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13. ABSTRACT (Maximum 200 Words) To develop a novel therapeutic approach for hormone-refractory prostate cancers, we proposed to knock down human androgen receptor (AR) gene using an RNAi-based technique. Based on our previous work, we generated a recombinant adeno-associated virus bearing a hairpin-structured small interfering RNA against the AR (ARHP8) for long-term expression of the AR siRNA. A control virus bearing the GFP gene only was also produced. We demonstrated that the resultant rAAV.ARHP8 knocked down the AR expression both in protein and mRNA levels in prostate cancer LNCaP cells. Next, we will use the virus to treat prostate cancer xenografts in a nude mice model.				
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Annual Report for the Project W81XWH-04-1-0214
February 2005

Project Title:

INHIBITION OF ANDROGEN-INDEPENDENT GROWTH OF PROSTATE CANCER BY siRNA-MEDIATED ANDROGEN RECEPTOR GENE SILENCING

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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 are 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 past year, we have accomplished all the tasks for the period as described in the Statement of Work (SOW) of the proposal.

To constitutively knockdown an endogenous *ar* gene product, a hairpin-structured construct containing an effective AR siRNA sequence (ARHP8) was generated for targeting human *ar* gene as shown in Fig 1A. To achieve a massive success in delivering the exogenous DNA to cultured mammalian cells or xenografts in animal model, an Adeno-associated virus (AAV) delivery system was chosen. To simplify the procedure of AAV preparation, we changed the plan outlined in the original proposal and used a commercial available Helper-free AAV construction system to create our ARHP8-bearing AAV. This system provides a *bi-cistronic* plasmid pAAV.IRES.GFP vector for monitoring the target gene expression after inserted in the upstream of IRES-GFP cDNA sequence, which fulfills our needs as described in the proposal. So, the U6-ARHP8 expression cassette for the AR #8 siRNA hairpin was isolated from *pU6.ARHP8* plasmid vector and then inserted into the pAAV.IRES.GFP to create the pAAV-ARHP8. The resultant pAAV-ARHP8 plasmid was co-transfected with other two helping vectors (pAAV.RC and pHelper) for packaging the recombinant AAV bearing the ARHP8 (rAAV.ARHP8). A control AAV (rAAV.GFP) was made by using pAAV.IRES.GFP instead of pAAV-ARHP8. The titer of rAAVs was determined in HT1080 cells according to the manual. About 4×10^{12} viral particles per ml were obtained. A representative picture of AAV infection in LAPC-4 was shown in Figure 1. To examine if the resultant rAAV.ARHP8 is effective on knocking down AR expression, LNCaP cells were infected with the AAV at 10^5 per ml viral particles and the AR protein and mRNA levels were measured 5 days later. As shown in Fig. 2, the rAAV.ARHP8 significantly reduced AR expression both in mRNA and protein levels.

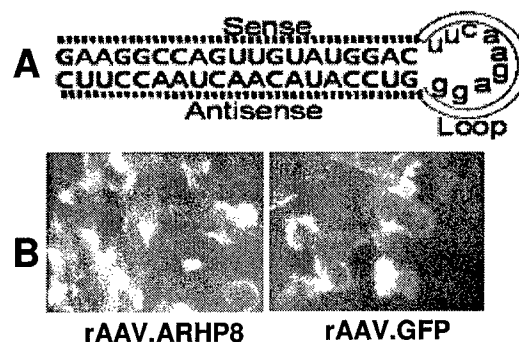


Fig. 1. AAV infection. (A) A hairpin-structured fragment used in AAV.ARHP8 preparation. (B) LAPC-4 cells were infected with AAV virus as indicated and photos were taken 3 days later under a fluorescent microscope.



Fig. 2. ARHP8 virus induces significant knockdown of AR gene expression. After infection with rAAV.GFP (lane 1) or rAAV.ARHP8 (lane 2) for 5 days, LNCaP cells were harvested and total RNA or cellular protein were extracted for RT-PCR (A) or Western blot (B) assays as described (Ref. 1-2).

2. Other Achievements

In addition to experimental progresses described above, this DOD-founded grant also provided a solid support for the P.I. Dr Benyi Li in submitting a patent application (*Appendix II*) in 2004. Also, two meeting abstracts based on this project were accepted for poster presentation as listed below (Ref 1-2), a manuscript (*Appendix I*) describing the major findings achieved from this project was accepted for publication in *Mol Cancer Therapeutics* this month and a R01 proposal of [*Bcl-xL and AR-dependent Survival*] based on the results from this project was submitted to NIH on Feb 1st 2005.

Key Research Accomplishments

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

1. Generation of a recombinant adeno-associate virus (rAAV) bearing the hairpin-structured small interfering RNA against human *androgen receptor* gene (ARHP);
2. Demonstration of the efficiency of the resultant rAAV.ARHP8 in knocking down the AR expression in mRNA and protein level in prostate cancer cell LNCaP cells that express an endogenous *AR* gene.
3. Generation of another rAAV (rAAV.GFP) that lacking the ARHP8 fragment for a negative control;
4. Determination of the efficiency of other siRNA sequences (including #1, #2, #7, #34) in knocking down the AR protein level in prostate cancer LNCaP and LAPC-4 cells. The corresponding rAAV shuttle vectors are created.

Reportable Outcomes

1. The rAAV.ARHP8 and a control rAAV.GFP
2. Functional AR siRNAs; including #1, #2, #5, #7, #8, #31, #34, sequence information is available upon request.
3. An accepted manuscript for publication in *Molecular Cancer Therapeutics*, 2005;
4. A patent application with a title of "METHOD FOR TREATING PROSTATE CANCER USING siRNA DUPLEX FOR ANDROGEN RECEPTOR".

Conclusion

In this first-year period, we conducted the experiments according to the State of Work (month 1-12), and also other related works. Based on our previous work, we generated the rAAV bearing the ARHP8 sequence and the resultant virus was demonstrated to knock down the AR in prostate cancer LNCaP cells, both at mRNA and protein levels. Also, we tested more siRNA sequences against the AR identified by the OligoEngine software. We determined a total of 7 (out of 34 sequences) siRNAs that are potent in knocking down the AR expression. We also generated the AAV shuttle vectors bearing those sequences for future experiments. Once we have any difficulty in knocking down the AR in animal model we may switch to these siRNA sequences.

Based on our current work, a manuscript was submitted and accepted for publication in *Molecular Cancer Therapeutics* and a US patent application was filed in 2004.

Reference

- (1) Benyi Li and Liao X. AR silencing induced by a siRNA duplex leads to apoptosis in prostate cancer. *AACR annual meeting 2004*, Orlando, FL.
- (2) Benyi Li and Liao X. Bcl-xL is a downstream effector of AR-mediated survival pathway in prostate cancer. *Keystone Symposium Feb 2005*, Monterey, CA.

Appendices

Xinbo Liao, Siqing Tang, J. Brantley Thrasher, Tomas Griebing, and Benyi Li. Small-interfering RNA-induced androgen receptor silencing leads to apoptotic cell death in prostate cancer. *Mol Cancer Ther.*, 2005.

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

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Running Title: AR silencing leads to apoptosis

Keywords: androgen receptor, apoptosis, gene silencing, prostate cancer, RNA interference, small-interfering RNA.

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Abbreviations: AR, androgen receptor; BH domain, Bcl-2 homology domain; CMV, cytomegalovirus; cFBS, charcoal-stripped fetal bovine serum; FACS, fluorescent-activated cell sorting; FCM, flow cytometry; GFP, green fluorescent protein; HEK, human embryonic kidney; LUC, luciferase; Neo, neomycin; PBS, phosphate-buffered saline; PI, propidium iodide; PARP, poly(ADP-ribose) polymerase; PI3K, phosphatidylinositol 3-kinase; PCR, polymerase chain reaction; PIAS, protein inhibitor of activated STAT; PS, phospholipid phosphatidylserine; Puro, Puromycin; RIPA, radio-immunoprecipitation assay; RNAi, RNA interference; RT, reverse transcription; SD, standard deviation; SEAP, secreted alkaline phosphatase; siRNA, small-interfering RNA.

Abstract

Prostate cancer is the second leading cause of cancer death in the United States, and so far, there has been no effective therapy for the treatment of hormone-refractory disease. Recently, the androgen receptor (AR) has been demonstrated to play a critical role in the development and progression of the disease. In this report, we demonstrated that knocking down the AR protein level by a small interfering RNA 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 PARP 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-x_L was significantly reduced that might account for the apoptotic cell death since ectopic enforced expression of Bcl-x_L protein partially inhibited apoptosis after AR silencing. Taken together, our data demonstrated that knocking down the AR protein level in prostate cancer cells leads to apoptosis by disrupting the Bcl-x_L-mediated survival signal downstream of AR-dependent survival pathway.

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; and 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, anti-androgen 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 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 demonstrated 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 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 mitochondria function and consist of both pro- and anti-apoptotic 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-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.

The *bcl-x* gene encodes multiple spliced mRNAs, of which Bcl-x_L is the major transcript (23-24). Like Bcl-2, Bcl-x_L protects cells from apoptosis by regulating mitochondria 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-x_L may prevent apoptosis *via* a cytochrome c-independent pathway (25). Although Bcl-x_L protein can be regulated post-transcriptionally, it is mainly controlled at the gene expression level (26-27). Bcl-x_L protein is detected in the epithelial cells of normal prostate gland and prostate cancers in an earlier report (28). The expression level of Bcl-x_L protein correlated with higher grade and stage of the disease, indicating an important role of Bcl-x_L in prostate cancer progression.

RNA interference (RNAi) is a recently discovered mechanism of post-transcriptional 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-x_L was also decreased in

parallel with AR silencing, and overexpression of exogenous Bcl-x_L controlled by a CMV 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 ATCC (Manassas, VA). The hormone-refractory prostate cancer cell LNCaP-Rf was a kind gift provided by Dr. Donald Tindall (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 (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-x_L 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 (Tampa, FL), and LNCaP/Puro 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 Biotech (Santa Cruz, CA). Antibodies against caspases, cytochrome c, Bcl-2 family members, XIAP, DFF45 and PARP were obtained from Cell Signaling (Beverly, MA). JC-1 fluorescent dye was obtained from Molecular Probes (Eugene, OR). Charcoal-stripped fetal bovine serum (cFBS) was obtained from Atlanta Biologicals (Norcross, GA). Other reagents were supplied by Sigma (Saint Louis, MO).

siRNA Synthesis, Labeling and Transfection

Sequence information regarding the human *AR* gene (GenBankTM accession NM_000044) was extracted from the NCBI Entrez nucleotide database. Up to 34 mRNA segments were identified using the *OligoEngine*TM 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 NCBI BlastN database. The siRNAs were prepared by a transcription-based method using the *Silencer*TM 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 RiboGreenTM fluorescence (Molecular Probes). A *Silencer*TM siRNA labeling kit using a fluorescent Cy3 dye (Ambion Inc.) was used for labeling the siRNA duplexes according to the manufacturer's instructions. The purified siRNA duplexes were transfected into cells with the *Oligofectamine*TM reagent (Invitrogen Co., Carlsbad, CA) in a medium supplied with 2% cFBS. The media were changed every 3 days. A scrambled negative siRNA duplex (Ambion Inc.) 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 phosphate-buffered saline (PBS) and lysed in a RIPA buffer supplied with protease inhibitors (CytoSignal, Irvine, CA). Western blot analysis was performed as described previously (32-35) to assess the protein expression level of target molecules. Blots were developed with a *SuperSignal West Dura*TM substrate kit (Pierce Biotech, Rockford, IL). Immunofluorescent staining was performed as previously described (34-35). The picture was taken under a fluorescence microscope (Nikon) set at 200× magnification.

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 min at room temperature in the dark and analyzed on a FACS Calibur 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 RT-PCR

Total RNA was prepared using *Trizol*TM reagent (Invitrogen Co., Carlsbad, CA). 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*TM kit (Ambion Inc. Austin, TX) per manufacturer's manual. The primers and PCR conditions were described as follow: for human *AR* gene (forward 5'-cctggcttcgcaacttacac-3'; backward 5'-ggacttgtgcatgcggtactca-3', adapted from Ref. 6); human *PSA* gene (forward 5'-gatgactccagccacgacct-3'; backward 5'-cacagacaccccatcctatc-3', Ref. 37); human *bcl-xl* gene (forward 5'-catggcagcagtaaagcaag-3'; backward 5'-gcattgttcccatagagtcc-3', Ref. 38). 28S ribozyme RNA (forward 5'-gttcacccactaatagggaac gtg-3'; backward 5'-gattctgacttagaggcggttcagt-3') was used as an internal control. The primers were synthesized by IDT (Coralville, IA). The amplification profile was as follows: 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min 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 min 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 using a *Fluoscan™* fluorescent reader as described previously (32-34).

Statistical Analysis

All experiments were repeated two or three times. 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

Since 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 been emerged as a novel strategy for prostate cancer therapy (13-15). Recently, RNA interference has been demonstrated 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 (#8, 5'-AAGAAGGCCAGUUGUAUGGAC-3'; #31, 5'-AAGACGCUUCUACCAGCUCAC-3') were identified in knocking down *AR* expression in the initial experiments when compared to others. The *AR* knockdown effect was further confirmed by checking the mRNA level followed by Western blot. A well-known androgen target prostatic specific antigen (PSA) was also down-regulated as determined by a RT-PCR assay. This knocking down effect was achieved as a sequence-specific event since a negative control siRNA with a scrambled sequence had no effect on *AR* protein or PSA mRNA level (Fig. 1A). Both #8 and #31 *AR* siRNAs significantly knocked down *AR* expression at a final concentration of 1.0-10 nM 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 LAPC-4 cells, which harbor an endogenous wild-type *AR* gene, were used (Fig. 1C). These results demonstrate 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 demonstrated 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 #8 *AR* siRNA or a pooled *AR* siRNA mixture (Figure 2A). However, the difference was that a massive cell death was observed if the cells were monitored for more than 4 days after siRNA transfection. To test if the cell death response is due to siRNA-mediated *AR* silencing, we performed a time-course experiment in LNCaP (hormone-sensitive) and C4-2 (hormone-refractory) cells. The cells were transfected with the #8 *AR* siRNAs or a scrambled negative siRNA in 2% cFBS. 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 to 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 to LNCaP cells.

Next, we asked if the AR siRNA-induced cell death was simply due to a cellular non-specific response to the double-strand siRNA, i.e. interferon response (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 #8 or #31 AR siRNAs significantly reduced the survival rate for more than 95% in LNCaP-Rf cells compared to the control siRNA. In contrast, the cell survival rate 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 non-specific cellular response to the double-strand 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 might not depend on the AR so that the cells remain survival.

AR siRNA-induced Cell Death Occurs Specifically in Prostate-derived Cells

In addition to those common-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 non-tumorigenic prostate epithelial cell line (41) while 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 non-tumorigenic RWPE-1 cell line demonstrated 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 the #31 AR siRNAs with a fluorescent dye (Cy3) and then transfected them into LNCaP cells. Cells were maintained in 2% cFBS 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 demonstrate 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 demonstrated that androgen ablation or antiandrogens induces apoptotic cell death in prostate epithelium and 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 (PS), 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 PS translocation, while the control siRNA had no effect.

Since 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 transfected with #8 AR siRNA (as pointed by arrows in Fig. 5B, panel c&d) while living cells remained normal membrane potential (red fluorescence as pointed with arrow-head 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 #8 AR siRNA was transfected into cells. Meanwhile, the apoptosis hall-marker PARP 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 the #31 AR siRNA into LNCaP cells induced significant reduction of the procaspase-3 and -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 significant increased when #31 AR siRNA was used compared to negative control siRNA (Fig. 6C). Thus, these data clearly demonstrated that the mitochondrial apoptotic mechanism is activated by the AR siRNAs.

Anti-apoptotic Protein Bcl-x_L is involved in AR-mediated Cell Survival

Having demonstrated the mitochondria 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, and subsequent assembly of an active apoptosome (22). These functions are promoted by the pro-apoptotic Bax or Bak, and are inhibited by the anti-apoptotic Bcl-2 and Bcl-x_L. 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 anti-apoptotic member Bcl-x_L dramatically decreased in the #8 AR siRNA transfected cells compared to the controls whereas another anti-apoptotic member Bcl-2 and the pro-apoptotic members Bax and Bak remained unchanged (Fig. 7A). To better illustrate the relationship of Bcl-x_L reduction with AR silencing, we conducted a time-course experiment (Fig. 7B). The protein levels of Bcl-x_L 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-x_L reduction that might lead to an imbalance between the pro- and anti-apoptotic members of the Bcl-2 family that in turn triggers apoptosis.

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

AR siRNA-induced Apoptosis was partially inhibited by Ectopic Bcl-x_L Expression

In view of the anti-apoptotic feature of Bcl-x_L protein, we hypothesized that the AR promotes cellular survival by up-regulating the *bcl-x* gene expression through a transcriptional mechanism in prostate cancer cells. Therefore, Bcl-x_L expression will decrease if the AR is knocked down, which subsequently results in apoptosis due to an imbalance between the pro- and anti-apoptotic members of the Bcl-2 family. Thus, we wondered if an enforced Bcl-x_L expression will protect cell from apoptosis while AR is silenced. To assess the protection effect of Bcl-x_L protein, a stable LNCaP subline over-expressing human Bcl-x_L protein controlled by a CMV promoter (LNCaP/Bcl-x_L) or a control subline with an empty vector (LNCaP/Puro) were established. Consistent with the results obtained from the parental cells (Fig. 7A), exposure of those LNCaP subline cells to #8 AR siRNA

resulted in a decrease of endogenous but not exogenous Bcl-x_L protein (Fig. 8A, lane 1 vs lane 2). Most significantly, as expected, enforced Bcl-x_L expression partially inhibited cell death induced by AR siRNA transfection in LNCaP/Bcl-x_L cells compared to the controls (Fig. 8A, lower panel). These data demonstrated that Bcl-x_L is involved in AR-mediated survival of prostate cancer, and the reduction of Bcl-x_L expression after AR silencing represents a mechanism for the AR siRNA-induced apoptosis.

In addition, while establishing a subclone for stable Bcl-x_L expression in LNCaP cells, an unexpectedly clone (LN#11) was obtained, in which Bcl-x_L expression was dramatically reduced for unknown reason, as confirmed by RT-PCR and Western blot (Fig. 8B, upper and middle panels). By taking the advantage of this particular clone of LNCaP cell subline, we further tested the involvement of Bcl-x_L in AR-mediated survival. Exposing the LN#11 subline cells to the #8 AR siRNAs resulted in a significant increase in AR siRNA-mediated cell death compared to the parental LNCaP cells and the untransfected controls (Fig. 8B, lower panel), although the LN#11 subline did not show a profound cell death with AR silencing. These data indicate that loss of Bcl-x_L expression enhances AR siRNA-induced cell death, and multiple downstream factors, besides Bcl-x_L, 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 their androgen sensitivity. Further analyses demonstrated that Bcl-x_L expression is transcriptionally dependent on the AR in prostate cancer cells, and siRNA-mediated AR silencing results in a reduction of Bcl-x_L expression

that accounts partially for the apoptotic response since enforced Bcl-x_L expression inhibited cell death after AR silencing. To the authors' knowledge, this is the first report showing AR involvement in Bcl-x_L 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 due to 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) since the AR protein still exists in the cell. It is believed that 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 demonstrated 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 account also for the different outcome.

It has been demonstrated 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 fully 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), while 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 or agonists. The ratio of death antagonists to agonists determines how a cell responds to an apoptotic signal. Like Bcl-2, Bcl-x_L 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, AP-1, Oct-1, Ets, Rel/NF- κ B, STATs (signal transducer and activator of transcription), and GATA-1, in which three transcription factor families, STATs, Rel/NF- κ B, and Ets family, have been demonstrated 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 interferon 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 interferon response than the chemically synthesized ones (our unpublished observation). 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 interferon-related effect. Moreover, it was reported that only TNF- α but not interferon down-regulates Bcl-x_L expression (54), suggesting that the reduction of Bcl-x_L protein is not due to the siRNA-triggered interferon response.

In conclusion, our results demonstrated 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-x_L expression because enforced Bcl-x_L 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 means of cure.

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Figure Legend

Figure 1. siRNA-mediated AR gene silencing in prostate cancer cells. **A**, Following transfection with the siRNA duplexes (final concentration at 10 nM in the medium) as indicated, cells were harvested at 48 h (upper panel) or 72 h (bottom panel) later. The mRNA levels of target genes as indicated were determined by RT-PCR assay (upper panel) and the AR protein was determined by western blot (lower panel) and actin blot served as loading control. The siRNA was omitted in the mock control (up panel). **B**, Cells were transfected with different among of AR #8 siRNA and then harvested at 72 h later. Western blot was done as above. **C**, LAPC-4 cells were transfected with the siRNA duplexes (10 nM in the media) 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 6-well plates were transfected with the siRNAs (10 nM in the media) as indicated. **A**, The total number of living (*white or unstained*) cells from each time point was counted by trypan blue exclusion assay. **B&C**, the survival rate (*white cells vs 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 nM in the media) as indicated. The clonogenic survival fraction of the cells was determined on day 7 post-transfection. Colonies were fixed, stained, and counted. The survival rate in control group was designated as 100%. Data represents three different experiments.

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 #8 AR siRNA at 10

nM in the culture medium supplied with 2% cFBS, and cell survival rate was determined 7 day later by trypan blue exclusion assay. **B**, Cells harvested from the experiments described in panel **A** were lysed for Western blot to determine the protein levels of the AR. Actin blot served as loading control.

Figure 4. Visualization of the Cy3-labeled AR siRNA-induced cell death. LNCaP cells were seeded in 6-well plates overnight and then transfected with Cy3-labeled siRNAs (10 nM in the media) as indicated and cell death was monitored daily. Pictures were taken at day 1 and day 4 after transfection. The Cy3-labeled siRNAs are seen as white dots in Cy3 panels (*b, d, f, h*). In panel *g* and *h*, white arrows indicate several living cells without the Cy3-labelling (negative transfection) while 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 nM in the media) as indicated for 4 days, LNCaP cells were harvested and the change of the membrane phospholipid phosphatidylserine (PS) was determined using FACS for FITC-labeled cells as described in the text. Data represents two different experiments. **B**, Following transfection with the siRNA duplexes (10 nM in the media) for 5 days, LNCaP cells were incubated with JC-1 (0.3 μ g/ml) for 15 min at 37°C. Pictures were taken under a fluorescent microscope (magnitude x 200).

Figure 6. AR siRNA induces cytochrome c release, caspase activation and cleavage of DFF45 and PARP. **A&B**, After 7 days 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, DFF45 and PARP cleavage were determined by Western blot. **C**, After 7 days 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. The mean value of the relative activity was shown from three independent experiments.

Figure 7. AR siRNA transfection leads to reduction of Bcl-x_L expression. **A&B**, After transfection with #8 AR siRNA or negative control siRNA (10 nM in the medium) as indicated, LNCaP cells were harvested (**A**) on day 7 or (**B**) at each time point, and the protein levels of AR, Bcl-2, Bcl-x_L, Bax, Bak and XIAP were assessed by Western blot. Data was reproducible in three independent experiments. **C**, After transfection with the indicated siRNAs (10 nM in the medium), LNCaP cells were harvested on day 7, and the total RNA was isolated and the Bcl-x_L mRNA level was assessed by RT-PCR as described in the text. **D**, Similar to panel **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.

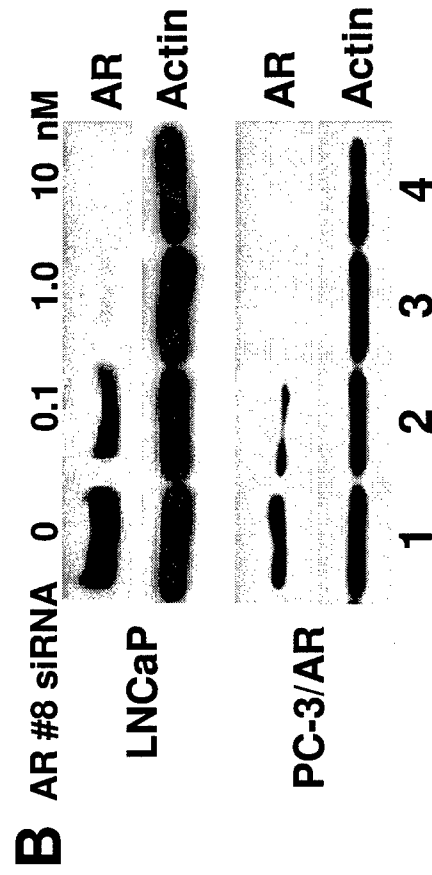
Figure 8. A, Ectopic enforced expression of *bcl-x_L* gene inhibits AR siRNA-induced cell death. LNCaP/Puro and LNCaP/Bcl-x_L cells were transfected with #8 AR siRNA for 7 days and the expression level of endogenous/exogenous *bcl-x_L* gene was determined by Western blot. Since the exogenous Bcl-x_L protein has a HA tag, the membrane was reprobed with anti-HA antibody to show the exogenous Bcl-x_L protein. Actin blot served as loading control. The cell death rate (*blue cells vs blue plus white cells*) was determined individually by trypan blue exclusion assay. The asterisk indicates a significant difference ($P < 0.05$) between LNCaP/Puro vs LNCaP/Bcl-x_L cells after the #8 AR siRNA transfection. Data represent three independent experiments. **B**, Lose of Bcl-x_L expression lead to a significant increase of AR siRNA-induced cell death. *Upper panel*: the parental LNCaP cells (*lane 1*), LNCaP subline LN#11 (*lane 2*) and a stable subclone bearing an empty vector (*lane 3*) were exponentially grown and harvested. Total RNA was isolated from Bcl-

x_L 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- x_L protein levels were assessed by Western blot, and anti-Actin blot served as loading control. Data represent two separate experiments. *Lower panel*: cells were transfected with negative siRNA (*black column*) or #8 AR siRNA (*shaded column*) at 10 nM in the culture medium supplied with 2% cFBS. Cell death rate [*dying cells vs (dying plus living cells)*] was determined 5 day later by trypan blue exclusion assay as described earlier. The asterisk indicates a significant difference ($P < 0.05$) between LNCaP subclone #11 (LN#11) vs the parental LNCaP cells.

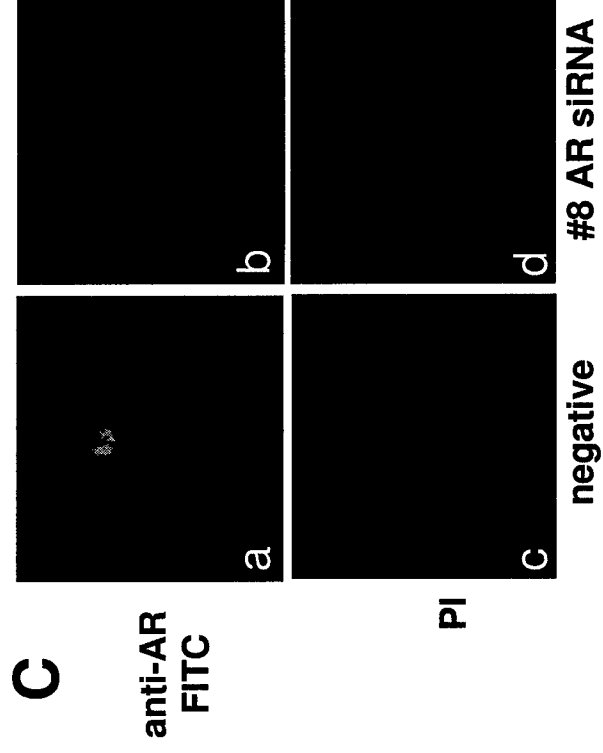
Table 1. Summary of cell lines used in this study

Cell Line	Origin and Modification	AR status	Hormone Response	Reference
LNCaP	human prostate cancer	Mutant	Yes	van Steenbrugge GJ, et al. 1991
LAPC-4	human prostate cancer	Wild type	Yes	Klein KA, et al. 1997
PC-3	human prostate cancer	Null	No	Kaighn ME, et al. 1994
C4-2	LNCaP co-engrafted with bone marrow cells	Mutant	No	Wu HC, et al. 1994
CWR22Rv1	human prostate cancer	Mutant	No	Sramkoski RM, et al. 1999
RWPE-1	human prostate epithelium transformed by HPV-18	Wild type	Yes	Bello D, et al. 1997
HEK293	human embryo kidney transformed by adenovirus 5	Null	No	Aiello L, et al. 1979
MCF-7	human breast cancer	Positive	Yes	Brooks SC, et al. 1973 Zava DT and McGuire WL, 1978
T47D	human breast cancer	Positive	No	Keydar I, et al. 1979

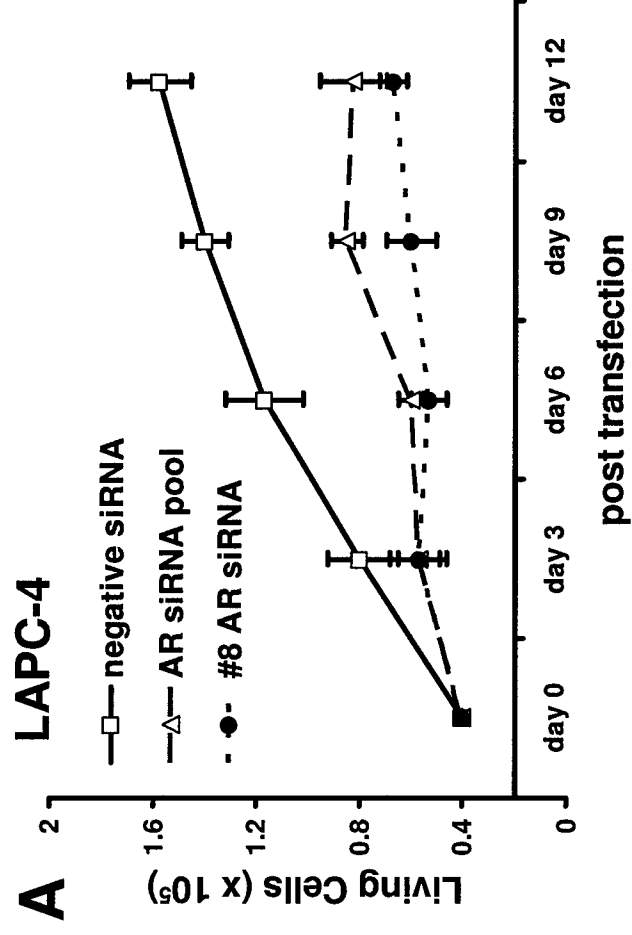
Liao, et al. Table 1



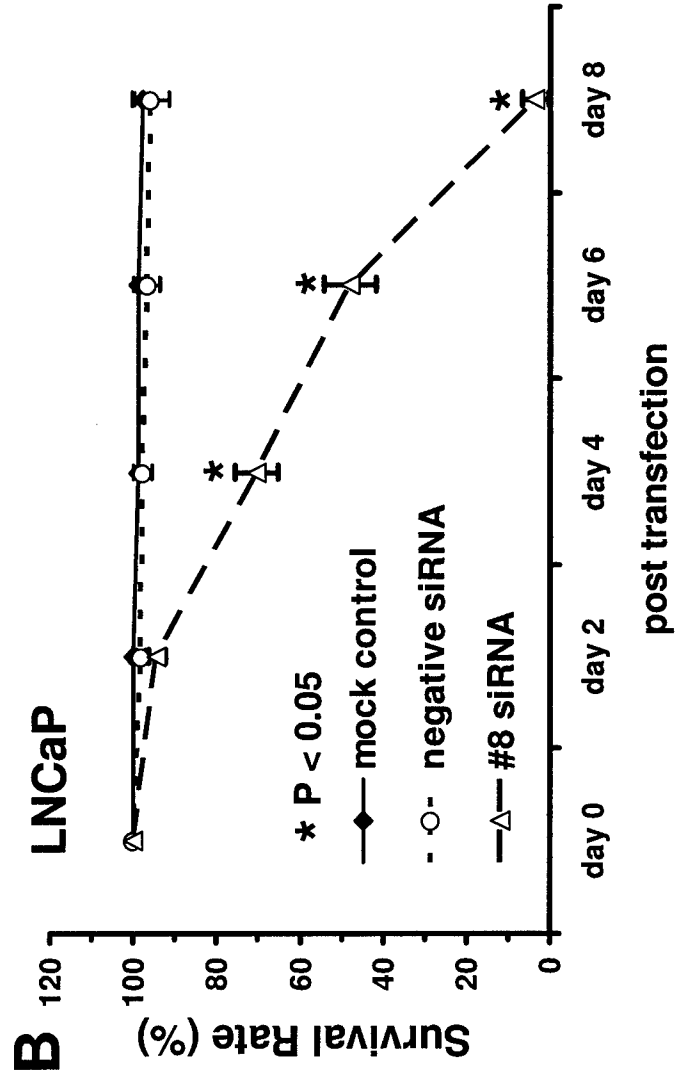
Liao, et al. Figure 1



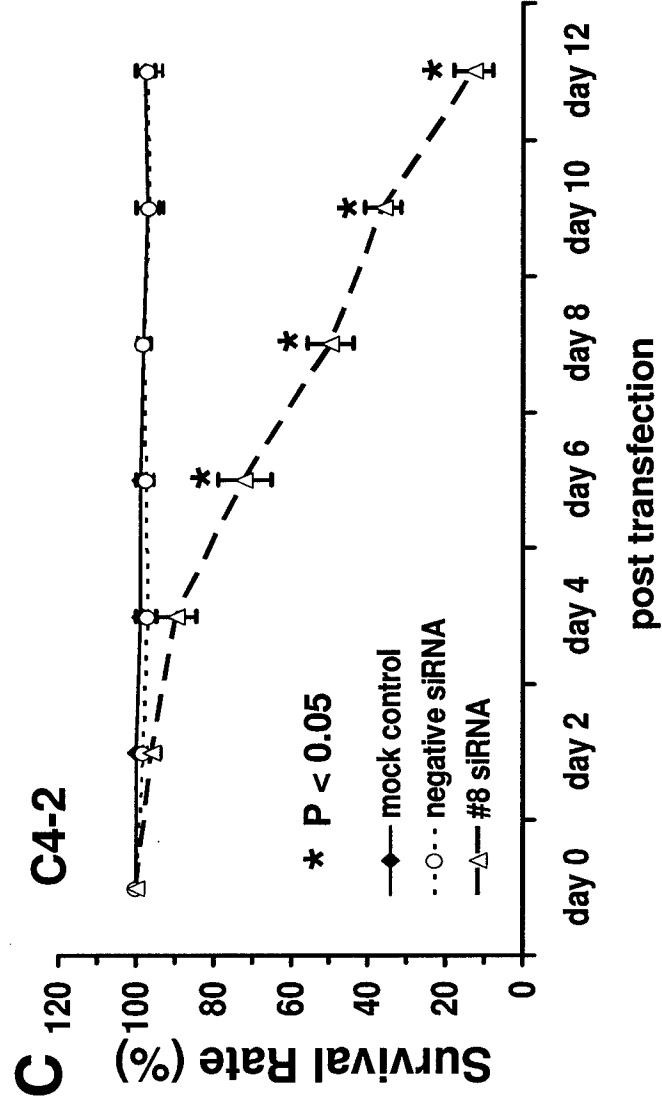
Liao, et al. Figure 1C



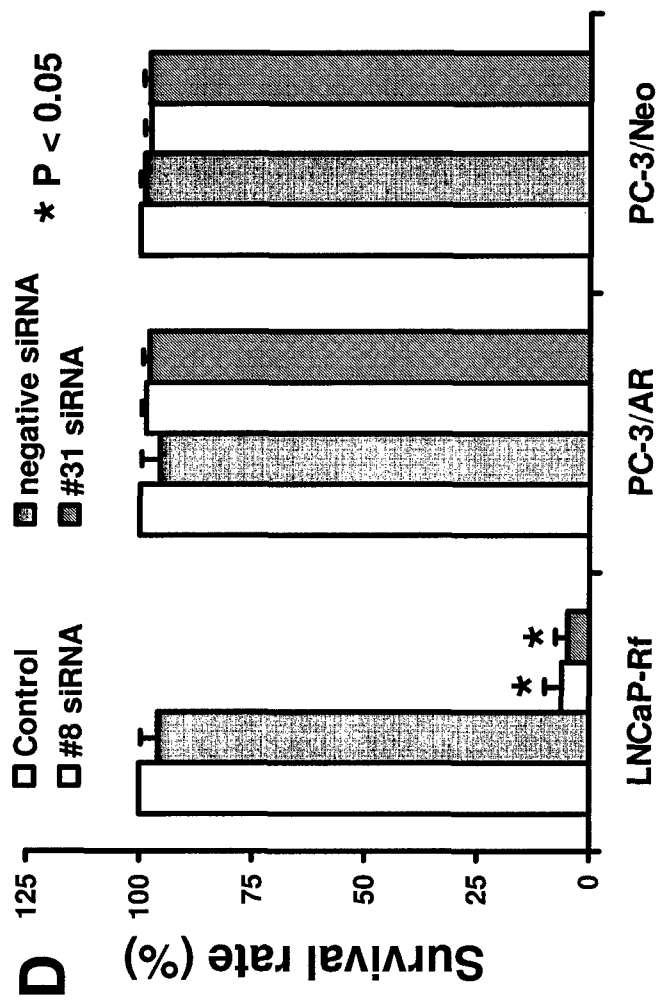
Liao, et al. Figure 2A



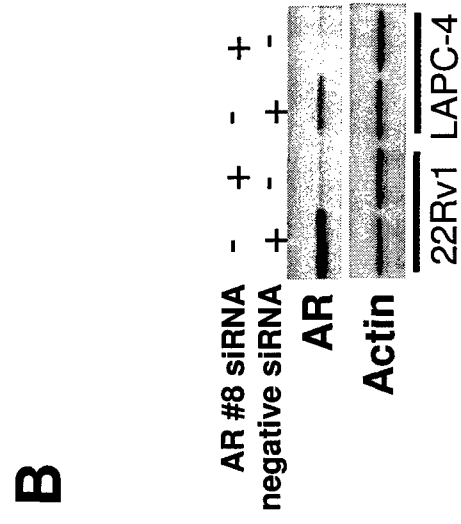
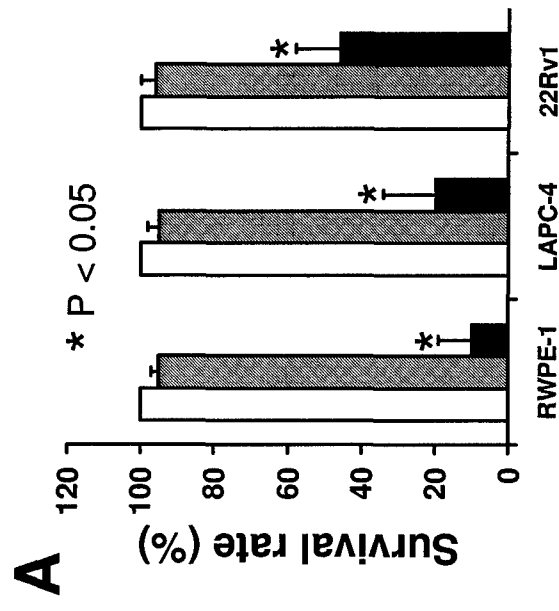
Liao, et al. Figure 2B



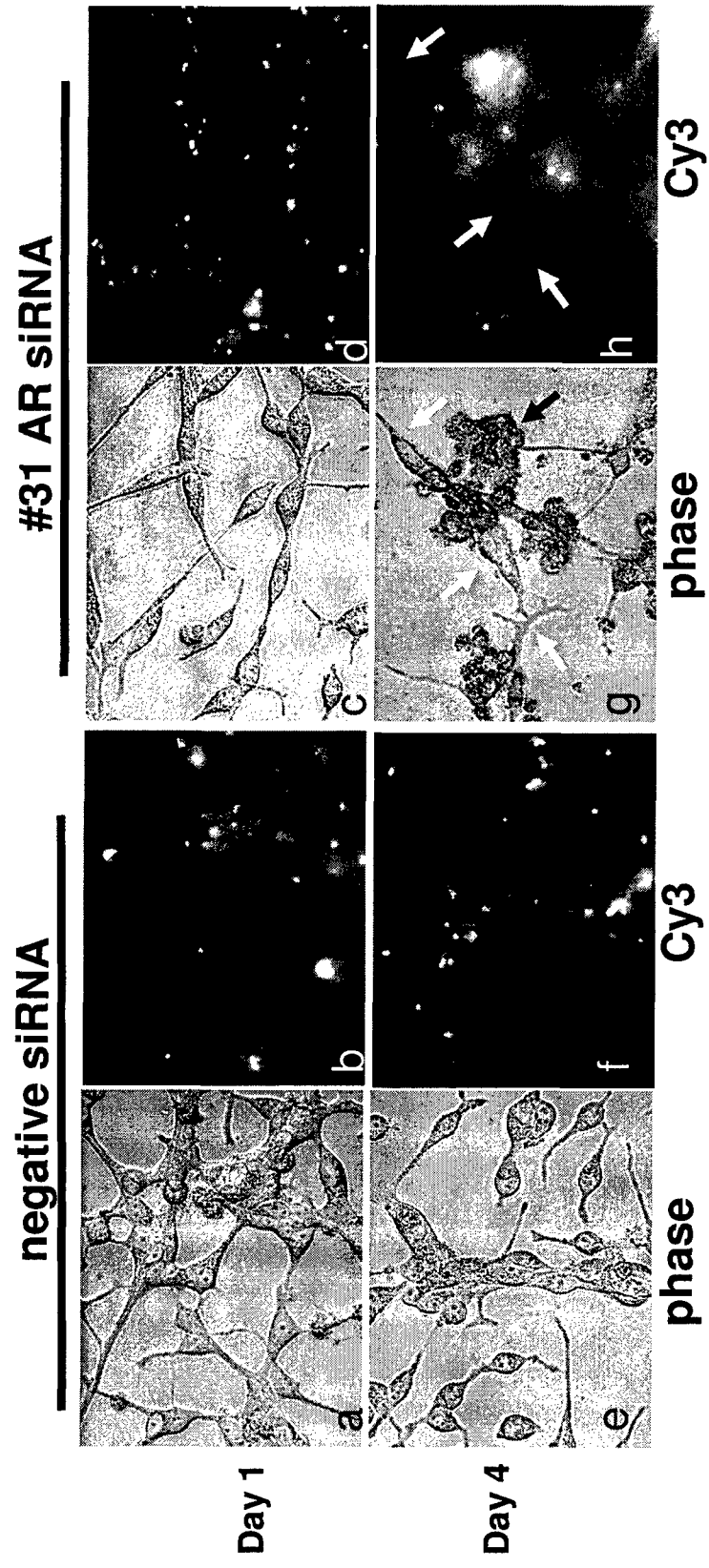
Liao, et al. Figure 2C



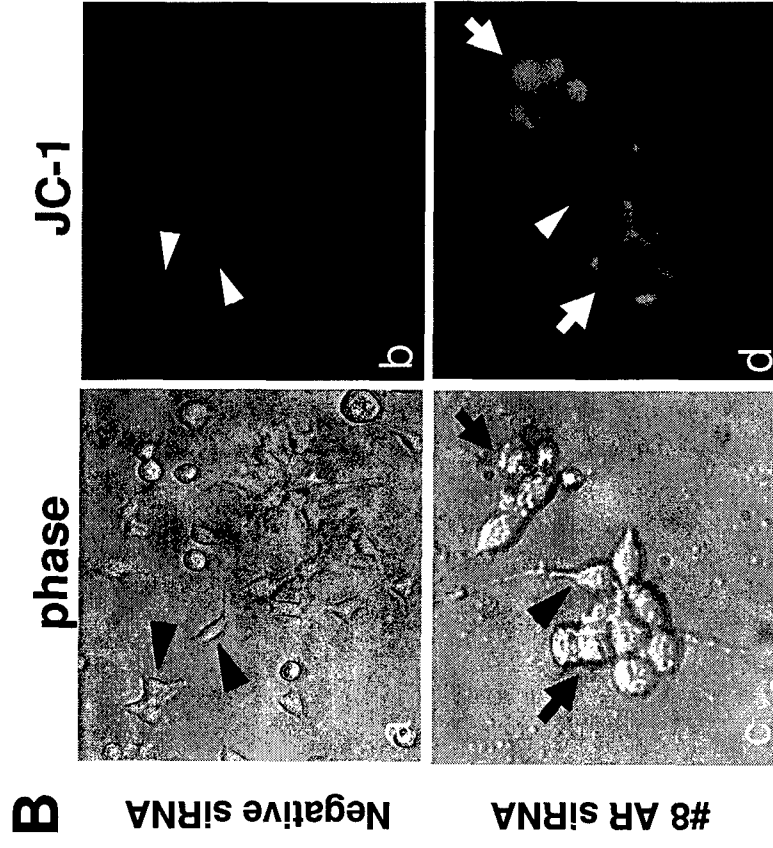
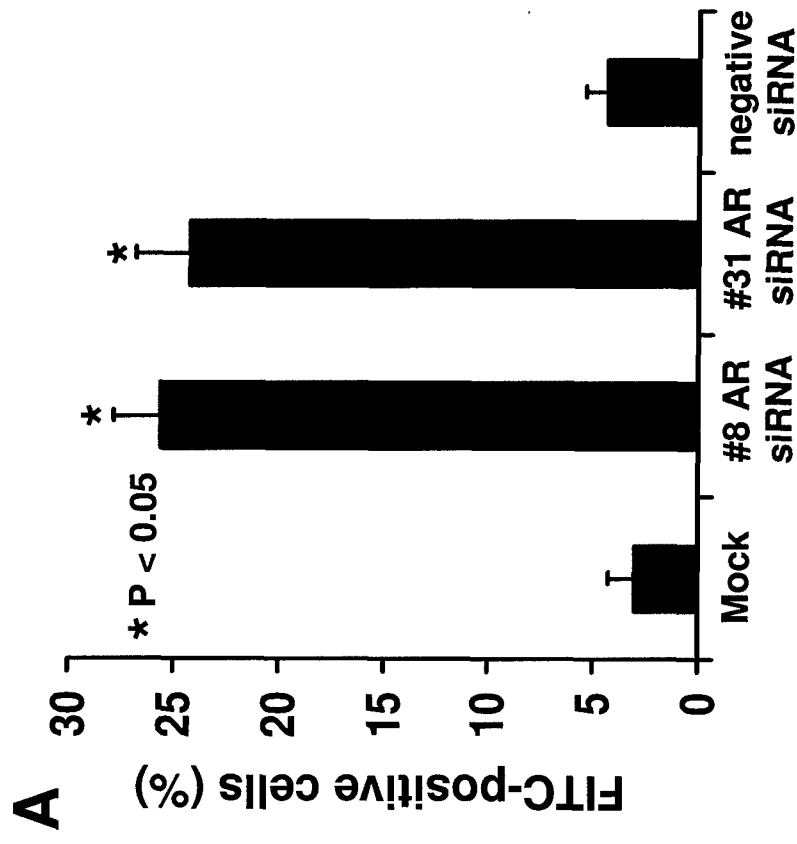
Liao, et al. Figure 2D



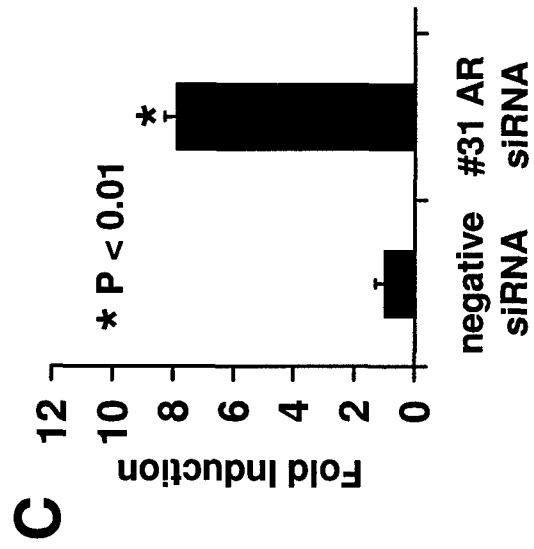
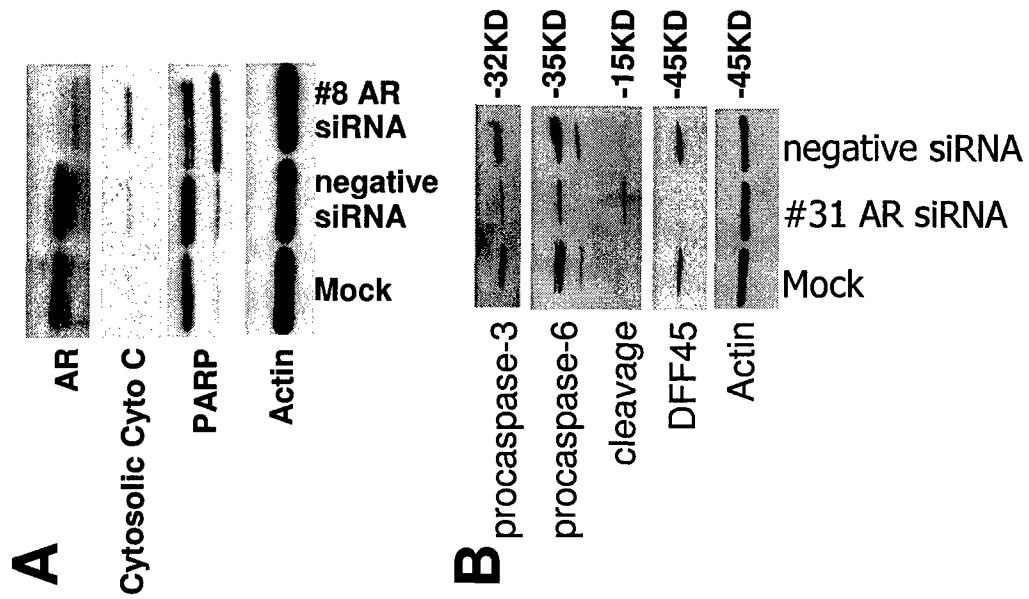
Liao, et al. Figure 3



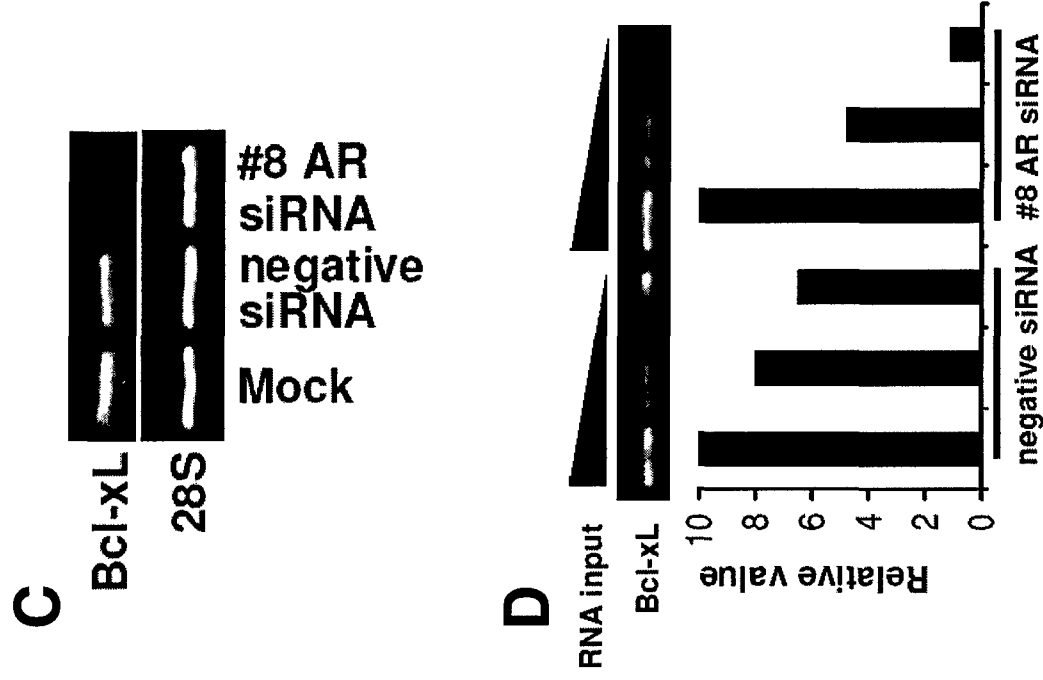
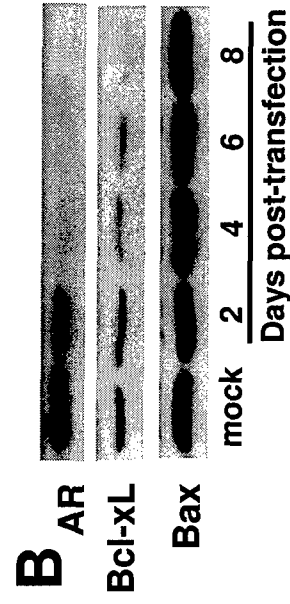
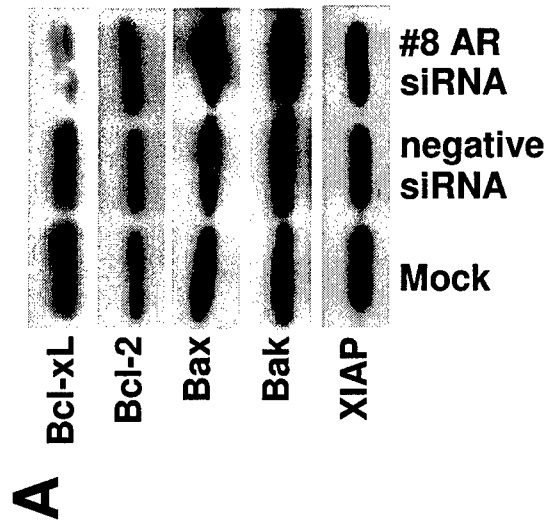
Liao, et al. Figure 4



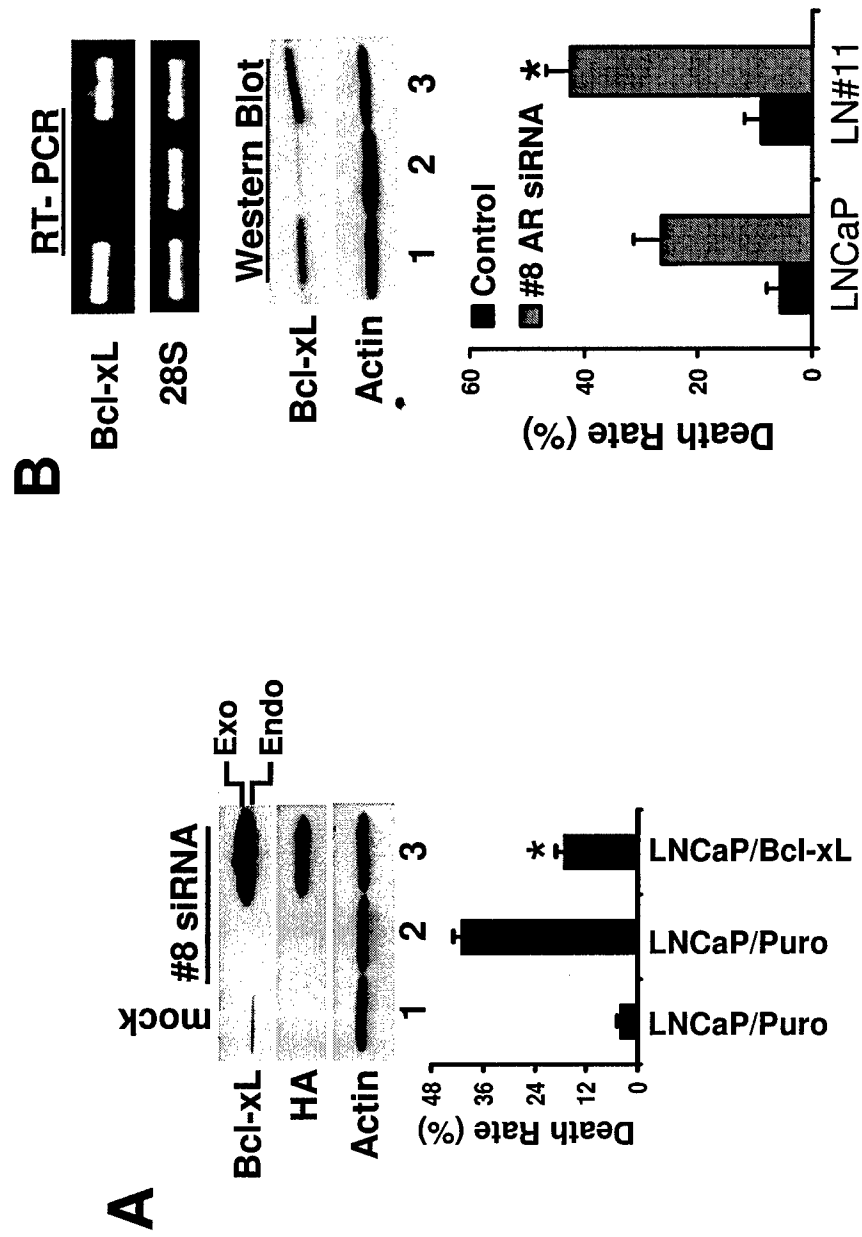
Liao, et al. Figure 5



Liao, et al. Figure 6



Liao, et al. Figure 7



Liao, et al. Figure 8