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$\alpha_v\beta_6$ Integrin Promotes Castrate-Resistant Prostate Cancer Through JNK1-Mediated Activation of Androgen Receptor

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Abstract

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Conflict of Interest: Paul H. Weinreb and Shelia M. Violette are employees and shareholders of Biogen Idec Inc. The other authors have declared that no conflict of interest exists.

Authors' Contributions

L.R. Languino, D.C. Altieri, R.J. Davis, H. Lu, T. Wang and J. Li designed the study.

H. Lu, T. Wang, J. Li, J. Zhang and C. Fedele. performed the experiments.

H. Lu, T. Wang, J. Li, J. Zhang and C. Fedele. acquired data.

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Androgen receptor signaling fuels prostate cancer and is a major therapeutic target. However, mechanisms of resistance to therapeutic androgen ablation are not well understood. Here, using a prostate cancer mouse model, *Pten*^{PC-/-}, carrying a prostate epithelial-specific *Pten* deletion, we show that the $\alpha_v\beta_6$ integrin is required for tumor growth *in vivo* of castrated as well as of non-castrated mice. We describe a novel signaling pathway that couples the $\alpha_v\beta_6$ integrin cell surface receptor to androgen receptor via activation of JNK1 and causes increased nuclear localization and activity of androgen receptor. This downstream kinase activation by $\alpha_v\beta_6$ is specific for JNK1 with no involvement of p38 or ERK kinase. In addition, differential phosphorylation of Akt is not observed under these conditions, nor is cell morphology affected by $\alpha_v\beta_6$ expression. This pathway, which is specific for $\alpha_v\beta_6$, since is not regulated by a different α_v containing integrin, $\alpha_v\beta_3$, promotes up-regulation of survivin which in turn supports anchorage-independent growth of $\alpha_v\beta_6$ expressing cells. Consistently, both $\alpha_v\beta_6$ and survivin are significantly increased in prostatic adenocarcinoma, but are not detected in normal prostatic epithelium. Neither XIAP nor Bcl-2 is affected by $\alpha_v\beta_6$ expression. In conclusion, we show that $\alpha_v\beta_6$ expression is required for prostate cancer progression including castrate resistant prostate cancer; mechanistically, by promoting activation of JNK1, the $\alpha_v\beta_6$ integrin causes androgen receptor increased activity in the absence of androgen, and consequent up-regulation of survivin. These preclinical results pave the way for further clinical development of $\alpha_v\beta_6$ antagonists for prostate cancer therapy.

Keywords

survivin; JNK1; castrate-resistant prostate cancer; mouse line with a prostate epithelial-specific *Pten* deletion (*Pten*^{PC-/-}); enzalutamide

Introduction

Despite considerable progress in the successful management of early stage disease, prostate cancer (PrCa) remains a life-threatening disease in its advanced stage (1–3). Most, if not all, of aberrant cellular signaling in PrCa can be traced back to aberrant androgen receptor (AR) signaling, which dictates disease onset and progression. AR belongs to the nuclear receptor gene superfamily and, in response to binding to its ligand, i.e. androgen, mediates transcription of various genes important for disease maintenance (4). The gain of resistance to therapeutic androgen ablation in its advanced phases, castrate-resistant prostate cancer (CRPC) (5, 6), does not indicate dispensability of AR activity, but rather the acquisition of ancillary mechanism(s) that fuel AR signaling in the absence of androgen (7), such as AR locus amplification, AR variants or mutants that display transcriptional activity without ligand binding, or inappropriate AR activation by ligand-independent and non-androgen-mediated pathways (4, 8).

Changes in the cell matrix interaction during prostate tumorigenesis are involved in disease behaviors and resistance to therapy (9, 10). In this context, PrCa cells often over-express members of the integrin family of adhesion receptors. Integrins not only mediate cell-to-cell communication, but also play a pivotal role in transducing environmental cues to downstream signaling molecules of cell proliferation, survival and invasiveness, including MAPK, focal adhesion kinase (FAK), src, Akt and JNK (11, 12). In particular, the $\alpha_v\beta_6$

integrin is associated with neoplastic and metastatic phenotypes in various cancers (13, 14). It functions as a receptor for extracellular matrix (ECM) proteins including Latency Associated Peptide Transforming Growth Factor β (LAP-TGF β), fibronectin (FN), tenascin (TN) and vitronectin (VN) and has been linked to activation of FAK and MAPK (15). However, the precise arrangement of integrin signaling contributes to the resistance to androgen ablation is still largely unknown.

Another downstream protein associated with integrin activation is survivin (16). Survivin is a member of the inhibitor of apoptosis (IAP) family whose expression is associated with conservation of cell division (17). Survivin is not usually expressed in normal prostate epithelium, but gradually increases during tumorigenesis and shows the highest level in lymph node metastasis (18). It has been also reported that androgen induces survivin expression and AR inhibitors prevent survivin expression (19). Intratumoral injection of an anti-sense reagent against survivin mRNA or an adenovirus expressing a dominant negative form of survivin, T34A, inhibits tumor growth and enhances sensitivity to androgen ablation (19, 20).

In this study, we investigated a potential cross-talk between integrins, AR signaling and survivin in PrCa models. We found that $\alpha_v\beta_6$ integrin expression is required for PrCa progression *in vivo*, including CRPC, and this pathway mediates activation of JNK1, nuclear accumulation and increased activity of AR in the absence of androgen.

Materials and Methods

Reagents and Antibodies (Abs)

Synthetic androgen R1881 was from Perkin-Elmer. Enzalutamide was from Selleck. The following rabbit Abs (pAbs) were used: AR (N20), phospho-ERK1 (Santa Cruz); Smad3 (Invitrogen); p-src (Y416), Akt, p-Akt, p-JNK and p-p38 (Cell Signaling); prostate specific antigen (PSA) (DAKO Cytomation). A goat Ab against p38 was from Santa Cruz. A rabbit monoclonal Ab against β_6 integrin, B1, was from Dr. Dean Sheppard. The following mouse monoclonal Abs (mAbs): AR (441) (Santa Cruz); β_1 integrin (C-18) and JNK (BD Biosciences); c-src (Cell Signaling); β_1 integrin (TS2/16) and β_3 integrin (AP3) are from ATCC; β_5 integrin (P1F6) from Life Technologies, Inc.; α_v integrin (VNR147) and α_5 integrin (P1D6); CK8 (Boehringer Mannheim), CK18 (Sigma); β_6 integrin Abs 6.2A1, ch2A1 and 6.3G9 were previously described (21); β_6 integrin Ab 10D5 from Chemicon. Purified non-immune mouse IgGs (mIgG) from Pierce, 1E6 (IgG1) from Biogen and 1C10 were used as negative controls.

Cells and culture conditions

LNCaP, C4-2B (UroCor), PC3 and RWPE cells were obtained from ATCC and cultured as previously described (22, 23). LNCaP stable cell lines expressing full-length β_3 , β_6 or empty vector have been described previously (24). PC3 and C4-2B cells stably transfected with human β_6 -integrin cDNA in pBABE retroviral vector were used (23). Authentication of the cell lines was provided with their purchase. Cellular morphology and anchorage-

independent growth assay were visualized using an Olympus IX71 inverted microscope with IPLab V3.55 (Scanalytics, Inc, Rockville, MD).

Human tissue specimens

All tissues in this study were discarded, coded and unidentifiable specimens. Forty-seven human adenocarcinoma (AdCa) tissue specimens from radical prostatectomies provided by: the Cooperative Human Tissue Network, which is funded by the National Cancer Institute (other investigators may have received specimens from the same subject); the Department of Pathology, University of Massachusetts Medical School and the Department of Pathology, Yale University and were processed according to Institution-approved protocols. Eight normal prostatic tissue sections were obtained from human biopsies. Thirteen additional prostate specimens were snap-frozen in liquid nitrogen for protein extraction. Tissues were processed as previously described (25).

Constructs and cell transfection

Adenoviruses, containing wild type survivin (WT), dominant negative survivin (T34A) or empty vector (pAd) have been described previously (26). $\alpha_v\beta_6$ -LNCaP cells were transiently transfected with adenoviral constructs for 17 hours. All three constructs contain the GFP gene for determination of transfection efficiency. C4-2B cells were stably transfected human β_6 and β_3 integrin cDNA generated as described before (24). Clones were selected and maintained in culture medium containing 5% FBS and 100 μ g/ml G418. Retroviral constructs, containing wild type JNK1 or an NH²-terminal JNK-binding domain (JBD), and the transient transfections have been described previously (27). $\alpha_v\beta_6$ -C4-2B, $\alpha_v\beta_3$ -C4-2B or mock-C4-2B cells were transiently transfected with WT-JNK- or JBD-viral construct to determine the effect of JNK on AR activity. WT-JNK (pBABE-puro-JNK1) or JBD containing retrovirus (babe-puro-JBD) were wrapped with VSVG and GAGPol using phoenix cells. The transient transfection was performed with 8 μ g/ml of polybrene for 48 hours, then cells were starved in 2% CS serum-containing medium for 24 hours followed by stimulation with 1 nM R1881 or ethanol for 24 hours.

siRNA transfection

Cells were transfected with β_6 integrin siRNA duplexes (IDT Inc.). Two β_6 siRNA duplexes, D1 and D2 are described before (22). siRNA duplexes were transfected using oligofectamine at a final concentration of 20 nM. Forty-eight hours after transfection, cells were harvested and analyzed by FACS to confirm down-regulation of β_6 integrin and to evaluate anchorage independent cell growth. For down-regulation of AR, siRNA smartpool and non-silencing siRNA (Dharmacon) was used according to manufacturer's instruction. The protocol used for down-regulation of survivin by siRNA in $\alpha_v\beta_6$ -LNCaP cells was based on published methods (28).

Luciferase assay and cell adhesion assay

Luciferase activity was measured as described (29). Cell adhesion assays used here have been described previously (30). For JNK activation assay, LNCaP, C4-2B or PC3 cells stably transfected with pBabe- β_6 -integrin or pBabe-empty vector were starved overnight in serum-

free RPMI culture medium. Cells were seeded on the culture plates coated with FN (3 $\mu\text{g}/\text{ml}$), LAP-TGF β_1 (0.5 $\mu\text{g}/\text{ml}$) or BSA. Cells (1×10^6) were plated on the substrate for 1 hour in serum-free RPMI culture medium before cell lysis.

Cell proliferation assay

Cells were seeded in a 96-well plate for 24 hours, starved with the medium containing 2% CS serum for 24 hours. Cells were stimulated with either 1 nM R1881 or same volume of ethanol and were cultured for 72 hours before performing Sulforhodamine B (SRB) staining.

Immunohistochemistry

IHC staining using formalin-fixed paraffin embedded specimens was performed as described elsewhere (31). For detection of survivin, IHC was performed according to published protocols (32).

Immunofluorescence

Primary epithelial cells isolated as described (30), were probed using Abs specific to CK8 or CK18, markers of prostate epithelial cells by indirect IF staining. AR nuclear localization was analyzed using IF staining. Cells were seeded on poly-L-Lysine (1 mg/ml) coated coverslips for 2 days. After starvation for 24 hours in 2% charcoal stripped (CS) serum contained medium, cells were grown in the presence of ethanol or R1881 (1 nM) for 2 hours. Cells were probed with an AR specific Ab N20 (1:500) to determine the localization of AR in nucleus.

FACS

FACS analysis was performed to determine surface expression of integrins using Abs specific to β_6 (10D5), β_3 (AP3), Non-immune mIgG, 12CA5 or IC10 were used as negative controls.

Generation of Pten^{PC-/-} mice and Castration

Prostate epithelial-specific Pten conditional knockout mice (Pten^{PC-/-}) mice are generated as previously reported (33). Pten^{PC-/-} mice were surgically castrated with published method (33).

Statistical analysis

Unless otherwise indicated, data in the figures are presented as mean $\pm$ SEM, and significant differences between experimental groups were determined using the 2-tailed Student's *t* test. Fisher's exact 2-tailed test was used to compare the percentage of β_6 positive cells. Mantel-extension of Mantel-Haenszel Statistic stratified by subject was used to test the correlation between β_6 expression and the types of lesion. Wilcoxon-Mann-Whitney test was used to analyze the difference in tumor growth between 6.3G9 and control group *in vivo*. To examine the association between β_6 and survivin expression in human specimens, the Spearman correlation coefficient was calculated and tested. A two-sided *p* value of less than 0.05 was considered statistically significant. SAS statistical software 9.1.2 (SAS Institute, Inc., Cary, North Carolina) was used for statistical analysis.

Results

$\alpha_v\beta_6$ integrin promotes castrate-resistant prostate cancer growth *in vivo*

To determine the effect of $\alpha_v\beta_6$ expression *in vivo*, we used a CRPC model that utilizes surgically castrated Pten^{PC-/-} as delineated in Fig. 1A. $\alpha_v\beta_6$ expression pattern in castrate resistant tumors (n=5) was compared by immunohistochemistry (IHC) with non-castrated tumors (n=5) in Pten^{PC-/-} mice. We show here that $\alpha_v\beta_6$ is expressed in mouse CRPC; specifically, we demonstrate that the expression of $\alpha_v\beta_6$ is highly heterogeneous (18% of the tumor cells) in tumors from non-castrated mice while homogenous (90% of tumor cells) in tumors from castrated mice, indicating a significant enrichment of this integrin upon castration. Accordingly, JNK nuclear localization is increased in $\alpha_v\beta_6$ positive cells in non-castrated tumors (3% of tumor cells), and becomes dominant in castrated tumors (75% of tumor cells) (Supplementary Fig. 1).

Upon intraperitoneal (i.p.) administration for 5 weeks of 6.3G9, a non ligand-mimetic blocking mAb that does not get internalized upon binding (21), to castrated Pten^{PC-/-} mice, tumor progression is inhibited and the prostate glands do not show evidence of invasive AdCa; in contrast, upon i.p. administration of a negative control mAb, 1E6, the prostate glands show invasive AdCa (Fig. 1B). The results show a significant decrease of tumor weight in castrated Pten^{PC-/-} mice treated with 6.3G9 (25.3 ± 1.8 mg) as compared with the group treated with 1E6 (39.8 ± 1.5 mg) (Fig. 1C). We also carried out xenograft tumor growth experiments in castrated athymic nude mice (Supplementary Fig. 2). For this purpose, we used AR⁺ LNCaP cells that stably express human β_6 ($\alpha_v\beta_6$ -LNCaP), human β_3 ($\alpha_v\beta_3$ -LNCaP) or empty vector (mock-LNCaP). These cells exhibit no difference in the expression of other endogenous integrin subunits (Supplementary Fig. 2A); upon subcutaneous (s.c.) injection, $\alpha_v\beta_6$ -LNCaP xenograft tumors continue to grow significantly (Supplementary Fig. 2B), suggesting that $\alpha_v\beta_6$ expression is sufficient to confer a resistant phenotype to PrCa cells, *in vivo*. In contrast, $\alpha_v\beta_3$ -LNCaP transfectants do not significantly grow as s.c. tumors in castrated mice (Supplementary Fig. 2B).

We in parallel investigated whether $\alpha_v\beta_6$ is required for PrCa growth in non-castrated mice. We used Pten^{PC-/-} mice to independently validate a requirement of $\alpha_v\beta_6$ for tumor growth *in vivo*. i.p. administration of the 6.3G9 Ab for 5 weeks to Pten^{PC-/-} mice results in acute disruption of epithelial layers of prostate AdCa, with appearance of necrotic cells filling the glandular lumen (Fig. 2A) and in a significant decrease in tumor weight as compared with mice treated with the 1E6 mAb ($P = 0.0042$) (Fig. 2B). In contrast, prostate specimens collected from Pten^{PC-/-} mice treated with 1E6 show an intact structure of transformed prostatic glands (Fig. 2A). In addition, histologically normal glands of Pten^{PC-/-} mice receiving 6.3G9 mAb do not show a disruption of epithelial layers (Fig. 2A). S.c. injection of $\alpha_v\beta_6$ -LNCaP cells in the flanks of immunocompromised SCID mice gives rise to exponentially growing tumors with a statistically significant difference ($P < 0.01$) compared to $\alpha_v\beta_3$ -LNCaP tumors starting at 54 days post-injection (Fig. 2C). In two additional independent experiments, $\alpha_v\beta_3$ -LNCaP cells or mock-LNCaP cells (Supplementary Fig. 3A) produce tumors with statistically slower growth kinetics ($P < 0.01$) as compared to $\alpha_v\beta_6$ -LNCaP tumors. Accordingly, expression of $\alpha_v\beta_3$ - or $\alpha_v\beta_6$ -integrins on the cell surface

was confirmed by FACS (Supplementary Fig. 3B). Tumors generated by the various transfectants retain the expression of the respective β_6 or β_3 integrins as detected by immunoblotting (IB), whereas tumors generated by mock-transfectants remain negative (Supplementary Fig. 3C). Together, these data demonstrate that $\alpha_v\beta_6$ is required for accelerated PrCa growth, *in vivo*.

$\alpha_v\beta_6$ integrin promotes PrCa cell proliferation and activates the AR pathway

To determine the potential basis for $\alpha_v\beta_6$ -mediated aggressive tumor behavior *in vivo*, we next measured the proliferation kinetics of integrin-expressing cells in the presence or absence of androgen. Using SRB proliferation assays, mock-LNCaP and $\alpha_v\beta_3$ -LNCaP cells exhibit increased proliferation in response to androgen (R1881) (Fig. 3A, left and right panels). Conversely, two independent clones of $\alpha_v\beta_6$ -LNCaP cells show increased rates of proliferation compared to mock-LNCaP or $\alpha_v\beta_3$ -LNCaP cells in the absence of androgen, and this response is unaffected by androgen (Fig. 3A, left panel). The $\alpha_v\beta_6$ -LNCaP, mock-LNCaP and $\alpha_v\beta_3$ -LNCaP cells do not show morphological differences (Fig. 3B). In an anchorage-independent assay, we down-regulated β_6 in AR+ RWPE cells using two independent siRNAs, D1 and D2 (Fig. 3C, right panels). β_6 silencing by two independent siRNAs, under these conditions, suppresses anchorage-independent tumor growth whereas non-targeting siRNA (NS) or oligofectamine (OF) alone has no effect (Fig. 3C, left panel).

To analyze the mechanism by which $\alpha_v\beta_6$ affects PrCa growth, we tested AR activity in the presence or absence of androgen in $\alpha_v\beta_6$ -LNCaP (Fig. 4A, left panel) or $\alpha_v\beta_6$ -C4-2B cells (Fig. 4B, left panel); the results show that $\alpha_v\beta_6$ -LNCaP exhibit constitutively high levels of PSA, an established AR target gene, without addition of exogenous androgen, and PSA levels are only minimally affected in the presence of androgen. In control experiments, mock-transfectants, $\alpha_v\beta_3$ -LNCaP or $\alpha_v\beta_3$ -C4-2B cells show negligible basal levels of PSA, whereas androgen (R1881) stimulation elevates PSA expression in these cells (Fig. 4A and 4B, left panels; also shown in Supplementary Fig. 4). Silencing AR by siRNA in $\alpha_v\beta_6$ -LNCaP cells (Fig. 4A, right panel) or $\alpha_v\beta_6$ -C4-2B cells (Fig. 4B, right panel) abolishes $\alpha_v\beta_6$ -induced PSA, confirming AR specificity of these findings. Similar results are obtained in the analysis of AR promoter activity; $\alpha_v\beta_6$ -LNCaP cells exhibit constitutive high levels of androgen responsive elements (ARE)⁴-luciferase activity, irrespective of androgen stimulation, as compared to mock-transfected cultures (Supplementary Fig. 5). In addition, neither expression of $\alpha_v\beta_6$ or $\alpha_v\beta_3$ integrin affects endogenous AR protein levels, in the presence or absence of androgen (Fig. 4C), indicating that the increase in AR activity observed in $\alpha_v\beta_6$ + cells does not reflect changes in AR protein expression. Immunofluorescence (IF) analysis shows increased nuclear localization of AR in the absence of androgen in $\alpha_v\beta_6$ -LNCaP cells (Fig. 4D). In contrast, AR reactivity is detected largely in the cytoplasm of unstimulated $\alpha_v\beta_3$ -LNCaP or mock-LNCaP cells, and becomes intense in the nuclei only in response to androgen stimulation.

The ability of $\alpha_v\beta_6$ to activate AR, however, does not promote resistance to enzalutamide, an AR antagonist developed for the treatment of metastatic CRPC patients. Mechanistically, enzalutamide inhibits translocation of AR to the nucleus and prevents binding of AR to DNA or to coactivator proteins (34). Based on this knowledge, we investigated whether this

compound may also inhibit ligand-independent AR activation mediated by $\alpha_v\beta_6$. We find that $\alpha_v\beta_6$ integrin expression does not promote resistance to enzalutamide at concentrations up to 100 μ M and that enzalutamide is effective in suppressing proliferation of $\alpha_v\beta_6$ -, $\alpha_v\beta_3$ - and mock- LNCaP cell transfectants in a comparable fashion (Fig. 4E).

$\alpha_v\beta_6$ activation of JNK1 regulates AR activity

To determine the mechanism by which $\alpha_v\beta_6$ activates AR, we analyzed the kinase cascades typically activated by integrin ligand binding. Adhesion of $\alpha_v\beta_6$ -LNCaP cells to the $\alpha_v\beta_6$ ligand LAP-TGF β_1 or FN results in JNK1 activation as evaluated by its phosphorylation pattern (Fig. 5A, top panel). In parallel, we observe that PSA is increased in $\alpha_v\beta_6$ -transfectants attached to LAP-TGF β_1 or FN while $\alpha_v\beta_3$ -transfectants show barely detectable PSA expression (Fig. 5A, middle panel). $\alpha_v\beta_3$ -LNCaP cells adhere to FN or LAP-TGF β_1 but fail to induce JNK1 phosphorylation (Fig. 5A, top panel). PC3 cells stably transfected with β_6 ($\alpha_v\beta_6$ -PC3) also exhibit increased JNK1 phosphorylation upon adhesion to FN or LAP-TGF β_1 , whereas mock-transfected cells are ineffective (Fig. 5A, bottom panel). The function-blocking Ab 6.3G9 inhibits attachment to LAP-TGF β_1 in a concentration-dependent manner, thus identifying $\alpha_v\beta_6$ as the predominant LAP-TGF β_1 receptor in PrCa cells (35) (Fig. 5A, graph). Conversely, ligand binding to $\alpha_v\beta_6$ does not activate related signaling pathways, including ERK, p38 (Supplementary Fig. 6, A and B) or JNK2 (data not shown) as determined by IB with phosphorylation specific Abs. Since JNK1 activation has been implicated in controlling AR activity via nuclear translocation (36), we infected $\alpha_v\beta_6$ -C4-2B cells with a retrovirus encoding a JNK-binding domain (JBD), which functions as a dominant negative JNK (27, 37). $\alpha_v\beta_6$ -C4-2B cells exhibit high PSA levels, which is not further modulated by androgen (R1881) stimulation (Fig. 5B; also shown in Supplementary Fig. 7), in agreement with the data presented above. In contrast, infection of decreasing concentrations of JBD inhibits the basal expression of PSA in $\alpha_v\beta_6$ -C4-2B cells, and completely abolishes PSA levels after androgen stimulation (Fig. 5B), whereas infection of WT-JNK1 does not affect PSA levels in $\alpha_v\beta_6$ -C4-2B cells, in the presence or absence of androgen. Androgen modulation of PSA in mock- or $\alpha_v\beta_3$ -C4-2B cells is unaffected by WT-JNK1 or JBD (Fig. 5B). To identify a potential link between JNK1 activity and AR-dependent gene expression, we next looked at potential changes in AR localization. IF staining shows that transfection of JBD in $\alpha_v\beta_6$ -LNCaP cells significantly impairs AR nuclear localization (Fig. 5C). Overall, this study suggests that upon ligand binding, $\alpha_v\beta_6$ enhances AR transactivation via JNK1 stimulation, which in turn controls AR nuclear trafficking.

Survivin is a downstream effector of the $\alpha_v\beta_6$ integrin

To identify potential downstream effectors of integrin-mediated PrCa progression, we focused on survivin, that has been implicated in PrCa maintenance and resistance to therapy (19, 38). $\alpha_v\beta_6$ -C4-2B cells exhibit higher levels of survivin, compared to $\alpha_v\beta_3$ -C4-2B (Fig. 6A), while XIAP or Bcl2, two other molecules known to inhibit apoptosis, are not affected (Fig. 6A). Similar results were obtained with LNCaP cells stably transfected with a $\beta_{3/6}$ integrin chimera (Fig. 6B and Supplementary Fig. 8), containing the β_6 intracellular domain and the β_3 extracellular domain expressed at the cell surface, suggesting that the β_6 cytoplasmic domain is required for survivin induction.

In this context, silencing of AR significantly reduces survivin levels in $\alpha_v\beta_6$ -LNCaP cells, whereas a non-targeting siRNA is ineffective (Fig. 6C). Inhibition of JNK activity in these cells via infection with JBD also decreases survivin levels independently of androgen, whereas expression of WT JNK1 moderately up-regulates survivin in $\alpha_v\beta_6$ -LNCaP cells (Fig. 6D). Silencing of survivin by siRNA, or interference with survivin function via adenoviral transduction of a survivin Thr34Ala dominant negative mutant (Fig. 6E, left panel), abolishes PrCa colony formation in soft agar, i.e. anchorage independent growth. In contrast, a non-targeting siRNA or adenoviral transduction of WT Survivin does not affect anchorage-independent growth under the same conditions (Fig. 6E, right panel). We also analyzed the expression of $\alpha_v\beta_6$ integrin in human primary specimens of prostatic AdCa or normal prostate. Using an Ab that specifically recognizes $\alpha_v\beta_6$, 6.2A1, we found by immunohistochemistry (IHC) that this integrin is abundant in PrCa (35 out of 47, 75%) (Fig. 7A), consistent with its epithelial-specific expression, although in a highly heterogeneous pattern with 5~31% of the glands being $\alpha_v\beta_6$ positive. Identical results were obtained with an independent mAb (B1) to $\alpha_v\beta_6$ (Supplementary Fig. 9). In contrast, $\alpha_v\beta_6$ is undetectable in 8 out of 8 cases of normal prostate (Fig. 7A), and does not correlate with Gleason Score (5–10), or patient age (range, 41–83 years). In addition, patient-derived primary PrCa cells, isolated as described (30) and reactive for cytokeratin (CK) 8 and 18 (Fig. 7B, middle panel), express high levels of $\alpha_v\beta_6$, as detected by FACS (Fig. 7B, top panel). This result was confirmed using 15 fresh prostatic AdCa tissue lysates by IB using an Ab to $\alpha_v\beta_6$ (6.2A1) which detects a 110 kDa protein consistent with β_6 reactivity (Fig. 7B, bottom panel, two representative specimens are shown) albeit with variable levels of expression. Survivin expression was also studied in 34 specimens and is found to be expressed in 97% of AdCa specimens (Fig. 7C, panels 1–4); the association between $\alpha_v\beta_6$ and survivin is statistically significant in 34 cases of AdCa ($P < 0.028$) and is independent of Gleason scores (5–10) or disease stage (T2-4) (Supplementary Table 1). Overall, our *in vitro* and IHC analysis show that survivin is a downstream effector of the $\alpha_v\beta_6$ integrin.

Discussion

In this study, we show that expression of an epithelial-specific integrin, $\alpha_v\beta_6$, in PrCa as well as in castrated tumors, is sufficient to promote aggressive tumor growth and CR disease. The molecular requirements of this pathway are centered on $\alpha_v\beta_6$ activation of JNK1, which in turn promotes AR nuclear shuttling, concomitant ligand-independent AR transcription and subsequent survivin-regulated tumor growth (Fig. 7D).

At the molecular level, this transition to CR is an as yet poorly understood process (4). Our data unravel a novel pathway of CR aggressive PrCa that originates in the tumor via $\alpha_v\beta_6$ integrin ligand binding, and ultimately promotes AR-modulated gene expression, independent of androgen.

There is ample precedent for the role of integrins in controlling steroid hormone receptor expression and function. In a PrCa model, our group recently found that $\alpha_v\beta_6$ is efficiently transferred via exosomes to $\alpha_v\beta_6$ -negative recipient cells and localizes to the PrCa cell surface, promoting cell adhesion and migration on LAP-TGF β , suggesting a systemic role of $\alpha_v\beta_6$ in tumor progression (39) in addition to its local effect. These effects, however, do not

require transfer of JNK via exosomes, since recent data show that JNK is not detected in exosomes (L.R. Languino *et al.*, unpublished observation). In this study, our data indicate that expression of $\alpha_v\beta_6$ in PrCa cells is sufficient to confer a “sub-optimal” androgen-independent phenotype, characterized by proliferation and PSA expression in the absence of exogenous androgen. This is further supported by our *in vivo* studies, showing a significant increase in the growth of $\alpha_v\beta_6$ -expressing tumors in castrated as well as non-castrated mice. Finally, since $\alpha_v\beta_6$ effect does not promote resistance to enzalutamide, targeting this integrin may thus be a potential adjuvant in enzalutamide treatment.

The molecular requirements of signal integration from $\alpha_v\beta_6$ to AR-dependent gene expression in PrCa remain to be fully elucidated. However, one of the key requirements in this pathway is $\alpha_v\beta_6$ integrin-mediated activation of the MAP kinase family member, JNK1. Despite its conflicting, and likely cell-type specific, roles in both cell survival and apoptosis (37), the JNK pathway has recently emerged as an important regulator of PrCa growth via AR (36). The JNK1 downstream target, c-Jun, strongly potentiates AR-mediated gene transcription (40, 41), and silencing of JNK1 inhibits PrCa tumor growth (42). The data presented here suggest that downstream kinase activation by $\alpha_v\beta_6$ may be specific for JNK1, with no involvement of p38 or ERK kinase. In addition, differential phosphorylation of Akt is not observed under these conditions consistent with a context-dependent role of Akt in AR activation as described by the following studies. Although PI3K/Akt signaling promotes AR activity (43), examples of PTEN regulation of JNK signaling independently of Akt have been reported (44); furthermore, JNK deficiency promotes androgen-independent progenitor cell growth and metastatic PrCa more rapidly than PTEN deficiency alone (33). Our data from several PrCa cell lines (LNCaP, C4-2B, PC3) show that JNK1 promotes AR nuclear translocation and activation. With respect to how active JNK1 modulates AR function in $\alpha_v\beta_6$ integrin-expressing cells, the data presented here are in line with several reports linking AR phosphorylation to its nuclear-cytoplasm shuttling. AR can be phosphorylated at tyrosine, serine and threonine residues by a wide range of kinases, such as cyclin-dependent kinases (CDK), Akt, PIM1, Fer, Aurora-A, Src, Etk, PKC, PAK6, JNK and p38 (45). Akt, for example, can phosphorylate AR at S210/S790 and inhibit androgen-induced apoptosis (46); CDK1 phosphorylates AR S81 and regulates AR stability (47); in addition, soluble factors derived from stroma activate ERK which phosphorylates AR at S81, promote AR-dependent transcription and anchorage-independent growth (48). The data presented in our study show that $\alpha_v\beta_6$ predominantly promotes AR nuclear translocation and activation through JNK1.

It should be stressed that AR is already activated in castrate resistant cells, such as C4-2B, but the activity and/or nuclei distribution are not saturated or irreversible. Upon proper stimulation (49), the fraction of AR in the nucleus can be further increased, as shown in $\alpha_v\beta_6$ transfectants in this study. Our data suggest that integrins promote a novel mechanism for castrate resistance, other than the mechanisms dependent on AR mutations. We believe the use of the castrate resistant cells in this paper supports the major conclusion that $\alpha_v\beta_6$ is one of the key factors promoting PrCa progression in castrate settings, in addition to its pro-tumor effect in androgen-sensitive cells such as RWPE cells.

An additional downstream effector of aggressive PrCa phenotype of $\alpha_v\beta_6$ integrin-expressing cells is identified here as survivin, a critical PrCa promoting molecule (18) regulated by PI3K/Akt signaling, as a mediator of drug resistance (19), and as an effector of tumor progression. Although Interleukin-4 induced survivin is independent of JNK (32), in our model JNK is required for up-regulation of survivin by $\alpha_v\beta_6$ integrin, suggesting a different mechanism in this context. Against this backdrop, survivin expression correlates here with $\alpha_v\beta_6$ integrin levels in primary PrCa specimens, is directly regulated by AR, and is required for anchorage-independent growth, in agreement with an emerging role of survivin in metastatic dissemination (50).

In summary, we have identified a novel signaling circuit linking integrins with modulation of nuclear receptor gene expression, critically important to confer a CR phenotype and aggressive behavior of PrCa in humans. The identification of mechanisms of CR amenable to therapeutic intervention is an urgent and as yet unmet need in advanced PrCa, a disease stage where therapeutic options are few and only minimally effective. The translational relevance of our findings is highlighted by the recognized role of integrins as bona fide therapeutic targets for drug development. The preclinical ability of a mAb to $\alpha_v\beta_6$ integrin to inhibit tumor growth and disrupt the epithelial architecture of PrCa, without toxicity to normal prostate in $Pten^{PC-/-}$ mice, as presented here, bodes well for the further clinical development of $\alpha_v\beta_6$ antagonists.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Inhibitory effect of mAb 6.3G9 on tumor growth in castrated $Pten^{PC-/-}$ mice. (A) Scheme of surgical castration and Ab treatment of $Pten^{PC-/-}$ mice. (B and C) mAb 6.3G9 against $\alpha_v\beta_6$ or a negative control mAb 1E6 were injected in castrated $Pten^{PC-/-}$ mice i.p. both at 10 mg/Kg weekly for 5 weeks; then mice ($n = 11$) were sacrificed and tumor weights were quantified. (B) H&E analysis of tumor specimens from 6.3G9 treated mice (upper panels) or 1E6 treated mice (lower panels). The arrow points at epithelial cells in the upper right panel. The arrowhead points at cancer cells sloughed in the lumen. The asterisk indicates micro-invasion in the lower right panel. Original magnification: left column, $\times 200$, right column, $\times 400$. (C) Individual tumor weight is plotted; lines are mean $\pm$ SEM. $P = 0.004$ (Wilcoxon rank-sum two-sided test).

Figure 2.

Inhibitory effect of mAb 6.3G9 on tumor growth in non-castrated Pten^{pc-/-} mice. (A and B) Pten^{pc-/-} mice (5–8 weeks) were i.p. injected with 6.3G9 or 1E6 as described in Fig. 1, then mice were sacrificed and the differences in tumor weight were quantified. (A) Representative prostate specimens (normal gland or AdCa) of Pten^{pc-/-} mice injected with mAb 6.3G9 or control mAb 1E6 were analyzed histologically by H&E staining. Arrow, points at cancer epithelial cells. Arrowhead, points at apoptotic cancer cells in the lumen. None: untreated. Original magnification: left column, ×200; right column, ×400. (B) Individual tumor weight is plotted (n = 8). Statistical significance was observed in tumor weight, $P = 0.0042$ (Wilcoxon rank-sum two-sided test). (C) α_vβ₆-LNCaP (filled circle) or α_vβ₃-LNCaP (open square) cells were injected s.c. into male CB17/SCID mice (16 mice/group), and differences in tumor volume were quantified. Statistical analysis shows significant differences in tumor growth between two cell lines from day 54 to day 85 (** $P < 0.01$). Data are shown as mean ± SEM.

Figure 3.

$\alpha_v\beta_6$ integrin expression enhances PrCa cell proliferation. (A) Mock-, $\alpha_v\beta_6$ -LNCaP (Clones 1 and 2) or $\alpha_v\beta_3$ -LNCaP cells were stimulated with R1881 (1 nM) or vehicle after starvation, and quantified for cell proliferation by SRB staining. (B) Mock-, $\alpha_v\beta_6$ - and $\alpha_v\beta_3$ -LNCaP cells were cultured in either tissue culture plates or FN (5 μ g/ml)-coated plates. After 72 hours, cellular morphology was visualized. (C) RWPE cells transfected with β_6 -directed (D1 or D2) or non-silencing (NS) siRNA were analyzed for anchorage-independent cell growth, and the total number of colonies in 20 fields was scored 14 days

after seeding (left panel). RWPE cells were transfected with β_6 integrin-directed siRNA and analyzed by FACS with a mAb to $\alpha_v\beta_6$ or nonbinding IgG (mIgG) (right panel). OF, oligofectamine. (A and C) Data are mean $\pm$ SEM. ** $P < 0.01$.

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Figure 4.

$\alpha_v\beta_6$ integrin regulates AR activity and nuclear trafficking. (A and B) Left: LNCaP and C4-2B transfectants were starved in 2% CS serum for 24 hours and stimulated with R1881 (1 nM) or ethanol for 24 hours. PSA level was analyzed by IB. Right: $\alpha_v\beta_6$ -LNCaP and $\alpha_v\beta_6$ -C4-2B cells were transfected with AR-directed siRNA and the PSA level was evaluated by IB. Original blots are shown in Supplementary Figure 4. (C) Mock-, $\alpha_v\beta_3$ -, $\alpha_v\beta_6$ - and parental (-) LNCaP cells were analyzed for AR expression with or without R1881 by IB. (A-C) ERK1/2 or Akt was used as a loading control. OF: oligofectamine. NS: non-

silencing. (D) Cellular distribution of AR in $\alpha_v\beta_6$ -, $\alpha_v\beta_3$ - and mock-LNCaP cells. Indicated cells were seeded on poly-L-Lysine (1 mg/ml) coated coverslips for 2 days, starved and treated with or without R1881 (1 nM). (E) LNCaP cell transfectants were treated with the indicated concentrations of enzalutamide in culture medium for 72 hours and cell proliferation rate was calculated by cell counting. Proliferation is valued as percentage of the control groups treated with DMSO only. Data are mean $\pm$ SD.

Figure 5.

JNK1 mediates transactivation of AR in $\alpha_v\beta_6$ integrin expressing cells. (A) $\alpha_v\beta_6$ -LNCaP or $\alpha_v\beta_3$ -LNCaP cells (top panel) were seeded on FN or LAP-TGF β_1 (LAP) coated plates, and analyzed for JNK1 phosphorylation by IB. White lines indicate that intervening lanes have been spliced out. PSA levels were also analyzed by IB. c-src was used as a loading control. PC3 cells stably transfected with pBabe- β_6 ($\alpha_v\beta_6$) or pBabe vector (mock) were seeded on FN, LAP or BSA coated plates and analyzed for JNK1 activation by detection of phospho-JNK1 (p-JNK1). p-JNK1 levels in parental cells (-) exposed to UV irradiation were used as a positive control for JNK1 activation. Total JNK1 was used as a loading control. PC3 cells (bottom panel) were incubated in the presence of the indicated concentrations of mAb 6.3G9 to $\alpha_v\beta_6$, and adhesion to LAP coated plates was quantified. Data are mean $\pm$ SEM. ** $P < 0.01$. (B) $\alpha_v\beta_6$ -, $\alpha_v\beta_3$ - or mock C4-2B cells were infected with WT-JNK or decreasing concentrations of JBD, and analyzed for PSA expression by IB. Akt or β -actin was used as a loading control. Uninfected cells (-) were used as controls. Original blots are shown in Supplementary Figure 7. (C) $\alpha_v\beta_6$ -LNCaP cells transiently infected with WT-JNK (WT) or

JBD were seeded on a poly-L-lysine coated coverslip for 24 hours, starved, and analyzed with an Ab to AR by IF (left panels). DAPI (right panels).

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Figure 6.

AR regulates survivin expression and tumorigenicity in $\alpha_v\beta_6$ integrin expressing cells. (A) $\alpha_v\beta_3$ - and $\alpha_v\beta_6$ -C4-2B cells were analyzed for survivin and XIAP expression by IB (top and middle panels). $\alpha_v\beta_3$ - and $\alpha_v\beta_6$ -C4-2B (clone 1 and 2) cells were stimulated with or without R1881 (1 nM) and analyzed for Bcl-2 levels by IB (bottom panel). (B) Mock-LNCaP (lane 1, 3), $\alpha_v\beta_6$ -LNCaP (lane 2) or chimera $\alpha_v\beta_3/6$ -LNCaP (lane 4) cells were analyzed for survivin expression by IB. The white line indicates that intervening lanes have been spliced out. (C) $\alpha_v\beta_6$ -LNCaP cells were transfected with non-silencing (NS) or AR-directed siRNA for 24 h, starved and analyzed for expression of AR or survivin by IB. (A-C) Akt or ERK1/2 was used as a loading control. OF: Oligofectamine. (D) $\alpha_v\beta_6$ -LNCaP cells were transiently infected with WT-JNK or JBD, and analyzed for survivin expression by IB. Akt was used as a loading control. Uninfected cells (-) were used as controls. (E) $\alpha_v\beta_6$ -LNCaP cells were infected with adenovirus containing survivin wild type (WT) or a survivin mutant (T34A)

and analyzed for anchorage-independent growth. The average of colony diameters (left panel) of the 3 groups $\pm$ SEM are plotted. $\alpha_v\beta_6$ -LNCaP cells were transiently transfected with NS or survivin-directed (SV) siRNA for 48 hours and analyzed for anchorage-independent growth. The average number of the colonies (right panel) in the 3 groups $\pm$ SEM are plotted.

Figure 7. $\alpha_v\beta_6$ integrin and Survivin are co-expressed in human prostatic lesions. (A) Human AdCa (panels 1–4), and normal prostate (panel 5) specimens were analyzed for $\alpha_v\beta_6$ expression by IHC using mAb 6.2A1 (panels 1–3) or mIgG was used as a negative control (panel 4). Representative cases are shown. Arrows point at $\alpha_v\beta_6$ expressing gland; arrowheads point at $\alpha_v\beta_6$ negative glands (panel 1 and 2). Original magnification, $\times 200$. The percentage of $\alpha_v\beta_6$ -expressing adenocarcinoma tissue specimens at different Gleason scores is shown. (B) Primary prostatic epithelial cells were isolated from three cancer patients and analyzed for

expression of $\alpha_v\beta_6$ by FACS analysis with mAb 10D5 (thick line; left panel). IF staining of primary prostatic epithelial cells was carried out using Abs to CK 8 or 18. Ab 1C10 against an endothelial marker was used as a negative control (right upper panel). Primary tumor extracts were analyzed for expression of β_6 integrin using mAb 6.2A1 (right lower panel). Akt was used as loading control. (C) Representative images of AdCa (panels 1–4) showing co-expression of $\alpha_v\beta_6$ integrin and survivin in the same gland. (D) Schematic diagram for integrin regulation of AR-mediated prostate tumorigenesis independently of androgen.