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14. ABSTRACT

Protein kinase C epsilon (PKCε), a member of the PKC family of phorbol ester/diacylglycerol receptors, is up-regulated in many human cancers, including prostate cancer. We recently demonstrated that PKCε is an essential mediator of NF-κB activation in prostate cancer (Garg et al., JBC, 287, 37570–37582, 2012). In this research, we wish to determine if PKCε regulates TNFα-signaling to mediate its effect on NF-κB activation. Using a specific PKCε antagonist, we demonstrated that PKCε plays essential role in the TNFα-induced phosphorylation of TNF receptor in prostate cancer cells.

We have previously identified that PKCε regulates NF-κB responsive genes in prostate cancer cells, including cyclooxygenase-2 (COX-2) (JBC, 2012). COX-2 has been reported to be up-regulated in metastatic prostate cancer. As PKCε plays an important role in prostate cancer cell survival and cooperates with other oncogenic insults, herein we aim to determine if PKCε regulates COX-2 activation during prostate tumorigenesis. In the previous funding period we have demonstrated that COX-2 is overexpressed in human prostate cancer cell lines similar to PKCε and NF-κB. It was found that PKCε regulates COX-2 activation and PGE2 levels in prostate cancer as well as normal human prostate epithelial cells.

In the present report, we present our continued efforts on the in-depth determination of the role of PKCε in COX-2 activation in prostate cancer and also to investigate if COX-2 mediates PKCε responses in prostate cancer. Our results showed that transgenic overexpression of PKCε in the mouse prostate causes preneoplastic lesions with elevated COX-2 levels. Interestingly, when we intercrossed the prostate-specific PKCε transgenic mice with mice haploinsufficient for Pten, a common genetic alteration in human prostate cancer, the resulting compound mutant mice (PB-PKCε;Pten+/- mice) developed fully invasive adenocarcinoma with NF-κB hyperactivation and high COX-2 levels. Likewise, stable overexpression of PKCε in mouse prostate epithelial cell lines that are either heterozygous (P8) or homozygous (CaP8) for Pten deletion, led to significant enhancements in cell proliferation, motility and invasiveness as well as in LPS-induced COX-2 mRNA expression compared to the respective control cells, and this effect is more pronounced in CaP8 cells. Studies using human prostate tumors revealed a co-existence of PKCε overexpression, NF-κB hyperactivation, and COX-2 up-regulation. Lastly, treatment of PKCε overexpressing P8 or CaP8 cells with the selective COX-2 inhibitor NS398 caused a pronounced growth inhibition. Overall, our study identified COX-2 as a PKCε-regulated gene. Our results suggest that COX-2 as a potential mediator of PKCε oncogenesis in prostate cancer, particularly in the context of Pten loss.

15. SUBJECT TERMS

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INTRODUCTION

Prostate cancer is the second leading cause of cancer-related deaths among men in the US. The genesis and progression of prostate cancer involves a series of genetic and epigenetic alterations that deregulate mitogenic and survival signaling. Most common alterations in prostate cancer include the functional inactivation/deletion of tumor suppressors (such as Pten, p53, or Nkx3.1), deregulation of growth factor signaling (including IGF-1R and ErbB receptors) and their effectors (such as PI3K)[1-7].

The altered balance in the expression of protein kinase C (PKC) isozymes has been fully recognized as a distinguished feature of cancer, including prostate cancer[8-13]. This family of Ser-Thr kinases has been classified into 3 classes based on their biochemical and structural properties. The calcium-dependent "classical" cPKCs (α/β/γ) and calcium independent "novel" nPKCs (δ/ε/η/θ) are the main targets for the phorbol ester tumor promoters and diacylglycerol (DAG), a lipid second messenger generated upon activation of tyrosine-kinase and G-protein coupled receptors. Atypical PKCs ζ and λ/ι are insensitive to DAG and phorbol esters[8-10]. Despite more than 3 decades of PKC research, there is still a huge gap in our understanding on how these kinases contribute to the initiation and maintenance of the cancer phenotype. Moreover, there is a limited availability of animal models that recapitulate changes in PKC isozyme expression/activity in disease and specific interactions of PKCs with oncogenic events.

A large body of data links PKC to tumor promotion and cancer progression. PKCs play important roles in the control of cell cycle progression, motility, and invasiveness, and they control prominent cancer signaling pathways, including Ras/Erk, PI3K/Akt, and NF-κB[8-12, 14-17]. PKCε, in particular has emerged as a potential oncogenic kinase[8, 13, 18, 19] and is up-regulated not only in prostate cancer but also in several other epithelial cancers including lung, breast, and thyroid cancer[8, 13, 20-26]. Studies from our laboratory and others have established that PKCε is a prosurvival and mitogenic kinase[23, 27-29]. In prostate cancer cells PKCε mediates survival through Bad-dependent and Bad-independent mechanisms and is involved in the transition to androgen-independence[27, 30]. PKCε up-regulation is observed in >95% of human prostate specimens[11, 12]. Up-regulated PKCε levels have also been reported in prostate tumors from TRAMP mice[12]. We have generated transgenic models to target PKC isozymes to the mouse prostate under the control of the androgen-responsive probasin (PB) promoter[29]. Interestingly, only PB-PKCε mice developed dysplastic changes characteristic of prostatic intraepithelial neoplasia (PIN). Strikingly, prostate-specific PKCε transgenic mice when intercrossed with the mice haploinsufficient for Pten, another common genetic alteration in human prostate cancer, resulted in a compound mutant mice (PB-PKCε;Pten+/- mice) that developed fully invasive adenocarcinoma. We recently demonstrated that transgenic overexpression of PKCε in the mouse prostate causes preneoplastic lesions with elevated NF-κB levels and that PKCε is an essential mediator of NF-κB activation in prostate cancer[16].

In the previous DOD funding period, we specifically identified a functional link between the oncogenic kinase PKCε and inducible cyclooxygenase-2 (COX-2), a well-known NF-κB responsive gene. Cyclooxygenase (COX) converts arachidonic acid to PGE2, PGD2, PGI2, PGF2α and thromboxane A2 (TXA2), collectively the prostanoids[31-33]. Studies demonstrated that COX-2 levels are increased in solid tumors including those of the prostate, and are associated with metastatic disease and reduced survival. Cell, animal and clinical studies established key roles for COX-2 in tumorigenesis and disease progression across a range

of cancers including prostate cancer. Concordantly, inhibition of COX with non-steroidal anti-inflammatory drugs (NSAIDs) reduces risk in human cancers[34-38].

In the last year progress report, we have demonstrated that:

a) COX-2 is overexpressed in human prostate cancer cell lines similar to PKCε and NF-κB.

b) PKCε regulates stimulated and constitutive activation of COX-2 in prostate cancer cells.

c) We also demonstrated that PKCε regulates PGE2 levels in prostate cancer cells.

d) Additionally, we found a marked induction of COX-2 and PGE2 levels in normal human prostate epithelial cells upon PKC overexpression.

The main goal of our continued research supported by DOD is to determine if PKCε regulates COX-2 activation during prostate tumorigenesis. We also aim to elucidate the effect of cooperativity between PKCε overexpression and Pten loss on COX-2 activation. Furthermore, we will determine if COX-2 mediates PKCε responses in prostate cancer.

BODY

1. **Targeted PKCε overexpression in normal prostate epithelium leads to COX-2 activation.** Prostates of transgenic mice overexpressing PKCε (PB-PKCε mice) display elevated nuclear NF-κB levels in PINs[13]. Moreover, in adenocarcinomas of the compound mice PB-PKCε-Pten+/- nuclear staining for NF-κB is remarkably high (unpublished observation). We carried out immunohistochemical analyses on formalin-fixed, paraffin-embedded sections of prostates from wild-type, PB-PKCε and PB-PKCε; Pten+/- mice sacrificed at 12 months using anti-COX-2 antibody (Cayman Chemical) and IgG as a negative control. As evident in Figure 1A enhanced COX-2 levels were observed in lesions from PB-PKCε mice and the effect is further enhanced in adenocarcinomas from PB-PKCε-Pten+/-mice. These findings are highly significant because both Pten loss and PKCε overexpression are common alterations in human prostate cancer. Figure 1B shows the incidence of hyperplasia, PIN lesions and adenocarcinoma in PB-PKCε, Pten and PB-PKCε; Pten+/- mice.

2. **Generation of mouse cellular models to study effect on COX-2 as a consequence of PKCε overexpression and Pten loss.** To begin dissecting the mechanistic aspects of the PKCε overexpression and Pten loss cooperativity, we generated mouse cell lines recapitulating the scenario in the compound transgenic mice. For the same, we took advantage of prostate epithelial cell lines derived from Pten KO mice by Dr. Hong Wu (UCLA), known as P2 and P8 (Pten positive), and CaP2 and CaP8 (the Pten-null counterparts)[39]. We established stable cell lines overexpressing PKCε (or LacZ as control) using lentiviruses generated in our laboratory (blasticidin selection) and described in the previous year annual report. Figure 2A depicts the western blot confirming PKCε overexpression achieved in the four sets of stable cell line thus generated. LacZ lentivirus was used as a control.

Hence, we have developed murine prostate cellular models that overexpress PKCε both in Pten+/- and Pten-/- background.

3. **PKCε overexpression and Pten loss cooperate for cell proliferation and growth**. Numerous evidences have implicated PKCε with multiple cellular processes including cell proliferation and survival in human cancers[13, 21, 23, 27]. We have previously reported that transient overexpression of PKCε in normal prostate human epithelial cells confers growth advantage[29]. Consistent with this we found that cell proliferation was significantly enhanced in murine P8 cells overexpressing PKCε vs. parental (P8) or LZ (P8-LZ) controls (Fig. 3A). Remarkably, loss of second allele of Pten in conjunction with PKCε overexpression in CaP8-PKCε and CaP2-PKCε cells resulted in significant enhancement in cell proliferation when compared to their respective parental controls or PKCε overexpressors with single Pten allele (P8-PKCε) (Fig. 3A). Besides, we found a remarkable cooperativity between PKCε overexpression and Pten loss for anchorage-dependent growth in clonogenic assays (Fig. 3B) and anchorage-independent growth in soft agar (Fig. 3C). Remarkably, CaP8-PKCε and CaP2-PKCε not only formed increased number of colonies but the relative size of colonies in soft agar was also larger than any other groups (Fig. 3C). Our findings thus undoubtedly establish that PKCε overexpression together with Pten loss confers enhanced transformation potential to prostate cells.

4. **PKCε overexpression and Pten loss cooperate for migration and invasiveness**. Next, we examined the ability of the prostate cell lines to migrate using a wound healing assay. Notably, in response to FBS, IGF or PDGF treatments CaP8-PKCε cells showed complete wound closure (100%) 14 h post-scratch formation, while under the same conditions P8-PKCε, CaP8 and parental P8 cells exhibited 75-80%, 55-67% and 30% of closure respectively (Fig. 4A). This implies CaP8-PKCε cells needed least time for wound healing indicating the collaborative phenomenon of PKCε overexpression and Pten loss in conferring enhanced cell motility. To further strengthen the above inferences, we used a Boyden chamber assay to examine the ability of murine prostate epithelial cells to invade through matrigel matrix that simulates a reconstituted basement membrane in vitro. As evident in Fig 4B, whereas P8 cells display a mild invasive phenotype, overexpression of PKCε or loss of single Pten allele alone markedly enhanced their invasiveness. Impressively, a dramatic enhancement in the invading capability could be seen when the two alterations (PKCε overexpression, Pten total loss) acted conjointly.

5. **PKCε overexpression and Pten loss cooperate for NF-κB activation**. The strong cooperativity between PKCε overexpression and loss of the Pten tumor suppressor gene for growth, motility and invasiveness suggests changes in key signaling events. All the four different types murine prostate cells were stimulated with LPS (5 μg/ml) for 1 h and effect of PKCε overexpression and Pten loss were analyzed on NF-κB activation using Western Blot analysis in total, cytoplasmic and nuclear fractions. Figure 5 shows that PKCε overexpression and Pten loss cooperate for the activation of p-IκBα, an established read-out for NF-κB activation[16]. Furthermore, PKCε overexpression and Pten loss together enhanced the translocation of NF-κB from cytosol to nuclear compartment (Fig. 4), thus recapitulating results observed in lesions from PB-PKCε;Pten+/- mice. ATF2 and vinculin were used as the loading control for the nuclear and cytoplasmic cellular fractions.

6. **PKCε overexpression and Pten loss cooperate for enhanced COX-2 activation and PGE2 production**. COX-2 is a well-known NF-κB regulated gene[16, 40]. Hence, we next intended to determine the effect of PKCε and Pten on COX-2 activation in murine prostate cells. As above, we observed a remarkable synergism between PKCε overexpression and Pten loss in enhancing LPS-mediated COX-2 protein and mRNA expression as measured by western blot or real time PCR (Fig. 6A and B). As cyclooxygenase is the rate limiting enzyme involved in the biosynthesis of prostaglandins[32, 33], we also measured the PGE2 synthesis in the four murine cells. Notably, a significant elevation in PGE2 production was observed in PKCε overexpressing P8 cells relative to controls and this effect is more pronounced in CaP8 cells (Fig. 6C). Overall, these results provide strong evidence for a cooperative role of PKCε and Pten in the COX-2 and PGE2 activation in the murine prostate epithelial cells.

7. **Impaired prostate cell growth and survival by COX-2 inhibition is dependent upon the expression of PKCε.** In order to determine the relevance of COX-2 in the context of PKCε overexpression, we used the COX-2 inhibitor NS398[41, 42]. When parental P8 cells were treated with NS398, there was essentially no effect on cell number. On the other hand, NS398 markedly reduced the cell viability in P8-PKCε and CaP8 cells. The effect was further intensified in CaP8-PKCε cells (Fig. 7A-C). Thus, the effectiveness of the COX-2 inhibitor markedly increased upon the expression of PKCε and is enhanced by Pten loss.

   Although NS398 induces apoptosis in prostate cancer cells, it is not known whether this relates to PKCε overexpression. Therefore, we screen the protein expression levels of various markers of apoptotic pathway in the murine prostate cells overexpressing PKCε with or without Pten. Interestingly, COX-2 inhibition by NS398 markedly reduced the survival of PKCε overexpressors but not normal cells as clearly evident from the increased expression of Bax, cleaved PARP, cytochrome C, cleaved caspase 3 and apoptotic index with concomitant decrease in Bcl-2 expression (Fig. 7D and E). These effects were further enhanced when PKCε overexpression acts together with Pten loss as seen in CaP8-PKCε cells (Fig. 7D and E)

8. **COX-2 inhibition reduced the PGE2 synthesis in PKCε overexpressors.** As can be seen in Fig. 8, NS398 significantly impaired the PGE2 synthesis in PKCε overexpressing cells and in particularly in the context of Pten loss, again emphasizing the selective effect of COX-2 inhibition on the presence of the two alterations (PKCε overexpression; Pten loss) in the cells.

9. **COX-2 inhibition decreased the invasiveness of PKCε overexpressors.** Apart from the effect on cell growth, survival and prostaglandin synthesis, we also found that inhibition of COX-2 by NS398 selectively impair the invasive potential of PKCε overexpressing cells and the effect is enhanced in context of Pten loss (Fig. 9).

10. **COX-2 inhibition inhibited the growth of CaP8-PKCε tumors in athymic nude mice and activates apoptotic pathway.** In order to assess the effect of CXO-2 inhibition on the tumorigenic potential of murine prostate epithelial cells in vivo, we chose to inject CaP8-PKCε cells (that are PKCε overexpressing and are Pten null) subcutaneously into athymic nude mice as in a separate study, our laboratory has determined that of the 4 murine prostate epithelial cells only CaP8-PKCε cells are

tumorigenic in nude mice. For this experiment, athymic nude mice were divided in 4 groups. Group 1 received the control laboratory diet. Group 2 mice were fed with rofecoxib (COX-2 inhibitor) diet. Group 3 mice were given intraperitoneal injections of vehicle control and Group 4 receive *i.p* injections of parthenolide (NF-κB inhibitor every other day and was stopped after 14 days of tumor appearance. Following a week of pretreatment with either COX-2 or NF-κB inhibitor, mice were injected subcutaneously with CaP8-PKCε cells mixed with Matrigel in a ratio of 1:1. Inoculation of CaP8-PKCε cells led to the formation of tumors with a latency of 2 weeks (Fig.10A). Notably, tumor growth of CaP8-PKCε cells was significantly hampered in mice fed with rofecoxib diet (Fig. 10A). Furthermore, lesions from mice fed with rofecoxib diet showed increased expression of apoptotic markers as compared to the tumors from mice receiving control diet (Fig. 10B).

11. **Correlation between PKCε levels, NF-κB hyperactivation and COX-2 upregulation in human prostate cancer specimens.** We recently initiated a collaborative project with Dr. Michael Feldman, from the UPenn Department of Pathology, to establish the relevance of PKCε overexpression in human prostate tumors. We successfully optimized the PKCε staining for IHC in human prostate cancer specimens, which took extensive testing of multiple commercial antibodies and validations using frozen pellets of cell lines expressing high PKCε (PC3 cells, + control) vs. those with low PKCε levels (RWPE-1, and PKCε RNAi depleted PC3 cells). Prostate tumors display a very strong PKCε staining. Consistent with most studies in human prostate cancer cell lines, including ours, as well as with studies in human tumors, we found NF-κB and COX-2 levels significantly elevated in human prostate cancer specimens (Fig. 11A). The quantification of PKCε, NF-κB and COX-2 stainings in 25 human prostate normal and tumor specimens is shown in Fig. 11B. Analysis revealed a coexistence of PKCε overexpression, NF-κB hyperactivation and COX-2 up-regulation in human prostate tumors.

KEY RESEARCH ACOMPLISHMENTS

(i) We successfully demonstrated that targeted PKCε overexpression in normal prostate epithelium leads to COX-2 activation.

(ii) We successfully generated mouse cellular models overexpressing PKCε both in Pten + and – background(s) to evaluate effect on COX-2 activation during prostate carcinogenesis.

(iii) We successfully demonstrated that PKCε overexpression and Pten loss cooperate for cell proliferation and growth.

(iv) We successfully demonstrated that PKCε overexpression and Pten loss cooperate for prostate cell migration and invasiveness.

(v) We successfully demonstrated that PKCε overexpression and Pten loss cooperate for NF-κB activation.

(vi) We successfully demonstrated that PKCε overexpression and Pten loss cooperate for enhanced COX-2 activation and PGE2 production.

(vii) We found that COX-2 inhibition specifically impaired the viability of PKCε overexpressing cells.

(viii) We found a critical role of COX-2 in PKCε mediated survival of murine prostate cells.

(ix) We successfully demonstrated that COX-2 inhibition reduced the PGE2 synthesis specifically in PKCε overexpressors.

(x) We successfully demonstrated that COX-2 inhibition decreased the invasiveness of PKCε overexpressors.

(xi) We successfully demonstrated that COX-2 inhibition inhibited the growth of CaP8-PKCε tumors in athymic nude mice and activates apoptotic pathway.

(xii) We successfully established a correlation between PKCε levels, NF-κB hyperactivation and COX-2 up-regulation in human prostate cancer specimens.

CONCLUSION

The main conclusions from the research carried out during the second year of DOD funding are as follows: (i) PKCε mediates the activation of COX-2, a well-known NF-κB responsive gene in prostate cancer. (ii) COX-2 mediates PKCε responses in prostate cancer. These findings suggest a crucial role of PKCε in several important cellular processes relevant to prostate cancer progression, including survival, proliferation, metastasis and invasion. Overall, our study identified COX-2 as a PKCε-regulated gene and also suggests that COX-2 is a potential mediator of PKCε oncogenesis in prostate cancer, particularly in the context of Pten loss. We will continue with our studies to determine if COX-2 inhibition could also affect the adenocarcinoma formation in the compound PKCε; Pten mice. loss, a common genetic alteration found in prostate cancer.

REPORTABLE OUTCOMES

1. Garg, R., Abera, M. B., Lal, P., Blando, J., Benavides, F., Feldman, M. D., Smyth, E. M., and Kazanietz, M.G. COX-2 as a mediator of oncogenic PKCε in prostate cancer. Late Breaking Abstract. Proceedings of the 104th Annual Meeting of American Association of Cancer Research in Cancer Res. 73(8), Suppl. 1; doi:10.1158/1538-7445.AM2013-LB-154 154 (2013).

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3. Garg, R., Lal, P., Blando, J., Benavides, F., Feldman, M. D., Smyth, E. M., and Kazanietz, M.G. COX-2 as a mediator of oncogenic PKCε in prostate cancer. Manuscript in preparation.

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APPENDICES

FIGURE LEGENDS:

Figure 1: Prostate-specific PKCε transgenic mice show enhanced COX-2 activation. (A) Immunohistochemical analyses for COX-2 staining was performed on ventral prostates from PB-PKCε , PB-PKCε;Pten or wild-type FVB/N male mice at 12 months. (B) Incidence of hyperplasia, PIN and adenocarcinoma in ventral prostate of PB-PKCε, Pten+/- and PB-PKCε;Pten+/- mice at 12 months.

Figure 2: Generation of Pten+ and Pten– murine prostate cell lines overexpressing PKCε. PKCε was expressed by infection with a PKCε lentivirus generated in our laboratory. A LacZ lentivirus was used as control.

Figure 3: PKCε overexpression and Pten loss cooperate for cell proliferation and growth. (A) Cells (1.5 x 10^4) were seeded in 12-well plates, grown in media with 5% FBS and counted using hemocytometer. (B) For clonogenic assay, cells (100 per 100 mm plate) were seeded and grown for 15 days. Colonies were stained with 0.7% methylene blue in 50% ethanol. (C) For soft agar assay 2 x10^3 cells were plated in 0.35% agar over a 0.5% agar layer. After 10 days plates were stained with MTS. For each well, number of colonies were counted in 5 different fields and averaged. Data are expressed as mean ± S.E.M of 3 individual experiments. *, $p<0.01$ **, $p<0.05$.

Figure 4: PKCε overexpression and Pten loss cooperate for migration and invasiveness. (A) Cells in confluent monolayers were treated with 5% FBS, IGF (50 ng/ml) or PDGF (60 ng/ml) and scratched. Wound closure was measured 14 h post-treatment. Representative micrographs are shown. (B) Cells were serum-starved for 24 h and then seeded in Boyden chamber with Matrigel-coated membranes. Invasiveness of prostate cells exposed to 5% FBS, IGF (50 ng/ml) or PDGF (30 ng/ml) was quantified 16 h later. Representative micrographs are shown.

Figure 5: PKCε overexpression and Pten loss cooperate for NF-κB activation. Cells were serum-starved for 48 h and then stimulated with LPS 5 μg/ml for 1 h. Effect of PKCε overexpression and Pten loss were analyzed on IκBα phosphorylation and nuclear translocation of NF-κB by Western Blot. Representative blots are shown.

Figure 6: PKCε overexpression and Pten loss cooperate for enhanced COX-2 activation and PGE2 production. Cells were serum-starved for 48 h and then stimulated with LPS 5 μg/ml for 4h. Effect of PKCε overexpression and Pten loss were analyzed on (A) COX-2 protein expression by Western Blot, (B) COX-2 mRNA expression by qPCR , and (C) PGE2 levels in the culture medium, normalized to total protein, using EIA kit. *, $p<0.05$ vs control; n=3.

Figure 7: Impaired prostate cell growth and survival by COX-2 inhibition is dependent upon the expression of PKCε. After 24 h of serum-starvation, cells were treated with the COX-2 inhibitor NS398 (0-100 μM) for (A) 24 h, (B) 48 h, and (C) 72 h and cell viability was determined by MTT assay. (D) Following serum starvation, cells were treated with 30 μM NS398 for 24 h and effect of PKCε overexpression was analyzed on the protein expression of apoptotic markers. (E) Extent of apoptosis was determined by calculating the ratio of normalized band density of Bax and Bcl2. *, $p<0.05$ vs P8 control; n=3.

Figure 8: COX-2 inhibition reduced the PGE2 synthesis in PKCε overexpressors. Cells were treated with NS398 at indicated concentrations for 24 h and then stimulated with LPS 5 μg/ml for 4 h. Effect of PKCε overexpression and Pten loss were analyzed on PGE2 levels in the culture medium using EIA kit. *, $p<0.05$ vs P8 control; n=3.

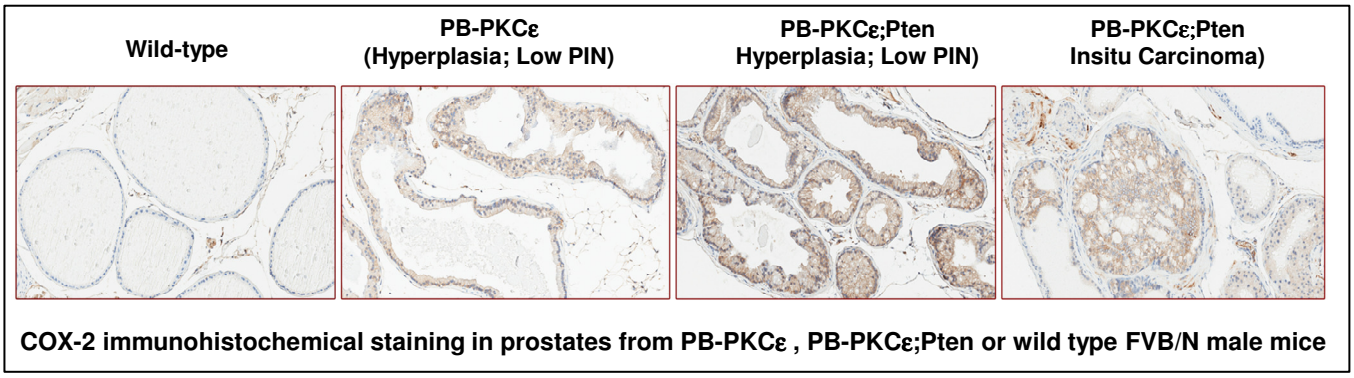
Figure 9: COX-2 inhibition decreased the invasiveness of PKCε overexpressors. Cells were treated with NS398 (1-3 μM) for 24 h and then seeded in a Boyden chamber with Matrigel-coated membranes. Invasiveness of prostate cells exposed to PDGF (30 ng/ml) was quantified 16 h later. Representative micrographs are shown.

Figure 10: COX-2 inhibition retarded the growth of CaP8-PKCε tumors in athymic nude mice and activates apoptotic pathway. Athymic nude mice were divided in 4 groups. Group 1 received the control laboratory diet. Group 2 mice were fed with rofecoxib diet. Group 3 mice were given intraperitoneal injections of vehicle control and Group 4 receive *i.p* injections of 400 μM parthenolide (NF-κB inhibitor every other day and was stopped after 14 days of tumor appearance. Following a week of pretreatment with either COX-2 or NF-κB inhibitor, mice were injected subcutaneously with 1×10^6 cells mixed with matrigel in a ratio of 1:1. (A) Once tumor started growing, their volume was calculated. Data are expressed as mean +/- S.D. (n=5). (B) Protein expression of apoptotic markers was analyzed by western blot in tumor extracts from mice fed with control or rofecoxib diet.

Figure 11: Correlation between PKCε levels, NF-κB hyperactivation and COX-2 up-regulation in human prostate cancer specimens. (A) Immunohistochemical staining of PKCε, NF-κB and COX-2 in 25 human prostate normal and tumor specimens (Gleason 6-7). Magnification x 40. Representative photomicrographs are shown. (B) H score is plotted for normal and cancer sample stainings of PKCε, NF-κB and COX-2. N = normal, C= cancer patient.

Figure 1

A



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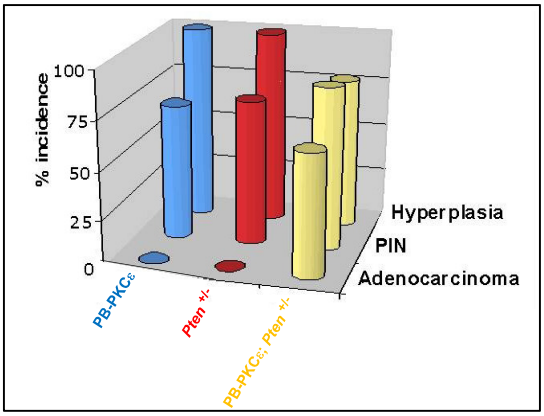

Figure 2

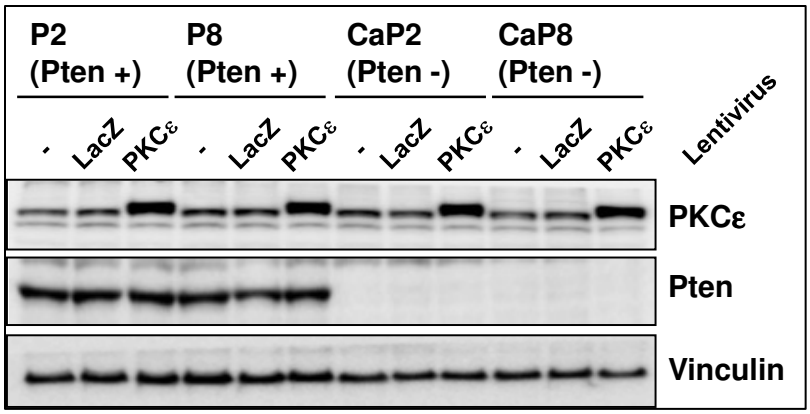

Figure 3

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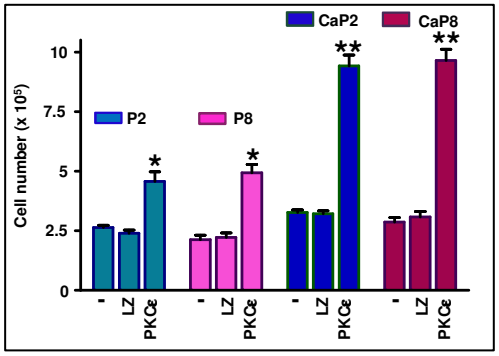
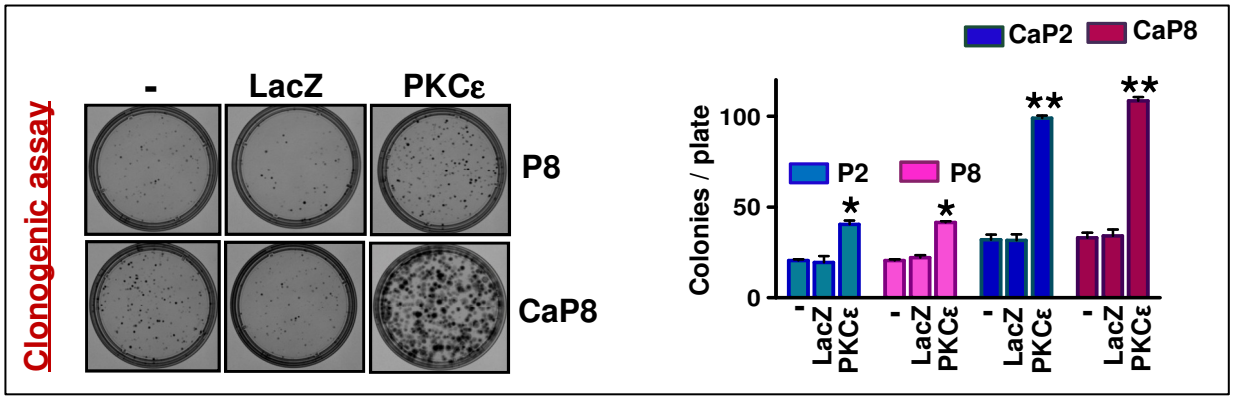

CaP2
CaP8
P2
P8
Cell number (x 10⁵)
10
7.5
5
2.5
0
-
LZ
PKCε

B


Clonogenic assay
-
LacZ
PKCε
P8
CaP8
CaP2
CaP8
P2
P8
Colonies / plate
100
50
0
-
LacZ
PKCε

C

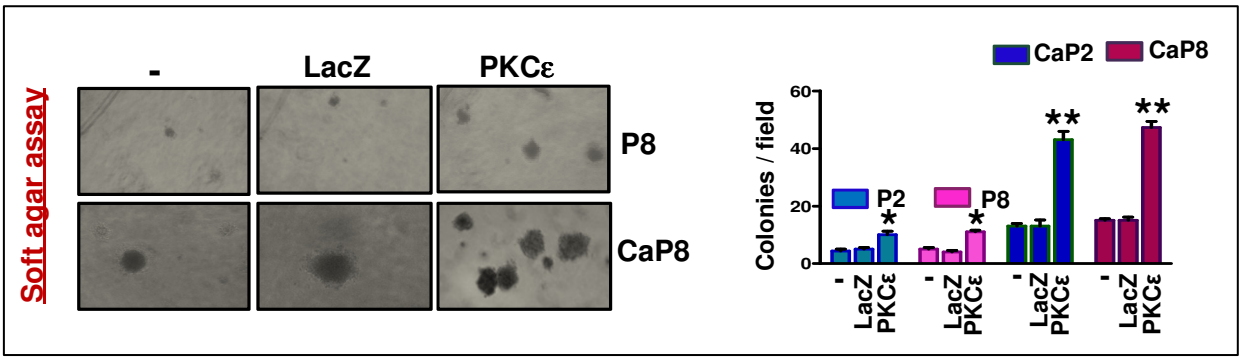
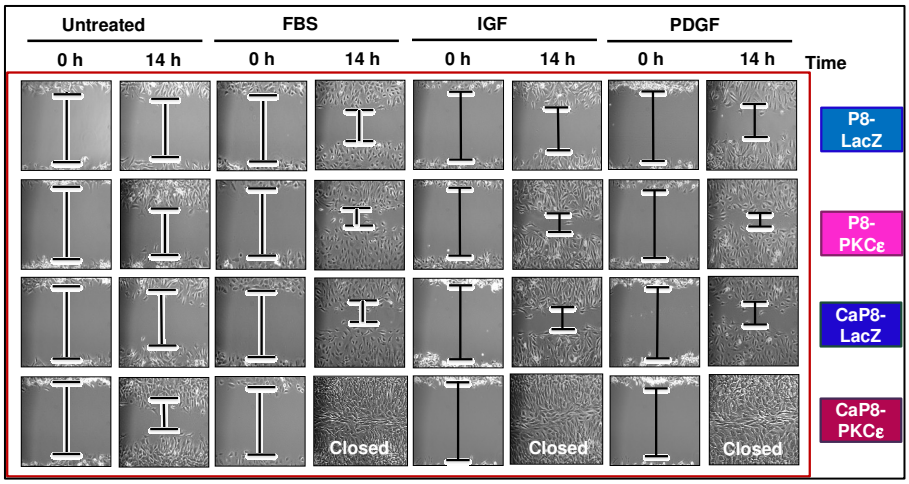

Soft agar assay
-
LacZ
PKCε
P8
CaP8
CaP2
CaP8
P2
P8
Colonies / field
60
40
20
0
-
LacZ
PKCε

Figure 4

A



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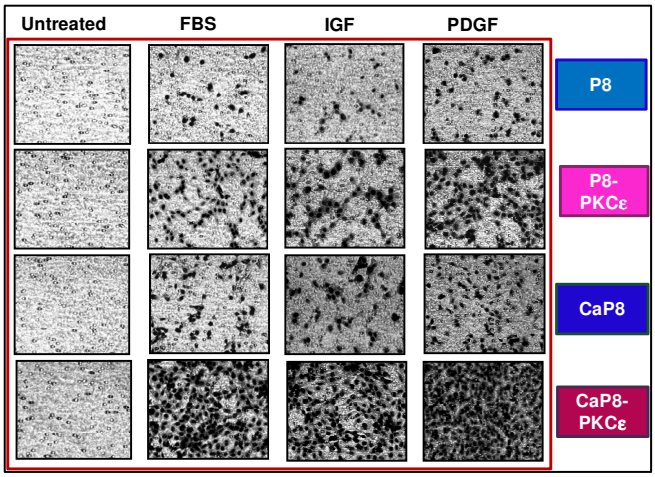

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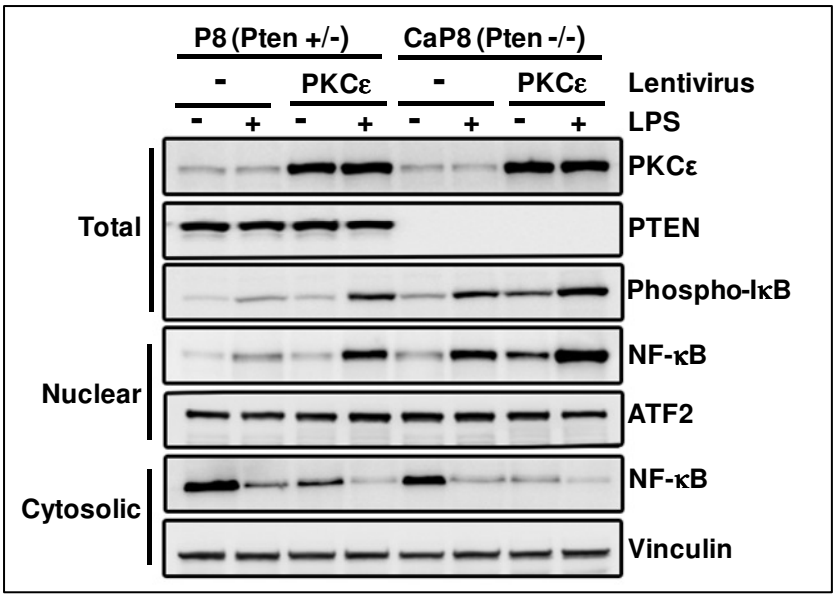

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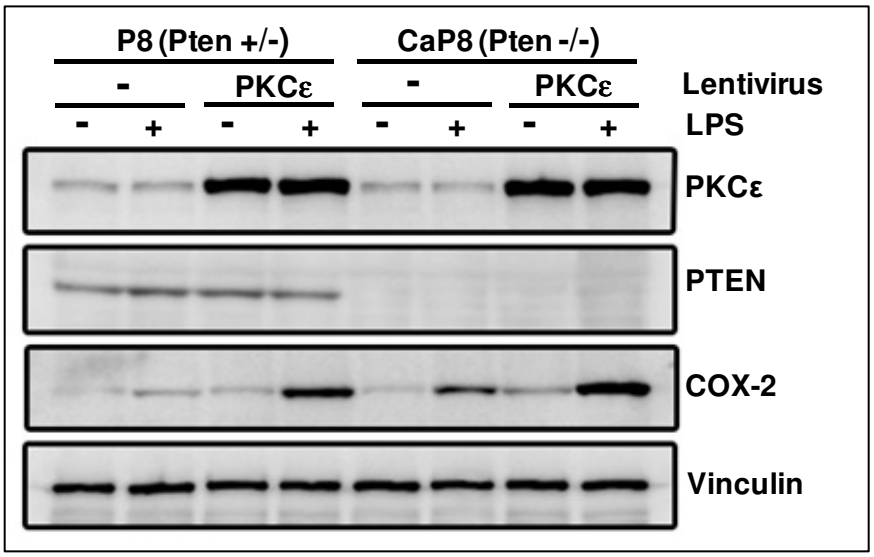
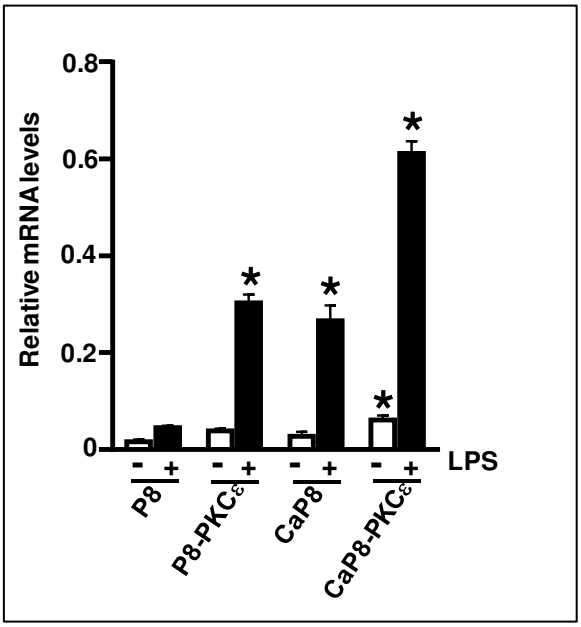

P8 (Pten +/-)
CaP8 (Pten -/-)
-
PKCε
-
PKCε
Lentivirus
- + - + - + - +
LPS
PKCε
PTEN
COX-2
Vinculin

B


Relative mRNA levels
0.8
0.6
0.4
0.2
0
*
- + - + - + - +
LPS
P8
P8-PKCε
CaP8
CaP8-PKCε

C

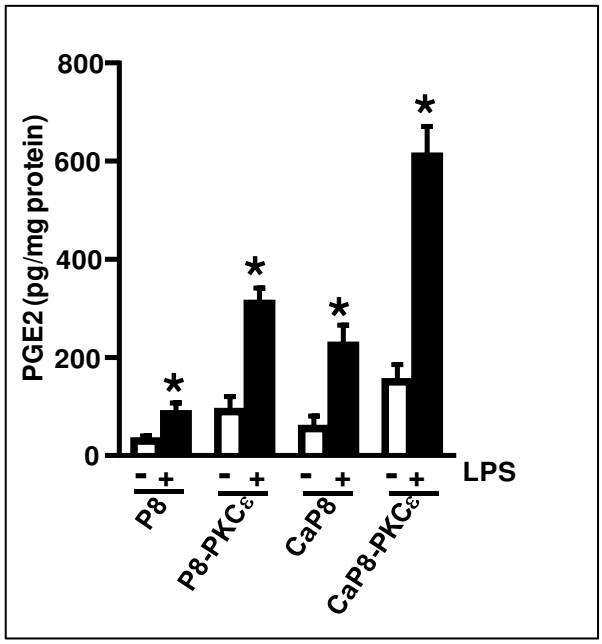

PGE2 (pg/mg protein)
800
600
400
200
0
*
- + - + - + - +
LPS
P8
P8-PKCε
CaP8
CaP8-PKCε

Figure 7

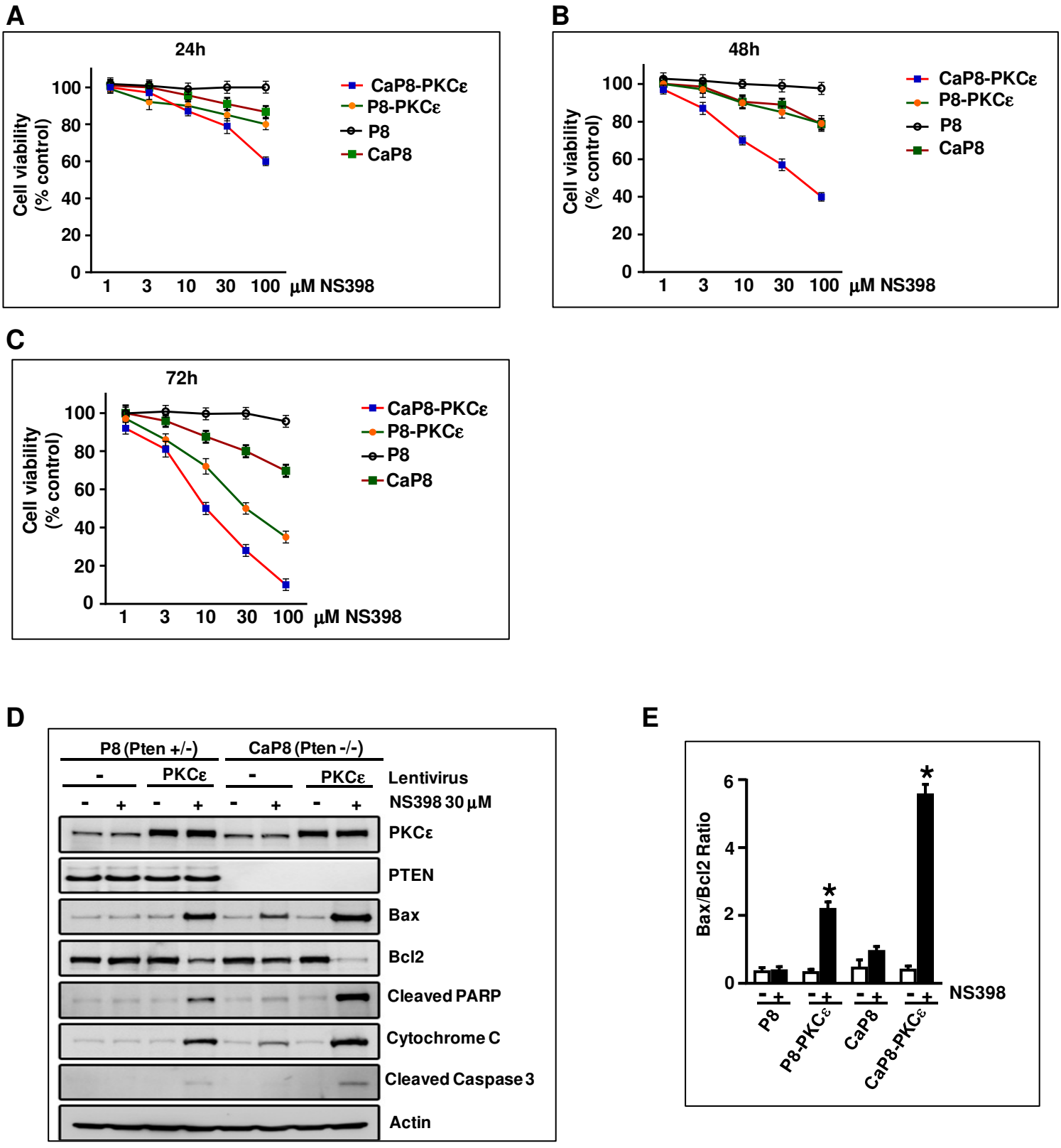
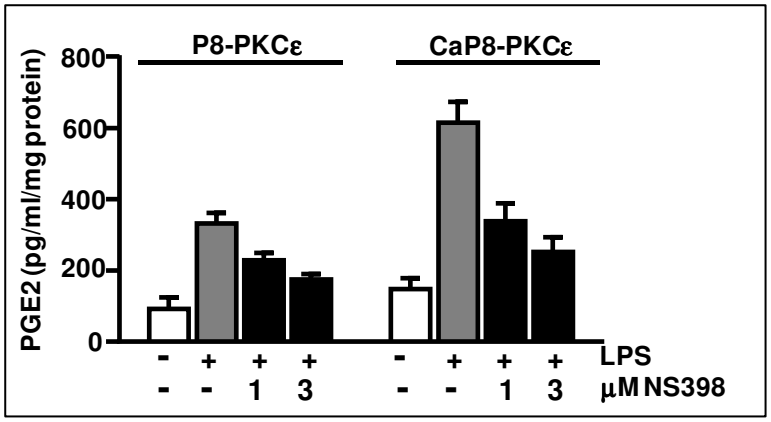

A
24h
Cell viability (% control)
100
80
60
40
20
0
1
3
10
30
100
μM NS398
CaP8-PKCε
P8-PKCε
P8
CaP8
B
48h
Cell viability (% control)
μM NS398
CaP8-PKCε
P8-PKCε
P8
CaP8
C
72h
Cell viability (% control)
μM NS398
CaP8-PKCε
P8-PKCε
P8
CaP8
D
P8 (Pten +/-)
CaP8 (Pten -/-)
-
PKCε
Lentivirus
NS398 30 μM
PKCε
PTEN
Bax
Bcl2
Cleaved PARP
Cytochrome C
Cleaved Caspase 3
Actin
E
Bax/Bcl2 Ratio
6
4
2
0
*
NS398
P8
P8-PKCε
CaP8
CaP8-PKCε

Figure 8



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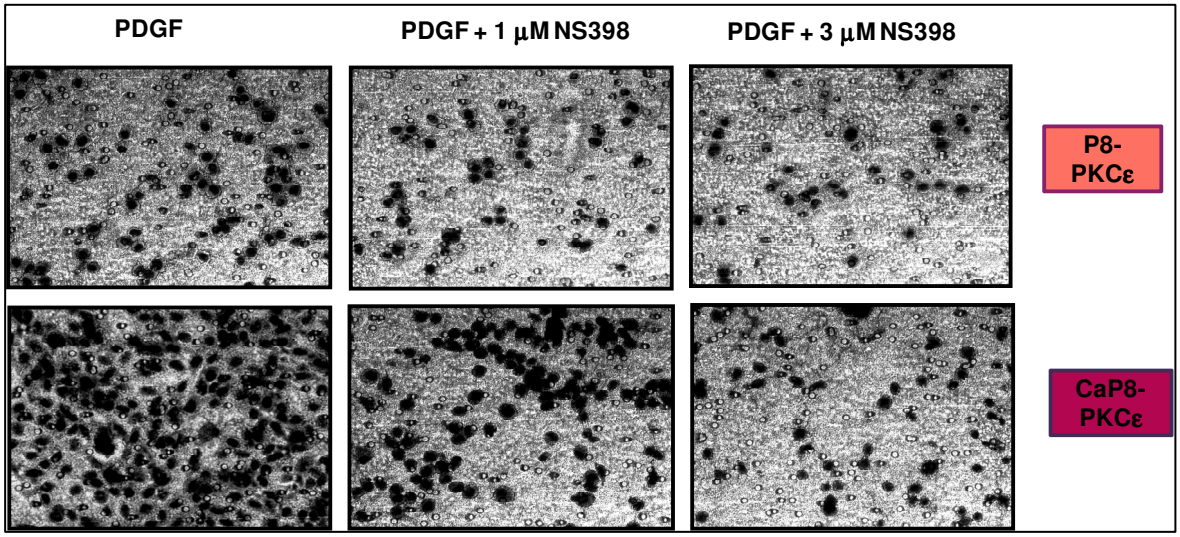

Figure 10

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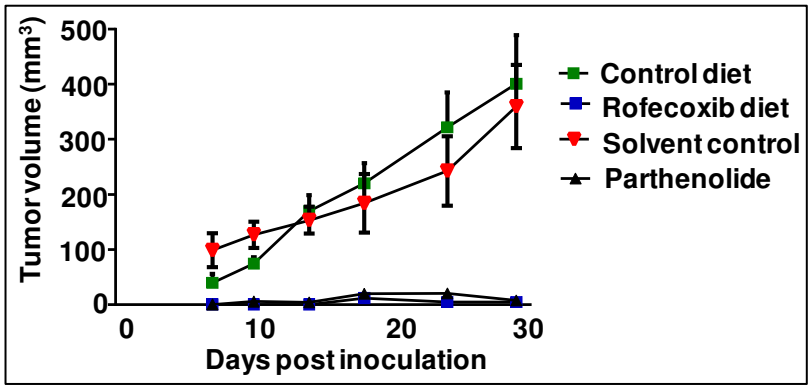

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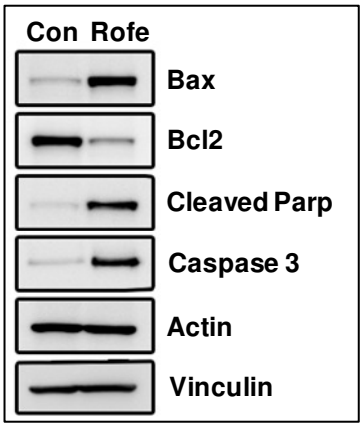

Figure 11

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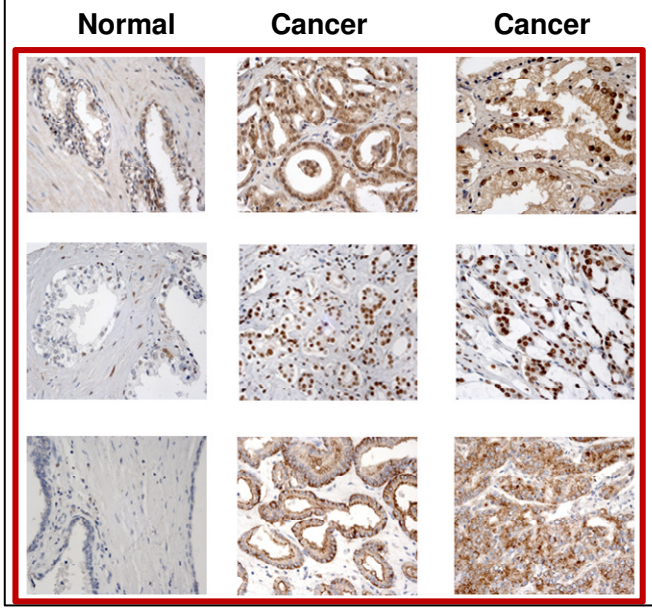

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