HHS Public Access
Author manuscript
Cancer Res. Author manuscript; available in PMC 2019 November 26.

Published in final edited form as:
Cancer Res. 2011 April 01; 71(7): 2739–2749. doi:10.1158/0008-5472.CAN-10-2745.

The Androgen Receptor Induces Integrin α6β1 to Promote Prostate Tumor Cell Survival via NF-κB and Bcl-xL Independently of PI3K Signaling

Laura E. Lamb[1,2], **Jelani C. Zarif**[1,2], **Cindy K. Miranti**[1]

[1]Laboratory of Integrin Signaling and Tumorigenesis, Van Andel Research Institute, Grand Rapids;

[2]Cell and Molecular Biology Program, Michigan State University, East Lansing, Michigan

Abstract

Recent studies indicate that androgen receptor (AR) signaling is critical for prostate cancer cell survival, even in castration-resistant disease wherein AR continues to function independently of exogenous androgens. Integrin-mediated adhesion to the extracellular matrix is also important for prostate cell survival. AR-positive prostate cancer cells express primarily integrin α6β1 and adhere to a laminin-rich matrix. In this study, we show that active nuclear-localized AR protects prostate cancer cells from death induced by phosphoinositide 3-kinase (PI3K) inhibition when cells adhere to laminin. Resistance to PI3K inhibition is mediated directly by an AR-dependent increase in integrin α6β1 mRNA transcription and protein expression. Subsequent signaling by integrin α6β1 in AR-expressing cells increased NF-κB activation and Bcl-xL expression. Blocking AR, integrin α6, NF-κB, or Bcl-xL concurrent with inhibition of PI3K was sufficient and necessary to trigger death of laminin-adherent AR-expressing cells. Taken together, these results define a novel integrin-dependent survival pathway in prostate cancer cells that is regulated by AR, independent of and parallel to the PI3K pathway. Our findings suggest that combined targeting of both the AR/α6β1 and PI3K pathways may effectively trigger prostate cancer cell death, enhancing the potential therapeutic value of PI3K inhibitors being evaluated in this setting.

Introduction

Androgen, acting through the androgen receptor (AR), is required for prostate cancer growth and survival. Therefore, chemical castration is initially an effective treatment option for advanced prostate cancer. However, patients ultimately relapse with castration-resistant tumors for which there are no effective treatments. Nonetheless, castration-resistant tumor cells are still dependent on AR, as inhibition of AR expression leads to cell death (1–3). How AR regulates survival of castration-resistant tumor cells is poorly understood.

Corresponding Author: Cindy K. Miranti, Van Andel Research Institute, 333 Bostwick Ave NE, Grand Rapids, MI 49503. Phone: 616-234-5358; Fax: 616-234-5359; cindy.miranti@vai.org.

Disclosure of Potential Conflicts of Interest
The authors have disclosed there are no conflicts of interest.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Integrins are heterodimeric cell surface receptors that mediate cell survival through adhesion to extracellular matrix (4, 5). Integrin signaling through various pathways regulates prosurvival and prodeath molecules and matrix detachment induces cell death (6). Integrin expression and signaling is aberrant in many cancers, including prostate cancer. In the normal human prostate, basal epithelial cells express 2 integrins, α6β4 and α3β4, which promote basal cell survival through adhesion to laminin 5 in the basement membrane (7, 8). Basal epithelial cells do not express AR but differentiate into AR-expressing secretory cells which downregulate integrins and no longer adhere to the basement membrane (9). Thus, integrin and AR expression are mutually exclusive in normal prostate epithelium. However, in prostate cancer the AR-expressing tumor cells exclusively express integrin α6β1 and adhere to a remodeled matrix containing the α6β1-specific substrate laminin 10 (10, 11). The predilection for α6β1 expression is preserved in lymph node metastases (12). Constitutive AR expression in immortalized prostate epithelial cells increases integrin α6 (13), suggesting that AR could be responsible for maintaining α6 expression in the cancer cells. In addition, the α6 promoter contains a steroid response element capable of stimulating α6 expression in response to progesterone (14). Thus, AR-mediated control of integrin α6 and the engagement of α6β1 in AR-expressing cells could provide a novel mechanism for prostate cancer cell survival.

Phosphoinositide 3-kinase (PI3K) signaling is required for survival of most prostate cancers. PTEN, a phosphoinositide phosphatase and negative regulator of PI3K signaling, is lost in approximately 30% of clinical prostate cancers and in approximately 60% of metastatic cancers, resulting in constitutive activation of PI3K (15, 16). Akt is a major downstream effector of PI3K signaling and regulates survival through inhibition of prodeath proteins, such as Bad, Bax, FOXO, DAP3, and caspase 9, and increased expression of the prosurvival protein survivin and stimulation of NF-κB and mTOR signaling (6, 17). Nonetheless, PI3K signaling is not the only survival pathway. The androgen-sensitive prostate cancer cell line LNCaP dies on PI3K/Akt inhibition; however, the addition of androgen can rescue this death (18, 19). In addition, long-term androgen ablation results in resistance to PI3K/Akt inhibition (20) and prostate regeneration studies show that AR and Akt can synergize to promote tumor formation even after androgen ablation (21). This suggests that AR, and in some contexts independent of exogenous androgen, promotes survival independent of PI3K. In this study, we tested the hypothesis that AR-dependent regulation of integrin α6β1 expression in prostate cancer cells promotes survival independent of PI3K.

Materials and Methods

Cell culture

PC3, DU145, LNCaP, and VCaP cells authenticated by DNA profiling were obtained from ATCC, (American Type Culture Collection). PC3 cells were grown in F-12K containing 10% charcoal-stripped serum (CSS) and dextran-treated FBS. DU145-AR cells were grown in Earle’s MEM (minimum essential medium) containing 10% CSS, nonessential amino acids, and sodium pyruvate. LNCaP cells were grown in RPMI-1640 supplemented with 10% FBS, 0.225% glucose, 10 mmol/L HEPES, and sodium pyruvate. VCaP cells were cultured in Dulbecco’s modified Eagle’s medium with sodium pyruvate and 10% FBS. An original stock of C4–2 cells was obtained from Dr. Leland Chung (22) and grown in RPMI-1640 and 10% FBS. LNCaP, C4–2, and VCaP cells were grown in phenol red–free media and 10% CSS 48 hours prior to experimental use. For all experiments, cells were plated on 10 μg/mL laminin 1 (Invitrogen; ref. 8, 23).

DNA constructs

pBabe-puro-hAR and pGL3-vector plasmids were provided by Dr. Beatrice Knudsen. pCSCG-AR-DNLS and pCSCG-ARN705S (ΔLBD) plasmids were obtained from Dr. Owen Witte (21, 24). pLKO.1 was provided by Dr. Jeff MacKeigan. pBabepuro-Bcl-xL was a gift from Dr. Douglas Green. pGL4.32-*luc2P*/NF-κB-RE and phRG-TK were purchased from Promega. All AR plasmids were sequenced verified. PC3-Puro, DU145-Puro, PC3-AR, DU145-AR, and PC3-Bcl-xL cells were generated by infecting cells with pBabe-puro, pBabe-puro-hAR, or pBabepuro-Bcl-xL retroviruses. Clones were selected and maintained in 2 μg/mL puromycin. PC3-pLKO, PC3-ΔNLS, and PC3-DLBD cells were generated by infecting cells with pLKO.1, pCSCG-AR-ΔNLS, or pCSCG-AR-N705S lentiviruses.

siRNA transfections

Pools of 4 siRNAs against AR, integrin α6, Bcl-xL, RelA, or a nontargeting sequence were purchased from Dharmacon. Cells were transfected with siRNA, using siLentFect lipid reagent (Bio-Rad). The lowest concentration of siRNA that reduced protein expression by more than 85% was used.

Reverse transcriptase PCR

Total RNA was isolated using TRIzol and chloroform. RNA was purified with RNase-free DNase and RNeasy Mini kits (Qiagen). Reverse transcriptase PCR (RT-PCR) was done on 1 μg RNA using the One-Step RT-PCR Kit (Qiagen). For quantitative PCR (qRT-PCR), 0.5 μg RNA was reversed transcribed with random primers, using a reverse transcription system (Promega). Synthesized cDNA was amplified for qRT-PCR, using SYBR green master mix (Roche) with gene-specific primers and an ABI 7500 RT-PCR system (Applied Biosystems). Gene expression was normalized to 18s rRNA by the $2^{-\Delta\Delta Ct}$ method (25). Specific primers were as previously published: Bcl-xL, glyceraldehyde 3-phosphate dehydrogenase (GAPDH; ref. 26), integrin α6 (27), and AR (28).

Reporter assays

Laminin-adherent cells were transfected with 1.25 μg pGL3-vector, pGL4.32-*luc2P*/NF-κB-RE, or pGL4-*luc2P*/ITGα6 (SwitchGear) and 0.5 μg phRG-TK, using Nanojuice Core Transfection Reagent and Booster Reagent (Novagen). After 48 hours, cells were lysed with the Dual-Luciferase Reporter Assay System (Promega) and luminescence was measured using the EnVision 2104 Multilabel Reader (PERKin Elmer) and Wallac EnVision Manager Software. Firefly luminescence activity was normalized to *Renilla* luciferase activity.

Immunoblotting

Total cell lysates were prepared for immunoblotting as described following lysis with MAPK (mitogen activated protein kinase) or RIPA (radioimmunoprecipitation assay) buffers

(8). Forty-five to 65 μg of protein was run on SDS polyacrylamide gels (Invitrogen) and transferred to polyvinylidene difluoride membranes. Membranes were blocked and processed as described (8) and visualized by chemiluminescence reagent with a CCD camera in a Bio-Rad Chemi-Doc Imaging system using Quantity One software (Bio-Rad).

Immunofluorescence

Cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 10% goat serum before incubation with AR (clone 411) antibody overnight at 4°C. Cells were incubated with secondary antibody and Hoechst 33258 (Sigma), washed, and mounted using Gel-Mount (Biomeda). Epifluorescent images were acquired on a Nikon Eclipse TE300 microscope using OpenLab software (Improvision).

Fluorescence-activated cell sorting

Suspended cells were washed (1% sodium azide/2% FBS/PBS) and incubated with primary antibodies or control immunoglobulin G (IgG) for 1 hour at 4°C and then with fluorescently labeled secondary antibodies for 1 hour at 4°C. Fluorescence was detected by a Becton-Dickinson FACSCalibur cytometer with CellQUEST Pro Software (Becton-Dickinson).

Antibodies

Polyclonal antibodies to Bcl-xL, phospho-IκBα S32 (14D4), phospho-NF-κB S536 (93H1), NF-κB p65-RelA, and monoclonal antibodies to IκBa (44D4) were purchased from Cell Signaling. Polyclonal antibodies to Nkx3.1 (H-50), PSA (prostate specific antigen; C-19), and monoclonal AR (411) were obtained from Santa Cruz and monoclonal anti-tubulin (DM1A) from Sigma. Integrin α6 (AA6A) was generously provided by Dr. Anne Cress and monoclonal TMPRSS2 (P5H9-A3) was provided by Dr. Pete Nelson. Monoclonal antibodies to integrin a2 (CBL477), a3 (MAB2056), and b4 (ASC-3) were purchased from Chemicon and a5 (P1D6) from Santa Cruz. Integrin α6 (GoH3) obtained from BD Pharmingen. Integrin b1 (AIIB2) monoclonal antibody, developed by Dr. Caroline Damsky (University of California San Francisco, San Francisco, CA), was obtained from the Developmental Studies Hybridoma Bank (University of Iowa).

Cell survival assays

Laminin-adherent cells were treated with 5 to 20 μmol/L of LY294002 (8). In some cases, ethanol, or 5 to 10 nmol/L each of dihydrotestosterone (DHT), R1881, Casodex, or RU486, was added. DHT was replenished every 24 hours. Cell viability was measured after 72 hours by collecting attached and floating cells and adding an equal volume of Trypan blue. Three separate cell counts per well were done on a hemocytometer; 2 to 3 wells were counted per condition.

Results

AR promotes PI3K-independent survival

To directly assess whether AR and integrin α6β1 cooperate to control prostate cancer survival, wild-type AR or 2 well-characterized AR mutants were introduced into PTENdeficient PC3 cells. AR expression in the PC3 clones was comparable with LNCaP cells (Fig. 1A). Wild-type AR localization was both cytoplasmic and nuclear (Fig. 1B). As previously observed, the ligand-binding mutant ΔLBD (N705S) was predominately nuclear (21). The ΔNLS mutant, defective in nuclear localization (24), was exclusively cytoplasmic (Fig. 1B). PC3-AR1 and PC3-AR2 cells expressed higher levels of the AR-target genes *Nkx3.1, PSA*, and the activated form of *TMPRSS2* (29) than the PC3-Puro control cells (Fig. 1C). Knockdown of AR in the clones reduced AR-target gene expression, indicating that AR is functional. Exogenous androgen was not required for AR-target gene expression, probably because AR is already nuclear localized in these cells (Fig. 1B).

Inhibition of PI3K with LY294002 in laminin-adherent PC3 cells induces cell death (8). To determine whether AR expression could protect cells from death induced by PI3K inhibition, cells were placed on laminin in the presence or absence of LY294002. Inhibition of PI3K induced cell death in 60% of the PC3-Puro control cells (Fig. 1D). In contrast, cell death was not induced by LY294002 in the PC3-AR cells. Similar results were obtained when cell death was measured by TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling) or propidium iodide staining (not shown). AR-dependent cell survival did not require exogenous androgen and was not observed when cells were plated on collagen, on plastic, or placed in suspension (Supplementary Fig. S1), indicating this response is specific to LM. Thus, in the context of LM, AR promotes cell survival independently of PI3K.

The difference in survival was not due to cell cycle status because PC3-AR1 cells grow at the same rate whereas PC3-AR2 cells grow slower than PC3-Puro cells (not shown). Nuclear localization of AR was required for resistance to PI3K inhibition, because the AR nuclear localization–defective mutant ΔNLS (Fig. 1E) could not confer resistance to PI3K inhibition. In contrast, the AR ligand–binding mutant ΔLBD (Fig. 1F) which localizes to the nucleus (Fig. 1B) conferred resistance to PI3K inhibition. Thus, nuclear-localized AR is required to promote survival on LM independently of PI3K.

AR promotes survival through upregulation of laminin integrin α6β1

Fluorescence-activated cell sorting (FACS) was used to compare integrin expression at the cell surface between PC3-AR and PC3-Puro cells. AR expression caused a 2-, 3-, and 6-fold reduction in integrin α2, α5, and α3, respectively, but increased integrin α6 levels 6-fold (Fig. 2A). There was a slight 1.5-fold decrease in integrin β1 (Fig. 2A) and a 4-fold decrease in integrin β4. Integrins are expressed as heterodimeric pairs on the cell surface, and integrin α6 pairs with either β1 or β4. The corresponding decrease in the integrin β1–specific a-subunits, that is, α2, α3, and α5, would generate free β1 integrin, making it available to dimerize with α6. The large decrease in β4 further indicates that α6 is paired with the β1. This predilection for integrin α6β1 mimics what is observed in prostate cancer patients (10, 11). An AR-dependent increase in integrin α6 was also observed in DU145 cells, whereas loss of endogenous AR in LNCaP, C4–2, or VCaP cells or in PC3-AR cells decreased integrin α6 (Fig. 2B).

The AR-dependent increase in integrin α6 expression suggested that it may be responsible for the increase in survival on LM. Reduction of α6 expression by siRNA had a negligible effect on AR expression (Fig. 2C) but completely reversed the sensitivity to cell death induced by PI3K inhibition (Fig. 2D). The effect of AR on α6 was not due to a clonal artifact, as loss of AR decreased α6 expression (Fig. 2E) and restored the sensitivity to PI3K inhibition (Fig. 2F). Thus, AR promotes survival on LM independently of PI3K by increasing integrin α6 expression.

AR stimulates integrin α6 transcription

The AR ΔNLS nuclear localization mutant could not protect cells from LY294002-induced death (see Fig. 1G), suggesting AR transcriptional activity is required. Correspondingly, integrin α6 mRNA is dramatically increased in AR-expressing PC3 cells and following androgen stimulation of LNCaP or C4–2 cells (Fig. 3A and B). Reciprocally, loss of AR suppresses α6 mRNA (Fig. 3C). Furthermore, cells expressing the ΔNLS mutant failed to upregulate α6 (Fig. 3D) and the AR transcriptional repressors Casodex and RU486 (30) decreased integrin α6 mRNA (Fig. 3E) and protein (not shown). Casodex also restored the sensitivity to cell death induced on PI3K inhibition (Fig. 3F). Thus, the transcriptional activity of AR is required to increase integrin α6 expression and confer resistance to cell death.

R1881-induced integrin α6 mRNA was observed as early as 6 hours (Fig. 3G) and peaked at 8 to 12 hours (Fig. 3H). Induction of α6 mRNA was resistant to cycloheximide treatment, indicating that the synthesis of other proteins is not required. Interestingly, combined R1881 and cycloheximide treatment enhanced α6 transcription, suggesting the presence of a protein synthesis-sensitive α6 repressor which is blocked in response to androgen. Expression of a luciferase reporter containing approximately 1 kb of the α6 promoter was elevated in PC3-AR cells relative to PC3-Puro cells (Fig. 3I) and stimulated by R1881 in LNCaP cells (Fig. 3J). These data indicate that AR directly stimulates integrin α6 transcription.

Bcl-xL is required for AR/α6β1-dependent survival

We have previously shown that adhesion to LM increases Bcl-xL expression (8). Therefore, we postulated that the AR-mediated increase in integrin α6 should increase Bcl-xL expression. Bcl-xL was dramatically upregulated in PC3-AR cells, and loss of α6 by siRNA decreased Bcl-xL whereas loss of AR decreased the expression of both α6 and Bcl-xL (Fig. 4A). Bcl-xL mRNA was also increased by AR (Fig. 4B). Stimulation of LNCaP, C4–2, or VCaP cells (Figs. 3H and 4C and D) with androgen or knockdown of AR (Fig. 4E) correspondingly altered α6 and Bcl-xL mRNA. Thus, AR stimulation of integrin α6 expression leads to increased Bcl-xL mRNA and protein expression.

Reduced Bcl-xL expression in PC3-AR cells by siRNA (Fig. 4F) restored the sensitivity to death induced by PI3K inhibition (Fig. 4G). Complete loss of Bcl-xL resulted in complete loss of viability of both PC3-Puro and PC3-AR cells (not shown). Conversely, overexpression of Bcl-xL in parental PC3 cells, to the levels seen in PC3-AR cells (Fig. 4H), was sufficient to confer resistance to PI3K inhibition (Fig. 4I). Thus, Bcl-xL promotes survival of LM-adherent prostate cancer cells independent of PI3K.

NF-κB signaling is required for PI3K-independent survival

Our data indicate that AR controls Bcl-xL expression indirectly through integrin α6 (see Fig. 4A and B). NF-κB has been reported to bind directly to the Bcl-xL promoter and drive its transcription, and α6 has been shown to regulate NF-κB (31–33). NF-κB p65-RelA activity was increased in PC3-AR cells (Fig. 5A and B) and inhibited upon AR knockdown in C4–2, VCaP, or PC3-AR cells (Fig. 5C–F). Conversely, NF-κB RelA activity was increased on androgen stimulation and its activity paralleled the increase in α6 and Bcl-xL expression, peaking at 24 hours (Fig. 5D and E). Increased phosphorylation of both IKKβ (IκB kinase b) and IκBa (inhibitor of NF-κB a) was also observed (Fig. 5G). Knockdown of integrin α6 in PC3-AR, C4–2, or LNCaP cells decreased RelA phosphorylation and Bcl-xL expression (Fig. 6A and B). Knockdown of RelA resulted in a partial loss of Bcl-xL (Fig. 6B and C) but was sufficient to sensitize C4–2 and PC3-AR cells to LY294002-induced death (Fig. 6D and E). Furthermore, the ability of androgen to rescue LNCaP or C4–2 cell death induced by PI3K inhibition, as previously reported (18, 19), is abrogated when AR, α6, or RelA expression is suppressed (Fig. 6F and G). Thus, NF-κB RelA activity is increased in an AR- and integrin α6–dependent manner and, in part, controls Bcl-xL expression downstream of integrin α6. This pathway is responsible for conferring resistance to death induced by PI3K inhibition when cells are adherent to LM.

Discussion

In this study, we identified an AR-dependent prostate cancer cell survival pathway that operates independently of PI3K when tumor cells are adherent to LM. Resistance to death induced by PI3K inhibition, mediated via AR-dependent transcriptional stimulation of integrin α6 mRNA, leads to increased α6β1 cell surface expression. Integrin α6β1 engagement of LM subsequently activates NF-κB and increases BclxL expression (Fig. 7). Downregulation of AR, integrin α6, NF-κB, or Bcl-xL resensitizes AR-expressing cells to PI3K-dependent survival.

Previous studies, in which AR was reexpressed in prostate tumor cell lines, reported reduced proliferation or cell survival due to activated AR (34–36). Therefore, extra precautions were taken to keep AR minimally active in our cells. First, the AR cDNA was sequence verified to be wild type and not an activated variant. Second, AR was not highly overexpressed but maintained at levels similar to LNCaP cells. Third, only low passage (<20) cells were used, as phenotypes can change with passage. Fourth, cells were isolated and constantly maintained in CSS and phenol red–reduced media to prevent overactivation of AR. Immunostaining indicates that even under these conditions a large portion of AR is nuclear localized in the absence of exogenous ligand. It is possible that the constitutive nuclear localization of AR in our cells is a reflection of the known steroidogenic activity present in PC3 cells resulting in intracellular synthesis of androgen (37–39). This could explain why addition of exogenous androgen to PC3-AR cells does not enhance AR function. Furthermore, continual addition of exogenous androgens in this system, such as propagation of cells in nonstripped serum, could hyperactivate AR such that it acts as a suppressor and thus explain why it might lead to suppressed growth and reduced survival as seen by others (40).

Loss of responsiveness to exogenous androgens in AR-expressing cells, in which AR is still active due to synthesis of intracellular androgens, is characteristic of castration-resistant tumors. Thus, the PC3-AR model may reflect events associated with castration-resistant cancers. In support of this, previous studies have linked increased NF-κB activity with prostate cancer progression and metastasis (41, 42), castration resistance (43, 44), poor prognosis (45), and biochemical failure (i.e., PSA relapse; ref. 46). Similarly, increased Bcl-xL expression is associated with prostate cancer progression and castration resistance (26, 47, 48). Furthermore, we observed that androgen-sensitive LNCaP cells have significantly less integrin α6 and Bcl-xL expression than the castration-resistant derived C4–2 subline. Our study indicates that AR is responsible for the increase in NF-κB activation as reported by others (42, 43), that this is mediated by AR-dependent stimulation of integrin α6β1 expression, and that LM-mediated activation of NF-κB contributes to Bcl-xL expression.

Oddly, while NF-κB or Bcl-xL knockdown was sufficient to completely resensitize cells to death induced by PI3K inhibition, NF-κB knockdown, unlike AR or integrin α6 loss, resulted only in a partial loss of Bcl-xL. The partial knockdown of Bcl-xL by NF-κB loss may be sufficient for AR-expressing cells to regain dependence on PI3K signaling. Alternatively, NF-κB may regulate other cell survival molecules whose loss on inhibition of NF-κB contributes to this phenotype.

Our finding that AR increases integrin α6 expression is consistent with the observation that constitutive AR expression in immortalized prostate epithelial cells leads to increased α6 (13) and its singular expression in prostate cancer tissues and metastases (11, 12). However, previous AR reexpression studies in PC3 or DU145 cells did not report an increase in integrin α6 expression (34, 35, 49). Possible explanations include differences in the level of AR reexpression, use of non-CSS for cultivation, duration of growth factor and serum starvation prior to experimental assays, and passage number used. However, the most significant difference was that the integrin expression assays in the other studies were done with cells plated on plastic whereas in our studies cells were adherent to LM. Adhesion to LM may result in increased integrin α6 stabilization, explaining this observed difference. Nonetheless, AR is still required in this context to control α6 expression. It is possible that in prostate cancer, elevated integrin α6β1 expression is also dependent on engagement of the integrin by LM. The preferred ligands for α6β1 are LM10 and LM1. LM10 is the expressed in adult tissues, whereas LM1 is predominantly embryonic. LM10 is present in prostate tumors and bone metastases. Because of lack of availability of purified LM10, we used LM1 in our studies. We assume that similar signaling pathways are activated on the two matrices, but it is possible there could be some differences.

The full range of transcriptional mechanisms that control integrin α6 expression has not been extensively studied. AR seems to directly regulate α6 transcription, because the response occurs within 6 hours and is not blocked by cycloheximide. In addition, the first kilobase (kb) of the α6 promoter is sufficient for activation by AR. However, this region does not contain canonical AR response elements (14, 50). Progesterone, but not estradiol, can increase α6 promoter activity via an imperfect steroid response element in this region (14). Our preliminary studies suggest that AR binds to a region containing this steroid response element.

Detection of the AR/α6β1 survival pathway requires that the constitutive PI3K signaling, due to PTEN loss, be simultaneously inhibited. Previous studies in PTEN-negative LNCaP cells suggested that survival of castration-resistant variants was mediated by augmenting PI3K signaling (51). We failed to detect an increase in PI3K signaling, as measured by Akt, Bad, survivin, or FOXO activation, above that seen in the vector control cells and LY294002 alone failed to induce any death above basal levels. It is possible that on adhesion to LM, the AR/α6β1 pathway precludes the need for survival signaling through PI3K. Inhibition of Src kinases also induces the death of LM-adherent PC3 cells (8). In addition to being resistant to PI3K inhibition, PC3-AR cells are also resistant to inhibition of Src kinases (Supplementary Fig. S2) but are not resistant to death induced by TNFα or staurosporine. Thus, other path ways may also be involved in controlling prostate tumor cell survival.

Interestingly, integrin α2b1, which mediates adhesion to collagen, was only slightly decreased in the PC3-AR cells, and when plated on collagen, both the control and PC3-AR lines were resistant to PI3K inhibition. These data indicate that integrin α2b1 also controls PC3 survival independent of PI3K but also independently of AR. The differences in survival mechanisms on specific matrices suggest that depending on the tumor microenvironment, different integrins may activate distinct signaling pathways to promote survival. These data have important therapeutic implications for treatment, whereby signaling of both AR/α6β1 and PI3K may need to be targeted to efficiently kill prostate cancer cells adherent to LM. On the other hand, if collagen is present, another pathway may be able to compensate.

In summary, we have identified an AR-dependent pathway acting through α6β1 that stimulates survival of LM-adherent prostate cancer cells independently of PI3K signaling. AR/α6β1 stimulates the activity of NF-κB and Bcl-xL, whose upregulation is highly associated with advanced hormone-refractory prostate cancer. Application of this new knowledge may lead to the development of better prostate cancer therapies and supports the importance of targeting more than one pathway to effectively treat prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Veronique Schulz, Rich West, Lia Tesfay, and Dr. Brendan Looyenga for technical assistance and laboratories of Developmental Cell Biology, Systems Biology, and Cell Structure and Signal Integration at the Van Andel Research Institute for their constructive suggestions. We also thank Drs. Beatrice Knudsen and Peter Nelson (Fred Hutchinson Cancer Research Center), Owen Witte (University of California at Los Angeles), Kenneth Pienta (University of Michigan), Jeff MacKeigan (Van Andel Research Institute), Douglas Green (St. Jude Children's Research Hospital), and Anne Cress (University of Arizona) for their generous supply of reagents and Dr. Aaron Putzke (Hope College) for AR sequencing primer design.

Grant Support

This work was supported by the Department of Defense Prostate Cancer Predoctoral Training Award (W81XWH-08-1-0058; to L.E. Lamb) and the American Cancer Society (RSG-05-245-01-CSM; to C.K. Miranti). Additional support was also provided by the generous gifts of the Van Andel Research Institute.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Liao X, Tang S, Thrasher JB, Griebling TL, Li B. Small-interfering RNA-induced androgen receptor silencing leads to apoptotic cell death in prostate cancer. Mol Cancer Ther 2005;4:505–15. [PubMed: 15827323]
2. Yang Q, Fung K-M, Day W, Kropp B, Lin H-K. Androgen receptor signaling is required for androgen-sensitive human prostate cancer cell proliferation and survival. Cancer Cell Int 2005;5:8. [PubMed: 15813967]
3. Cohen MB, Rokhlin OW. Mechanisms of prostate cancer cell survival after inhibition of AR expression. J Cell Biochem 2009;106:363–71. [PubMed: 19115258]
4. Hynes R Integrins: bidirectional, allosteric signaling machines. Cell 2002;110:673–8. [PubMed: 12297042]
5. Miranti C, Brugge J. Sensing the environment: a historical perspective on integrin signal transduction. Nat Cell Biol 2002;4:83–90. [PubMed: 11744924]
6. Reddig PJ, Juliano RL. Clinging to life: cell to matrix adhesion and cell survival. Cancer Metastasis Rev 2005;24:425–39. [PubMed: 16258730]
7. Knudsen BS, Miranti CK. The impact of cell adhesion changes on proliferation and survival during prostate cancer development and progression. J Cell Biochem 2006;99:345–61. [PubMed: 16676354]
8. Edick MJ, Tesfay L, Lamb LE, Knudsen BS, Miranti CK. Inhibition of integrin-mediated crosstalk with EGFR/Erk or Src signaling pathways in autophagic prostate epithelial cells induces caspase-independent death. Mol Biol Cell 2007;18:2481–90. [PubMed: 17475774]
9. Lamb LE, Knudsen BS, Miranti CK. E-cadherin-mediated survival of androgen-receptor-expressing secretory prostate epithelial cells derived from a stratified in vitro differentiation model. J Cell Sci 2010;123:266–76. [PubMed: 20048343]
10. Bonkhoff H, Stein U, Remberger K. Differential expression of α6 and α2 very late antigen integrins in the normal, hyperplastic, and neo-plastic prostate: simultaneous demonstration of cell surface receptors and their extracellular ligands. Hum Pathol 1993;24:243–8. [PubMed: 7681030]
11. Cress AE, Rabinovitz I, Zhu W, Nagle RB. The α6β1 and α6b4 integrins in human prostate cancer progression. Cancer Metastasis Rev 1995;14:219–28. [PubMed: 8548870]
12. Pontes-Junior J, Reis ST, Dall'oglio M, Neves de Oliveira LC, Cury J, Carvalho PA, et al. Evaluation of the expression of integrins and cell adhesion molecules through tissue microarray in lymph node metastases of prostate cancer. J Carcinog 2009;8:3. [PubMed: 19240373]
13. Whitacre DC, Chauhan S, Davis T, Gordon D, Cress AE, Miesfeld RL. Androgen induction of in vitro prostate cell differentiation. Cell Growth Differ 2002;13:1–11. [PubMed: 11801526]
14. Nishida K, Kitazawa R, Mizuno K, Maeda S, Kitazawa S. Identification of regulatory elements of human α6 integrin subunit gene. Biochem Biophys Res Commun 1997;241:258–63. [PubMed: 9425259]
15. Dong JT. Chromosomal deletions and tumor suppressor genes in prostate cancer. Cancer Metastasis Rev 2001;20:173–93. [PubMed: 12085961]
16. Schmitz M, Grignard G, Margue C, Dippel W, Capesius C, Mossong J, et al. Complete loss of PTEN expression as a possible early prognostic marker for prostate cancer metastasis. Int J Cancer 2007;120:1284–92. [PubMed: 17163422]
17. Duronio V The life of a cell: apoptosis regulation by the PI3K/PKB pathway. Biochem J 2008;415:333–44. [PubMed: 18842113]
18. Carson JP, Kulik G, Weber MJ. Antiapoptotic signaling in LNCaP prostate cancer cells: a survival signaling pathway independent of phosphatidylinositol 3′-kinase and Akt/protein kinase B. Cancer Res 1999;59:1449–53. [PubMed: 10197612]
19. Li P, Nicosia SV, Bai W. Antagonism between PTEN/MMAC1/TEP-1 and androgen receptor in growth and apoptosis of prostatic cancer cells. J Biol Chem 2001;276:20444–50. [PubMed: 11278645]
20. Pfeil K, Eder IE, Putz T, Ramoner R, Culig Z, Ueberall F, et al. Long-term androgen-ablation causes increased resistance to PI3K/Akt pathway inhibition in prostate cancer cells. Prostate 2004;58:259–68. [PubMed: 14743465]

21. Xin L, Teitell MA, Lawson DA, Kwon A, Mellinghoff IK, Witte ON. Progression of prostate cancer by synergy of AKT with genotropic and nongenotropic actions of the androgen receptor. Proc Natl Acad Sci U S A 2006;103:7789–94. [PubMed: 16682621]
22. Wu HC, Hsieh JT, Gleave ME, Brown NM, Pathak S, Chung LW. Derivation of androgen-independent human LNCaP prostatic cancer cell sublines: role of bone stromal cells. Int J Cancer 1994;57:406–12. [PubMed: 8169003]
23. Miranti CK. Application of cell adhesion to study signaling networks. Methods Cell Biol 2002;69:359–83. [PubMed: 12071005]
24. Chen C, Welsbie D, Tran C, Baek SH, Chen R, Vessella R, et al. Molecular determinants of resistance to antiandrogen therapy. Nat Med 2004;10:33–9. [PubMed: 14702632]
25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(DC(T)) method. Methods 2001;25:402–8. [PubMed: 11846609]
26. Sun A, Tang J, Hong Y, Song J, Terranova PF, Thrasher JB, et al. Androgen receptor-dependent regulation of Bcl-xL expression: implication in prostate cancer progression. Prostate 2008;68:453–61. [PubMed: 18196538]
27. Tapia A, Salamonsen LA, Manuelpillai U, Dimitriadis E. Leukemia inhibitory factor promotes human first trimester extravillous tropho-blast adhesion to extracellular matrix and secretion of tissue inhibitor of metalloproteinases-1 and −2. Hum Reprod 2008;23:1724–32. [PubMed: 18492704]
28. Xu LL, Srikantan V, Sesterhenn IA, Augustus M, Dean R, Moul JW, et al. Expression profile of an androgen regulated prostate specific homeobox gene NKX3.1 in primary prostate cancer.J Urol 2000;163: 972–9. [PubMed: 10688034]
29. Afar DEH, Vivanco I, Hubert RS, Kuo J, Chen E, Saffran DC, et al. Catalytic cleavage of the androgen-regulated TMPRSS2 protease results in its secretion by prostate and prostate cancer epithelia. Cancer Res 2001;61:1686–92. [PubMed: 11245484]
30. Hodgson MC, Astapova I, Cheng S, Lee LJ, Verhoeven MC, Choi E, et al. The androgen receptor recruits nuclear receptor CoRepressor (N-CoR) in the presence of mifepristone via its N and C termini revealing a novel molecular mechanism for androgen receptor antagonists. J Biol Chem 2005;280:6511–9. [PubMed: 15598662]
31. Chen C, Edelstein LC, Gelinas C. The Rel/NF-κB family directly activates expression of the apoptosis inhibitor Bcl-x(L). Mol Cell Biol 2000;20:2687–95. [PubMed: 10733571]
32. Glasgow JN, Wood T, Perez-Polo JR. Identification and characterization of NF-κB binding sites in the murine bcl-x promoter. J Neurochem 2000;75:1377–89. [PubMed: 10987817]
33. Friedland JC, Lakins JN, Kazanietz MG, Chernoff J, Boettiger D, Weaver VM. α6β4 integrin activates Rac-dependent p21-activated kinase 1 to drive NF-κB-dependent resistance to apoptosis in 3D mammary acini. J Cell Sci 2007;120:3700–12. [PubMed: 17911169]
34. Bonaccorsi L, Carloni V, Muratori M, Salvadori A, Giannini A, Carini M, et al. Androgen receptor expression in prostate carcinoma cells suppresses α6β4 integrin-mediated invasive phenotype. Endocrinology 2000;141:3172–82. [PubMed: 10965888]
35. Evangelou A, Letarte M, Marks A, Brown TJ. Androgen modulation of adhesion and antiadhesion molecules in PC-3 prostate cancer cells expressing androgen receptor. Endocrinology 2002;143:3897–904. [PubMed: 12239101]
36. Heisler LE, Evangelou A, Lew AM, Trachtenber J, Elsholtz HP, Brown TJ. Androgen-dependent cell cycle arrest and apoptotic death in PC-3 prostatic cell cultures expressing a full-length human androgen receptor. Mol Cell Endocrinol 1997;126:59. [PubMed: 9027364]
37. Dillard PR, Lin MF, Khan SA. Androgen-independent prostate cancer cells acquire the complete steroidogenic potential of synthesizing testosterone from cholesterol. Mol Cell Endocrinol 2008;295:115–20. [PubMed: 18782595]
38. Locke JA, Guns ES, Lubik AA, Adomat HH, Hendy SC, Wood CA, et al. Androgen levels increase by intratumoral de novo steroidogenesis during progression of castration-resistant prostate cancer. Cancer Res 2008;68:6407–15. [PubMed: 18676866]
39. Stanbrough M, Bubley GJ, Ross K, Golub TR, Rubin MA, Penning TM, et al. Increased expression of genes converting adrenal androgens to testosterone in androgen-independent prostate cancer. Cancer Res 2006;66:2815–25. [PubMed: 16510604]

40. Tararova ND, Narizhneva N, Krivokrisenko V, Gudkov AV, Gurova KV. Prostate cancer cells tolerate a narrow range of androgen receptor expression and activity. Prostate 2007;67:1801–15. [PubMed: 17935158]

41. Ismail HA, Lessard L, Mes-Masson AM, Saad F. Expression of NF-κB in prostate cancer lymph node metastases. Prostate 2004;58:308–13. [PubMed: 14743471]

42. Setlur SR, Royce TE, Sboner A, Mosquera JM, Demichelis F, Hofer MD, et al. Integrative microarray analysis of pathways dysregulated in metastatic prostate cancer. Cancer Res 2007;67:10296–303. [PubMed: 17974971]

43. Chen CD, Sawyers CL. NF-κB activates prostate-specific antigen expression and is upregulated in androgen-independent prostate cancer. Mol Cell Biol 2002;22:2862–70. [PubMed: 11909978]

44. Jin RJ, Lho Y, Connelly L, Wang Y, Yu X, Saint Jean L, et al. The NF-κB pathway controls the progression of prostate cancer to androgen-independent growth. Cancer Res 2008;68:6762–9. [PubMed: 18701501]

45. Lessard L, Karakiewicz PI, Bellon-Gagnon P, Alam-Fahmy M, Ismail HA, Mes-Masson AM, et al. Nuclear localization of NF-κB p65 in primary prostate tumors is highly predictive of pelvic lymph node metastases. Clin Cancer Res 2006;12:5741–5. [PubMed: 17020979]

46. Domingo-Domenech J, Oliva C, Rovira A, Codony-Servat J, Bosch M, Filella X, et al. Interleukin 6, a NF-κB target, predicts resistance to docetaxel in hormone-independent prostate cancer and NF-κB inhibition by PS-1145 enhances docetaxel antitumor activity. Clin Cancer Res 2006;12:5578–86. [PubMed: 17000695]

47. Castilla C, Congregado B, Chinchon D, Torrubia FJ, Japon MA, Saez C. Bcl-xL is overexpressed in hormone-resistant prostate cancer and promotes survival of LNCaP cells via interaction with proapoptotic Bak. Endocrinology 2006;147:4960–7. [PubMed: 16794010]

48. Krajewska M, Krajewski S, Epstein JI, Shabaik A, Sauvageot J, Song K, et al. Immunohistochemical analysis of Bcl-2, Bax, Bcl-x, and Mcl-1 expression in prostate cancers. Am J Pathol 1996;148:1567–76. [PubMed: 8623925]

49. Nagakawa OAT, Hayakawa Y, Junicho A, Koizumi K, Fujiuchi Y, Furuya Y, et al. Differential expression of integrin subunits in DU-145/AR prostate cancer cells. Oncology Rep 2004;12:837–41.

50. Lin CS, Chen Y, Huynh T, Kramer R. Identification of the human α6 integrin gene promoter. DNA Cell Biol 1997;16:929–37. [PubMed: 9303435]

51. Murillo H, Huang H, Schmidt LJ, Smith DI, Tindall DJ. Role of PI3K signaling in survival and progression of LNCaP prostate cancer cells to the androgen refractory state. Endocrinology 2001;142:4795–805. [PubMed: 11606446]

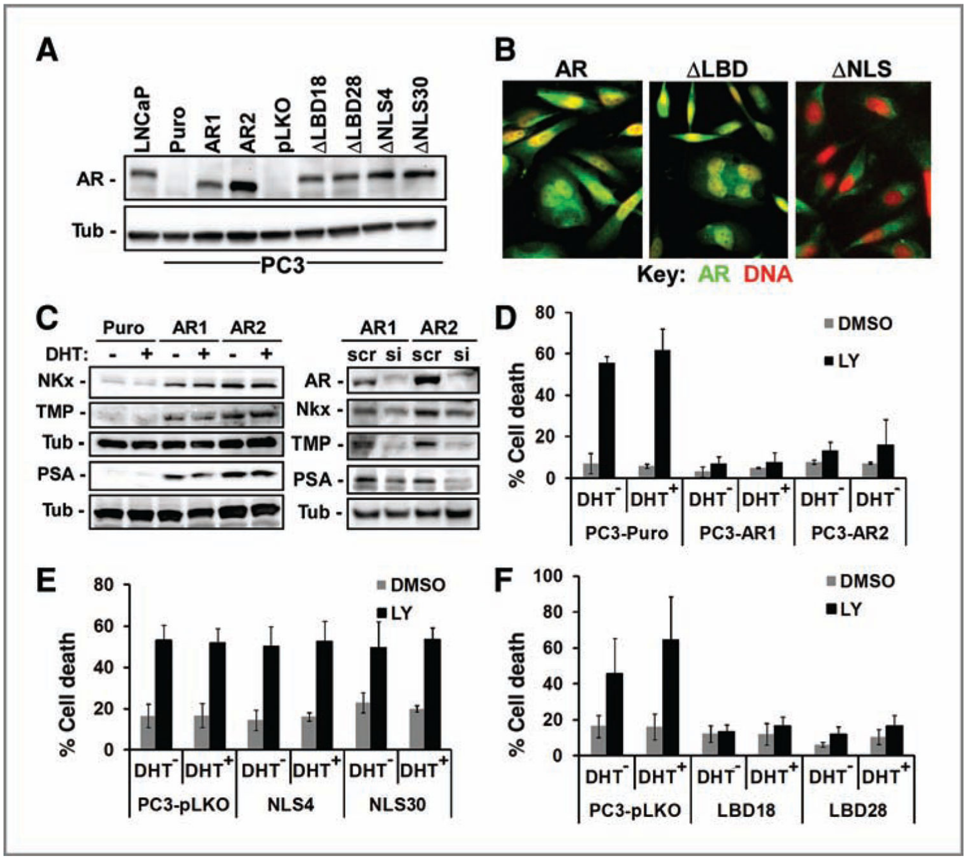

Figure 1.

AR stimulates cell survival independently of PI3K. A, AR and tubulin (Tub) expression in LNCaP, PC3 vector controls (Puro or pLKO), and PC3 cells expressing wild-type AR (AR), a ligand-binding mutant (ΔLBD), or a nuclear-localization mutant (ΔNLS) monitored by immunoblotting. B, PC3-AR1 (AR), ΔLBD28 (ΔLBD), and ΔNLS4 (ΔNLS) cells immunostained for AR (green) and counterstained for DNA (red). Yellow indicates colocalization. C, Nkx3.1 (Nkx), TMPRSS2 (TMP), PSA, AR, and tubulin (Tub) expression in PC3-Puro, AR1, and AR2 cells treated with vehicle (−) or 10 nmol/L DHT (+) or treated with AR (si) or control (scr) siRNA. D, viability of PC3-Puro, AR1, AR2 or (E and F) PC3-pLKO, ΔNLS, or ΔLBD cells plated on LM, treated with vehicle (−) or 10 nmol/L DHT in the presence of DMSO or LY294002 (LY). Error bars, SD; $n = 3–5$.

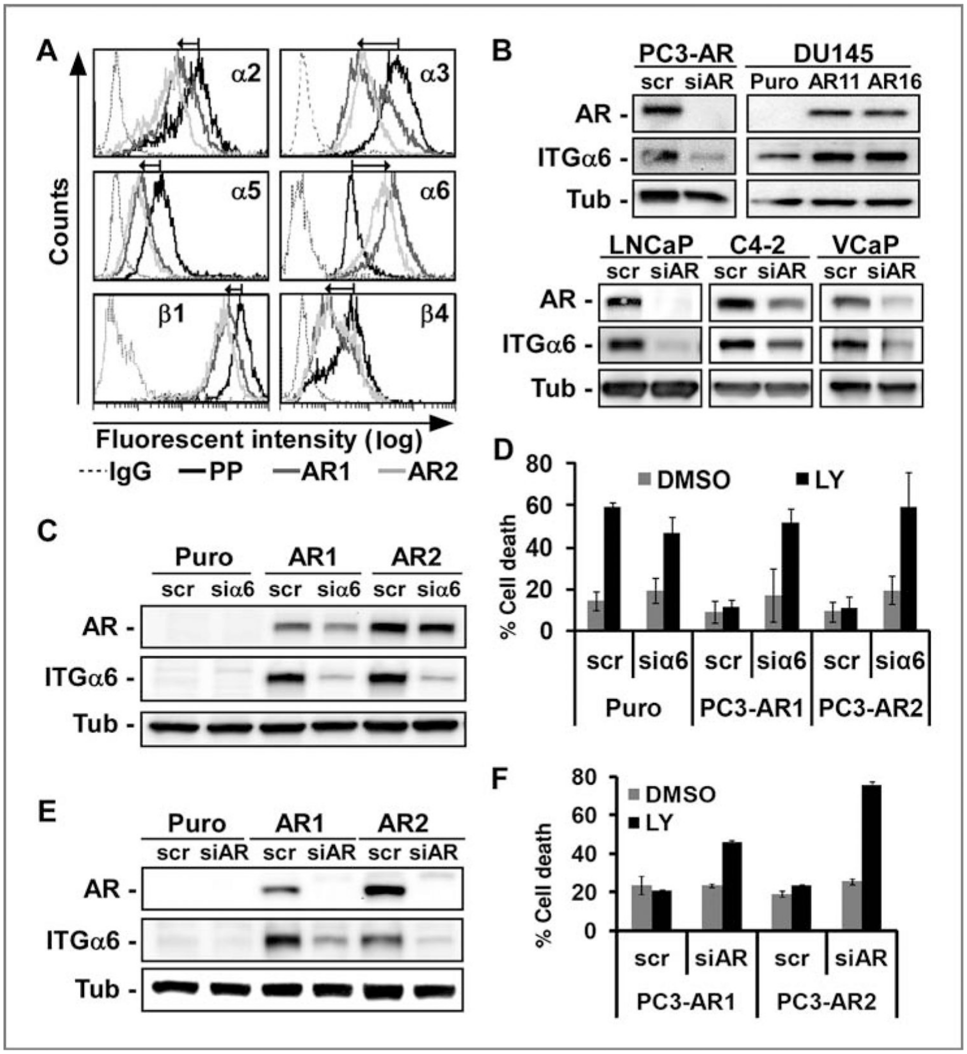

Figure 2.

AR promotes survival through upregulation of integrin α6β1. A, FACS analysis of integrin expression in LM-adherent PC3-Puro (PP; solid black), AR1 (solid dark gray), and AR2 (solid light gray) cells. IgG control is dashed line. Small arrows indicate direction of peak shifts. *N* = 5–8. B, AR, integrin α6 (ITGα6), and 1/4 tubulin expression in PC3-AR, LNCaP, C4–2, or VCaP cells treated with AR (siAR) or control (scr) siRNA or in DU145 clones expressing AR monitored by immunoblotting. C–F, PC3-Puro, AR1, and AR2 cells treated with integrin α6 (siα6), AR, or control siRNA. C and E, integrin α6, AR, and tubulin immunoblots. D and F, viability after DMSO or LY294002 (LY) treatment.

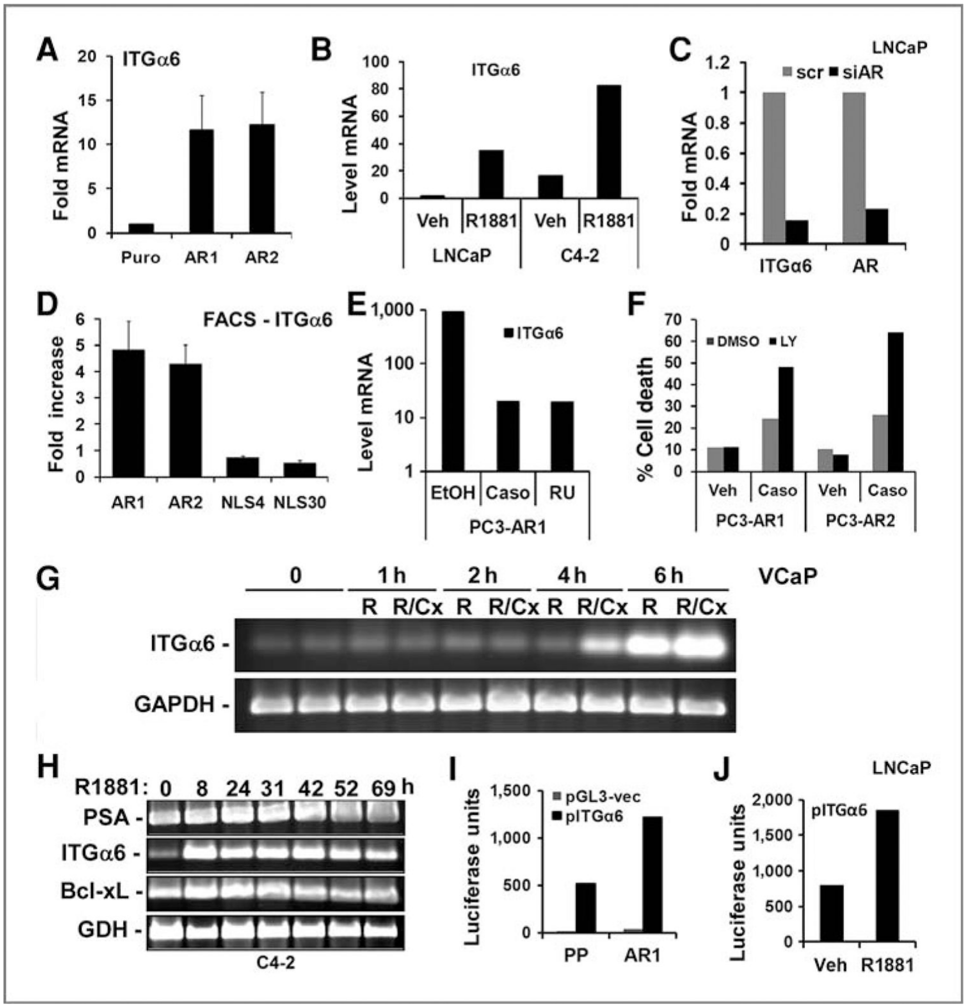

Figure 3.

AR transcriptionally regulates integrin α6β1. A, integrin α6 (ITGα6) mRNA measured by qRT-PCR in PC3-Puro, AR1, and AR2 cells. B and C, integrin α6 or AR mRNA measured by qRT-PCR in (B) LNCaP and C4–2 or (C) LNCaP cells (B) treated 24 hours with vehicle (Veh) or 5 nmol/L R1881 or (C) 48 hours with AR or control siRNA. D, FACS analysis of α6 expression in AR1, AR2, ΔNLS-AR4, and ΔNLS-AR30 cells. Values are normalized to vector control cells. E, integrin α6 mRNA measured by qRT-PCR in PC3-AR1 cells treated with vehicle (EtOH), 10 nmol/L Casodex (Caso), or 10 nmol/L RU486 (RU). F, viability of PC3-AR1 or AR2 cells treated with Casodex in the absence or presence of LY294002 (LY). G, time course of α6 and GAPDH mRNA in VCaP cells stimulated with 5 nmol/L R1881 (R) in the absence or presence of 10 μg/mL cycloheximide (Cx). H, time course of PSA, α6, Bcl-xL, and GAPDH mRNA in C4–2 cells stimulated with 5 nmol/L R1881. I, luciferase activity in PC3-Puro, PC3-AR1, or (J) LNCaP cells transiently transfected with vector (pGL3-vec) or integrin α6 reporters (pITGα6). LNCaPs were treated with vehicle (Veh) or 5 nmol/L R1881 for 24 hours.

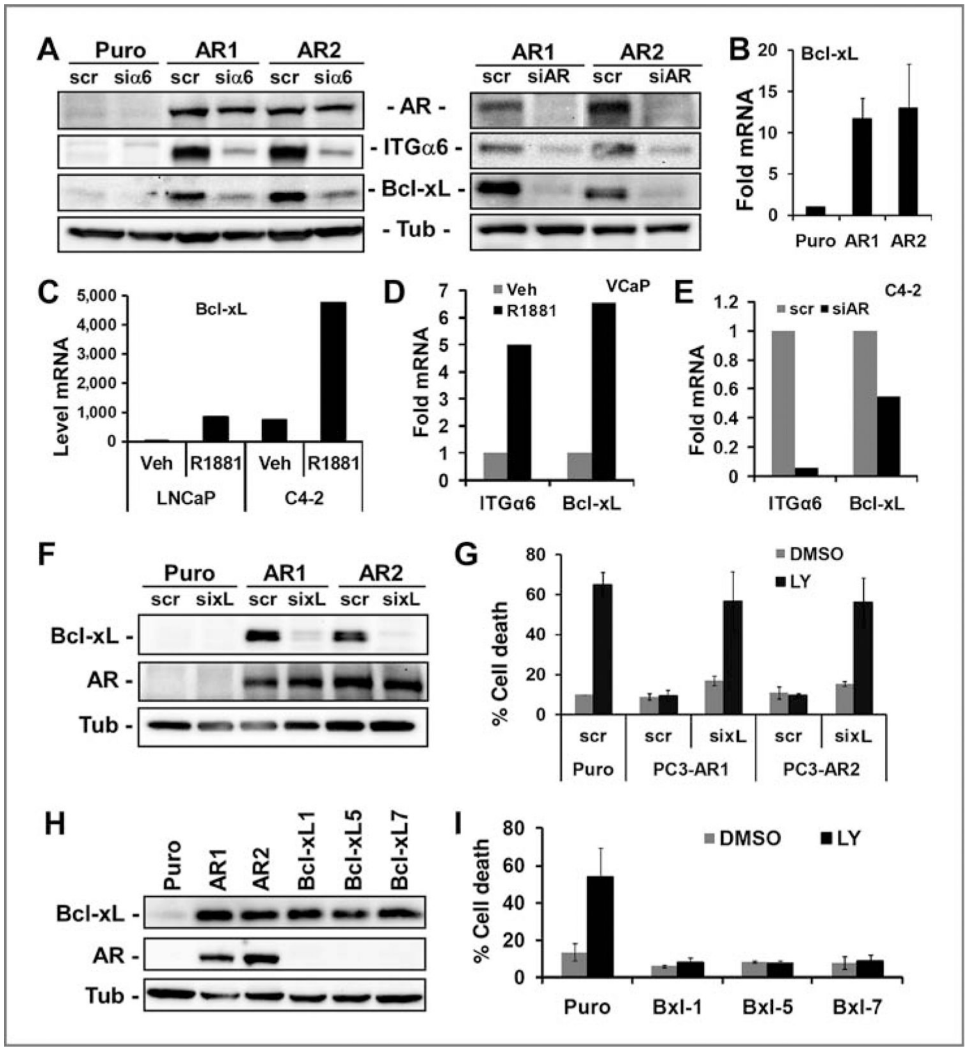

Figure 4.

Bcl-xL promotes AR/α6β1-dependent survival independent of PI3K. A, AR, α6, Bcl-xL, and tubulin (Tub) expression in PC3-Puro, AR1, and AR2 cells treated with α6, AR, or control siRNA monitored by immunoblotting. B–E, Bcl-xL or α6 mRNA measured by qRT-PCR in (B) PC3-Puro, AR1, AR2, (C and E) LNCaP, C4–2, or (D) VCaP cells treated with (C and D) R1881 or (E) siRNA; Veh, vehicle. F and G, cells treated with Bcl-xL (sixL) or control siRNA. F, Bcl-xL, AR, and tubulin (Tub) immunoblots. G, viability of DMSO- or LY294002-treated cells. H, Bcl-xL, AR, and tubulin (Tub) expression in PC3 cells stably overexpressing Bcl xL. I, viability of PC3-Puro and Bcl-xL (Bxl) clones treated with DMSO or LY294002 (LY).

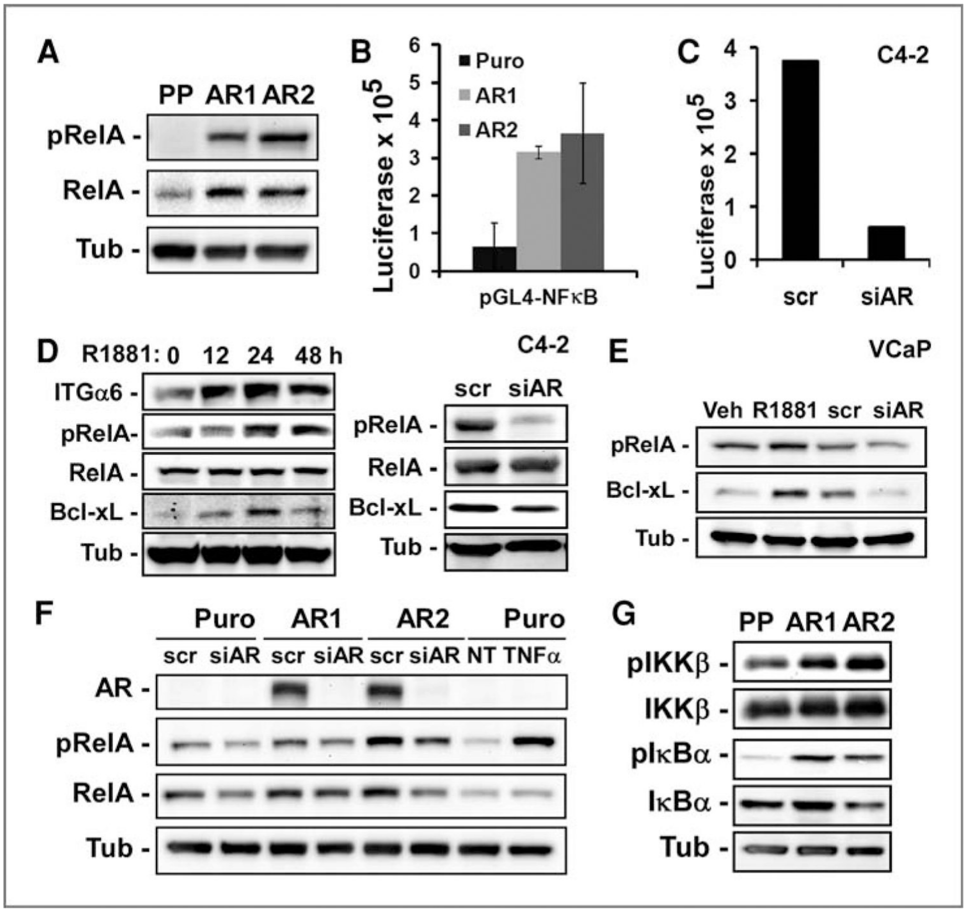

Figure 5.

AR stimulates NF-κB activity. A, B, and F, PC3-Puro (PP), AR1, and AR2 cells, or (C–E) C4–2 and VCaP cells treated with AR or control siRNA, or treated with vehicle (Veh) or R1881. NF-κB activity measured by (A, D–F) immunoblotting for phosphorylated RelA (pRelA) or (B, C) transfection of an NF-κB luciferase reporter. Integrin α6, Bcl-xL, or total RelA measured by immunoblotting. F, control cells not treated (NT) or treated with 10 ng/mL TNFα for 1 hour. G, IKKβ (pIKKβ) and IκBα (pIκBα) phosphorylation monitored by immunoblotting of immunoprecipitated IKKβ or IκBα in total cell lyastes with phospho-specific antibodies; Tub, tubulin.

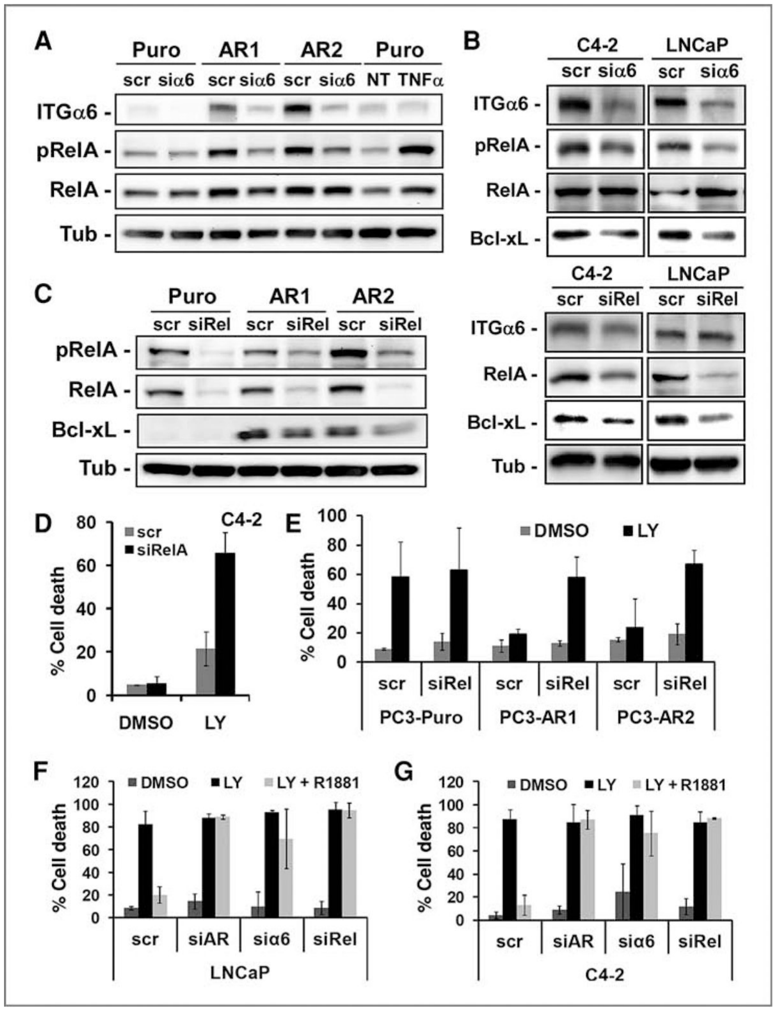

Figure 6.

Integrin α6 stimulates NF-κB activity and survival. A–C, PC3-Puro, AR1, AR2, LNCaP, or C4–2 cells treated with α6, RelA (siRel), or control siRNA. Control cells not treated (NT) or treated with 10 ng/mL TNFα. RelA phosphorylation, total RelA, AR, α6, Bcl-xL, or tubulin (Tub) was monitored by immunoblotting. D and E, viability of RelA siRNA–transfected C4–2, PC3-Puro, AR1, or AR2 cells treated with DMSO or LY294002 (LY). F and G, viability of LNCaP or C4–2 cells transfected with control, AR, α6, or RelA siRNA and subsequently treated with DMSO, LY294002, or LY294002 + R1881.

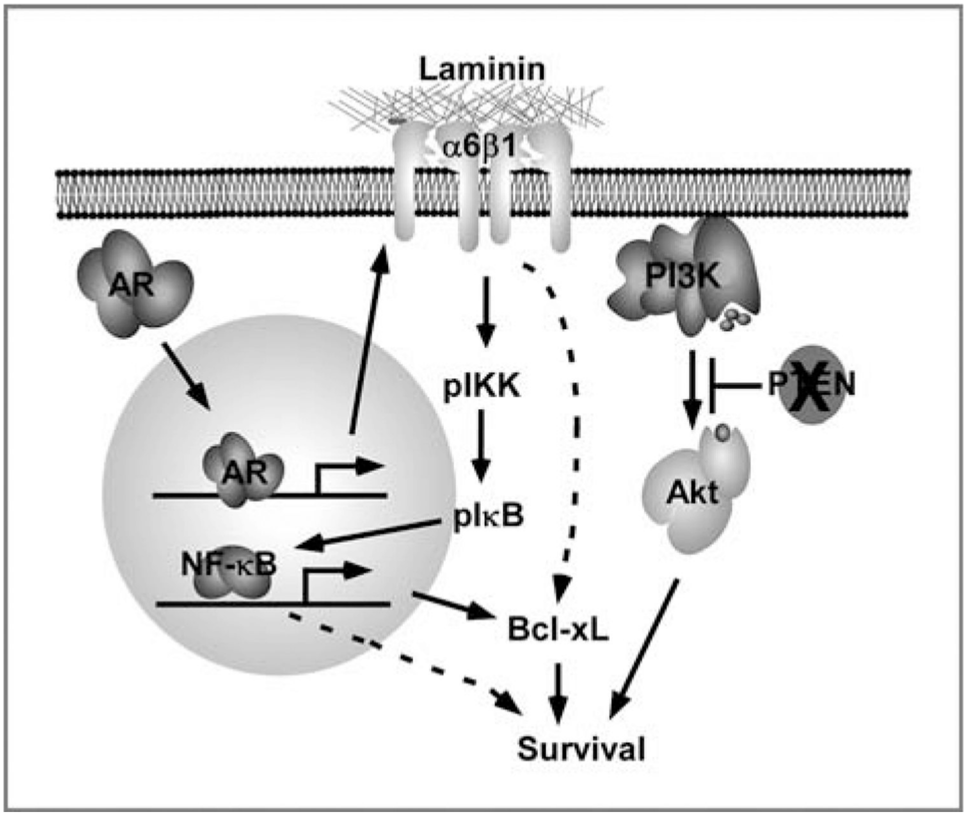

Figure 7.
Model for AR/α6β1-mediated survival. AR stimulates integrin α6 transcription and expression leading to canonical activation of NF-κB and upregulation of Bcl-xL. NF-κB, in part, increases Bcl-xL expression. NF-κB and Bcl-xL are required for survival on laminin independent of PI3K.