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13. SUPPLEMENTARY NOTES

14. Abstract:
In current period, We first used a signal intra-tumoral injection of different amount of AAV particles to define a proper dosage for efficient virus distribution and knockdown of AR expression. The defined dose was 5.0 x 10^6 AAV particles per 100 mm^3 of tumor volume. Next, we used this dose to treat prostate cancer xenografts. We found that intra-tumoral injection of the ARHP8 but not GFP AAVs abolished tumor growth in LNCaP- and C4-2-derived xenografts in both castrated and sham-operated mice. Immunostaining results confirmed that the AR expression was dramatically down-regulated in AAV.ARHP8-injected tumors. In addition, a significant increase of apoptosis index (TUNEL assay) and dramatic decrease of proliferation index (BrdU incorporation assay) were found in AAV.ARHP8-injected tumors compared to the GFP control. These results demonstrated that the AR is critical for androgen-dependent survival and tumor growth in prostate cancer. Next year, we will repeat these experiments using two more prostate cancer cell line-derived xenografts.

15. SUBJECT TERMS
Prostate Cancer Xenograft; RNA Interference, siRNA, Androgen Receptor; Adeno-Associated Virus; Prostate Cancer Cell Line; Cell Culture; Apoptosis

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Introduction

Prostate cancer is the second leading cause of cancer death among American men. Medical treatment for metastatic prostate cancer has relied heavily on androgen ablation. However, most patients treated by androgen ablation ultimately relapse to more aggressive androgen-independent cancer with no means to cure. The mechanism(s) involved in androgen-independent progression of prostate cancer is(are) not fully known, but if it were better understood, perhaps new therapies or existing ones could be used to better control prostate cancer cells. Many theories regarding the mechanism(s) for androgen-independent progression of prostate cancer have emerged, but lacking is convincing evidence to support any one of these hypotheses as the definitive mechanism. Clinically, nearly all prostate cancers retain a functional androgen receptor (AR) signaling pathway. Current evidence favors a model where activation of intracellular signal transduction pathways that stimulate the AR in the absence of ligand or in the presence of androgen antagonist. A recent report demonstrated that disruption of the androgen receptor function suppresses cellular proliferation of both androgen-dependent and -independent prostate cancer cells in an in vitro cell-based assay. Thus, it may be more clinically relevant to shift the therapeutic target from androgen to its receptor, the androgen receptor. To this end, it is extremely critical and urgent to determine if the androgen receptor is essential in androgen-independent progression of prostate cancer cells in vivo. The proposed studies would seek to answer the question of whether the AR signaling is essential for prostate cancer progression despite androgen deprivation. By studying what will happen if constitutively knocking down the AR expression or insight into the mechanism of androgen-independent prostate cancer will be gained. The newly developed RNAi approach gives us a powerful tool to knock down gene expression of interest per se, for example, the AR gene. The RNAi approach will determine the essential need of the AR signaling for prostate cancer cells to proliferate independent of androgen. In our preliminary studies, we found that AR siRNA against human AR gene knocked down AR protein expression in both androgen-sensitive LNCaP and androgen-insensitive PC-3/AR cells. Also, cell growth and survival were dramatically reduced after AR siRNA transfection (as shown in *Appendix* **I**).

The objective of this proposal is to determine if AR gene silencing in prostate cancer cells via RNA interference mechanism leads to disruption of androgen-independent progression. We plan to accomplish the objective of this application by pursuing the following ***specific aims***:
(1): Generation of a recombinant AAV for long-term expression of a hairpin-structured AR siRNA *in vivo*.
(2): Determination of the essential role of the androgen receptor in androgen-independent growth of prostate cancer.

We will generate a recombinant adeno-associate virus (rAAV) for expressing the AR siRNA hairpin in a prostate cancer xenograft of animal model. Then, we will use the resultant rAAV to inject into prostate cancer xenograft established in nude mouse to determine the effect of AR gene silencing on androgen-independent growth of prostate cancer.

Report Body

The tasks for the first year of grant support were to generate a recombinant AAV for the AR siRNA hairpin expression (Months 1-6) and then to evaluate the efficiency of the resultant rAAV.ARHP8 for AR gene silencing (Months 7-12). In the second (past, Months 13-24) year, we have accomplished all the tasks for the period as described in the Statement of Work (SOW) of the proposal.

1. Determination of proper viral dosage for knocking down AR expression in xenograft

We first determined a proper dose for efficient distribution and knockdown AR expression in xenograft tumors, which was generated in nude mice using prostate cancer cell line PC-3AR. This cell line was described in our recent publication (Ref. 1). A total of 2.0 x 10^6 viable cells, as determined by trypan blue exclusion, was resuspended in RPMI-1640/10% fetal bovine serum (FBS) mixed with a 4:1 v/v ratio of MatriGel^{TM} (Catalog#356237, BD Bioscience) *vs* cells and then injected subcutaneously (27-gauge needle, 1-ml disposable syringe, total volume 0.1 ml/site at 2 sites per mouse) into the rear flank of six-week old athymic male mice (Balb/c, Charles River Laboratories). Four weeks later when the tumor was palpable (around 50 mm^3 in size), 7 different doses (log-dilution, 5 x 10^3 – 5 x 10^9 viral particles in 10 μl total volume) of the recombinant rAAV.ARHP8 produced during the first year of work was injected into the tumor (multiple sites per tumor). In addition, two other animals received control virus rAAV.GFP (2 x 10^9 viral particles in 10 μl) or 10 μl PBS as the negative controls. One week later, xenograft tumors were harvested and frozen sections were viewed for GFP expression under fluorescent microscopy. The intensity of GFP expression was gradually increased along with increasing dosage of the AAV.ARHP8 particles injected. No GFP expression was observed in PBS-injected xenograft section. Furthermore, Western blot results showed a consistent pattern in GFP protein levels (Fig 1, middle row in lower panel). Figures are listed in the end of this report.

In addition, we checked AR mRNA and protein levels after extracting total RNAs and proteins from xenograft specimens. As shown in Fig 1, the AR mRNA (RT-PCR) and protein levels (western blot) did not change in AAV.GFP virus-injected xenograft compared to the PBS control. However, AAV.ARHP8 virus-injected xenogarfts lost the AR expression in a virus-dosage-dependent manner. The peak effect was seen at the virus dose of 5 x 10^6 particles per 100 mm^3. Thereafter, we used this dose level for the following experiments.

2. AAV.ARHP8 suppresses androgen-dependent tumor growth of LNCaP xenografts

We used the androgen-dependent prostate cancer LNCaP cell line to test if the AR is essential for tumor relapse after castration in xenograft experiments. LNCaP cells have an androgen-independent relapse phenotype after a short arresting period caused by castration (Ref. 2). Exponential growing LNCaP cells were inoculated into nude mice as described above and allowed to establish xenografts for 4-6 week. Once xenografts were palpable, animals were divided into two groups to receive bilateral castration or sham operation, respectively. One day after operation, we injected the AAV.ARHP8 or AAV.GFP virus into the xenograft tumors and monitored tumor growth for another 8 weeks. One hour before sacrifice, animals were received an intraperitoneal injection of 0.5 ml BrdU solution (10 mM, a commercial kit from Roche

Diagnostics, Indianapolis, IN) for evaluating proliferation rate in the xenografts. We also measured apoptosis by means of TUNEL analysis (ApoAlert® DNA fragmentation assay kit, Cat#K2024-1, Clontech) in the tumors.

As shown in Fig 2, after castration tumor growth was arrested for 3-4 week but resumed later on in AAV.GFP virus-injected xenografts; however, tumor growth was abolished by intratumoral injection of AAV.ARHP8 virus in both castrated and sham-operated animals.

As shown in Fig 3, a diffused GFP distribution was achieved in either AAV.ARHP8 or AAV.GFP viruses-injected xenografts. AR expression and BrdU incorporation decreased significantly in ARHP8 virus-injected but not in GFP virus-injected xenografts. A significant higher apoptotic index (TUNEL assay) was found in ARHP8 virus-treated xenografts compared to GFP virus-treated xenografts.

3. AAV.ARHP8 suppresses androgen-independent tumor growth of C4-2 xenografts

We used the androgen-independent prostate cancer C4-2 cell line to test if the AR is essential for androgen-independent tumor growth in xenograft experiments. C4-2 cells form tumors in castrated nude mice (Ref. 3). Exponential growing C4-2 cells were inoculated into castrated nude mice as described above and allowed to establish xenografts for 4-6 week. Once xenografts were palpable, animals were divided into two groups to the AAV.ARHP8 or AAV.GFP virus injection and monitored tumor growth for another 8 weeks. BrdU incorporation assay was performed as described above. As shown in Fig 4, ARHP8 virus injection significantly abrogated tumor growth compared to GFP virus injection. Similar to that seen in LNCaP xenograft experiments as shown above, AR expression and BrdU labeling decreased dramatically in ARHP8 virus-injected xenografts compared to that in GFP virus-injected xenografts. Also, apoptotic index (TUNEL assay) was significantly higher in ARHP8 virus-injected xenografts than that in GFP virus-injected xenografts. Quantitative data was presented in Fig 5.

Other Achievements

1. In addition to the tasks described above, this DOD-founded grant also provided a solid support for the P.I. Dr Benyi Li in submitting a R01 proposal of [***Membrane-associated Signaling cascades in AR transactivation***] to NIH on Feb 1st 2006.

2. One invited review article (Book chapter) with a title of “Androgen receptor and cellular survival in prostate cancer” was published under the support of this grant.

3. One original article with the title of “Small-interfering RNA-induced androgen receptor silencing leads to apoptotic cell death in prostate cancer.” was published based on the results from this project.

4. Two meeting abstracts were accepted for poster presentation as listed in the Appendix.

5. A manuscript with a title of “Silencing androgen receptor gene down-regulates serum/glucorcorticoid-induced kinase (SGK) and abolishes tumor growth in prostate cancer”, describing the major findings achieved from this project, is currently under preparation for possible submission next month.

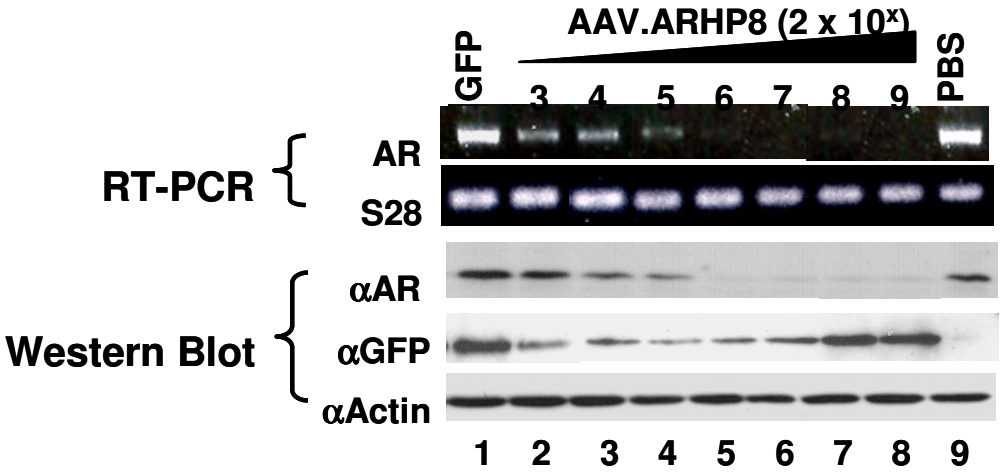

Fig 1. Determination of a proper dose of AAV.ARHP8 particle. A total of 2.0 x 106 PC-3AR cells per injection (s. c.) was used to established xenograft tumors in nude mice. Four weeks later when xenografts were palpable, 7 different doses (log-dilution, 2 x 10^3 – 2 x 10^9 viral particles in 10 μl, lane 2-8) of rAAV.ARHP8 was injected into the tumor (multiple sites per tumor). Empty virus rAAV.GFP (2 x 10^9 particles, lane 1) and PBS (10 μl, lane 9) were used as controls. One week later, xenograft tumors were harvested. Total RNA and protein extracts were used for detecting AR mRNA (RT-PCR assay) and AR protein (Western blot) levels as described previously (Ref 1). Meanwhile, RT-PCR for S28 gene and anti-Actin western blot served as loading control. GFP expression was also evaluated by western blot with an anti-GFP antibody (Santa Cruz Biotech).

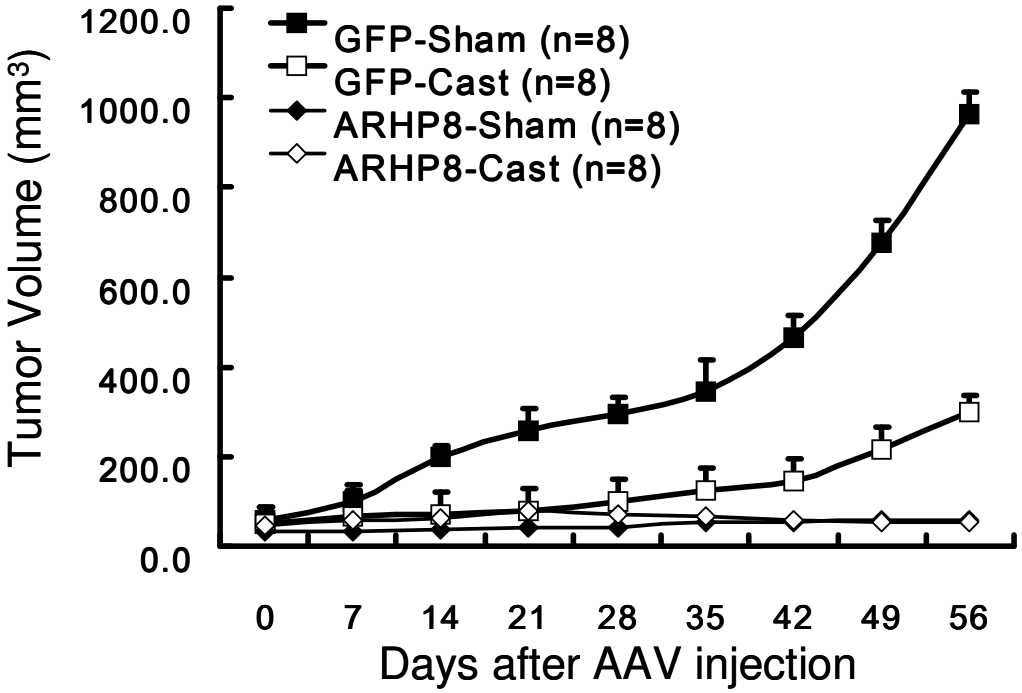

Fig 2. AAV.ARHP8 suppressed tumor growth in LNCaP xenogarfts. A total of 2 x 10^6 LNCaP cells were used to establish xenografts in nude mice and the animals were castrated or sham-operated once the xenografts were palpable. One day later, AAV.ARHP8 or AAV.GFP were injected as indicated (day 0) at a dose of 5 x 10^6 viral particles per 100 mm^3 tumor volume. Tumor growth was monitored for 8 weeks.

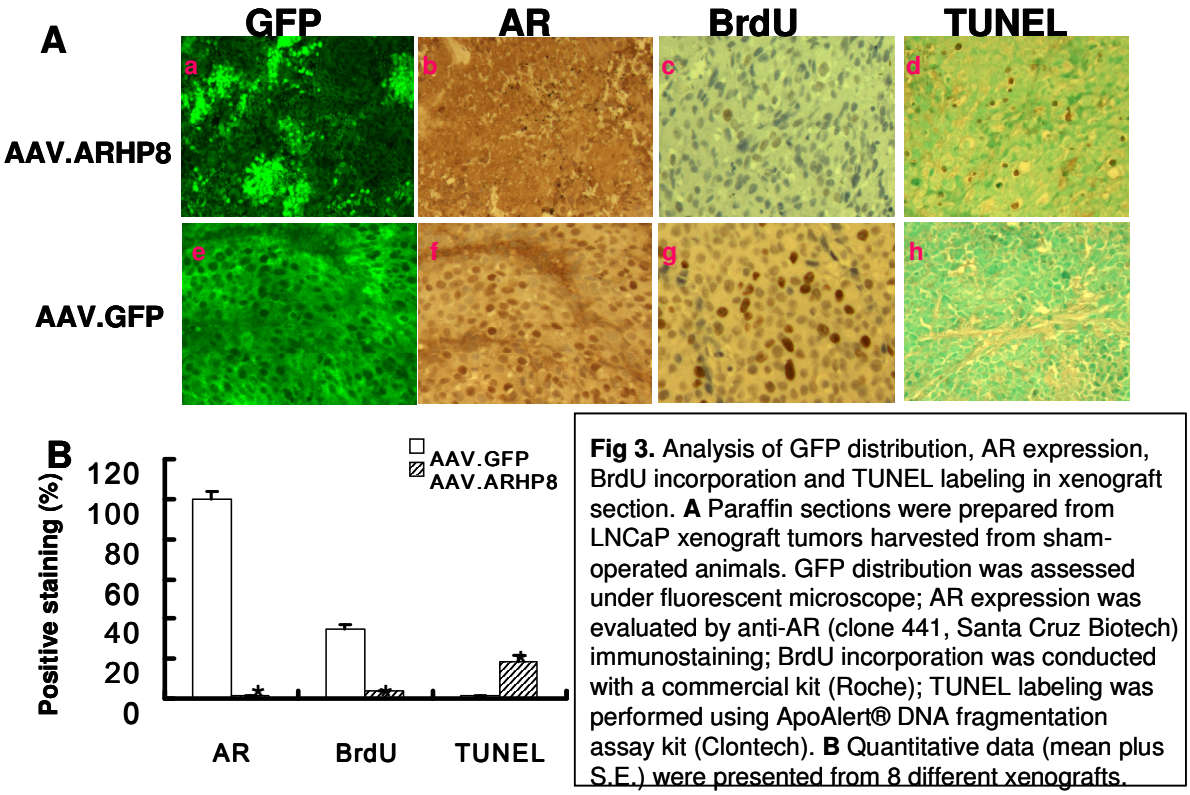

Fig 3. Analysis of GFP distribution, AR expression, BrdU incorporation and TUNEL labeling in xenograft section. **A** Paraffin sections were prepared from LNCaP xenograft tumors harvested from sham-operated animals. GFP distribution was assessed under fluorescent microscope; AR expression was evaluated by anti-AR (clone 441, Santa Cruz Biotech) immunostaining; BrdU incorporation was conducted with a commercial kit (Roche); TUNEL labeling was performed using ApoAlert® DNA fragmentation assay kit (Clontech). **B** Quantitative data (mean plus S.E.) were presented from 8 different xenografts.

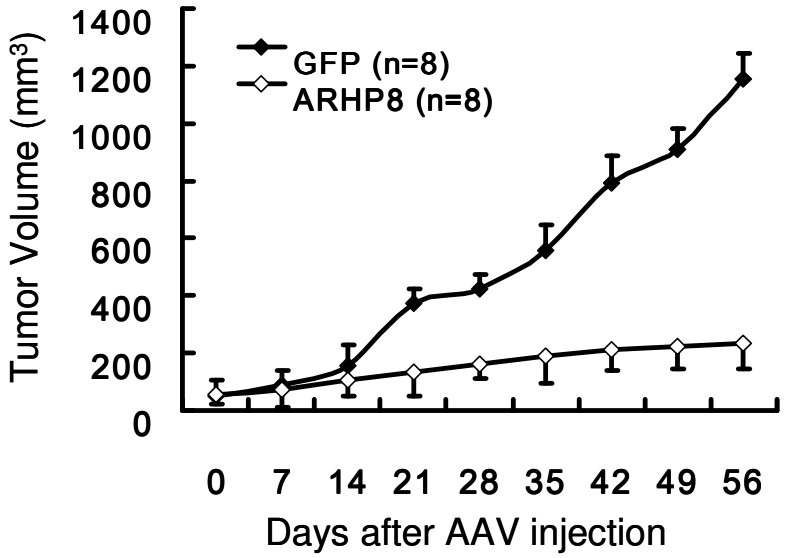

Fig 4. AAV.ARHP8 suppressed androgen-independent tumor growth in C4-2 xenogarfts. A total of 2 x 10^6 C4-2 cells were used to establish xenografts in castrated nude mice once the xenografts were palpable, AAV.ARHP8 or AAV.GFP were injected at a dose of 5 x 10^6 viral particles per 100 mm^3 tumor volume at day 0. Tumor growth was monitored for 8 weeks.

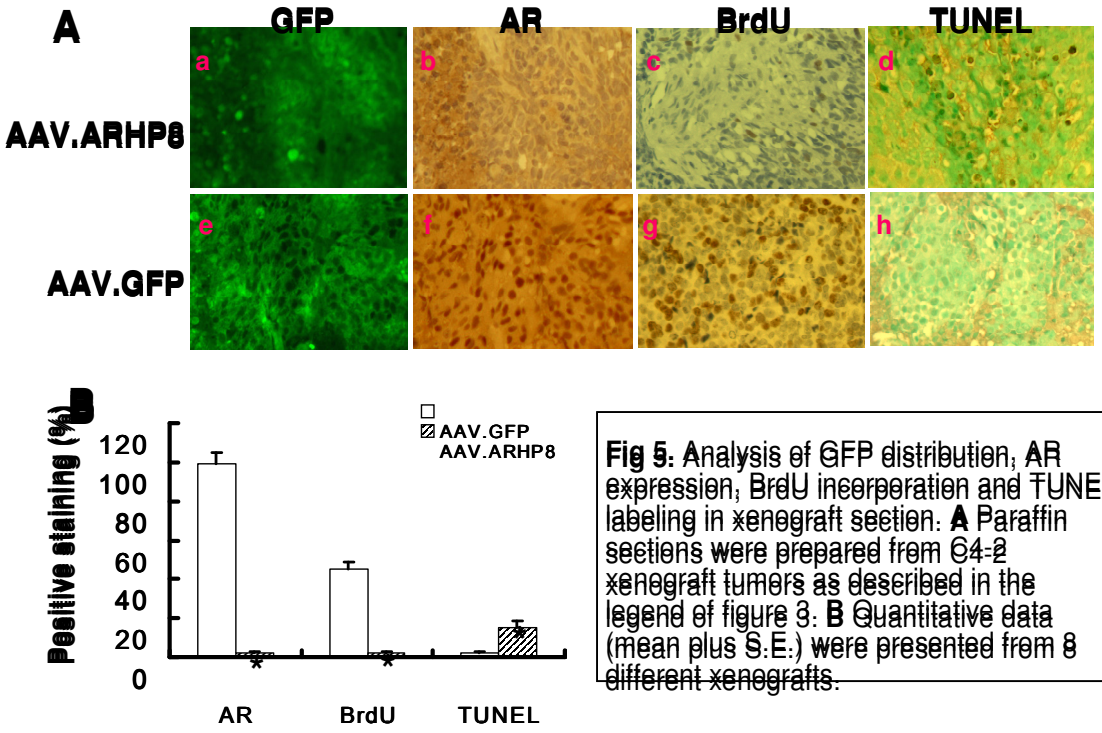

Fig 5. Analysis of GFP distribution, AR expression, BrdU incorporation and TUNEL labeling in xenograft section. **A** Paraffin sections were prepared from C4-2 xenograft tumors as described in the legend of figure 3. **B** Quantitative data (mean plus S.E.) were presented from 8 different xenografts.

Key Research Accomplishments

For the second year of this project according to the proved State of Work, we accomplished:

1. A proper dose of AAV particle for efficient distribution and knockdown of AR gene in mouse xenograft model;

2. A total suppression of androgen-dependent tumor growth and androgen-independent transition was observed in prostate cancer LNCaP cells-derived xenografts after silencing AR gene by intra-tumoral injection of AAV.ARHP8 virus.

3. A profound suppression of androgen-independent tumor growth was achieved in prostate cancer C4-2 cells-derived xenografts after silencing AR gene by injection of AAV.ARHP8 virus.

4. Determination of two downstream effecters, Bcl-xL and SGK, in AR-mediated survival pathway.

Reportable Outcomes

1. An invited review article was published by Transworld Research Network in a book of <Recent Research and Development in Cancer>, 7(2005): ISBN: 81-7895-185-1.
2. An original article was published in Molecular Cancer Therapeutics, 2005;

Conclusion

In this second-year period, we conducted the experiments according to the State of Work (month 13-24), and also other related works. Based on the outlines in the proposal, we first defined a proper dose of the rAAV bearing the ARHP8 sequence to efficiently knock down AR expression in xenografts in vivo. Then, using this dose level, we demonstrated that knocking down AR expression abolished androgen-dependent tumor growth and blocked androgen-independent transition in LNCaP cell-derived xenografts. Also, we observed that AR silencing resulted in a significant decrease of androgen-independent tumor growth in C4-2 cell-derived xenografts in castrated mice. These results were consistent to our recent in vitro data as decribed in Ref. 1. We analyzed the mechanisms for AR siRNA-induced cell death and identified two anti-apoptotic proteins, Bcl-xL and SGK, as the downstream effecters of AR-mediated survival pathway. We published two articles and presented two abstracts in national annual meetings based on the work supported by this grant. We submitted a new R01 grant application to NIH within the scope of AR's role in prostate cancer progression related to this project.

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Appendices

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3. ***Benyi Li*, Xinbo Liao, J Brantley Thrasher.*** ANDROGEN RECEPTOR-MEDIATED REGULATION OF BCL-XL EXPRESSION: IMPLICATION IN CELLULAR SURVIVAL OF PROSTATE CANCERS. ***AUA annual meeting 2005***, San Antonio, TX. **Discussed Poster - Sunday, May 22, 2005, 1:00 PM - 5:00 PM**

4. Hyewon Youn*, J. Brantley Thrasher. **Gα12 is required for Androgen Receptor transactivation in prostate cancer.** ***AUA annual meeting 2006***, Atlanta, GA. **Discussed Poster - Monday, May 22, 2006.**

Small-interfering RNA–induced androgen receptor silencing leads to apoptotic cell death in prostate cancer

Xinbo Liao,[1] Siqing Tang,[2] J. Brantley Thrasher,[1,3] Tomas L. Griebling,[1,4] and Benyi Li [1,2,3]

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Abstract

Prostate cancer is the second leading cause of cancer death in the United States and, thus far, there has been no effective therapy for the treatment of hormone-refractory disease. Recently, the androgen receptor (AR) has been shown to play a critical role in the development and progression of the disease. In this report, we showed that knocking down the AR protein level by a small interfering RNA (siRNA) approach resulted in a significant apoptotic cell death as evidenced by an increased annexin V binding, reduced mitochondrial potential, caspase-3/6 activation, and DFF45 and poly(ADP-ribose) polymerase cleavage. The apoptotic response was specifically observed in those siRNA-transfected cells that harbor a native *AR* gene. No cell death was found in the AR-null prostate cancer cell PC-3 or its subline that has been reconstituted with an exogenous *AR* gene, as well as two breast cancer cell lines that are AR positive. Moreover, in parallel with the siRNA-induced AR silencing, the anti-apoptotic protein Bcl-xL was significantly reduced, which might account for the apoptotic cell death because ectopic enforced expression of Bcl-xL protein partially inhibited apoptosis after AR silencing. Taken together, our data showed that knocking down the AR protein level in prostate cancer cells leads to apoptosis by disrupting the Bcl-xL–mediated survival signal downstream of AR-dependent survival pathway. [Mol Cancer Ther 2005; 4(4):505–15]

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Introduction

Prostate cancer is a significant risk for men in the United States (1). Sixty years ago, it was found that androgens were required for prostate epithelial cells to proliferate, differentiate, and survive; apoptotic cell death has been found in the prostate after androgen withdrawal (2, 3). Because of this insight, androgen ablation has been widely accepted as a major medical treatment for metastatic prostate cancer. However, most patients treated by androgen ablation ultimately relapse to more aggressive incurable hormone refractory prostate cancer (4). Moreover, antiandrogen withdrawal syndrome is another concern for androgen antagonist therapy (5). The etiology of hormone-refractory relapse may have various molecular causes, but in each scenario the androgen receptor (AR) is expressed and its function is maintained (6–11), suggesting that androgen-independent AR signaling is involved. In a transgenic mouse model, AR overexpression in prostate epithelium resulted in marked increases in epithelial proliferation and focal areas of intraepithelial neoplasia in the ventral prostate and dorsolateral prostate (12). Recently, the critical role of the AR for cellular proliferation *in vitro* or tumor growth *in vivo* of prostate cancer has been shown by different approaches, including disruption of AR function by anti-AR antibody, inhibition of AR expression by AR-specific ribozyme or antisense oligonucleotides, as well as knocking down AR expression by the RNA interference (RNAi) approach (8, 13–15). However, the mechanisms of AR-dependent cellular survival remain unclear in prostate cancer progression although some survival mechanisms involved in hormone-resistant progression of prostate cancer have been proposed (16–20).

Apoptosis, or programmed cell death, is a well-conserved process whose basic tenets remain common to all metazoans (21, 22). Intracellular organelles, like mitochondria, are key participants in apoptosis. The main aspects of mitochondrial involvement in apoptotic process include two critical events, the onset of multiple parameters of mitochondrial dysfunction, such as loss of membrane potential and the release of mitochondrial proteins including cytochrome *c*. The Bcl-2 family proteins are critical regulators that directly control the mitochondrial function and consist of both proapoptotic and antiapoptotic members (23). Bax, Bak, and Bok are proapoptotic members, as are the BH3 domain only members, such as Bad, Bik, and Bid. Antiapoptotic members include Bcl-2 and Bcl-xL, Bcl-w, Mcl-1, etc. It is believed that the relative levels of proapoptotic and antiapoptotic members are the key determinants in the regulation of cell death and survival.

The *bcl-x* gene encodes multiple spliced mRNAs, of which Bcl-xL is the major transcript (23, 24). Like Bcl-2, Bcl-xL protects cells from apoptosis by regulating mitochondrial membrane potential and volume, and subsequently prevents the release of cytochrome *c* and other mitochondrial factors from the intermembrane space into cytoplasm. In addition, Bcl-xL may prevent apoptosis via a cytochrome *c*–independent pathway (25). Although Bcl-xL protein can be regulated posttranscriptionally, it is mainly controlled at the gene expression level (26, 27). Bcl-xL protein is detected in the epithelial cells of normal prostate gland and prostate cancers in an earlier report (28). The expression level of Bcl-xL protein correlated with higher grade and stage of the disease, indicating an important role of Bcl-xL in prostate cancer progression.

RNAi is a recently discovered mechanism of posttranscriptional gene silencing in which double-stranded RNA corresponding to a gene (or coding region) of interest is introduced into an organism, resulting in degradation of the corresponding mRNA (29, 30). Unlike antisense technology, the RNAi phenomenon persists for multiple cell divisions before gene expression is regained, and is more efficient than antisense oligonucleotides. RNAi is, therefore, an extremely powerful, simple method for assaying gene function (31).

In an effort to dissect the mechanism of AR-dependent survival and to develop novel therapeutic strategies for prostate cancer, we knocked down the AR protein expression in prostate cancer cells that harbor the *AR* gene using the RNAi technique. Surprisingly, in addition to cell arrest, we found a significant apoptotic cell death when AR expression was knocked down by a small interfering RNA (siRNA) duplex. Most interestingly, the antiapoptotic protein Bcl-xL was also decreased in parallel with AR silencing, and overexpression of exogenous Bcl-xL controlled by a cytomegalovirus promoter partially rescued the cells from AR siRNA-induced apoptosis.

Materials and Methods

Cell Lines and Reagents

The human prostate cancer LNCaP, LAPC-4, PC-3, C4-2 and CWR22Rv1 cells, and HEK293 cells were described previously (32–34). The cell line information is briefly summarized in Table 1. Prostate epithelial cell RWPE-1 and breast cancer cell lines MCF-7 and T47D were obtained from American Type Culture Collection (Manassas, VA). The hormone-refractory prostate cancer cell LNCaP-Rf was a kind gift provided by Dr. Donald Tindall (Department of Biochemistry, Mayo Clinic, Rochester, MN; ref. 13). PC-3/AR subline was established by stably transfecting the AR-null PC-3 cells with a vector bearing the human *AR* gene obtained from Dr. Fahri Saatcioglu (Department of Biology, University of Oslo, Oslo, Norway). PC-3/Neo subline was established when an empty vector was used. The stable clones were selected in G418 and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). LNCaP/Bcl-xL subline was established by stably transfecting the LNCaP cells with a vector bearing the human *bcl-xl* cDNA sequence with a HA-tag obtained from Dr. Hong-gang Wang (Moffitt Cancer Center, University of South Florida, Tampa, FL) and LNCaP/puromycin subline was established when an empty vector was used. The stable clones were selected in a puromycin-containing culture medium. Antibodies against human AR, actin, and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against caspases, cytochrome *c*, Bcl-2 family members, XIAP, DFF45, and poly(ADP-ribose) polymerase were obtained from Cell Signaling (Beverly, MA). JC-1 fluorescent dye was obtained from Molecular Probes (Eugene, OR). Charcoal-stripped FBS was obtained from Atlanta Biologicals (Norcross, GA). Other reagents were supplied by Sigma (St. Louis, MO).

siRNA Synthesis, Labeling, and Transfection

Sequence information regarding the human *AR* gene (Genbank accession no. NM_000044) was extracted from the National Center for Biotechnology Information Entrez nucleotide database. Up to 34 mRNA segments were identified using the OligoEngine software (OligoEngine, Inc., Seattle, WA), which fulfill the requirements for potentially triggering RNAi according to the literature (31). The *AR* gene specificity was confirmed by searching the National Center for Biotechnology Information BlastN database. The siRNAs were prepared by a transcription-based method using the *Silencer* siRNA construction kit (Ambion, Austin, TX) according to the manufacturer's instructions. The 29-mer sense and antisense DNA oligonucleotide templates (21 nucleotides specific to the targets and 8 nucleotides specific to T7 promoter primer sequence 5′-CCTGTCTC-3′) were synthesized by IDT (Coralville, IA). The quality of the synthesized siRNA was estimated by agarose gel analysis and found to be very clean. RNAs were quantified by using RiboGreen fluorescence (Molecular Probes). A Silencer siRNA labeling kit using a fluorescent Cy3 dye (Ambion) was used for labeling the siRNA duplexes according to the manufacturer's instructions. The purified siRNA duplexes were transfected into cells with the Oligofectamine reagent (Invitrogen, Co., Carlsbad, CA) in a medium supplied with 2% charcoal-stripped FBS. The media were changed every 3 days. A scrambled negative siRNA duplex (Ambion) was used as control. A pooled chemically synthesized AR siRNA mixture was purchased from Upstate (Charlottesville, VA).

Western Blotting and Immunofluorescence Staining

For Western blot, cells were washed in PBS and lysed in a radioimmunoprecipitation assay buffer supplied with protease inhibitors (CytoSignal, Irvine, CA). Western blot analysis was done as described previously (32–35) to assess the protein expression level of target molecules. Blots were developed with a SuperSignal West Dura substrate kit (Pierce Biotech, Rockford, IL). Immunofluorescent staining was done as previously described (34, 35). The picture was taken under a fluorescence microscope (Nikon Inc., Melville, NY) set at ×200 magnification.

Table 1. Summary of cell lines used in this study

Cell line	Origin and modification	AR status	Hormone response	Reference
LNCaP	Human prostate cancer	Mutant	Yes	55
LAPC-4	Human prostate cancer	Wild type	Yes	56
PC-3	Human prostate cancer	Null	No	57
C4-2	LNCaP coengrafted with bone marrow cells	Mutant	No	58
CWR22Rv1	Human prostate cancer	Mutant	No	42
RWPE-1	Human prostate epithelium transformed by HPV-18	Wild type	Yes	41
HEK293	Human embryo kidney transformed by adenovirus 5	Null	No	59
MCF-7	Human breast cancer	Positive	Yes	60, 61
T47D	Human breast cancer	Positive	No	62

Cytotoxicity Assays and Flow Cytometry

Typically, cell viability was assessed with a trypan blue exclusion assay as described in our previous publication (33). Apoptotic cell death was determined using an annexin V-FITC Apoptosis Detection kit (BD PharMingen, San Diego, CA) according to the manufacturer's manual. Briefly, cells were harvested and washed with ice-cold PBS and then suspended in annexin V binding buffer. Then, cells were stained for 15 minutes at room temperature in the dark and analyzed on a FACSCalibur flow cytometer using CELLQuest software. For clonogenic survival assay, 10^3 cells were seeded in a 35 mm dish and transfected with the siRNAs as indicated in the figure legend. The media were changed every 3 days and the cultures were observed daily for colony formation. On day 7, the cultures were washed with PBS, fixed, and stained as previously described (36). The colonies were counted under an inverted microscope.

mRNA Expression Analysis and Reverse Transcription-PCR

Total RNA was prepared using Trizol reagent (Invitrogen). To assess mRNA expression, a semiquantitative reverse transcription-PCR (RT-PCR) method was used as described previously (35). RT-PCR was done using a RETROscript kit (Ambion) per manufacturer's instructions. The primers and PCR conditions were described as follows: for human *AR* gene (forward 5′-cctggcttccgcaacttacac-3′; backward 5′-ggacttgtgcatgcggtactca-3′; adapted from ref. 6); human *PSA* gene (forward 5′-gatgactccagccacgacct-3′; backward 5′-cacagacaccccatcctatc-3′; ref. 37); and human *bcl-xl* gene (forward 5′-catggcagcagtaaagcaag-3′; backward 5′-gcattgttcccatagagttcc-3′; ref. 38). 28S ribozyme RNA (forward 5′-gttcacccactaatagggaac gtg-3′; backward 5′-gattctgacttagaggcgttcagt-3′) was used as an internal control. The primers were synthesized by IDT. The amplification profile was as follows: 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute running in a total of in 25 cycles. After 25 amplification cycles, the expected PCR products were size fractionated onto a 2% agarose gel and stained with ethidium bromide.

Mitochondrial Membrane Potential and Caspase Activity

The siRNA-transfected cells were incubated in the presence of JC-1, which was added to the culture medium at a final concentration of 0.3 μg/mL for 15 minutes at 37°C. Thereafter, the cells were analyzed under a fluorescent microscope. The caspase activity was measured using an Apo-ONE Homogeneous Caspase-3/7 Assay kit obtained from Promega (Madison, WI) per the manufacturer's manual. Briefly, the cells were washed in ice-cold PBS and then suspended in the assay buffer containing the substrate rhodamine 110 (Z-DEVD-R110) provided by the supplier. The amount of fluorescent product generated is measured at 480/520 nM (wavelength) using a Fluoscan fluorescent reader as described previously (32, 34).

Statistical Analysis

All experiments were repeated twice or thrice. Western blot results are presented from a representative experiment. The mean and SD from two experiments for cell viability are shown. The number of viable/dying cells or cell colonies in the control group or the initial time point was assigned a relative value of 100%. The significant differences between groups were analyzed using the SPSS computer software (SPSS, Inc., Chicago, IL).

Results

Knocking Down AR Expression via RNA Interference Approach in Prostate Cancer Cells

Because the AR has been shown to play a critical role in hormone-refractory progression of prostate cancer (6–17), targeting the *AR* gene by reducing its translation or blocking its function via antisense approach has emerged as a novel strategy for prostate cancer therapy (13–15). Recently, RNA interference has been shown to be a better strategy in blocking gene expression in cultured cells or animal model (29–31). To explore the feasibility of the RNAi technique in knocking down AR expression in prostate

cancer cells that harbor the *AR* gene, we designed and synthesized a panel of siRNAs against human *AR* gene. Two relatively potent siRNAs (AR siRNA 8, 5′-AAGAAG-GCCAGUUGUAUGGAC-3′; AR siRNA 31, 5′-AAGACG-CUUCUACCAGCUCAC-3′) were identified in knocking down AR expression in the initial experiments when compared with others. The AR knockdown effect was further confirmed by checking the mRNA level followed by Western blot. A well-known androgen target prostate-specific antigen was also down-regulated as determined by a RT-PCR assay. This knocking down effect was achieved as a sequence-specific event because a negative control siRNA with a scrambled sequence had no effect on AR protein or prostate-specific antigen mRNA level (Fig. 1A). Both AR siRNAs 8 and 31 significantly knocked down AR expression at a final concentration of 1.0 to 10 nmol/L in culture media after 4 days in LNCaP cells that harbor an endogenous mutant *AR* gene, as well as in PC-3/AR cells that were reconstituted with an exogenous wild-type *AR* gene (Fig. 1B). Moreover, the knocking down effect of the AR protein was further verified using an immunofluorescent staining approach where LAPC4 cells, which harbor an endogenous wild-type *AR* gene, were used (Fig. 1C). These results show that the RNAi machinery is functional in prostate cancer cells, which is consistent with two recent reports (8, 39), and can be activated by a siRNA duplex.

siRNA-Mediated AR Silencing Leads to Dramatic Cell Death

It is shown that the AR is a key factor for cell proliferation *in vitro* (13, 14) or tumor growth *in vivo* (15) in prostate cancer. Consistent with two recent reports showing a reduced cell proliferation after AR protein was knocked down via the RNAi approach (8, 39), we also found that cell growth was largely reduced after transfection of LAPC-4 cells with either AR siRNA 8 or a pooled AR siRNA mixture (Fig. 2A). However, the difference was that a massive cell death was observed if the cells were monitored for >4 days after siRNA transfection. To test if the cell death response is due to siRNA-mediated AR silencing, we did a time course experiment in LNCaP (hormone-sensitive) and C4-2 (hormone-refractory) cells. The cells were transfected with AR siRNA 8 or a scrambled negative siRNA in 2% charcoal-stripped FBS. The relative survival rate of the cells was determined every 2 days using a trypan blue exclusion assay. Transfection of the cells with the AR siRNA duplexes resulted in a significant cell death in which LNCaP cells (Fig. 2B) showed a quicker response compared with C4-2 cells (Fig. 2C). In contrast, the negative control siRNA did not cause cell death. These data suggest that the AR siRNA induces cell death regardless of hormone sensitivity, although C4-2 cells showed a delayed response compared with LNCaP cells.

Next, we asked if the AR siRNA–induced cell death was simply due to a cellular nonspecific response to the double-stranded siRNA (i.e., IFN response; ref. 40) or those degraded AR mRNA produced by the RNAi machinery. The experiments were conducted using PC-3/AR, PC-3/Neo (empty vector control subline), and LNCaP-Rf (hormone-refractory, ref. 13) cell lines. As shown in Fig. 2D, either AR siRNA 8 or 31 significantly reduced the survival rate for >95% in LNCaP-Rf cells compared with the control siRNA. In contrast, the cell survival rate

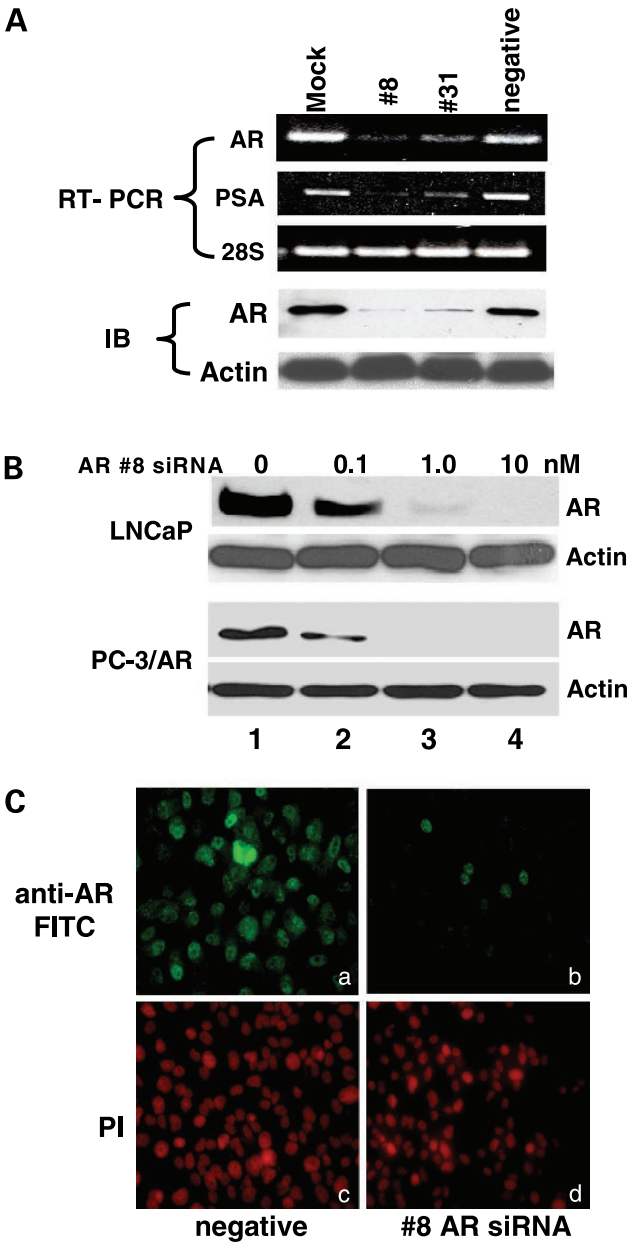

Figure 1. siRNA-mediated *AR* gene silencing in prostate cancer cells. **A,** following transfection with the siRNA duplexes (final concentration at 10 nmol/L in the medium) as indicated, cells were harvested at 48 h (*top*) or 72 h (*bottom*) later. The mRNA levels of target genes as indicated were determined by RT-PCR assay (*top*) and the AR protein was determined by Western blot (*bottom*). Actin blot served as loading control. The siRNA was omitted in the mock control. **B,** cells were transfected with different concentrations of AR siRNA 8 and then harvested 72 h later. Western blot was done as above. **C,** LAPC-4 cells were transfected with the siRNA duplexes (10 nmol/L in the medium) as indicated for 72 h and then subjected to immunofluorescent staining as described in the text.

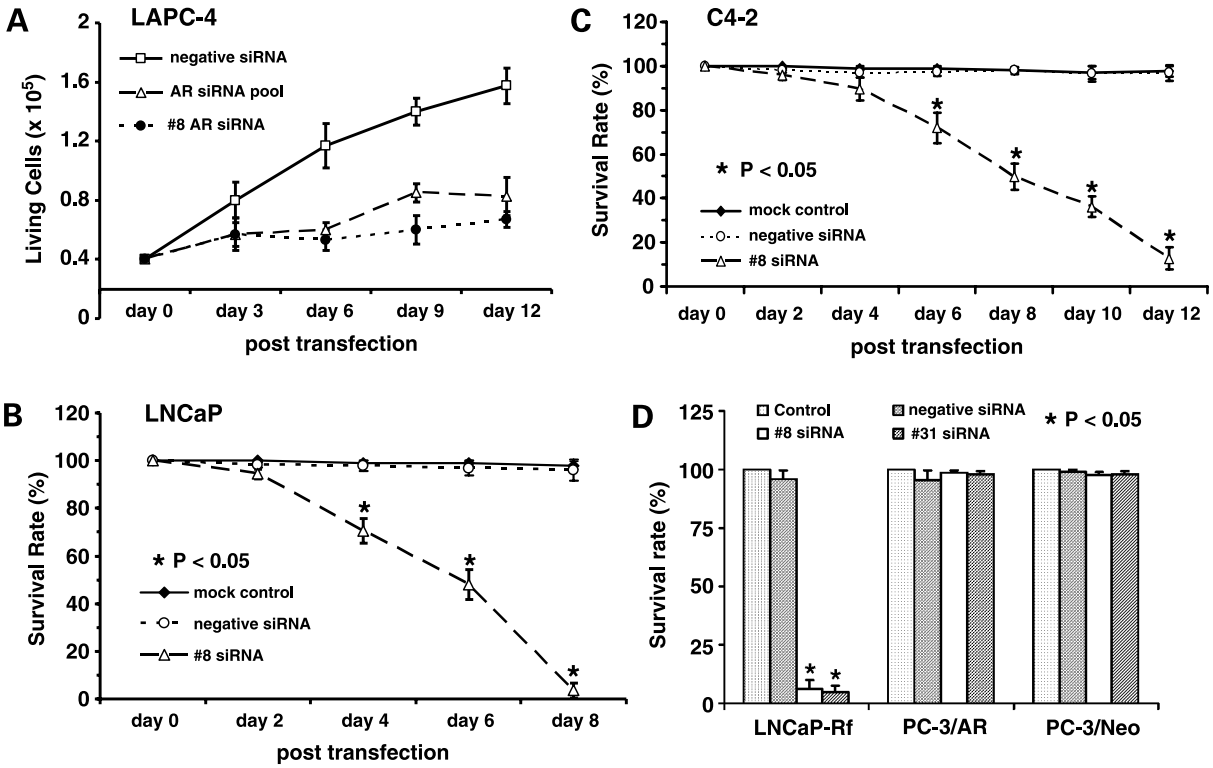

Figure 2. AR siRNA induces cell arrest and death. Cells seeded in six-well plates were transfected with the siRNAs (10 nmol/L in the medium) as indicated. **A,** the total number of living (white or unstained) cells from each time point was counted by trypan blue exclusion assay. **B** and **C,** the survival rate (white cells versus white plus blue cells) was determined in each time point by trypan blue exclusion assay, and then the relative survival rate was calculated by normalizing the data from late time points against the initial time point group that was set as 100%. **D,** cells were seeded in 35 mm dishes at a density of 10^3 cells per dish overnight and then transfected with the siRNA duplexes (10 nmol/L in the medium) as indicated. The clonogenic survival fraction of the cells was determined on day 7 posttransfection. Colonies were fixed, stained, and counted. The survival rate in control group was designated as 100%. Data are from three different experiments.

was not affected in either PC-3/AR or PC-3/Neo cells after the siRNA transfection. These data suggest that the AR siRNA–induced cell death in the native AR-harboring cells is not a nonspecific cellular response to the double-stranded siRNA or siRNA-mediated AR mRNA degradation but due to a disruption of the survival machinery that depends on the AR. In the AR-null cells, like PC-3/Neo or PC-3/AR cells where an exogenous *AR* gene is expressed, the survival machinery of the cells might not depend on the AR.

AR siRNA–Induced Cell Death Occurs Specifically in Prostate-Derived Cells

In addition to those commonly used prostate cancer cells as mentioned above, we also tested the cell death response to the AR siRNA in two more prostate epithelial cell lines (RWPE-1 and CWR22Rv1) and breast cancer cell lines (MCF-7 and T47D) to verify the specificity of AR siRNA–induced cell death. The RWPE 1 is a nontumorigenic prostate epithelial cell line (41), whereas the CWR22Rv1 is a hormone-refractory prostate cancer cell derived from CWR22 xenograft (42). Although the CWR22Rv1 cells, like C4-2 cells, showed a delayed response to AR siRNA–induced cell death, the nontumorigenic RWPE-1 cell line showed a rapid death response even faster than LAPC-4 (Fig. 3A) and LNCaP cells (Fig. 2B). However, the two breast cell lines did not show any cell death response to the AR siRNA although they are also harboring an endogenous AR (data not shown). A selected data for AR siRNA–induced AR protein knockdown in CWR22Rv1 and LAPC-4 cells was shown in Fig. 3B.

To visualize the specificity of the AR siRNA–induced cell death, we labeled AR siRNA 31 with a fluorescent dye (Cy3) and then transfected into LNCaP cells. Cells were maintained in 2% charcoal-stripped FBS and cell death was monitored daily under a fluorescent microscope. As shown in Fig. 4, the Cy3-labeled siRNA was seen in a large population of the cells, indicating a successful transfection. Most interestingly, only the dying cells (round and detached from the plastic) showed a positive Cy3 labeling (Fig. 4, *black arrow*); however, living cells (spreading and attached cells) showed no Cy3 labeling (Fig. 4, *white arrow*). These data show the specific effect of the AR siRNA–induced cell death only on the transfected cells.

Mitochondrial Apoptotic Mechanism Is Involved in AR siRNA–Induced Cell Death

It has been shown that androgen ablation or antiandrogens induces apoptotic cell death in prostate epithelium and

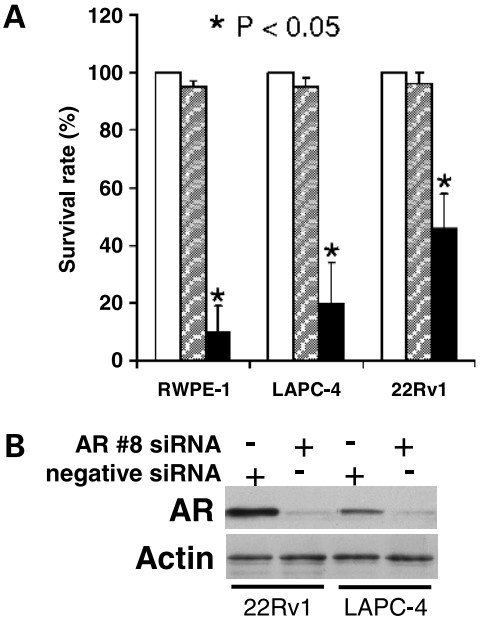

Figure 3. AR siRNA specifically induces cell death in other prostate-derived cells. **A,** three prostate cell lines (RWPE-1, LAPC-4, and CWR22Rv1) were transfected with AR siRNA 8 at 10 nmol/L in the culture medium supplied with 2% charcoal-stripped FBS, and cell survival rate was determined 7 days later by trypan blue exclusion assay. **B,** cells harvested from the experiments described in (**A**) were lysed for Western blot analysis to determine the protein levels of the AR. Actin blot served as loading control.

prostate cancer cells (3). To determine if AR siRNA–induced cell death is an apoptotic response, we first detected the change of the membrane phospholipid phosphatidylserine, which is translocated from the inner to the outer leaflet of the plasma membrane during the earlier phase of apoptosis (43). As shown in Fig. 5A, transfection of the cells with the AR siRNAs induced significant phosphatidylserine translocation, whereas the control siRNA had no effect.

Because loss of mitochondrial transmembrane potential ($\Delta\psi_m$) is considered to be one of the central events in apoptotic death that leads to incapacitation of the mitochondria, release of cytochrome *c*, and activation of the caspase pathway, we tested the integrity of this signaling event using the fluorescent dye JC-1 as described elsewhere (44). Upon entering the mitochondrial negative transmembrane potential in healthy cells, JC-1 forms red fluorescent aggregates. When the transmembrane potential is low, as in many cells undergoing apoptosis, JC-1 exists as a monomer and produces green fluorescence. Consistent with this notion, green fluorescence was observed in dying cells after being transfected with AR siRNA 8 (as pointed by arrows in Fig. 5B, *c* and *d*), whereas living cells remained normal membrane potential (red fluorescence as pointed with arrowhead in Fig. 5B).

The presence of cytochrome *c* in the cytosol is a critical event required for the correct assembly of the apoptosome, subsequent activation of the executioner caspases, and induction of cell death (45). To evaluate the release of cytochrome *c*, cytosolic fraction of the cellular protein was collected 6 days after siRNA transfection. As shown in Fig. 6A, in parallel with the AR knocking down, cytochrome *c* was detected in the cytosolic fraction when AR siRNA 8 was transfected into cells. Meanwhile, the apoptosis hallmarker poly(ADP-ribose) polymerase cleavage fragment was also detected. Finally, the proteolytic processing of inactive procaspases, the essential component of the death pathway in many cells (21), and their catalytic activity were also analyzed. As shown in Fig. 6B, transfection with AR siRNA 31 into LNCaP cells induced significant reduction of procaspase-3, procaspase-6, and DFF45 (evidence for proteolytic activation or cleavage). Similar results were also seen when LAPC-4 or C4-2 cells were used (data not shown). Consistently, the catalytic activity of caspase 3/7 was significantly increased when AR siRNA 31 was used compared with negative control siRNA (Fig. 6C). Thus, these data clearly showed that the mitochondrial apoptotic mechanism is activated by the AR siRNAs.

Antiapoptotic Protein Bcl-xL Is Involved in AR-Mediated Cell Survival

Having shown the mitochondrial involvement in AR siRNA–induced cell death, we next focused on the Bcl-2 family members because they are the major regulators of mitochondrial function in the aspect of apoptosis by facilitating or inhibiting cytochrome *c* release to cytosol

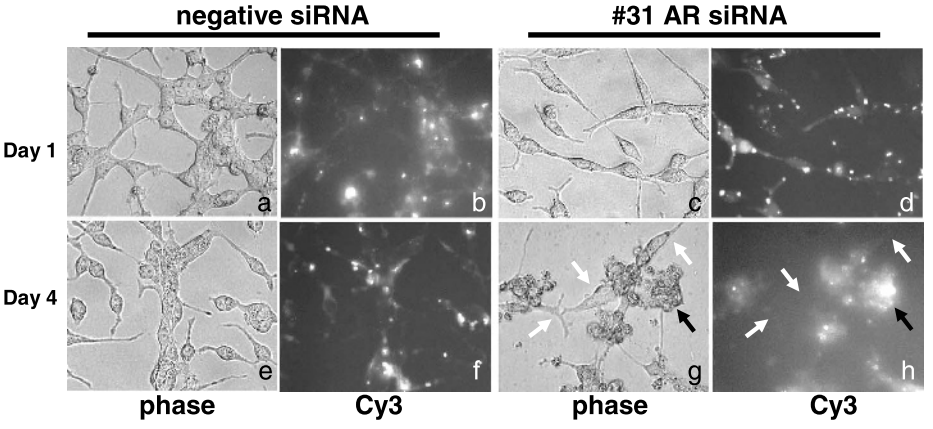

Figure 4. Visualization of the Cy3-labeled AR siRNA–induced cell death. LNCaP cells were seeded in six-well plates overnight and then transfected with Cy3-labeled siRNAs (10 nmol/L in the medium) as indicated. Cell death was monitored daily. Pictures were taken at days 1 and 4 after transfection. The Cy3-labeled siRNAs are seen as white dots in Cy3 (*b*, *d*, *f*, and *h*). In (*g*) and (*h*), white arrows indicate several living cells without the Cy3 labeling (negative transfection), whereas black arrows indicate a cluster of dying cells (round and detached) with strong Cy3 labeling (positive transfection).

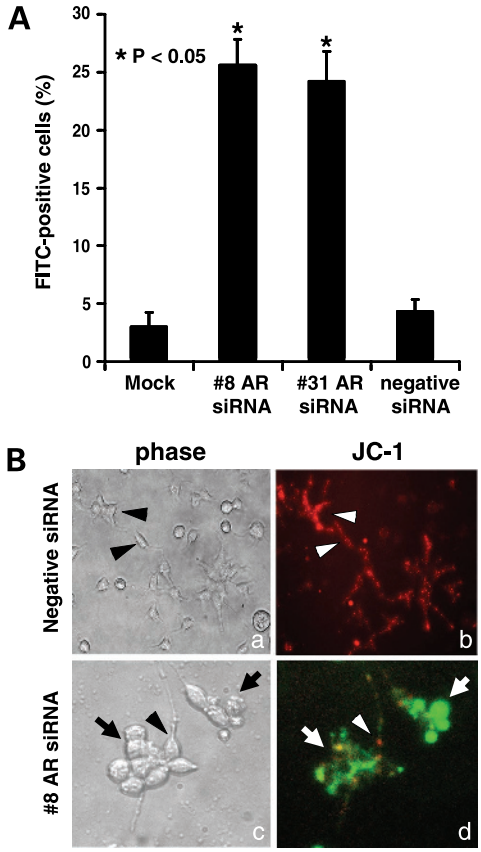

Figure 5. AR siRNA induces apoptotic cell death. **A,** after transfection with the siRNA duplexes (10 nmol/L in the medium) as indicated for 4 d, LNCaP cells were harvested and the change of the membrane phospholipid phosphatidylserine was determined using fluorescence-activated cell sorting for FITC-labeled cells as described in the text. Data are from two different experiments. **B,** following transfection with the siRNA duplexes (10 nmol/L in the medium) for 5 d, LNCaP cells were incubated with JC-1 (0.3 μg/mL) for 15 min at 37°C. Pictures were taken under a fluorescent microscope (magnitude ×200).

and subsequent assembly of an active apoptosome (22). These functions are promoted by the proapoptotic Bax or Bak and are inhibited by the antiapoptotic Bcl-2 and Bcl-xL. We determined whether protein expression of these Bcl-2 family members is altered after the AR siRNA transfection. Interestingly, we found that the protein level of the antiapoptotic member Bcl-xL dramatically decreased in the AR siRNA 8–transfected cells compared with the controls, whereas another antiapoptotic member, Bcl-2, and the proapoptotic members, Bax and Bak, remained unchanged (Fig. 7A). To better illustrate the relationship of Bcl-xL reduction with AR silencing, we conducted a time course experiment (Fig. 7B). The protein levels of Bcl-xL decreased in a time-dependent manner following the AR siRNA transfection; however, Bax protein remained consistent during the time course. These data indicate that AR silencing results in Bcl-xL reduction that might lead to an imbalance between the proapoptotic and antiapoptotic members of the Bcl-2 family that, in turn, triggers apoptosis.

To shed light onto the mechanistic basis underlying the response of Bcl-xL reduction to AR silencing, we also examined Bcl-xL expression at the mRNA level by RT-PCR assay. As shown in Fig. 7C, the Bcl-xL mRNA level decreased significantly after AR siRNA 8 transfection compared with the controls, indicating that the reduction of Bcl-xL protein after AR silencing is via a transcriptional mechanism.

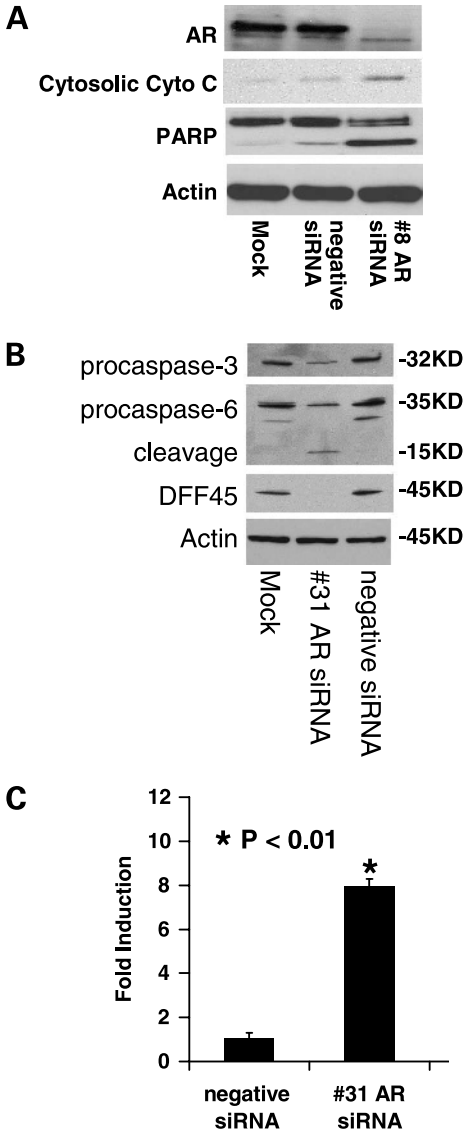

Figure 6. AR siRNA induces cytochrome *c* release, caspase activation, and cleavage of DFF45 and poly(ADP-ribose) polymerase. **A** and **B,** after 7 d of transfection with the siRNAs as indicated, LNCaP cells were harvested and the cytosolic occurrence of cytochrome *c*, proteolytic process of caspase-3 and caspase-6, and DFF45 and poly(ADP-ribose) polymerase cleavage were determined by Western blot. **C,** after 7 d of transfection with the siRNAs as indicated, LNCaP cells were washed with ice-cold PBS and then harvested. Caspase activity was measured as described in the text. *Columns,* mean value of relative activity from three independent experiments.

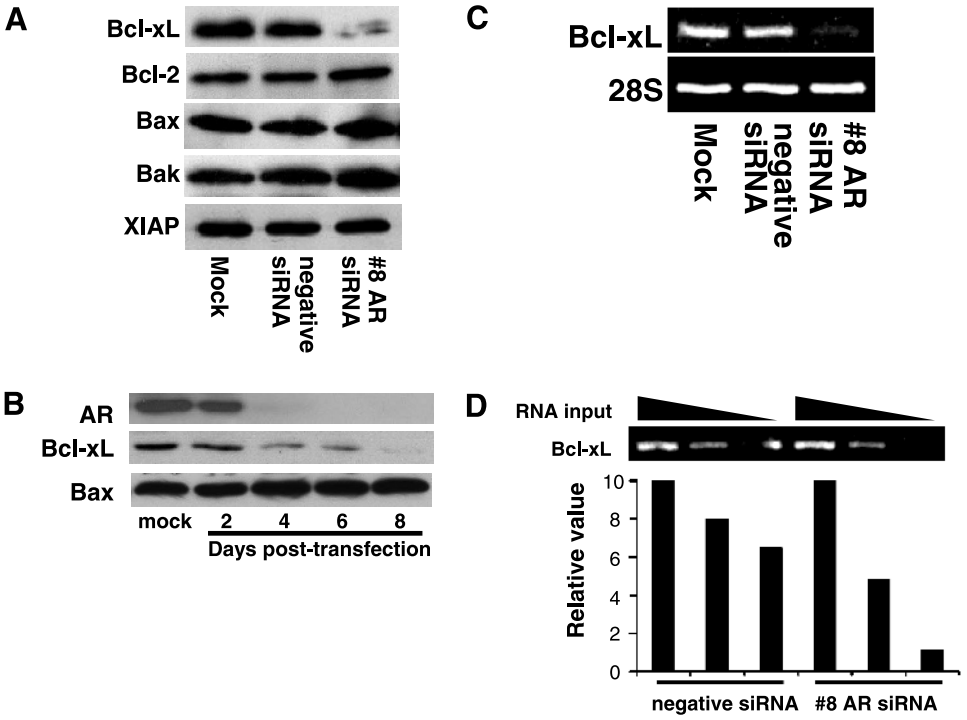

Figure 7. AR siRNA transfection leads to reduction of Bcl-xL expression. **A** and **B,** after transfection with AR siRNA 8 or negative control siRNA (10 nmol/L in the medium) as indicated, LNCaP cells were harvested on day 7 (**A**) or at each time point (**B**), and the protein levels of AR, Bcl-2, Bcl-xL, Bax, Bak, and XIAP were assessed by Western blot. Data was reproducible in three independent experiments. **C,** after transfection with the indicated siRNAs (10 nmol/L in the medium), LNCaP cells were harvested on day 7, the total RNA was isolated, and the Bcl-xl mRNA level was assessed by RT-PCR as described in the text. **D,** similar to (**C**), but a serial 10-fold dilution of the total RNA input was made for the first-strand cDNA synthesis in the RT-PCR assay. Relative band density in each lane was determined and graphed.

AR siRNA–Induced Apoptosis Was Partially Inhibited by Ectopic Bcl-xL Expression

In view of the antiapoptotic feature of Bcl-xL protein, we hypothesized that the AR promotes cellular survival by upregulating the *bcl-x* gene expression through a transcriptional mechanism in prostate cancer cells. Therefore, Bcl-xL expression will decrease if the AR is knocked down, which subsequently results in apoptosis due to an imbalance between the proapoptotic and antiapoptotic members of the Bcl-2 family. Thus, we wondered if an enforced Bcl-xL expression will protect cell from apoptosis while AR is silenced. To assess the protection effect of Bcl-xL protein, a stable LNCaP subline overexpressing human Bcl-xL protein controlled by a cytomegalovirus promoter (LNCaP/Bcl-xL) or a control subline with an empty vector (LNCaP/puromycin) were established. Consistent with the results obtained from the parental cells (Fig. 7A), exposure of those LNCaP subline cells to AR siRNA 8 resulted in a decrease of endogenous but not exogenous Bcl-xL protein (Fig. 8A, *lane 1* versus *lane 2*). Most significantly, as expected, enforced Bcl-xL expression partially inhibited cell death induced by AR siRNA transfection in LNCaP/Bcl-xL cells compared with the controls (Fig. 8A, *bottom*). These data showed that Bcl-xL is involved in AR-mediated survival of prostate cancer, and the reduction of Bcl-xL expression after AR silencing represents a mechanism for the AR siRNA–induced apoptosis.

In addition, while establishing a subclone for stable Bcl-xL expression in LNCaP cells, an unexpected clone [LNCaP subclone 11 (LN11)] was obtained, in which Bcl-xL expression was dramatically reduced for unknown reason, as confirmed by RT-PCR and Western blotting (Fig. 8B, *top* and *middle*). By taking advantage of this particular clone of LNCaP cell subline, we further tested the involvement of Bcl-xL in AR-mediated survival. Exposing LN11 subline cells to AR siRNA 8 resulted in a significant increase in AR siRNA–mediated cell death compared with the parental LNCaP cells and the untransfected controls (Fig. 8B, *bottom*), although the LN11 subline did not show a profound cell death without AR silencing. These data indicate that loss of Bcl-xL expression enhances AR siRNA–induced cell death, and multiple downstream factors, besides Bcl-xL, are mediating AR survival signal.

Discussion

In this report, we identified two siRNA duplexes that induce a strong AR silencing in prostate cancer cells. Most importantly, we found that siRNA-mediated AR silencing subsequently leads to a massive cell death through a mitochondrial apoptotic pathway. AR siRNA–induced apoptosis only occurs in prostate cancer cells that harbor an endogenous AR regardless of their androgen sensitivity. Further analyses showed that Bcl-xL expression is transcriptionally dependent on the AR in prostate cancer cells, and siRNA-mediated AR silencing results in a reduction of Bcl-xL expression that accounts partially for the apoptotic response because enforced Bcl-xL expression inhibited cell death after AR silencing. To the authors' knowledge, this is the first report showing AR involvement in Bcl-xL expression and apoptotic response to siRNA-mediated AR silencing in prostate cancer.

Our results are somewhat different from other approach-induced AR blockage as mentioned above (8, 13–15, 39), in which only cell arrest or reduced tumor growth, but no cell death, were reported. The plausible reason might be the difference in the extent of AR blockage or protein knockdown. For example, the AR-specific antibody might not totally block the AR function as used in a previous report (13) because the AR protein still exists in the cell. It is believed that the RNAi approach is more potent than the ribozyme (13) or antisense approach in terms of gene silencing (14, 15); therefore, our RNAi approach might have induced a more efficient knocking down of the AR protein than the former approach of AR ribozyme or antisense oligonucleotides. In addition, current experiences in the field of RNAi technology showed that the siRNAs targeted to different regions of a gene transcript may not function equally (31), which may be responsible for the different findings between our results and others (8, 39). Finally, the strategies in the experimental condition used between ours and other groups (8, 39) might also account for the different outcome.

It has been shown that androgen or other factors as critical survival stimuli play an important role via the AR in prostate cancer progression. Although AR-dependent functional repression of FKHR and related FOXO forkhead proteins was reported as a possible pathway (18), the survival pathway of AR-dependent mechanism is not clear. PI3K-Akt is a major cellular survival factor that is negatively regulated by the PTEN phosphatase (46). In LNCaP cells, Akt is constitutively active due to PTEN mutational inactivation (47), whereas LAPC-4 cells maintain a wild-type PTEN (48). Here, we observed that AR siRNA induced cell death in both of the cell lines, suggesting that the AR-dependent survival pathway is via an AKT-independent mechanism, which was also proposed by previous reports (49, 50). In addition, we observed the apoptotic response from those native AR-harboring cells (RWPE-1, LAPC-4, LNCaP, CWR22Rv1, and C4-2), but not from the AR-null PC-3 or its subline PC-3/AR cells, which was reconstituted with an exogenous *AR* gene. These findings indicate that the AR is a critical survival factor for prostate epithelium-derived cells and remains as an important survival factor even in those hormone-refractory cancer cells, although they might have developed additional survival mechanism. However, the AR-null prostate cancer cells already escaped from the AR-regulated survival control.

The Bcl-2 family proteins reside immediately upstream of mitochondria and function as either death antagonists

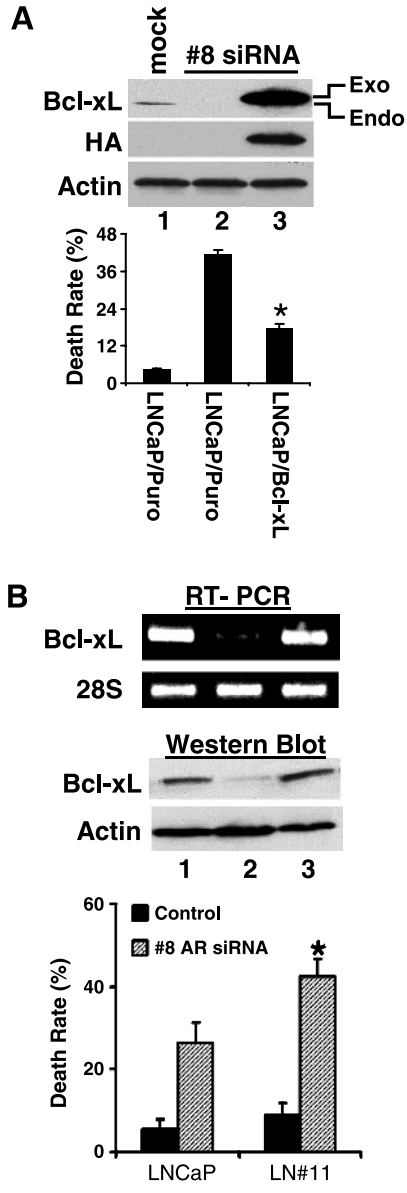

Figure 8. **A,** ectopic enforced expression of *bcl-xl* gene inhibits AR siRNA–induced cell death. LNCaP/puromycin and LNCaP/Bcl-xL cells were transfected with AR siRNA 8 for 7 d and the expression level of endogenous/exogenous *bcl-xl* gene was determined by Western blot. Because the exogenous Bcl-xL protein has a HA tag, the membrane was reprobed with anti-HA antibody to show the exogenous Bcl-xL protein. Actin blot served as loading control. The cell death rate (blue cells versus blue plus white cells) was determined individually by trypan blue exclusion assay. The asterisk indicates a significant difference ($P < 0.05$) between LNCaP/puromycin versus LNCaP/Bcl-xL cells after AR siRNA 8 transfection. Data are from three independent experiments. **B,** loss of Bcl-xL expression lead to a significant increase of AR siRNA–induced cell death. *Top,* the parental LNCaP cells (*lane 1*), LNCaP subline LN11 (*lane 2*), and a stable subclone bearing an empty vector (*lane 3*) were exponentially grown and harvested. Total RNA was isolated and Bcl-xL mRNA levels were determined by RT-PCR and 28S gene served as internal control for the RT-PCR assay. Cellular proteins were extracted and Bcl-xL protein levels were assessed by Western blot. Antiactin blot served as loading control. Data are from two separate experiments. *Bottom,* cells were transfected with negative siRNA (*black columns*) or AR siRNA 8 (*gray columns*) at 10 nmol/L in the culture medium supplied with 2% charcoal-stripped FBS. Cell death rate [dying cells versus (dying plus living cells)] was determined 5 d later by trypan blue exclusion assay as described earlier. The asterisk indicates a significant difference ($P < 0.05$) between LN11 versus the parental LNCaP cells.

or agonists. The ratio of death antagonists to agonists determines how a cell responds to an apoptotic signal. Like Bcl-2, Bcl-xL is another major apoptotic antagonist and its expression is mainly regulated through transcriptional mechanisms (21, 22). The *bcl-x* promoter contains consensus motifs for a number of transcription factors, including Sp1, activator protein-1, Oct-1, Ets, Rel/NF-κB, signal transducers and activators of transcription (STAT), and GATA-1, in which three transcription factor families, STATs, Rel/NF-κB, and Ets family, have been shown to play an important role in the regulation of the *bcl-x* gene expression (26, 27). Recently, other steroid hormone receptors, including receptors for glucocorticoid and progesterone, have been reported to bind to the mouse *bcl-x* promoter (51, 52). In this report, our data suggest that the AR is involved in the transcriptional regulation of *bcl-x* gene expression, although the underlying mechanism is under further investigation by our group.

Recently, siRNA-mediated IFN response has emerged as a big concern regarding the use of siRNA in mammalian cells (40, 53). In our system, we also observed the similar response in which the transcriptionally made siRNAs induced more significant IFN response than the chemically synthesized ones.[5] However, similar responses were observed in all of those prostate cancer cells used in our study, indicating that the apoptotic effect of AR siRNA in the AR-harboring cells is independent of the IFN-related effect. Moreover, it was reported that only tumor necrosis factor-α, but not IFN, down-regulates Bcl-xL expression (54), suggesting that the reduction of Bcl-xL protein is not due to the siRNA-triggered IFN response.

In conclusion, our results showed for the first time that knocking down the AR protein by a siRNA duplex induces apoptosis in native AR-positive prostate cells regardless their hormone sensitivity. The apoptotic response induced by the AR siRNAs is partially due to reduction of Bcl-xL expression because enforced Bcl-xL expression inhibits AR siRNA-induced cell death. Currently, the underlying mechanism for AR-mediated up-regulation of the *bcl-x* gene is under further investigation. The siRNA-mediated AR silencing may be implicated as a novel approach in the future for curing the hormone-refractory prostate cancers that are currently considered as a condition with no cure.

Acknowledgments

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[5] Unpublished observation.

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Androgen receptor and cellular survival in prostate cancer

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Introduction

Prostate cancer is the most common cancer diagnosed after skin cancers and the second leading cause of cancer death in the US. It is estimated that there will be about 232,090 new cases and 30,350 men will die because of this disease in 2005 (1). Since it is a severe health threat to men, extensive studies have been conducted on this disease in recent years and the research advances were summarized by numerous excellent review articles in all aspects including general mechanism (2-9), cancer genetics and epidemiology (10-18), androgen receptor (AR) biology (19-40), AR coregulators (41-42), AR cross-talk with other signal pathways (43-47), antiandrogen

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and novel therapies (48-60), as well as new biomarkers for prognosis and diagnosis (61-63). In this review, thus, we will only discuss the survival pathways related to the AR signaling in prostate cancers.

Androgen receptor is the key molecule for prostate cancer progression

Since the seminal work of Huggins and Hodges in 1941 (64), it has been widely accepted that androgens play a critical role not only in the physiological development of the prostate but also in the genesis of prostate cancer. Currently, androgen ablation is the major therapeutic approach for advanced disease. However, most patients treated by androgen ablation ultimately relapse to more aggressive androgen-independent or so-called hormone-refractory (because it is resistant to hormone ablation therapy) prostate cancers. The etiology of hormone-refractory progression may have various molecular causes, but the AR is expressed and its function is maintained in each scenario (reviewed in Ref. 19-40), suggesting that androgen-independent AR signaling is involved. Since the original cloning of the AR gene in 1988 (65), the role of AR in prostate development and cancer progression has been extensively studied. Especially, the essential role of the AR for hormone-refractory progression was demonstrated recently (66-69).

The first report showing a direct connection between the AR and cell growth in androgen-independent prostate cancer cells under androgen deprivation condition was published in 2002 by Dr Tindall's group (66). In a cell-based experiment, disruption of AR activity using an AR antibody to block its function or AR-specific ribosome to suppress its protein translation caused a significant growth inhibition either in androgen-dependent or -independent prostate cancer cells that harbor a native *AR* gene. However, there was no significant inhibition of cell growth upon exposure of prostate cancer DU145 cells that are null of *AR* gene to those procedures. This initial observation was supported by a study using xenograft model of prostate cancer (67). The transition from androgen-dependent to -independent stage was established using human prostate cancer LAPC-9 xenograft line in severe combined immunodeficiency (SCID) mice. Then, AR function was determined using androgen responsive reporter gene assay and AR-DNA binding (chromatin immunoprecipitation, ChIP) analysis. In androgen-dependent stage, the AR localized to the nucleus and bound to the endogenous prostate-specific antigen (PSA, a typical androgen response gene) enhancer. Following castration, the AR disassociated from the chromatin and moved out of the nucleus. However, in recurrent tumors (androgen-independent stage), the AR re-entered into the nucleus and rebound to PSA enhancer region on the chromatin. Reporter assays also showed that the AR was re-activated upon

tumor re-occurrence. This study clearly demonstrated that the AR is fully functional in recurrent androgen-independent tumor after androgen withdrawal. Later on, the critical role of the AR molecule was further determined in a more comprehensive analysis (68). Firstly, AR expression at both transcriptional and translational levels was determined in seven xenograft models, and the results showed that the most profound alteration after castration was a dramatic increase of AR mRNA and protein levels. Secondly, the causative role of increased AR expression on androgen-independent transition was tested. As expected, enhanced AR expression significantly promoted cell growth under androgen deprivation condition in cell culture, castration condition in xenograft mice model, or even in the presence of anti-androgens. Thirdly, the mechanism involved in AR-mediated progression was studied, and the data indicated that the AR was still ligand dependent for its transactivation and the genomic effect is necessary for AR-mediated androgen-independent transition. Taken together, these studies established that the AR is playing a critical role in prostate cancer progression.

In considering its functional role of the AR in prostate cancer progression, targeting the AR but not the androgenic hormone becomes more realistic in terms of therapeutic strategy. Most recently, in an effort to develop novel therapies for prostate cancer, we used the RNA interference technique to silence the AR expression in prostate cancer cells. Several small-interfering RNA (siRNA) duplexes were created and tested for knocking down AR protein. In addition to cell arrest as reported previously (68), surprisingly, a massive apoptotic cell death was observed after siRNA transfection in AR-native prostate cancer cells regardless of their androgen dependency (69). This is the first report showing a direct link between AR molecule and cell death in prostate cancer cells, which further confirmed previous reports that the AR is the key molecule in cellular survival of prostate cancer cells.

Androgen receptor and its expression

The androgenic hormones transmit their regulatory signals to the nucleus through the cognate androgen receptor (AR), which is a ligand-activated transcription factor and a member of the nuclear receptor superfamily. The AR is a member of nuclear receptor superfamily produced from a single-copy 90-kilobase pair (kbp) gene located on the X chromosome at Xq11-12 (65). Without androgen stimulation, the AR is inactive and sequestered in the cytoplasm as a multiprotein complex with heat shock proteins and immunophilins. After the receptor binds androgens, the AR is disassociated from the multiprotein complex, and it undergoes a conformational change, homodimerization such that a nuclear localization signal (NLS) present in the receptor structure is exposed, allowing import of the ligand bound AR to the nucleus. Binding of importins to the exposed NLS is needed for this translocation

process. The transcriptionally active AR accumulates within a subnuclear compartment that is microscopically visualized as nuclear foci (70).

During the development and progression of prostate cancer, multiple alterations occur in the AR molecule and its signaling pathway, including genetic mutations, gene amplification, protein overexpression and functional deregulations. In clinical settings, almost all the prostate cancers express the AR except for the small cell carcinomas, and no correlation was found between the AR expressing levels and clinical outcome. However, compared to primary cancers, hormone-refractory cancers display an increased AR expression at both mRNA and protein levels (20, 72-73). In a recent study using matched paired hormone-sensitive and resistant tumors (74), the copy numbers of AR locus on X chromosome was found to increase in 20% (10 out of 49) cases while only 2% (1 out of 48) of primary tumors displayed multiple AR copies. Consistently, tumors with higher copies of the AR gene showed significant high level of AR protein level in those matched cases. In addition, overexpression of AR protein was also detected in 35% of cases lacking AR gene amplification, indicating that alternative mechanisms exist for the increase of AR protein levels (i.e. decrease in degradation or increase in stabilization). In deed, enhanced stabilization of AR protein was found to be associated with the development of hormone-refractory tumor in a mouse xenograft model (75), resulting in hypersensitivity to castrated levels of androgens. In a well-designed PCR-based study (76), AR expression at the mRNA level was determined based on the cell type using cell type-specific RNA references. Compared to normal prostate tissues and localized primary tumors, epithelial levels of AR mRNA were significantly higher in hormone-refractory tumors. However, there is no significant difference in AR levels between normal tissues and primary tumors. Another group also reported similar results based on a cDNA microarray analysis using patient cancer tissues (77). Since expression of some AR-regulated genes (i.e. PSA and PAP) were not up-regulated in those cases with AR amplification (74), it is not clear if the increased AR expression levels reflect the likelihood of hormone refractory transition. Otherwise, increased AR expression might be involved in other signaling pathways that are responsible for hormone refractory progression of the disease.

Androgen-stimulated AR transactivation

Currently, the regulation of ligand-induced AR transactivation in a target cell is only partially understood, and most of the proposed mechanisms for AR transactivation are based on the work for the GR, a glucocorticoid receptor, which is a close-related member in structure to the AR (21). Classically, in response to androgen stimulation (binding), cytoplasmic localized AR is dissociated from the chaperon heat shock protein 70/90, phosphorylated and

then translocated into nuclear compartment for its genomic actions after interaction with chromatin although a DNA binding-independent mechanism for AR transactivation was also reported (78), and a recent review article exclusively summarized this part of current understanding (79).

In a tempt to dissect the mechanisms involved in androgen-induced AR transactivation, we recently demonstrated that androgen-induced AR transactivation involves two major cellular signaling molecules, phosphoinositide 3-OH kinase (PI 3-kinase, PI3K) and glycogen synthesis kinase-3β (GSK-3β) (80-81), which is consistent with previous reports (82-83). Inhibition of their function using specific inhibitors or using small interference RNA approach to knock down GSK-3β protein expression resulted in a suppression of androgen-mediated gene reporters or endogenous gene expression but not nuclear translocation of the receptor. Androgen treatment increased GSK-3β tyrosine phosphorylation that is associated with its enzymatic activity, while pretreatment with PI3K inhibitor abolished androgen-induced GSK-3β activation. The mechanism involved in androgen-mediated PI3K activation is under further investigation; however, a possible involvement of guanine nucleotide-binding proteins (G-protein) has emerged (84-86). Androgen-induced AR-responsive reporter activity was enhanced by overexpression of a Rho guanine nucleotide dissociation inhibitor (RDIα) (84-85), which is a negative regulator of small G-protein RhoA. Previous reports showed that RhoA can down-regulate PI3K activity (87-88). In addition, the trimeric G-protein subunits alpha-s and -q were reported to enhance AR transactivation when only a trace level of androgen is available (86), suggests that G-proteins are providing both positive and negative signals to androgen-mediated AR transactivation (89-90).

Androgen-regulated genes

As a transcription factor, ligand-activated AR interacts with chromatin at the androgen response element (ARE) in a given target gene promoter/enhancer region and regulates gene expression in cooperation with the general transcription machinery. The molecular communication between the AR and transcription machinery was recently reviewed elsewhere (91), thus we will focus on the target gene groups regulated by the AR.

As discussed above, androgens, acting via the AR, are central to prostate development and carcinogenesis, and understanding AR-regulated genes at the molecular basis in prostate cancer will potentially improve patient care and outcome. Using a high-through put genomic scale analysis, AR-regulated genes in prostate cancer, cell lines or patient tissues, were determined by several research teams (77, 92-94), and the listed genes were verified at the transcription level by RT-PCR or at protein level by Western blot and

immunostaining. In general, there are five groups of genes stimulated by androgen treatment, including cell proliferation, differentiation, apoptosis, metabolism, and secretory activity (79, 92-94). All prostate-derived AR-positive cancer cell lines, regardless of their androgen-dependency or AR mutation, displayed similar pattern of gene expression after androgen stimulation, indicating conservation of specific androgen responsiveness (92). The majority of androgen-stimulated genes are those involved in prostatic secretory fluid, which is the main function of the prostate gland (92). In two other reports, changes of AR-regulated genes were determined after androgen ablation or AR elimination (77, 95). In human prostate cancer cell LNCaP, androgen ablation or AR elimination by antisense approach resulted in several concordances in terms of gene expression alteration, including genes related to proliferative and cell cycle machinery, and fatty acid metabolism. However, some differences were also revealed. For example, AR elimination led to profound downregulation of insulin-like growth factor binding protein 2 (IGFBP-2) and the phosphatidylinositol-4-phosphatase 5-kinase type I alpha (PIP5KIA), while androgen blockage induced a dramatic change in the expression of the prostate overexpressed gene 1 and the S100 calcium binding protein P (S100P). These results suggest that a functional difference exits between androgen ablation and AR elimination (95).

Progressing to hormone refractory is currently a critical issue for curing prostate cancers, and understanding the genes that abnormally expressed in those late stage tumors will provide potential therapeutic targets. A recent study performed a genome-wide analysis of human prostate cancers during androgen ablation therapy (77), and they found that the hormone-refractory tumors showed a similar overall expression profiles as the untreated primary tumors in a hierarchical clustering algorithm analysis. These results indicate that a reversal event occurred when the tumor progresses to hormone refractory stage. In this process the effect of androgen ablation on prostate cancer cells disappeared and the AR signaling is reactivated (77, 94). However, a unique set of genes was also identified from those refractory tumors, in which increased AR expression is the most profound one that may contribute to the reactivation of the AR signal pathway. Another intriguing finding is that several genes involved in steroid precursor synthesis also increased in hormone refractory tumors.

Androgen receptor-mediated survival pathways

Apoptosis, or programmed cell death is a well-conserved process whose basic tenets remain common to all metazoans (96-97). It is usually controlled by two major execution pathways: the death receptor pathway or so-called extrinsic pathway; and the mitochondrial or intrinsic pathway. The extrinsic pathway is activated by binding of ligand to the death receptors located at the

plasma membrane such as the receptors for Tumor Necrosis Factor α (TNF-α), Fas ligand and TNF-α-related apoptosis-inducing ligand (TRAIL). Intracellular organelles, like mitochondria, are key participants in apoptosis. The main aspects of mitochondrial involvement in apoptotic process include two critical events, the release of mitochondrial proteins such as cytochrome c and the onset of multiple parameters of mitochondrial dysfunction such as loss of membrane potential.

In addition to the development in embryonic stage, the proliferation and differentiation in puberty and the physiological function in adulthood of the prostate gland, androgens are also critical for survival to the prostatic epithelial cell throughout its entire life. It is well demonstrated that the prostate gland responding to castration in rodent models or human cell lines responding to androgen deprivation or antiandrogens have shown a rapid apoptosis of the prostate epithelial cells, resulting in an extensive glandular regression (98-100). There is now evidence for a critical role of AR-mediated signaling pathway in cellular survival, although other AR bypassing pathways including castration-reduced blood flow are also emerging (101-102). The castration effects on blood flow to the prostate gland seem to be related to vascular degeneration associated with apoptosis of a subset of prostate endothelial cells (103). However we will mainly discuss the AR-mediated signaling pathways involved in cellular survival of prostate cancer. Currently only a few downstream targets involved in this pathway are identified.

Functioning as a nuclear transcription factor, the AR mainly exerts its effect through regulation of gene expression or so-called genomic effect although a DNA binding-independent mechanism was reported for AR transactivation by triggering of AR coactivators in some circumstances (78). Consistent with this notion, introduction of synthetic “decoy” androgen response element (ARE) oligonucleotides induced cell death in human prostate cancer LNCaP cells (104). On the other hand, a non-genomic effect of the AR through a protein-protein interaction mechanism on cell survival was also reported recently (105). Based on this knowledge AR-mediated survival pathways might be summarized in two aspects: genomic and non-genomic effects.

Cell cycle progression and cellular proliferation are controlled by cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CDKIs). CDKs are sequentially activated upon association with their partner cyclins. There are two checkpoints in each cell cycle: the G1/S checkpoint controls initiation and completion of DNA replication, and the G2/M checkpoint controls mitosis and cell division (106-107). Functional regulation of cyclin/CDK complexes by its inhibitors (CDKIs) is critical for cell cycle regulation at or before the checkpoints. There are two groups of CDKIs that control the checkpoints. The first one comprises $p16^{INK4a}$ and $p15^{INK4b}$, as well as p18 and p19 (106). Each

of these genes encodes a protein that specifically inhibits CDK4 and CDK6. Another group of CDKIs consists of $p21^{WAF1/CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$, which has considerable sequence homology and can inhibit all CDKs (106). In addition to G1 phase cyclins (cyclin A and B1) and CDKs such as CDK1, CDK2, CDK4 (95-96), CDKI $p16^{INF4}$ and $p21^{WAF1/CIP1}$ are also regulated by the AR in prostate cancer cells (108-112).

The CDK inhibitor $p21^{WAF1/CIP1}$ is a multi-function protein involving in cell proliferation, DNA repair and survival (106, 113-114). Although there are some evidences from the clinical studies and mouse xenograft models that $p21^{WAF1/CIP1}$ is involved in prostate cancer progression (116-122), its actual role in this process is still controversial. In experimental situations, opposite findings were reported (110-111, 114, 123). Earlier studies showed that $p21^{WAF1/CIP1}$ expression is stimulated by androgen treatment at a transcriptional level and an androgen responsive element (ARE) was identified in the promoter region of *p21* gene. Meanwhile, the basal level of $p21^{WAF1/CIP1}$ protein increased and was further enhanced by androgen stimulation in androgen-independent LNCaP sublines compared to its parental cell line (110-111). The authors stated that AR-mediated $p21^{WAF1/CIP1}$ expression might participate in androgen-induced antiapoptotic effect. On the other hand, some other reports provided evidences that p21 is inversely correlated with cell survival during prostate cancer progression (114, 123). Overexpression of $p21^{WAF1/CIP1}$ in prostate cancer cells not only resulted in cell arrest but also caused a significant apoptotic cell death (114). During the transition from androgen dependent to independent stage, AR expression increases but $p21^{WAF1/CIP1}$ level decreases in parallel in LNCaP cells. Meanwhile, a functional link between the AR and $p21^{WAF1/CIP1}$ expression was established by the fact that androgen treatment reduced largely the $p21^{WAF1/CIP1}$ protein level and inhibited $p21^{WAF1/CIP1}$ promoter activity via the putative ARE motif located in the promoter region. Furthermore, elimination of AR protein by an antisense oligonucleotide enhanced $p21^{WAF1/CIP1}$ promoter activity and increased $p21^{WAF1/CIP1}$ protein level in androgen-independent LNCaP cells (123). These evidences indicate $p21^{WAF1/CIP1}$ as an inducer of apoptotic cell death in prostate cancer cells, which is totally controversial with the aforementioned conclusion and is just exactly like the role of $p21^{WAF1/CIP1}$ itself in cell survival or death depending on cellular condition and stimuli (123-124).

The second downstream target involved in AR-mediated survival is the forkhead transcription factors in rhabdomyosarcoma (FKHR) family member FOXO1 (105, 125). The FKHR family proteins play an important role in many cellular processes including cell proliferation through regulation of CDK inhibitor $p27^{kip1}$ and D-type cyclones (126-127), cyclin B and polo-like kinase (128), as well as cyclin G2, EXT1 and Wip1 (129). This group of proteins is also involved in cell survival by up-regulating pro-apoptotic proteins such as Fas ligand, the insulin-like growth factor-binding protein 1 (IGFBP-1) and

Bcl-2 family protein Bim (129-135), as well as interacting with peroxisome proliferative activated receptor-gamma co-activator 1 (PGC-1, a transcription co-activator). In deed, overexpression of active FKHR leads to apoptotic cell death in human prostate cancer LNCaP cells (131, 137).

Two different mechanisms were proposed for AR-mediated survival through inhibition of FKHR action, AR interaction with FKHR (105) and AR-mediated FKHR degradation (125). In AR-null prostate cancer DU145 cells, overexpressed AR inhibited FKHR-mediated gene expression and cell death, which was not due to a competition of AR with FKHR for transcription co-factors but due to a complex formation between AR and FKHR. This interaction was also confirmed in an *in vitro* protein binding assay (105). However, AR-mediated enhancement of FKHR degradation was reported in LNCaP cells that express a native mutant *AR* gene. When the cells were treated for a rather long period of time (48-72 hours) with androgen, a cleavage fragment (p60) of FKHR protein was detected while a 24-hr treatment of androgen did not cause any changes of FKHR protein (105). Proteomic approach confirmed that the fragment is a c-terminal truncation of FKHR protein, which was cleaved by lysosomal acidic cysteine protease. This truncated form of FKHR in turn inhibits the function of the intact form of FKHR (125). Due to the heterogeneous feature of prostate cancers (138), both mechanisms for AR-mediated FKHR inhibition needs further verification.

The Bcl-2 family proteins are critical regulators that directly control the mitochondria function and consist of both pro- and anti-apoptotic members (reviewed in Ref. 139). Bax, Bak, and Bok are proapoptotic members, as are the BH3-domain only members such as Bad, Bik, Bim, and Bid. Antiapoptotic members include Bcl-2 and Bcl-x_L, Bcl-w, Mcl-1, and etc. It is believed that the relative levels of pro- and anti-apoptotic members are the key determinants in the regulation of cell death and survival. Bcl-2 protein was once reported to be elevated by androgen treatment in LNCaP cells in an earlier study (140), which was not confirmed by later reports (141-143). In contrast, Bcl-2 expression was found to increase after androgen ablation therapy, serving as a potential mechanism for androgen-independent progression of prostate cancer (reviewed in Ref. 144).

As another major anti-apoptotic member of Bcl-2 family, Bcl-x_L was recently identified by our group as a downstream effecter of AR-mediated cell survival pathway (69). As described earlier, in parallel to AR silencing and cell death, Bcl-x_L was the only member of Bcl-2 family proteins that was down-regulated. Consistently, enforced overexpression of Bcl-x_L partially protected cell death induced by the AR siRNA. More convincingly, our unpublished data showed that androgen treatment increased Bcl-xL expression at the transcription level, and the AR was found to bind to a putative androgen response element in the promoter region of human *bcl-x* gene (Fig 1). Taken

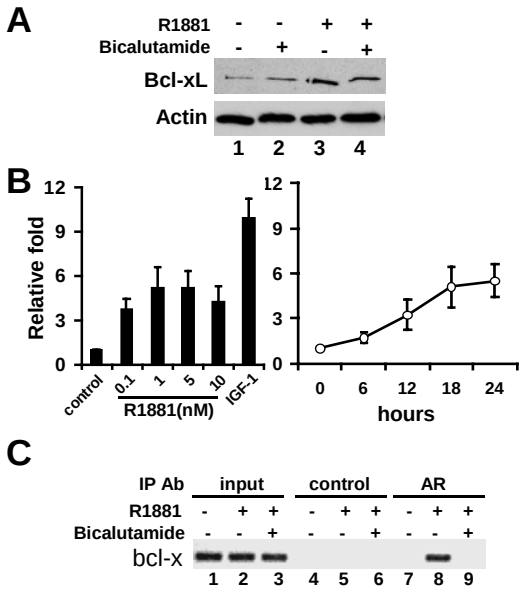

Figure 1. Androgen stimulates Bcl-xL gene expression *via* an AR-dependent mechanism. (A) After serum starvation for 24 h, LNCaP cells were treated with R1881 (1.0 nM) in the present or absent of bicalutamide (10 μM) for another 24 h. Cells were harvested and Bcl-xL protein level was determined by Western blot, and Actin blot served as loading control. **(B)** LNCaP cells were co-transfected with a luciferase reporter construct pBcl-xL-LUC together with an internal control reporter construct pCMV-SEAP overnight and then were serum-starved for 24 h. The solvent ethanol (control), R1881 in different doses as indicated or IGF-1 (10 ng/ml) alone was added once in the culture media containing 2% cFBS for another 24 h (left-half panel) or for several different time-points as indicated (right-half panel). Luciferase or SEAP activities were measured, and the luciferase activity was presented as fold induction against control sample after normalized with protein content and SEAP activity. **(C)** ChIP assay. LNCaP cells were serum-starved for 24 h and then untreated or treated with R1881 (1.0 nM) for 18 h in the presence or absence of the antiandrogen bicalutamide (10 μM). Binding of AR to the bcl-x promoter was determined with the ChIP assay as described in the text (lanes 7-9). As controls, sample lysates were also incubated with a normal rabbit serum IgG (lanes 4 and 6). Lanes 1 and 3 represent input signals obtained from 1% input chromatin. IP Ab, immunoprecipitation antibody.

together, our work demonstrated that Bcl-xL is a downstream target involved in AR-mediated cell survival in prostate cancer cells.

The fourth downstream target in AR-mediated survival is the c-FLIP (cellular FLICE-like inhibitory protein). FLICE [FADD (Fas-associated death

domain)-like interleukin-1β-converting enzyme] is one of the former names for caspase-8, which is one of the initial caspases (Caspase-2, -8, -10) involved in death receptor-mediated apoptosis (145-146). There are two variants of c-FLIP, short and long isoforms and most of the studies were dealt with the long form (c-$FLIP_L$) (146). Like caspase-8, c-$FLIP_L$ contains N-terminal tandem death effecter domains (DEDs), but its caspase domain is enzymically inactive that differs from caspases-8. In contrast, c-$FLIP_S$ only has two N-terminal DEDs that are very similar to the prodomains of caspase-8. Since the structural similarity, c-FLIPL is believed as an ideal inhibitor of death receptor-mediated apoptosis pathway. This concept was supported by data such that c-FLIP knockout mouse embryonic fibroblasts (MEFs) are more sensitive to death-receptor-induced apoptosis (147).

Almost all prostate-derived cancer cell lines are resistant to death receptor-mediated apoptosis compared to leukemia cells (148); however, this resistance can be reversed by additional reagents, for example, glycogen synthesis kinase-3β (GSK-3β) inhibitors as seen in our recent publication (149). In attempt to identify any gene alterations during castration-induced apoptosis in rat prostate, mRNA levels of genes encoding components of death receptor-mediated apoptosis pathway were analyzed using a quantitative real-time RT-PCR technique (150). The mRNA level for c-FLIP was the only one that reduced dramatically within the first 12 hours after castration, while the mRNA level of its interaction partner caspases-8 was not changed. Most interestingly, androgen administration to castrated animals restored the c-FLIP expression to normal levels, indicating an androgen-dependent regulation. Consistently, another report showed that androgen treatment activates c-FLIP promoter activity and gene expression in prostate cancer cells; and the AR is indeed recruited to the promoter region of human c-FLIP gene and several putative androgen response elements were identified (151). Overexpression of c-FLIP in prostate cancer cells confers its resistance to death receptor-induced apoptosis and promotes tumor progression in a mouse xenograft model (151-152). These reports are also somewhat in agreement to our findings that prostate cancer cells can be sensitized to death-receptor-mediated apoptosis by suppression of GSK-3β activity (149); meanwhile, GSK-3β activity is required for androgen-induced AR transactivation (81) although c-FLIP expression was not determined in our work.

Androgen receptor-related survival pathways

In recent years, androgen receptor-mediated non-genomic effect on multiple signaling pathways has emerged as an alternative mechanism for androgen action (153). In androgen-sensitive epithelial cells including prostate cancer LNCaP cells, the AR interacts and activates a major cellular signaling

cascade, the phosphoinositide 3-OH kinase (PI 3-kinase)-protein kinase B (also called Akt) signaling pathway (154-157). It has been very clear that PI-3k-Akt pathway plays an important role in providing cells with a survival signal that allows them to withstand apoptotic stimuli (reviewed recently in ref. 158-159). Upon activation, PI3K produces more lipid products such as phosphatidylinositol 3, 4, 5-trisphosphate and phosphatidylinositol 3, 4-bisphosphate on the inner surface of the plasma membrane, which is responsible for Akt activation (160-162). In contrast, a dual phosphatase PTEN (phosphatase and tensin homologue deleted from chromosome 10) dephosphorylates lipid products produced by the PI3K (163), resulting in inactivation of PI3K-Akt cascade. Somatic mutation or deletion of PTEN is a rather common event in solid tumors including prostate cancer (164-165), causing elevated levels of PI 3-kinase products and overactive Akt cascade. As a result those tumor cells are relatively resistant to apoptosis (166).

In early studies, the PI3K-Akt cascade was demonstrated as a dominant survival signal in a cell culture or mouse xenograft model based on LNCaP line harboring a mutant PTEN gene (167-169). Inhibition of the PI3K-Akt pathway but not other signal pathways caused a rapid apoptotic response, which was attenuated by androgen addition. This observation was confirmed by other reports (170-171). During the androgen independent transition after removal of androgens from the culture, LNCaP cells showed an increased level of PI3K-Akt cascade activity, indicating a compensational change had emerged. Increased Akt activity, enhanced phosphorylation of Akt downstream targets including pro-apoptotic Bcl-2 protein Bad, and diminished expression of CDK inhibitor $p27^{kip1}$ were observed in androgen-independent tumors, indicating their association with prostate cancer progression (168-169, 171). Although the mechanism responsible for androgen protection of PI3K inhibitor-induced apoptosis remains largely unknown, a possible role of Bcl-x_L was suggested (172). Ectopic overexpression of Bcl-x_L protein protected LNCaP cells from PI3K inhibitor-induced cell death, indicating Bcl-x_L is mediating a PI3K/Akt-independent survival cascade. In our recent study we demonstrated that Bcl-x_L is a downstream target of the AR survival pathway (69), which provides a possible mechanism in androgen protection against PI3K inhibition-induced cell death.

Targeting the AR as a novel strategy in prostate cancer therapy

Currently androgen-independent (or so-called hormonal refractory) phenotype is the major obstacle to curing prostate cancers. Androgen ablation by chemical or surgical approaches and AR blockage by anti-androgens have been shown to fail in controlling the disease (173). In certain circumstance, removal

of anti-androgens has been reported to result in regression of the disease (termed as androgen withdrawal syndrome, reviewed in ref. 51). Thus novel therapeutic strategies that target the disease at a molecular level are desirable to prevent or disrupt its progression.

As discussed above, the AR has been demonstrated as a critical molecule in prostate cancers, and then shifting the therapeutic target from androgens to the AR itself will be more realistic and efficient in terms of erasing its action on disease progression (174). A group from Austria reported recently that using an antisense oligonucleotide approach against the AR resulted in a significant inhibition of cell proliferation *in vitro* (175) and tumor growth *in vivo* (176). Among various oligonucleotides with different sizes and targeting segments on the AR sequence, a 15-base oligonucleotide targeting the CAG repeats was identified to be most effective in suppressing AR protein level in an LNCaP cell-based *in vitro* assay. Treatment of the cells with the particular oligonucleotides for 24 hours caused a more than 90% reduction of AR expression. Cell proliferation and PSA secretion were significantly inhibited. A similar effect was also observed in LNCaP-derived subline that has an androgen-independent phenotype. This antisense oligonucleotide was also used in a mouse xenograft model derived from LNCaP cells. Surgical implantation of a diffusion pump containing the oligonucleotides near the xenograft for 7 weeks resulted in around 40% reduction in tumor weight compared to control groups. However, no significant cell death was observed after antisense oligonucleotides treatment compared to control group although the cell proliferation maker Ki67 was found to be correlated with tumor size. These pioneer works pointed out that targeting the AR might be a permissive approach for curing prostate cancers although more improvements are needed to enhance its efficiency.

Targeting the AR for prostate cancer treatment is also the major focus of our group. Using a more efficient and gene-specific silencing approach, RNA interference, we demonstrated that silencing AR expression in native AR-positive prostate cancer cells led to a massive cell death (69). Using a computer-based design tool (*OligoEngine*™, www.oligoengine.com), we synthesized several small interfering RNA (siRNA) duplexes and tested them on different prostate cancer cell lines. Of 34 siRNA duplexes, we identified 4 duplexes that efficiently knocked down AR expression in mRNA and protein levels by 3-4 days after transfection. To our surprise, in addition to cell arrested as reported by others using AR siRNA (68, 177), a massive apoptotic cell death was observed when the siRNA-transfected cells were kept in a hormone-free condition for up to 4 days. Only a tract percentage of cells could survive after 12 days under the condition. These phenomena occurred in all AR-positive prostate cancer cells regardless of their androgen dependency. However, no cell death was induced in AR-null prostate cancer cells or such

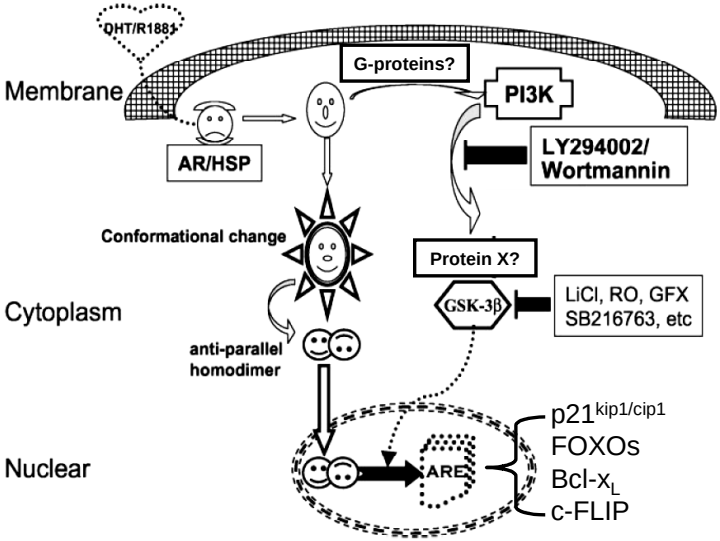

Figure 2. Schematic illustration of the proposed mechanism of AR transactivation in prostate cancer. Upon androgen binding, the AR is dissociated from the chaperon heat shock proteins (HSP) and then translocated into the nuclear as an antiparallel homodimer after a conformational change. In addition, androgen treatment also results in PI3K activation, possibly via G-proteins, which in turn leads to GSK-3β tyrosine phosphorylation through unknown mechanism. Finally, activated GSK-3β regulates the assembly of AR-mediated transcriptional complex in the nuclear.

cells that were even reconstituted with an exogenous *AR* gene, indicating the AR-null cells escaped already from AR control for survival. In addition, no cell death was found in breast cancer cells although they are AR-positive, suggesting a cell-specificity for AR-mediated survival. These results suggested that targeting the AR with the siRNA approach is a powerful therapeutic measurement to treat prostate cancers at any stage as long as they express the AR.

Conclusion

Prostate cancer is a major health threat to Western men, and currently no efficient cure exists when it becomes resistance to androgen ablation therapy. The AR gene as well as its protein product is the only one constantly and profoundly upregulated during progression of the disease. In prostate epithelium or its cancerous compartment, the AR controls most of the aspects of cell fate including proliferation, differentiation, secretion and survival.

During the development and progression of prostate cancer, the AR is always a critical molecule. Overexpressed AR promotes cell growth under a trace level of androgen *via* its genomic effects. Under castration condition, the AR is still active in cancer cells after reoccurrence *in vivo* but the mechanisms are not fully clear. Blocking its function using a specific antibody or reducing its expression using a gene-specific ribosome or antisense oligonucleotides led to growth inhibition both *in vitro* and *in vivo*. Silencing the AR gene using a more efficient approach, RNA interference, could induce a massive cell death after a short period of growth inhibition. AR siRNA-induced cell death was observed in both androgen-dependent and -independent prostate cancer cell lines but not in AR-null prostate cancer cells or none-prostate cells. The siRNA-based approach might be a permissive measurement for curing prostate cancers although further testing in a mouse xenograft model is desirable.

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Gα12 is required for Androgen Receptor transactivation in prostate cancer

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Introduction and Objective: The Androgen Receptor (AR) is a ligand-dependent transcription factor that plays a critical role in the development and progression of prostate cancer by regulating target genes involved in cellular proliferation and survival. In attempt to dissect the mechanisms involved in androgen-induced AR transactivation, our group demonstrated the involvement of two major signaling molecules, phosphoinositide 3-OH kinase (PI3K) and glycogen synthesis kinase-3β (GSK-3β). Also, a possible involvement of guanine nucleotide-binding proteins (G-protein) or their relative members in AR activation has emerged recently. To identify novel components that participate in the AR transactivation, we tested if Gα proteins or its related members are involved in AR activation. Methods: Two prostate cancer cell lines, LNCaP and LAPC-4, were used in this study. Two androgen responsive gene reporter constructs, ARE-LUC and Probasin-SEAP, were utilized to access the signaling pathways involved in AR transactivation. To determine the involvement of Gα-proteins on the AR transactivation, we used a targeted expression of constitutively activated forms of Gα proteins and a knock-down expression of Gα proteins using small interference RNA (siRNA). Transient transfection of Green fluorescent protein (GFP) conjugated AR was performed to investigate nuclear translocation of AR after activation. Results: Over expressing constant active mutants of Gα proteins such as Gα12, Gαs, Gαi and Gαq showed different effect on the basal level or androgen (R1881)-induced reporter activities. Consistent with a recent report, only active Gαs but not others enhanced the basal reporter activity. Active Gαq overexpression resulted in a significant inhibition of androgen-induced reporter activities, but Gα12 and Gαi enhanced androgen-induced reporter activities. In the knockdown experiments using a siRNA approach, we found that knockingdown Gα12 and Gαs reduced androgen-induced reporter activities, but Gαq and Gαi did not showed a dramatic effect. In the AR nuclear translocation experiments, we found that knocking down Gα12 suppressed AR nuclear translocation after androgen treatment. Finally, we demonstrated that Gα12 silencing impaired androgen-induced activation of PI3K and GSK-3β, which were required for AR activation. Conclusions: Taken together, our findings clearly demonstrated that Gα12 is required for androgen-induced AR transactivation in prostate cancer cells, which provides a novel target for better management of prostate cancer.
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[2005] [390] ANDROGEN RECEPTOR-MEDIATED REGULATION OF BCL-X_L EXPRESSION: IMPLICATION IN CELLULAR SURVIVAL OF PROSTATE CANCERS

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INTRODUCTION AND OBJECTIVE: Recently, the androgen receptor (AR) has been demonstrated to play a critical role in the development and progression of prostate cancer. However, the precise pathway for AR-mediated survival is unclear. We sought to identify Bcl-x_L, an anti-apoptotic member of Bcl-2 family, as one of the downstream effectors responsible for AR-mediated survival in prostate cancer cells.
METHODS: We first demonstrated that expression of the *bcl-x* gene is upregulated upon androgen treatment in prostate cancer cells and the AR is bond to the promoter region of the *bcl-x* gene. This suggests that the AR is involved in the transcriptional regulation of the gene. We then knocked down the AR protein level by using a small interfering RNA approach.
RESULTS: Reduction in AR protein level resulted in a profound decrease of Bcl-x_L transcript and protein levels. In parallel, a significant apoptotic cell death occurred after AR silencing, as evidenced by an increased Annexin V binding, reduced mitochondrial potential, enhanced caspase-3/6 activation, and cleavage of DFF45 and PARP proteins. The apoptotic response was only found in those siRNA-transfected cells that harbor a native *AR* gene, but not in the AR-null prostate cancer cell PC-3 or its subline that has been reconstituted with an exogenous *AR* gene. Most interestingly, ectopic enforced expression of Bcl-x_L protein partially inhibited apoptosis resulted from AR silencing, while reducing Bcl-xL expression significantly enhanced AR siRNA-triggered apoptosis in prostate cancer cells.
CONCLUSIONS: Our data demonstrated that Bcl-x_L is involved in AR-mediated survival pathway in prostate cancer cells that may play an essential role in prostate cancer progression.

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