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14. ABSTRACT
Although prostate cancer patients initially respond to androgen ablation therapy, they ultimately relapse and the tumor no longer responds to androgen, offering little hope for long-term disease-free survival. However, inhibition of AR expression in cells leads to cell death. This suggests that prostate cancer cells are still dependent on AR for survival, even if the cells are no longer responding to physiological levels of androgen. We have demonstrated that expression of AR in PC3 prostate tumor cells can rescue cells from death induced by inhibition of PI3K. Expression of AR in PC3 cells leads to increased expression of integrin α6β1 and Bcl-xL along with increased activation of NF-κB. Blocking each these components individually concurrent with inhibition of PI3K led to death of the AR-expressing cells, suggesting that AR regulates cell survival through enhancement of α6β1/NF-κB/Bcl-xL signaling. We have also confirmed that this pathway is activated in prostate cancer cell lines expressing endogenous AR, including LNCaP, C4-2 and VCaP cells.

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Cell biology, integrins, androgen receptor (AR), signal transduction, survival signaling, differentiation

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INTRODUCTION

Prostate cancer is the second leading cause of cancer death in men and death is due to metastasis. While primary prostate tumors are often curable, metastatic tumors are not. Androgen ablation therapy has been the most commonly prescribed treatment for metastatic prostate cancer for the last sixty years. Androgen ablation therapy prevents androgen function by inhibiting both the production of androgen and its binding to its receptor, androgen receptor (AR). Although patients initially respond to treatment, they ultimately relapse and the tumor no longer responds to androgen, offering little hope for long-term disease-free survival. However, inhibition of AR expression or its DNA binding activity even in androgen independent (i.e. non-responsive) cells inhibits their proliferation and leads to cell death. This suggests that prostate cancer cells are still dependent on AR for survival, even if the cells are no longer responding to physiological levels of androgen. Thus targeting AR directly or its downstream effectors that regulate survival would be a more effective therapeutic approach for targeting and killing prostate cancer cells. Development of new strategies for more effective treatment of prostate cancer is limited by an incomplete understanding of the mechanisms regulating cell survival of either normal prostate or prostate cancer cells.

Prostate cancer arises from the epithelial layer of the prostate. The normal prostate epithelium consists of two types of cells, basal cells and secretory cells. In the basal cells, which do not express AR, adhesion to the extracellular matrix in the basement membrane is required for cell survival. In the secretory cells, which do express AR, survival is independent of matrix and is suggested to be regulated by AR since these cells die during androgen ablation therapy. In normal prostate epithelial, adhesion to matrix and AR expression are mutually exclusive events. However, in prostate cancer, the tumor cells express AR and are adherent to matrix, allowing for interactions between these two signaling pathways. **My hypothesis was that the interaction of cancer cells with the matrix and the integration of signals from integrins and AR regulate their survival, while AR regulates survival of normal cells independently of integrins.** The objective of these studies is to identify the AR- and integrin-mediated mechanisms which regulate survival in AR expressing tumor and normal prostate cells. By understanding the activities that lie downstream of AR that directly regulate survival of the tumor cells versus normal cells, a specific approach to disrupt AR-dependent actions only in the tumor cells can be developed, which will lead to the death of tumor cells without harming normal prostate tissues.

BODY

My working hypothesis is that the interaction of cancer cells with the matrix and the integration of signals from integrins and AR regulate their survival, while survival of normal cells is regulated independently of integrins. To accomplish the tasks outlined in the statement of work, AR expressing prostate cancer cells and AR expressing normal cells first had to be generated as previously described and validated in the original submission.

Summary of Aim 1:

The goal of Aim 1 in our Statement of Work was to determine how AR signaling mediates survival in prostate cancer cells *in vitro*. My *working hypothesis* was that AR activation will independently regulate the same downstream survival targets as those regulated by the PI3K/Akt pathway, such as survivin, such that inhibition of signaling from either PI3K/Akt or AR can be rescued by the other pathway**.** **Task A** was to determine if AR expression affects integrin-mediated survival signaling pathways in DU145s. Along with another graduate student, Jelani Zarif, I began these studies. Similar to what we found in PC3 cells (2008 report), we found that integrin α6 expression is up-regulated in AR expressing DU145 cells (see attached paper) [1]. However, due to time constraints and scientific feedback from presentations and

meetings, it was suggested that I should instead focus on determining if the AR/integrin α6 pathway I found in PC3 cells expressing AR (Fig. 1) also existed in cell lines that expressed endogenous AR. For these studies, I used LNCaP, C4-2, and VCaP cells. Using these models, I was able to confirm that AR regulated integrin α6 expression, and subsequently up-regulated NF-κB activity and Bcl-xL expression [1]. In LNCaP cells, PI3K signaling is required for cell survival, however androgen stimulation can promoted cell survival even when PI3K signaling is lost [2]. Inhibition of AR, integrin α6, or NF-κB RelA by siRNA was sufficient to induce cell death in the presence of PI3K loss and androgen stimulation [1], suggesting that androgen mediates its pro-survival effects via AR, integrin α6, and NF-κB. Emphasis was also placed on preparing and submitting a manuscript describing these results and those obtained for **Task B**. This manuscript [1] has been submitted to and tentatively accepted by *Cancer Research* pending editorial revisions and is attached as a pdf.

Lamb, L.E., Zarif, J.C., and Miranti, C.K., *E-Cadherin-Mediated Survival of Androgen Receptor Expressing Secretory Prostate Epithelial Cells Derived from a Stratified In Vitro Differentiation Model.* Cancer Research, 2011. In Revisions.

As such, **Task B** is complete.

In **summary** of Aim 1, we demonstrated that re-expression of wild type AR in PC3 cells prevented the cell death normally induced upon inhibition of PI3K signaling, independent of androgen. Re-expression of AR in PC3 cells lead to increased expression of integrin α6 and subsequent activation of NF-κB and increased expression of the pro-survival protein Bcl-xL (Fig. 1). Loss of AR, integrin α6, NF-κB, or Bcl-xL re-sensitized AR-expressing PC3 cells to PI3K-dependent survival. Treatment of AR expressing PC3 cells with the AR inhibitor RU486 or AR specific siRNA, or expression of AR mutants lacking the ability to translocate to the nucleus (ΔNLS), but not to bind ligand (ΔLBD), largely restored the parental PC3 phenotype, including PI3K dependent survival. These results are supported by siRNA knock-down of endogenous AR, integrin α6, or NF-κB in LNCaP, C4-2, and VCaP cells. Thus AR can support prostate tumor cell survival on laminin via enhanced expression of α6β1 integrin, leading to elevated Bcl-xL levels, by a mechanism that is independent of PI3K.

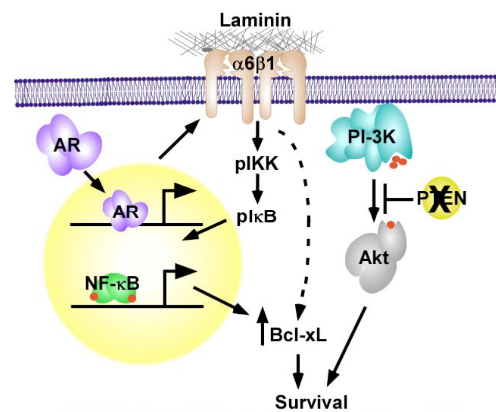

FIGURE 1. Model for AR signaling in PC3 cells. AR promotes cell survival independent of DHT or PI3K signaling. AR regulates survival via the integrin α6, which leads to phosphorylation of IKK and IκB and subsequent up-regulation of NF-κB signaling. This leads to increased Bcl-xL expression, which regulates survival independent of the PI3K pathway.

Summary of Aim 2:

The goal of Aim 2 is to determine how AR mediates survival in normal primary prostate epithelial cells *in vitro.* My *working hypothesis* was that the integrin-mediated survival pathway in primary prostate epithelial cells will shift from being dependent on EGFR to being dependent on AR. In addition, AR regulates survival by directly regulating survivin. **Task A** was to determine whether AR expression affects integrin-mediated survival signaling pathways in PECs. **Task B** was to determine if survivin mediate survival in PECs expressing AR. **Task C** was to determine if integrins mediate survival in AR expressing PECs. I had proposed to complete the tasks in the second to third year of funding. However, I made significant progress on Aim 2 in the first two years of funding (2008 and 2009 Report). These results have been published in the below reference [3] that has also been attached as a pdf.

Lamb, L.E., Knudsen, B.S., and Miranti, C.K., *E-Cadherin-Mediated Survival of Androgen Receptor Expressing Secretory Prostate Epithelial Cells Derived from a Stratified In Vitro Differentiation Model.* J. Cell Science, 2010. **123**: p. 266-276

KEY RESEARCH ACCOMPLISHMENTS

1. Generated the new cell lines/models PC3-AR, PC3-ΔNLS-AR, and PC3-ΔLBD-AR.
2. Determined that nuclear localization, but not ligand binding, is required for AR-mediated survival.
3. Determined that survivin levels were not affected by adhesion to different matrices, androgen, AR expression, or treatment with the PI-3K inhibitor LY294002 in AR expressing PC3 cells.
4. Demonstrated that expression of AR in PC3 cells regulates survival via integrin α6/Bcl-xL signaling.
5. Demonstrated that expression of AR in PC3 cells up-regulates Src signaling independent of integrin α6.
6. Demonstrated that AR expression in PC3 cells results in altered cell morphology including increased filopodia expression and increases cell migration.
7. Generated and characterized an *in vitro* differentiation model of the prostate epithelium which generates differentiated AR-expressing secretory cells that recapitulate many *in vivo* characteristics.
8. Demonstrated that secretory-like cells are dependent on E-cadherin and PI-3K signaling, but not androgen, integrins, or EGFR signaling, for survival.

REPORTABLE OUTCOMES

The following items have been generated due to the research carried out in the last year or in the funding year indicated.

1. In the first year, the following abstracts were presented at scientific meetings as poster presentations.

Lamb, L.E., Zarif, J.C., Miranti, C.K. 2008. Integrin Regulation of Survival in Prostate Tumor Cells Expressing Androgen Receptor. Gordon Research Conference: “Signaling by Adhesion Receptors”, South Hadley, MA, June 29-July 4.

Lamb, L.E., Zarif, J.C., Miranti, C.K. 2008. Integrin Regulation of Survival in Prostate Tumor Cells Expressing Androgen Receptor. Gordon Graduate Research Seminar: “Signaling by Adhesion Receptors”, South Hadley, MA, June 28-29.

Lamb, L.E., Zarif, J.C., Miranti, C.K. 2008. AR Regulates Bcl-xL and Integrins in Prostate Tumor Cells Expressing Androgen Receptor. Michigan Prostate Research Colloquium: “Frontiers in Urologic Cancer: Molecular Mechanisms and Therapeutic Strategies”, Grand Rapids, MI, May 2-3.

In the second year, the following abstracts were presented at scientific meetings as poster presentations.

Lamb, L.E., Zarif, J.C., Miranti, C.K. 2009. AR-Enhanced α6β1 Integrin and Bcl-x_L Expression Promotes Androgen-Independent Prostate Tumor Cell Survival Independently of PI3K Signaling**.** American Association for Cancer Research: “Advances in Prostate Cancer Research”, San Diego, CA, January 21-24.

Lamb, L.E., Knudsen, B.S., Miranti, C.K. 2009. E-Cadherin-Mediated Survival of Androgen Receptor Expressing Secretory Prostate Epithelial Cells Derived from a Stratified In Vitro Differentiation Model. Michigan Prostate Research Colloquium: “Prostate Tumor Microenviroment and Metastasis”, Detroit, MI, May 30.

Also won a Third Place Poster Award.

Lamb, L.E., Knudsen, B.S., Miranti, C.K. 2009. A Novel *In Vitro* Differentiation Model to Study Survival Signaling of Androgen Receptor Expressing Prostate Secretory Cells**.** American Association for Cancer Research: "Advances in Prostate Cancer Research", San Diego, CA, January 21-24.

In the second year, the following abstract was also presented as a talk at a meeting.

Lamb, L.E., Knudsen, B.S., Miranti, C.K. 2009. E-Cadherin-mediated Survival of Androgen Receptor Expressing Secretory Prostate Epithelial Cells Derived from a Stratified *In Vitro* Differentiation Model. 2nd Salk Institute Mechanisms and Models of Cancer meeting, La Jolla, CA, August 12-16.

In the past year, two abstracts were presented at scientific meetings as poster presentations. A copy of one of the abstracts is included in the appendix.

Lamb, L.E., Zarif, J.C., Miranti, C.K. 2010. AR-Enhanced α6β1 Integrin and Bcl-xL Expression And NF-κB Activation Promotes Androgen-Independent Prostate Tumor Cell Survival Independently of PI-3K Signaling**.** Michigan Urological Oncology Research Colloquium: "Animal Models for Bladder and Prostate Cancer", Ann Arbor, MI, June 11-12.

Lamb, L.E., Zarif, J.C., Miranti, C.K. 2010. Androgen Receptor-Enhanced Integrin α6β1 Regulation of Prostate Tumor Survival and Invasion on Laminin**.** Metastasis Research Society and American Association of Cancer Researchers Joint Meeting: "Metastasis and the Tumor Microenvironment", Philadelphia, PA, September 12-15.

2. Last year, I had the following paper accepted in Journal of Cell Science.

   Lamb, L.E., Knudsen, B.S., and Miranti, C.K., *E-Cadherin-Mediated Survival of Androgen Receptor Expressing Secretory Prostate Epithelial Cells Derived from a Stratified In Vitro Differentiation Model.* J. Cell Science, 2010. **123**: p. 266-276.

   I submitted the following paper in the last year to *Cancer Research* and is tentatively accepted pending editorial revisions. A copy is in the appendix.

   Lamb, L.E., J. Zarif, and C.K. Miranti. *AR-Enhanced α6β1 Integrin and Bcl-xL Expression Promotes Androgen-Independent Prostate Tumor Cell Survival Independently of PI-3K Signaling.* Cancer Res., 2011. *In Review.*

3. Last year, I co-filed a patent based on the newly published work.

   A Method for Inducing Differentiation of Androgen Receptor-Expressing Prostate Epithelial Cells
   U.S. Patent Office Application No. 61173783, April 29, 2009.

4. In the last year, I joined the professional society, American Association for Cancer Research.

5. I graduated from Michigan State University with my Ph.D. Spring 2010.

6. I obtained a post-doctoral fellow position in the laboratory of Dr. Raphael Kopan at Washington University in Saint Louis.

CONCLUSIONS

These studies have demonstrated that expression of AR in PC3 cells can rescue cells from death induced by inhibition of PI3K when adherent to laminin 1. This is important because of the proposed role for laminin in tumor invasion and metastasis [4]. Expression of AR in PC3 cells leads to increased expression of integrin α6β1 and Bcl-xL along with increased activation of NF-κB. Blocking each of these components individually concurrent with inhibition of PI3K led

to death of the AR-expressing cells, suggesting that AR regulates cell survival through enhancement of α6β1/NF-κB/Bcl-xL signaling. To assess the role of AR in normal cell survival, we generated a novel in vitro differentiation model. Confluent primary human prostate epithelial cell cultures were treated with KGF and androgen (DHT). After two weeks, a suprabasal cell layer formed in which cells no longer expressed integrins, p63, K5/14, EGFR, FGFR2IIIb, or Bcl-2, but instead expressed AR and androgen-induced differentiation markers, including K18/19, TMPRSS2, Nkx3.1, PSMA, KLK2 and secreted PSA. Differentiated prostate cell survival depended on E-cadherin and PI3K, but not KGF, DHT, AR or MAPK. Therefore, while in the prostate tumor cell line PC3, AR and integrin α6β1 cooperate to drive cell survival, neither AR nor integrins were required for survival of differentiated prostate epithelial cells. Furthermore, targeting the PI3K pathway or AR alone will be ineffective in treating metastatic prostate cancer; rather both these pathways, possibly in combination with integrin α6β1, NF-κB, and/or Bcl-xL, must be targeted in order to effectively kill metastatic prostate cancer. Lastly, I have developed an in vitro differentiation model that will allow further investigation into a cell population previously unavailable for in vitro studies and can be utilized to predict the effects of new prostate cancer therapies on the normal AR expressing epithelial cells of the prostate.

LIST OF PERSONNEL PAID FROM RESEARCH EFFORT

Laura Lamb 100% effort

REFERENCES

1. **Lamb, L.E.**, J. Zarif, and C.K. Miranti. *AR-Enhanced α6β1 Integrin and Bcl-xL Expression Promotes Androgen-Independent Prostate Tumor Cell Survival Independently of PI-3K Signaling.* Cancer Res., 2011. *In Review.*
2. Lin, J., Adam, R.M., Santiestevan, E., and Freeman, M.R. *The phosphatidylinositol 3'-kinase pathway is a dominant growth factor-activated cell survival pathway in LNCaP human prostate carcinoma cells.* Cancer Res., 1999. **59**(12): p. 2891-2897.
3. **Lamb, L.E**., Knudsen, B.S., and Miranti, C.K. *E-Cadherin-Mediated Survival of Androgen Receptor Expressing Secretory Prostate Epithelial Cells Derived from a Stratified In Vitro Differentiation Model.* J. Cell Science, 2010. **123**: p. 266-276.
4. Cress, A. E., Rabinovitz, I., Zhu, W., and Nagle, R. B. The alpha 6 beta 1 and alpha 6 beta 4 integrins in human prostate cancer progression. Cancer Metastasis Rev., 1995 **14**: p. 219-228.

APPENDIX

ABSTRACT

Poster Presentation:

Metastasis Research Society and American Association of Cancer Researchers Joint Meeting: "Metastasis and the Tumor Microenvironment", Philadelphia, PA, September 12-15.

AR-Enhanced α6β1 Integrin and Bcl-xL Expression and NF-κB Activation Promotes Androgen-Independent Prostate Tumor Cell Survival Independently of PI-3K Signaling

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Development of strategies for more effective treatment of prostate cancer is limited by an incomplete understanding of the mechanisms regulating survival of prostate cancer cells. Androgen receptor (AR) signaling plays an important role in regulating cell survival in prostate cancer, even in castration-resistant patients where AR continues to function independently of exogenous androgens to promote prostate cancer survival. Likewise, adhesion to matrix through integrins is required for survival of epithelial cells. In prostate cancer, the AR-positive tumor cells express primarily integrin α6β1 which they use to adhere to a laminin-rich matrix in the tumor. Our hypothesis is that the interaction of cancer cells with the matrix and the integration of signals from integrins and AR regulate their survival. We have previously demonstrated that PC3 prostate tumor cell lines require PI3K signaling for cell survival on laminin matrix. In this study, we demonstrated that expression of wild type AR in PC3 cells rescues cells from death induced by inhibition of PI3K when adherent to laminin. Rescue of cell death occurrs independently of androgen, requires nuclear-localized AR, and is blocked by AR antagonists. Expression of AR in PC3 cells leads to increased transcription and expression of integrin α6β1 and Bcl-xL along with increased activation of PAK2 and NF-κB signaling. Blocking integrin α6, NF-κB, or Bcl-xL individually concurrent with inhibition of PI3K is sufficient and necessary to induce death of the AR-expressing cells. Therefore on laminin, AR regulates cell survival through enhancement of α6β1/NF-κB/Bcl-xL signaling. These findings have significant implications for therapeutic targeting of androgen-independent prostate cancer cells; inhibition of both the PI3K and AR/α6β1 pathways will minimally be required to effectively treat prostate cancer.

E-cadherin-mediated survival of androgen-receptor-expressing secretory prostate epithelial cells derived from a stratified in vitro differentiation model

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Summary

The androgen receptor (AR) is expressed in differentiated secretory prostate epithelial cells in vivo. However, in the human prostate, it is unclear whether androgens directly promote the survival of secretory cells, or whether secretory cells survive through androgen-dependent signals from the prostate stroma. Biochemical and mechanistic studies have been hampered by inadequate cell-culture models. In particular, large-scale differentiation of prostate epithelial cells in culture has been difficult to achieve. Here, we describe the development of a differentiation system that is amenable to functional and biochemical analysis and its application to deciphering the survival pathways in differentiated AR-expressing epithelial cells. Confluent prostate epithelial cell cultures were treated with keratinocyte growth factor (KGF) and dihydrotestosterone. After 2 weeks, a suprabasal cell layer was formed in which cells no longer expressed α2, α3, α6, αv, β1 or β4 integrins or p63, K5, K14, EGFR, FGFR2IIIb or Bcl-2, but instead expressed AR and androgen-induced differentiation markers, including K18, K19, TMPRSS2, Nkx3.1, PMSA, KLK2 and secreted prostate-specific antigen (PSA). Differentiated prostate cell survival depended on E-cadherin and PI3K, but not KGF, androgen, AR or MAPK. Thus survival of differentiated prostate epithelial cells is mediated by cell-cell adhesion, and not through androgen activity or prostate stroma-derived KGF.

Key words: Prostate, Epithelial, Androgen receptor, Secretory cells, Survival, Differentiation

Introduction

Epithelial cells serve several vital functions. For instance, all epithelial cells act as a barrier to protect organs from external environmental assault, as exemplified by the skin. Intestinal epithelial cells are required for the absorption of nutrients, and mammary and prostate epithelial cells are primarily secretory. Proper regulation of epithelial differentiation is crucial for the development and maintenance of barrier and organ function. Differentiation of epithelial cells has been extensively characterized in the epidermis. The basal layer of the epidermis consists of proliferating keratinocytes that adhere to a basement membrane via integrins. Loss of basal cell adhesion through integrin β1 initiates terminal differentiation, resulting in flattening of the cells, expression of differentiation proteins, and subsequent cornification, which ultimately produces several distinct stratified cell layers that make up the epidermis (Levy et al., 2000; Lippens et al., 2005).

The epithelium of the human prostate consists of two cell layers, a basal layer and a secretory layer. Similar to other stratified epithelium, prostate basal cells are mitotic and adhere to a basement membrane (Knox et al., 1994; Uzgare et al., 2004; van Leenders and Schalken, 2003). Prostate basal cells give rise to terminally differentiated secretory cells (Knox et al., 1994; Uzgare et al., 2004; van Leenders and Schalken, 2003). However, unlike other epithelia, prostate epithelial cell differentiation is regulated by androgen signaling (Berger et al., 2004; Cunha et al., 1987; Heer et al., 2007; Ling et al., 2001; Whitacre et al., 2002). The androgen receptor (AR) is a nuclear transcription factor activated in response to the steroid hormone androgen (Lamb et al., 2001). AR is expressed only in the differentiated secretory cells and not in the basal cells (Lamb et al., 2001). It is unclear exactly how androgen regulates epithelial differentiation. However, tissue combination studies from AR-null mice suggest that androgen stimulation of AR in the early developing mesenchyme, and not the epithelium, is solely responsible for the induction of epithelial morphogenesis in vivo (Cunha et al., 2004).

Androgen also appears to be important for secretory cell survival, in that anti-androgen therapies specifically kill the secretory cells, leaving the basal cells intact (Denis and Griffiths, 2000). Furthermore, restoration of androgens results in regeneration of the secretory cell compartment. However, tissue recombination experiments, as well as studies using conditional knockout mice that lack AR only in prostate epithelium, suggest that AR does not directly regulate epithelial survival (Cunha et al., 2004; Wu et al., 2007). Instead, androgen stimulation of the AR-positive stromal cells of the prostate might induce secreted factors that regulate secretory cell survival. Keratinocyte growth factor (KGF) and FGF10 are two factors secreted by the stromal cells, though not in an androgen-dependent manner (Alarid et al., 1994; Cunha et al., 2004; Sugimura et al., 1996; Thomson, 2001). KGF and FGF10 are both involved in murine prostate organogenesis and can induce differentiation of isolated prostate epithelial cells (Alarid et al., 1994; Cooke et al., 1991; Cunha, 1996; Donjacour et al., 2003; Heer et al., 2006; McKeenhan, 1991; Sugimura et al., 1996). In some cases, KGF can substitute for androgens and it is likely that KGF and AR signaling pathways interact (Thomson et al., 1997). KGF has also been reported to promote differentiation and survival of the epithelium of the skin, lung and eye (Geiger et al., 2005; Marchese et al., 1997; Ray et al., 2003). KGF acts specifically on epithelial

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cells and has been reported to activate p38 MAPK signaling (Heer et al., 2006).

Clarification of the roles of androgen and KGF in prostate epithelial differentiation and survival has been hampered by our inability to culture normal differentiated AR-expressing secretory cells in vitro. Prostate epithelial cells (PECs) cultured from normal human prostate tissue consist primarily of AR-negative basal cells and their transient amplifying derivatives. Previous studies in our lab have demonstrated that survival of cultured PECs is specifically mediated through $\alpha3\beta1$-integrin-dependent adhesion (Edick et al., 2007). Similarly, basal keratinocytes are dependent on $\alpha3\beta1$ integrin for their survival (Manohar et al., 2004). During keratinocyte differentiation, basal cells lose integrin expression as well as adhesion to matrix as they are extruded to the upper layers of the skin (Watt, 2002). In suprabasal keratinocytes, as well as in other epithelia, cell-cell adhesion structures such as E-cadherin appear to promote survival through phosphoinositide 3-kinase (PI3K) signaling, and when PI3K signaling is lost these cells die (Calautti et al., 2005; Espada et al., 2009; Rivard, 2009). Whether the same survival mechanisms are operative in differentiated secretory prostate epithelial cells is unknown, and the role of KGF or androgen in prostate epithelial cell survival remains unresolved.

In this study, confluent cultured primary prostate basal epithelial cells were induced to differentiate following treatment with KGF and androgen. After 2 weeks, differentiated AR-expressing secretory cells appeared as a secondary cell layer above the basal cells. This model was used to identify the signaling pathways important for prostate secretory cell survival. This new model will serve as a valuable tool for understanding the biology of prostate secretory epithelial cells, a cell population previously not available for extensive analysis.

Results

Differentiation of confluent PECs by KGF and DHT

Previous studies have demonstrated that KGF might be an important epithelium differentiation factor in many tissues, including prostate epithelium (Alarid et al., 1994; Cunha, 1996; Heer et al., 2006; Peehl et al., 1996; Sugimura et al., 1996). Androgen, acting via the androgen receptor, also plays an important role in prostate epithelial cell differentiation (Berger et al., 2004; Cunha et al., 1987; Heer et al., 2007; Ling et al., 2001; Whitacre et al., 2002). To determine if the combination of KGF and androgen is sufficient to induce differentiation of prostate cells grown in culture, human primary basal prostate epithelial cells (PECs) grown to confluency in monolayer cell cultures were treated with 10 ng/ml KGF and 5-10 nM androgen (DHT). Culturing the cells for 10-15 days with KGF and DHT resulted in the formation of stratified cell patches consisting of at least two cell layers, resembling the bilayer of basal and secretory cells observed in the prostate epithelium in vivo (Fig. 1A-C).

To determine if the stratified cells expressed differentiation markers specific to prostate secretory cells, expression of AR and the AR-target protein prostate-specific antigen (PSA) were examined by fluorescence confocal microscopy. Cells in a higher z-plane than the bottom cells, stained positive for AR and PSA (Fig. 1B). AR expression was both nuclear and cytoplasmic, whereas the secreted protein PSA had the expected cytoplasmic localization (Fig. 1B). AR expression was uniform throughout the top cells, whereas PSA expression was often concentrated at the upper membrane of the top-most cells, consistent with that of a secreted protein (not shown). Neither AR nor PSA was found in the bottom cells (Fig. 1B). Additionally, the AR-regulated proteins Nkx3.1 and TMPRSS2 (Bowen et al., 2000; Lin et al., 1999a; Murtha et al., 1993; Young et al., 1992) were expressed in top cells and not in bottom cells (Fig. 1C).

To determine the extent to which androgen stimulation contributes to PEC differentiation, PECs were treated for 10-14 days with KGF in the presence or absence of DHT, and the expression of AR, AR-target proteins, and differentiation cell markers was

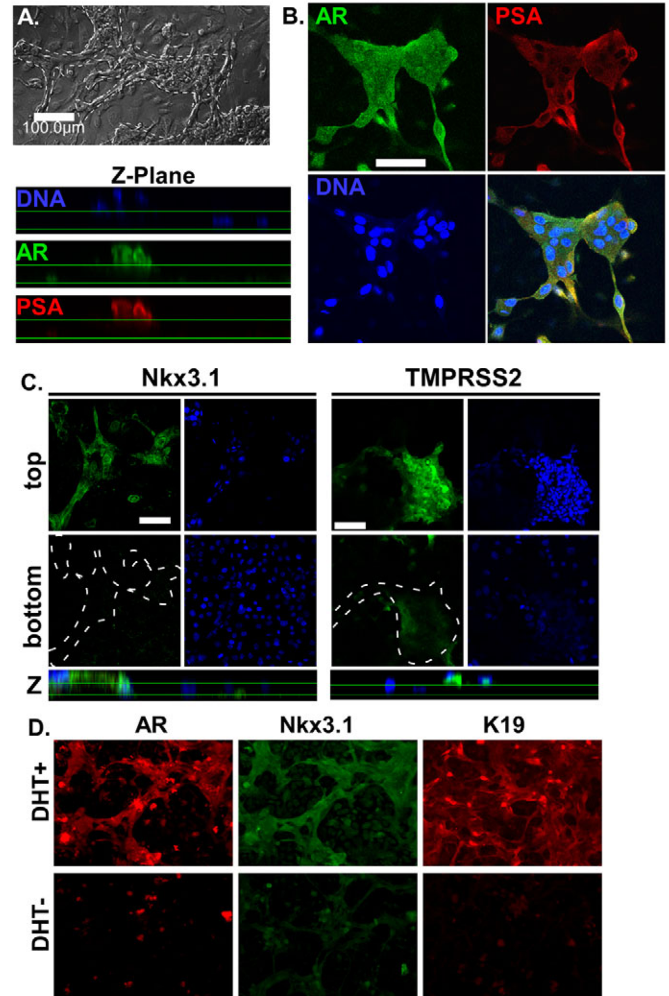

Fig. 1. AR and AR-dependent proteins are present in the differentiated cultures. Confluent primary prostate epithelial cells (PECs) were induced to differentiate with 10 ng/ml KGF and 5 nM DHT for 10-14 days. (**A**) DIC image of a differentiated culture shows an upper layer of cells (outlined with dashed white line) on top of a confluent bottom cell layer. Scale bars: 100 μm. (**B**) A 14-day differentiated culture was immunostained for AR (green) and PSA (red). Nuclei (blue) were visualized by Hoechst 33258 staining. (Left panel) A z-section image was compiled from 30 confocal x-y sections representing a thickness of 38.0 μm. Horizontal lines demarcate top and bottom cell layers. (Right panels) Confocal images of top cells in 14-day differentiated cultures. Scale bar: 50 μm. (**C**) Differentiated PEC cultures were immunostained for Nkx3.1 and TMPRSS2 (green) and imaged by confocal microscopy. Nuclei were stained with Hoechst 33258 (blue). Representative top and bottom cells and z-plane images (Z) are shown below. Scale bar: 100 μm. (**D**) PECs were induced to differentiate for 14 days with KGF in the presence or absence of 10 nM DHT. Cells were immunostained with AR, Nkx3.1 and K19 and imaged by epifluorescence microscopy.

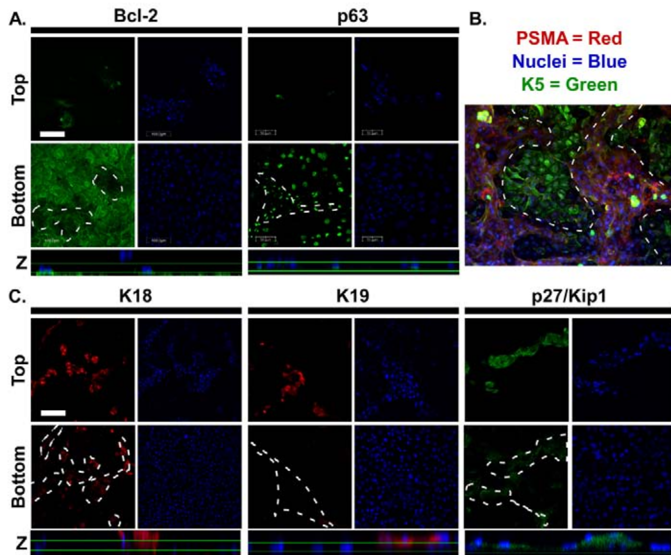

Fig. 2. Differentiation-specific epithelial markers present in the top cells of differentiated cultures. 10- to14-day differentiated cultures were immunostained for (**A**) Bcl-2, p63 (green), (**B**) K5 (green), PMSA (red), (**C**) K18, K19 (red), and p27 (Kip1; green) expression, and images were captured by confocal microscopy (A,C) or epifluorescence (B). Nuclei were stained with Hoechst 33258 (blue). Representative top and bottom cells are shown. Representative z-section images (Z) were compiled from 10-15 confocal x-y sections representing a thickness of 17.04 (±3.27) μm. Horizontal lines demarcate top and bottom cells. Scale bars: 100 μm.

monitored. PSA, Nkx3.1 and TMPRSS2 were only expressed when DHT was present (Fig. 1D, PSA and TMPRSS2 not shown). Intriguingly, cytokeratin markers, K18 and K19, were also expressed only in the presence of androgen (Fig. 1D, K18 data not shown). Furthermore, there was a dramatic increase in AR expression itself when DHT was present.

KGF, in the absence of DHT, was sufficient to induce formation of stratified cells, with maximal formation occurring between10 and 15 days. PECs treated with KGF in the presence of KGF-blocking antibody did not stratify. Confluency of the cultures was essential. Subconfluent cells treated with KGF and DHT did not form stratified clusters. KGF-induced stratification occurred equally efficiently, with or without the supplementary bovine pituitary extract (BPE) and EGF in the culture medium. Occasionally, a few small stratified clusters appeared in BPE-containing medium without KGF treatment, suggesting the presence of low levels of KGF and/or an additional unknown factor(s) in BPE that can promote differentiation at a low efficiency. KGF-blocking antibodies prevented the appearance of these occasional clusters. The optimal concentration of KGF was 10 ng/ml. Lower doses (1-5 ng/ml) resulted in fewer clusters and higher doses (20-50 ng/ml) did not generate more clusters. DHT alone was not sufficient to induce stratification. DHT plus KGF treatment dramatically increased the number of top cells seen after 15 days. DHT was required for expression of androgen-dependent markers in the top cells. FGF10, a functionally related FGF family member shown to be important for prostate development in vivo (Donjacour et al., 2003; Igarashi et al., 1998), could also induce PEC differentiation in the presence of DHT. Differentiation was reproducibly observed in cells derived from two different patients at three different passage numbers (passages 2, 3 and 4). It was observed however, that once cells reached passage 5, the efficiency of differentiation was dramatically reduced. Furthermore, we were able to induce differentiation in an immortalized cell line derived from a third patient. We observed that these more proliferative immortalized cultures took a few days longer to reach maximal differentiation.

Stratified cells express additional differentiation markers

Markers specific to basal and differentiated epithelial cells populations were examined in the stratified cultures. The basal markers Bcl-2, K5 and K14 (McDonnell et al., 1992; Wang et al., 2001) were expressed predominantly in the bottom cells; occasionally a few K5- and K14-positive cells were seen in the top cells (Fig. 2A,B, K14 not shown). Basal marker p63 (Parsons et al., 2001; Signoretti et al., 2000) was associated only with bottom cells (Fig. 2A). EGFR, which is predominately expressed in basal cells (Sherwood and Lee, 1995), was associated primarily with bottom cells (not shown). Epithelial cell markers K19 and PMSA were expressed only in the top cells and not in the bottom cells (Fig. 2B,C). K18, as well as the cell cycle inhibitor p27 (Kip1) (Peehl et al., 1994; Tsihlias et al., 1998; Wernert et al., 1987; Yang et al., 1998), was expressed predominately in the top cells (Fig. 2C).

Differentiation induces integrin loss

Consistent with previous observations of differentiating epithelium in vitro and in vivo (Gustafson et al., 2006; Heer et al., 2006; Levy et al., 2000; Li et al., 2008), epifluorescence and confocal imaging revealed that the subpopulation of the cells undergoing differentiation lost expression of many integrins, including α2, α3, α6, β1 and β4 (Fig. 3A,B). Basal cells also expressed αv-, but not β3- or β5-integrin subunits. None of these integrins were present in the differentiated cells (not shown). Cultured PECs secrete and organize a laminin 5 (LM5)-rich matrix (Yu et al., 2004); the differentiating cell population that lost integrin expression also no longer produced LM5 (Fig. 3A,B). Although it appears, by confocal imaging, that the cells directly below the top cells do not express integrin or LM5, it is possible that there is incomplete antibody penetrance into the lower cells. To address this, a timecourse study was performed. We observed a decrease in LM5 expression as early as 3 days after KGF and DHT treatment and a complete loss after 8 days. At 8 days decreased β1 integrin expression was observed in LM5-negative cells prior to formation of the second cell layer (supplementary material Fig. S1A). Therefore, cells directly underneath the top layer also lose LM5 and integrin expression. LM5 loss might be the trigger that initiates differentiation.

Differentiated cells respond to androgen

AR expression could be detected by immunoblotting of cell lysates from whole cultures treated with KGF and DHT (Fig. 4A). Expression of the androgen-dependent secreted proteins, KLK2 and PSA, was monitored in differentiated cultures by RT-PCR. KLK2 and PSA mRNAs were present only when DHT was present in the culture (Fig. 4B). Furthermore, secreted PSA, up to 0.8 ng/ml, could be detected by ELISA (Fig. 4C). PSA secretion required androgen and increased with increasing DHT concentration. The expression and secretion of an androgen-regulated protein in an androgen-dependent manner indicates the presence of differentiated prostate secretory cells in the culture, and that AR is functional and regulates expression of differentiation markers.

Overall, this in vitro differentiation model recapitulates many aspects of in vivo differentiation as assessed by the specific markers

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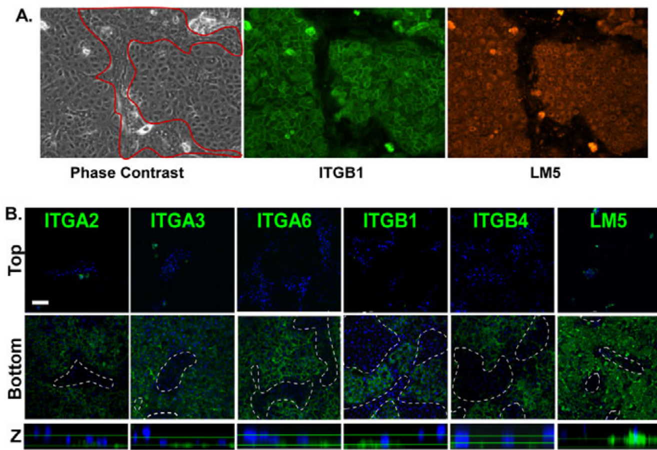

Fig. 3. Prostate epithelial differentiation is accompanied by loss of integrin expression. (**A**) Integrin β1 (ITGB1; green) and laminin 5 (LM5; red) expression in 10-day differentiated cultures were monitored by DIC (left panel) and epifluorescence microscopy following immunostaining. (**B**) 14-day differentiated cultures were immunostained to detect expression (green) of integrins α2 (ITGA2), α3 (ITGA3), α6 (ITGA6), β1 (ITGB1), β4 (ITGB4) and laminin 5 (LM5) and imaged by confocal microscopy. Nuclei (blue) were visualized by Hoechst 33258 staining. Representative confocal x-y sections of the top and bottom cells are shown. The area located directly beneath the top layer of differentiated cells is indicated with dashed white lines in the bottom image. Representative z-section images (Z) were compiled from 10-15 confocal x-y sections representing a thickness of 17.04 (±3.27) μm. Horizontal lines demarcate top and bottom cells.

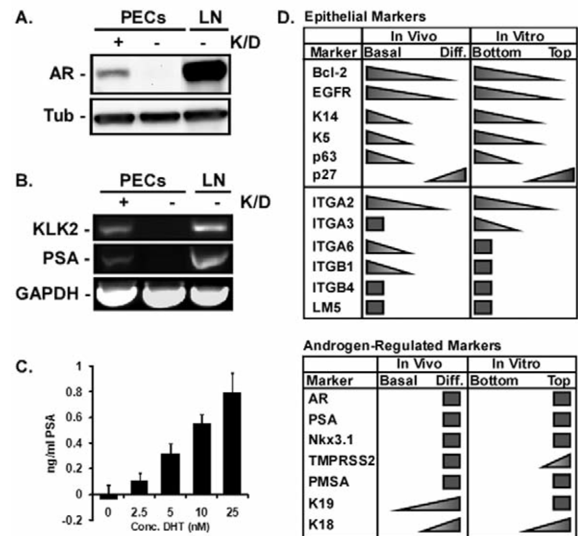

Fig. 4. Differentiated cells respond to androgen. (**A**) Immunoblot for AR expression in cultures of PECs treated with or without KGF and DHT(K/D) for 16 days. LNCaP cells (LN) were used as a positive control for AR expression. Total levels of protein in the lysates were monitored by immunoblotting with anti-tubulin. (**B**) Levels of KLK2 and PSA mRNA isolated from 14-day differentiated (K/D) cultures were analyzed by PCR and compared to LNCaP (LN) cells. GAPDH served as a control. (**C**) Levels of secreted PSA from 14-day differentiated cultures treated with KGF and increasing amounts of DHT were determined by ELISA. (**D**) Summary of epithelial and androgen-dependent markers observed and their relative expression in the lower (bottom) and upper (top) cells. Expression observed in vitro is compared with that reported in vivo.

(Fig. 4D). In addition to the induction of markers common to most differentiating epithelial cells, the presence of DHT markedly stimulates the expression of markers unique to prostate secretory epithelial cells. Hereafter when referring to this model, the AR-expressing top cells will be referred to as secretory-like cells and the AR-negative bottom cells as basal cells.

Isolation of secretory-like cells

Treatment of differentiated cultures with dissociation buffer preferentially dislodges the secretory-like cells. FACS analysis indicates that 96.6% (±0.8%) of the isolated dislodged population is negative for cell surface α6 integrin, whereas 97.19% (±1.70%) of the cells not dislodged are positive for α6 integrin (Fig. 5A). Further FACS sorting based on surface staining of α6 integrin and TMPRSS2 revealed that on average 87.92% (±3.71%) of the α6-integrin-negative cells were positive for TMPRSS2. A representative example is provided in Fig. 5B. Immunoblotting of separated cells indicated that some remaining basal cells expressed AR as well as full-length TMPRSS2 protein; however, only the secretory-like cells expressed the cleaved and activated form of TMPRSS2 (Fig. 5C) (Wilson et al., 2005). Conversely, only the basal cells expressed Bcl-2 and EGFR, whereas K5 was predominately found in the basal cells (Fig. 5D).

Secretory cell survival is dependent on PI3K and E-cadherin, but not KGF or androgen

In previous studies, we demonstrated that integrin-mediated activation of EGFR and downstream signaling to ERK, but not PI3K signaling, is required for the survival of basal PECs (Edick et al., 2007). However, the differentiated secretory-like PECs have lost integrin expression, no longer adhere to the LM5 matrix, and have significantly lower levels of EGFR, suggesting that other survival pathways must be important for secretory cell survival. It has been suggested that secretory cell survival might be dependent on stromal-derived growth factors, including KGF (Kurita et al., 2001). One possibility is that the KGF used to induce differentiation, might also be necessary for survival. To test this, the KGF receptor FGFR2IIIb (Giri et al., 1999) mRNA levels were analyzed in the isolated secretory-like cells and basal cells by RT-PCR. Only the basal cells expressed FGFR2IIIb mRNA (Fig. 6A). Furthermore, removal of KGF after 15 days of differentiation did not induce cell death (not shown). Thus it is unlikely that KGF is regulating cell survival in the secretory-like cells.

Dissociated secretory-like cells and the remaining basal cells were screened for ERK and AKT activation by immunoblotting. Active ERK was present only in the basal cells, but not in the secretory-like cells (Fig. 6B). Activated AKT was present in both types of cells (Fig. 6C). Thus, ERK signaling probably does not regulate survival in differentiated cells, whereas the PI3K pathway could. Since the differentiated cells remain adherent to the bottom basal cells, we also investigated whether there is an increase in expression of the cell-cell adhesion molecule E-cadherin in the secretory-like cells. Compared with the basal cells, E-cadherin levels were elevated in the secretory cell population that also does not express α6β1 integrin (Fig. 6D). E-cadherin can lead to activation of PI3K signaling in skin and colonic epithelium as well as in some tumor cell lines (Calautti et al., 2005; Hofmann et al., 2007; Pang et al., 2005). Blocking antibodies to E-cadherin

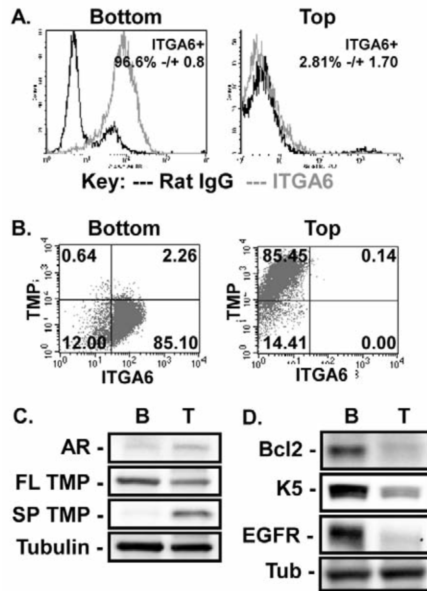

Fig. 5. Isolation of secretory-like cells. (**A**) Following treatment of 14-day cultures with dissociation buffer the separated upper (Top) and lower (Bottom) cells were subjected to FACS to measure cell surface α6-integrin expression (gray line) versus control antibody (black line). Dead cells were excluded using PI staining. Values are the percentage α6-integrin-positive cells. (**B**) Cells isolated and sorted for α6 integrin as in A were further sorted based on TMPRSS2 (TMP) surface expression. Values are the percentage of positive cells in each quadrant. Data is from one typical experiment. (**C**,**D**) Bottom (B) and top (T) cells, obtained after treatment with dissociation buffer, were analyzed by immunoblotting for AR and TMPRSS2 (C), and Bcl-2, K5 and EGFR (D) expression. Tubulin immunoblotting served as a loading control.

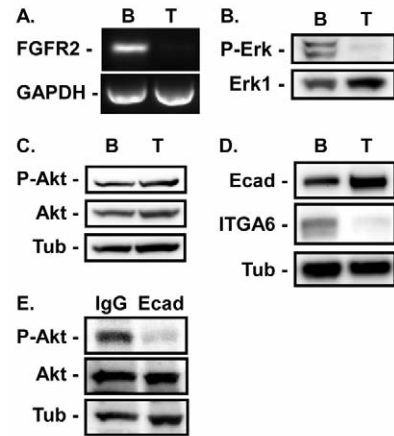

Fig. 6. Signaling pathways in secretory-like cells. Following treatment of 14-day cultures with dissociation buffer, mRNA or protein was isolated from the lower (B) and upper cells (T). (**A**) Levels of FGFR2IIIb mRNA (FGFR2) were assessed by PCR. GAPDH served as a control. (**B**) Levels of ERK activation (P-Erk) in the lower and upper cells were monitored by immunoblotting of cell lysates with phosphospecific ERK antibodies. Total levels of ERK and tubulin in the lysates were measured by immunoblotting. (**C**) Levels of AKT activation (P-Akt) in the lower and upper cells were monitored by immunoblotting of cell lysates with phosphospecific AKT antibodies. Total levels of AKT and tubulin in the lysates were measured by immunoblotting. (**D**) Levels of E-cadherin (Ecad) and α6 integrin (ITGA6) in the lower and upper cells were analyzed by immunoblotting. Tubulin immunoblots served as loading controls. (**E**) 14-day cultures were treated with control IgG or E-cadherin-blocking antibody (Ecad) for3 hours. Levels of AKT activation (P-Akt) in the isolated upper cells were monitored by immunoblotting of cell lysates with phosphospecific AKT antibodies. Total levels of AKT and tubulin in the lysates were measured by immunoblotting.

suppressed AKT activity in both the secretory-like (Fig. 6E) and the basal cells (not shown).

The relative importance of the different signaling pathways on secretory-like cell survival was investigated. Fourteen-day KGF and DHT-differentiated cultures were placed in KGF- and DHT-free basal medium without any pituitary extract or EGF supplement for 72 hours to reduce any signaling induced by the growth medium (Fig. 7A). Visually, the starved cell cultures appeared viable, and the upper secretory-like cell layer remained intact (data not shown). Then the starved differentiated cultures were treated with specific inhibitors in the presence or absence of freshly added DHT or KGF and analyzed over a 72-hour timecourse. Cell death was measured in the upper secretory-like cell layer by immunostaining for active caspase 3/7, TUNEL staining or propidium iodide (PI) uptake. Staining was quantified as described in Materials and Methods. Inhibition of PI3K signaling with LY294002 resulted in maximal secretory-like cell death at 72 hours, where 60% of the cells stained positive for PI (Fig. 7B). Furthermore, inhibition of PI3K, but not EGFR, induced a 7.0- to 7.5-fold increase in secretory cell caspase 3 activity (Fig. 7C), and a 5.5- to 5.7-fold increase in TUNEL staining (Fig. 7D; supplementary material Fig. S1B). Maximal annexin V staining was observed 66 hours after LY294002 treatment (not shown). Secretory-like cell survival was not dependent on DHT or KGF, and addition of DHT or KGF was unable to promote cell survival in the absence of PI3K signaling (Fig. 7B-D). Although KGF should not be present in the media, and prostate epithelial cells have been reported not to produce KGF, KGF-blocking antibodies were used to prevent any endogenous or remaining KGF from promoting cell survival. KGF-blocking antibodies had no effect on cell survival (data not shown). KGF has been reported to activate p38, and Jnk can promote survival during stress (Heer et al., 2006; Leppä and Bohmann, 1999; Mehta et al., 2001). Inhibiting p38 with SB202190, JNK with 420119, or ERK with PD98059 did not result in cell death, suggesting these pathways are not critical for secretory cell survival (supplementary material Fig. S1C). The lack of effect of the inhibitors on cell survival was not due to a failure to inhibit signaling, as the concentrations of drugs used here did effectively block signaling to their specific targets in basal cells.

Cell-cell adhesion via E-cadherin was inhibited by treatment of differentiated cells with two different preparations (lots) of E-cadherin-blocking antibodies. Inhibition of cell-cell adhesion with one lot of E-cadherin-blocking antibody resulted in maximal cell death at 48 hours with over 80% of the cells staining positive for PI (Fig. 7E). By 66 hours, no secretory-like cells remained in the cultures. A second lot of E-cadherin antibody resulted in a seven- to eightfold increase in TUNEL staining 72 hours after treatment (Fig. 7F). The presence of DHT or KGF could not protect cells from death due to loss of E-cadherin function. No cell death was observed in the lower basal cells. Furthermore, blocking E-cadherin lead to a decrease in AKT activation (see Fig. 6E), indicating that cell-cell adhesion mediated by E-cadherin promotes secretory-like cell survival through PI3K signaling.

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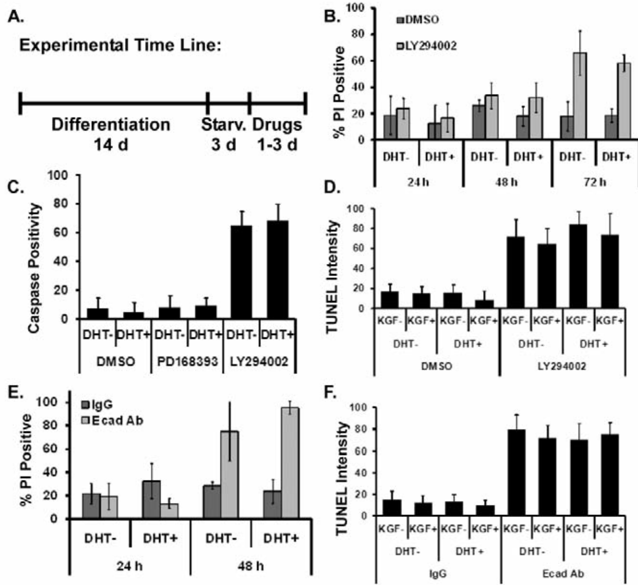

Fig. 7. Secretory-like cells are dependent on PI3K and E-cadherin, but not androgen or KGF, for survival. (**A**) PECs were treated with KGF and DHT for 14 days (Differentiation), then starved of growth factors and DHT for 3 days (Starv.), and then treated with pharmacological inhibitors (Drugs) for 1-3 days. In some cases DHT or KGF were also added back with the inhibitors. (**B**) Differentiated cultures were treated with vehicle (DMSO) or PI3K inhibitor LY492004 in the presence or absence of DHT for 24, 48 or 72 hours. Cell viability in the top cells was measured by quantifying the number of cells with high PI staining and expressed as percentage PI-positive cells. (**C**,**D**) Differentiated cultures were treated with vehicle (DMSO), EGFR inhibitor PD168393, or PI3K inhibitor LY492004 in the presence or absence of DHT or KGF. After 72 hours cell viability in the top cells was assessed using (C) cleaved caspase-3 or (D) TUNEL staining. Total DNA was stained with PI. Six fields per experiment and condition were examined and positive pixels counted using the software program Imagine as outlined in the Material and Methods. TUNEL- or cleaved caspase-3-positive pixels were normalized to the total number of stained DNA pixels in the region of interest and expressed as relative intensity of caspase-3 or TUNEL staining. Error bars indicate standard deviation. *n*=3. (**E**) Differentiated cultures were treated with non-specific mouse IgG (IgG) or with E-cadherin-blocking antibody (Ecad Ab; lot 2) in the presence or absence of DHT for 24 or 48 hours. Cell viability was measured by PI staining. (**F**) Cell viability of differentiated cultures treated with non-specific mouse IgG (IgG) or with E-cadherin-blocking antibody (Ecad Ab; lot 1) in the presence or absence of DHT or KGF for 72 hours was measured by TUNEL staining.

Although DHT was not important for survival of the differentiated secretory-like cells, it is theoretically possible that AR, acting via an androgen-independent mechanism might still be important for cell survival. To address this, 14-day KGF- and DHT-differentiated cultures were transfected with an AR-specific siRNA pool or a scrambled siRNA sequence. Confocal imaging of the transfected cells 72 hours later demonstrated the absence of AR expression in the upper cells (Fig. 8A). Absence of AR expression also resulted in loss of androgen-dependent cell markers such as Nkx3.1 and K19 (Fig. 8A). Cell viability of the AR siRNA-treated cells was assessed by TUNEL staining. Loss of AR had no effect on secretory-like cell viability (Fig. 8B). Thus, AR and androgen signaling are not required to maintain the viability of differentiated secretory-like cells derived from our in vitro culture system.

Discussion

By treating cultured primary prostate basal epithelial cells with androgen and KGF, we have established an in vitro differentiation model of the prostate epithelium. The differentiated cells in our culture system possess the important features of terminally differentiated secretory prostate epithelial cells in vivo: they do not proliferate, they adhere to a basal cell layer and not to the basement membrane, they express AR protein, and they respond to DHT by inducing AR-dependent genes. Specifically, the cells express androgen-sensitive proteins, such as KLK2, PSA, Nkx3.1, PMSA and TMPRSS2. In addition, cleaved TMPRSS2 is present in the upper, but not the lower cells and PSA is secreted into the culture medium. Furthermore, cytokeratin K18 and K19 expression was found to be dependent on androgen. K18 expression has previously been reported to be regulated by androgen (Heer et al., 2007; Ling et al., 2001), and K19 has been suggested to be responsive to estrogen (Choi et al., 2000); however, both K18 and K19 promoters lack classical androgen response elements, making the mechanism of regulation unclear.

Further evidence for terminal differentiation is that the cells did not revert to basal cells when isolated and re-plated, and they failed to reattach, probably because of continued loss of integrin and/or matrix expression. Furthermore, after 21-25 days in culture the upper cells sloughed off and a few activated caspase-3-positive cells were seen in the aging cultures (data not shown), similar to what is observed in vivo. Oddly, no more differentiated cells reappeared. Only about 20% of the cells appeared to be capable of undergoing differentiation, suggesting that the differentiated cells are derived from a distinct subpopulation of basal cells. The lack of continued differentiation after 25 days may indicate depletion of these special cells and a lack of ability to renew. The population of differentiation-competent cells is not likely to be stem cells, since 20% of the cells are capable of undergoing differentiation. However, we cannot rule out the possibility that these cells arose from some stem cell-like progenitor within the culture. Further analysis would be required to determine if the progenitors are analogous to the Nkx3.1-positive luminal stem cell recently described (Wang et al., 2009). However, whatever the progenitor, it apparently cannot renew in the context of our culture conditions.

Although many aspects of the differentiated cells recapitulate what is observed in vivo, there still remain some differences. For instance, the distribution of AR demonstrates a significant amount of cytoplasmic expression in the in vitro culture system, whereas in vivo AR is primarily nuclear. Another difference is the absence of columnar cells. In addition, a few K5- and/or K14-positive cells were sometimes seen in the upper layer, which has also been reported in another differentiation model (van Leenders et al., 2000). Hence, we cannot unequivocally say whether our secretory-like cells represent completely terminally differentiated prostate cells and there are still some distinctive morphological differences between our cultures and what is seen in the prostate gland in vivo.

Other studies have reported on prostate epithelial differentiation in vitro. Although these studies were informative, they were limited since AR and AR-regulated proteins were not expressed (Dalrymple et al., 2005; Danielpour, 1999; Garraway et al., 2003; Gu et al., 2006; Gustafson et al., 2006; Yasunaga et al., 2001). A few studies have reported seeing stratified layering similar to ours after treating prostate epithelial cells in vitro with retinoic acid, FGF and/or insulin (Gustafson et al., 2006; Peehl et al., 1994; Robinson et al., 1998; van Leenders et al., 2000); however, in these models the top layer of cells either failed to express AR or still expressed basal markers.

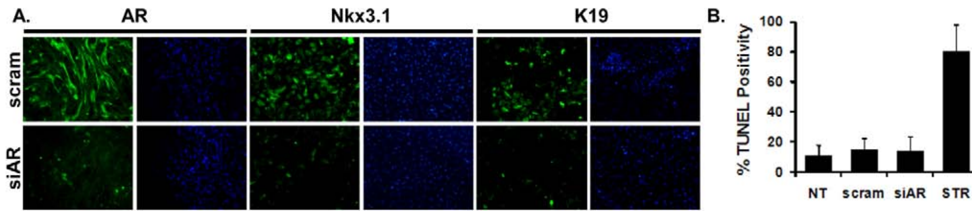

Fig. 8. AR is not required for secretory-like cell survival. PECs were treated with KGF and DHT for 14 days and then transfected with scrambled siRNA (scram) or AR-specific siRNA (siAR) to block AR expression. (**A**) Cells were immunostained with AR, Nkx3.1 and K19 (green) and imaged by epifluorescence microscopy. Nuclei were stained with Hoechst 33258 (blue). (**B**) Differentiated cultures left untreated (NT) or treated with scrambled siRNA (scram), AR siRNA (siAR), or staurosporine (STR) were measured for cell viability by TUNEL staining.

In our model, the top secretory-like cells expressed AR and lost basal marker expression. In one case, gland-like buds and extensions were observed to form from confluent cell cultures, reminiscent of acini structures in overall shape but without lumens (van Leenders et al., 2000). We have also observed cases where cells appear to form mounds. By confocal imaging, some of them appear to have formed a hollow mound (data not shown). A recent study demonstrated that co-treatment of prostate basal cells with the monoamine oxidase A inhibitor clorgyline, 1,25-dihydroxyvitamin D_3, all-trans retinoic acid and TGF-β1 induced AR expression and loss of basal marker K14 (Zhao et al., 2008), suggesting that there might be alternative mechanisms to inducing prostate epithelial cell differentiation.

In contrast to other published systems, we have demonstrated that our model can be utilized for biochemical and genetic manipulation. It is amenable to treatment with pharmacological inhibitors or siRNA to study signaling and biological pathways. Furthermore, exploitation of differential cell surface markers and adhesion properties can be used to separate basal from secretory-like cells to separately analyze RNA and protein expression.

It is unknown whether AR represses integrin expression or whether loss of integrin expression must precede expression of AR. Unpublished data from our laboratory and others demonstrates that re-expression of AR in prostate cancer cell lines results in decreased integrin expression (Bonaccorsi et al., 2000; Nagakawa et al., 2004). However, in our model we observed that not all integrin-negative cells were AR positive, suggesting that integrin loss might precede AR expression. Furthermore, LM5 matrix loss preceded integrin loss, which preceded stratification and robust AR expression in our timecourse studies. Heer et al. have demonstrated that blocking integrin β1 is sufficient to induce partial differentiation; however, cells do not reach terminal differentiation since the cells do not express AR-regulated genes (Heer et al., 2006). This suggests that loss of adhesion can initiate early differentiation and may even be required, but that integrin loss alone is not sufficient for terminal differentiation. By contrast, unbound integrin β1 is sufficient to initiate terminal differentiation in keratinocytes (Levy et al., 2000; Watt, 2002). In mammary epithelium, however, loss of integrin β1 suppresses differentiation (Naylor et al., 2005).

Interestingly, in most of the reported prostate differentiation models (including ours), confluent cultures were necessary for stratification. In addition, previous studies suggest that cell cycle inhibition is a prerequisite for expression of secretory cell markers K18, K19 and AR (Danielpour, 1999; Garraway et al., 2003; Gustafson et al., 2006; Litvinov et al., 2006). We similarly saw a loss in cell proliferation in the differentiating cell population (data not shown). This led us to develop the following model for prostate differentiation (Fig. 9). Basal cells are proliferative and a subset begins to undergo growth arrest once the cells are confluent. Treatment with KGF causes a select population of cells, perhaps those that express higher levels of the KGF receptor FGFR2IIIb (Giri et al., 1999), to lose LM5 and then integrin expression, causing the cells to detach. Integrin loss and detachment might then trigger low AR expression. AR expression was not detectable by immunostaining in cultures treated with only KGF, in which integrin expression was lost; however, some AR expression was detectable in the basal cells from the differentiated cultures by immunoblotting. The presence of androgen in the culture appears to be necessary to allow the integrin-deficient cells to express AR at a higher level, which then turns on AR-dependent differentiation-specific genes.

Work by Heer et al. suggests that AR might be expressed at low levels in primary prostate epithelial cells and is rapidly degraded by the proteosome (Heer et al., 2007); hence androgen treatment might stabilize and/or help drive production of AR protein. In fact AR mRNA has been detected in some cultured prostate epithelial cells (Litvinov et al., 2006). However, in our studies and those of others, androgen alone is not very effective in inducing AR expression (Litvinov et al., 2006). Thus, additional events are required to induce stable AR expression even in the presence of androgen. Reduced cell proliferation caused by strong growth suppression or loss of cell adhesion, which is also growth suppressive, might be necessary. Significant increases in AR

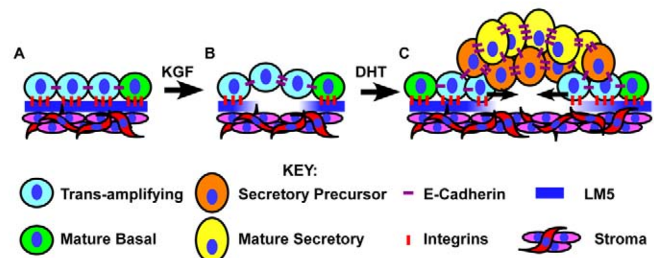

Fig. 9. Model of differentiation. (**A**) Confluent primary prostate basal cells secret and adhere to a matrix rich in LM5 via integrins, which physically separates the epithelial cells from the stromal cells. (**B**) After treatment with KGF, a sub-population of transient amplifying cells loses expression of LM5 and subsequently integrins, resulting in loss of adhesion. Concurrently, there is increased cell-cell adhesion via E-cadherin. (**C**) Secretory-like precursor cells arise in concert with androgen treatment, which induces their differentiation into mature secretory cells. Transient amplifying cells at the edge continue to proliferate to fill in the space generated by detachment of cells and movement into the top layer. After 10-14 days, cells become stratified as more transient amplifying cells are committed to terminal differentiation.

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expression can be detected in isolated suspended cells in the presence of androgen (Heer et al., 2007), thus supporting cell detachment as a potential mechanism required for stabilizing AR.

Previous work from our laboratory has demonstrated that integrin-mediated survival of primary prostate basal cells requires integrin-induced EGFR signaling to ERK, but not PI3K signaling (Edick et al., 2007). In this study we have expanded our analysis of survival mechanisms to secretory-like prostate epithelial cells and demonstrated that secretory-like cells depend on a non-integrin-dependent mechanism for cell survival that involves cell-cell interactions through E-cadherin. Interestingly, there is switch from ERK-dependent survival in the basal cells to PI3K-dependent survival in the secretory-like cells. In the secretory-like cells EGFR levels dropped dramatically and EGFR-dependent signaling to PI3K was not required for survival (blocking EGFR had no effect on secretory cell survival). Interestingly, in prostate cancer, there appears to be a strong dependence on PI3K signaling for survival, as these cells tend to acquire mutations in Pten, a negative regulator of PI3K signaling (Bertram et al., 2006; Edick et al., 2007; Lin et al., 1999b; Wen et al., 2000). This suggests that prostate cancer might arise from a more differentiated cell that has already acquired dependence on PI3K for its survival.

In our studies, secretory cell survival was not dependent on the presence of androgen, and knockdown of AR with siRNA in differentiated cells did not induce their death. The lack of dependence on androgen or AR for secretory cell survival in our human culture system is in agreement with genetic and tissue recombination studies in mice. Conditional knockout of AR in mature mouse prostates results in decreased numbers of secretory cells without inducing cell death, suggesting that AR functions to increase secretory cell numbers by promoting differentiation rather than cell survival in mature glands (Wu et al., 2007). Tissue recombination experiments using mesenchyme and epithelium from AR-negative or wild-type mice demonstrate that AR expression in the epithelium is not required for early prostate development, indirectly ruling out a role for AR in epithelial cell survival in newly formed glands (Cunha et al., 2004). Thus, in both models, as well as ours, androgen is responsible for the synthesis of secretory proteins and the secretory function of the prostate.

If androgen and AR do not act cell autonomously to control epithelial cell survival, then why do only the AR-expressing epithelial cells die upon castration-induced androgen deprivation (Evans and Chandler, 1987; Mirosevich et al., 1999)? One possibility is that AR signaling in the stromal cells promotes survival by paracrine factors that act on the epithelial cells (Verhoeven and Swinnen, 1999). In our model the paracrine function of KGF, known to be expressed by stromal cells in vivo, was required for differentiation; however, it was dispensable for cell survival in committed differentiated cells. Thus, the nature of the paracrine survival factor(s) remains undetermined. In our in vitro model, survival was highly dependent on E-cadherin-based cell-cell adhesion and signaling to PI3K. Whether paracrine factors in vivo are responsible for maintaining survival via E-cadherin or whether they act on other pathways remains to be determined.

Our study supports a simpler concept that the role of stromal-derived paracrine factors is to act primarily on the stem and/or basal cells, whose proliferation and regenerative capacity is driven by these factors. As terminally differentiated cells are sloughed into the lumen, basal cells are triggered to proliferate and differentiate to replace the lost cells. Under androgen-ablative conditions, the loss of paracrine factors in the stroma prevents stem cell and/or basal cell renewal and the terminally differentiated cells eventually slough off and are not replaced. Re-administration of androgen restores basal cell proliferation and differentiation, and subsequent restoration of secretory cells. This model would preclude the need for stromal factors acting directly on the secretory cells.

An alternative model to explain castration-induced loss of prostate secretory cells involves the observation that castration reduces blood flow and microvasculature collapse in the gland, inducing a state of hypoxia (Buttyan et al., 2000). It would appear that secretory cells are much more sensitive to such stress than the basal or stromal cells. This might be related to a lack of extracellular matrix support that provides additional survival signaling cues to the basal and stromal cells. Alternatively, hypoxia might affect the production of the paracrine factors required for maintenance of epithelial differentiation or survival.

In summary, we have established an in vitro differentiation model of human prostate epithelium composed of stratified cells that recapitulates many in vivo characteristics of basal and secretory cells, including AR-dependent differentiation and function. This model can be treated with pharmacological inhibitors and siRNA to study biochemical and genetic effects and the differentiated secretory-like cells can be isolated for further analysis. We have further established that although KGF, AR and androgen are important for initiating the differentiation process and AR is important to maintain the androgen-dependent phenotype of secretory-like cells, these factors are not required for survival of the committed differentiated cells. The primary critical mechanism driving cell survival is E-cadherin-based cell-cell adhesion and subsequent activation of the PI3K signaling pathway.

Materials and Methods

Cell culture

Human primary prostate epithelial cells (PECs) derived from prostectomy specimens were isolated, cultured, and verified to be free of stromal contamination as described previously (Edick et al., 2007; Gmyrek et al., 2001). Specific patient samples used in this study were again verified to be negative for the stromal cell marker smooth muscle actin by immunostaining. PECs were grown in keratinocyte-SFM medium (Invitrogen) supplemented with bovine pituitary extract (BPE) and epidermal growth factor (EGF). Experiments were reproducibly performed in cells derived from two different patients at three different passage numbers (passage 2, 3 and 4). In addition, at least three separate primary cultures from each patient were used. Experiments were verified at least three times for each of the two patients. We were also able to induce differentiation in an immortalized cell line derived from a third patient. The AR-positive prostate cancer cell line LNCaP was purchased from ATCC. LNCaP cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 IU penicillin, 50 μg/ml streptomycin, 0.225% glucose, 10 mM HEPES, and 1 mM sodium pyruvate.

Differentiation assay

To induce differentiation, a 10-cm culture dish of confluent PECs was divided equally between three eight-chambered slides (Lab-Tek). Cells were grown in keratinocyte-SFM supplemented with BPE, EGF, 10 ng/ml keratinocyte growth factor (KGF; Calbiochem), and 5-10 nM dihydrotestosterone (DHT; Sigma) for 10-18 days. KGF and DHT were replenished three and five times a week, respectively. For larger-scale experiments, three 10-cm plates of confluent PECs were combined onto one 10-cm dish and treated with KGF and DHT for 21-30 days.

KGF blocking experiments

KGF-FGF7 blocking antibody (clone 29522) was purchased from R&D Systems. 2 μg/ml KGF blocking antibody or IgG control was added immediately prior to KGF addition. Differentiation of PECs was then assessed by immunofluorescent staining for differentiation markers.

Cell surface integrin and TMPRSS2 expression analysis

Whole cultures of differentiated PEC cultures were placed in suspension by washing the cells twice with PBS, treating with cell dissociation buffer (Gibco, Invitrogen) for 5 minutes, then adding TrypLE Express trypsin (Gibco, Invitrogen). Cells were then washed with wash buffer (1% sodium azide, 2% FBS-PBS) and incubated with primary antibodies or control IgG molecules for 1 hour at 4°C. Cells were washed twice and incubated with fluorescently labeled secondary antibodies for 1 hour at

4°C in the dark. Cells were washed twice more, and fluorescence was detected using a Becton-Dickinson FACSCalibur four-color flow cytometer with CellQUEST Pro Software v5.2.1 (Becton-Dickinson).

Isolation of differentiated cells

Differentiated PEC cultures were washed with 1 mM EDTA in PBS without calcium or magnesium, and then incubated for 5 minutes with 1 mM EDTA-PBS. Cells were then incubated with cell dissociation buffer (Gibco, Invitrogen) for 6-8 minutes. The top layer of cells could then be removed by pipetting the cell dissociation buffer over the cells; the bottom confluent cell layer remained attached to the culture vessel. The isolated cells were used directly or undifferentiated α6-integrin-expressing cells were separated from the differentiated cells using α6 integrin antibodies and FACS as described above using fluorescently conjugated integrin α6 antibody (BD Pharmingen). Cells were sorted on a Becton-Dickinson FACSAria special order system 12-color flow cytometer using FACSDiVa software v5.2 (Becton-Dickinson).

Immunoblotting

Total cell lysates were prepared for immunoblotting as previously described (Edick et al., 2007; Miranti, 2002). Briefly, cells were lysed with Triton X-100 lysis buffer and 45-75 μg of total cell lysates in 2× SDS sample buffer were boiled for 10 minutes. Samples were run on SDS polyacrylamide gels following standard SDS-PAGE protocols and transferred to PVDF membrane. Membranes were blocked in 5% BSA in TBST for 2 hours at room temperature, then were probed with primary antibody overnight at 4°C. Membranes were washed three times, and incubated with horseradish peroxide-conjugated secondary antibodies (Bio-Rad) in 5% BSA in TBST for 1 hour at room temperature. After washing an additional three times, signals were visualized using a chemiluminescence reagent with a CCD camera in a Bio-Rad Chemi-Doc Imaging System using Quantity One software v4.5.2 (Bio-Rad).

Immunoblotting antibodies

Antibodies for phospho-specific AKT (S473) or phospho-specific ERK1/2 (T202/Y204) were purchased from Cell Signaling. Antibodies for total ERK were from Becton-Dickinson Transduction Labs and total AKT antibodies have been described previously (Bill et al., 2004). α6 integrin and TMPRSS2 antibody were gifts from Anne Cress (University of Arizona, Phoenix, AZ) and Peter Nelson (Fred Hutchinson Cancer Research Institute, Seattle, WA) (Lucas et al., 2008), respectively. Androgen receptor antibody (441) was purchased from Santa Cruz Biotechnology. E-cadherin antibody (clone HECD1) was purchased from Zymed. Tubulin antibody (clone DM1A) was purchased from Sigma.

Immunofluorescence

Differentiated PEC cultures were fixed with 4% paraformaldehyde (Mallinckrodt Chemicals) for 10 minutes and permeabilized for 4 minutes with 0.2% Triton X-100 (EMD) at room temperature. Cells were then blocked with 10% normal goat serum (Pierce) for 2 hours at room temperature before incubation with primary antibodies overnight at 4°C. Cells were incubated with appropriate secondary antibodies for 1 hour at room temperature. DNA was visualized by staining with Hoechst 33258 (Sigma) for 10 minutes at room temperature. Cells were washed three times with PBS between all steps. Coverslips were mounted on the slides using Gel-Mount (Biomeda).

Specific antibodies against proteins of interest were obtained as indicated in supplementary material Table S1 and used for immunofluorescent (IF) staining at the stated dilutions. Whole IgG antibodies for controls were purchased from Pierce. Species appropriate Alexa Fluor 488 or 546 antibodies (Molecular Probes, Invitrogen) were used as secondary antibodies for indirect fluorescence.

Microscopy

Epifluorescence images were acquired using a Nikon Eclipse TE300 fluorescence microscope with OpenLab v5.5.0 image analysis software (Improvision). Confocal images were acquired by sequential detection using a Zeiss 510 Meta NLO v4.2, or Olympus FluoView 1000 LSM using FluoView software v5.0.

PSA quantification

Differentiated PEC cultures in eight-chambered slides were grown in the presence or absence of DHT for 72 hours in 200 μl per well of growth medium. To quantify PSA concentrations in conditioned medium, a human PSA ELISA kit (Abzyme) was used according to the manufacturer's directions with the following modifications: the entire 200 μl samples were incubated 50 μl at a time per well for 1 hour each. PSA standards were added to coated wells during the final 50 μl of sample incubation.

Reverse transcription PCR (RT-PCR) for differentiation markers

Human KLK2, human KLK3 (PSA), FGFR2IIIb and GAPDH mRNA levels were quantified in differentiated cells by RT-PCR. Total RNA was isolated from upper and lower cell populations of dissociated cells from differentiated cultures or from LNCaP cells using TRIzol (Gibco) and chloroform (Sigma-Aldrich). Contaminating DNA was then removed using a RNAse-free DNAse kit (Qiagen) following manufacturer's directions. RT-PCR was performed on 1-2 μg RNA with the primers listed in supplementary material Table S2 using the One-Step RT-PCR kit (Qiagen) following manufacturer's directions. RT-PCR products were analyzed on a 2% agarose-TBE gel and DNA was visualized with ethidium bromide and a CCD camera in a Bio-Rad Chemi-Doc Imaging System using Quantity One software v4.5.2 (Bio-Rad).

Small interfering RNA transfections

A pool of four small interfering RNAs (siRNA) against androgen receptor (siGENOME SMARTpool) or a non-targeting sequence were purchased from Dharmacon. Differentiated cultures were transfected with 20 nM siRNA in keratinocyte-SFM medium using siLentFect lipid reagent (Bio-Rad) and Opti-MEM (Invitrogen) medium following manufacturer's directions. The medium was changed 16 hours after transfection.

Cell survival assays

Differentiated PECs were starved of growth factor in keratinocyte-SFM medium containing no supplements, KGF, or DHT for 72 hours. Then DMSO (control; Sigma), pharmacological inhibitors 0.5 μM PD168393, 2 μM LY294002, 20 μM PD90859, 10 μM SB209102, 10 μM 420119 (all purchased from Calbiochem), 1 μM staurosporine (Promega) or 1 μg/ml E-cadherin-blocking antibody (SHE78-7, Calbiochem) or non-specific mouse IgG (Sigma) was added; in some experiments, siRNAs were used to knock down AR expression (Dharmacon). Cells were incubated for 24, 48, 66 or 72 hours after drug, antibody or siRNA addition. LY294002 was replenished 48 hours after its initial addition. To assess cell viability, cells were fixed and DNA fragmentation was monitored using terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) following the protocol of the APO-BrdU TUNEL Assay Kit (BD Pharmingen). On several occasions, cleaved caspase 3 (Asp175) staining with antibody clone 5A1 from Cell Signaling was also used to measure cell viability of fixed cells. TUNEL and caspase activity were quantified using Imagine software (Qian et al., 2006). Total TUNEL- or caspase-positive pixels were normalized to total propidium iodide-stained DNA pixels in fixed cells and expressed as relative intensity of TUNEL staining. This quantification is based on pixel counts and does not necessarily reflect the percentage of positive cells, but rather the relative intensity of TUNEL or caspase 3 staining between treated and untreated cultures. As an alternative method for measuring cell viability, unfixed cells were treated with propidium iodide (PI). High intensity PI staining of dead, i.e. permeabilized cells, was quantified on a per cell basis and expressed as the percentage PI-positive cells.

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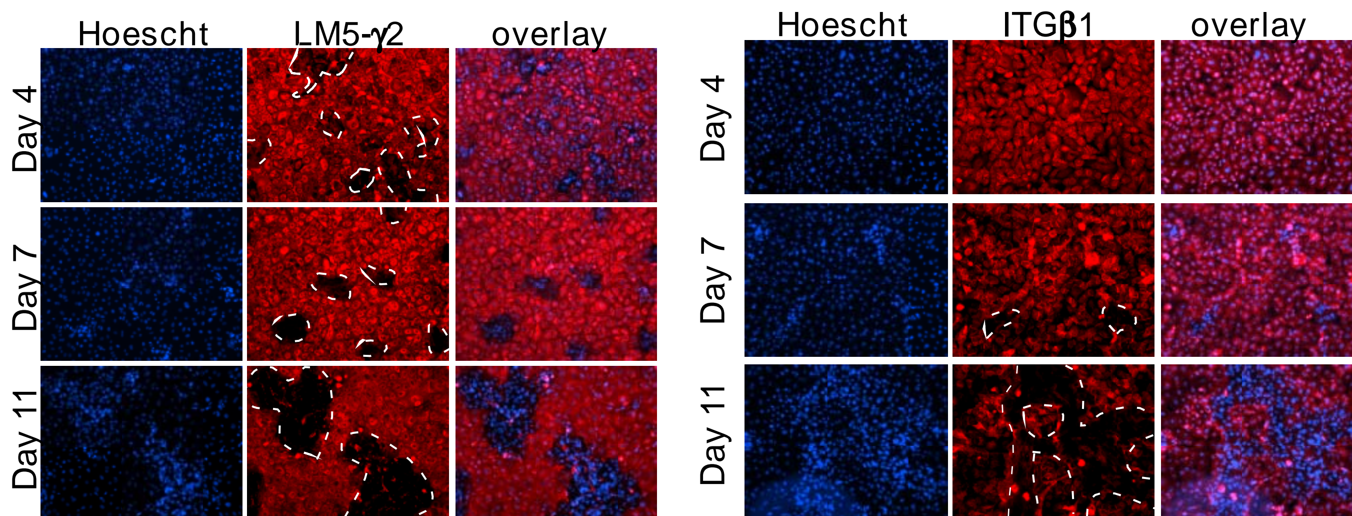
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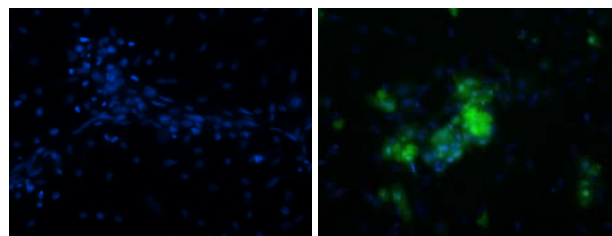
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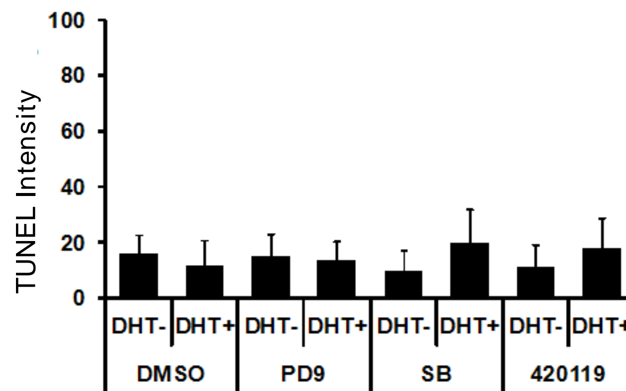
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Table S1. Immunofluorescent antibodies

Protein	Clone	IF dilution	Company
goatAb PSA	C-19	1:100	Santa Cruz Biotechnology
mAb AR	411	1:500	Santa Cruz Biotechnology
mAb Bcl-2	100	1:50	Santa Cruz Biotechnology
mAb E-cadherin	HECD-1	1:100	Zymed/Invitrogen
mAb EGFR	Ab12	1:200	Neomarkers
mAb ITGA2	P1H5-E9	1:10	Gift from W.G. Carter
mAb ITGA2	PIE6-1-1	1:10	Gift from W.G. Carter
mAb ITGA3	P1F2-1-1	1:10	Gift from W.G. Carter
mAbITGAV	272-17E6	1:250	AbCam
mAb K18	CY-90	1:100	Sigma
mAb K19	A53-BIA2	1:50	Sigma
mAb LM5 (γ2 chain)	D4B5	1:100	Chemicon
mAb p63	4A4	1:100	Santa Cruz Biotechnology
mAb PSA	18127	1:100	R&D Systems
mAb PSMA	YPSMA-1	1:250	AbCam
mAb SMA	1A4	1:100	Zymed/Invitrogen
mAbTMPRSS2	P5H9-A3	1:250	Gift from P.S. Nelson
rAb K5	AF138	1:500	Convance
rAb Ki67		1:200	Zymed/Invitrogen
rAb Kip1/p27	G173-324	1:100	Pharmigen
rAb Nkx3.1	H-50	1:500	Santa Cruz Biotechnology
ratAb ITGA6	GoH3	1:100	BD Pharmingen
ratAb ITGB1	AIIB2	1:100	Iowa State Univ. Hybridoma Bank
ratAb ITGB4	P4GH-1	1:10	Gift from W.G. Carter

Table S2. RT-PCR primers

Target	Fwd Primer (5′→3′)	Rev Primer (5′→3′)	Ref
ITGA6	GCTGGTTATAATCCTTCAATATCAATTGT	TTGGGCTCAGAACCTTGGTTT	(Tapia et al., 2008)
ITGB1	GTGGTTGCTGGAATTGTTCTTATT	TTTTCCCTCATACTTCGGATTGAC	(Tapia et al., 2008)
AR	TTTTCAATGAGTACCGCATGC	TCTCGCAATAGGCTGCACG	(Heer et al., 2007)
FGFR2IIIb	ATTGTTCTCCTGTGTCTG	CTTTTCAGCTTCTATATCC	(Mehta et al., 2000)
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	(Sun et al., 2008)
KLK2	GGCAGGTGGCTGTGTACAGTC	CAACATGAACTCTGTCACCTTCTC	(Shaw and Diamandis, 2007)
KLK3 (PSA)	CCCACTGCATCAGGAACAAAAGCG	GGTGCTCAGGGGTTGGCCAC	(Shaw and Diamandis, 2007)

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

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Keywords: Prostate Cancer, Androgen Receptor, Integrin α6β1, NF-κB, Bcl-xL

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ABSTRACT

Recent studies indicate that androgen receptor (AR) signaling is critical for prostate cancer cell survival; even in castration-resistant disease where 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 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 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.

Integrins are heterodimeric cell surface receptors that mediate cell survival through adhesion to extracellular matrix (4, 5). Integrin signaling through various pathways regulates pro-survival and pro-death 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 two integrins, α6β4 and α3β1, 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 down-regulate 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 ~30% of clinical prostate cancers and in ~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 pro-death proteins, such as Bad, Bax, FOXO, DAP3, and caspase 9, and increased expression of the pro-survival 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 upon PI3K/Akt inhibition; however, 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 demonstrate 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. PC3 cells were grown in F-12K containing 10% charcoal-stripped and dextran-treated FBS (CSS). DU145-AR cells were grown in MEM Earles containing 10% CSS, non-essential amino acids, and sodium pyruvate. LNCaP cells were grown in RPMI-1640 supplemented with 10% FBS, 0.225% glucose, 10mM HEPES, and sodium pyruvate. VCaP cells were cultured in DMEM 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 10μg/mL Laminin 1 (Invitrogen) (8, 23).

DNA Constructs. pBabe-puro-hAR and pGL3-vector plasmids were provided by Dr. Beatrice Knudsen. pCSCG-AR-ΔNLS and pCSCG-AR-N705S (ΔLBD) plasmids were obtained from Dr. Owen Witte (21, 24). pLKO.3pg was provided by Dr. Jeff MacKeigan. pBabe-puro-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-Bclxl cells were generated by infecting cells with pBabe-puro, pBabe-puro-hAR, or pBabe-puro-Bclxl retroviruses. Clones were selected and maintained in 2μg/mL puromycin. PC3-pLKO, PC3-ΔNLS, and PC3-ΔLBD cells were made by infecting cells with pLKO.3pg, pCSCG-AR-ΔNLS, or pCSCG-AR-N705S lentiviruses.

siRNA Transfections. Pools of four siRNAs against AR, integrin α6, Bcl-xL, RelA, or a non-targeting sequence were purchased from Dharmacon. Cells were transfected with siRNA using siLentFect lipid reagent (Bio-Rad). Lowest concentration of siRNA able to reduce protein expression by over 85% was used.

RT-PCR. Total RNA was isolated using TRIzol and chloroform. RNA was purified with RNase-free DNase and RNeasy Mini Kits (Qiagen). RT-PCR was performed on 1μg RNA using the

One-Step RT-PCR kit (Qiagen). For qRT-PCR, 0.5µg 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, GAPDH (26), integrin α6 (27), and AR (28).

Reporter Assays. Laminin-adherent cells were transfected with 1.25µg pGL3-vector, pGL4.32-*luc2P*/NF-κB-RE, or pGL4-*luc2P*/ITGα6 (SwitchGear) and 0.5µg phRG-TK using Nanojuice Core Transfection Reagent and Booster Reagent (Novagen). After 48 hours, cells were lysed with Dual-Luciferase Reporter Assay System (Promega) and luminescence measured using 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 or RIPA buffers (8). 45-65µg of protein was run on SDS polyacrylamide gels (Invitrogen) and transferred to PVDF 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% TritonX-100, and blocked with 10% goat serum before incubation with AR (clone 411) antibody (Santa Cruz) 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).

FACS. Suspended cells were washed (1% sodium azide/2% FBS/PBS) and incubated with primary antibodies or control 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 IκBα (44D4) were purchased from Cell Signaling. Polyclonal antibodies to Nkx3.1 (H-50), PSA (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 α2 (CBL477), α3 (MAB2056), and β4 (ASC-3) were purchased from Chemicon, and α5 (P1D6) from Santa Cruz. Integrin α6 (GoH3) came from BD Pharmingen. Integrin β1 (AIIB2) monoclonal antibody, developed by Dr. Caroline Damsky (UC San Francisco, CA), was obtained from the Developmental Studies Hybridoma Bank (University of Iowa).

Cell Survival Assays. Laminin-adherent cells were treated with 5-20μM of LY294002 (8). In some cases, ethanol or 5-10nM each of 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 performed on a hemocytometer; two to three wells 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 two well-characterized AR mutants were introduced into PTEN-deficient PC3 cells. AR expression in the PC3 clones was comparable to 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). Knock-down of AR in the clones reduced AR-target gene expression, indicating 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 if AR expression could protect cells from death induced by PI3K inhibition, cells were placed on laminin (LM) 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 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 since PC3-AR1 cells grow at the same rate, while PC3-AR2 cells grow slower, than PC3-Puro cells (not shown). Nuclear localization of AR was required for resistance to PI3K inhibition, since the AR nuclear localization defective mutant ΔNLS (Fig. 1E) was unable to 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 up-regulation of laminin integrin α6β1. 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 alpha subunits, i.e. α2, α3, and α5, would generate free β1 integrin making it available to bind α6. The large decrease in β4, further indicates that α6 is pairing 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, while 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 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 was unable to 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,B). Reciprocally, loss of AR suppresses α6 mRNA (Fig. 3C). Furthermore, cells expressing the ΔNLS mutant failed to up-regulate 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 upon 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 at 6 hours (Fig. 3G) and peaked at 8-12 hours (Fig. 3H). Induction of α6 mRNA was resistant to cycloheximide treatment, indicating 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 ~1kb 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 AR directly stimulates integrin α6 transcription.

Bcl-xL is required for AR/α6β1-dependent survival. We previously demonstrated that adhesion to LM increases Bcl-xL expression (8). Therefore, we postulated the AR-mediated increase in integrin α6 should increase Bcl-xL expression. Bcl-xL was dramatically up-regulated in PC3-AR cells, and loss of α6 by siRNA decreased Bcl-xL while loss of AR decreased both α6 and Bcl-xL expression (Fig. 4A). Bcl-xL mRNA was also increased by AR (Fig. 4B). Stimulation of LNCaP, C4-2, or VCaP cells (Fig. 4C-D, 3H) with androgen or knock-down 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, over expression 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,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-B) and inhibited upon AR knock-down in C4-2, VCaP, or PC3-AR cells (Fig 5C-F). Conversely, NF-κB-RelA activity was increased upon androgen-stimulation and its activity paralleled the increase in α6 and Bcl-xL expression, peaking at 24 hours (Fig 5D-E). Increased phosphorylation of both IKKβ and IκBα was also observed (Fig. 5G). Knock-down of integrin α6

in PC3-AR, C4-2, or LNCaP cells decreased RelA phosphorylation and Bcl-xL expression (Fig. 6A-B). Knock-down of RelA resulted in a partial loss of Bcl-xL (Fig. 6B-C), but was sufficient to sensitize C4-2 and PC3-AR cells to LY294002-induced death (Fig. 6D-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,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 is mediated via AR-dependent transcriptional stimulation of integrin α6 mRNA lead to increased α6β1 cell surface expression. Integrin α6β1 engagement of LM subsequently activates NF-κB and increases Bcl-xL expression (Fig. 7). Down-regulation of AR, integrin α6, NF-κB, or Bcl-xL re-sensitizes AR-expressing cells to PI3K-dependent survival.

Previous studies, in which AR was re-expressed 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 over-expressed, but maintained at levels similar to LNCaP cells. Third, only low passage (<20) cells were used, since phenotypes can change with passage. Fourth, cells were isolated and constantly maintained in charcoal-stripped serum and phenol red-reduced media to prevent over activation 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 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 non-stripped serum, could hyper-activate AR such that it acts a suppressor and thus explain why it might 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) (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 knock-down was sufficient to completely re-sensitize cells to death induced by PI3K inhibition, NF-κB knock-down, unlike AR or integrin α6 loss, resulted in only a partial loss of Bcl-xL. The partial knock-down 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 upon 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 re-expression 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 re-expression, use of non-charcoal stripped serum 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, while 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, while LM1 is predominantly embryonic. LM10 is present in prostate tumors and bone metastases. Due to lack of availability of purified LM10, we used LM1 in our studies. We assume 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 appears to directly regulate α6 transcription, since the response occurs within 6 hours and is not blocked by cycloheximide. In addition, the first kilobase 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 upon 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 (Supplemental Fig. S2), but are not resistant to death induced by TNFα or staurosporine. Thus, other pathways may also be involved in controlling prostate tumor cell survival.

Interestingly, integrin α2β1, 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 α2β1 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 both AR/α6β1 and PI3K signaling 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 up-regulation 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.

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FIGURE LEGENDS

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 co-localization. **C)** Nkx3.1 (Nkx), TMPRSS2 (TMP), PSA, AR, and tubulin (Tub) expression in PC3-Puro, AR1, and AR2 cells treated with vehicle (-) or 10nM DHT (+) or treated with AR (si) or control (scr) siRNA. **D)** Viability of PC3-Puro, AR1, AR2 or **E-F)** PC3-pLKO, ΔNLS, or ΔLBD cells plated on LM, treated with vehicle (-) or 10nM DHT in the presence of DMSO or LY294002 (LY). Error bars are S.D.; $n = 3\text{-}5$.

FIGURE 2. AR promotes survival through up-regulation of integrin α6β1. A) FACS analysis of integrin expression in LM-adherent PC3-Puro (PP) (solid black), AR1 (solid dark grey), and AR2 (solid light grey) cells. IgG control is dashed line. Small arrows indicate direction of peak shifts. $n = 5\text{-}8$. **B)** AR, integrin α6 (ITGα6), and 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,E)** Integrin α6, AR, and tubulin immunoblots. **D,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-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 5nM 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), 10nM Casodex (Caso), or 10nM 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 5nM R1881 (R) in the absence or presence of 10μg/ml cycloheximide (Cx). **H)** Time course of PSA, α6, Bcl-xL and GAPDH mRNA in C4-2 cells stimulated with 5nM 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 5nM R1881 for 24 hours.

FIGURE 4. Bcl-xL promotes AR/α6β1-dependent survival independent of PI3K. A) AR, α6, Bcl-xL, and tubulin 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,E)** LNCaP, C4-2, or **D)** VCaP cells treated with **C,D)** R1881 or **E)** siRNA. **F-G)** Cells treated with Bcl-xL (si-xL) or control siRNA. **F)** Bcl-xL, AR, and tubulin immunoblots. **G)** Viability of DMSO- or LY294002-treated cells. **H)** Bcl-xL, AR, and tubulin expression in PC3 cells stably over-expressing 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,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 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 10ng/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.

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 10ng/mL TNFα. RelA phosphorylation, total RelA, AR, α6, Bcl-xL, or tubulin monitored by immunoblotting. **D-E)** Viability of RelA siRNA-transfected C4-2, PC3-Puro, AR1, or AR2 cells treated with DMSO or LY294002 (LY). **F-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 up-regulation 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.

FIGURE 1

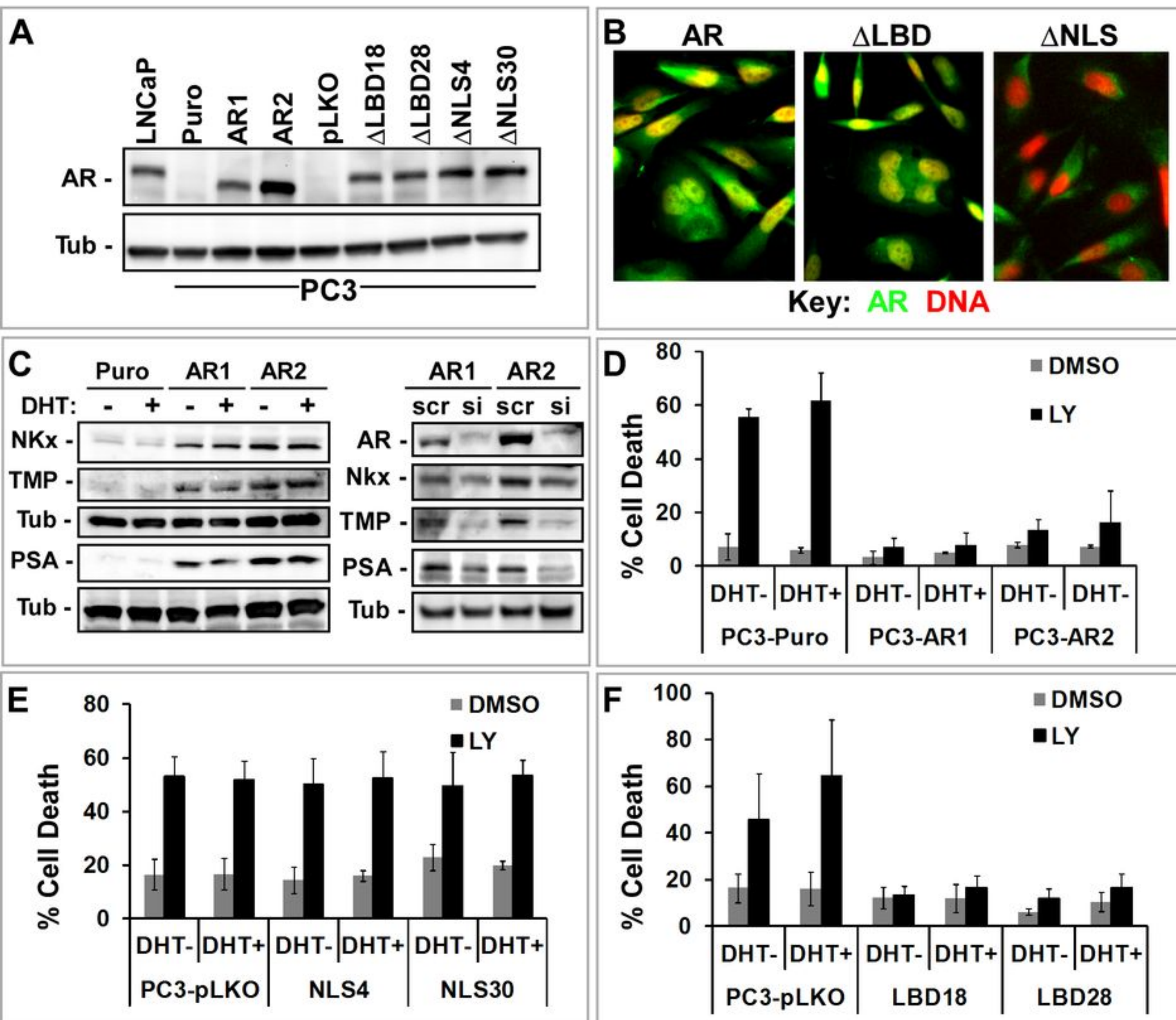
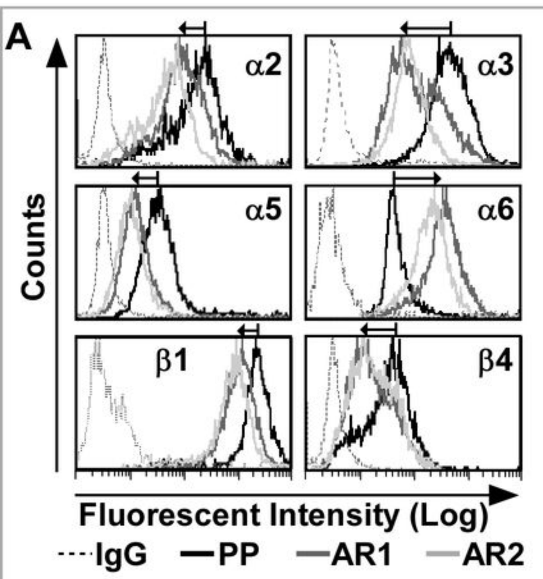
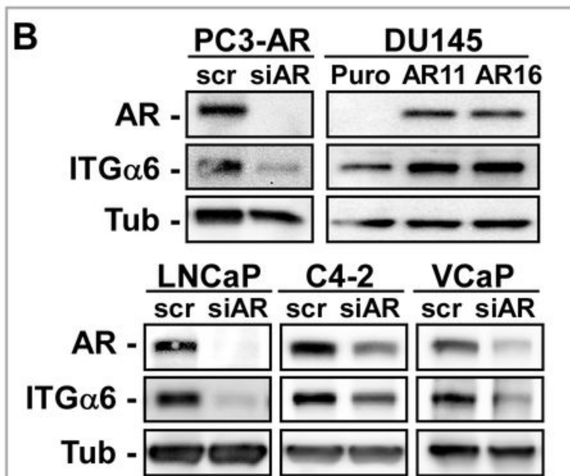
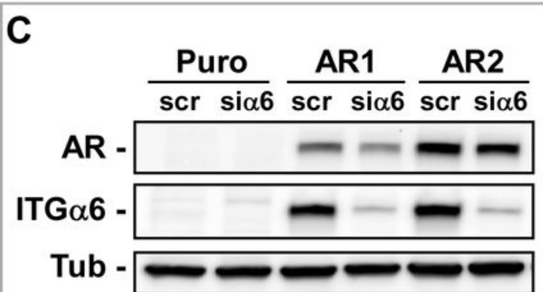
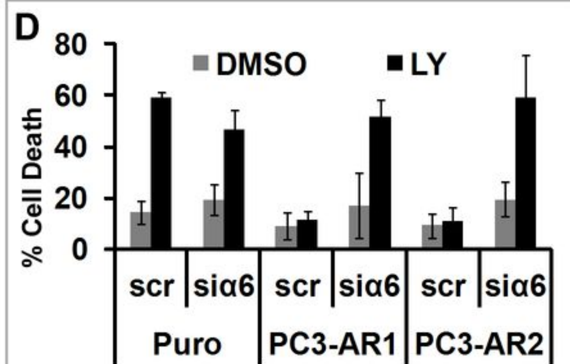


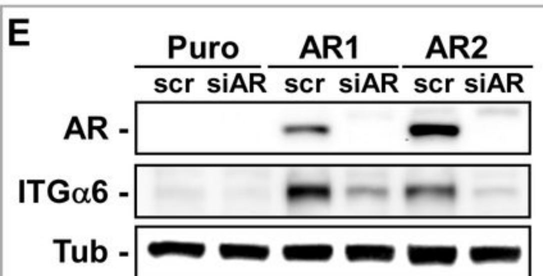
FIGURE 2











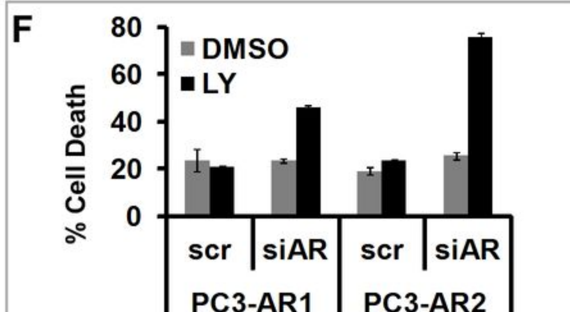


FIGURE 3

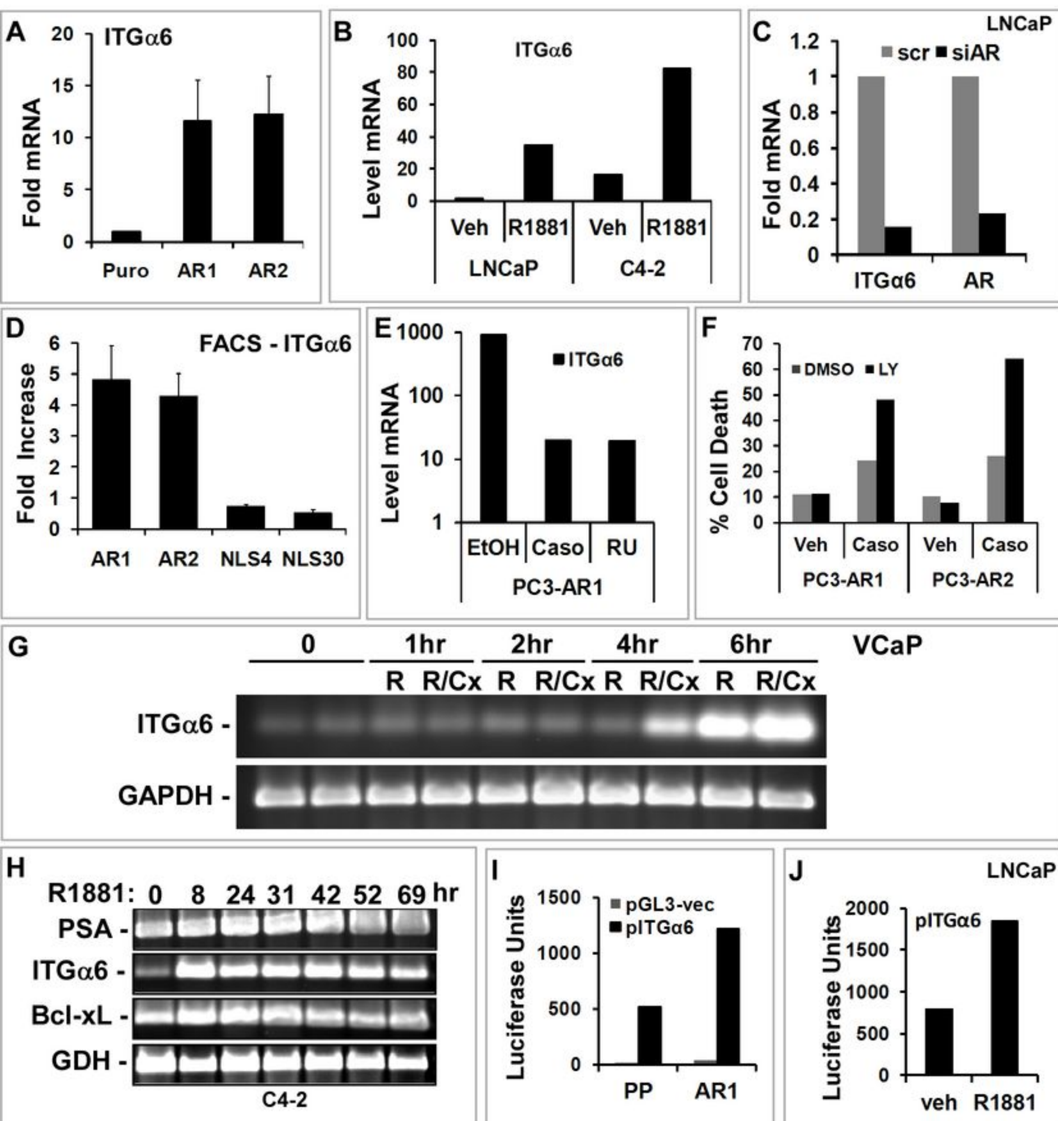


FIGURE 4

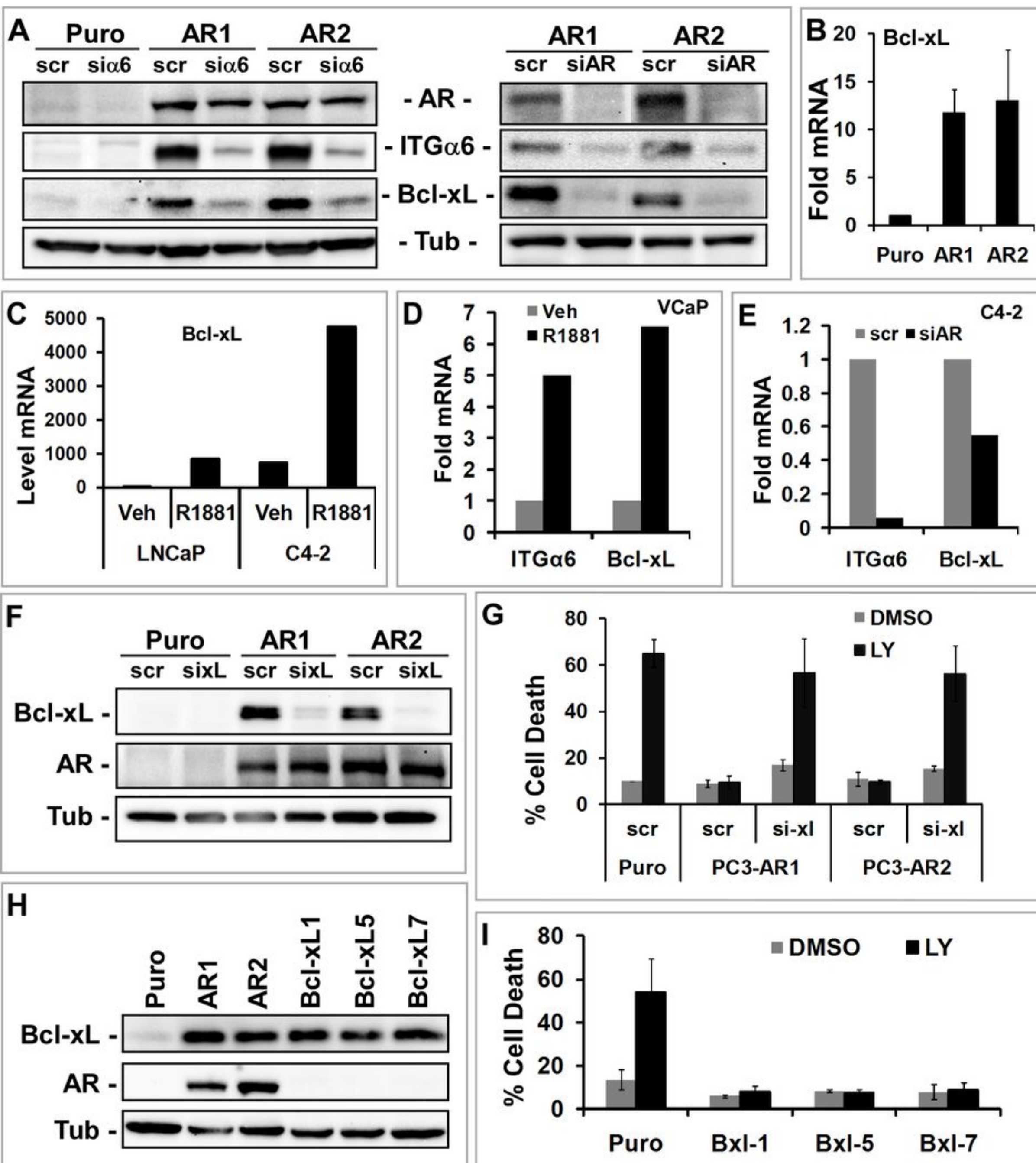


FIGURE 5

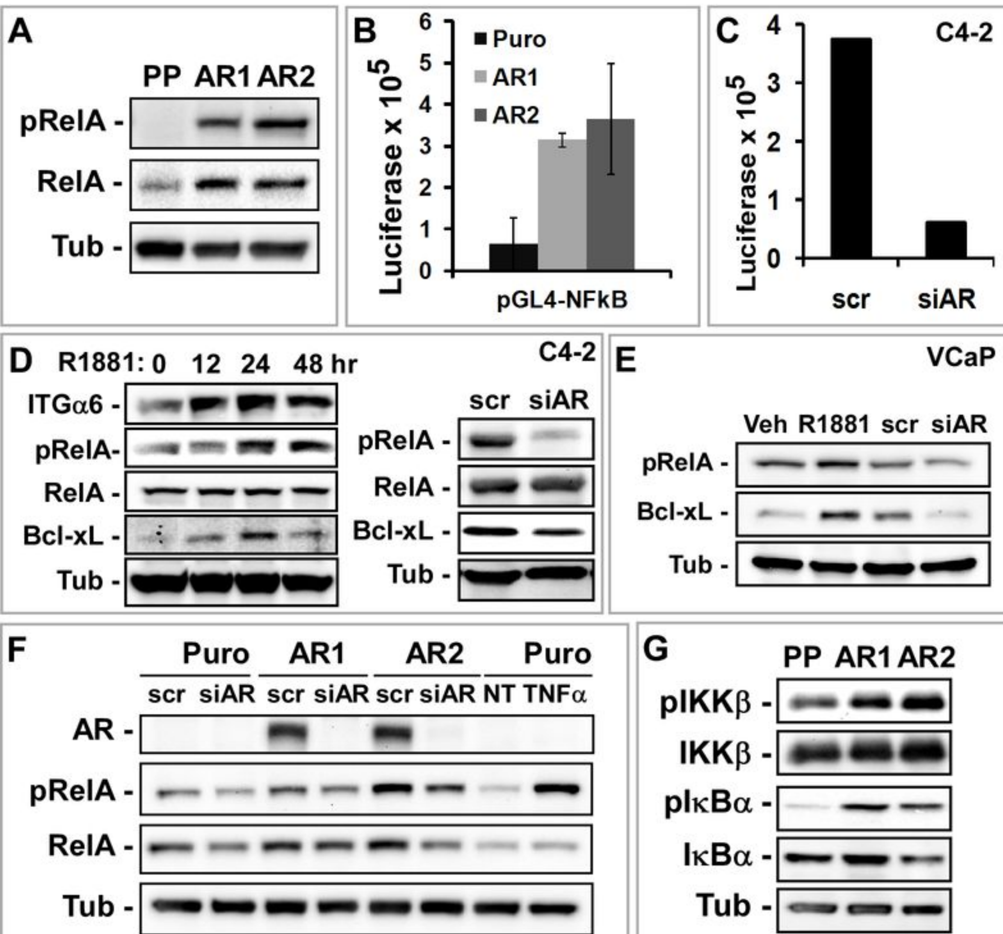


FIGURE 6

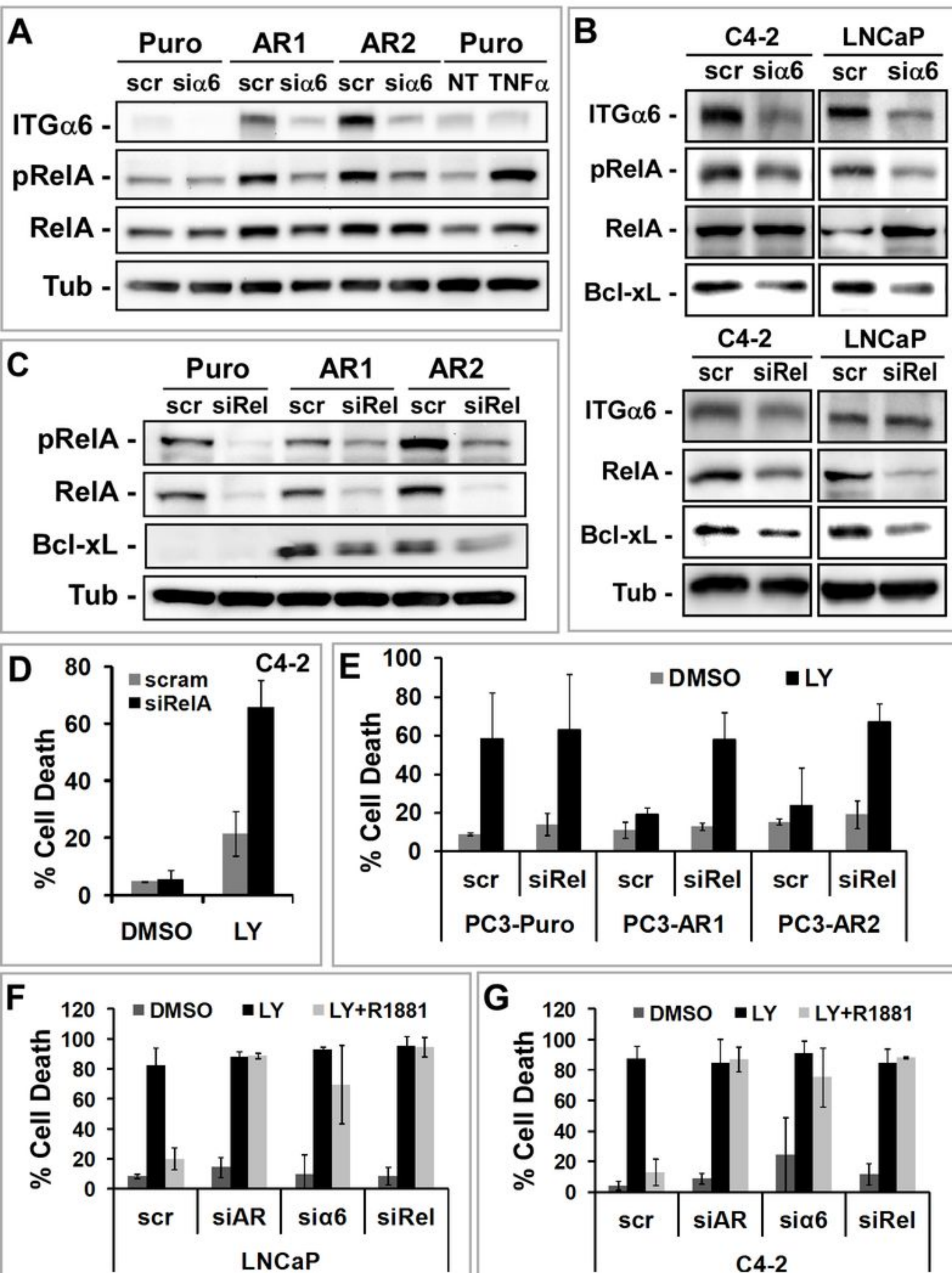


FIGURE 7