Conditional Akt activation promotes androgen-independent progression of prostate cancer

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Aggressive androgen-independent (also termed as hormone-refractory) prostate cancer is a major clinical obstacle because there is no means to cure. Previous studies have shown that Akt activation is associated with prostate cancer progression from androgen-dependent to androgen-independent stage. However, its causative role in this process has not been established. One of the major limitations is the lack of a well-controlled inducible system to study Akt involvement. Recently, we developed a novel inducible Akt (iAKT) system based on a chemically induced dimerization (CID) approach. This system allows for conditional activation of Akt in a physiological setting. Utilizing this iAKT system, we found that Akt activation prevented cell death after serum withdrawal and promoted cell proliferation in the absence of androgen in vitro in human prostate cancer LNCaP cells, which should stop growing after androgen withdrawal or even die after serum starvation. The iAKT-induced death protection and growth promotion were further demonstrated in vivo using a transgenic mouse model that expresses the iAKT system conditionally in the prostate epithelium. Most importantly, in a mouse xenograft model derived from LNCaP cells, iAKT activation promoted tumor growth in castrated animals by enhancing cell proliferation and inhibiting apoptosis. Together, our data suggest that Akt activation is playing a causative role in androgen-independent progression of prostate cancer. This study provides a significant relevance of Akt-targeted therapy for hormone-refractory prostate cancers.

Introduction

Currently, medical treatment for metastatic prostate cancer heavily relies on androgen ablation. However, most patients treated by androgen ablation ultimately relapse to more aggressive androgen-independent (or termed as hormone-refractory) prostate cancers with no means to cure (1–3). The molecular alterations that facilitate human prostate cancer progression and the emergence of androgen-independent tumor cells are not fully clear. Androgen withdrawal triggers apoptosis in both normal and malignant androgen-dependent prostate epithelial cells. However, androgen-independent prostate cancer cells do not undergo apoptosis (2–3); suggesting that androgen-independent prostate cancer cells may utilize alternative cellular pathways for their survival. Under the condition of androgen ablation, successful progression by prostate cancer cells requires an ability to overcome two major roadblocks, survival and proliferation.

Akt, also known as protein kinase B, represents a subfamily of the serine/threonine protein kinases. Akt was originally cloned as a retroviral oncogenic product, v-Akt, which can transform rodent cells (4–5). To date, three human isoforms of Akt have been identified, Akt-1, -2 and -3 (6), which are expressed in the majority of cancer cell lines, primary cancers and normal tissues. Akt is activated by a variety of stimuli in a phosphatidylinositol 3-kinase (PI3K)-dependent manner. Its activation by growth factors depends on the integrity of the pleckstrin homology (PH) domain, which binds to phospholipids [PI(3,4,5)P3 or PIP3], the PI3K products. For full activation, Akt is phosphorylated on two key sites of T308/S473 by phosphatidylinositol-dependent kinase 1 (PDK1) and other kinases, respectively (6–8).

Recent work has demonstrated that overexpression of constitutively active Akt mutants in most cell types promotes cellular transformation by two distinct mechanisms: promoting proliferation under conditions in which cells should normally be growth-arrested; and inhibiting apoptosis by virtually all cell-death-inducing agents, thus promoting survival of tumor cells under conditions in which they should die. It was shown that Akt-1 is expressed in a wide array of prostate cancer cell lines and its kinase activity is frequently elevated in human prostate cancer specimens (7). Akt-3 expression is exclusive to the more advanced, androgen-independent prostate cancer cell lines (8). Increased Akt activity and decreased cell cycle regulator p27Kip1 were observed in androgen-independent prostate cancer cells compared to androgen-dependent ones, and pharmacological inhibition of PI3K blocked progression to the androgen-independent state of androgen-dependent prostate cancer LNCaP cells (9,10). Although there is an association between Akt activation and androgen-independent progression in

Abbreviations: AR, androgen receptor; BrdU, 5-bromo-2-deoxyuridine; CDK, cyclin-dependent kinase; cFBS, charcoal-stripped FBS; CID, chemically induced dimerization; IACUC, institutional Animal Care and Use Committee; iAKT, inducible Akt; IGF, insulin-like growth factor; PDK1, phosphatidylinositol-dependent kinase 1; PI3K, phosphatidylinositol 3-kinase; PH, pleckstrin-homology domain; PIN, prostatic intraepithelial neoplasia; PSA, prostate-specific antigen; PTEN, phosphatase and tensin homolog; RIPA, radio-immunoprecipitation assay.
prostate cancer cells (3,11–13), it remains unclear if Akt is playing a causative role in this process. In this study, we utilized our newly developed inducible Akt (iAKT) system to study the role of active Akt in androgen-independent progression of prostate cancer. The iAKT system contains three components, the membrane docking molecule (FRB\_2) and the fusion protein of PH domain-deleted Akt mutant (Δph.AKT) with drug binding domain (F\_pa3), as well as, the chemically induced dimerization (CID). We have demonstrated that CID-mediated membrane association of the inducible Akt mutant leads to Akt activation, which protects cells from apoptosis induced by various stimuli (14). In the present study, we determined that Akt activation is playing a causative role in androgen-independent progression of prostate cancer by promoting survival and stimulating cell proliferation.

Materials and methods

**Cell culture, constructs and reagents**

The human prostate cancer LNCaP cell line and its maintenance have been described previously (15). Antibodies for HA, Akt (clone B1), phospho-BAD S136, cyclin D1, proliferating cell nuclear antigen (PCNA), p21\_waf1, p27\_kip1, green fluorescent protein (GFP) and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for phospho-specific Akt at S473 and T308, phospho-specific glyceron synthase kinase 3 (GSK-3) at serine t21/199 and plain GSK-3, as well as, cleaved caspase-3 were obtained from Cell Signaling (Beverly, MA). Polyclonal antibody for Par-4 was purchased from Abcam Inc. (Cambridge, MA). Charcoal-stripped fetal bovine serum (cFBS, steroid-depleted) was obtained from Atlanta Biologicals (Norcross, GA). Insulin-like growth factor (IGF)-1 and other reagents (Roche Diagnostics) were purchased from Caltag Laboratories (San Francisco, CA). Bovine-serum albumin was obtained from Invitrogen (Carlsbad, CA). DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). The mammalian expression constructs pBJS-Neo and pBJS-Neo.ΔAKT have been described previously (14). In the bicistronic vector pBJS-Neo.ΔAKT, the two components of the iAKT system were linked via a poliovirus internal ribosome entry sequence (14). A kinase-dead mutant of this inducible Akt was generated by site-directed mutagenesis (QuickChange\® Site-Directed Mutagenesis Kit, Stratagene) of protein kinase-conserved lysine 179 (K179M) within the catalytic domain, which produces an inactive form of the kinase (16). This attenuated construct was designated as pBJS-Neo.ΔAKTkm and used as negative control. The stable LNCaP sublines Neo.iAKT and Neo.iAKTkm were established and expanded by in vitro cell culture. The human prostate cancer LNCaP cell line and its maintenance have been described previously (15). Antibodies for HA, Akt (clone B1), phospho-BAD S136, cyclin D1, proliferating cell nuclear antigen (PCNA), p21\_waf1, p27\_kip1, green fluorescent protein (GFP) and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for phospho-specific Akt at S473 and T308, phospho-specific glyceron synthase kinase 3 (GSK-3) at serine t21/199 and plain GSK-3, as well as, cleaved caspase-3 were obtained from Cell Signaling (Beverly, MA). Polyclonal antibody for Par-4 was purchased from Abcam Inc. (Cambridge, MA). Charcoal-stripped fetal bovine serum (cFBS, steroid-depleted) was obtained from Atlanta Biologicals (Norcross, GA). Insulin-like growth factor (IGF)-1 and other reagents (Roche Diagnostics) were purchased from Caltag Laboratories (San Francisco, CA). Bovine-serum albumin was obtained from Invitrogen (Carlsbad, CA). DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). The mammalian expression constructs pBJS-Neo and pBJS-Neo.ΔAKT have been described previously (14). In the bicistronic vector pBJS-Neo.ΔAKT, the two components of the iAKT system were linked via a poliovirus internal ribosome entry sequence (14). A kinase-dead mutant of this inducible Akt was generated by site-directed mutagenesis (QuickChange\® Site-Directed Mutagenesis Kit, Stratagene) of protein kinase-conserved lysine 179 (K179M) within the catalytic domain, which produces an inactive form of the kinase (16). This attenuated construct was designated as pBJS-Neo.ΔAKTkm and used as negative control. The stable LNCaP sublines Neo.iAKT and Neo.iAKTkm were established and expanded by in vitro cell culture.

**Immunoprecipitation, in vitro Akt kinase assay and western blot analysis**

Protein G immunoprecipitation kit (Sigma) was used according to manufacturer’s recommendation. Briefly, cells were harvested from 100 mm dishes, and cellular lysates were prepared with the RIPA (radioimmunoprecipitation assay) buffer containing protease inhibitors from the kit. A total of 200 µg protein and 2.0 µg purified anti-HA antibody were mixed for 4 h at 4°C with rotation followed by incubation with protein G beads overnight at 4°C, mixing by inversion. Eluted precipitates were subjected for western blot analysis. The construct pCX1-GFP-iAKT was generated to establish the first line transgenic mouse (Figure 3A). This vector was generated based on pBS-CXI-LeL vector that contains a hybrid promoter composed of the CMV immediate early enhancer and a chicken β-globin promoter, and the GFP gene is flanked by two LoxP sites (20). The blunted NotI/MfeI iAKT fragment from pSH1/M-FRB\_2-ires-F\_pa3-3-phaAkt (14) was subcloned into the blunted EcoRI site on pARR2PB-KBPA (obtained from Dr. David Spencer, Baylor College of Medicine) to make pKBPA-ARR2PB.IAKT. Then, the HindIII fragment containing the iAKT cassette from pKBPA-ARR2PB.IAKT was ligated into the HindIII site behind the second LoxP sequence on pBS-CXI-LeL vector to make pCX1-GFP-iAKT. Finally, the KpnI/XbaI fragment from pCX1-GFP-iAKT construct was released and ligated into nuclear injection in C57B6/SJL strain-derived stem cells. Several founder lines (termed as CGX-GFP\_iAKT) were established and expanded using a standard protocol.

To establish a prostate-specific expression of the iAKT system (Figure 4A), the first line CGX-GFP\_iAKT mice were cross-bred with a parastatic-specific Cre expressing line PB-Cre\_4 mouse (21), which was obtained from NCI Mouse Models of Human Cancers Consortium Repository. Dual transgenic male mice, termed as PBcre-iAKT mice, were subjected for next experiments. All of the mice were kept in pathogen-free conditions in KUMC animal facility with an IACUC approved protocol. Male mice at 12-weeks age were castrated or sham-operated through scrotal incision. After 3 days, the animals were injected with AP21967 at a dose of 2.0 mg/kg body wt (18), which was formulated in the solvent containing 50% N, N-dimethylacetamide and 50% PEG-400/Tween-80 (9:1), provided by Ariad Pharmaceutical. The treatment was repeated three times a week. The tumor volumes were determined by measuring the length (L) and the width (W) and calculating the volume (V = L × W\(^2\)), as described previously (17).

**Mouse xenograft experiments, immunohistochemistry and TUNEL assay**

Athyric male mice (Charles River, Wilmington, MA) were maintained in accordance with the Institutional Animal Care and Use Committee (IACUC) procedures and guidelines. LNCaP.iAKT cells were harvested, resuspended in PBS, and injected subcutaneously (s.c.) into the right and left flanks (2 × 10\(^6\) cells/flank) of 6-week-old mice as described previously (17). When tumors were palpable (~40–50 mm\(^3\) in 4–6 weeks), animals were castrated or sham-operated through scrotal incision. There were eight mice in each group. Three days later, animals were treated intraperitoneally (i.p.) with AP21967 at a dose of 2.0 mg/kg body wt (18), which was formulated in the solvent containing 50% N, N-dimethylacetamide and 50% PEG-400/Tween-80 (9:1), provided by Ariad Pharmaceutical. The treatment was repeated three times a week. The tumor volumes were determined by measuring the length (L) and the width (W) and calculating the volume (V = L × W\(^2\)), as described previously (17).

**Statistical analysis**

All cell line-based experiments were repeated two or three times. Western blot results were presented from a representative experiment. The mean and standard error from two or more experiments are shown. The significance of the differences between treatment and control was analyzed using the SPSS software (SPSS, Chicago, IL).
RESULTS

Conditional activation of the iAKT system in prostate cancer cells

To study the causative role of Akt activation in the transition from androgen-dependent to androgen-independent state, we established several stable cell lines expressing the iAKT system in human prostate cancer LNCaP cell line. LNCaP cells are responsive to androgen-stimulated growth and survival (24). Protein levels of the iAKT system (FRBl2, 29 kDa and Fpk3, DphaKT, HA-tagged, 91 kDa) were verified by western blot assay (Figure 1A).

Akt is fully activated after phosphorylation at two sites, T308 and S473 (5,6). Thus, we examined iAKT (Fpk3, DphaKT) phosphorylation at these two sites after CID (AP21967) treatment. First, we used serum-free condition to avoid any effect of serum-derived factors on iAKT phosphorylation. LNCaP.iAKT cells were serum starved for 24 h and then treated with AP21967 for up to 30 min. Chimeric Fpk3, DphaKT proteins were immunoprecipitated with the anti-HA antibody and the eluted immunoprecipitates were subjected for western blot analysis with phospho-specific Akt antibodies. As shown in Figure 1B, without AP21967 addition, the iAKT was not phosphorylated at either site. Upon AP21967 addition, iAKT phosphorylation at S473 or T308 was dramatically increased in a time-dependent manner.

Then, we evaluated iAKT phosphorylation in the presence of steroid-depleted serum (10% Charcoal-stripped FBS, cFBS) for up to 6 h after AP21967 treatment. Cell lysates were directly used for western blot assay in order to examine the phosphorylation status of both iAKT and endogenous (cellular) Akt at the same time. As shown in Figure 1C, AP21967-induced iAKT phosphorylation was sustained over the 6-h period. For endogenous cellular Akt, there was a basal level of S473 phosphorylation, which was slightly increased after AP21967 addition, suggesting that AP21967-induced iAKT activation might lead to activation of cellular Akt, possibly due to an intermolecular event.

Lastly, we examined Akt enzymatic activity with an in vitro IP-based kinase assay. GSK-3α/β ‘cross-tide’ was used as the substrate as described in our previous publication (14). The phosphorylation level of GSK-3 ‘cross-tide’ was determined by immunoblotting with an antibody against phospho-specific GSK-3 at serine sites α21/β9. As shown in Figure 1D, CID treatment induced a dose-dependent phosphorylation on GSK-3 ‘cross-tide’. Taken together, CID addition induced iAKT activation, which also led to activation of endogenous Akt.

iAKT promotes survival induced by serum withdraw in vitro

Since successful progression from androgen-dependent to androgen-independent stage by prostate cancer cells requires an ability to survive first in the absence of androgens. Thus, we went on to determine if CID-mediated Akt activation promotes survival in LNCaP sublines.

Since cFBS addition induced cellular Akt phosphorylation (Figure 1C), we used serum-free condition to trigger apoptosis in LNCaP subline cells so as to eliminate the basal activity of cellular Akt. Annexin-V labeling assay was used to examine apoptotic response after serum starvation. LNCaP.iAKT cells were kept in serum-free culture for 7 days in the presence or absence of AP21967 and the apoptosis rate was analyzed (Figure 2A).
absence of AP21967. As shown in Figure 2A, serum-free conditions triggered a dramatic apoptotic response (middle panel) compared with FBS condition. AP21967 treatment significantly reduced apoptotic events induced by serum starvation (right panel). This pro-survival effect of iAKT activation was further evaluated among LNCaP.iAKT, LNCaP.iAKTkm or LNCaP.Neo cells. These cells were kept in serum-free culture for 3 days after AP21967 addition and then harvested for apoptosis assay. As shown in Figure 2B, without AP21967 addition, serum starvation induced a significant apoptotic response in all the three sublines compared with the FBS control. However, AP21967 addition significantly reduced apoptotic responses in LNCaP.iAKT but not in LNCaP.Neo and LNCaP.iAKTkm cell lines. These results indicated that AP21967-mediated iAKT activation promoted survival in LNCaP.iAKT cells.

iAKT stimulates cell proliferation in the absence of androgens in vitro

In addition to survive, the second requirement of successful progression to androgen-independent stage is to proliferate in the absence of androgens. Therefore, we assessed whether iAKT activation stimulates cell proliferation in the absence of androgens using MTT assays. LNCaP subline cells were serum starved for 24 h and then treated with or without AP21967 in charcoal-stripped serum (cFBS, steroid-depleted). Cell proliferation was monitored for up to 3 days after AP21967 addition. Cells treated with IGF-1 or the synthetic androgen R1881 were used as positive controls. In a low serum condition (2% cFBS), IGF-1/R1881 were able to significantly stimulate cell proliferation compared with the solvent control in all three LNCaP sublines, however, AP21967 treatment did not obviously alter cell proliferation in this low cFBS condition (Figure 2C). Then, we increased cFBS supplement to a regular level of 10% concentration. As shown in Figure 2D, 10% cFBS alone moderately increased cell proliferation when compared with the conditions of serum-free (panel AP21967) but it was weaker than the full serum condition (panel FBS alone), conversely, AP21967 plus 10% cFBS significantly increased cell proliferation in LNCaP.iAKT but not in other two control cells compared with serum-free condition (panel AP21967), indicating that iAKT activation, together with other serum-derived genotrophic factors, stimulated cell proliferation.

**Fig. 2.** Conditional iAKT activation promotes survival and stimulates cell proliferation in the absence of androgens. (A) LNCaP.iAKT cells were serum starved for 24 h and then treated with the solvent or AP21967 (200 nM) in serum-free media. Control cells (panel FBS) were grown in 10% FBS-containing media. Seven days later, cells were harvested and apoptotic cell death was assessed with a flow cytometry-based Annexin V-FITC binding assay (15). V, viable cells; A, early apoptotic cells; N, late apoptotic cells; D, dead cells. Numbers with underline indicated the percentage in total cell population. Representative data charts were shown from two separate experiments. (B) LNCaP subline cells were serum starved for 24 h and then treated with the solvent or AP21967 (200 nM) in serum-free media. Control cells (panel FBS) were kept in 10% FBS-containing media. Three days later, cells were harvested and apoptotic cell death was analyzed using Annexin V-FITC binding assay as described earlier. Data represent the average value and error bars indicate standard error (SE) from three different experiments. The asterisk indicates a significant difference (P < 0.05, t-test) compared to the control group (panel FBS) for individual sublines. (C) LNCaP subline cells were serum starved for 24 h and then treated with the solvent, AP21967, IGF-1 and R1881 as indicated in 2% cFBS for 72 h. Cell proliferation was assessed with MTT assay. The assay readings in the solvent-treated LNCaP.Neo cells were set as 100% (the control), and others were presented as relative ratios against this control. Error bars represent SE from three experiments. The asterisk indicates a significant difference (P < 0.05, t-test) compared with the control. (D) LNCaP subline cells were serum starved and then treated with AP21967 in serum-free condition (panel AP21967), 10% cFBS alone, AP21967 plus 10% cFBS, as well as 10% full serum (panel FBS) for 72 h. Cell proliferation was assessed with MTT assay. The assay readings from LNCaP.Neo cells treated with AP21967 alone (panel AP21967) were set as 100% (the control), and others were presented as relative ratios against this control. Error bars represent standard error (SE) from three experiments. The asterisk indicates a significant difference (P < 0.05, t-test) compared with the control.
Androgen-independent iAKT expression and activation in mouse prostate epithelium

Then, we took this iAKT system into in vivo setting to determine if Akt activation promotes survival and stimulates proliferation of normal prostate epithelial cells after castration. Usually, an androgen-responsive gene promoter, such as prostate-specific antigen (PSA) or rat probasin (PB), is employed to establish prostate-specific transgenic mouse model (reviewed in ref. 25). However, this kind of strategy can not be used in studies of androgen-independent regulation, because removing androgens from the animal (i.e. castration) will shut down the transgene expression. To express the iAKT system in mouse prostate for studying iAKT activation in androgen-ablated situation, a conditional Cre-LoxP knock-in strategy was used. As illustrated in Figure 3A, to generate the prostate-specific transgenic mouse

Fig. 3. Conditional iAKT expression and activation in transgenic mouse prostate. (A) Schematic of iAKT transgenic strategy. Prostate-specific Cre line (PB-Cre4 mice, 21) was obtained from NCI MMHCC Repository; CX-GFPLoxP-iAKT line was generated in house with a transgenic construct pCX1-GFP-iAKT; PBCre-iAKT line represents the dual transgenic mice of PB-Cre4 crossed with CX1-GFPLoxP-iAKT mice, resulting the prostate-specific expression of the iAKT system after deletion of the LoxP-flanked GFP gene. The iAKT construct was described previously (14). (B) Protein extracts from micro-dissected mouse prostates were obtained from five different founder lines of PBCre-iAKT mice as indicated. A littermate control mouse of CX-GFPLoxP-iAKT was included as the control. The expression of the transgenes were assessed using anti-GFP antibodies and anti-HA antibodies for the HA epitope on the iAKT construct. Actin blot served as the protein loading control. (C) Paraffin-embedded mouse prostate sections from the lateral lobes were stained with H&E method. Micrographics were taken under a visible (H&E) and fluorescent light source (GFP). Magnification was set at 400. Prostate epithelium-specific deletion of GFP gene was evidenced in PBCre-iAKT (panel d) but not in CX-GFPLoxP-iAKT mice (panel b). (D) Castrated or sham-operated PBCre-iAKT mice at age 12 weeks were injected i.p. with the solvent or AP21967 (2.0 mg/kg bodywt) every other day for three times. Micro-dissected prostate ventral–lateral lobes were homogenized and protein extracts were used for western blot analysis with the antibodies as listed on the left side. The identities of bands on the blots were indicated on the right side. Actin blot served as protein loading control.
model bearing the iAKT system (termed as PBCre-iAKT model), homozygous CX-GFP\textsuperscript{LoxP}\textsuperscript{-iAKT} lines were crossed with a prostate-specific Cre expressing line PB-Cre4 (21). Expression levels of the iAKT proteins in mouse prostates were compared among five different founder-derived offspring lines. A Cre negative iAKT littermate (CX-GFP\textsuperscript{LoxP}\textsuperscript{-iAKT}) served as a negative control. Protein extracts from prostate tissues were used to examine the iAKT (HA-F\textsubscript{pk3.3\textgreek{d}pAkt}) expression levels. As expected, the Cre negative littermate showed a positive GFP but negative iAKT expression (Figure 3B). All PBCre-iAKT lines expressed HA-tagged F\textsubscript{pk3.3\textgreek{d}pAkt} (anti-HA blot) at variable levels. Line no. 11 and no. 22 showed the highest level of the transgene iAKT. In subsequent experiments, the no. 11 line was used for assessment of the iAKT activation in prostate.

Cre-mediated GFP deletion in prostate epithelia was confirmed by fluorescence microscopy in PBCre-iAKT mice derived from founder no. 11. Since the Cre transgene was expressed at the highest level in ventral–lateral lobes of mouse prostate (21), we focused our analysis mainly in these lobes. Paraffin-embedded tissue sections were stained with H&E to evaluate the histological structure. As shown in Figure 3C, CX-GFP\textsuperscript{LoxP}\textsuperscript{-iAKT} mice retained GFP expression but PBCre-iAKT mice lost GFP expression in the prostatic epithelium. No structural lesions were observed.

Next, we examined AP21967-induced Akt activation (S473 phosphorylation) in the prostate of PBCre-iAKT transgenic mice. The 12-week-old mice were castrated or sham operated and were then injected i.p. with AP21967 or the solvent every other day for three times. Mice were sacrificed and the prostates were removed 6 h after the last injection. Akt S473 phosphorylation was assessed with phospho-specific antibodies in western blot assay. In the prostates obtained from sham-operated mice, as expected, the endogenous but not the transgenic Akt was phosphorylated at S473 without AP21967 treatment, however, Akt S473 phosphorylation was largely increased after AP21967 injection. In the prostates obtained from castrated mice, the endogenous Akt was only weakly phosphorylated without AP21967 treatment, but Akt S473 phosphorylation was strongly increased upon AP21967 injection compared with the control animal (Figure 3D). Meanwhile, Akt S473 phosphorylation of the endogenous Akt in castrated animals also slightly enhanced after AP21967 treatment, which was consistent with our in vitro data (Figure 1C). These results demonstrated that the transgene iAKT is activated after AP21967 treatment in PBCre-iAKT mouse prostate.

iAKT promotes survival of prostatic epithelial cell in vivo after castration

Castration-induced apoptosis in prostate epithelium is a well-documented event (26). Therefore, we used PBCre-iAKT transgenic mice to evaluate the pro-survival effect of iAKT activation in vivo. PBCre-iAKT mice at 12 weeks of age were castrated, followed by treatment with the solvent or AP21967 for 3 weeks. The urogenital tracts were first examined macroscopically. In the solvent-treated animals, castration resulted in a dramatic regression of the mouse urogenital organs, as evidenced by a significant shrinkage of seminal vesicles and prostate lobes. In contrast, AP21967-treated mice exhibited large seminal vesicles and healthy prostatic glands, and the seminal vesicles were fluid-filled. Histologically, comparing with the prostate in gonad-intact animals (Figure 4A, panel a), castration induced a significant prostatic atrophy as evidenced by much fewer and reduced luminal size of prostatic ducts, a thinner epithelial layer and inflammation response in prostatic epithelia (Figure 4A, panel b). In contrast, there was no apparent atrophic change in AP21967-treated animal after castration (Figure 4A, panel c). These animals showed a very similar histological structure with thick epithelia and large luminal duct as seen in the gonad-intact mice (panel a). Because castration-induced prostatic atrophy is due to apoptotic cell death in prostatic epithelia, we examined the apoptotic events using in situ TUNEL assay. Semi-quantitative data were presented in Figure 4C. Comparing to the solvent control, AP21967 treatment significantly reduced castration-induced apoptotic events in the prostate gland. These data indicated that iAKT activation promoted survival of prostate epithelial cells after castration.

iAKT stimulates cell proliferation of prostatic epithelium in gonad-intact mouse

Next, we determined if iAKT activation stimulates cell proliferation or even induces neoplasia-like lesion in the prostate, as seen in the MPAKT mouse model (27). In that MPAKT model, a constitutively active Akt was overexpressed in mouse prostate epithelia, which resulted in prostatic intraepithelial neoplasia (PIN). In our castrated PBCre-iAKT mice, after AP21967 treatment for up to 6 weeks, there was no any evidence of hyperplasia or neoplasia, as assessed by histological evaluation after H&E staining and BrdU labeling assay (data not shown). However, in gonad-intact PBCre-iAKT mice, AP21967 treatment for 3 weeks significantly increased the wet weight of the prostate lobes compared with the solvent controls (81.25 ± 0.4 versus 67.75 ± 1.4 mg, n = 5, t-test, \( P < 0.05 \)).

Fig. 4. Conditional iAKT activation promotes survival and stimulates proliferation in transgenic mouse. (A) PBCre-iAKT mice at 12-week age were castrated, followed by i.p. injection of the solvent or AP21967 (2.0 mg/kg body wt) three times a week for 2 weeks (n = 5 per treatment). Prostate ventral–lateral lobes were dissected and processed for histological evaluation. Paraffin sections from the ventral–lateral lobes were stained with H&E method. Note that comparing with the solvent-treated sham-operated control (panel a), prostate glandular ducts and their luminal size are largely reduced in solvent-treated castrated animal (panel b). In contrast, AP21967-treated prostates remained unchanged in castrated animal (panel c) compared with sham-operated animal (panel a). Magnification ×200. (B) PBCre-iAKT mice at 12-week age were i.p. injected with the solvent or AP21967 (2.0 mg/kg body wt) three times a week for 6 weeks (n = 5 per treatment). One hour before sacrifice, animals were received i.p. injection of BrdU solution. Micro-dissected prostate ventral–lateral lobes were weighted and processed for paraffin embedding. Representative H&E micrographs were shown from different treatments as indicated. Please note that AP21967-treated animal exhibited a moderate hyperplastic lesion in the ventral–lateral glands as indicated by black arrows (panel b) while the solvent-treated control showed a normal structure (panel a). Magnification ×200. In addition, cell proliferation was assessed with anti-BrdU immunostaining in these animals (panel c and d). Note that there much more BrdU-positive cells as indicated by white arrows in AP21967-treated animal (panel d) compared to the solvent control (panel c). Semi-quantitative data of BrdU-positive staining from 10 different microscope fields were presented in panel (C) Error bars represent SE. The asterisk indicates a significant difference (\( P < 0.05 \), t-test, n = 5) compared to the solvent control. (D) Prostate tissue sections obtained from the castrated animals described in panel a were subjected to TUNEL analysis for apoptotic events. Semi-quantitative data of positive TUNEL signals were presented from each group. Error bars represent SE. * significant difference (\( P < 0.05 \), t-test, n = 5) between AP21967 treatment and the solvent control.
Histologically, a hyperplasia-like lesion was observed in prostate epithelia from the lateral lobes after a 6-week treatment with AP21967, as evidenced by increased cell layers and more luminal branching (Figure 4B, panels a and b), but no atypical cells were observed. Proliferation was assessed with BrdU labeling assay. In the solvent-treated mice, only few cells were labeled by BrdU in the basal layer of prostate, in contrast, AP21967 treatment significantly increased the numbers of BrdU-labeled cells in both basal and luminal layers (Figure 4B, panels c and d). The semi-quantitative data indicated a significant difference between AP21967-treated mice and the solvent controls (Figure 4C).

These data, iAKT activation-stimulated cell proliferation of prostate epithelium in gonad-intact but not in castrated animals, suggested that active iAKT may need to work in concert with androgen-mediated genotropic factors to enhance cell proliferation in prostate epithelia in vivo, which is supported by a recent study using an in vivo prostate regeneration system (28).

iAKT promotes xenograft tumor growth in castrated mice

Previous studies have shown the association of Akt activation with androgen-independent progress of prostate cancer [using] in vitro cell-based assays and in vivo mouse model (9–12,29). So far, however, it is not clear if Akt activation plays a causative role in the androgen-independent transition of prostate cancer cells in vivo. Therefore, we went on to address this issue with our iAKT system. LNCAP.iAKT cell-derived xenografts were established in gonad-intact male nude mice. When xenografts tumors became palpable, the animals received bilateral castration or sham operation by scrotal incision. After 3 days, when endogenous androgens were eliminated, mice were injected i.p. with either AP21967 or the solvent three times a week for 4 weeks. Tumor size in volume was followed daily. As shown in Figure 5, tumor growth of LNCAP.iAKT xenografts in castrated animals was static in the solvent-treated group, reflecting the androgen responsiveness of LNCAP cells, similar to most of primary prostate cancers in human after androgen ablation therapy. In contrast, xenograft tumor growth was significantly increased in AP21967-treated animals compared to the solvent control (Figure 5A and B). In sham-operated animals, AP21967 slightly increased xenograft tumor growth but no statistically significant difference was observed compared to the solvent control. These data demonstrated that Akt activation promotes xenograft tumor growth in the condition of castration, which is further supported by a study that shows a hyperplasia-like lesion was observed in prostate epithelia from the lateral lobes after a 6-week treatment with AP21967, as evidenced by increased cell layers and more luminal branching (Figure 4B, panels a and b), but no atypical cells were observed.

To understand the mechanism of iAKT activation-induced tumor growth in the absence of androgens, we evaluated cell proliferation in xenograft tumors using BrdU labeling and anti-Ki67 immunoassays. As shown in Figure 5C and D, both Ki-67 and BrdU-positive cells were significantly higher in the xenografts obtained from AP21967-treated animals than that in the solvent control after castration. However, there was no significant difference between AP21967 treatment and the solvent control from sham-operated animals, which was consistent with the tumor volume data (Figure 5A). These data suggested that iAKT activation promoted androgen-independent tumor growth by increasing cell proliferation.

Then, AP21967-induced iAKT phosphorylation was evaluated in xenograft tumors from castrated animals. As expected, a strong pAkt S473 expression was detected by the phosho-specific antibody in AP21967-treated tumor section but not in the solvent control (Figure 6A, panels a and b). In addition, iAKT activation-induced GSK-3 phosphorylation, a well-known Akt downstream target, was evaluated by western blot analysis with the phospho-specific GSK-3 antibodies. As shown in Figure 6B, GSK-3 phosphorylation at serine sites (α21/β9) was largely increased in AP21967-treated xenografts compared to the solvent control. Taken together, these data indicated that AP21967 treatment activated iAKT in the xenografts.

Akt activation has been shown to promote cell cycle progression (reviewed in ref. 30). Thus, we assessed the changes of cell cycle-related genes, including cyclin D1, PCNA, p21<sup>wafl</sup> and p27<sup>kip1</sup>. Protein extracts from four xenografts of castrated animals were used for western blot assay. As shown in Figure 6B, AP21967-treated tumors expressed more cyclin D1 and PCNA proteins compared to the solvent-treated xenografts. Conversely, p21<sup>wafl</sup> and p27<sup>kip1</sup> levels were hardly detectable in AP21967-treated xenografts but were expressed at high levels in the solvent-treated xenografts. These data suggested that iAKT activation promoted androgen-independent tumor growth by increasing the expression of cyclin D1 and PCNA, and by inhibiting the expression of p21<sup>wafl</sup> and p27<sup>kip1</sup>, leading to cell cycle progression.

To determine if iAKT activation promoted survival after castration, we evaluated the apoptotic events in the xenografts using the TUNEL assay. As shown in Figure 6C, AP21967 treatment dramatically reduced castration-induced apoptotic events compared to the solvent control in the xenografts. In addition, we examined caspase-3 cleavage, which is another hallmark for apoptotic cell death, in the xenografts. As shown in Figure 6A (panels g and h), cleaved caspase-3 was clearly detected in the solvent-treated but not in AP21967-treated xenograft, indicating an inhibition of caspase-3 cleavage by AP21967 treatment. These data suggested that iAKT activation promoted androgen-independent tumor growth by promoting survival.

Lastly, we evaluated the modifications of other Akt downstream targets, including Par-4 (31) and BAD (16,32) that are involved in apoptosis, by immunostaining analysis in the xenografts obtained from castrated animals. As shown in Figure 6A, Par-4 was mainly localized within nuclei compartment in the solvent-treated xenograft (panel c) that showed a massive apoptosis by TUNEL assay (Figure 6C, panel b), reflecting its role in androgen withdrawal-induced apoptosis as reported previously (33). Conversely, Par-4 was detected in cytoplasm compartment in AP21967-treated xenograft (Figure 6A, panel d), indicating an inhibitory effect of iAKT activation on Par-4 nuclear translocation (31). Meanwhile, we observed an increased level of BAD S136 phosphorylation in AP21967-treated xenograft compared to the solvent control (Figure 6A, panels e and f), which is supported by a previous study that Akt exhibits its anti-apoptotic effect by inducing BAD S136 phosphorylation (32). Taken together, these data suggested that iAKT activation promoted survival by blocking Par-4 nuclear translocation and causing BAD phosphorylation.

DISCUSSION

In the present study, using the inducible system of Akt activation, we demonstrated that active Akt plays a functional...
role in androgen-independent progression of prostate cancer. As discussed earlier, prostate cancer cells need to survive after androgen withdrawal and to proliferate in the absence of androgens in order to progress as androgen-independent cancer. In this study, we showed that conditional Akt activation promoted survival and proliferation of androgen-responsive LNCaP cells in vitro and tumor growth of LNCaP-derived xenografts in castrated animals. These results clearly indicated that active Akt is a likely causative factor in androgen-independent (hormone-refractory) progression of prostate cancer.

What is the mechanism for Akt activation-induced survival and proliferation in prostate cancer cells? As a well-demonstrated survival factor, Akt has been shown to promote survival via multiple mechanisms (3,5,6), such as blocking Par-4 nuclear translocation (31), inactivating pro-apoptotic...
protein BAD through phosphorylation (16,32). Consistent with these previous reports, we found that iAKT activation resulted in a cytoplasm localization of Par-4 proteins and BAD S136 phosphorylation, which were associated with increased survival.

Besides promoting survival, Akt has been previously shown to stimulate cell proliferation (reviewed in refs 30 and 34). For example, Akt regulates the expression of cyclin-dependent kinase (CDK) inhibitors p21\(^{Cip1/Waf1}\) and p27\(^{Kip1}\) (35,36). Overexpression of a constitutively active Akt mutant decreases the cellular levels of p27\(^{Kip1}\), thereby stimulating cell proliferation (9,37). Akt increases the rate of translation of D-type cyclins and stabilizes cyclin D1 protein that is a major driving force for cell cycle progression (38,39). In this study, we found that iAkt activation dramatically reduced the protein levels of p21\(^{Cip1/Waf1}\) and p27\(^{Kip1}\) but largely increased cyclin D1 expression in prostate cancer xenografts (Figure 6B), suggesting that accumulation of cyclin D1 protein and elimination of CDK inhibitors are involved in iAKT-induced androgen-independent progression of prostate cancer.

In addition to the cancer cell-based system, we used a transgenic mouse model to study the effect of iAKT activation on cell survival and proliferation of normal prostate epithelium. The iAKT system was targeted specifically to mouse prostate epithelium in a unique androgen-independent manner. Using this iAKT mouse model, we demonstrated that iAKT activation promoted survival of prostate epithelial cells after castration and stimulated cell proliferation in prostate epithelia of gonad-intact animals. After 3–6 weeks of iAKT activation, however, we did not notice any atypical lesions, which were observed in a previously reported MPAKT mouse model (27). The phenotype differences between these two models might be due to the use of different strategies and transgenes. Our iAKT model uses a CID-mediated dimerization system coupling with a PH-domain deleted Akt mutant molecule. In contrast, the MPAKT model used a myristoylation sequence to target the wild-type Akt molecule onto plasma membrane for auto-activation. The PH domain is important for both protein–protein and protein–lipid interaction in signaling transduction (40,41), therefore, it is plausible that its deletion may eliminate certain signaling events in our iAKT system,
which might be the reason for lacking of neoplasia lesions in our iAKT model but were seen in the MPAKT model (27).

Most significantly, we demonstrated that iAKT activation promoted androgen-independent progression of LNCaP-derived xenografts that were androgen responsive before iAKT activation (Figure 5A). As mentioned earlier, previous studies showed the association of elevated Akt activation with prostate cancer progression (8–13,29). Recent studies also demonstrated that Akt is critical for tumor development in mouse prostate after PTEN (phosphatase and tensin homolog) gene knockout (42). In a transgenic model, active Akt induced neoplasia lesions (tumor development) in mouse prostate epithelium (27). In a prostate regeneration system, Akt synergizes androgen receptor-initiated prostate carcinogenesis through both genotoxic and non-genotoxic mechanisms (28). Nonetheless, these studies did not address the issue of Akt-caused androgen-independent progression from its androgen-dependent stage. As the authors are aware, the present study is the first report to show the causative role of Akt activation in androgen-independent progression of prostate cancer.

A previous study reported a failure in establishing xenograft tumors in castrated animal using LNCaP cells carrying an active Akt mutant (9). It is plausible that the stable transfection process for overexpressing the active mutant Akt-1 (T308D/S473D) in LNCaP cells might select a strong androgen-dependent subclone. In contrast, as described in our previous publication (14) and this study, our iAKT system is inactive during the process of stable clone screening, therefore, the behavior of LNCaP subline cells with iAKT expression remains unchanged before adding the CID AP21967, as seen in Figure 5A. Second, our iAKT system mimics exactly the physiological activation process of Akt kinase and is independent of environmental factors, i.e. serum did not induce iAKT activation (Figures 1C and 3D). Thus, we successfully established the xenografts in nude mice.

In conclusion, we demonstrated that Akt activation is a causative factor in androgen-independent progression of prostate cancer by promoting survival and stimulating cell proliferation. This is significant because Akt has been considered as a major target for anti-cancer therapy (3,43). Further studies are desirable to determine the identities of Akt-stimulated genes responsible for cell proliferation and survival in mouse prostate epithelium and human prostate cancers.

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