

Transgenic overexpression of PKC ϵ in the mouse prostate induces preneoplastic lesions

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It is well established that protein kinase C (PKC) isozymes play distinctive roles in mitogenic and survival signaling as well as in cancer progression. PKC ϵ , the product of the *PKRCE* gene, is upregulated in various types of cancers including prostate, lung and breast cancer. To address a potential role for PKCs in prostate cancer progression we generated three mouse transgenic lines expressing PKC α , PKC δ or PKC ϵ in the prostate epithelium under the control of the rat probasin (PB) promoter. Whereas PB-PKC α and PB-PKC δ mice did not show any evident phenotype, PB-PKC ϵ mice developed prostate hyperplasia as well as prostate intraepithelial neoplasia (PIN) that displayed enhanced phospho-Akt, phospho-S6 and phospho-Stat3 levels, as well as enhanced resistance to apoptotic stimuli. PKC ϵ overexpression was insufficient to drive neoplastic changes in the mouse prostate. Notably, overexpression of PKC ϵ by adenoviral means in normal immortalized RWPE-1 prostate cells confers a growth advantage and hyperactivation of Erk and Akt. Our results argue for a causal link between PKC ϵ overexpression and prostate cancer development.

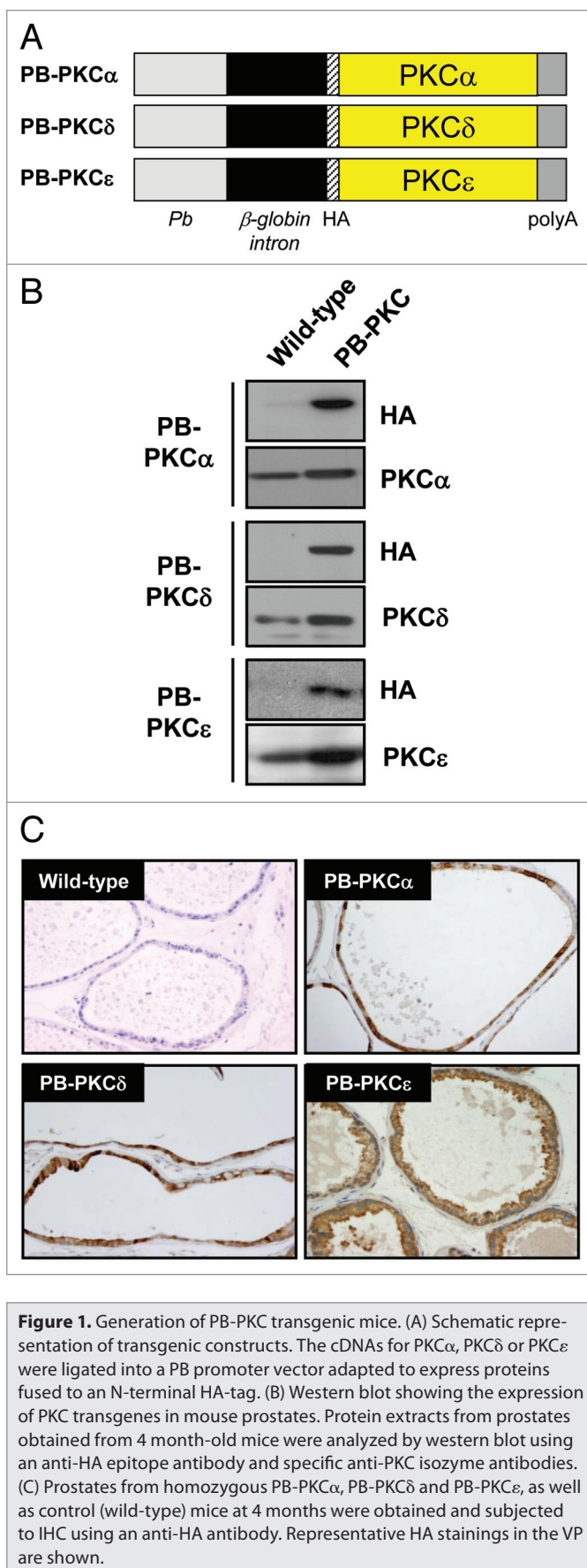
Introduction

The protein kinase C (PKC) isozymes are a family of serine-threonine kinases widely implicated in cancer progression. Members of the classical PKCs (cPKCs α , β and γ) and novel PKCs (nPKCs δ , ϵ , η and θ) are main cellular targets for the phorbol ester tumor promoters and the second messenger diacylglycerol (DAG) generated in response to the activation of growth factor receptors.^{1,2} Studies in cancer cell models have established essential roles for DAG/phorbol ester-responsive PKCs either as positive or negative regulators of proliferation and survival. While there is remarkable cell specificity in the responses triggered by activation of individual PKCs, it is well established that PKC ϵ overexpression confers a growth advantage in a number of cellular models, and eventually transform cells to a malignant state, as described for fibroblasts and colonic epithelial cells. It is remarkable that, on the other hand, the related nPKC δ mostly conveys anti-proliferative and apoptotic signals, and suppressing PKC δ expression or activity promotes a transformed phenotype.³⁻⁷ cPKCs are able to exert either pro- or anti-mitogenic/tumorigenic effects.^{1,8} The diversity in PKC isozyme function has been associated with their differential ability to relocalize to distinct intracellular compartments and access specific substrates, which ultimately leads to signaling specificity.

The altered balance in the expression of PKC isozymes is a distinguished feature of cancer. One of the most notable alterations

in epithelial cancers is the upregulation of PKC ϵ , the product of the *PKRCE* gene. PKC ϵ has emerged as a potential oncogene and tumor biomarker,^{4,5,9-11} however, little is known regarding a potential causality between its upregulation and cancer development. PKC ϵ overexpression is a prominent feature of human prostate cancer, as determined by several studies using clinical specimens.¹²⁻¹⁴ Although the implications of PKC ϵ upregulation in prostate cancer in vivo are yet to be determined, studies in cellular models have demonstrated central roles for this nPKC in prostate cancer cell survival. Our laboratory recently found that PKC ϵ protects LNCaP prostate cancer cells against phorbol ester- and TNF α -induced apoptosis through a mechanism that involves Bad phosphorylation.¹⁵ PKC ϵ also interacts with Bax in prostate cancer cells to neutralize apoptotic signals propagated through the mitochondrial death-signaling pathway.¹⁶ There is evidence that PKC ϵ links integrin to the Akt survival pathway in recurrent prostate cancer cells.¹⁷ PKC ϵ has been also implicated in the transition to androgen independence and was found to be consistently elevated in recurrent androgen-independent tumors.^{17,18} More recently, studies identified a microRNA that exerts tumor suppressive functions in human prostate through PKC ϵ downregulation,¹⁹ arguing for a potential role for this PKC in prostate cancer development. Unlike PKC ϵ , activation of PKC α and PKC δ positively modulates apoptotic pathways and their activation leads to programmed cell death in LNCaP

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cells.^{7,8,20-22} It is therefore likely that alterations in the balance of expression of PKC isozymes in prostate cells may lead to remarkably distinct phenotypic changes.

The aim of this study was to determine the phenotypic consequences of overexpressing PKC ϵ in the normal prostate in vivo. To tackle this issue we generated a transgenic mouse model in which PKC ϵ was expressed specifically in the mouse prostate under the control of a probasin (PB) promoter. Our studies revealed that targeted PKC ϵ overexpression, but not PKC α and PKC δ overexpression, leads to the development of pre-neoplastic lesions in the prostate, therefore suggesting a potential link between PKC ϵ and prostate cancer development.

Results

Generation of PB-PKC α , PKC δ and PKC ϵ mice. To study the role of PKC ϵ overexpression in prostate tumor development, we targeted this PKC to mouse epithelial prostate cells using the androgen-responsive ARR₂PB promoter.²³ As the roles of other DAG/phorbol ester-responsive PKCs in the prostate in vivo have not yet been determined (as well as for control of specificity), we also generated two additional prostate-specific transgenic models for PKC α and PKC δ . The cDNAs for PKC α , PKC δ or PKC ϵ were ligated into a PB promoter vector that we previously adapted to express proteins fused to an N-terminal HA-tag (Fig. 1A). After the DNA pronuclear microinjections, founder mice were identified by PCR genotyping using primers that amplify the rabbit beta-globin sequence. Three positive male founders were identified for PB-PKC α , two for PB-PKC δ and two for PB-PKC ϵ . A founder was selected for each one and a line established. Protein extracts from prostates obtained from 4-month old transgenic and wild-type mice were analyzed by western blot using an anti-HA epitope antibody. HA immunoreactivity was detected in prostates from PB-PKC α , PB-PKC δ and PB-PKC ϵ transgenic mice but not in prostates from wild-type mice. A representative experiment is shown in Figure 1B. Using specific anti-PKC antibodies we could observe significant overexpression of each corresponding PKC transgene, which normally ranged from 3- to 5-fold over the endogenous protein (Fig. 1B). Using a real-time PCR specific for the rabbit beta-globin sequence present in all transgenic constructs, we developed one homozygous line (Tg/Tg) for each PKC isoform and confirmed the expression by immunohistochemistry (IHC) (Fig. 1C). Consistent with previous studies using this PB promoter,²⁴ transgene expression for the three PKC isozymes was detected specifically in the epithelial compartment of the ventral prostate (VP), with lower expression in the dorsal prostate (DP) (data not shown).

Differential effects of PKC isozymes in the mouse prostate: only PKC ϵ overexpression leads to a preneoplastic phenotype. Homozygous male animals for PB-PKC α , PB-PKC δ and PB-PKC ϵ mice were sacrificed at 12 months. Age-matched wild-type FVB mice were used as controls. All PKC transgenic mice appeared normal at birth and did not show any signs of abnormalities to adulthood. Prostates were isolated, sectioned and stained with hematoxylin and eosin (H&E) for morphologic

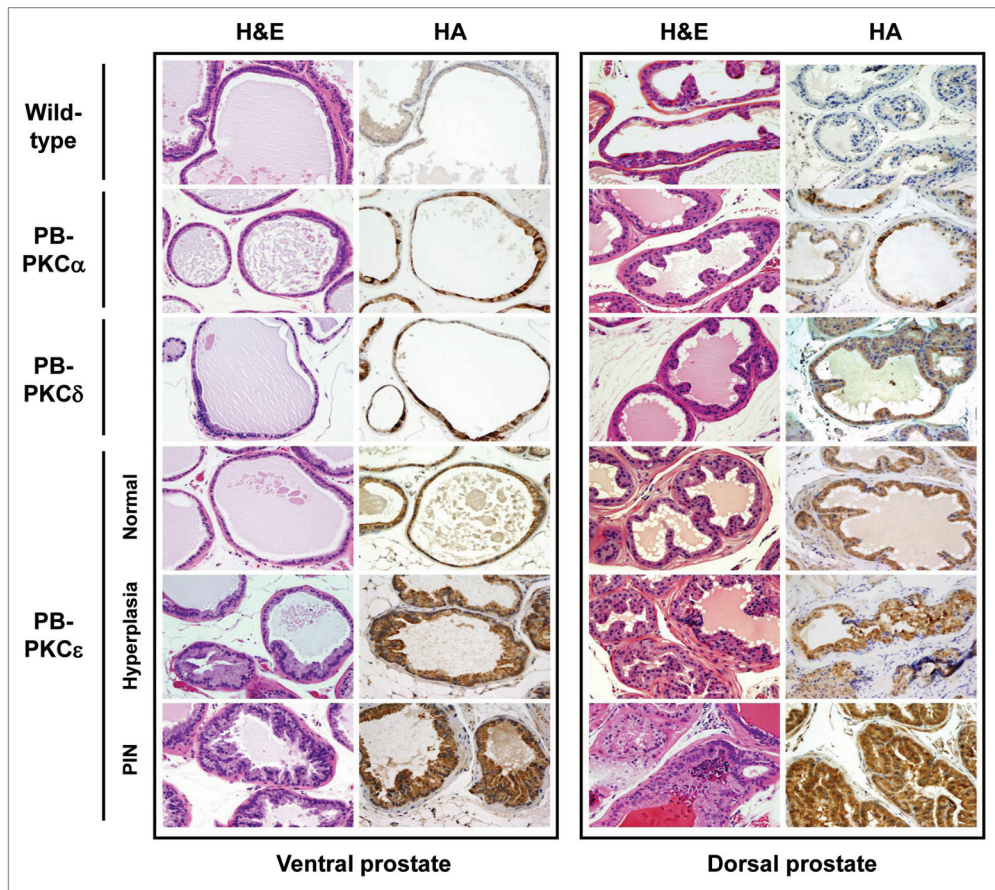


Figure 2. Phenotypes of PB-PKC transgenic mice: PKC ϵ overexpression leads to a preneoplastic phenotype. Homozygous males for PB-PKC α , PB-PKC δ , PB-PKC ϵ and non-transgenic FVB control mice at 12 months old were analyzed. Histological analyses revealed significant epithelial hyperplasia and PIN lesions in both VP and DP from PB-PKC ϵ mice. Representative H&E and HA stainings are shown. All microphotographs are 20x magnification.

Table 1. Incidence of hyperplasia and PIN lesions in VP and DP of PB-PKC transgenic mice

Mice	Age (months)	n	Ventral prostate		Dorsal prostate	
			Hyperplasia, number (%)	PIN, number (%)	Hyperplasia, number (%)	PIN, number (%)
Wild-type	12	14	0 (0%)	0 (0%)	0 (0%)	0 (0%)
PB-PKC α	12	8	1 (12%)*	0 (0%)	1 (12%)*	0 (0%)
PB-PKC δ	12	6	1 (8%)	0 (0%)	0 (0%)	0 (0%)
PB-PKC ϵ	8	7	7 (100%)	6 (86%)	6 (86%)	5 (71%)
PB-PKC ϵ	12	17	17 (100%)	17 (100%)	17 (100%)	17 (100%)
PB-PKC ϵ	14	4	4 (100%)	4 (100%)	4 (100%)	4 (100%)
PB-PKC ϵ	16	3	3 (100%)	3 (100%)	3 (100%)	3 (100%)

*Mild hyperplasia.

assessment. Histological analyses revealed significant epithelial hyperplasia both in the ventral and dorsal lobes of PB-PKC ϵ mice. In addition, a high proportion of mice developed dysplastic changes characteristic of PINs both in VP and DP. On the other hand, PB-PKC α and PB-PKC δ mice did not present any noticeable phenotypic change in the prostate. Only one mouse in each PB-PKC α and PB-PKC δ group showed hyperplasia (Fig. 2 and Table 1). Additional representative low- and high-magnifications photomicrographs of H&E-stained lesions in the VP

and DP are shown in Figure 3. PIN lesions in VP and DP glands exhibited tufting to micropapillary pattern, and some VP glands also presented a cribriform pattern. Cells with karyomegaly and cytomegaly, nuclear atypia with apical localization and chromatin condensation were observed. The presence of one or more prominent nucleoli was also observed. Lesions were seen merely in the prostate, and no other lesions were observed in the remaining of the genito-urinary track, with the exception of mild hyperplasia in few anterior prostate lobes (data not shown). Analysis

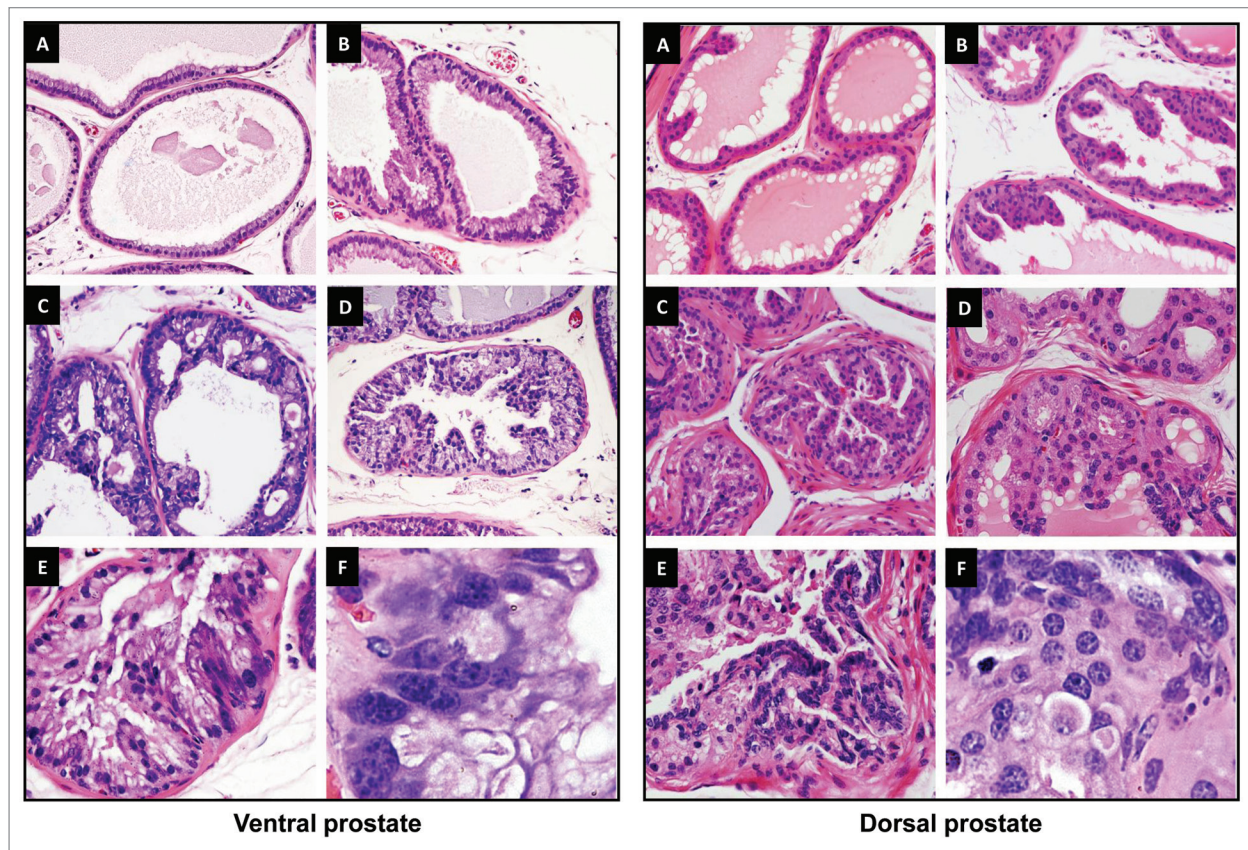


Figure 3. Representative lesions in PB-PKC ϵ mice. Left panels. H&E stained sections of VP from PKC ϵ mice. (A) (20x) VP showing a single stratum of luminal epithelial cells with normal lower flat and cylindrical shaped cells projecting into the gland. (B) (20x) VP showing luminal epithelial hyperplasia, few cells present nuclear atypia. (C and D) (20x) Representative examples from VP glands presenting PIN lesions with cribriform pattern (C) and a mixed of tufting with micropapillary pattern (D). Cells display from moderate to strong cellular atypia. Note also the nucleus adopting an apical localization. (E) (40x) This lesion displays a mild to moderate dysplastic luminal epithelium and the presence of nuclear atypia. Kariomegaly and kariocyto-megaly are common findings. (F) (100x) Clusters of cells presenting severe dysplasia. Note the difference in the shape and size of the nucleus as well as the large number of nucleoli. Right panels. H&E stained sections of the DP from PKC ϵ mice. (A) (20x) Normal glands showing cubical shaped cells with normal mucosal folding projected into the lumen. (B) (20x) DP showing luminal epithelial hyperplasia with increased cellular tufting but without cellular atypia. (C) (20x) A mixed hyperplasia with cellular atypia and PIN lesion with mild dysplasia. (D) (40x) Some of the PIN lesions display many small "intraluminal glands." (E) (40x) Highly dysplastic PIN lesion with increased epithelial tufting and micropapillary pattern. (F) (100x) Higher magnification from a PIN showing dysplastic cells with enlarged nucleus presenting one or more large nucleoli.

of PB-PKC ϵ mice at different ages showed that both hyperplasia and PINs were evident at 8 months. Notably, no evidence of invasive or in situ carcinomas were seen up to 16 month of age (Table 1), although PINs in 12 months of age and older present a high degree of dysplastic cells (data not shown). BrdU incorporation index is generally very low in the prostatic epithelium, however a slight increase could be observed in VPs from 12 month old PB-PKC ϵ mice relative to control mice (wild-type: 0.26 ± 0.10 %, PB-PKC ϵ : $0.43 \pm 0.07\%$, see Sup. Fig. 1). Taken together, these results suggest that overexpression of PKC ϵ in mouse prostatic epithelial cells for several months causes preneoplastic lesions. Moreover, there is a remarkable selectivity for PKC isozymes to confer such phenotypic changes.

PIN lesions from PB-PKC ϵ mice display hyperactivation of the Akt pathway. The appearance of preneoplastic lesions in prostates of PB-PKC ϵ mice suggests major dysregulations in survival signals. Studies in different cellular models

identified the Akt pathway as a downstream effector of PKC ϵ .⁵ Hyperactivation of Akt is an established signature of prostate cancer, as revealed in mouse models of prostate cancer models as well as in clinical specimens. The activation status of Akt in hyperplasia and PIN lesions from 12 month old PB-PKC ϵ mice was determined by IHC using an anti phospho-Ser473 (active state) Akt antibody. While total Akt staining was similar among all samples (data not shown), phospho-Akt staining was clearly elevated in hyperplasia and PINs from PB-PKC ϵ mice. There was a significant degree of nuclear phospho-Akt staining, particularly in PIN lesions, both in VP and DP. Furthermore, PIN lesions show a significant hyperactivation of the Akt effector S6 relative to normal tissue, as determined with anti-phospho-S6 antibody (Fig. 4).

A physical and functional association between PKC ϵ and Stat3 has been recently reported in human cancer. PKC ϵ modulates the transcriptional activity of Stat3 in prostate cancer cells.^{9,14} Remarkably, when we determined the expression levels of

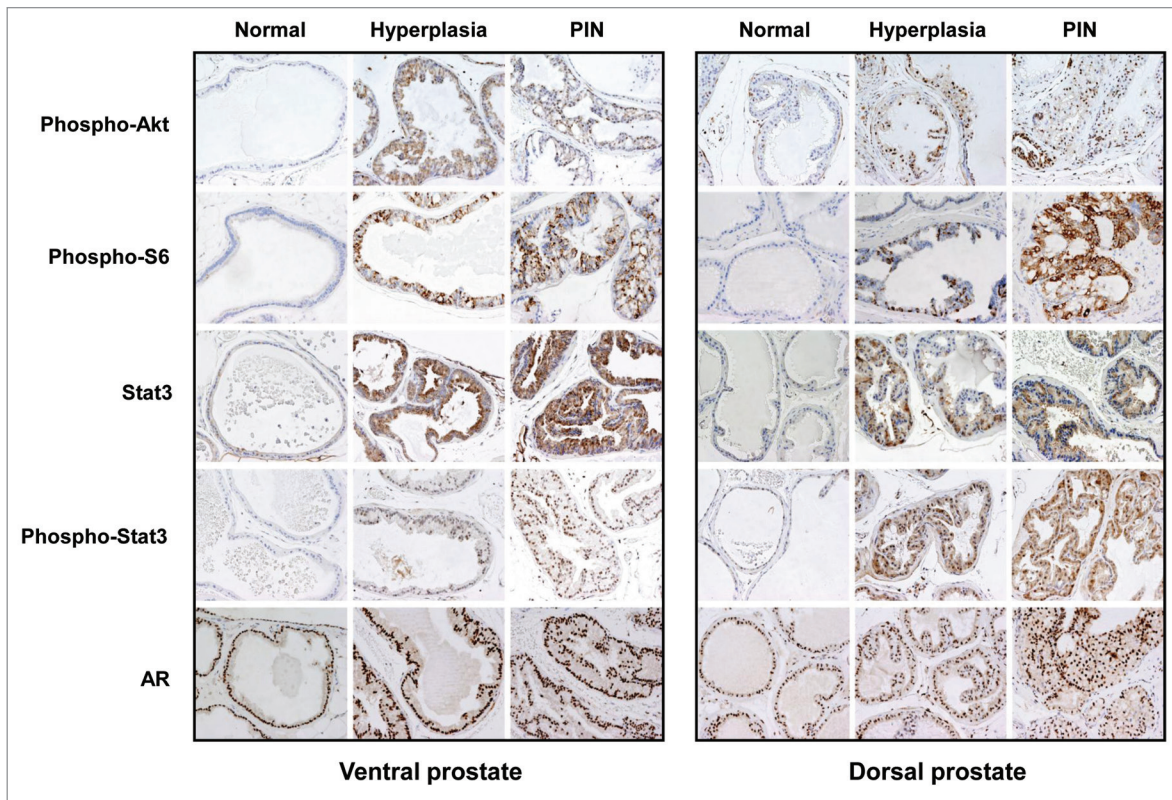


Figure 4. Immunohistochemical analysis of signaling markers in prostates from PB-PKC ϵ mice. Paraffin-embedded prostate tissue sections from 12 month old wild-type or PB-PKC ϵ mice were subject to IHC for the signaling markers indicated in the figure. Representative figures are shown. All microphotographs are 20x magnification.

Stat3 and its phosphorylation status in prostates from 12 month old PB-PKC ϵ mice we observed that they were both markedly elevated compared to prostates from control mice. Total Stat3 staining in prostates from PB-PKC ϵ mice was detected in the cytoplasm of the hyperplastic lesion in both ventral and dorsal lobes and elevated in cytoplasmic and also nuclear compartments in the PIN lesions of VP glands, whereas its expression was slightly lower in the PIN lesions of DP glands. Phosphorylated (active) Stat3-Tyr705 staining shows a clear nuclear expression and light cytoplasmic staining in hyperplasias and PINs of both VP and DP glands. Androgen receptor (AR) expression was strongly expressed in all stages in VP and DP, from normal glands to PIN lesions and no obvious changes could be detected throughout all the stages (Fig. 4).

PKC ϵ overexpression confer resistance to apoptosis induced by androgen ablation. To assess a potential role for PKC ϵ in prostate cell survival in vivo, we investigated if PKC ϵ overexpression confers resistance to apoptosis induced by castration. It is well established that androgen ablation leads to prostate involution characterized by an extensive apoptotic response. A general drawback of the PB-driven models is that transgene expression is reduced upon androgen ablation. Despite this limitation, it has been found that transgenes driven by the ARR₂PB promoter remain expressed, although at lower levels, several days after castration (data not shown). Eight-week old PB-PKC ϵ mice and control FVB littermates were castrated as described in Experimental

Procedures, and the prostates removed after 7 or 14 days and subject to active caspase-3 staining. Notably, the apoptotic index was significantly lower in prostates from PB-PKC ϵ mice (Fig. 5). These data suggest that PKC ϵ has pro-survival activity when expressed in the mouse prostate.

PKC ϵ confers a growth advantage to prostate epithelial cells in culture. Next, we investigated the role of PKC ϵ overexpression in RWPE-1 cells, a model of normal immortalized prostate epithelial cells that has been widely used to assess pro-mitogenic and transforming actions of oncogenes.²⁵⁻²⁷ RWPE-1 cells express very low levels of PKC ϵ relative to prostate cancer cell lines (Fig. 6A), which is in concordance with data in human specimens.¹²⁻¹⁴ To overexpress PKC ϵ we used an adenoviral approach. RWPE-1 growing either in complete medium or in medium deficient in supplements (EGF/bovine pituitary extract) were infected with increasing MOIs (0.1–10 pfu/cell) of an AdV for PKC ϵ (PKC ϵ AdV), and cell number determined for 1, 2 or 3 days after infection. As shown in Figure 6B, infection with the PKC ϵ AdV leads to an increase in cell number, and this effect was proportional to the MOIs. The effect was observed both in RWPE-1 cells growing in complete or incomplete medium. On the other hand, a control LacZ AdV (MOI = 10 pfu/cell) had no effect on RWPE-1 cell number. PKC ϵ expression levels are shown in Figure 6C. Note that the elevation in cell number was observed at expression levels even lower than those observed in prostate cancer cells.

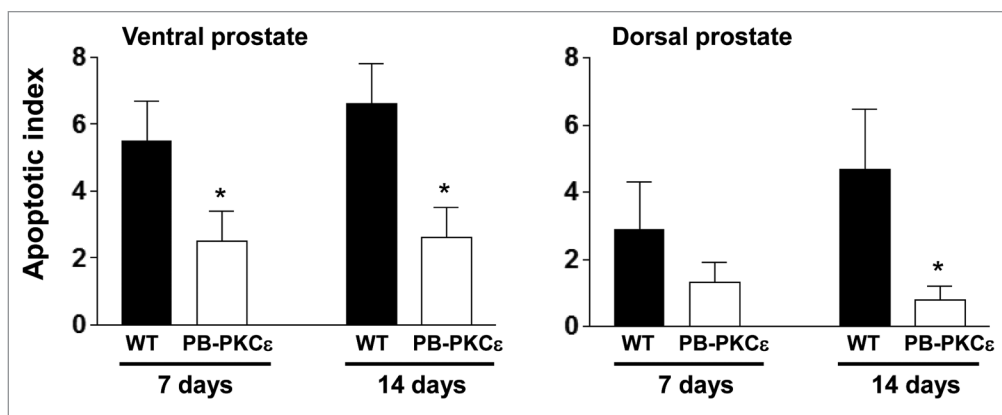


Figure 5. PKC ϵ overexpression confers resistance to apoptosis after castration. Eight-week old mice were castrated as described in Experimental Procedures. Prostate lobes were collected 7 or 14 days later. Apoptosis was detected by IHC using an antibody specific for activated caspase-3. Data are expressed as mean \pm SD. Similar results were observed in 2 independent experiments. WT, wild type; * p < 0.05, Student's t test.

Finally, we examined if PKC ϵ overexpression in RWPE-1 cells could lead to changes in the activation status of Erk and Akt. Cells growing in complete medium were infected with either PKC ϵ AdV or LacZ AdV as described above, and phospho-Erk and phospho-Akt levels were assessed by western blot. Notably, PKC ϵ overexpression led to a marked activation of Erk and Akt in RWPE-1 cells. The effect was proportional to the expression levels of PKC ϵ achieved by increasing MOIs for the PKC ϵ AdV, and was noticeable even at MOIs which confer only a slight overexpression of PKC ϵ (Fig. 6C). Therefore, upregulation of PKC ϵ in prostate epithelial cells is sufficient to elicit changes in markers of mitogenesis and survival.

Discussion

PKC isozymes regulate multiple cellular processes including apoptosis, proliferation, chemoresistance, migration and invasion, and have been widely studied in the context of tumorigenesis, particularly as intracellular receptors for the phorbol ester tumor promoters. Several years of extensive research helped delineating the specific functions of individual PKCs in different cellular contexts. Members of the nPKC family play paradoxical biological roles: PKC δ signals in most cases for apoptosis or inhibition of mitogenesis whereas PKC ϵ promotes growth advantage or can even confer in some cell lines a malignant phenotype upon overexpression.²⁻⁹ This divergence in PKC function is also well exemplified in mouse models of skin cancer. Targeted overexpression of PKC δ in the epidermis inhibits tumor promotion, whereas PKC ϵ overexpression promotes the development of skin metastatic squamous cell carcinomas. On the other hand PKC α overexpression in skin does not affect tumor promotion.²⁸⁻³⁰ In prostate cancer models in culture, both PKC α and PKC δ mediate apoptotic responses.^{7,8,20-22} While the role of PKC ϵ in prostate cancer cells was controversial, mounting evidence implicates PKC ϵ in prostate tumorigenesis through its stimulatory effects on proliferation, survival and invasiveness.¹⁴⁻¹⁸ The recent identification of a microRNA that exerts tumor suppressive functions in human prostate through PKC ϵ downregulation¹⁹ reinforces this concept.

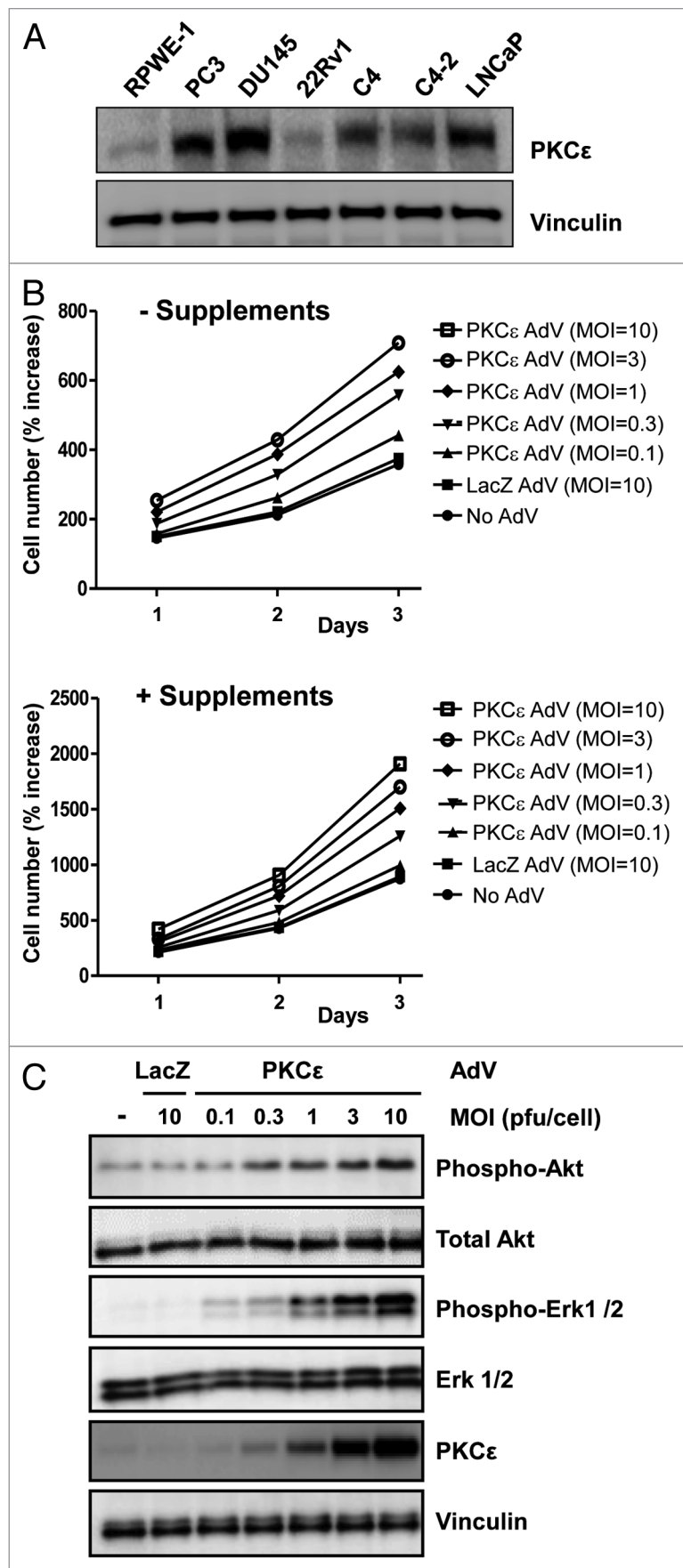
PKC ϵ overexpression is a hallmark of several cancers, most notably prostate cancer. Early studies by Conford et al.¹² showed the upregulation of PKC ϵ in early prostate neoplasia, a finding later confirmed by others.^{13,14} The question that remained to be addressed is whether PKC ϵ upregulation was capable of conferring a preneoplastic or neoplastic phenotype in the prostate *in vivo*. The present study clearly tackled this issue and established that targeted overexpression of PKC ϵ in the mouse prostate leads to the development of a hyperplastic state. Substantial PIN lesions, but not carcinomas, could be observed in prostates from PB-PKC ϵ mice. Consistent with the inability of PKC α and PKC δ to activate proliferative and survival signals in prostate cells, no phenotypic changes could be observed in prostates from PB-PKC α and PB-PKC δ mice. Thus, PKC ϵ overexpression is sufficient to initiate the development of early stage preneoplastic lesions but is incapable to lead to prostate cancer. Unlike the skin PKC ϵ transgenic mice, PB-PKC ϵ mice do not show carcinomas in the prostate. It is important to highlight that our prostate mouse model presents moderate overexpression, unlike the skin transgenic mice in which PKC ϵ has been massively overexpressed.²⁹ It is conceivable that PKC ϵ overexpression cooperates with other genetic alterations to promote prostate cancer. Interestingly, preliminary studies from our laboratory revealed that PKC ϵ synergizes with Pten deficiency for the development of prostate cancer. Indeed, PB-PKC ϵ /Pten^{-/-} compound mice show a high incidence of invasive adenocarcinoma of the prostate (unpublished observations). Cooperative interactions with Pten deficiency have been previously described in prostate mouse models, such as with fibroblast growth factor 8b (FGF8b) overexpression or loss of the homeobox gene Nkx3.1, as well as in other cancer types, such as with Wnt-1 in breast cancer.³¹⁻³³ Coincidentally, PKC ϵ is an effector of mitogenic and tumorigenic pathways, including the Wnt and FGF receptor pathways.³⁴⁻³⁶

The present results also uncovered a role for PKC ϵ in prostate cancer survival *in vivo*. PINs from PB-PKC ϵ mice display hyperactivation of the Akt pathway. Moreover, we found that PKC ϵ overexpression protects against apoptosis induced by androgen

Figure 6. Overexpression of PKC ϵ in RWPE-1 cells enhances proliferation and leads to Erk and Akt activation. (A) Comparison of the expression of PKC ϵ between RWPE-1 cells and several prostate cancer cell lines, as determined by western blot. (B) RWPE-1 cells were infected with increasing multiplicities of infection (MOI) of PKC ϵ AdV or LacZ AdV (control). Cell number was determined at different times after infection. Data from triplicate samples are expressed as mean \pm SD (n = 3). (C) Cell extracts were prepared 72 h after infection with either PKC ϵ AdV or LacZ AdV. Erk and Akt (total and phosphorylated) levels were determined by western blot. Similar results were observed in 3 experiments.

deprivation. These results support the conclusions from our recent studies in LNCaP cells showing that PKC ϵ RNAi depletion enhances the apoptotic activity of phorbol esters and TNF α .¹⁵ Together with reports in other cell types, we conclude that PKC ϵ has a broader survival function in response to physiological and pharmacological stimuli. Indeed, PKC ϵ protects cells against the apoptotic effect of ceramide, TRAIL and chemotherapeutic drugs such as cisplatin. Increased levels of PKC ϵ have been associated with chemoresistance in non-small cell lung cancer (NSCLC) and ovarian cancer cells, and ectopic expression of PKC ϵ in small cell lung cancer (SCLC) cells, which normally present low PKC ϵ levels, confers resistance to etoposide and doxorubicin. In cellular models PKC ϵ enhances Akt phosphorylation and activity in response to diverse stimuli, including growth factors, insulin and stress (reviewed in ref. 5). It has been shown that cardiac-specific overexpression of PKC ϵ in mice leads to elevations in phospho-Ser473-Akt levels and protects against ischemia-reperfusion injury.³⁷ The mechanisms by which PKC ϵ activates Akt in prostate cancer cells remain to be elucidated. Studies in other cellular models suggest that PKC ϵ and Akt form a complex, and their association results in high phospho-Ser473 Akt levels. PKC ϵ can also activate Akt by enhancing its interaction with DNA-PK.^{5,38} Another interesting observation in our study was the elevation in activated Stat3 levels in lesions from PB-PKC ϵ mice. Stat3 has been widely implicated in apoptosis resistance of cancer cells.^{39,40} Activation of Stat3 through phosphorylation is associated with reduced patient survival and the development of hormone-refractory prostate cancer.⁴¹ Moreover, there is a positive correlation between PKC ϵ , Stat3 levels and prostate cancer progression in humans.¹⁴ It remains to be determined if a positive correlation also exists between PKC ϵ overexpression and activated Akt/Akt effectors in human prostate tumors.

We found that overexpression of PKC ϵ in normal immortalized RWPE-1 cells leads to a proliferative response and augmented phospho-Erk levels. In support of our studies, it has been reported that the acquisition of androgen independence of LNCaP cells by PKC ϵ overexpression is linked to the constitutive activation of Erk, cyclin D1 upregulation and Rb



hyperphosphorylation.¹⁸ Activation of Erk in prostate cancer cells by PKC ϵ overexpression may conceivably proceed through the activation of Raf, as described in other cell types.^{4,5} Interestingly, oncogenic Ha-ras leads to the transcriptional activation of cyclin D1 via an Erk-PKC ϵ -dependent manner.⁴² As PKC ϵ overexpression promotes the secretion of mitogenic factors,⁴³ an attractive possibility is that the activated Erk (and Akt) status may occur through an autocrine pathway.

PKC ϵ may represent an appealing target for the treatment of prostate cancer or other cancers. PKC antagonists designed as anti-cancer drugs had only partial success possibly due to their inability to discriminate between PKC isozymes triggering disparate effects. Notably, PKC ϵ -specific modulators have been generated for other purposes, such as inflammation, ischemia and cardiovascular diseases.⁴⁴⁻⁴⁶ We have recently observed that the tumorigenic activity of non-small lung cancer cells (which express very high PKC ϵ levels) in nude mice can be impaired by continuous delivery of the selective PKC ϵ antagonist ϵ V1-2 (unpublished observations). In summary, our studies warrant the development of highly specific inhibitors against PKC ϵ . Such agents could be valuable in combination therapies with inhibitors of the PI3K/Akt pathway or other survival and mitogenic pathways, and may help overcome the resistance of prostate cancer cells to chemotherapeutic agents.

Materials and Methods

Animal care. FVB/N inbred mice (used in maintenance of transgenic lines) were acquired from Charles River Laboratories Inc., (strain code 207). Mice were housed in individually ventilated cages on autoclaved hardwood bedding in an AAALAC accredited facility at the MD Anderson Cancer Center, Science-Park-Research Division. Controlled room conditions were as follows: temperature 20–22°C, humidity 60–70% and light 14/10 h = light/dark. Commercial rodent pelleted food and autoclaved R.O. water were available ad libitum. All procedures were in compliance with the Public Health Service Guide for the Care and Use of Laboratory Animals.

Generation of PKC overexpressing transgenic mice. To achieve prostate-specific expression of PKC α , PKC δ or PKC ϵ , their full-length cDNAs were placed under the control of the ARR₂PB promoter, which targets prostate-specific transgene expression.^{23,47} The PB vector was adapted with a cassette that includes an upstream HA tag and *Xho*I-*Mlu*I sites for unidirectional ligation of PKC inserts. In vitro studies have shown that this tag does not affect the basal activity or activation of PKCs (data not shown). Transgenic mice were developed by means of pronuclear microinjection of FVB/N fertilized eggs in the Transgenic Core Facility of the Department of Carcinogenesis, M.D. Anderson Cancer Center, Smithville, Texas. Expression analysis of the transgene was performed by western blot, using an anti-HA antibody (HA.11, catalog # MMS-101P) and the following specific anti-PKC antibodies: anti-PKC α (Millipore Corporation, catalog # 05-154), anti-PKC δ (Cell Signaling Technology Inc., catalog # 2058) and anti-PKC ϵ (Santa Cruz Biotechnology Inc., catalog # sc-214). Based on expression

analysis, we selected one founder per isoform to develop a transgenic line. The genotype of the animals was carried out by PCR for the detection of the reporter rabbit beta globin sequence present in the transgenic construct, as previously described in reference 48. The standard nomenclature for these new lines is as follows: FVB/N-*Tg*(*Pbsn-Prkca*), FVB/N-*Tg*(*Pbsn-Prkcd*) and FVB/N-*Tg*(*Pbsn-Prkce*) (referred to as PB-PKC α , PB-PKC δ and PB-PKC ϵ , respectively). In order to develop homozygous lines (Tg/Tg), we crossed hemizygous (Tg/0) mice and genotyped the progeny using beta globin-specific quantitative real-time PCR.⁴⁸

Analysis of phenotype. For each transgenic line (PB-PKC α , PB-PKC δ and PB-PKC ϵ), we studied homozygous Tg/Tg males on a pure FVB/N background and age-matched wild-type FVB/N males at different times. Complete necropsy, macroscopic examination, tissue collection and processing were carried out as described in reference 49. Mice were sacrificed with CO₂ and their entire genitourinary tract removed. Prostates were submitted en bloc and processed in standard formalin-fixed, paraffin-embedded sections. To avoid compromising subsequent immunohistochemical analyses, tissues were switched overnight to 70% ethanol. Formalin-fixed paraffin-embedded tissue sections were stained with H&E. Histological examination was performed by two independent pathologists and was based on the “Basic Protocols in Characterization of Prostate Lesions in GEM” from the *Consensus Report from the Bar Harbor Meeting of the Mouse Models of Human Cancer Consortium Prostate Pathology Committee*.⁵⁰

For cell proliferation, mice were injected intraperitoneally with the thymidine analog 5'-bromo-2'-deoxyuridine (BrdU) (60 μ g/g; Sigma, catalog # B5002) 30 min before sacrifice. BrdU incorporation was detected by a standard 3-step immunoperoxidase detection using mouse anti-BrdU monoclonal antibody (Becton-Dickinson Immunocytometry System, catalog # 347580), biotin F(ab') rabbit anti-mouse IgG (Accurate Chemical, catalog # JZM066045) and streptavidin peroxidase (BioGenex, catalog # HK-330-9K). Diaminobenzidine (BioGenex, HK-153-5K) was used as a chromogen for visualization.

For IHC analysis on paraffin-embedded prostate tissue sections we used the following primary antibodies: phospho-Akt (Ser473) (1:50), phospho-S6 (S235/236) (1:50), Stat3 (1:50) and phospho-Stat3-Tyr705 (1:50; Cell Signaling Technologies, catalog # 9145) and anti-androgen receptor (1:200, Abcam, catalog # ab2742). Antibodies were detected with biotinylated secondary antibodies, followed by peroxidase-conjugated avidin/biotin (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) and DAB substrate (Dako, Carpinteria, CA).

Castration experiments. Bilateral orchiectomy was performed in 8-week old FVB/N-PB-PKC ϵ males and FVB/N wild-type controls (10 mice/group). Combined ketamine and xylazine was used as anesthetic (1.5 ml of 100 mg/ml xylazine and 10 ml of 100 mg/ml ketamine; diluted 1:4 with saline, at 0.1 ml/20 g of body weight). A small surgical incision was made in the center of the scrotum and each testicle was exposed through the surgical orifice. The ductus deferens and main arteries and veins were isolated and ligated. Subsequently, the duct and blood vessels were severed allowing the testicles and epididymis to be removed.

The incision was closed, sutured and swabbed with povidone-iodine solution and post-operative procedures were applied when necessary. Prostate lobes were collected at different times after castration and subject to IHC for active caspase-3 using an anti-activated caspase-3 antibody (R&D Systems, Minneapolis, MN).

Experiments with RWPE-1 cells. Low passage (1.5×10^4) RWPE1 cells were seeded in 12-well plates in complete K-SFM medium. After 24 h, cells were infected with either PKC ϵ adenovirus (AdV) or LacZ AdV at different multiplicities of infection (MOI) in K-SFM medium without supplements (EGF and bovine pituitary extract). Four hours later the AdV were removed by extensive washing and cells were given either complete K-SFM medium or supplement-free K-SFM medium. Cell number was determined at 24, 48 and 72 h.

For western blot experiments, 2×10^5 cells were seeded in 6-well plates and infected with AdVs as described above. Cell extracts were prepared at different times and equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis, transferred to PVDF membranes and immunostained with the following antibodies: anti-phospho-ERK1/2 (Cell Signaling

Technology Inc., catalog # 9101), anti-total ERK1/2 (Cell Signaling Technology Inc., catalog # 9102), anti-phospho-Akt (Ser473) (Cell Signaling Technology Inc., catalog # 9271) and anti-total Akt, (Cell Signaling Technology Inc., catalog # 4691), anti-PKC ϵ (Santa Cruz Biotechnology, catalog # sc-214) or vinculin (Sigma, catalog # V9131). Bands were visualized by using the enhanced chemiluminescence (ECL) western blotting detection system.

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Note

Supplemental materials can be found at: www.landesbioscience.com/journals/cc/article/14469

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