-fold by DHT treatment and repressed to less than 0.75-fold by bicalutamide and siAR treatment in both LNCaP and VCaP cell lines. Nine transcripts were common in both cell lines using the GENCODE annotation and two in the NONCODE annotation (Fig. 1*A*). We examined these 11 transcripts and found that they corresponded to lncRNAs transcribed from five different genes (Table 1).

SOCS2-AS1 Is an Androgen-induced lncRNA Highly Expressed in Castration-resistant Prostate Cancer Cells—Next, we performed qRT-PCR to analyze the expression of five lncRNAs in both LNCaP and VCaP and their LTAD cells. We validated their androgen induction as observed in RNA-seq data (Fig. 1, *B* and *C*). Among these five lncRNAs, we found that *SOCS2-AS1* was highly expressed in LTAD and VCaP-LTAD compared with the parental cell lines by RNA-seq and qRT-PCR analysis (Figs. 1, *A*–*C*, and Fig. 2*A*). Therefore, we chose this lncRNA for further study. Our ChIP-seq data (26, 27) revealed ARBS in the promoter region (Chr12: 93,963,629–93,965,465 (hg19), +1.5 kbp–291 bp from the transcriptional start site (TSS) of NR_038263). Interestingly, although its expression was repressed after 48 h of androgen treatment in LNCaP cells, it was further induced even after 72 h of androgen stimulation in LTAD cells (Fig. 2*B*). Its sense gene, *SOCS2*, showed a similar pattern of induction (Fig. 2, *B* and *C*). These data indicate a distinct pattern of induction in castration-resistant prostate cancer cells compared with hormone-naive prostate cancer.

SOCS2-AS1 is an antisense lncRNA transcribed from the opposite strand of the protein coding the *SOCS2* gene. *SOCS2* is one of the eight members of the SOCS family that are induced by cytokine stimulation through the Janus kinase (JAK/STAT) signaling. *SOCS* genes contribute to cytokine inhibition by reducing JAK or STAT phosphorylation, inhibiting the same cascade that initiated their production through a negative feedback mechanism (28–30). We identified ARBSs at a common promoter region of both *SOCS2-AS1* and *SOCS2*, as well as in the intronic region of *SOCS2-AS1*, suggesting that their expressions are directly regulated by *AR* (Fig. 2*A*). Consistently, *AR* knockdown by siRNA and pretreatment with the anti-androgen bicalutamide repressed *SOCS2-AS1* and *SOCS2* mRNA induction by androgen in both LNCaP and VCaP cells (Fig. 3, *A* and *B*). We reported previously the enhancement of AR expression and sensitivity in LTAD cells compared with parental cells (31). We analyzed whether high sensitivity of AR to a low concentration of DHT is involved in the induction of *SOCS2-AS1* in these cell lines. We observed that low concentration of DHT increased the *SOCS2-AS1* expression level in LTAD cells (Fig. 3*C*). In addition, AR binding to the promoter was observed in the absence of DHT and increased by low concentration of DHT (Fig. 3*D*). These results indicate one possibility that enhanced AR sensitivity in LTAD cells induce the overexpression of *SOCS2-AS1*.

SOCS2-AS1 Induces Prostate Cancer Cell Growth—To investigate the role of *SOCS2-AS1* in prostate cancer cells, we designed two siRNAs to reduce androgen-induced *SOCS2-AS1* expression levels in LNCaP and LTAD prostate cancer cell lines, as well as one siRNA targeting *SOCS2* (Fig. 4, *A*–*C*). We found that both *SOCS2-AS1* and *SOCS2* knockdown decreased cell proliferation in LNCaP and LTAD cells (Fig. 4*D*). In addition, we transfected si*SOCS2-AS1* or si*SOCS2* to LNCaP and LTAD cells and counted cell numbers over time after androgen treatment. In line with the results of the MTS assays, cell proliferation of si*SOCS2-AS1*- or si*SOCS2*-transfected cells was repressed compared with control siRNA-transfected cells, supporting the idea that *SOCS2-AS1* and SOCS2 increase cell proliferation rate (Fig. 4*E*).

In addition, we established LNCaP cells stably overexpressing *SOCS2-AS1* or SOCS2 (Fig. 5, *A* and *B*). *SOCS2-AS1* is located on chromosome 12 (93,959,404–93,965,174 (hg19) reverse strand, NR_038263) overlapping the promoter region of *SOCS2* (Fig. 2*A*). Given this close proximity, we hypothesized that *SOCS2-AS1* may regulate SOCS2. However, SOCS2 mRNA and protein levels were not altered with *SOCS2-AS1* overexpression (Fig. 5*B*), and the negative regulation of SOCS2 protein expression by antisense transcript was marginally detected but not evident (Fig. 5*C*).

We performed cell proliferation assays using normal culture medium and androgen-deprived medium. We found that the increase in the cell proliferation rate of *SOCS2-AS1* stable cells was higher than that of SOCS2 stable cells, not only in the normal medium (Fig. 5*D*) but also in androgen-deprived

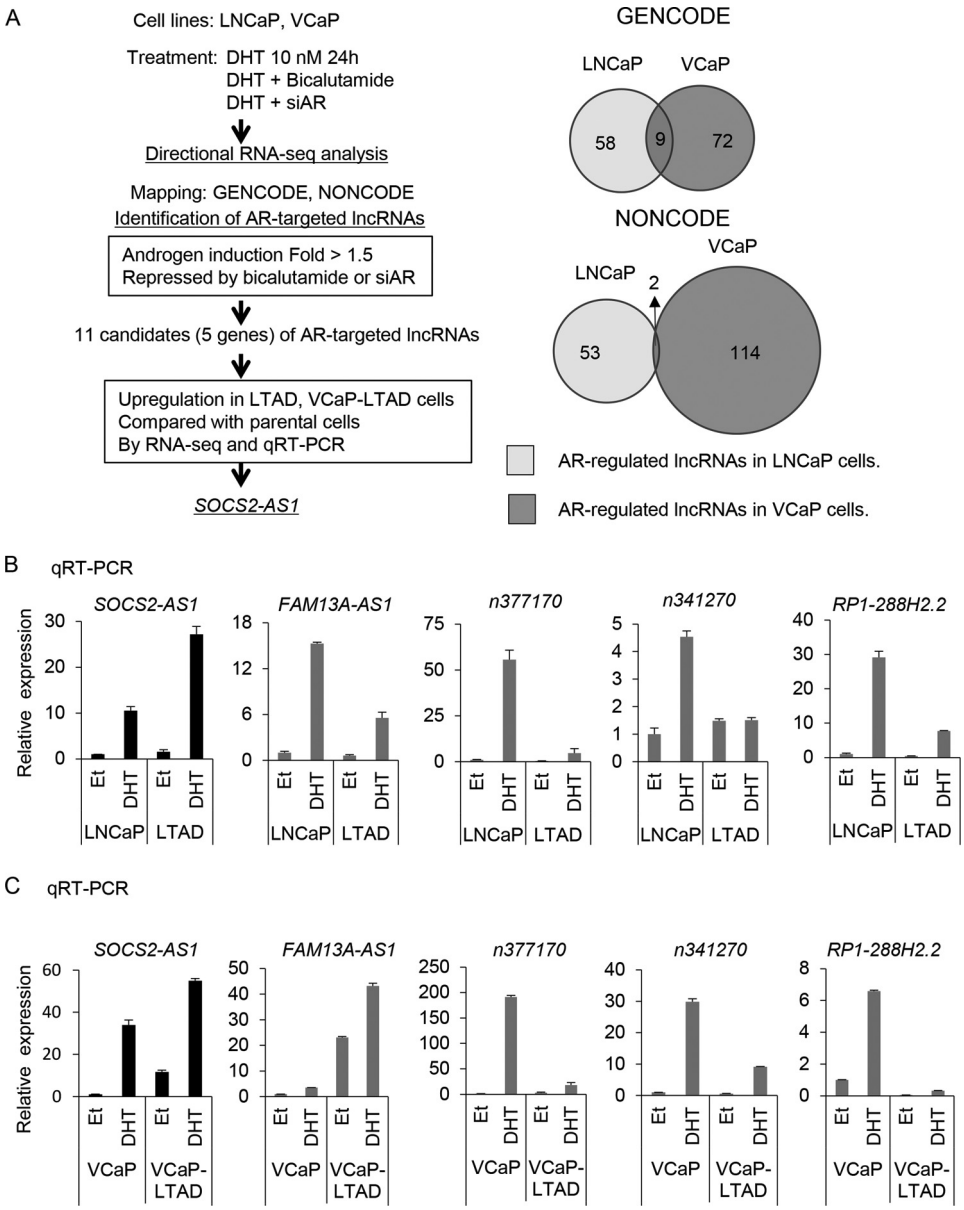


FIGURE 1. **Analysis of androgen-induced lncRNAs in prostate cancer and identification of *SOCS2-AS1* as a lncRNA highly expressed in castration-resistant prostate cancer.** *A*, flow diagram for the identification of *SOCS2-AS1* as an AR-targeted lncRNA up-regulated in LTAD cells. Venn diagram representing overlap of lncRNAs induced by androgen (10 nM DHT) and repressed by 1 mM bicalutamide and 10 nM siAR treatment for 24 h in LNCaP and VCaP cell lines. *B*, qRT-PCR analysis of androgen-induced lncRNAs by qRT-PCR in LNCaP cells. *C*, qRT-PCR analysis of androgen-induced lncRNAs by qRT-PCR in VCaP cells. Cells were treated with 100 nM DHT or ethanol (*Et*) for 24 h. The expression level of each lncRNA was determined by qRT-PCR. $n = 3$. Expression levels are presented relative to the value of *GAPDH* as the reference gene. Values represent mean ± S.D.

medium (Fig. 5*E*). These results suggest that *SOCS2-AS1* plays a central role in promoting both hormone-dependent and castration-resistant prostate cancer cell growth.

SOCS2-AS1 Induces Prostate Cancer Cell Migration—Next, we performed migration assay in LNCaP and LTAD cells transfected with si*SOCS2-AS1* or negative control siRNA. Both cell lines were cultivated for 2 days in androgen-deprived medium, transfected with siRNA for 24 h, transferred into 8-μm pore seize inserts. The number of cells migrated among *SOCS2-AS1* knockdown cells was lower compared with control cells (Fig. 6, *A* and *B*). We also performed migration assay using LNCaP cells stably expressing *SOCS2-AS1*, *SOCS2* and control cells. The number of cells migrated among *SOCS2-AS1*-overexpressing cells was higher than in SOCS2-overexpressing cells or control cells (Fig. 6, *C* and *D*). These data suggest that *SOCS2-AS1* may enhance the metastatic potential of prostate cancer cells.

SOCS2-AS1 Regulates the Expression of Apoptosis-associated Genes Such as TNFSF10—We performed microarray analysis to investigate the *trans*-regulatory effects of *SOCS2-AS1* in LNCaP cells by comparing the gene expression profiles in cells treated with si*SOCS2-AS1* or si*SOCS2* or with negative control siRNA (siNC) (Fig. 7*A*). We then found that *SOCS2-AS1* and SOCS2 have distinct downstream signals, suggesting the different biological responses by both genes were induced by such different gene regulations. Genes differentially expressed with si*SOCS2-AS1* transfection in DHT-treated cells were analyzed using the DAVID bioinformatics platform (32, 33). Gene ontology (GO) term analysis showed an enrichment of genes

TABLE 1

List of androgen-induced lncRNAs

lncRNAs induced by more than 1.5-fold with 10 nM DHT compared with ethanol (Et) treatment and repressed to less than 0.75-fold by bicalutamide (Bic) and siAR in LNCaP and VCaP cell lines are listed. RPKM, reads per kilobase per million mapped reads.

								RPKM			
	Database	Transcript Name	ID	Locus	Strand	Sense Strand Gene	Length	Et	DHT	DHT Bic	DHT siAR
LNCaP	Genecode	SOCS2-AS1-001	ENST00000499137.2	chr12:93936239-93965544	-	SOCS2	3325	0.3369	1.1402	0.3453	0.5240
		SOCS2-AS1-002	ENST00000500986.1	chr12:93936240-93965544	-	SOCS2	1655	0.6158	2.1971	0.6424	0.9953
		SOCS2-AS1-003	ENST00000547845.1	chr12:93945209-93960955	-	SOCS2	555	1.3896	5.8784	1.4941	2.5494
		SOCS2-AS1-004	ENST00000551626.1	chr12:93959360-93965174	-	SOCS2	618	1.2480	5.2792	1.3418	3.9970
		SOCS2-AS1-005	ENST00000549723.1	chr12:93960515-93965066	-	SOCS2	354	2.1786	9.2162	2.3425	2.2895
		FAM13A-AS1-001	ENST00000511543.1	chr4:89642866-89649837	+	FAM13A	2453	0.1160	0.7876	0.3676	0.0818
		FAM13A-AS1-002	ENST00000500765.1	chr4:89630940-89651254	+	FAM13A	3210	0.0887	0.7211	0.4074	0.1217
		RP1-288H2.2	ENST00000547538.1	chr12:52486269-52498081	-	-	512	0.6635	1.5663	0.2907	0.0825
		CTB-167B5.2	ENST00000585152.1	chr7:87900207-87903065	-	-	2859	0.0000	0.4622	0.0223	0.0443
	Noncode	CTB-167B5.2	n341270	chr7:87900206-87903065	-	-	2859	0.0000	0.4622	0.0223	0.0443
		TCONS_00029024	n377170	chr21:42953358-42954625	+	-	1182	0.0000	0.8558	0.2429	0.0000
VCaP	Genecode	SOCS2-AS1-001	ENST00000499137.2	chr12:93936239-93965544	-	SOCS2	3325	0.5654	4.0296	1.2633	2.3647
		SOCS2-AS1-002	ENST00000500986.1	chr12:93936240-93965544	-	SOCS2	1655	0.0760	7.9838	2.4825	6.4103
		SOCS2-AS1-003	ENST00000547845.1	chr12:93945209-93960955	-	SOCS2	555	0.1528	19.7518	6.1828	5.7733
		SOCS2-AS1-004	ENST00000551626.1	chr12:93959360-93965174	-	SOCS2	618	0.3796	17.6279	5.5525	10.0214
		SOCS2-AS1-005	ENST00000549723.1	chr12:93960515-93965066	-	SOCS2	354	0.3409	30.6916	9.6933	7.2389
		FAM13A-AS1-001	ENST00000511543.1	chr4:89642866-89649837	+	FAM13A	2453	0.1149	0.3946	0.0858	1.1556
		FAM13A-AS1-002	ENST00000500765.1	chr4:89630940-89651254	+	FAM13A	3210	0.0988	0.5959	0.1918	0.2155
		RP1-288H2.2	ENST00000547538.1	chr12:52486269-52498081	-	-	512	5.6580	65.0695	22.7286	18.6027
		CTB-167B5.2	ENST00000585152.1	chr7:87900207-87903065	-	-	2859	0.2248	30.7869	10.6454	7.2389
	Noncode	CTB-167B5.2	n341270	chr7:87900206-87903065	-	-	2859	0.2248	30.7869	10.6454	7.2389
		TCONS_00029024	n377170	chr21:42953358-42954625	+	-	1182	0.1693	29.9912	8.8549	9.6232

involved in apoptosis ($p = 4.4E\text{-}09$) for 594 genes up-regulated by si*SOCS2-AS1* transfection compared with control siRNA in LNCaP cells (Fig. 7*B*). Interestingly, Tumor necrosis factor superfamily 10 (*TNFSF10*), which is involved in apoptosis signaling, was the most up-regulated gene by si*SOCS2-AS1* transfection. Moreover, we also found that genes related to cell proliferation or cell cycling are major sources of *SOCS2-AS1* downstream signaling.

We further performed microarray analysis using si*SOCS2-AS1*-transfected LTAD cells (Fig. 7*C*). We identified genes regulated by *SOCS2-AS1* in LTAD cells (We obtained 301 up-regulated genes and 413 down-regulated by *SOCS2-AS1* knockdown in the presence of DHT). Interestingly, 37% of up-regulated and 81% of down-regulated genes were found to be LTAD-specific *SOCS2-AS1* targets (Fig. 7*D*). GO analysis of these genes indicated that *SOCS2-AS1* regulates a greater variety of signals, such as cell migration and transcriptional regulation in addition to apoptosis, cell cycle, and cell proliferation (Fig. 7*E*). We also found LTAD-specific target genes associated with apoptosis such as apoptosis-inducing factor 2 (*AIF2*) and checkpoint kinase 2 (*CHEK2*), suggesting the role of other signaling for apoptosis in LTAD cells.

TNFSF10 belongs to a small subset of pro-apoptotic protein ligands in the TNF superfamily. The TNF superfamily consists of proteins involved in proliferation, differentiation, and apoptosis, including TNF and Fas ligand (FasL) (34, 35). We also found that TRAIL receptor 1 (*DR4* or *TNFRSF10A*), as well as the cell death surface receptor *Fas*, was up-regulated by si*SOCS2-AS1* (Fig. 8*A*), indicating that *SOCS2-AS1* may repress the apoptotic pathways. We confirmed that *TNFSF10* expression in LNCaP cells stably overexpressing *SOCS2-AS1* was lower than in control cells (Fig. 8*B*). In contrast, *TNFSF10* expression was higher in LNCaP cells treated with si*SOCS2-AS1* knockdown cells (Fig. 8*B*). We confirmed by qRT-PCR that TRAIL receptor 1 and *Fas* were up-regulated, supporting the idea that *SOCS2-AS1* may repress the apoptotic pathway by modulating the expression of these genes (Fig. 8*B*). We also observed that si*SOCS2-AS1* treatment up-regulated *TNFSF10* expression in the presence of DHT in VCaP cells (Fig. 8*C*).

Interestingly, *TNFSF10* expression was totally repressed in LTAD cells (Fig. 8, *D* and *E*) and it was induced with *SOCS2-AS1* knockdown (Fig. 8*F*), suggesting the role of *SOCS2-AS1* in repressing *TNFSF10* in CRPC. Most of the other apoptosis-related genes in LNCaP cells (Fig. 8*A*) were also repressed in LTAD compared with LNCaP cells and up-regulated by si*SOCS2-AS1* in LTAD cells (Fig. 8*G*), suggesting the role of *SOCS2-AS1* up-regulation in the anti-apoptotic ability in CRPC cells.

SOCS2-AS1 Inhibits Apoptosis—To examine whether *SOCS2-AS1* is involved in apoptosis, we performed a cell proliferation assay in LNCaP and LTAD cells treated with docetaxel or dimethyl sulfoxide (DMSO) transfected previously

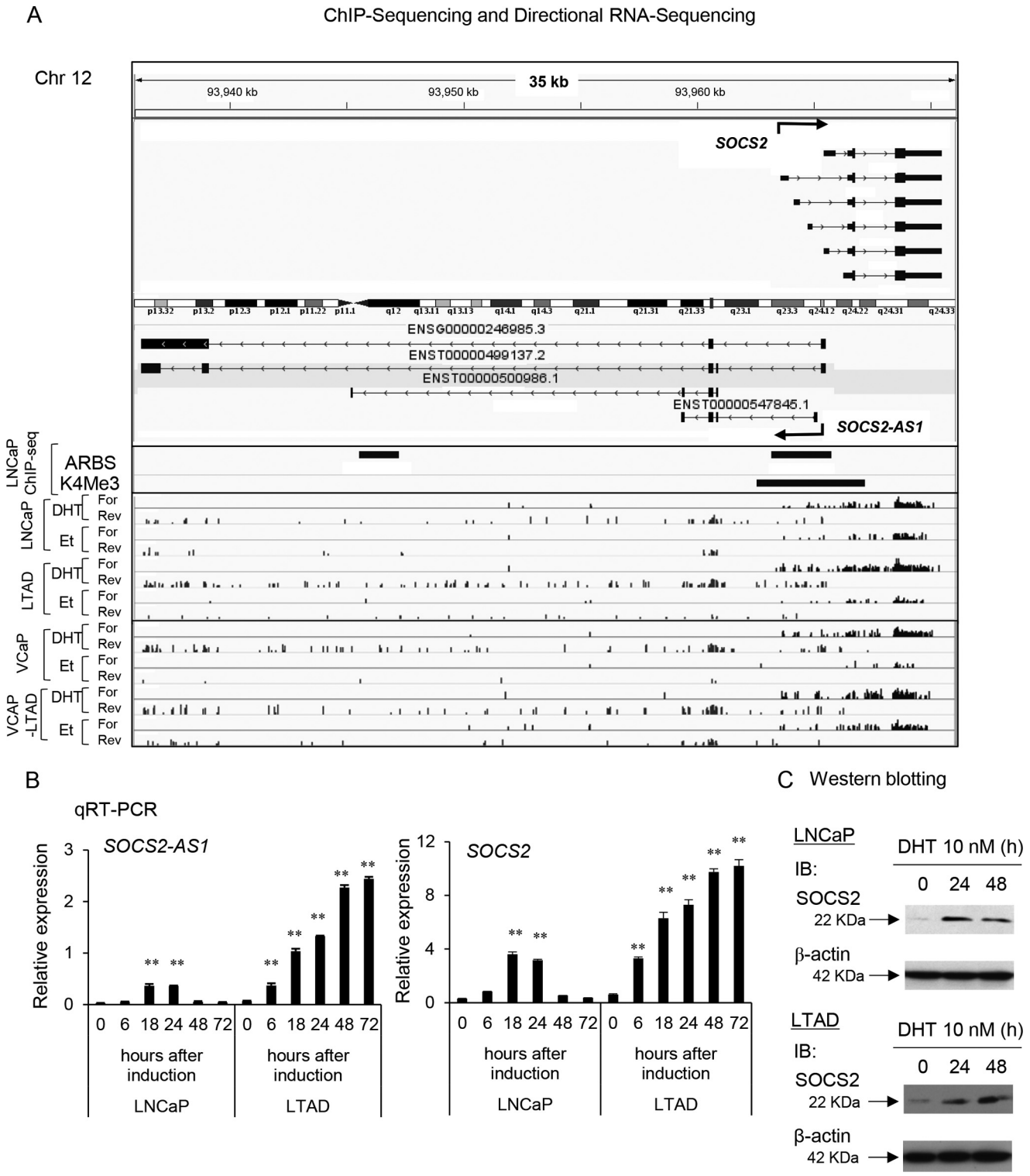


FIGURE 2. **SOCS2-AS1 is induced by androgen in LNCAP cells and is highly expressed in castration-resistant prostate cancer model LTAD cells.** *A*, RNA-seq and ChIP-seq analysis of *SOCS2-AS1* and *SOCS2* in LNCaP and VCaP cell lines treated with 10 nM DHT or ethanol (*Et*) for 24 h. *B*, time course analysis of *SOCS2-AS1* and *SOCS2* mRNA after DHT treatment in LNCaP and LTAD cell lines determined by qRT-PCR. *C*, Western blotting analysis of SOCS2 after 10 nM DHT treatment at indicated time points. β-Actin was used as an internal control. *IB*, immunoblot. Values represent mean ± S.D.; **, $p < 0.01$.

with si*SOCS2-AS1* or si*SOCS2*. LNCaP cells were treated with 1 nM docetaxel and LTAD cells with 2 nM docetaxel for 24 h. In addition, we showed further repression of *SOCS2-AS1* levels after 7 days of siRNA transfection (Fig. 9*A*). Cell viability was suppressed in the presence of docetaxel in *SOCS2-AS1* or *SOCS2* knockdown LNCaP cells and *SOCS2-AS1* knockdown LTAD cells (Fig. 9*B*). Next, we performed a TUNEL assay with LNCaP and LTAD cells transfected with si*SOCS2-AS1*, si*SOCS2*, or siNC. Green fluorescence-stained cells, representing cells undergoing apoptosis, were observed in both si*SOCS2-*

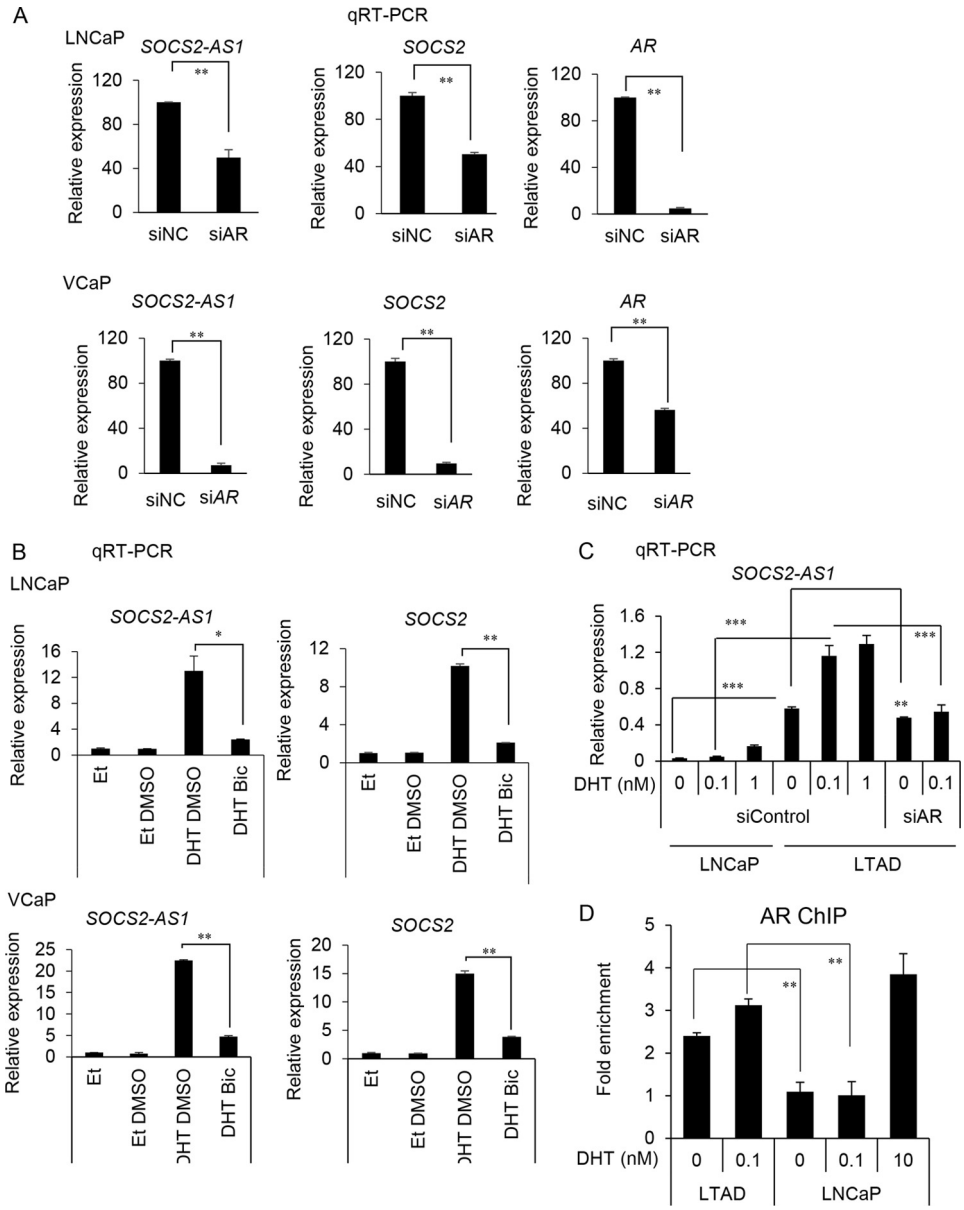


FIGURE 3. **AR knockdown and bicalutamide treatment represses *SOCS2-AS1* expression.** *A*, qRT-PCR analysis of *SOCS2-AS1*, *SOCS2* mRNA, and *AR* mRNA levels following 10 nM siAR treatment and subsequent androgen treatment (10 nM DHT for 18 h) in LNCaP and VCaP cell lines ($n = 3$). *B*, *SOCS2-AS1* and *SOCS2* mRNA levels following 1 μM bicalutamide or DMSO and 10 nM DHT or ethanol treatment for 24 h in LNCaP and VCaP cell lines by qRT-PCR ($n = 3$). RNA expression levels are presented relative to the value of *GAPDH* as the reference gene. *C*, qRT-PCR analysis of *SOCS2-AS1* mRNA level after low dose androgen treatment (0, 0.1, and 1 nM DHT for 18 h) in LNCaP and LTAD cell lines ($n = 3$). *D*, ChIP analysis of AR recruitments to the ARBS in the *SOCS2-AS1* promoter region by low level of androgen. LNCaP and LTAD cells were treated with ethanol or 0.1 nM DHT for 24 h. 10 nM DHT was used as a positive control. -Fold enrichment over control region (*GAPDH*) was measured by qPCR. Values represent mean ± S.D.; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

AS1- and si*SOCS2*-transfected LNCaP cells and in si*SOCS2-AS1*-transfected LTAD cells, whereas apoptotic cells were not significantly observed in control cells (Fig. 9, *C* and *D*). We further examined the role of *SOCS2-AS1* in apoptosis using VCaP cells treated with DMSO or 2 nM docetaxel and obtained similar results (Fig. 9, *E* and *F*).

Conversely, *SOCS2-AS1*-overexpressing cells showed resistance to docetaxel-induced inhibition of cell proliferation compared with control cells (Fig. 10*A*). In addition, the TUNEL assay using *SOCS2-AS1* and SOCS2-overexpressing cells treated with 2 nM docetaxel for 48 h showed that cells overexpressing *SOCS2-AS1* and *SOCS2* were more resistant to apoptosis (Fig. 10, *B* and *C*). Furthermore, Western blotting analysis showed that the expression of cleaved-form PARP was significantly reduced in *SOCS2-AS1* stably expressing cells compared with control cells when treated with 1 nM docetaxel for 48 h (Fig. 10*D*). Thus, these data suggest that *SOCS2-AS1*, as well as SOCS2, inhibits apoptosis and promotes prostate cancer cell survival.

SOCS2-AS1 Regulates Androgen-mediated Gene Expression—We further analyzed whether *SOCS2-AS1* regulates AR activity, because we found more than half of the up-regulated genes by si*SOCS2-AS1* in LNCaP cells were repressed by androgen treatment, and *TNFSF10* was also repressed by androgen (Fig. 7*A*). We selected 559 androgen-induced (>1.5-fold) genes. Among them, 267 (48%) genes were repressed (<0.8-fold) by si*SOCS2-*

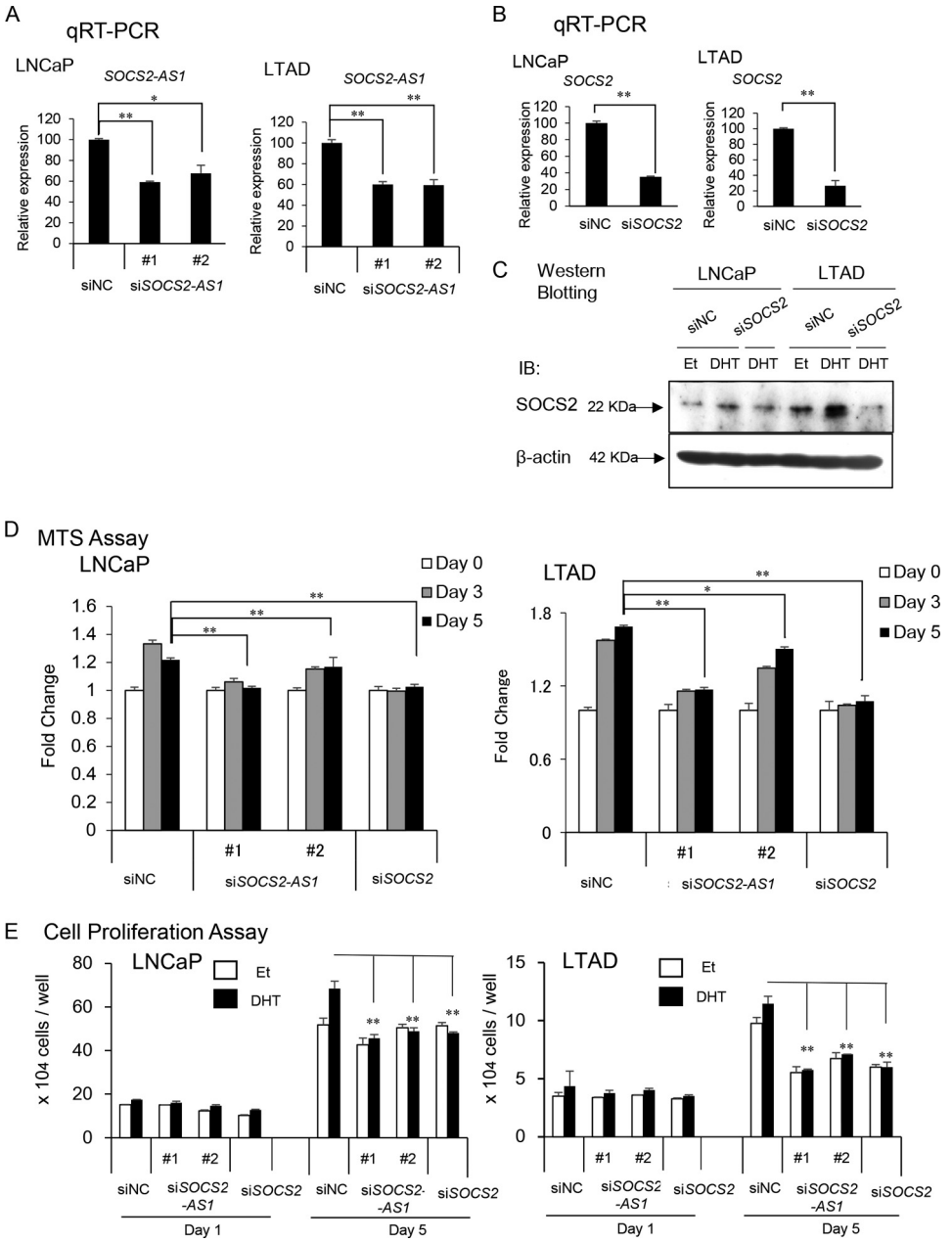


FIGURE 4. **SOCS2-AS1 knockdown inhibits LNCaP and LTAD cell growth.** *A*, knockdown efficiency of *SOCS2-AS1* by siRNA. LNCaP and LTAD cells were transfected with siNC (control) siRNA or si*SOCS2-AS1* #1 and #2 for 48 h and then treated with 10 nM DHT or ethanol for 18 h. The expression level of *SOCS2-AS1* was analyzed by qRT-PCR ($n = 3$). *B*, knockdown efficiency of *SOCS2* by siRNA analyzed by qRT-PCR ($n = 3$) in LNCaP and LTAD cells treated with 10 nM DHT for 18 h. *C*, knockdown efficiency of *SOCS2* by siRNA analyzed by Western blotting in LNCaP and LTAD cells, treated with 10 nM DHT or ethanol (*Et*) for 18 h. *IB*, immunoblot. *D*, MTS assay in LNCaP and LTAD cell lines transfected with 20 nM siNC, si*SOCS2-AS1*, or si*SOCS2* for 24 h following androgen treatment. *E*, cell proliferation assay in LNCaP and LTAD cell lines transfected with 20 nM siNC, si*SOCS2-AS1*, or si*SOCS2* for 24 h following androgen (10 nM DHT) treatment. Values represent mean ± S.D.; *, $p < 0.05$; **, $p < 0.01$.

AS1 in the presence of DHT (Fig. 11*A*). In addition, among 521 androgen-repressed genes (<0.7-fold), 186 (35%) genes were induced (>1.4-fold). This repression of androgen signaling was not evident in *SOCS2* knockdown samples. By qPCR analysis, we observed that androgen-dependent induction of *ACSL3* and *TMPRSS2* (both are representative androgen-regulated genes) was enhanced in LNCaP cells overexpressing *SOCS2-AS1* (Fig. 11*B*). In addition, we also confirmed that *SOCS2-AS1* knockdown inhibits these androgen-mediated gene inductions in both LNCaP and VCaP cells (Fig. 11, *C* and *D*).

Next, we further investigated whether AR function is regulated by *SOCS2-AS1*. Among genes up-regulated by *SOCS2-AS1* knockdown, we found AR-binding genes were significantly enriched compared with background by analyzing ChIP-seq data (Fig. 12*A*). Then we explored the regulatory mechanism of AR signaling by *SOCS2-AS1*. We found that 8 of 14 representative genes associated with apoptosis, including *TNFSF10*, have ARBSs in their vicinity. In the intron region of *TNFSF10*, we found a strong AR binding region and recruitment of CtBP2, a cofactor important in prostate cancer progression (28), to the

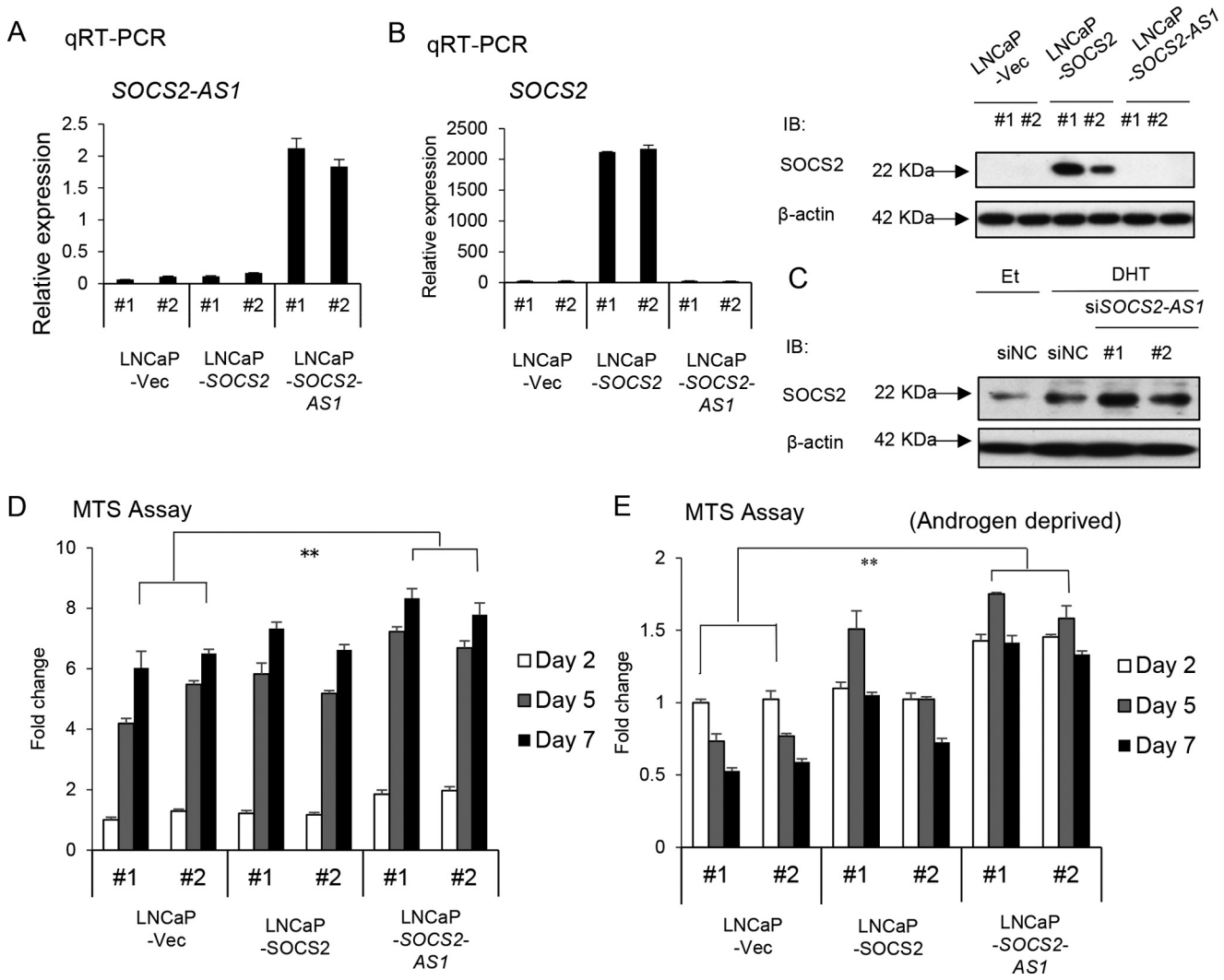


FIGURE 5. **SOCS2-AS1 overexpression enhances cell growth.** *A*, *SOCS2-AS1* expression in stable cells analyzed by qRT-PCR ($n = 3$). *B*, qRT-PCR ($n = 3$) and Western blotting analysis of *SOCS2* expression in stable cell lines. *IB*, immunoblot; *Et*, ethanol. *C*, *SOCS2* protein expression analyzed by Western blotting in LNCaP cells transfected with 20 nM si*SOCS2-AS1* or siNC for 24 h following androgen (10 nM DHT) or ethanol treatment for 18 h. *IB*, immunoblot. *D* and *E*, MTS assays of stable cells cultured with normal medium (*D*) or phenol-free RPMI supplemented with 10% charcoal stripped FBS (*E*). Values represent mean ± S.D.; *, $p < 0.05$; **, $p < 0.01$.

ARBS (Fig 12*B*). In addition, androgen-dependent repression of *TNFSF10* was reversed by AR knockdown, indicating the regulation of *TNFSF10* by AR (Fig. 12*C*). However, by Western blot analysis, we found that the protein levels of AR or phosphorylated AR (Ser-81), an important modification for AR binding to genome (36), were not affected by si*SOCS2-AS1* (Fig. 12*D*). In this ARBS, we observed histone deacetylation, and recruitment of histone deacetylase to this region was inhibited by *SOCS2-AS1* knockdown (Fig. 12, *E* and *F*), suggesting that *SOCS2-AS1* is involved in AR activity by modulating the epigenetic function.

The Involvement of SOCS2-AS1 in AR-mediated Cofactor Recruitments—Therefore, we investigated how *SOCS2-AS1* regulates AR epigenetic function by chromatin immunoprecipitation (ChIP) assay in three ARBSs (*TNFSF10*, *ACSL3*, and *TMPRSS2*). Interestingly, androgen-mediated recruitment of CtBP2 was repressed by *SOCS2-AS1* knockdown, although AR bindings were not influenced (Fig. 13*A*). SRC1 is associated with gene induction by enhancing histone acetylation (37). We observed recruitment of SRC1 only to ARBSs of androgen-induced genes, and it was repressed by si*SOCS2-AS1* treatment. Androgen-dependent histone acetylation was also inhibited by si*SOCS2-AS1* (Fig. 13*B*), suggesting that *SOCS2-AS1*-mediated epigenetic control in AR signaling could affect both AR-mediated gene induction and repression. Moreover, we analyzed whether *SOCS2-AS1* is involved in AR activity by interacting with AR. By qRT-PCR analysis, we showed that *SOCS2-AS1* is enriched in the nucleus of LNCaP cells (Fig. 13*C*). By RNA immunoprecipitation (RIP) assay using nuclear lysates, we observed that AR associates with *SOCS2-AS1* in the presence of androgen (Fig. 13*D*). These results suggest that *SOCS2-AS1* interacts with AR in the nucleus and modulates AR activity by regulating cofactor recruitment for epigenetic controls. As an AR target gene, *TNFSF10* is repressed by androgen, the expression of which may be modulated by the action of *SOCS2-AS1* on AR.

To evaluate the importance of *SOCS2-AS1* in the clinical setting, we investigated the expression of *SOCS2-AS1* as well as its potential target, *TNFSF10*, in clinical samples (38). *SOCS2-AS1* was up-regulated in some metastatic prostate cancers com-

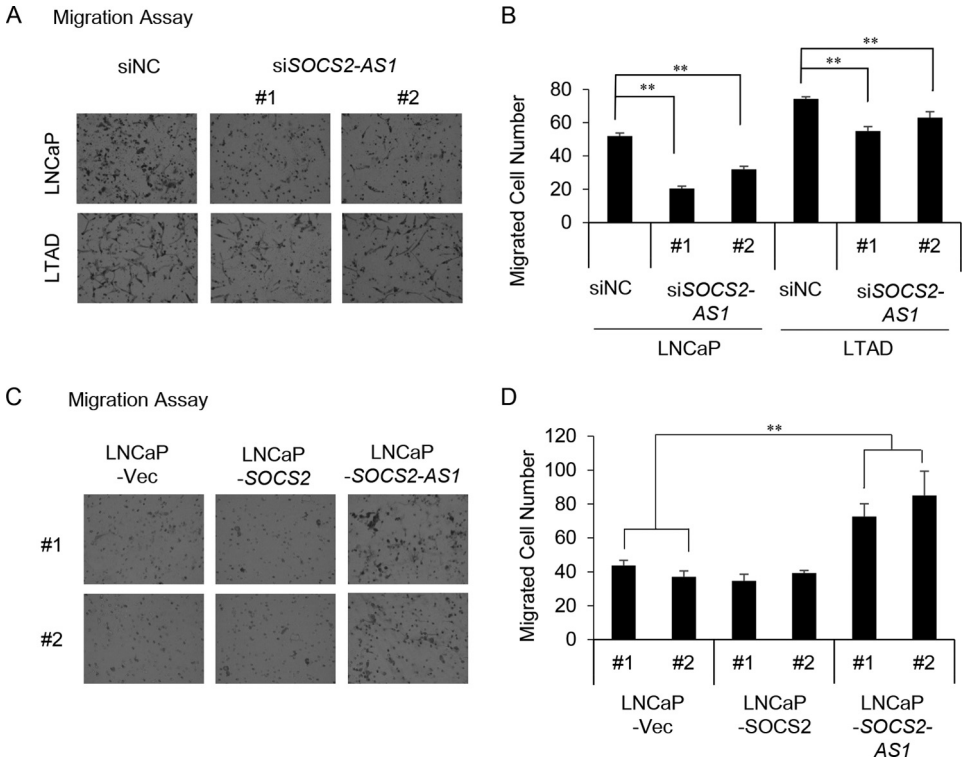


FIGURE 6. ***SOCS2-AS1* overexpression enhances cell migration.** *A*, migration assay of si*SOCS2-AS1* (#1 and #2) and control siRNA-transfected cells in 10 nM DHT medium. *B*, quantification of migrated cells. Five random fields were counted using a microscope, and the average number of cells per field was calculated. *C*, migration assay of *SOCS2* (#1 and #2), *SOCS2-AS1* (#1 and #2) and control (#1 and #2) stable cells. *D*, quantification of migrated cells. Four random fields were counted using a microscope, and the average number of cells per field was calculated. Data represent the average of four different views. *Vec*, vector. Values represent mean ± S.D. **, $p < 0.01$.

pared with clinically localized primary prostate cancer, whereas *TNFSF10* inversely correlated with *SOCS2-AS1* expression, supporting the oncogenic role of *SOCS2-AS1* (Fig. 14, *A* and *B*). Taken together, our findings provide a link between an androgen-induced lncRNA, *SOCS2-AS1*, and the apoptosis pathway, which may be mediated by *TNFSF10* (Fig. 14*C*).

Discussion

Previous studies have reported that several lncRNAs are certainly vital for the molecular chain of events that leads to prostate cancer development and progression. Therefore, RNA-based therapy could be a potential strategy for CRPC. In the present study, we investigated lncRNAs induced by androgen in two AR-positive prostate cancer cells using directional RNA-seq method. Among these lncRNAs, we found that the expression level of *SOCS2-AS1* was higher in CRPC model LTAD cells than in parental androgen-dependent LNCaP cells. *Socs2* knock-out mice, as well as transgenic mice, have been reported to display gigantism (39, 40), suggesting a dual role for *SOCS2* in growth regulation. In this study we demonstrated that *SOCS2-AS1* overexpression promoted castration-resistant and androgen-dependent cell growth and migration. On the other hand, *SOCS2-AS1* knockdown inhibited cell growth of CRPC model cells as well as parental prostate cancer cells. Thus, our results indicate that *SOCS2-AS1* may be a useful target for treating CRPC.

Several studies have addressed the regulation of bidirectional transcription (41–46). The expression of two genes from a bidirectional promoter suggests a shared upstream transcriptional regulator-mediated control of both genes, and implies that their functions might contribute independently to the same cellular response. Here we identified *SOCS2-AS1* and *SOCS2* as a pair of transcripts for which expression may be regulated by the same promoter/enhancer, in the opposite direction. Although our Western blot analysis of SOCS2 in LNCaP cells transfected with si*SOCS2-AS1* suggests the putative negative sense/antisense regulation, further experiments with the stability of the transcript, for instance, are necessary to show the relationship. Importantly, *SOCS2-AS1* induction by androgen was concordant with its sense strand gene, *SOCS2*, and both transcripts were repressed by the anti-androgen bicalutamide and siAR treatment.

In contrast to *SOCS2-AS1*, SOCS2 overexpression did not enhance cell proliferation and migration (Figs. 5, 6, and 10*A*), and the effect of knockdown is relatively small for inducing apoptosis in the LTAD or VCaP TUNEL assay (Fig. 9, *D* and *E*). Although *SOCS2-AS1* and *SOCS2* are induced by androgen and knockdown of both decreased the cell proliferation rate, they affect different genes and pathways, as suggested in the present study (Fig. 7*A*). These results support the idea that both *SOCS2-AS1* and SOCS2 confer specific biological response to cell growth and apoptosis by modulating different downstream signaling.

SOCS2-AS1 works as a regulator at the transcriptional level to affect the gene expression profiles in several pathways linked to cancer progression such as apoptosis. *TNFSF10*, which mediates apoptosis in cancer cells and leaves normal cells intact (47, 48), was found as a candidate *SOCS2-AS1* target. *TNFSF10* expression was repressed in CRPC cells, and repression of

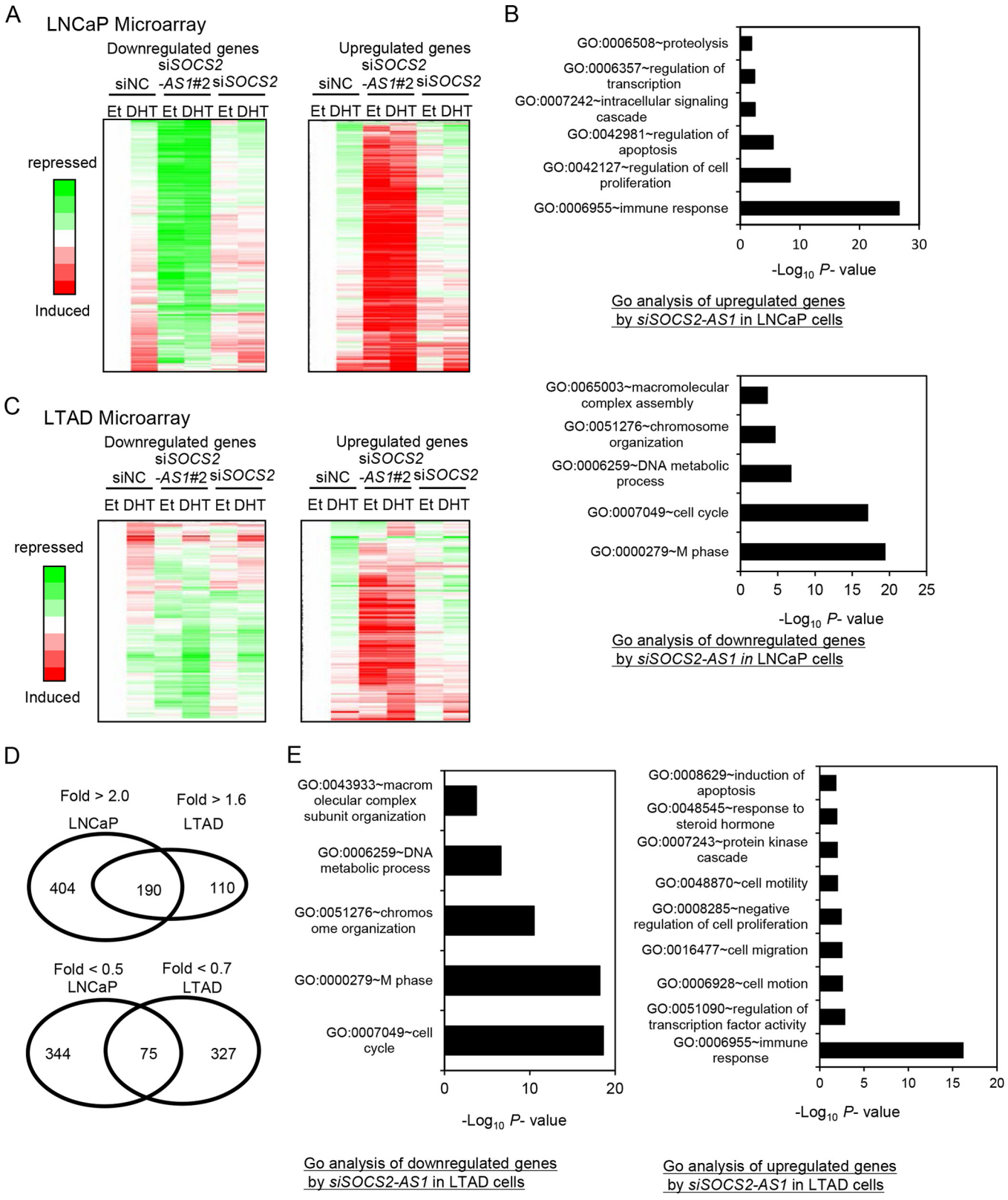


FIGURE 7. **SOCS2-AS1 inhibits the apoptosis pathway.** *A*, microarray analysis of LNCaP cells transfected with 20 nM si*SOCS2-AS1* (#2) or siNC for 24 h. Cells were treated with ethanol (*Et*) or DHT for 18 h. Genes repressed or induced more than 2-fold by si*SOCS2-AS1* transfection in the presence of androgen were selected as *SOCS2-AS1* downstream signals. Expression values displayed in a gradient of *red* or *green* are the ratio between log2-transformed gene expression values in si*SOCS2-AS1*, si*SOCS2*, and siNC-transfected cells. *B*, GO term analysis of up-regulated or down-regulated *SOCS2-AS1* signals. The most significantly enriched functions were presented. *C*, microarray analysis of LTAD cells transfected with 20 nM si*SOCS2-AS1* (#2) or siNC for 24 h. Cells were treated with ethanol or DHT for 18 h. Genes repressed less than 0.7-fold or induced more than 1.6-fold by si*SOCS2-AS1* transfection in the presence of androgen were selected as *SOCS2-AS1* downstream signals. *D*, Venn diagram showing the overlap of *SOCS2-AS1* target genes in LNCaP and LTAD cells. *E*, GO term analysis of up-regulated or down-regulated *SOCS2-AS1* signals in LTAD cells. The most significantly enriched functions were presented.

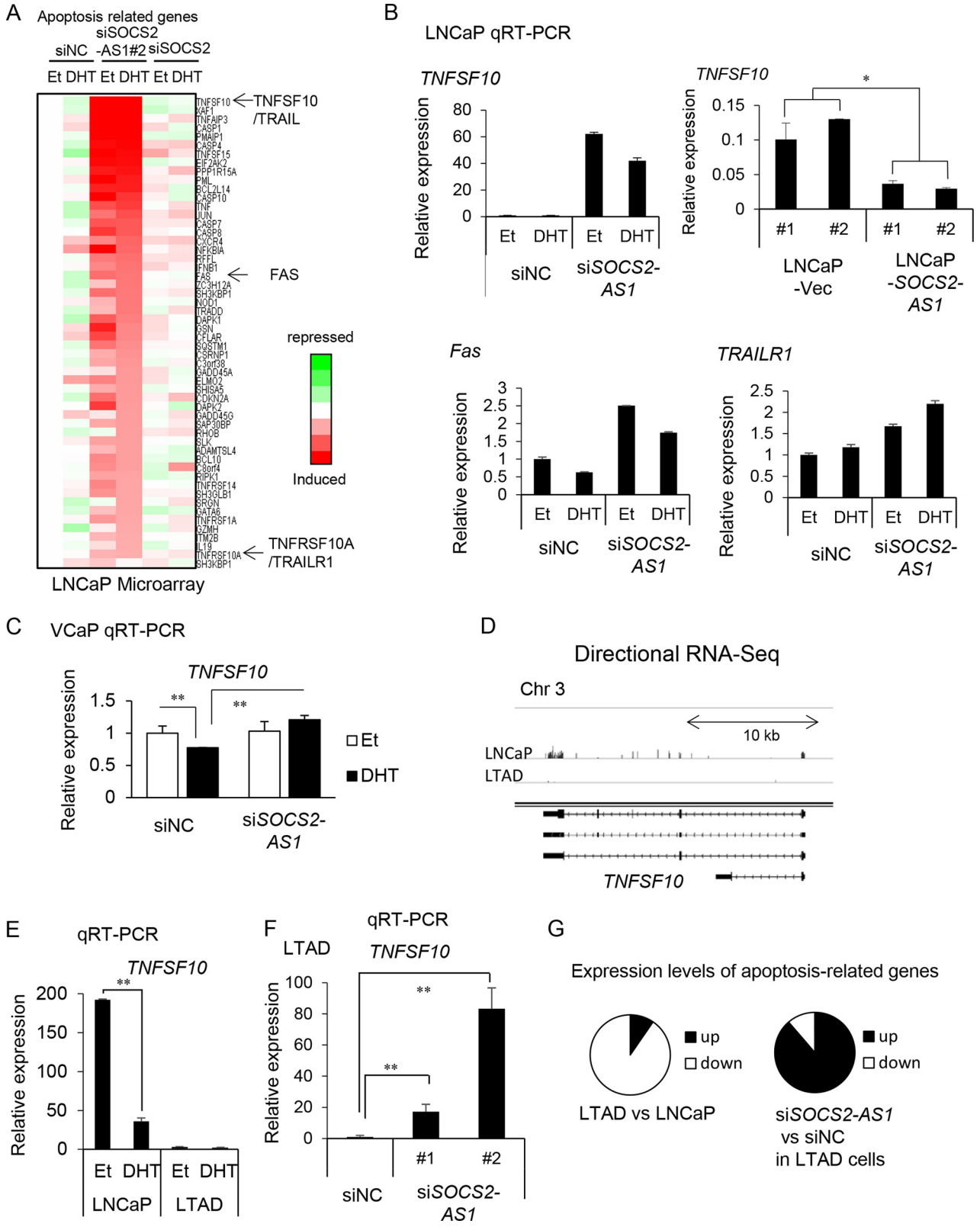
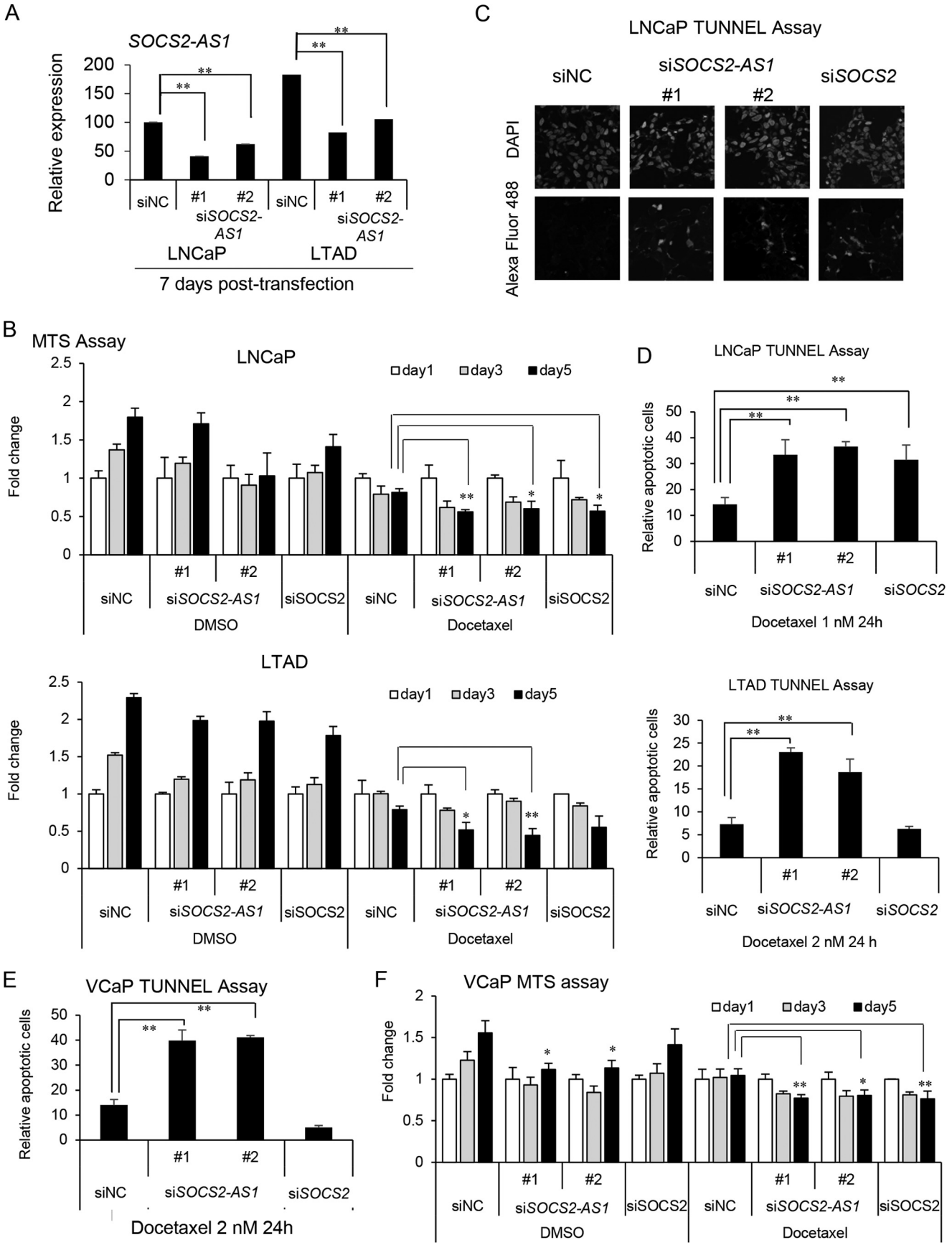


FIGURE 8. **SOCS2-AS1 modulates *TNFSF10* expression.** *A*, expression levels of apoptosis-related genes included in *SOCS2-AS1*-regulated genes in LNCaP microarray data. Representative genes *TNFSF10*, *FAS*, and *TRAILR1* are shown by *arrows*. *B*, *TNFSF10* mRNA expression in *SOCS2-AS1* stable cells analyzed by qRT-PCR; and *TNFSF10*, *Fas*, and *TRAILR1* mRNA expression in 20 nM si*SOCS2-AS1* (si*SOCS2-AS1*#2) or siNC-transfected cells for 24 h in LNCaP cells. *C*, *TNFSF10* mRNA expression in 20 nM si*SOCS2-AS1* (si*SOCS2-AS1*#2) or siNC-transfected cells for 24 h in VCaP cells. *D*, *TNFSF10* mRNA expression in LNCaP and LTAD cell lines analyzed by directional RNA-seq using Integrative Genomics Viewer (IGV browser). *E*, *TNFSF10* mRNA expression in LNCaP and LTAD cell lines analyzed by qRT-PCR. *F*, *TNFSF10* mRNA expression in si*SOCS2-AS1*-transfected LTAD cells. Values represent mean ± S.D.; *, $p < 0.05$; **, $p < 0.01$. *G*, *left*, expression levels of apoptosis related genes (Fig. 8*A*) in LTAD cells were compared with LNCaP cells using microarray data. *Right*, we compared expression levels of these genes in LTAD cells treated with si*SOCS2-AS1* with those in siNC.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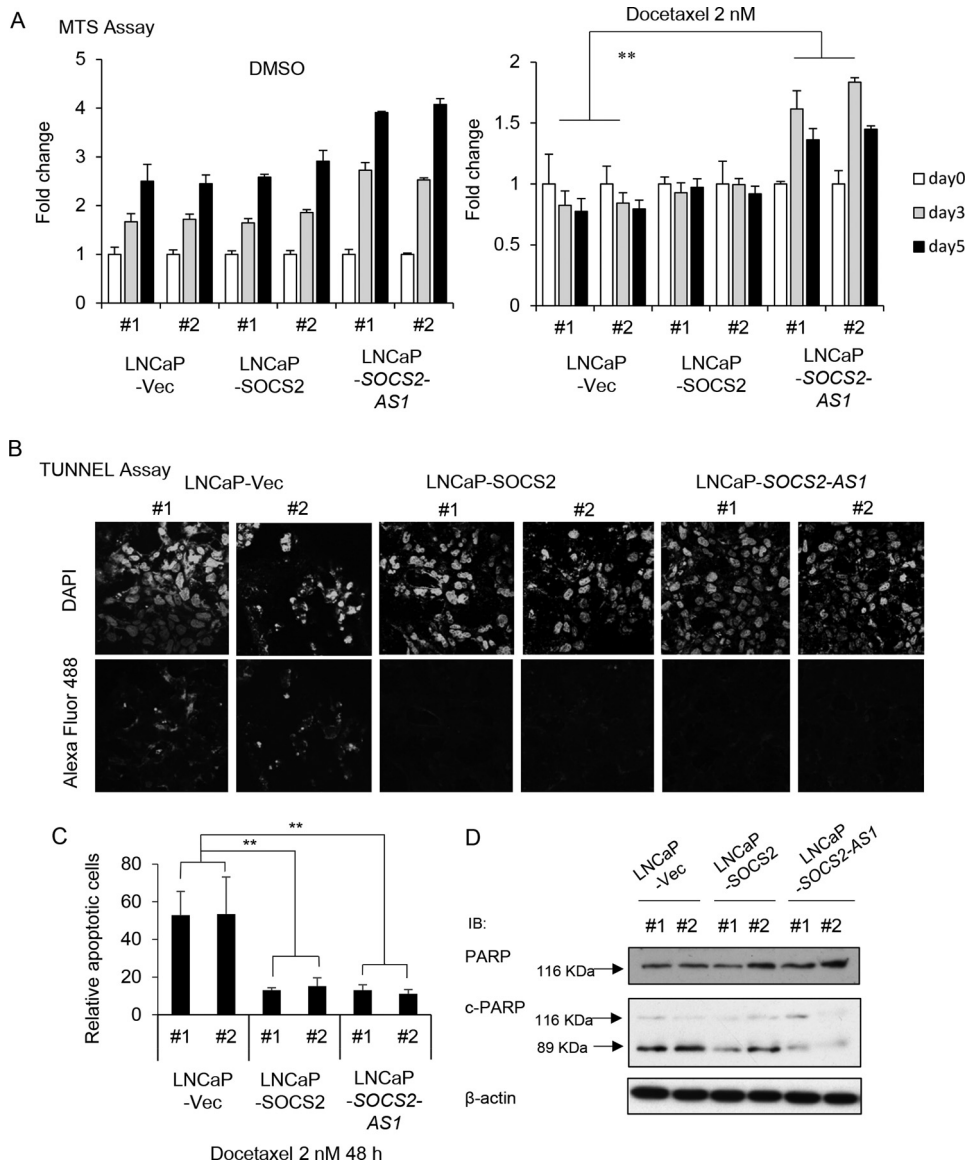

FIGURE 10. **SOCS2-AS1-overexpressing cells are more resistant to docetaxel treatment and apoptosis.** *A*, MTS assay of LNCaP stable cells treated with 2 nM docetaxel or DMSO for the indicated times. *B*, TUNEL assay in LNCaP stable cells treated with 2 nM docetaxel for 48 h. Shown are DAPI (*upper panels*)- and Alexa Fluor 488 (*lower panels*)-stained docetaxel-treated stable cells. *C*, quantification of apoptotic cells of TUNEL assay. Data represent the average of three different views ($n = 3$). *Vec*, vector. Values represent mean ± S.D. **, $p < 0.01$. *D*, docetaxel-induced apoptosis is inhibited in *SOCS2-AS1* stably expressing cells. PARP cleavage was analyzed by Western blotting in LNCaP cells stably expressing *SOCS2-AS1* or *SOCS2* and control cells treated with 1 nM docetaxel for 48 h. PARP and cleaved PARP antibodies were used. PARP proenzyme (116 kDa) and the cleaved subunit, c-PARP (89 kDa), are indicated on the *left* by *arrows*. β-Actin was used as a loading control. *IB*, immunoblot.

SOCS2-AS1-sensitized prostate cancer cells to docetaxel-induced apoptosis. These findings suggest that *SOCS2-AS1* plays a key role in the development of CRPC by repressing *TNFSF10* and apoptosis. Furthermore, si*SOCS2-AS1* down-regulated several known oncogenic genes known such as Forkhead box protein M1 (*FOXM1*) and its target gene, centromere protein F (*CENPF*), which are highly expressed in prostate cancer (49). *FOXM1* encodes a Forkhead domain transcription factor involved in the regulation of cell growth, DNA damage, genomic stability, drug resistance, and metastasis. In addition, coexpression of *FOXM1* and *CENPF* activates PI3K and MAPK signal pathways (50, 51). Together, these data support a role for *SOCS2-AS1* in prostate cancer progression and provide a link for this lncRNA to cancer-specific genes.

FIGURE 9. **SOCS2-AS1 knockdown sensitizes cells to docetaxel treatment and increases apoptosis.** *A*, knockdown efficiency of *SOCS2-AS1* by siRNA as analyzed by qRT-PCR ($n = 3$) in LNCaP and LTAD cells treated with docetaxel for 7 days. *B*, MTS assay of LNCaP and LTAD cells transfected with 20 nM si*SOCS2-AS1*, si*SOCS2*, or siNC for 24 h following 1 nM docetaxel or DMSO treatment for the indicated times. *C* and *D*, TUNEL assay in LNCaP and LTAD cells transfected with 20 nM si*SOCS2-AS1*, si*SOCS2*, or siNC for 24 h following 1 or 2 nM docetaxel treatment for 24 h. *C*, representation of DAPI (*upper panels*) and Alexa Fluor 488 (*lower panels*) signals detected in LNCaP cells treated with docetaxel. *D*, *graph* representing the quantification of apoptotic cells in LNCaP and LTAD cells. *E*, TUNEL assay in VCaP cells. VCaP cells transfected with 20 nM si*SOCS2-AS1*, si*SOCS2*, or siNC for 24 h following 2 nM docetaxel treatment for 24 h. *F*, MTS assay of VCaP cells transfected with 20 nM si*SOCS2-AS1*, si*SOCS2*, or siNC for 24 h following 2 nM docetaxel or DMSO treatment for the indicated times. Data represent the average of three different views ($n = 3$). Values represent mean ± S.D. *, $p < 0.05$; **, $p < 0.01$ (*versus* siNC).

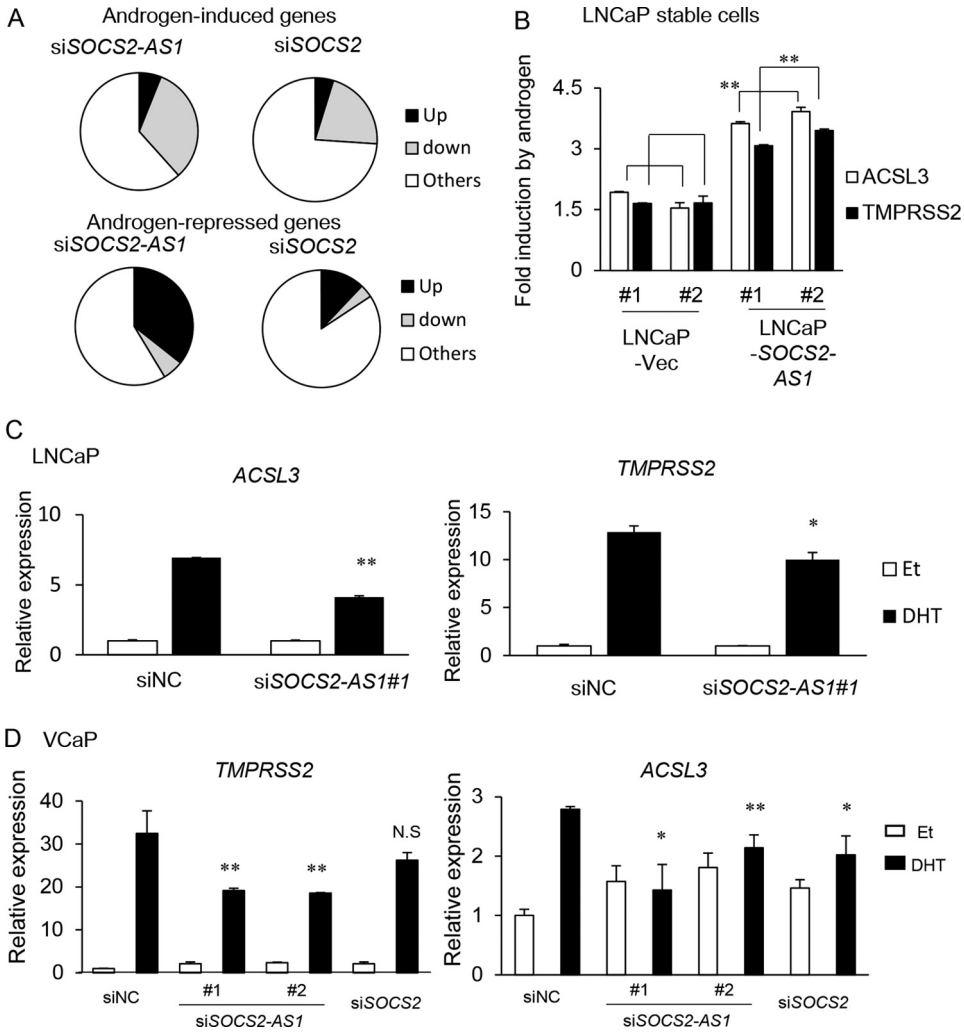

FIGURE 11. **SOCS2-AS1 promotes androgen-mediated gene regulation.** *A*, global effect of *SOCS2-AS1* knockdown on androgen-mediated gene induction and repression. Microarray data from LNCaP cells transfected with 20 nM si*SOCS2-AS1* (#2) or siNC for 24 h was analyzed. Among DHT-induced genes (>1.5-fold), those repressed (<0.8-fold) with si*SOCS2-AS1* (#2) or si*SOCS2* transfection are showed as the *gray fraction* (*upper panel*). Similarly, among DHT-repressed genes (<0.7-fold), those induced (>1.4-fold) with si*SOCS2-AS1* (#2) or si*SOCS2* transfection are showed as the *black fraction* (*lower panel*). *B*, LNCaP cells overexpressing *SOCS2-AS1* or vector control cells were treated with ethanol or 10 nM DHT for 24 h. Expression levels of *ACSL3* and *TMPRSS2* were measured by qPCR. -Fold inductions over vehicle (*Et*, ethanol) control are shown. *C*, LNCaP cells were treated with siNC or si*SOCS2-AS1*#1 for 24 h and then treated with ethanol or 10 nM DHT for 24 h. Expression levels of *ACSL3* and *TMPRSS2* were measured by qPCR. *D*, VCaP cells were treated with siNC, si*SOCS2-AS1*, or siSOCS2 for 48 h and then treated with ethanol or 10 nM DHT for 24 h. Expression levels of *ACSL3* and *TMPRSS2* were measured by qPCR. Values represent mean ± S.D. *, $p < 0.05$; **, $p < 0.01$.

The role of *SOCS2* in cancer has been controversial (52–55). In prostate cancer, it was reported that SOCS2 protein levels are reduced in CRPC and that SOCS2 inhibits the oncogenic events caused by growth hormone, such as cell proliferation and invasion (56). In contrast, another report revealed that SOCS2 protein expression was increased in prostate tumor tissues compared with benign tissues and that SOCS2 has a growth-promoting role *in vitro* and *in vivo* (57). Our present study is in line with the latter report, supporting the oncogenic role of SOCS2 in prostate cancer progression, in concert with *SOCS2-AS1*. *SOCS2* knockdown also repressed or induced genes related to the apoptosis (data not shown), suggesting that both transcripts may cooperate to inhibit apoptosis and promote prostate cancer cell survival.

Moreover, our microarray data suggest that *SOCS2-AS1* confers oncogenic signaling by transcriptional regulation of genes associated with apoptosis, cell cycle, proliferation, and migration. We found that genes related to apoptosis and up-regulated by si*SOCS2-AS1* transfection in DHT-treated LNCaP cells have ARBSs in the vicinity by ChIP-seq analysis, suggesting that AR may modulate the expression of these genes (Fig. 12*C*). *TNFSF10* was the representative gene, with a marked ARBS in the intronic region. We further determined the effects of *SOCS2-AS1* on the AR pathway by analyzing the effects of *SOCS2-AS1* knockdown on the expression of androgen-regulated genes. Microarray data indicate that *SOCS2-AS1* depletion inhibited the androgen-mediated gene regulations (Fig. 11*A*). We further analyzed the role of *SOCS2-AS1* in AR binding and epigenetic regulation by ChIP assay. We demonstrated *SOCS2-AS1* modulate the cofactor recruitments and histone modifications in *TNFSF10* ARBS. Interestingly, we also observed that *SOCS2-AS1* associated with AR by RIP assay in the nucleus. Thus, the transcriptional regulations by *SOCS2-AS1* may be caused by modulating AR epigenetic action. These results suggest that high AR signaling in castration-resistant prostate cancer induces strong *SOCS2-AS1* expression, which

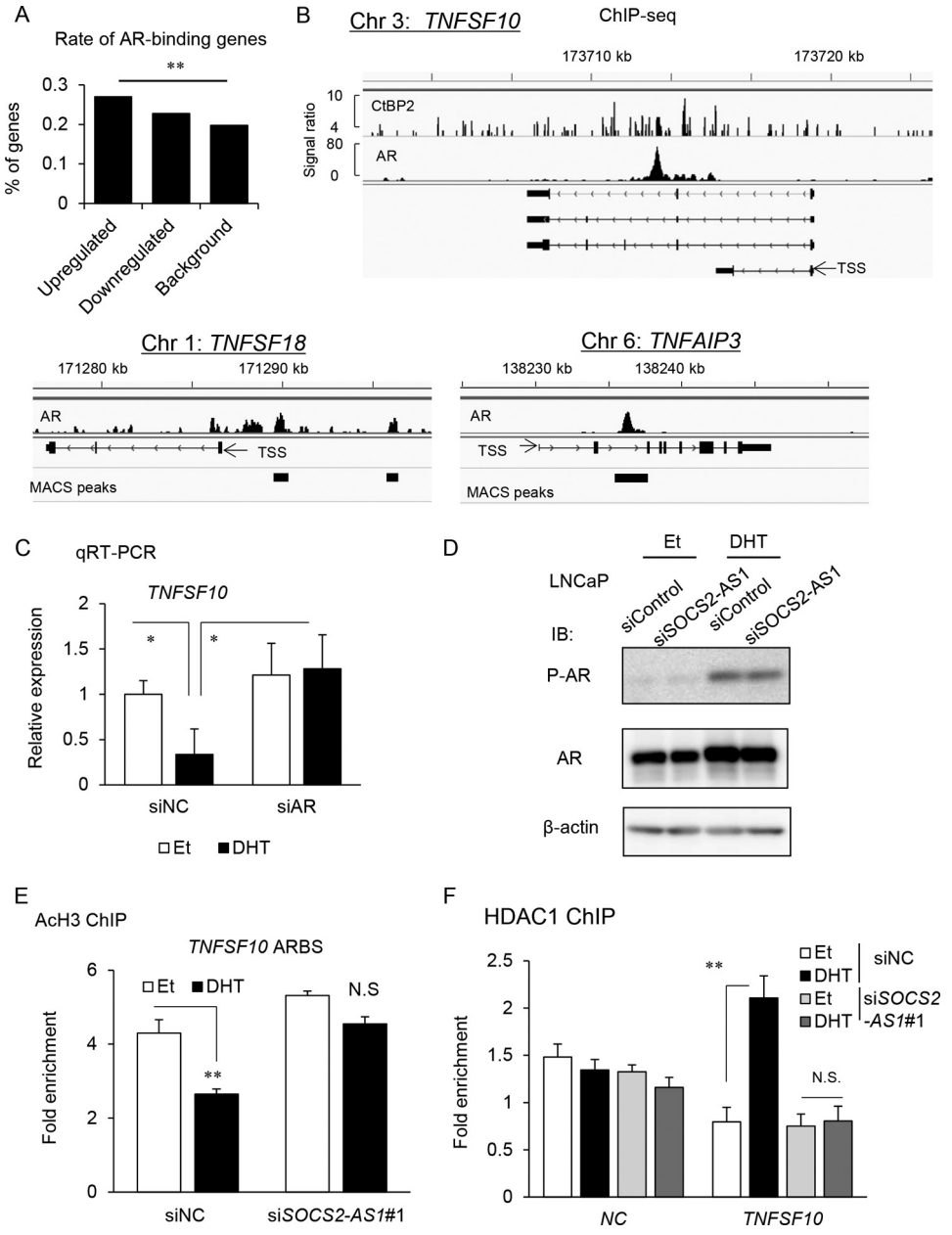

FIGURE 12. **Androgen-dependent epigenetic control at *TNFSF10* ARBS is modified by *SOCS2-AS1*.** *A*, AR-binding genes were significantly enriched in *SOCS2-AS1* downstream signals identified by microarray analysis (Fig. 7*A*). AR-binding genes were determined based on our AR ChIP-seq data in LNCaP cells (28). Among genes up-regulated or down-regulated by si*SOCS2-AS1* treatment in LNCaP cells, the number of AR binding genes was counted. A Chi-square test was performed to determine the *p* value. *B*, identification of ARBSs in the vicinity of apoptosis-related genes. The results of AR ChIP-seq are shown in the genomic loci of the representative genes, *TNFSF10*, *TNFSF18*, and *TNFAIP3*. In *TNFSF10*, the result of CtBP2 ChIP-seq data (31) is also shown. *C*, qRT-PCR analysis of *TNFSF10* mRNA following 10 nM si*AR* treatment and subsequent androgen treatment (10 nM DHT for 18 h) in LNCaP cell lines ($n = 3$). *D*, LNCaP cells were transfected with si*SOCS2-AS1* #1 or siNC for 48 h and then treated with ethanol (*Et*) or DHT for 24 h. Protein levels of phosphorylated AR (Ser-81) and total AR were analyzed by Western blotting. *IB*, immunoblot. β-Actin was used as a loading control. *E*, ChIP analysis of histone acetylation (*AcH3*) was performed in LNCaP cells. Cells were transfected with siNC or si*SOCS2-AS1* #1 for 48 h and treated with ethanol or DHT for 18 h. -Fold enrichments over *GAPDH* exon locus were calculated by qPCR ($n = 3$). *F*, ChIP analysis of histone deacetylase 1 (*HDAC1*) was also performed. *NC*, negative control (*myoglobin* locus). ($n = 3$). Values represent mean ± S.D. *, $p < 0.05$; **, $p < 0.01$; *N.S.*, not significant.

can activate high levels of anti-apoptosis signals and cell growth and lead to metastasis by enhancing AR signaling. Further studies will be necessary to address the mechanisms of *SOCS2-AS1* for transcriptional regulation.

Recent studies have identified several metastases-associated lncRNAs; their overexpression may signify the propensity of a tumor to metastasize. In this study, *SOCS2-AS1* enhanced the migration potential of prostate cancer cells. A possible mechanism for such migration and the cell growth-promoting ability of *SOCS2-AS1* may be the modulation of AR signaling by *SOCS2-AS1*. As in several lncRNAs such as *HOTAIR* (22) and *PCGEM1* (58), Our ChIP analysis indicated that *SOCS2-AS1* may be involved in the modification of AR for recruiting epigenetic modifiers without changing the AR protein level or binding to genome. However, *TNFSF10* is up-regulated even in the absence of DHT by *SOCS2-AS1* knockdown (Fig. 8*A*), suggest-

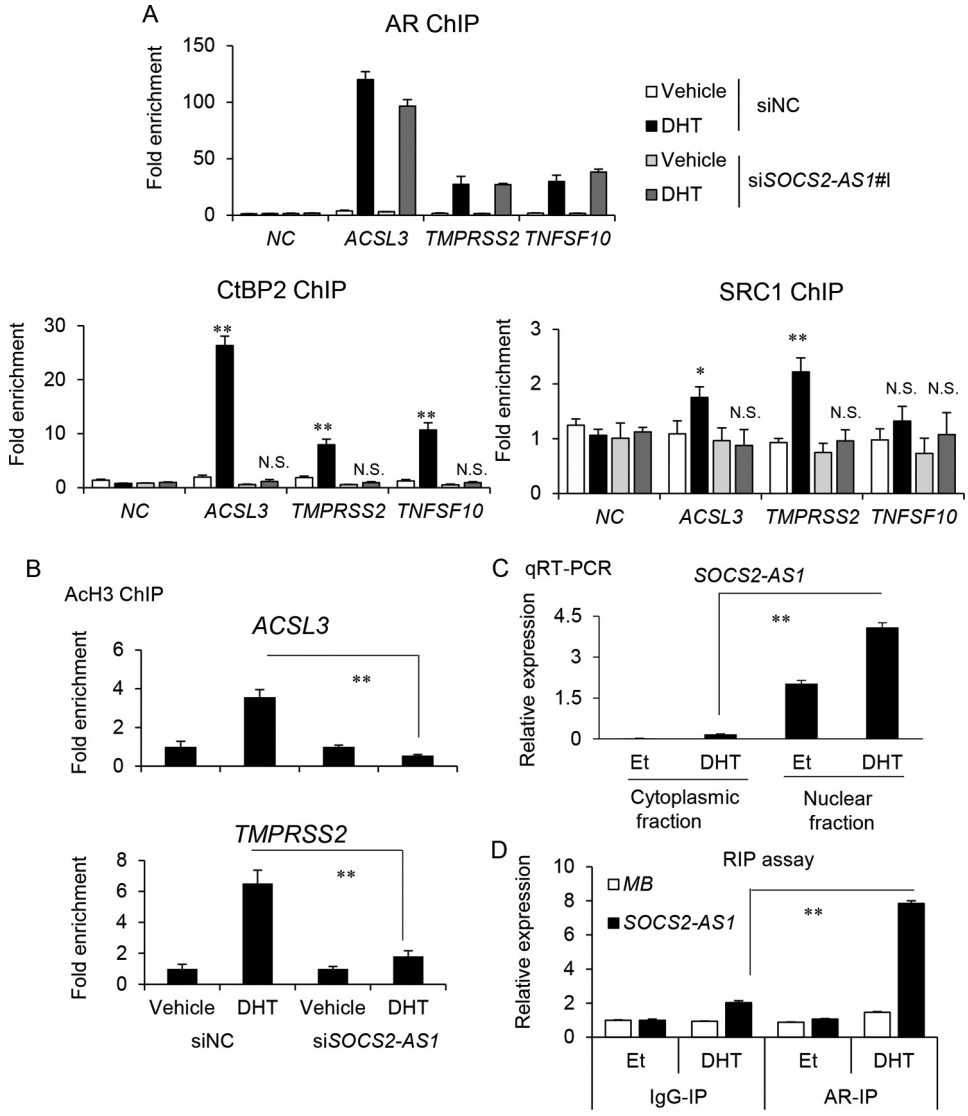

FIGURE 13. **SOCS2-AS1 interacts with AR and controls the association of AR cofactors for histone modification.** *A*, ChIP analysis of AR, CtBP2, and SRC1 was performed in LNCaP cells. Cells were transfected with siNC or si*SOCS2-AS1* #1 for 48 h and treated with ethanol or DHT for 18 h. -Fold enrichments over the *GAPDH* exon locus were calculated by qPCR. *NC*, negative control (*myoglobin* locus). ($n = 3$). *B*, ChIP analysis of histone H3 acetylation (*AcH3*) was performed in LNCaP cells. Cells were transfected with siNC or si*SOCS2-AS1* #1 for 48 h and treated with ethanol or DHT for 18 h. -Fold enrichments over ethanol control (*Et*) were calculated by qPCR ($n = 3$). *C*, LNCaP cells were treated with ethanol or DHT for 18 h. Total RNA was extracted from nuclear and cytosolic fractions. Expression level of *SOCS2-AS1* was determined by qRT-PCR ($n = 3$). *D*, RIP analysis of AR was performed in LNCaP cells. Cells were treated with ethanol or DHT for 18 h. Nuclear lysates were obtained, and then immunoprecipitation using AR antibody (*AR-IP*) was performed ($n = 3$). Associated RNA was isolated, and then the RNA level of *SOCS2-AS1* was determined by qRT-PCR. *MB*, myoglobin. Values represent mean ± S.D. *, $p < 0.05$; **, $p < 0.01$; *N.S.*, not significant.

ing other possible mechanisms of functions for *SOCS2-AS1* such as the recruitment of other transcriptional factors to the chromatin site, like *CTBP1-AS* (24), or the coactivation of cancer-related genes such as c-*myc*, like *PCGEM1* (59). The investigation of associated transcription factors or epigenetic factors will be required to clarify how *SOCS2-AS1* interferes with AR and modulates AR signaling in prostate cancer. To show more of the molecular mechanisms of *SOCS2-AS1*, investigating the interaction partners by mass spectrometry would be interesting. Additionally, by comparing the results of the microarray, we identified LTAD-specific target genes of *SOCS2-AS1*. A previous study (6) has demonstrated different AR binding sites in CRPC model cells from hormone-naive cells. Therefore, changes in AR or other transcription factor binding sites in CRPC cells may influence the action of *SOCS2-AS1*, which interacts with AR for transcriptional regulation.

In summary, we show that an androgen-induced lncRNA, *SOCS2-AS1*, which is more highly expressed in CRPC model LTAD cells than in parental androgen-dependent LNCaP cells, promotes castration-resistant and androgen-dependent cell growth and cell migration. *SOCS2-AS1* modulates genes related to the apoptosis pathway, including *TNFSF10*, suggesting that it may play a key role in the development of CRPC by repressing apoptosis.

Experimental Procedures

Cell Culture and Reagents—LNCaP cells were grown in RPMI medium supplemented with 10% FBS. VCaP cells were

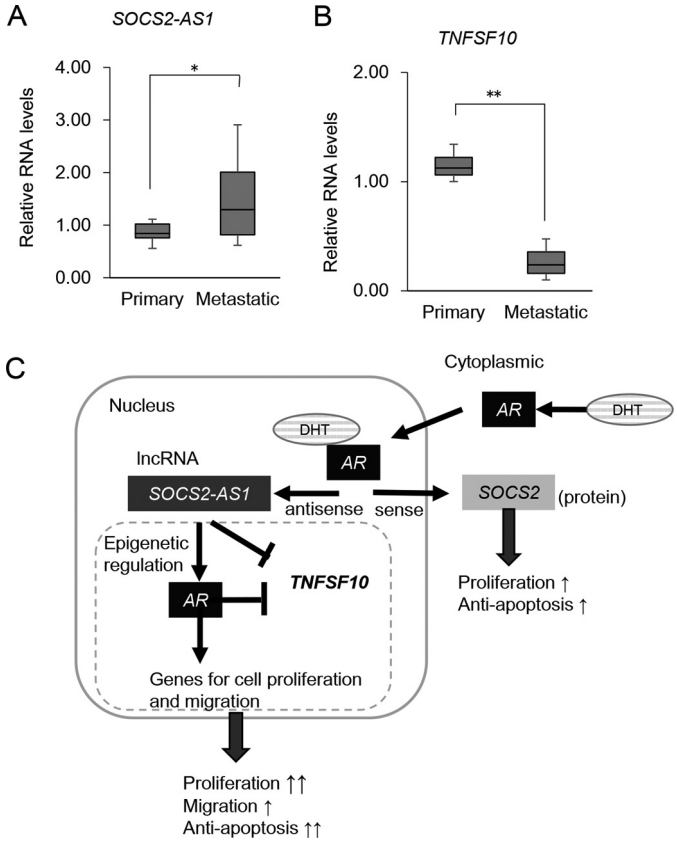

FIGURE 14. ***SOCS2-AS1* and *TNFSF10* expression in clinical samples.** *A* and *B*, *SOCS2-AS1* and *TNFSF10* expression in publicly available data sets (GEO accession No. GSE3325). *SOCS2-AS1* (*A*) and *TNFSF10* (*B*) microarray data were comparatively plotted in clinically localized primary prostate cancer and metastatic prostate cancer. *Box plots* are shown with the lower and upper bounds of the *boxes* representing the 25th and 75th quartiles, respectively. Values represent normalized RNA expression levels relative to the value of *GAPDH* as the reference gene. *, $p < 0.05$; **, $p < 0.01$. *C*, schematic model of *SOCS2-AS1* androgen induction and cancer progression in prostate cancer.

TABLE 2
List of siRNAs and PCR primers used for RNA knockdown, qRT-PCR, ChIP-qPCR analysis, and *SOCS2-AS1* expression vector construction

siRNAs	
si*SOCS2-AS1* #1	UUAUCACUCAUCAUUUCAGAA (forward)
	CUGAAAUGAUGAGUGAUAAUG (reverse)
si*SOCS2-AS1* #2	GACCUGUAUGGUCAUUAUCACUC (forward)
	GUGAUAAUGACCAUACAGGUCAA (reverse)
si*SOCS2*	UAUAUUCUUCCAAGUAAUCUU (forward)
	GAUUACUUGGAAGAAUAUAAA (reverse)
PCR primers	
SOCS2-AS1	CCATACAGGTCAACTTTTCCACCAC (forward)
	CCAACCTCAGCTCTGCTCTCTT (reverse)
SOCS2	AACCGCTCTACACGTCAGCA (forward)
	TGGTAAAGGCAGTCCCCAGA (reverse)
TNFSF10	AGACCTGCGTGCTGATCGTG (forward)
	CAAGCAATGCCACTTTTGGA (reverse)
GAPDH	GGTGGTCTCCTCTGACTTCAACA (forward)
	GTGGTCGTTGAGGGCAATC (reverse)
PCR primers for cloning of *SOCS2-AS1*	
SOCS2-AS1	CTCAAGCTTAATTTGGGAACGAGATGCCCGGGGA (forward)
SOCS2-AS1	CCCTCTAGATAGAAAGCTCCAGGTGTGTTTTATTTTA (reverse)
ChIP qPCR primers	
SOCS2-AS1 ARBS	TGGGACCGACGTTTAACTCT (forward)
	GGGGTATCCTGAGGCTGTG (reverse)
TNFSF10 ARBS	TCCGTACAGCTTGTGTGGAG (forward)
	TTGGTGGTTAGGGATCAAGC (reverse)

grown in DMEM supplemented with 10% FBS. LTAD cells were grown in phenol red-free RPMI medium supplemented with 10% charcoal-dextran-stripped FBS. For androgen deprivation, cells were cultured for 3 days in phenol red-free RPMI medium (Nacalai Tesque, Kyoto, Japan) with 2.5% charcoal-dextran-stripped FBS. All cells were maintained at 37 °C in 10% O_2 and 5% CO_2. LNCaP and VCaP cells were purchased from American Type Culture Collection. DHT and bicalutamide (Casodex) were purchased from Sigma.

RNA-seq—RNA library preparation was performed according to the manufacturer's protocol for the OP-Illumina-Directional_RNA_seq-single_end-v1.0 (Illumina, San Diego). Sequencing libraries was constructed starting from 2 μg of total RNA. Sample DNA for cluster generation was prepared according to the manufacturer's protocol for the OP-HiSeq2000-sequencing-cBot-v3.0. The RNA-seq readings were analyzed using whole transcriptome software tools from Illumina. Matching locations were subsequently used to generate counts for annotated features, exons, transcripts, or genes using RefSeq genes to determine the exons genomic locations of known transcripts or coverage files (wiggle format). Finally, genes expression was determined as the number of reads per kilobase of exon model per million mapped reads (RPKM). RNA-seq data were uploaded to the GEO database (GSE82225). The Integrative Genomics Viewer, version 2.2 (IGV browser) was used for visualization.

qRT-PCR—Total RNA was isolated using the RNeasy kit (Qiagen). First-strand cDNA was generated using a PrimeScript RT reagent kit (Takara, Kyoto, Japan). Expression levels were quantified by qPCR using KAPA SYBR FAST ABI Prism 2× qPCR master mix and ABI StepOne system (Life Technologies). Relative mRNA levels were determined by normalization to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA level. Primer sequences are listed in Table 2.

ChIP Assay—ChIP was performed as described previously (23, 24). The fold enrichment relative to the *GAPDH* locus was quantified by qPCR using SYBR Green PCR master mix and the ABI StepOne system (Life Technologies). We used the *myoglobin* locus as a negative control. Antibodies for ChIP have been described previously. The primer sequences for ChIP qPCR have been described previously (23, 24) or are listed in Table 2.

RIP Assay—Confluent LNCaP cells in 15-cm dishes were harvested, and nuclear fractions were lysed in RIP buffer as described previously (24). Specific antibody was added to the supernatant, and the mixture was incubated overnight with rotation at 4 °C. Thirty microliters of protein G beads was added followed by incubation at 4 °C for 2 h with gentle rotation. The beads were washed three times with lysis buffer and resuspended in 1 ml of Isogen (Nippon Gene). Co-precipitated RNA was isolated and used for qRT-PCR analysis.

siRNA Transfection—siRNAs were designed using siDirect version 2.0 and purchased from Sigma Genosys. Cells were transfected with siRNAs using Lipofectamine RNAiMax transfection reagent (Thermo Fisher Scientific) at a final concentration of 20 nM according to the manufacturer's protocol. siRNA sequences are listed in Table 2.

MTS Assay—Cells were plated at 2×10^3 cells/well in 96-well plates. For RNA interference experiments, cells were transfected with 20 nM siRNA 24 h after plating. Cell viability was

assessed at different time points using a CellTiter 96 AQueous One Solution cell proliferation assay kit (Promega, Madison, WI). Plates were incubated at 37 °C for 50 min, and optical density was measured at 490 nm using a microplate-spectrophotometer (Benchmark Plus, Bio-Rad). Relative cell survival (mean ± S.D. of triplicate determinations) was calculated by standardizing the nontreated cell survival to 100%.

Cell Proliferation Assay—Cells were plated at 5×10^4 cells/well in 24-well plates. The following day, cells were transfected with 20 nM siRNA, and the cell number was counted at different time points as described (24).

Western Blotting—Whole cell lysates were prepared using Nonidet P-40 lysis buffer. Protein concentration was measured by BCA assay (Pierce). Cell lysates were fractionated on 8 or 12% SDS-PAGE and transferred onto Immobilon-P transfer membrane (Millipore, Billerica, MA). Membranes were incubated with primary antibodies at 4 °C overnight and with secondary antibodies at room temperature for 1 h, using Blocking One buffer (Nacalai). Antibody-antigen complexes were detected with the ECL Plus detection system (GE Healthcare). Antibodies used in this study are SOCS2 rabbit antibody (2779S, Cell Signaling, Danvers, MA), PARP rabbit antibody 46D11 (9532S , Cell Signaling), cleaved PARP rabbit antibody D214 (9541S , Cell Signaling), and β-actin (A5316, Sigma).

Migration Assay—Cell migration assays were performed using a 24-well plate with Matrigel-coated invasion chambers (BD Biosciences). Briefly, 5×10^4 cells were suspended in serum-free RPMI medium and transferred into suspended 8-μm pore inserts. RPMI medium supplemented with 10% FBS was placed at the bottom of the wells. After 24 h of culturing, cells that migrated to the opposite side of the membrane were fixed with methanol and stained with Giemsa. Five random fields were counted using an Olympus CKX41 microscope (×10 objective), and the average number of cells per field was calculated.

Microarray Analysis—For expression microarrays, a GeneChip Human Exon 1.0 ST array (Affymetrix, Santa Clara, CA) was used according to the manufacturer's protocol (24). Data analysis was performed using Affymetrix Microarray Suite software. To compare arrays, normalization was performed on data from all probe sets. GO term and Pathway analysis was performed using DAVID (25, 26). Microarray data were uploaded to the GEO database (GSE82225).

Plasmid Construction—To overexpress *SOCS2-AS1,* we performed PCR with specific primers, listed in Table 2. cDNA synthesized using total RNA extracted from androgen-treated LTAD was used as template. We chose the most abundant PCR product, which corresponded to the length of *SOCS2-AS1* variant 2 (ENST00000499137.2) and cloned it into pcDNA3 expression vector.

A *SOCS2*-expressing vector was constructed by transferring its protein coding sequence from pDNR-LIB-SOCS2 vector (cDNA clone MGC:13697 IMAGE:4277425) to FLAG-tagged pcDNA3. Constructs were sequenced using the ABI PRISM 310 genetic analyzer (Life Technologies).

Establishment of SOCS2- and SOCS2-AS1-overexpressing Cells—*SOCS2-AS1*, *SOCS2*-expressing vectors, and empty vector were transfected into LNCaP cells using FuGENE HD (Promega) according to the manufacturer's protocol. Transfected cells were treated with 500 μg/ml G418 (Geneticin disulfate solution, Life Technologies) for selection of stably transfected cells. The surviving colonies were picked up and grown as stably transfected cells.

TUNEL Assay—For RNA interference experiments, 5×10^4 LNCaP, LTAD, or VCaP cells were reverse-transfected with 20 nM si*SOCS2-AS1* (#1 and (#2), si*SOCS2*, or siNC (negative control) and suspended on poly-L-lysine-coated slides placed in each well of a 24-well plate. For *SOCS2-AS1* and *SOCS2* stably expressing cells, 5×10^4 cells were suspended on poly-L-lysine-coated slides placed in each well of a 24-well plate. At 24 h after plating, cells were treated with 1 or 2 nM docetaxel and cultured for another 24 h. A TUNEL assay was performed using the DeadEnd™ fluorometric TUNEL system (Promega). Briefly, slides were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, equilibrated with buffer, and stained with the reaction mixture. The reaction was stopped with 2× SSC, and cells were stained with 1 mg/ml DAPI (Nacalai Tesque). Cover glasses were mounted onto glass slides, and positively stained cells were counted using an Olympus FluoView (FV10i) microscope (×60 objective). Images were obtained through FluoView software (version 2.0).

Statistical Analysis—For the cell proliferation assay, we analyzed four wells. For the growth assay using stable cell lines, we performed two-way analysis of variance at each time point. For other cell line experiments, the statistical differences (p values) among groups were obtained using a two-sided Student's t test. All experiments were performed at least twice, and similar results were obtained. A Chi-square test was performed to analyze the ratios of AR binding, and p values less than 0.05 were considered statistically significant. Statistical procedures were performed using GraphPad Prism 5 software (GraphPad Software, San Diego) or MS Excel.

Author Contributions—K. T. designed the study and performed RNA-seq. A. M. and K. T. performed experiments and analyzed the data. S. I. supervised the study. A. M., K. T., T. U., and S. I. wrote the manuscript.

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