Endocrinology

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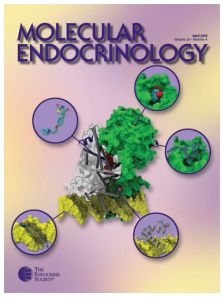
Endocrinology 2007 148:1340-1349 originally published online Nov 16, 2006; , doi: 10.1210/en.2006-0716



0013-7227/07/$15.00/0
Printed in U.S.A.

Endocrinology 148(3):1340–1349

doi: 10.1210/en.2006-0716

Suppression of Androgen Receptor Transactivation and Prostate Cancer Cell Growth by Heterogeneous Nuclear Ribonucleoprotein A1 via Interaction with Androgen Receptor Coregulator ARA54

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The androgen receptor (AR) requires coregulators for its optimal transactivation. Whether AR coregulators also need interacting proteins to modulate their function remains unclear. Here we describe heterogeneous nuclear ribonucleoprotein (hnRNP) A1 as an associated negative modulator for the AR coregulator ARA54. hnRNP A1 selectively suppressed ARA54-enhanced wild-type and mutant AR transactivation via interruption of AR-ARA54 interaction and ARA54 homodimerization. Stable transfection of hnRNP A1 in the LNCaP cells suppressed AR-mediated cell growth and the expression of prostate-specific antigen, and this suppressive effect was abolished by the addition of ARA54-small interfering RNA. Small interfering RNA knockdown of endogenous hnRNP A1 enhanced cell growth and prostate-specific antigen expression in LNCaP cells. These results not only suggest that the loss of hnRNP A1 expression might activate the ARA54-enhanced cell growth and contribute to the prostate cancer progression, but also demonstrate the dual functional roles for ARA54 as an AR coregulator directly and as a mediator for the suppressive effect of hnRNP A1 indirectly. The novel finding that a protein can modulate AR function without direct interaction with AR might provide a new therapeutic approach to battle prostate cancer by targeting AR indirectly with fewer side effects. (*Endocrinology* 148: 1340–1349, 2007)

PROSTATE CANCER CONTINUES to pose a great challenge to public health in the United States. Each year about 31,000 men in the United States lose their lives because of this disease (1). Surgical or medical castration combined with antiandrogens targeting to block the androgen receptor (AR) signaling pathway, named as total androgen blockade, is the cornerstone for the therapy of late-stage patients (2). Androgen and the AR signaling pathway play pivotal roles throughout prostate cancer development, including the androgen independent state (3–7). AR, which is a member of the nuclear receptor (NR) superfamily, is a ligand-dependent transcription factor (8, 9). Dissecting the precise molecular mechanism of how AR signaling is regulated in normal and malignant cells, and how its aberrant signaling might contribute to the progression and development of prostate cancer might greatly help in combating this androgen-related disease.

Numerous studies showed that upon ligand binding, AR needs coregulators for its optimal function (10). Several AR coregulators, including the ARA54, ARA55, ARA67, ARA70, hRad9, cAMP response element binding protein binding protein (CBP), steroid receptor coactivator (SRC)-1, and phosphatase and tensin homolog have been identified, and their potential pathophysiological roles in prostate cancer progression have been studied (10–24). Although the detailed molecular mechanism of AR signaling regulation is still not well documented, it is well known that the interaction of these protein factors with AR influences almost every aspect of its function, including ligand-binding, N-C interaction, translocation to the nucleus, and DNA binding capacity, which affects the functional transcription of AR target genes, like the prostate-specific antigen (PSA) (10). The aberrant function of AR coregulators might contribute to the development of androgen independent prostate cancer as well as the antiandrogen withdrawal syndrome.

ARA54 is an AR coactivator that interacts with AR and further induces AR transactivation up to 6-fold in human prostate cancer cell lines. It also enhances the mutant AR amino acid 877 mutation from threonine to alanine (mtART877A) transactivation in response to 17β-estradiol (E2) and hydroxyflutamide (HF), suggesting its role in the

First Published Online November 16, 2006

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Abbreviations: AR, Androgen receptor; CBP, cAMP response element binding protein binding protein; CT value, threshold cycle value; DHT, dihydrotestosterone; E2, 17β-estradiol; FBS, fetal bovine serum; GST, glutathione-S-transferase; HF, hydroxyflutamide; hnRNP, heterogeneous nuclear ribonucleoprotein; MMTV-LUC, mouse mammary tumor virus luciferase; mtART877A, mutant AR derived from the prostate cancers, codon 877 mutation threonine to alanine; MTT, methyl thiazolyl tetrazolium; NR, nuclear receptor; PSA, prostate-specific antigen; si, small interfering; SRC, steroid receptor coactivator.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

development of the antiandrogen withdrawal syndrome (15). The study of its dominant-negative mutant further reveals the functional role of ARA54 in prostate cancer (25). Recent studies also confirmed the increasing expression level of ARA54 in the clinical prostate cancer specimens (26–28). These findings revealed the important roles played by ARA54 in the regulation of AR signaling and prostate cancer progression.

Recent studies showed that some protein factors can indirectly modulate the AR activity by their interaction with the AR coregulators (29). These findings led us to hypothesize the presence of some unknown protein factors that might interact with ARA54 and modulate the signaling function of AR indirectly. By using the yeast two-hybrid assay system, we were able to isolate heterogeneous nuclear ribonucleoprotein (hnRNP) A1 as an ARA54-associated negative modulator for AR transactivation. hnRNP A1, which belongs to the large family of hnRNPs, functions as a mRNA-binding protein shuttling between the nucleus and cytoplasm (30–32). It plays important roles in the mRNA splicing (33, 34), telomere elongation (35), mRNA turnover, and translation (36). Here we report that hnRNP A1 functions as an ARA54-interacting protein and a suppressor of ARA54-enhanced AR transactivation, which results in the growth suppression of LNCaP cells.

Materials and Methods

Materials and plasmid

Dihydrotestosterone (DHT) and E2 were obtained from Sigma-Aldrich (St. Louis, MO), and HF was from Schering-Plough Corp. (Kenilworth, NJ). The pACT2-prostate cDNA library and Marathon-Ready prostate cDNA library were purchased from Clontech (Palo Alto, CA) and pGEMTeasy vector was from Promega Corp. (Madison, WI). pAS2-ARA54 containing the full-length ARA54 fused to the GAL4 DNA binding domain (amino acids 1–147) was constructed by inserting the *Bam*HI fragment of ARA54 in frame into the pAS2 plasmid. pGEX-GST-ARA54 was constructed as described previously (37). pCMX-GAL4-AR and pCMX-VP16-AR were constructed for mammalian two-hybrid assay (13, 38). pCMV-mtART877A has been described previously (39). pM-ARA54 and pVP16-ARA54 were constructed by inserting the *Bam*HI fragment of ARA54 in frame to the pM and pVP16 vectors, respectively. pSG5-ARA54, mouse mammary tumor virus luciferase (MMTV-LUC), pG5-LUC, and pRL-TK have been described previously (25). Full-length hnRNP A1 cDNA was amplified from Marathon-Ready prostate cDNA library using PCR and cloned into the pGEMTeasy vector and subcloned into pSG5 expression vector via the *Eco*RI site. pcDNA3-Flag-hnRNP A1 and pVP16-hnRNP A1 were generated by insertion of the full-length human hnRNP A1 cDNA via *Eco*RI sites. Sequencing confirmed all the constructs. Anti-ARA54 and anti-PSA antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-Flag antibody was from Sigma-Aldrich. Anti-hnRNP A1 antibody was purchased from Abcam (Cambridge, MA). pSG5-ARA70N, pSG5-ARA55, pSG5-gelsolin, pSG5-supervillin, pSG5-SRC-1, and pSG5-CBP have been described previously (12, 13, 40–43). pM-53 and pVP16-T were purchased from Clontech. The LNCaP, 3T3, DU145, and COS-1 cells were obtained from the American Type Culture Collection (Manassas, VA). Cell culture media RPMI-1640 and DMEM were obtained from Life Technologies, Inc (Rockville, MD).

Yeast two-hybrid screening

A fusion protein (GAL4-ARA54) containing GAL4 DNA-binding domain and full-length ARA54 was used as bait to screen 1.26×10^6 transformants of MATCHMAKER human prostate cDNA library (Clontech). Transformants were selected for growth on nutrition selection plates containing synthetic dropout media lacking histidine, leucine, and tryptophan (−3SD) with 20 mM 3-aminotriazole. Colonies were also filter-assayed for β-galactosidase activity. DNA from positive clones was recovered from yeast using PCR. The PCR primers were sense: 5′-GGG TTT TTC AGT ATC TAC GAT TCA TAG A-3′, and antisense: 5′-CTA TTC GAT GAT GAA GAT ACC CCA CCA AAC CC-3′. The annealing temperature was 58 C. The PCR products were sequenced and then digested with *Bgl*II and cloned into pVP16 vector via the *Bam*HI site. The full-length cDNA of candidate genes were generated by PCR using the Clontech Marathon-Ready cDNA library as template, the PCR primers were sense: 5′-ACC CTG CCG TCA TGT CTA AG-3′, and antisense: 5′-CTG TAG CTT CCC TGT CAC TTC T-3′. The PCR product was cloned into the pGEMTeasy vector, and then further inserted into the pSG5, pVP16, and pcDNA3-Flag vector.

Cell culture, transient transfections, and reporter gene assays

COS-1, 3T3, LNCaP, and DU145 cells were grown in appropriate medium at $1–4 \times 10^5$ cells in 24-well plates. Transfections were performed using SuperFect (QIAGEN, Inc., Valencia, CA) according to the manufacturer's procedure. After incubation for 2–3 h, the medium was changed, and cells were treated with ethanol or DHT for another 24 h and then harvested for luciferase assay. In each experiment, the total amount of transfected DNA/well was maintained as a constant by the addition of empty backbone vectors (pSG5, pVP16, or pcDNA3-Flag) or scrambled small interfering (si) RNA. The MMTV-LUC plasmid was used as the reporter gene. pRL-TK was used as an internal control. The Dual-luciferase reporter 1000 assay system (Turner Designs, Inc., Sunnyvale, CA) was used to measure luciferase activity. The activity of the experimental reporter was normalized to the activity of the internal control to minimize the experimental variability caused by differences in cell viability or transfection efficiency.

Mammalian two-hybrid assay

3T3 cells were transiently cotransfected with a GAL4-hybrid expression plasmid, a VP16-hybrid expression plasmid, the reporter plasmid pG5-LUC, and the pRL-TK internal control plasmid. Cells were treated with DHT or ethanol for 24 h and then harvested for luciferase assay. The activities of renilla luciferase were used to normalize any variations in transfection efficiency.

In vitro glutathione-S-transferase (GST) pull-down assay

GST pull-down assays were performed as described previously (17). Briefly, the full-length hnRNP A1 was *in vitro* translated in the presence of [^{35}S] methionine using T7 polymerase and the coupled transcription/translation kit (Promega). pGEX-GST-ARA54 plasmids expressing the GST-ARA54 fusion protein were transformed into BL21(DE3) bacteria strain, and 0.4 mM isopropyl-β-D thiogalactopyranoside was added into LB medium containing transformed bacteria when the OD_{600} reached 0.5. Bacteria were further cultured at 30 C for 3 h and lysed by 4 cycles of freezing-thawing in NENT buffer (20 mM Tris/pH 8.0, 0.5% Nonidet P-40, 100 mM NaCl, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 8% glycerol, and 1 mM phenylmethylsulfonyl fluoride). The GST-ARA54 fusion proteins were purified with glutathione-beads at 4 C. For *in vitro* interaction, mixtures of glutathione bead-bound GST fusion proteins and 5 μl [^{35}S]methionine-labeled input proteins in 100 μl interaction buffer (50 mM HEPES, 100 mM NaCl, 20 mM Tris-Cl/pH 8.0, 0.1% Tween 20, 10% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM NaF, and 0.4 mM sodium vanadate) at 4 C for 2 h. The beads were then washed with NENT buffer four times, resuspended in SDS-PAGE loading buffer, and resolved on 12% SDS-PAGE gel electrophoresis followed by autoradiography.

Co-immunoprecipitation of hnRNP A1 and ARA54

Co-immunoprecipitation of hnRNP A1 and ARA54 was performed as described previously (44). Briefly, LNCaP cells cultured in RPMI-1640 supplemented with 10% nonheated-inactivated fetal bovine serum (FBS) were lysed using lysis buffer [1% Nonidet P-40, 10% glycerol, 135 mM NaCl, 40 mM Tris/pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1× protease inhibitor cocktail (Hoffmann-La Roche

Inc., Nutley, NJ)]. Lysates were clarified by centrifugation, and then 500 μg protein was incubated with 2 μg anti-Flag antibody or IgG for 4 h at 4 C with agitation. A total of 35 μl protein A/G plus agarose was added to each sample and incubated for 1 h. After washing three times with RIPA buffer, the complex was resolved on a 10% SDS-polyacrylamide gel. The separated proteins were transferred to a polyvinylidene difluoride membrane, and then blotted with anti-ARA54 and anti-hnRNP A1 antibody. An alkaline phosphatase detection kit (Bio-Rad Laboratories, Hercules, CA) resolved the bands.

Construction of DNA vector-based RNA interference plasmids

siRNA target sites were selected by scanning the cDNA sequence of hnRNP A1 and ARA54 using the Oligoengine (Seattle, WA) siRNA designing software and BLAST search to eliminate any sequence with significant homology to other genes. The siRNA inserts, containing selected 19-nt coding sequences, followed by a 9-nt spacer, an inverted repeat of the coding sequence, plus 5 Ts, were generated as double-stranded DNAs with *Hin*dIII and *Bgl*II sites, and then cloned into plasmid pSUPERIOR.retro.puro (Oligoengine). The corresponding oligos for generating hnRNP A1 siRNA were 5′-GAT CCC CTG AGA GCC TGA GGA GCC ATT TCA AGA GAA TGG CTC CTC AGG CTC TCA TTT TTA-3′ and 5′-AGC TTA AAA ATG AGA GCC TGA GGA GCC ATT CTC TTG AAA TGG CTC CTC AGG CTC TCA GGG-3′ (position 80–99). A nonfunctional scrambled siRNA was constructed as a negative control, which contains nucleotide substitutions at the 19-nt targeting sequence of siRNA. The corresponding oligos for generating this scrambled siRNA control were 5′-GAT CCC CGA GAG TGT CCA CCT ACA TAT TCA AGA GAT ATG TAG GTG GAC ACT CTC TTT TTA-3′ and 5′-AGC TTA AAA AGA GAG TGT CCA CCT ACA TAT CTC TTG AAT ATG TAG GTG GAC ACT CTC GGG-3′. The corresponding oligos for generating ARA54 siRNA sequences were: 5′-GAT CCC CAT GCC TCA ACT GCC CAG AAT TCA AGA GAT TCT GGG CAG TTG AGG CAT TTT TTA-3′, 5′-AGC TTA AAA AAT GCC TCA ACT GCC CAG AAT CTC TTG AAT TCT GGG CAG TTG AGG CAT GGG-3′ (position 782–801).

Establishment of cell lines stably transfected with the plasmid encoding siRNA against ARA54 and hnRNP A1

The pSUPERIOR.retro.puro (Oligoengine) vector based siRNA against the ARA54 and hnRNP A1 was constructed and delivered into LNCaP cells using retrovirus-mediated transfection. Briefly, 10 μg siRNA were transfected into Phoenix packaging cells using SuperFect. After 48-h transfection, the cell culture medium was filtered through a 0.45-μm filter. The viral supernatant was used for infection of cells after addition of 4 μg/ml polybrene. Cells were infected for 6 h and allowed to recover for 24 h with fresh medium. Infected cells were selected with puromycin (3 μg/ml for 48 h).

Establishment of cell lines stably transfected with the plasmid encoding hnRNP A1

The pcDNA3-Flag vector contains a selective marker conferring resistance to neomycin for the generation of stable cell lines. We first constructed pcDNA3-Flag-hnRNP A1 and then transfected it into LNCaP cells using SuperFect transfection reagent. After transfection, cells were cultured in the presence of 300 μg/ml neomycin (Invitrogen, Corp., Carlsbad, CA) to select for stably transfected cells that had incorporated the pcDNA3-Flag based construct. After selection for an additional 2 wk, individual clones were picked. We then confirmed the stable expression of hnRNP A1 in the selected clones using real-time RT-PCR and Western blotting.

Cell proliferation assay

LNCaP cells were seeded in 24-well plates at a density of 4000 cells/cm^2 in medium containing 10% CD-FBS with 1 nM DHT. At the indicated time points, the medium was replenished, and cell proliferation was determined by methyl thiazolyl tetrazolium (MTT) assay (Sigma-Aldrich). Serum-free medium containing MTT (0.5 mg/ml) was added into each well. After 4 h incubation at 37 C, the stop solution was added to solubilize the formazan product, and the absorbance was recorded.

Real-time RT-PCR

Total RNA was extracted from tissue or cells using TRIZOL (Invitrogen). We performed RT with the SuperScript II kit (Invitrogen) and PCR amplifications with SYBR Green PCR Master Mix on an iCycler IQ multicolor real-time PCR detection system (Bio-Rad Laboratories). The PCR was performed as follows: initial denaturation at 95 C for 10 min, and 45 cycles of denaturation at 95 C for 30 sec, annealing at 60 C for 30 sec, and extension at 72 C for 30 sec. Primer sequences were: hnRNP A1: sense: 5′-CTC TTC ATT GGA GGG TTG-3′, antisense: 5′-TGT GGC CTT GCA TTC ATA-3′; ARA54: sense: 5′-CTA GTC TTC TAC CTC ATC TTC-3′, antisense: 5′-CGA AAT GAA TAC CTG CAA GCG-3′; β-actin, sense 5′-TGT GCC CAT CTA CGA GGG GTA TGC-3′ and antisense 5′-GGT ACA TGG TGG TGC CGC CAG ACA-3′. ΔThreshold cycle (ΔCT) values were calculated by subtracting the CT value from the corresponding β-actin CT (internal control) value from each time point. Agarose-gel electrophoresis confirmed the absence of nonspecific amplification products.

Western blotting assay

Protein samples collected from the cells were separated on SDS-10% PAGE and transferred to polyvinylidene difluoride membrane. Membranes were blocked with 5% nonfat milk in TBST buffer (150 mM NaCl, 10 mM Tris/pH 8.0, and 0.5% Tween 20) at room temperature for 1 h. The membranes were then immunoblotted with primary antibodies for 2 h at room temperature or overnight at 4 C, followed by incubation with secondary antibodies for 1 h at room temperature. Blots were developed with the AP color developing reagents (Bio-Rad Laboratories), and the Western data were quantified by Quantity one software (Bio-Rad Laboratories).

Results

Identification of hnRNP A1 as an ARA54-interacting protein

We screened the human prostate cDNA library using the Clontech MATCHMAKER yeast two-hybrid assay system. Full-length ARA54 fused to the GAL4 DNA-binding domain was used as bait to screen 1.26×10^6 transformants of MATCHMAKER human prostate cDNA library. Full-length cDNA of three positive clones were then amplified and cloned into the pSG5 expression vector for further characterization. Sequence analysis determined that one of the candidate clones matched the hnRNP A1 sequence, and its interaction with ARA54 was further confirmed in the yeast growth assay and colony lift assay for β-galactosidase on −3SD nutrition selection plates (data not shown), as well as mammalian two-hybrid assay. Figure 1A shows that in 3T3 cells, the full-length hnRNP A1 protein interacts strongly with ARA54 in the absence or presence of androgen (Fig. 1A, lane 5), while it showed little interaction with AR in the same mammalian two-hybrid assay system (Fig. 1A, lane 6). Using the GST pull-down assay, we further tested the interaction between hnRNP A1 and ARA54 *in vitro*. As depicted in Fig. 1B, the GST-ARA54 fusion protein can pull down the *in vitro* translated [^{35}S]-labeled hnRNP A1. The *in vivo* co-immunoprecipitation test further showed that the endogenous hnRNP A1 can be co-immunoprecipitated with the endogenous ARA54 in LNCaP cells (Fig. 1C). We were then interested to know if hnRNP A1 can be complexed to AR directly. Using AR antibody, we were able to co-immunoprecipitate ARA54, but not hnRNP A1 (Fig. 1D). Together, the yeast and mammalian two-hybrid assays, *in vitro* GST pull-down, and *in vivo* co-immunoprecipitation assay all indicate that hnRNP A1 can interact with ARA54. In contrast, the hnRNP A1

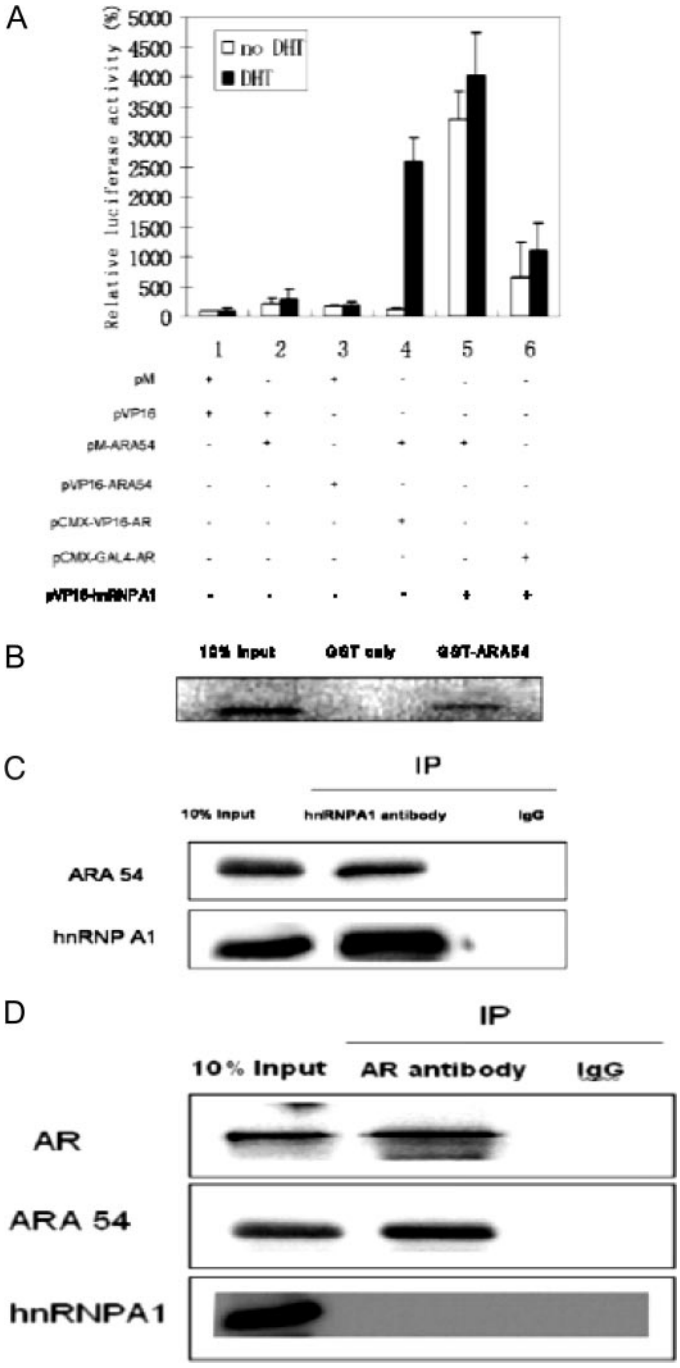

FIG. 1. ARA54 interacts with hnRNP A1 *in vitro* and *in vivo*. A, Mammalian two-hybrid assay: 3T3 cells cultured in 24-well plates were co-transfected with 0.5 μg pVP16, pM, pM-ARA54, pVP16-ARA54, pCMX-GAL4-AR, pCMX-VP16-AR, and pVP16-hnRNP A1 as indicated together with 0.4 μg pG5-LUC reporter plasmid and 0.5 ng pRL-TK internal control plasmid. The luciferase activity of the interaction between pM and pVP16 without ethanol treatment (lane 1) was set as 100%. All values represent the mean ± SD of three independent experiments. B, ARA54 interacts with hnRNP A1 *in vitro*. GST pull-down assay was performed with purified GST control protein and GST-ARA54 fusion protein with 5 μl of [^{35}S] methionine-labeled full-length hnRNP A1. C, hnRNP A1 co-immunoprecipitated with ARA54. LNCaP cells were cultured in RPMI-1640 supplemented with 10% non-heat-inactivated FBS. Anti-ARA54 antibody or normal goat IgG was added to 500 μg LNCaP cell lysate for 4 h at 4 C to immunoprecipitate the endogenous ARA54 and hnRNP A1 complex. D, hnRNP A1 cannot be co-immunoprecipitated with AR. LNCaP cells were cultured in RPMI-1640 supplemented with 10% non-heat-inactivated FBS. Anti-AR antibody or normal goat IgG was added to 500 μg LNCaP cell lysate for 4 h at 4 C to immunoprecipitate the endogenous ARA54 and hnRNP A1 complex.

showed little interaction with AR in the mammalian two-hybrid system.

hnRNP A1 selectively suppressed the ARA54-enhanced AR transactivation

We then tested the effect of hnRNP A1 on the AR transactivation in mammalian cells. In 3T3, which is an ARA54-negative cell line (see Fig. 3A), ARA54 can further enhance the AR transactivation 4–6-fold (Fig. 2A, lanes 5 and 9 *vs.* 2). The addition of hnRNP A1 significantly suppressed the ARA54-enhanced AR transactivation (Fig. 2A, lanes 6, 7, and 8 *vs.* 5, lanes 10, 11, and 12 *vs.* 9). Similar suppression effects were also observed when we replaced 3T3 cells with DU145 cells, another ARA54-negative prostate cancer cell line (15, 25) (Fig. 2A). These data suggested that hnRNP A1 might function as a negative regulator for AR via interaction with ARA54. We also tested the suppressive effect of hnRNP A1 on the ARA54-enhanced mtART877A transactivation induced by HF and E2. As shown in Fig. 2B, addition of hnRNP A1 could effectively suppress the ARA54-enhanced mtART877A transactivation induced by E2 and HF in COS-1 cells (lanes 13 and 14 *vs.* 12). We further tested the effect of hnRNP A1 on the AR transactivation enhanced by other AR coregulators, such as the ARA70, ARA55, SRC-1, supervillin, gelsolin, and CBP. As shown in Fig. 2C, hnRNP A1 showed little effect on the AR transactivation enhanced by these coregulators except for ARA54. These data suggested that hnRNP A1 might function as a relatively specific modulator for ARA54.

ARA54 is essential for hnRNP A1's suppressive effect on AR transactivation

Because the hnRNP A1 showed little effect on AR transactivation without exogenously transfected ARA54 (Fig. 2A, lanes 3 and 4 *vs.* 2), and hnRNP A1 showed little interaction with AR (Fig. 1A, lane 6), we were interested to know whether ARA54 is essential for the suppressive effect of hnRNP A1 on the AR transactivation. First, we used ARA54-negative cell line 3T3 and the ARA54-positive cell line LNCaP to test the effect of hnRNP A1 on the AR transactivation without exogenously transfected ARA54. Fig. 3A showed the differential expression of endogenous ARA54 in LNCaP and 3T3 cell lines. In the ARA54-negative 3T3 cell lines, the addition of hnRNP A1 did not significantly suppress the AR transactivation (Fig. 3B), while the addition of hnRNP A1 to the ARA54-positive LNCaP cells significantly suppressed the AR transactivation (Fig. 3C). The differential effect of hnRNP A1 on the AR transactivation in ARA54-positive and ARA54 negative cell lines suggested that hnRNP A1 requires interaction with ARA54 to mediate its suppressive effect on AR transactivation. To confirm further our hypothesis that ARA54 is necessary for the suppression of AR activity by hnRNP A1, we used retrovirus-delivered siRNA against ARA54 to abolish endogenous ARA54 ex-

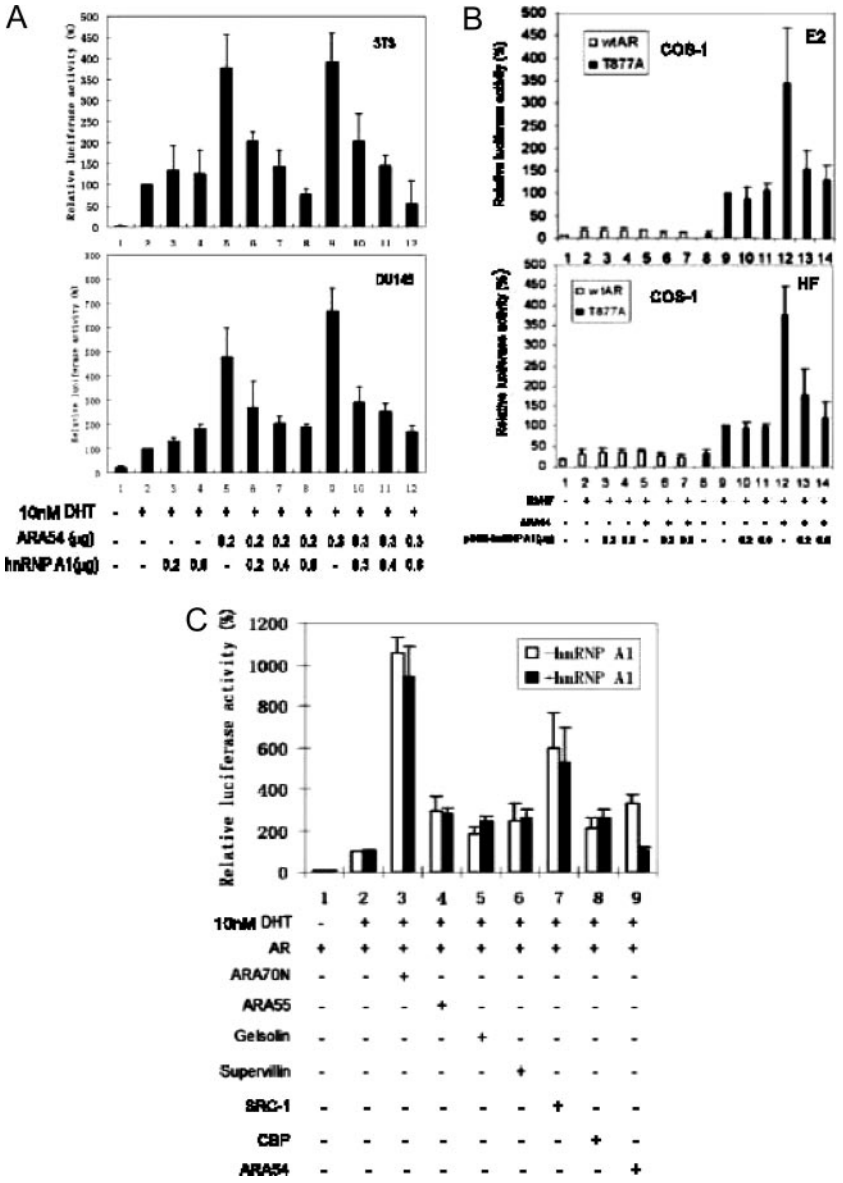

FIG. 2. hnRNP A1 selectively suppressed ARA54-enhanced AR transactivation. A, 3T3 (*upper panel*) and DU145 (*lower panel*) cells cultured in 24-well plates were co-transfected with 0.075 μg pSG5-AR and 0.2 or 0.3 μg pSG5-ARA54, together with different doses of pSG5-hnRNP A1 as indicated. A total of 0.225 μg MMTV-LUC was used as reporter plasmid and 5 ng pRL-TK as the internal control. The luciferase activity in the presence of DHT without ARA54 or hnRNP A1 (lane 2) was set as 100%. Values represent the mean ± SD of at least three determinations. B, COS-1 cells were co-transfected with 0.225 μg MMTV-LUC, 0.075 μg pCMV-mtART877A, or pSG5-AR, 0.2 μg pSG5-ARA54, and different doses of pSG5-hnRNP A1 as indicated. After transfection, cells were cultured for an additional 24 h in the presence or absence of 10 nM E2 or 10 μM HF. The luciferase activity is presented relative to that of pCMV-mtART877A with E2 or HF (lane 2), respectively. C, COS-1 cells were transfected with 0.225 μg MMTV-LUC, 0.075 μg pSG5-AR, 0.3 μg each AR coactivator (ARA70N, SRC-1, gelsolin, supervillin, ARA55, CBP, or ARA54) and 0.4 μg pSG5- hnRNP A1. The luciferase activity is presented relative to that of vector alone with DHT in the absence of hnRNP A1 (lane 2). Values represent the mean ± SD of at least three determinations.

pression in LNCaP cells. Figure 3D showed the silencing of endogenous ARA54 in the siRNA stably transfected LNCaP cell lines. In the ARA54-siRNA transfected LNCaP cells, the suppressive effect of hnRNP A1 was abolished (Fig. 3E, *upper panel*, lanes 3–6 *vs.* 2), while in the scrambled siRNA transfected cells, hnRNP A1 can still suppress the AR transactivation (Fig. 3E, *lower panel*, lanes 3–6 *vs.* 2). These results confirmed our hypothesis that hnRNP A1 might require interaction with ARA54 to suppress the AR transactivation.

hnRNP A1 blocked the interaction between ARA54 and AR, as well as the ARA54 homodimerization

To explore the possible molecular mechanisms of how hnRNP A1, an ARA54-associated protein, can suppress the ARA54-enhanced AR transactivation, we proposed that hnRNP A1, by interacting with ARA54, an important coactivator of AR, might reduce the ARA54 interaction with AR. Figure 4A showed that the addition of pcDNA3-Flag-hnRNP A1 significantly suppressed the interaction between AR and ARA54 in a dose-dependent manner (Fig. 4A, lanes 4–6 *vs.* 3). One important step in the coregulation of ARA54 on AR function is the homodimerization of ARA54 (25). We hypothesized that another possible molecular mechanism is that this protein might block the ARA54 homodimerization. In the same mammalian two-hybrid system, we co-transfected the pM-ARA54 and pVP16-ARA54. By the addition of pcDNA3-Flag-hnRNP A1, the ARA54 homodimerization was significantly suppressed (Fig. 4B, lanes 4–6 *vs.* 3). In the same mammalian two-hybrid assay systems, we see little effect of hnRNP A1 on the interaction between pM-53 and pVP16-T (Fig. 4A and 4B, lanes 7–9 *vs.* 6). Although they cannot exclude the involvement of auxiliary proteins, these preliminary data suggested that hnRNP A1, by its interaction with ARA54, might suppress the interaction between ARA54 and AR, as well as the ARA54 homodimerization. This effect is not the result of suppression on the reporter gene by hnRNP A1.

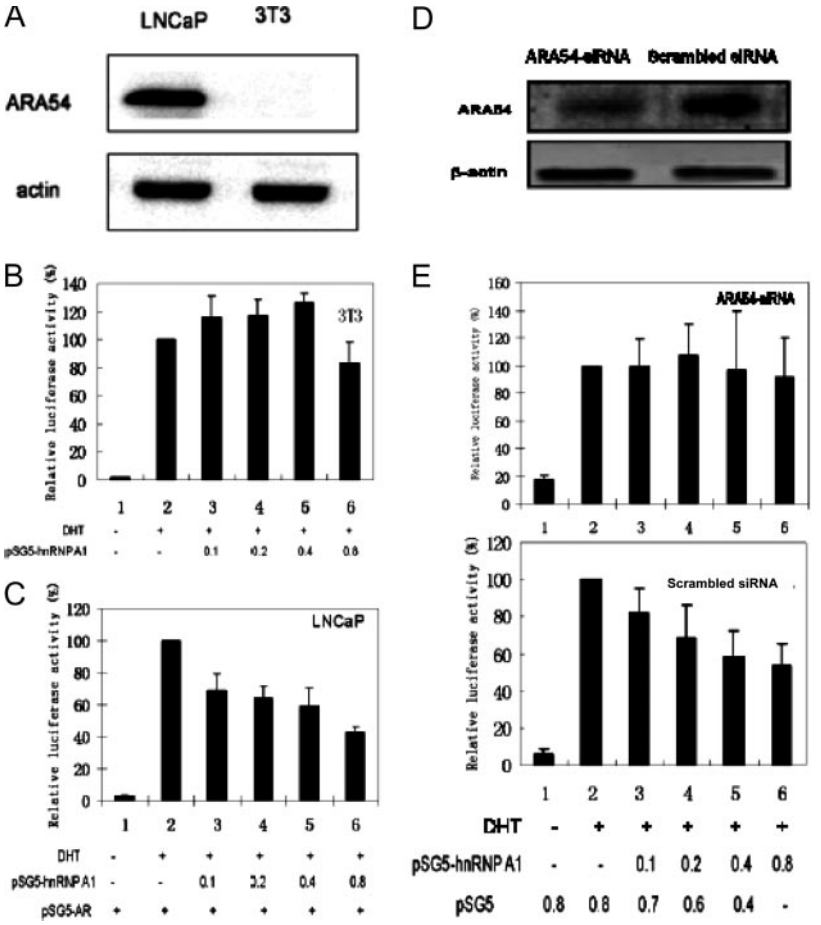

FIG. 3. ARA54 is essential for hnRNP A1's suppressive effect on AR transactivation. A, Western blot detection of endogenous ARA54 mRNA in LNCaP and 3T3 cells. B, 3T3 cells seeded in 24-well plates were co-transfected with 0.225 μg MMTV-LUC reporter plasmid, 5 ng pRL-TK internal control plasmid, as well as different doses of pSG5-hnRNP A1 as indicated. C, LNCaP cells seeded in 24-well plates were co-transfected with 0.225 μg MMTV-LUC reporter plasmid, 5 ng pRL-TK internal control plasmid, and 0.075 μg pSG5-AR, as well as different doses of pSG5-hnRNP A1 as indicated. The luciferase activity with DHT treatment and vector alone (lane 2) was set as 100%. All values represent the mean ± SD of three independent experiments. D, Western data show the endogenous ARA54 level in ARA54-siRNA and scrambled siRNA stably transfected LNCaP cells. E, ARA54-siRNA (*upper panel*) and scrambled siRNA (*lower panel*) stably transfected LNCaP cells seeded in 24-well plates were co-transfected with 0.225 μg MMTV-LUC reporter plasmid, 5 ng pRL-TK internal control plasmid, as well as different doses of pSG5-hnRNP A1 as indicated. The luciferase activity with DHT treatment and vector alone (lane 2) was set as 100%. All values represent the mean ± SD of three independent experiments.

The silencing of endogenous hnRNP A1 enhanced the AR transactivation in LNCaP cells

To further reduce the possible artificial effects caused by the exogenously transfected hnRNP A1 on the AR transactivation, we applied siRNA to suppress the expression of endogenous hnRNP A1 and observed the effect of hnRNP A1 silencing on the AR transactivation. There were three candidates of siRNA against the hnRNP A1 constructed, and their silencing effect on endogenous hnRNP A1 was evaluated using real-time RT-PCR assay (data not shown). As shown in Fig. 5A, the endogenous hnRNP A1 can be reduced to about 20% by the transient transfection of hnRNP A1-siRNA. In this assay, the vector containing the scrambled sequence of siRNA was used as a control. As shown in Fig. 5B, the AR transactivation was increased 2–3-fold by the suppression of endogenous hnRNP A1. This result suggests that the silencing of endogenous hnRNP A1 might activate the coactivator function of endogenous ARA54 to enhance AR transactivation.

Addition of hnRNP A1 suppressed AR-mediated LNCaP cell growth and PSA expression

We then stably transfected hnRNP A1 into the LNCaP cells and used neomycin to select the stable clones. After selection, Western blotting confirmed the expression of hnRNP A1 in stably transfected cells (Fig. 6A, *left panel*). After 1 nM 5α-DHT treatment for 48 h, the PSA expression of the hnRNP A1-transfected LNCaP cells was significantly lower, while the AR expression was not affected compared with the vector-transfected cells (Fig. 6A, *right panel*). Quantitation of Western blotting showed that PSA expression was reduced to 54% comparing to vector alone (Fig. 6A). We then seeded the pcDNA3-Flag-hnRNP A1 and pcDNA3-Flag vector LNCaP sublines in 24-well plates and treated them with 1 nM DHT for 7 consecutive days. The growth assay test in Fig. 6B showed that the addition of hnRNP A1 had a significant suppressive effect on the LNCaP cell growth. These data suggested that in accordance with the suppression of ARA54-enhanced AR transactivation, the AR-mediated LNCaP cell growth and PSA expression were suppressed by addition of hnRNP A1. As the suppressive effect of hnRNP A1 on the AR function is ARA54-dependent (Fig. 3), we would like to determine whether the growth suppressive effect of hnRNP A1 on the LNCaP is also ARA54 dependent. We used retrovirus-delivered siRNA to silence the endogenous ARA54 in hnRNP A1 and vector stably transfected LNCaP cells. Stable ARA54-siRNA transfected cells were selected by puromycin. In the 7-d growth assay, we found that the growth suppression effect of hnRNP A1 was reduced upon the silencing of endogenous ARA54 (Fig. 6C).

Silencing of endogenous hnRNP A1 via siRNA promoted the growth of LNCaP cells and PSA expression

We then used siRNA to silence the endogenous hnRNP A1 in LNCaP cells to confirm the suppressive effect of hnRNP

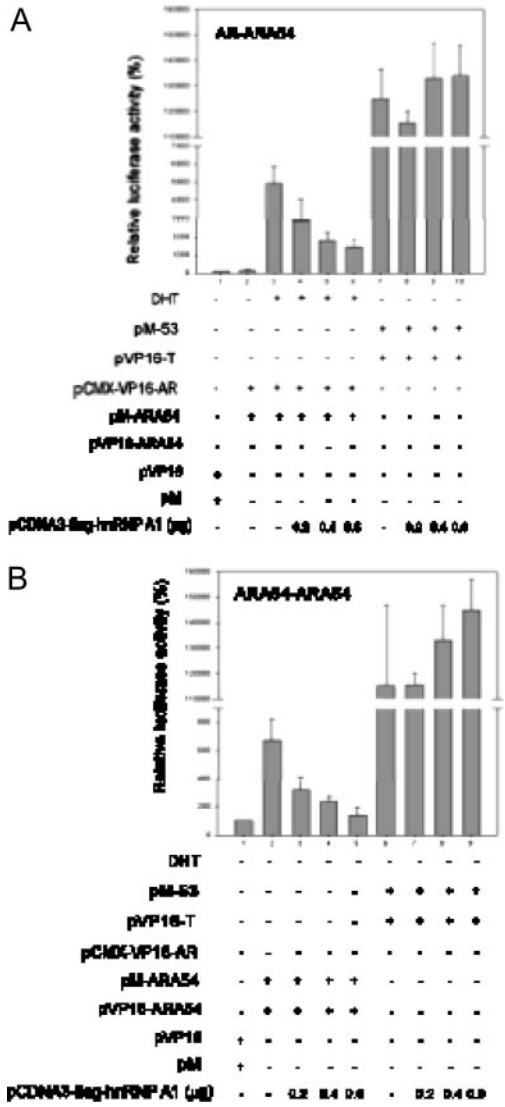

FIG. 4. Molecular mechanisms of hnRNP A1's suppressive effect. hnRNP A1 blocked the interaction between ARA54 and AR (A) as well as the homodimerization of ARA54 (B), but not the interaction between pM-53 and pVP16-T. 3T3 cells were transiently co-transfected with 0.4 μg pG5-LUC reporter plasmid, 0.5 ng pRL-TK internal control plasmid, 0.2 μg pM-ARA54 and pVP16-AR, or 0.2 μg pM-ARA54 and pVP16-ARA54, or 0.2 μg pM-53 and pVP16-T, with different doses of pcDNA3-Flag-hnRNP A1 as indicated. The luciferase activity of the sample transfected with pM and pVP16 without DHT treatment (lane 1) was set as 100%. All values represent the mean ± SD of three independent experiments.

A1 on cell growth and PSA expression. We stably transfected hnRNP A1-siRNA into the LNCaP cells and selected them with puromycin. After selection, Western blotting confirmed the expression of hnRNP A1 in stably transfected cells (Fig. 7A). After 1 nM DHT treatment for 48 h, the PSA expression in the hnRNP A1-siRNA-transfected LNCaP cells was significantly increased, while the AR expression was not affected compared with the scrambled siRNA-transfected cells (Fig. 7A, *left panel*). Quantitation of Western blotting showed that PSA expression increased to 196% (Fig. 7A, *right panel*). We then seeded the hnRNP A1-siRNA or scrambled siRNA LNCaP sublines in 24-well plates and treated them with 1 nM

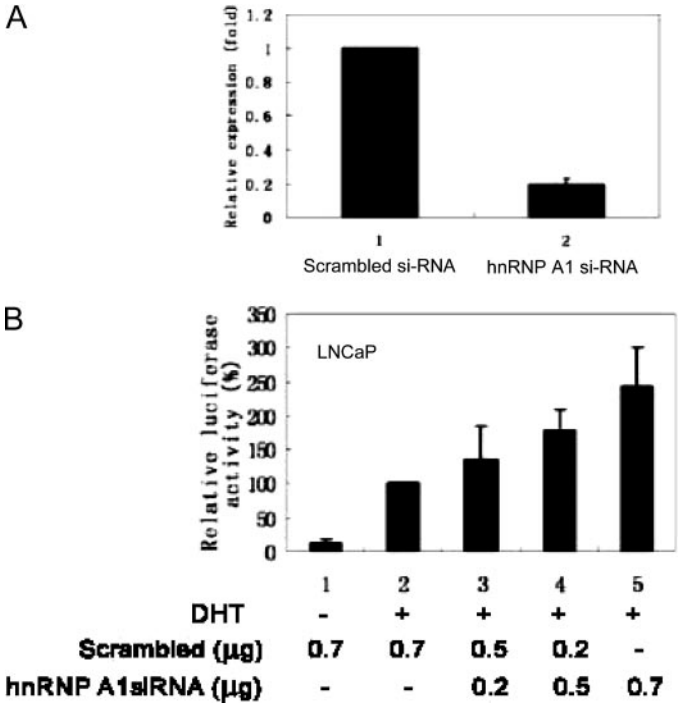

FIG. 5. Suppression of endogenous hnRNP A1 by siRNA resulted in enhanced AR transactivation in LNCaP cells. A, Expression of hnRNP A1 by real-time RT-PCR in the siRNA transiently transfected LNCaP cells. A total of 10 μg scrambled siRNA or hnRNP A1-siRNA was transfected into LNCaP cells cultured in 100-mm dishes. Total RNA was extracted and real-time RT-PCR was used to assay the relative expression level of hnRNP A1 mRNA. B, LNCaP cells were cultured in 24-well plate and transfected with 0.225 μg MMTV-LUC, and 5 ng pRL-TK internal control plasmid, together with scrambled siRNA or hnRNP A1-siRNA as indicated. Values represent the mean ± SD of at least three determinations.

DHT for 7 consecutive days. As expected, stable transfection of hnRNP A1-siRNA enhanced LNCaP cell growth (Fig. 7B). Together, both the addition and silencing of hnRNP A1 demonstrated that hnRNP A1 might suppress prostate cancer cell growth via inhibition of ARA54-enhanced AR activity and AR-mediated LNCaP cell growth.

Discussion

Sustained blocking of the AR signaling pathway is critical for controlling prostate cancer progression. Although multiple strategies have been used to reduce serum levels of androgens or interfere with their function via AR, combined androgen blockade using medical or surgical castration with antiandrogens is currently a standard treatment for advanced prostate cancer (45). One serious problem for current androgen ablation therapy is the severe side effects caused by the systemic suppression of AR signaling in all types of cells expressing functional AR, while regulated AR signaling is essential for the proper function of many important organs, like the male fertility, cardiovascular system, and bone. Severe side effects, like the loss of libido, erectile dysfunction, male infertility, osteoporosis, and increased incidence of cardiovascular diseases, usually result from the undifferentiated and sustained blocking of AR signaling in these organs. How to suppress AR function more specifically in the malignant cells with minimal influence

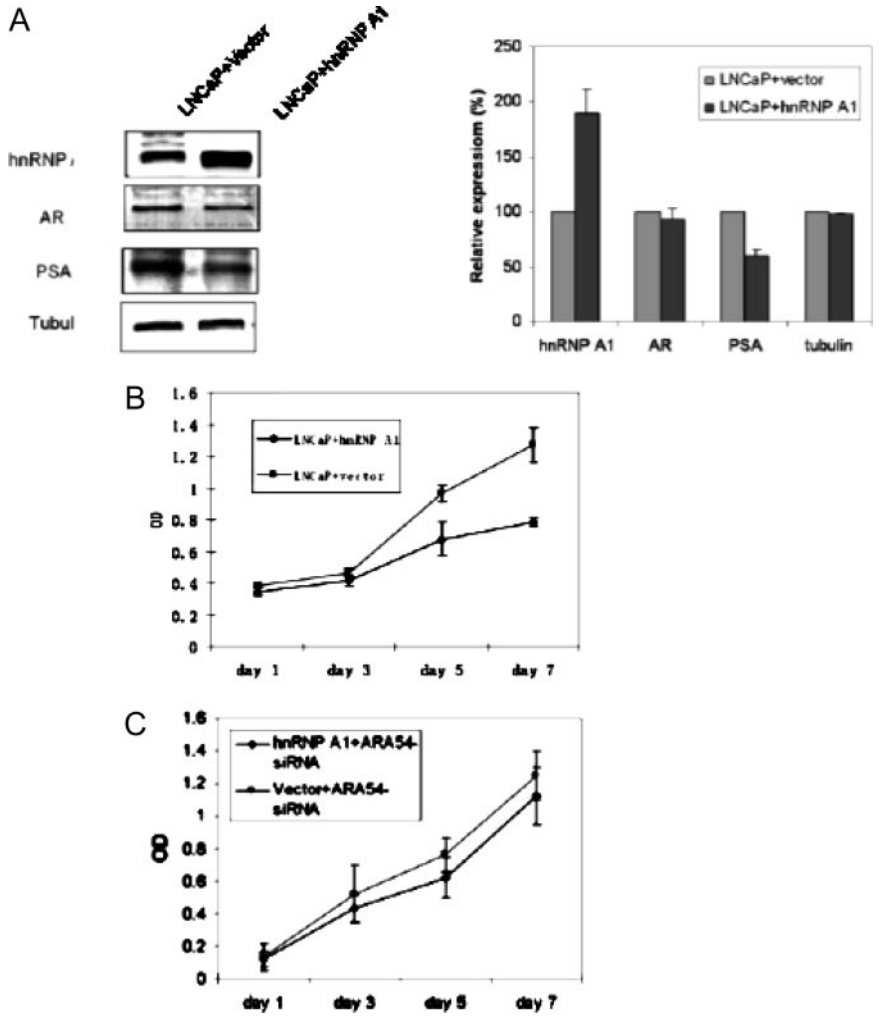

FIG. 6. The ARA54-dependent suppression of hnRNP A1 on LNCaP cell growth and PSA expression. A, LNCaP cells stably transfected with pcDNA3-Flag-hnRNP A1 or pcDNA3-Flag were treated with 1 nM DHT for 48 h. The protein levels of hnRNP A1, AR, and PSA were assayed using Western blot. B, The stable LNCaP clones with pcDNA3-Flag-hnRNP A1 or pcDNA3-Flag were cultured with RPMI-1640 medium supplemented with 10% CD FBS and 1 nM DHT. Cell growth was determined using MTT assay. C, hnRNP A1 and vector stably transfected LNCaP cell lines were further stably transfected with retrovirus-delivered ARA54-siRNA. Cells were cultured in RPMI-1640 medium supplemented with 10% CD FBS and 1 nM DHT. Cell growth was determined using MTT assay.

to the normal cells and organs poses a severe challenge to successful treatment. The differential distribution and expression of AR coregulators and their modulators in different cell types allows the modulation of AR signaling in a cell type-specific manner. Accumulating evidence showed that the progression of prostate cancer and its acquisition of androgen independence were accompanied with changing expression profiles of AR coregulators. In our study we showed the increased expression of ARA54 in prostate cancer tissues compared with the normal tissues (Yang, Z., and Y.-J. Chang, unpublished observations). If the therapy targets specific AR coregulators, like ARA54, and their modulators instead of directly targeting to AR, only the prostate cancer cells expressing specific AR coregulators will be suppressed, while the AR signaling in normal cells with low or no expression of this coregulator would be unaffected. The identification and characterization of AR coregulators and their interacting proteins may pave the way to find potential therapeutic strategies of suppressing AR function by interrupting the interaction between AR and its coregulators. hnRNP A1 shows a suppressive effect on AR function via selective suppression of ARA54-enhanced AR transactivation and AR-mediated prostate cancer cell growth. Molecular mechanism dissection showed that this suppressive effect might be via the interruption of AR-ARA54 heterodimerization and ARA54 homodimerization. hnRNP A1 inhibited AR transactivation in cancer cells expressing ARA54, while hnRNP A1 did not affect AR signaling in fibroblast 3T3 cells without ARA54 expression. These findings suggested that new approaches targeting AR coregulator's modulator, without directly affecting AR, might have better therapeutic effects with fewer side effects in the treatment of prostate cancer.

Although initially effective, total androgen blockade therapy used in the treatment of prostate cancer may eventually fail to sustain its efficacy when continued beyond 1–2 yr (46). Cancer cells become resistant to ablation therapy, and AR loses its specificity and becomes activated by antiandrogens. Earlier studies suggested AR mutations and aberrant expression of AR coregulators as reasons for the antiandrogen withdrawal syndrome (10, 47–49). Our previous study showed that ARA54, in addition to enhancing the DHT-induced wtAR transactivation, also enhanced mtART877A transactivation induced by E2 and HF (15). Interestingly, in this study the ARA54-enhanced mtART877A transactivation induced by E2 and HF was abrogated by hnRNP A1, suggesting that the effect of hnRNP A1 might be helpful in the suppression of mutant AR transactivation induced by antiandrogens.

In this study we demonstrated the dual functional roles played by ARA54 in the AR signaling regulation. In addition

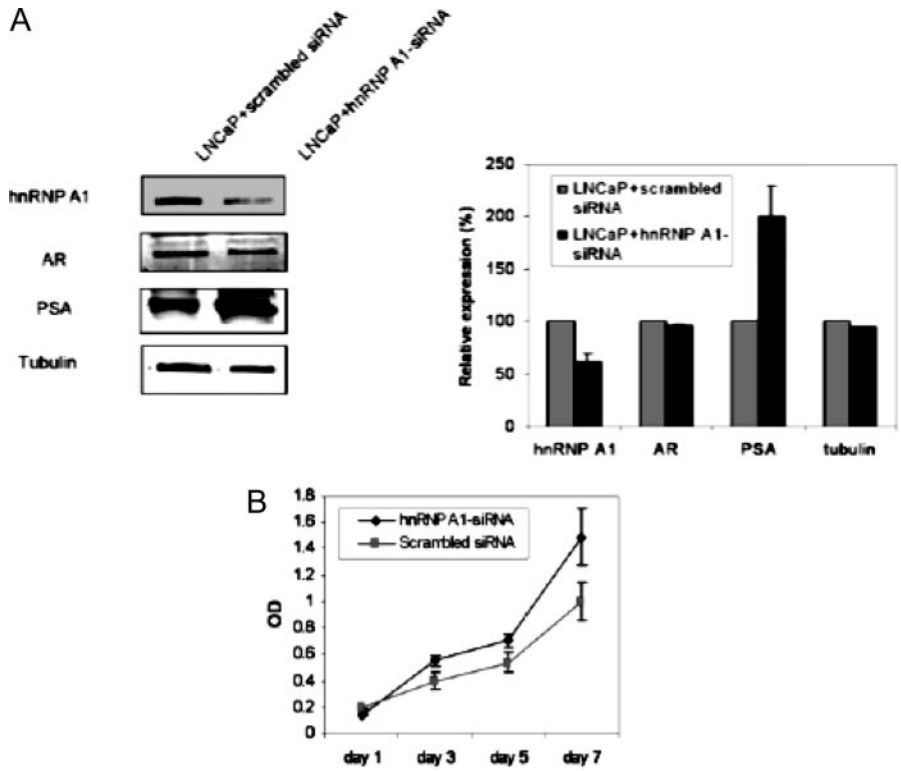

FIG. 7. Silencing of endogenous hnRNP A1 by siRNA enhanced the LNCaP cell growth and PSA expression. A, LNCaP cells stably transfected with hnRNP A1-siRNA or scrambled siRNA were treated with 1 nM DHT for 48 h. The protein levels of hnRNP A1, AR, and PSA were assayed using Western blot. B, The stable cell lines transfected with hnRNP A1-siRNA or scrambled siRNA were cultured with RPMI-1640 medium supplemented with 10% CD FBS and 1 nM DHT. Cell growth was determined using MTT assay.

to its direct AR coregulator function, ARA54 also mediates the suppressive effect of hnRNP A1 on the AR function. These findings further strengthened the *in vivo* coregulator role of ARA54. AR coregulators like ARA54 might influence AR function via direct coregulator activity as well as mediating its interacting protein(s) modulator function. To further identify and characterize modulators for AR coregulators and their effect on the AR signaling pathway might shed light on the complicated molecular mechanism of AR signaling regulation. The detailed molecular mechanistic dissection may pave the way to better understanding the functional roles played by AR signaling pathway in the androgen-related diseases, like the prostate cancer.

The overexpression of hnRNP A1 might be used as a gene therapy strategy for the treatment of prostate cancer. Our findings of expressing ectopic hnRNP A1 in a human prostate cancer cell line suppressed its growth, and the expression of PSA suggests its potential suppressor role in prostate cancer development. It is likely that the loss of hnRNP A1 suppression in prostate cancer might activate the ARA54 coactivator function, which might result in the AR-mediated prostate cancer progression. The study of hnRNP A1 and ARA54 in prostate cancer might provide insights into the malignant process and potentially generate both diagnostic and prognostic markers, as well as future treatment strategies.

To compare hnRNP A1 effects with other AR corepressors, we transfected hnRNP A1 in the presence and absence of other repressors, such as SMRT, in LNCaP cells. The results showed only little additive effects on the suppression of AR transactivation (data not shown), suggesting that these two corepressors might have their own distinct mechanisms to suppress AR transactivation. In addition, we found that overexpressing hnRNPA1 in AR negative DU145 cells results in little influence on the cell viability, suggesting that AR signals might play important roles in the hnRNP A1 effects. Finally, we also examined the hnRNP A1 effects on other NRs. Early results indicated that hnRNP A1 had different effects on different NRs. For example, hnRNP A1 showed little effects on estrogen receptor-mediated transactivation in MCF-7 cells (supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). We are on the way to study further the effects of hnRNP A1 on the other NRs.

In conclusion, hnRNP A1 can selectively repress the ARA54-enhanced AR transactivation by interrupting the interaction between AR and ARA54, as well as ARA54 homodimerization. hnRNP A1 overexpression suppresses the AR-mediated prostate cancer cell growth and PSA expression in LNCaP cells. Our study will lead to a further understanding of the complexity of the molecular mechanism of androgen action in prostate, and support the important roles played by hnRNP A1 and ARA54 in the development and progression of prostate cancer.

Acknowledgments

We thank Karen Wolf for help in editing the manuscript and Jing Ni for real-time RT-PCR.

Received May 30, 2006. Accepted November 8, 2006.

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This work was supported by NIH Grants DK067686 and DK60948 and the George Whipple Professorship Endowment and Dr. Shu-Shi Chang Neuroendocrinology Fund.

Disclosure Statement: None of the authors have anything to declare.

References

1. **Jemal A, Siegal R, Ward E, Murray T, Xu J, Smigal C, Thun MJ** 2006 Cancer statistics, 2006. CA Cancer J Clin 56:106–130
2. **Crawford ED, Eisenberger MA, McLeod DG, Spaulding JT, Benson R, Dorr FA, Blumenstein BA, Davis MA, Goodman PJ** 1989 A controlled trial of leuprolide with and without flutamide in prostatic carcinoma. N Engl J Med 321:419–424
3. **Chodak GW, Kranc DM, Puy LA, Takeda H, Johnson K, Chang C** 1992 Nuclear localization of androgen receptor in heterogeneous samples of normal, hyperplastic and neoplastic human prostate. J Urol 147:798–803
4. **Hobisch A, Culig Z, Radmayr C, Bartsch G, Klocker H, Hittmair A** 1996 Androgen receptor status of lymph node metastases from prostate cancer. Prostate 28:129–135
5. **Mohler JL, Chen Y, Hamil K, Hall SH, Cidlowski JA, Wilson EM, French FS, Sar M** 1996 Androgen and glucocorticoid receptors in the stroma and epithelium of prostatic hyperplasia and carcinoma. Clin Cancer Res 2:889–895
6. **Sadi MV, Walsh PC, Barrack ER** 1991 Immunohistochemical study of androgen receptors in metastatic prostate cancer. Comparison of receptor content and response to hormonal therapy. Cancer 67:3057–3064
7. **van der Kwast TH, Schalken J, Ruizeveld de Winter JA, van Vroonhoven CC, Mulder E, Boersma W, Trapman J** 1991 Androgen receptors in endocrine-therapy-resistant human prostate cancer. Int J Cancer 48:189–193
8. **Chang CS, Kokontis J, Liao ST** 1988 Molecular cloning of human and rat complementary DNA encoding androgen receptors. Science 240:324–326
9. **Chang CS, Kokontis J, Liao ST** 1988 Structural analysis of complementary DNA and amino acid sequences of human and rat androgen receptors. Proc Natl Acad Sci USA 85:7211–7215
10. **Heinlein CA, Chang C** 2002 Androgen receptor (AR) coregulators: an overview. Endocr Rev 23:175–200
11. **Hsiao PW, Chang C** 1999 Isolation and characterization of ARA160 as the first androgen receptor N-terminal-associated coactivator in human prostate cells. J Biol Chem 274:22373–22379
12. **Fujimoto N, Yeh S, Kang HY, Inui S, Chang HC, Mizokami A, Chang C** 1999 Cloning and characterization of androgen receptor coactivator, ARA55, in human prostate. J Biol Chem 274:8316–8321
13. **Yeh S, Chang C** 1996 Cloning and characterization of a specific coactivator, ARA70, for the androgen receptor in human prostate cells. Proc Natl Acad Sci USA 93:5517–5521
14. **Wang X, Yeh S, Wu G, Hsu CL, Wang L, Chiang T, Yang Y, Guo Y, Chang C** 2001 Identification and characterization of a novel androgen receptor coregulator ARA267-α in prostate cancer cells. J Biol Chem 276:40417–40423
15. **Kang HY, Yeh S, Fujimoto N, Chang C** 1999 Cloning and characterization of human prostate coactivator ARA54, a novel protein that associates with the androgen receptor. J Biol Chem 274:8570–8576
16. **Zhang Y, Yang Y, Yeh S, Chang C** 2004 ARA67/PAT1 functions as a repressor to suppress androgen receptor transactivation. Mol Cell Biol 24:1044–1057
17. **Wang L, Hsu CL, Ni J, Wang PH, Yeh S, Keng P, Chang C** 2004 Human checkpoint protein hRad9 functions as a negative coregulator to repress androgen receptor transactivation in prostate cancer cells. Mol Cell Biol 24:2202–2213
18. **Lin HK, Hu YC, Lee DK, Chang C** 2004 Regulation of androgen receptor signaling by PTEN (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor through distinct mechanisms in prostate cancer cells. Mol Endocrinol 18:2409–2423
19. **Aarnisalo P, Palvimo JJ, Janne OA** 1998 CREB-binding protein in androgen receptor-mediated signaling. Proc Natl Acad Sci USA 95:2122–2127
20. **Fronsdal K, Engedal N, Slagsvold T, Saatcioglu F** 1998 CREB binding protein is a coactivator for the androgen receptor and mediates cross-talk with AP-1. J Biol Chem 273:31853–31859
21. **Onate SA, Tsai SY, Tsai MJ, O'Malley BW** 1995 Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 270:1354–1357
22. **Chakravarti D, LaMorte VJ, Nelson MC, Nakajima T, Schulman IG, Juguilon H, Montminy M, Evans RM** 1996 Role of CBP/P300 in nuclear receptor signalling. Nature 383:99–103
23. **Rowan BG, Weigel NL, O'Malley BW** 2000 Phosphorylation of steroid receptor coactivator-1. Identification of the phosphorylation sites and phosphorylation through the mitogen-activated protein kinase pathway. J Biol Chem 275:4475–4483
24. **Alen P, Claessens F, Verhoeven G, Rombauts W, Peeters B** 1999 The androgen receptor amino-terminal domain plays a key role in p160 coactivator-stimulated gene transcription. Mol Cell Biol 19:6085–6097
25. **Miyamoto H, Rahman M, Takatera H, Kang HY, Yeh S, Chang HC, Nishimura K, Fujimoto N, Chang C** 2002 A dominant-negative mutant of androgen receptor coregulator ARA54 inhibits androgen receptor-mediated prostate cancer growth. J Biol Chem 277:4609–4617
26. **Fujimoto N, Mizokami A, Harada S, Matsumoto T** 2001 Different expression of androgen receptor coactivators in human prostate. Urology 58:289–294
27. **Mestayer C, Blanchere M, Jaubert F, Dufour B, Mowszowicz I** 2003 Expression of androgen receptor coactivators in normal and cancer prostate tissues and cultured cell lines. Prostate 56:192–200
28. **Nessler-Menardi C, Jotova I, Culig Z, Eder IE, Putz T, Bartsch G, Klocker H** 2000 Expression of androgen receptor coregulatory proteins in prostate cancer and stromal-cell culture models. Prostate 45:124–131
29. **Wang X, Yang Y, Guo X, Sampson ER, Hsu CL, Tsai MY, Yeh S, Wu G, Guo Y, Chang C** 2002 Suppression of androgen receptor transactivation by Pyk2 via interaction and phosphorylation of the ARA55 coregulator. J Biol Chem 277:15426–15431
30. **Pinol-Roma S, Dreyfuss G** 1992 Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. Nature 355:730–732
31. **Siomi MC, Eder PS, Kataoka N, Wan L, Liu Q, Dreyfuss G** 1997 Transportin-mediated nuclear import of heterogeneous nuclear RNP proteins. J Cell Biol 138:1181–1192
32. **Iervolino A, Santilli G, Trotta R, Guerzoni C, Cesi V, Bergamaschi A, Gambacorti-Passerini C, Calabretta B, Perrotti D** 2002 hnRNP A1 nucleocytoplasmic shuttling activity is required for normal myelopoiesis and BCR/ABL leukemogenesis. Mol Cell Biol 22:2255–2266
33. **Hamilton BJ, Nagy E, Malter JS, Arrick BA, Rigby WF** 1993 Association of heterogeneous nuclear ribonucleoprotein A1 and C proteins with reiterated AUUUA sequences. J Biol Chem 268:8881–8887
34. **Izaurralde E, Jarmolowski A, Beisel C, Mattaj IW, Dreyfuss G, Fischer U** 1997 A role for the M9 transport signal of hnRNP A1 in mRNA nuclear export. J Cell Biol 137:27–35
35. **Ford LP, Wright WE, Shay JW** 2002 A model for heterogeneous nuclear ribonucleoproteins in telomere and telomerase regulation. Oncogene 21:580–583
36. **Svitkin YV, Ovchinnikov LP, Dreyfuss G, Sonenberg N** 1996 General RNA binding proteins render translation cap dependent. EMBO J 15:7147–7155
37. **Hsu CL, Chen YL, Yeh S, Ting HJ, Hu YC, Lin H, Wang X, Chang C** 2003 The use of phage display technique for the isolation of androgen receptor interacting peptides with (F/W)XXL(F/W) and FXXLY new signature motifs. J Biol Chem 278:23691–23698
38. **Thin TH, Kim E, Yeh S, Sampson ER, Chen YT, Collins LL, Basavappa R, Chang C** 2002 Mutations in the helix 3 region of the androgen receptor abrogate ARA70 promotion of 17β-estradiol-induced androgen receptor transactivation. J Biol Chem 277:36499–36508
39. **Yeh S, Miyamoto H, Shima H, Chang C** 1998 From estrogen to androgen receptor: a new pathway for sex hormones in prostate. Proc Natl Acad Sci USA 95:5527–5532
40. **Ting HJ, Yeh S, Nishimura K, Chang C** 2002 Supervillin associates with androgen receptor and modulates its transcriptional activity. Proc Natl Acad Sci USA 99:661–666
41. **Nishimura K, Ting HJ, Harada Y, Tokizane T, Nonomura N, Kang HY, Chang HC, Yeh S, Miyamoto H, Shin M, Aozasa K, Okuyama A, Chang C** 2003 Modulation of androgen receptor transactivation by gelsolin: a newly identified androgen receptor coregulator. Cancer Res 63:4888–4894
42. **Rahman MM, Miyamoto H, Takatera H, Yeh S, Altuwaijri S, Chang C** 2003 Reducing the agonist activity of antiandrogens by a dominant-negative androgen receptor coregulator ARA70 in prostate cancer cells. J Biol Chem 278:19619–19626
43. **Yeh S, Kang HY, Miyamoto H, Nishimura K, Chang HC, Ting HJ, Rahman M, Lin HK, Fujimoto N, Hu YC, Mizokami A, Huang KE, Chang C** 1999 Differential induction of androgen receptor transactivation by different androgen receptor coactivators in human prostate cancer DU145 cells. Endocrine 11:195–202
44. **Xie S, Lin HK, Ni J, Yang L, Wang L, di Sant'Agnese PA, Chang C** 2004 Regulation of interleukin-6-mediated PI3K activation and neuroendocrine differentiation by androgen signaling in prostate cancer LNCaP cells. Prostate 60:61–67
45. **Hellerstedt BA, Pienta KJ** 2002 The current state of hormonal therapy for prostate cancer. CA Cancer J Clin 52:154–179
46. **Kelly WK, Scher HI** 1993 Prostate specific antigen decline after antiandrogen withdrawal: the flutamide withdrawal syndrome. J Urol 149:607–609
47. **Culig Z, Hobisch A, Cronauer MV, Cato AC, Hittmair A, Radmayr C, Eberle J, Bartsch G, Klocker H** 1993 Mutant androgen receptor detected in an advanced-stage prostatic carcinoma is activated by adrenal androgens and progesterone. Mol Endocrinol 7:1541–1550
48. **Taplin ME, Bubley GJ, Shuster TD, Frantz ME, Spooner AE, Ogata GK, Keer HN, Balk SP** 1995 Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. N Engl J Med 332:1393–1398
49. **Taplin ME, Bubley GJ, Ko YJ, Small EJ, Upton M, Rajeshkumar B, Balk SP** 1999 Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist. Cancer Res 59:2511–2515

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