Published OnlineFirst February 1, 2008; DOI: 10.1158/1541-7786.MCR-07-0354

Sterol Regulatory Element–Binding Protein-1c Represses the Transactivation of Androgen Receptor and Androgen-Dependent Growth of Prostatic Cells

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Abstract

Sterol regulatory element-binding protein-1c (SREBP-1c) is a basic helix-loop-helix transcription factor that plays an important role in lipid homeostasis. Here, we show that SREBP-1c regulates androgen receptor (AR) transactivation through direct interaction with AR and represses androgen-dependent growth of prostatic cells. Transient transfection studies show that SREBP-1c specifically inhibits the transactivation of AR. Chromatin immunoprecipitation assays reveal that SREBP-1c is recruited with AR onto the endogenous AR target promoter. Moreover, adenovirus-mediated overexpression of SREBP-1c decreases the mRNA level of the *prostate-specific antigen* gene, an endogenous target gene of AR, supporting SREBP-1c modulation of AR transactivation. *In vivo* and *in vitro* protein interaction assays show that SREBP-1c directly interacts with AR through the activation function-1 domain of AR. In addition, transfection studies and glutathione *S*-transferase pull-down competition experiments reveal that the SREBP-1c–mediated repression of AR transactivation is accomplished through competition with certain AR coactivators for AR interaction. The SREBP-1c–mediated inhibition of AR transactivation also involves the recruitment of histone deacetylase 1. Finally, adenovirus-mediated overexpression of SREBP-1c inhibits androgen-induced proliferation of prostatic cells *in vitro* and *in vivo*, and small interfering RNA–mediated down-regulation of SREBP-1 enhances androgen-induced proliferation of prostatic cells as well as the transactivation of AR. Taken together, these results suggest that SREBP-1c acts as an AR corepressor and may play an important role in the regulation of AR-dependent prostatic cell growth. (Mol Cancer Res 2008;6(2):314–24)

Received 7/27/07; revised 10/15/07; accepted 11/1/07.
Grant support: Korea Science and Engineering Foundation grant funded by the Korea government (MOST), R01-2006-000-10378-0.
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doi:10.1158/1541-7786.MCR-07-0354

Introduction

Androgen receptor (AR) is a ligand-dependent transcription factor that is a member of the steroid hormone receptor superfamily. AR shares a characteristic structure with other steroid hormone receptors and contains three distinct functional domains: a variable NH_2-terminal activation function-1 domain (AF-1), a central DNA-binding domain (DBD), and a COOH-terminal ligand-binding domain (1). Upon binding with ligand, AR translocates into the nucleus, where it binds to specific DNA sequences, referred to as androgen response elements (ARE), and regulates the expression of its target genes (2, 3). The transcriptional activity of AR is modulated by coregulators that are known as coactivators and corepressors. Coactivators enhance ligand-dependent transactivation of the receptor, including direct interaction with basal transcription machinery and covalent modification of histones and other proteins (4-7). In contrast, corepressors repress AR transactivation through diverse mechanisms. The mechanism of repression by corepressors may involve the alteration of chromatin structure by the recruitment of histone deacetylase (8, 9), the inhibition of coactivator recruitment to the receptor complex (10, 11), or the inhibition of nuclear translocation and DNA binding of AR (12, 13).

AR is an important mediator in the growth of prostate cancers as well as in the development and maintenance of the prostate (14-16). AR is expressed in primary prostate cancer and is detected throughout the progression of cancer in both androgen-dependent and androgen-independent prostate cancers (17-19). Moreover, the knockdown of the *AR* gene by small interfering RNA (siRNA) decreases prostate-specific antigen (PSA) expression and cell proliferation in both androgen-dependent and androgen-independent prostate cancer cells (20-22), which suggests that AR has an important function in the progression of both androgen-dependent and androgen-independent prostate cancers.

The sterol regulatory element-binding proteins (SREBP) are a family of basic helix-loop-helix leucine zipper transcription factors that play important roles in cholesterol and fatty acid homeostasis (23, 24). Three SREBP isoforms have been identified thus far, SREBP-1a, SREBP-1c, and SREBP-2. SREBP-1a and SREBP-1c are produced from the same gene through the alternative splicing of the first exon (23). SREBPs are synthesized as a 125-kDa precursor protein and bound in the endoplasmic reticulum, forming a complex with the SREBP cleavage-activating protein (25, 26). The mature amino-terminal segments (active forms) are cleaved from the precursor SREBPs by a sequential two-step cleavage process and move

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into the nucleus where they bind to the promoters of target genes (27-29). Besides being a transcription factor that is involved in adipogenesis, SREBPs have been also reported to modulate the function of numerous nuclear receptors (30-33).

Androgen increases lipogenic gene expression in prostate cancer cells through the activation of the SREBP pathway (34). AR directly binds to ARE within the intron of the *SCAP* gene, and increases both the mRNA and protein levels of SREBP cleavage-activating protein (35, 36). In addition, long-term (2 days) androgen treatment of prostate cancer cells enhances the production of mature SREBPs in the nucleus, resulting in the induction of lipogenic gene expression (34, 36). These findings suggest that AR may have a role as a cooperator with the SREBP pathway in prostate cancer cells. Although the SREBP pathway is activated by androgen/AR in prostate cancer cells, the functions of SREBPs in the development and progression of prostate cancers remain largely unknown.

Here, we investigated the role of SREBP-1c in the modulation of AR transcriptional activity in prostatic cells. We show that the mature SREBP-1c inhibits AR transactivation and its target gene expression through direct physical interaction. SREBP-1c represses AR transactivation both by outcompeting AR coactivators and by actively recruiting histone deacetylases (HDAC). Moreover, overexpression of SREBP-1c inhibits the proliferation of prostate cancer cells and the growth of tumors in an animal tumor model. This report may provide new insight into the function of SREBP-1c in prostatic cells.

Results

SREBP-1c Represses the Transcriptional Activity of AR

Precursor SREBP-1c, which is bound in the endoplasmic reticulum, is expressed in prostate cancer cells, and long-term (2 days) exposure of androgens stimulates the production of mature SREBPs (active form of SREBPs) and enhances lipogenic gene expression (34-36). To investigate the effect of SREBP-1c on AR signaling in the nucleus, we did reporter assays in LNCaP cells, AR-positive cancer cells, using AR-responsive ARE_2-TATA-luc that contains two copies of the AR-binding element. When LNCaP cells were transfected with the expression vector of SREBP-1c (1-403 amino acids, a mature form), SREBP-1c strongly repressed the transcriptional activity of AR in a dose-dependent manner (Fig. 1A). SREBP-1c also repressed AR transactivation in PPC-1 cells, AR-negative prostate cancer cells, when the cells were cotransfected with AR and SREBP-1c expression vectors (Fig. 1B). To confirm the effect of SREBP-1c on the transactivation of AR, we repeated reporter assays using a natural AR target promoter-directed reporter, PSA-luc, in LNCaP cells. SREBP-1c significantly repressed the androgen-induced PSA promoter activity in a dose-dependent manner (Fig. 1C). Taken together, these results suggest that SREBP-1c inhibits the transcriptional activity of AR in prostatic cells.

SREBP-1c Is Recruited onto the AR Target Promoter with AR and Represses the Expression of the AR Target Gene

To investigate whether SREBP-1c is recruited onto the natural AR promoter with AR to repress the transactivation of AR, we did chromatin immunoprecipitation assays using LNCaP cells, which were cotransfected with the expression vectors of AR and hemagglutinin-tagged SREBP-1c in the absence or presence of dihydrotestosterone. In the absence of dihydrotestosterone, neither AR nor SREBP-1c was recruited to the PSA promoter (Fig. 1D). However, in the presence of dihydrotestosterone, SREBP-1c and AR were both associated with the ARE-containing region of the PSA promoter (Fig. 1D), which suggests the androgen-dependent recruitment of SREBP-1c onto the AR target gene promoter.

Because SREBP-1c represses AR transactivation, we assessed whether SREBP-1c inhibits the expression of the *AR* target gene. In the presence of dihydrotestosterone, adenovirus-mediated overexpression of SREBP-1c protein in LNCaP cells significantly decreased the level of PSA mRNA (Fig. 1E). The level of PSA mRNA was androgen dependently increased in LNCaP cells infected with null adenovirus (data not shown). Taken together, SREBP-1c is recruited onto the AR target promoter and represses the expression of the AR target gene *in vivo*.

The Repression of AR Transactivation by SREBP1c Is Specific

To investigate whether SREBP-1c represses the transcriptional activity of other nuclear receptors as well as AR, we did transient transfection assays with the expression vectors of AR, ERα, and glucocorticoid receptor in 293T cells. SREBP-1c again repressed the transcriptional activity of AR in an androgen-dependent manner in 293T cells (Fig. 2A). However, SREBP-1c enhanced the transcriptional activity of ERα in both ligand-independent and ligand-dependent manners as reported previously (33), and exerted little effect on glucocorticoid receptor transactivation (Fig. 2A). Furthermore, AR transactivation was not significantly affected by the coexpression of mature ATF6, a protein that is processed for its activation in a similar manner to those of SREBPs in the endoplasmic reticulum (ref. 37; Fig. 2B). On the other hand, other SREBP isoforms, SREBP-1a and SREBP-2, repressed the transcriptional activity of AR in a manner similar to that of SREBP-1c (Fig. 2C). Taken together, these results suggest that SREBP-1c represses the transcriptional activity of AR, but not ER or glucocorticoid receptor, and all three isoforms of SREBP repress AR transactivation.

SREBP-1c Physically Interacts with AR in an Androgen-Dependent Manner

To determine whether the SREBP-1c–induced repression of AR transactivation is mediated through physical interaction, we did coimmunoprecipitation assays using 293T cells cotransfected with AR and SREBP-1c expression vectors. When the AR complex was precipitated with an anti-AR antibody, SREBP-1c was detected with AR in the precipitate in the presence, but not in the absence, of dihydrotestosterone (Fig. 3A). These results suggest that SREBP-1c physically interacts with AR in an androgen-dependent manner. To confirm the interaction between SREBP-1c and AR, we did *in vitro* glutathione *S*-transferase (GST) pull-down assays. The [^{35}S]methionine-labeled AR protein synthesized by *in vitro* translation was incubated with bacterially expressed GST or GST–SREBP-1c protein. AR protein was retained by GST–SREBP-1c, but not by GST protein (Fig. 3B), which suggests that SREBP-1c directly interacts with AR *in vitro*.

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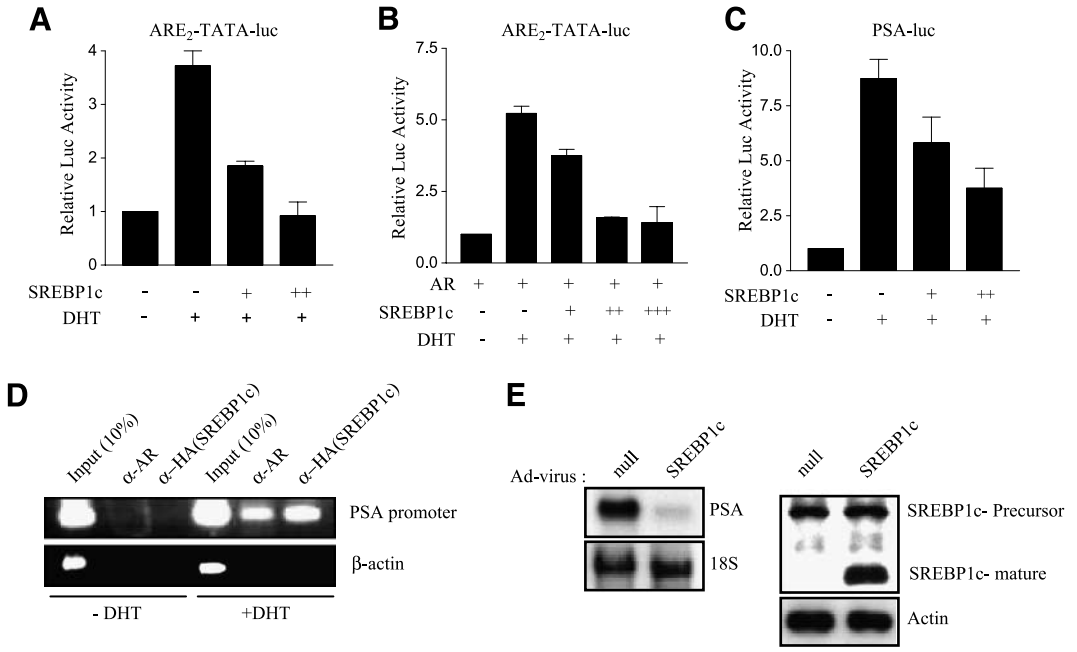

FIGURE 1. SREBP-1c represses AR transactivation and is recruited onto the endogenous AR target gene promoter. **A** and **C.** LNCaP cells were cotransfected with pcDNA3HA-SREBP-1c (a mature form, 100 and 200 ng) and 150 ng of ARE_2-TATA-luc (**A**) or PSA-luc (**C**). **B.** PPC-1 cells were cotransfected with 100 ng of pcDNA3-mAR that expressed mouse AR, pcDNA3HA-SREBP-1c (100, 200, and 300 ng), and 150 ng of ARE_2-TATA-luc. Cells were cultured in medium containing 5% charcoal-stripped serum with or without 10 nmol/L dihydrotestosterone (*DHT*) for 24 h and were then assayed for luciferase activity. Data are representative of at least three independent experiments with similar results. Columns, mean of duplicate samples; bars, SD. **D.** LNCaP cells were transfected with pcDNA3HA-SREBP-1c and treated with or without 10 nmol/L dihydrotestosterone for 16 h after transfection. Anti-AR and anti-hemagglutinin (*HA*) antibodies were used for immunoprecipitation. DNAs isolated from the immunoprecipitates were analyzed by PCR using a pair of specific primers spanning the region containing AREs of the PSA promoter. A control PCR for nonspecific immunoprecipitation was done using primers specific to the β-actin coding region. **E.** Overexpression of SREBP-1c decreases the endogenous PSA mRNA level. LNCaP cells were infected with mature SREBP-1c–expressing or null adenovirus (multiplicity of infection 50) in the absence or presence of 10 nmol/L dihydrotestosterone. Total RNA was isolated from cells, and the PSA mRNA levels were determined by Northern blot analysis. Precursor and mature SREBP-1c protein levels were confirmed by Western blot analysis. 18S rRNA and actin protein were used as internal controls.

To determine the domains within AR, which are required for the interaction with SREBP-1c, we did GST pull-down assays using a GST-fusion protein of SREBP-1c. The [^{35}S]methionine-labeled AR domain mutants (Fig. 3C) produced by *in vitro* translation were incubated with GST–SREBP-1c protein. GST–SREBP-1c interacted with AR-AF1+DBDh protein, but did not interact with AR-DBDh or AR-ligand binding domain (Fig. 3D). To map the SREBP-1c domains involved in the interaction with AR, [^{35}S]methionine-labeled AR was incubated with the GST-fusion proteins of SREBP-1c deletion mutants, N1, N2, and N3 (Fig. 3E). SREBP-1c N1 (1-153 amino acids) contains the proline-rich domain, whereas SREBP-1c N3 (308-403 amino acids) contains the HLH domain. AR interacted with GST–SREBP-1c N1 and N3, but not with GST–SREBP-1c N2 (Fig. 3F). Taken together, these results suggest that SREBP-1c directly interacts with AR in an androgen-dependent manner, and the AF-1 region of AR and both the NH_2-terminal region and COOH-terminal region of mature SREBP-1c are involved in their interaction.

SREBP-1c Inhibits AR Transactivation by Competing with Coactivators

Previous studies showed that SREBP-1 competes with the peroxisome proliferator-activated receptor-γ coactivator-1 for the modulation of hepatocyte nuclear factor-4α or liver receptor homologue-1 transactivation (30, 31). To determine whether SREBP-1c regulates the coactivator-enhanced transcriptional activity of AR, 293T cells were cotransfected with SREBP-1c and coactivators such as ART-27 and SRC-1, which bind to the AR AF-1 region. Coexpression of SREBP-1c significantly decreased the ART-27– or SRC-1–enhanced transcriptional activity of AR (Fig. 4A). In addition, the SREBP-1c–mediated repression of AR transactivation was almost fully recovered by overexpression of ART-27 or SRC-1 (Fig. 4B). These results suggest that SREBP-1c may interfere with coactivators in the modulation of AR transactivation. However, SREBP-1c seemed to interfere with only certain coactivators, because overexpression of p300 did not recover the SREBP-1c–mediated repression of AR transactivation at all (Fig. 4C).

To determine whether SREBP-1c physically competes with coactivators to bind to AR, we assessed GST pull-down competition assays *in vitro* using ^{35}S-labeled ART-27 and ^{35}S-labeled SREBP-1c. ART-27 interacted with GST-AR AF1+DBDh fusion protein as expected (38), and the interaction showed competition with SREBP-1c binding to GST-AR AF1+DBDh in a dose-dependent manner (Fig. 4D). In addition, SREBP-1c also interfered with the interaction between AR and SRC-1 (data not shown). Taken together, these results suggest that SREBP-1c inhibits the transcriptional activity of AR by interfering with the binding of AR to its coactivators, which include ART-27 and SRC-1.

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HDAC1 Is Involved in the SREBP-1c–Mediated Repression of AR Transactivation

One of the mechanisms by which corepressors inhibit AR transactivation is through the recruitment of HDAC. Therefore, we tested whether SREBP-1c repression of AR transactivation also involves HDAC activity using a HDAC inhibitor, trichostatin. In 293T cells, the repression of AR transactivation by SREBP-1c coexpression was recovered by TSA treatment in a dose-dependent manner (Fig. 5A). These results suggest the involvement of HDACs in the SREBP-1c–mediated suppression of AR transactivation.

To further confirm the involvement of HDACs in the repression of AR transactivation by SREBP-1c, we coexpressed HDAC1 and HDAC4 and assessed their effects on the repression of AR. As shown in Fig. 5B, the SREBP-1c repression of AR transactivation was further suppressed by the coexpression of HDAC1 but not by HDAC4. Expression of only HDAC1 or HDAC4 showed little effect on AR transactivation in the presence of dihydrotestosterone (Fig. 5B). Taken together, these results suggest that HDAC1, at least, is involved in the SREBP-1c–mediated repression of AR transactivation.

SREBP-1c Regulates Androgen-Dependent Growth of Prostate Cancer Cells

Because SREBP-1c represses the function of AR, we did [^{3}H]thymidine incorporation assays to determine whether the overexpression of SREBP-1c inhibits the proliferation of LNCaP cells, which shows androgen-dependent cell growth. As shown in Fig. 6A, dihydrotestosterone-induced thymidine incorporation was inhibited by the overexpression of SREBP-1c. The overexpression of SREBP-1c little affected cell proliferation in the absence of dihydrotestosterone. These results show that the overexpression of SREBP-1c inhibits androgen-induced growth of prostatic cells.

To confirm the role of SREBP-1c in the modulation of androgen-dependent growth of prostatic cells and AR transactivation, we examined the effect of endogenous SREBP-1 knockdown using siRNA. Both precursor and mature SREBP-1c expression was significantly reduced by SREBP-1 siRNA (data not shown). As shown Fig. 6B, siRNA-mediated down-regulation of SREBP-1c increased androgen-induced growth of LNCaP cells. The androgen-induced transactivation of AR was also increased by the down-regulation of endogenously expressed SREBP-1c with SREBP-1 siRNA, when tested with PSA-luc reporter in LNCaP cells (Fig. 6C). Taken together, these results suggest that the level of endogenous SREBP-1c regulates the tansactivation of AR and androgen-induced proliferation of prostatic cells.

To confirm the effect of SREBP-1c overexpression on androgen-induced cell proliferation, we did soft agar colony formation assays. In the absence of dihydrotestosterone, a few small colonies of LNCaP cells formed and adenovirus-mediated overexpression of SREBP-1c slightly affected the number and size of colonies. However, in the presence of dihydrotestosterone, the number of colonies of LNCaP cells increased ~7-fold

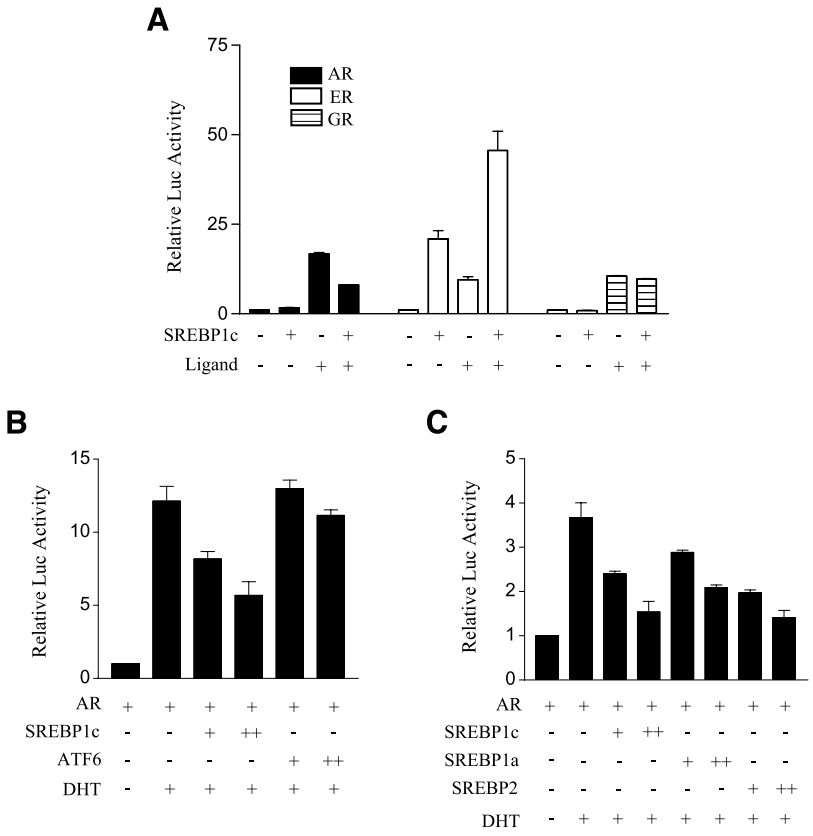

FIGURE 2. SREBP-1c represses the transactivation of AR, but not that of ER or glucocorticoid receptor (*GR*). **A.** 293T cells were cotransfected with 150 ng of the indicated reporter, 100 ng of the corresponding steroid receptor, and 100 ng of mature SREBP-1c expression constructs. **B** and **C.** 293T cells were cotransfected with 150 ng of ARE_2-TATA-luc, 100 ng of pcDNA3-mAR, and 100 ng of mature SREBPs or ATF6 expression constructs. Cells were cultured in medium containing 5% charcoal-stripped serum with or without cognate ligand for each receptor and were then assayed for luciferase activity after 24 h. Data are representative of at least three independent experiments with similar results. Columns, mean of duplicate samples; bars, SD.

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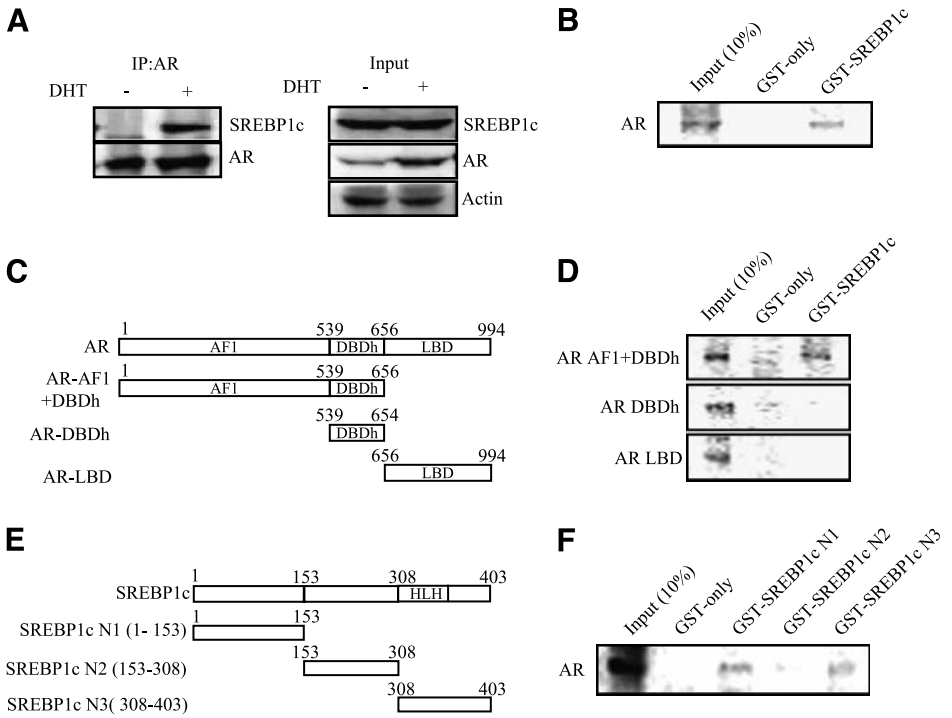

FIGURE 3. SREBP-1c physically interacts with AR in an androgen-dependent manner. **A.** Coimmunoprecipitation (*IP*) assays were done using 293T cells cotransfected with pcDNA3-mAR and pcDNA3HA-SREBP-1c constructs. Cells were treated with or without 10 nmol/L dihydrotestosterone for 12 h. Coimmunoprecipitation was done with anti-AR antibody, and Western blot analyses were carried out using anti–SREBP-1c and anti-AR antibodies. Expression levels of mature SREBP-1c, AR, and actin were confirmed by Western blot analyses of inputs. **B.** In GST pull-down assays, mAR labeled with [^{35}S]methionine by *in vitro* translation was incubated with glutathione *S*-Sepharose beads containing bacterially expressed GST alone or GST–SREBP-1c (a mature form) fusion proteins in the presence of dihydrotestosterone. **C** and **E.** Schematic representations of the full-length and deletion constructs of AR (**C**) and mature SREBP-1c (**E**) used in GST pull-down assays. **D** and **F.** In GST pull-down assays, proteins labeled with [^{35}S]methionine by *in vitro* translation were incubated with glutathione *S*-Sepharose beads containing bacterially expressed GST alone or GST-fusion protein. The input lane represents 10% of the total volume used in the binding assay.

in addition to the magnificent increase of colony size, and the overexpression of SREBP-1c strongly decreased both the number and the size of colonies (Fig. 6D), which is consistent with the results of [^{3}H]thymidine incorporation assays.

We also examined whether SREBP-1c regulates tumor growth in a xenograft tumor model using mouse TRAMP-C2 (transgenic adenocarcinoma of mouse prostate) cells, which show similarities to human prostate cancer cells, including the progression to adenocarcinoma (39-41). TRAMP-C2 cells were infected with adenovirus–SREBP-1c or adenovirus-null, and were injected subcutaneously into two flank sites of C57BL/6 male mice. As shown in Fig. 6E, the tumor growth rate in the mouse group injected with adenovirus–SREBP-1c-infected cells was much lower than that in the control group (Ad-null). These results suggest that the overexpression of SREBP-1c suppresses the growth of prostate cancer cells *in vivo* as well as *in vitro*.

Discussion

In this study, we show that mature SREBP-1c represses androgen signaling through the inhibition of AR transactivation. SREBP-1c directly interacts with AR and competes with coactivators for AR binding to modulate the transactivation. It also seems to recruit HDAC activity for the repression of AR transactivation. Moreover, adenovirus-mediated overexpression of SREBP-1c decreases the growth of prostate cancer cells, and down-regulation of SREBP-1c by siRNA increases the growth of prostate cancer cells. These current findings suggest that SREBP-1c misregulation may play a physiologic role in the development of prostate cancers.

Previous studies have shown that SREBPs modulate the function of several nuclear receptors. In particular, SREBP-1 and SREBP-2 function as negative regulators to repress the transactivation of hepatocyte nuclear factor-4α and liver receptor homologue-1 by interfering with the recruitment of peroxisome proliferator-activated receptor-γ coactivator-1, a coactivator of hepatocyte nuclear factor-4α and liver receptor homologue-1 (30, 31). Therefore, in terms of competition with coactivators, SREBP-1c works on AR in a similar manner, at least competing with ART27 and SRC-1 (Fig. 4). In addition, we show that the SREBP-1c–mediated repression of AR involves the recruitment of HDAC1 (Fig. 5), which suggests that the repression mechanisms of SREBP-1c may vary depending on its interaction partner. Meanwhile, SREBP-1a enhances the activity of ER-dependent LDL-R SR-BI promoter by directly binding to ERE in this promoter or directly interacting with ER (33). Although the mechanism of the cross-talk between ER and SREBP-1a remains to be elucidated, the role of SREBP-1a in the function of ER is opposed to that in the function of AR (Fig. 2). Therefore, SREBP-1 seems to function differently as a specific coregulator depending on its binding partner. It would be worthwhile to

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investigate whether SREBP-1 has different functions in prostate cancers and breast cancers, in which AR and ER play a crucial role in cell proliferation, respectively.

Previous reports have suggested that SREBP-1c, SREBP-1a, and SREBP-2 have different functions in lipid homeostasis. SREBP-1c is preferentially involved in fatty acid synthesis, whereas SREBP-2 is preferentially involved in cholesterol synthesis. Meanwhile, SREBP-1a is implicated in both fatty acid and cholesterol synthesis (42, 43). Although three SREBP isoforms have different roles in lipid homeostasis, our data showed that all SREBP isoforms are able to repress AR transactivation in prostate cancer cells (Fig. 2E). These results suggest that a common feature of the SREBP isoforms is important for their functions as AR coregulators.

In the interaction with nuclear receptors, the transactivation domain of SREBP-1a is necessary for the interaction with hepatocyte nuclear factor-4α (30), whereas the HLH domain of SREBP-2 is required for the interaction with liver receptor homologue-1 (31). However, our results show that both the transactivation domain and the basic helix-loop-helix domain of SREBP-1c are capable of interacting with AR independently (Fig. 3). This suggests that the transactivation domains or basic helix-loop-helix domains of SREBPs may play a role in its cross-talk with nuclear receptors, although the interaction domain of SREBPs with nuclear receptors differs depending on their interaction partners.

Overexpression of mature SREBP-1c represses androgen-induced growth of prostate cancer cells and inhibits tumor growth in a xenograft tumor model (Fig. 6), which suggests that SREBP-1c may interfere with the androgen signaling that governs androgen-dependent tumor growth *in vivo*. Prostate cancer cells show high lipid contents resulting from active lipogenesis (44). Several reports have shown that lipogenic enzymes such as fatty acid synthase and acetyl-CoA carboxylase were overexpressed in many cancer types, including breast and ovary cancers as well as prostate cancer (45-47). E2 and progesterone enhance the gene expression of fatty acid synthase in breast cancer cells (48, 49). Moreover, progesterone regulates the gene expression of fatty acid synthase through the modulation of *SREBP-1c* gene expression (49). In the case of the prostate, several studies have shown that androgen/AR enhances lipogenic gene expression through the activation of SREBP pathways in androgen-dependent prostate cancer cells (34). Androgen increases the expression of SREBP-1c and SREBP-2 precursors as well as the nuclear accumulation of mature SREBPs (36). In this study, we show that mature SREBP-1c represses AR function. Therefore, under conditions of androgen-induced production of mature SREBP-1c, AR is subject to feedback inhibition by SREBP-1c, thereby repressing further activation of SREBP pathways and also blocking androgen signaling for the growth of prostate cancer cells. Such feedback regulation may provide cells with a subtle balance between the production and consumption of cell components such as lipids for cell growth.

SREBP-1c, which is also called adipocyte determination and differentiation-dependent factor 1, promotes adipocyte differentiation through its correlation with peroxisome proliferator-activated receptor γ (50). It was previously reported that

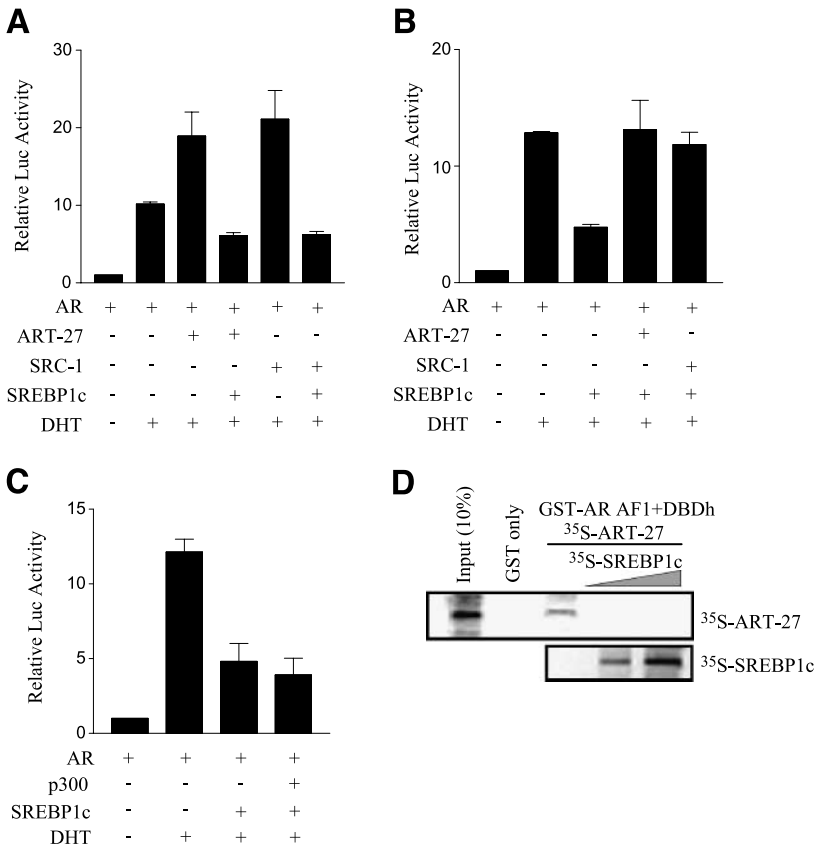

FIGURE 4. SREBP-1c represses AR transactivation by competition with AR coactivators for binding with AR. **A** and **B.** 293T cells were cotransfected with 150 ng of ARE_2-TATA-luc reporter, 100 ng of pcDNA3-mAR, 300 ng of pcDNA3HA-ART-27 or 300ng of pcDNA3-SRC-1, and 150 ng of pcDNA3HA-SREBP-1c. **C.** 293T cells were cotransfected with 150 ng of ARE_2-TATA-luc, 100 ng of pcDNA3-mAR, 150 ng of pcDNA3-SREBP-1c, and 300 ng of pcDNA3-p300. **D.** SREBP-1c interferes with the interaction between AR and ART-27. [^{35}S]methionine-labeled ART-27 was incubated with bead-bound GST-AR AF1+DBDh fusion protein in the presence of increasing amounts of *in vitro* translated SREBP-1c. Input represents 10% of the total volume of ^{35}S-labeled ART-27 used in the assay. **A** to **C.** Cells were treated with or without dihydrotestosterone for 24 h and assayed for luciferase activity. Data are representative of at least three independent experiments with similar results. Columns, mean of duplicate samples; bars, SD.

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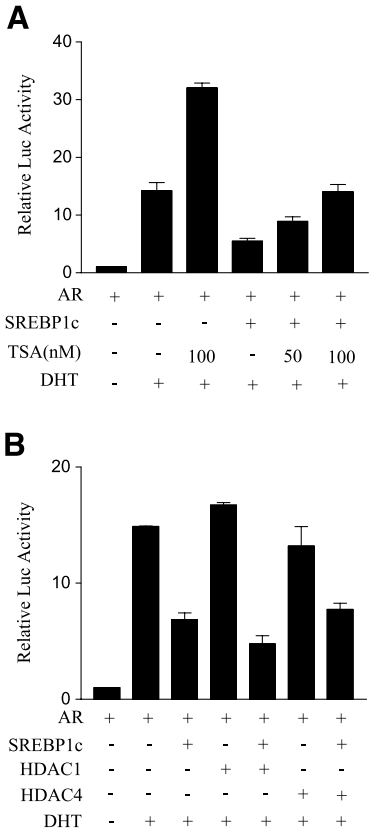

FIGURE 5. Involvement of HDAC in the SREBP-1c repression of AR transactivation. **A.** 293T cells were cotransfected with 150 ng of ARE_2-TATA-luc, 100 ng of pcDNA3-mAR, and 100 ng of pcDNA3HA-SREBP-1c. Cells were treated with or without 50 or 100 nmol/L of trichostatin A in the presence or absence of 10 nmol/L dihydrotestosterone. **B.** 293T cells were cotransfected with 150 ng of ARE_2-TATA-luc, 100 ng of pcDNA3-mAR, 100 ng of pcDNA3HA-SREBP-1c, and 400 ng of HDAC1 or HDAC4 expression plasmids. Cells were treated with or without 10 nmol/L dihydrotestosterone. Data are representative of at least three independent experiments with similar results. Columns, mean of duplicate samples; bars, SD.

androgen blocks adipogenic differentiation through the activation of Wnt signaling (51) and the down-regulation of peroxisome proliferator-activated receptor γ and CCAAT/enhancer-binding protein α expression (52). Our data showed that AR directly interacts with SREBP-1c in a ligand-dependent manner, thus suggesting that androgen/AR may regulate the transcriptional activity of SREBP-1c. Such suppression of SREBP-1c transactivation by AR could be another mechanism by which androgen inhibits adipogenic differentiation.

In conclusion, our data show that mature SREBP-1c represses the functions of AR, which inhibits the expression of AR target genes and androgen-dependent growth of prostate cancer cells. These findings suggest that SREBP-1c may play an important role in cell growth as well as in the control of lipogenesis in prostate cancer cells, therefore proposing SREBP-1c activation pathways as potential therapeutic targets for prostate cancers.

Materials and Methods

Animals

C57BL/6 male mice, 8 weeks of age, were purchased from a commercial supplier (Damul Science). Animals were kept in a cage and given water and chow *ad libitum*, and were maintained under controlled conditions (12-h light and dark photoperiod, 50% humidity, 22°C). The ethical treatment of animals in the present study was carried out according to NIH standards.

Plasmids

The mammalian expression vectors of pcDNA3-mouse AR, pcDNA3-SRC-1, and pcDNA3-p300 have been described previously (53, 54). The mammalian expression vectors of HDAC1 and HDAC4, $pARE_2$-TATA-luc reporter, and PSA-luc reporter plasmid were also described previously (55). The mammalian expression vectors of pcDNA3-ERα and pcDNA3-glucocorticoid receptor, mouse mammary tumor virus-luc reporter, and ERE-luc reporter plasmid were gifts from J.W. Lee (Baylor College of Medicine; ref. 56). Expression vectors of the mature forms of SREBP-1c, SREBP-1a, and SREBP-2 (1-403 amino acids) were described previously (57). The mammalian expression vector of ATF6 was kindly provided as a gift from Ron Prywes (Columbia University, New York, NY; ref. 37). The ART-27 construct was cloned into the pcDNA3HA vector digested with *Eco*RI and *Xho*I. pcDNA3HA-mAR deletion mutants were generated by cloning the regions spanning AF1+DBDh, DBDh, and ligand-binding domain of mouse AR into vector B42 digested with *Eco*RI and *Xho*I restriction enzymes. The GST–SREBP-1c (1-403 amino acids) and GST–SREBP-1c deletion mutants were described previously (58, 59).

Cell Culture and Transient Transfection Assays

293T and PPC-1 cell lines were maintained in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. The LNCaP cell line was purchased from the American Type Culture Collection and maintained in RPMI 1640 (Life Technologies) supplemented with L-glutamine and 10% fetal bovine serum. The TRAMP-C2 cell line was purchased from the American Type Culture Collection and maintained in DMEM (Life Technologies) supplemented with 5% fetal bovine serum, 5% NuSerum (BD Biosciences), 5 μg/mL bovine insulin, and 10 nmol/L dehydroisoandrosterone. Cells were plated in 24-well plates and transfected with the indicated amounts of expression plasmids, a reporter plasmid, and the control *lacZ* expression plasmid, pCMV-β (Clontech), by using the Superfect or Effectene reagent (Qiagen) according to the instructions of the manufacturer. Total amounts of expression vectors were kept constant by the addition of appropriate amounts of empty pcDNA3 vector. Cells kept in 5% charcoal-stripped serum were treated with 10 nmol/L dihydrotestosterone for 24 h after transfection. Trichostatin A was applied 20 h before harvesting. Luciferase and β-galactosidase activities were assayed as described previously (60). The levels of luciferase activity were normalized to *lacZ* expression.

Chromatin Immunoprecipitation Assay

LNCaP cells were transfected with pcDNA3HA-SREBP-1c (1-403 amino acids, a mature form) expression plasmid, treated with or without 10 nmol/L dihydrotestosterone for 16 h after 24 h of transfection, and cross-linked with 1% formaldehyde.

Published OnlineFirst February 1, 2008; DOI: 10.1158/1541-7786.MCR-07-0354

After incubating the samples with TSE I [100 mmol/L Tris-HCl (pH 9.4) and 10 mmol/L DTT] for 20 min at 30°C, the cells were washed and processed for chromatin immunoprecipitation assays as described previously (60). Anti-AR or anti-hemagglutinin antibodies (Santa Cruz Biotechnology) were used for immunoprecipitation. Immunoprecipitated DNA and input-sheared DNA were subjected to PCR using a PSA primer pair (forward, 5′-TGAGAAACCTGAGATTAGGA-3′ and reverse, 5′-ATCTCTCTCAGATCCAGGCT-3′), which amplified a 229-bp region (−4271 to −4043) spanning the ARE of the PSA promoter. As a negative control, PCRs were done using an actin primer pair (sense, 5′-GAGACCTTCAACACCCCAGCC-3′, and antisense, 5′-CCGTCAGGCAGCTCATAGCTC-3′), which amplified a 362-bp region spanning exon 4 of the *β-actin* gene.

Northern Blot Analysis

Total RNA was isolated using TRI Reagent (Sigma). Twenty micrograms of total RNA were fractionated by electrophoresis on 1% agarose gel containing formaldehyde and transferred to nylon membranes (Zeta-probe, Bio-Rad) by capillary blotting with 10× sodium citrate-sodium chloride. After UV cross-linking and prehybridization, membranes were hybridized overnight at 42°C in a solution containing 50% formamide, 10% dextran sulfate, 5× sodium citrate-sodium chloride, 1 mmol/L EDTA, 10 mg/mL denatured salmon sperm DNA, and 2×10^6 to 4×10^6 cpm of ^{32}P-labeled probes. After hybridization, membranes were washed twice for 5 min at room temperature in 2× sodium citrate-sodium chloride and 0.1% SDS, followed by 30 min in 0.5× sodium citrate-sodium

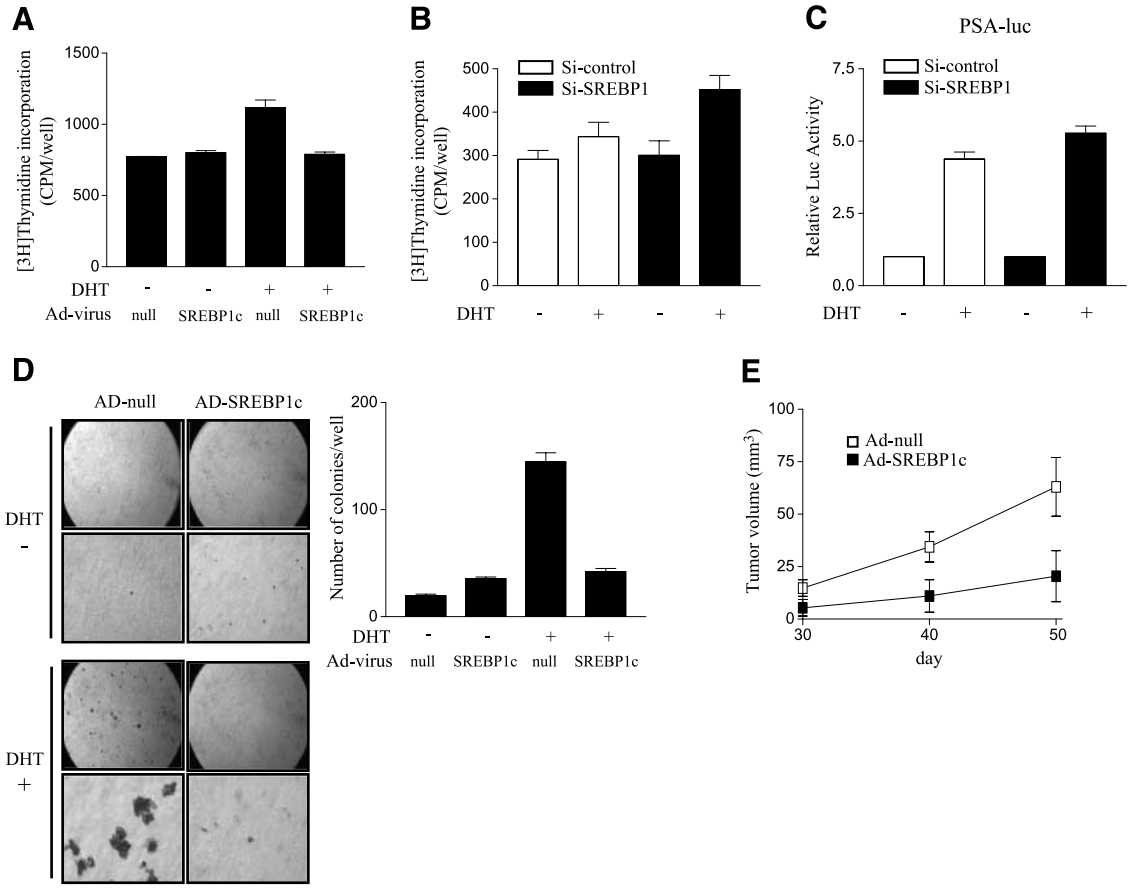

FIGURE 6. Effects of SREBP-1c overexpression and down-regulation on the proliferation of prostate cancer cells and AR transactivation. **A.** The overexpression of SREBP-1c inhibits androgen-induced proliferation of LNCaP cells. LNCaP cells were infected with SREBP-1c–expressing or null adenovirus (multiplicity of infection 50) in the absence or presence of 1 nmol/L dihydrotestosterone for 72 h. Cell proliferation was determined by the incorporation of [^{3}H]thymidine during the last 4 h of culturing. Columns, mean of experiments done in triplicate; bars, SD. Data are representative of at least three independent experiments. **B.** Down-regulation of the endogenous SREBP-1c by SREBP-1 siRNA increases androgen-induced proliferation of LNCaP cells. LNCaP cells were transfected with control or SREBP-1 siRNA in the absence or presence of 1 nmol/L dihydrotestosterone for 48 h. Cell proliferation was determined by the incorporation of [^{3}H]thymidine during the last 4 h of culturing. Columns, mean of experiments done in duplicate; bars, SD. Data are representative of at least two independent experiments. **C.** Down-regulation of the endogenous SREBP-1c by SREBP-1 siRNA increases the transactivation of AR. LNCaP cells were transfected with control or SREBP-1 siRNA. Cells were cultured in medium containing 0.5% charcoal-stripped serum with or without 10 nmol/L dihydrotestosterone for 48 h, and were then assayed for luciferase activity. Data are representative of at least two independent experiments with similar results. Columns, mean of duplicate samples; bars, SD. **D.** Effect of SREBP-1c overexpression on the size and number of colonies. LNCaP cells were infected with SREBP-1c–expressing or null adenovirus (multiplicity of infection 50) for 24 h. The cells were plated in soft agar and incubated for 3 wk in the absence or presence of dihydrotestosterone. Columns, mean of duplicate determinations; bars, SD. Data are representative of at least two independent experiments. **E.** The overexpression of SREBP-1c inhibits tumor growth in an animal tumor model using TRAMP-C2 cells infected with SREBP-1c–expressing or null adenovirus (multiplicity of infection 50). After infection, the cells were injected subcutaneously into the left and right flanks of each of the C57BL/6 male mice (8 mice per each group). Tumor volume was assessed with the formula: (length × width^2) / 2. Bars, SE.

Published OnlineFirst February 1, 2008; DOI: 10.1158/1541-7786.MCR-07-0354

chloride and 0.1% SDS at 65°C. Membranes were then exposed using Kodak RX films (Eastman Kodak Co.) for 12 to 24 h at −70°C. The signals were normalized to the 18S rRNA internal control.

Coimmunoprecipitation and Western Blot Assays

Coimmunoprecipitation assays were done with 293T cells that were transfected with 1 μg of AR and 1 μg of SREBP-1c expression plasmids. Cells were treated with or without 10 nmol/L dihydrotestosterone for 12 h after transfection, and were harvested in radioimmunoprecipitation cell lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 2.5 mmol/L EGTA, 1% Triton X-100, 50 mmol/L NaF, 10 mmol/L $Na_4P_2O_7$, 10 mmol/L Na_3VO_4, 1 μg/mL aprotinin, 0.1 μg/mL leupeptin, 1 μg/mL pepstatin, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L DTT]. Whole-cell lysate (400 μg) was incubated with 2 μg of anti-AR (Santa Cruz Biotechnology) for 4 h at 4°C, and was further incubated for another 4 h after the addition of 20 μL of protein A-agarose bead slurry (Invitrogen). Agarose beads were washed thrice with RIPA buffer at 4°C, and bound proteins were separated by SDS-PAGE. Proteins on the gels were transferred to a nitrocellulose membrane (Sigma); subjected to Western blot analysis with anti–SREBP-1c, anti-AR, and anti–β-actin antibodies (Santa Cruz Biotechnology); and then detected using an ECL kit (Amersham Pharmacia).

GST Pull-Down Assay

GST and GST–SREBP-1c (a mature form) fusion proteins were expressed in *Escherichia coli* BL21 cells and isolated with glutathione-Sepharose-4B beads (Pharmacia Biotech AB). Immobilized GST fusion proteins were then incubated with [^{35}S]methionine-labeled AR or AR domain mutant proteins produced by *in vitro* translation using a TNT-coupled transcription-translation system (Promega). Binding reactions were carried out in 250 μL of GST-binding buffer [20 mmol/L Tris-HCl (pH 7.9), 100 mmol/L NaCl, 10% glycerol, 0.05% NP40, 5 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, 1 mmol/L DTT, and 1.5% bovine serum albumin] for 4 h at 4°C. The beads were washed thrice with 1 mL of GST-binding buffer. Bound proteins were eluted by the addition of 20 μL of SDS loading buffer, and were analyzed by SDS-PAGE and autoradiography.

For *in vitro* competition assays, [^{35}S]methionine-labeled mature SREBP-1c protein produced by *in vitro* translation was added to the binding reaction, and beads were washed thrice with the binding buffer, resuspended in 2× SDS loading buffer, separated by SDS-PAGE, and visualized using a Phosphorimager (BAS-1500, Fuji). The GST-fused proteins used in each reaction were analyzed by SDS-PAGE and quantified by Coomassie blue staining. Ten percent of the *in vitro* translated SREBP-1c and ART-27 used in each reaction was loaded as inputs.

siRNA Experiment

SREBP-1 siRNAs were chemically synthesized (Bioneer Co.), deprotected, annealed, and transfected according to the manufacturer's instructions. LNCaP cells were transfected with siRNA using Oligofectamine reagent (Invitrogen Life Technologies). The sequences of siRNA are as follows: SREBP-1 siRNA, sense 5′-CCACCGTTTCTTCGTGGATd-TdT-3′.

Soft Agar Colony Formation Assay

LNCaP cells were infected with mature SREBP-1c–expressing or null adenovirus. After 24 h of infection, cells were trypsinized and seeded at 5×10^3 per well in six-well culture dishes containing 0.35% agar over a 0.7% agar layer. Cells were fed with medium containing 5% charcoal-stripped serum with or without 1 nmol/L dihydrotestosterone. Fresh media were changed every 2 to 3 days for 3 weeks. Colonies with a diameter larger than 50 μm were scored.

Thymidine Incorporation

LNCaP cells were cultured in 96-well plates at a density of 2×10^4 per well, and were infected with mature SREBP-1c–expressing (multiplicity of infection 50) or null adenovirus (multiplicity of infection 50). After 24 h of infection, the cells were treated with or without 1 nmol/L dihydrotestosterone for 72 h, and were then pulse-labeled with [^{3}H]thymidine (10 μCi/mL, specific activity 80 Ci/mmol; Perkin-Elmer Life Sciences) for 4 h. Cells were harvested onto a glass microfiber filter (Whatman, Inc.) and intensively washed with distilled water. The incorporation of thymidine into DNA was measured by counting the filters with a scintillation counter.

Tumor Xenograft In vivo

TRAMP-C2 cells were infected with adenovirus expressing mature SREBP-1c or null adenovirus for 12 h. C57BL/6 male mice were subcutaneously injected with the adenovirus-infected TRAMP-C2 cells (1×10^6 per flank) into the right and left hind flanks. Tumor size was measured every 10 days from day 30 to day 50 using a caliper. Tumor volumes were calculated using the formula (length × width^2) / 2. Eight mice were used for each group.

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Published OnlineFirst February 1, 2008; DOI: 10.1158/1541-7786.MCR-07-0354

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Published OnlineFirst February 1, 2008; DOI: 10.1158/1541-7786.MCR-07-0354

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Sterol Regulatory Element–Binding Protein-1c Represses the Transactivation of Androgen Receptor and Androgen-Dependent Growth of Prostatic Cells

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Mol Cancer Res 2008;6:314-324. Published OnlineFirst February 1, 2008.