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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. We observe that PKCε RNAi depletion diminishes constitutive and stimulated COX-2 mRNA expression and PGE2 levels in prostate cancer cells. Conversely, PKCε overexpression by adenoviral means potentiates COX-2 expression in LNCaP cells. Thus, our study characterizes a novel molecular link between PKCε and NF-κB/COX-2 and its implication in survival pathways in prostate cancer.

15. SUBJECT TERMS
PKC Epsilon, Prostate Cancer, COX-2, TNF receptor

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INTRODUCTION

The protein kinase C (PKC) isozymes represent one of the most studied Ser/Thr kinases in signaling, mitogenesis and transformation [1-2]. The PKC family has been classified into 3 classes based on their structural and biochemical properties: classical PKCs (calcium-dependent cPKCs α, βI, βII, and γ), novel PKCs (calcium-independent nPKCs δ, ε, η, and θ) and atypical PKCs (aPKCs ζ and ι) [3]. The first 2 groups are activated by the phorbol ester tumor promoters and the lipid second messenger diacylglycerol (DAG), a lipid generated by phospholipase C (PLC) in response to tyrosine-kinase and G-protein-coupled receptor (GPCR) activation [3]. Despite our extensive knowledge on PKC regulation and function, the specific roles for individual PKC isozymes in the context of cancer, particularly in prostate cancer, is only partially understood. Prostate cancer cells express three phorbol ester/DAG-responsive PKCs: cPKCα, and nPKCs δ and ε [4]. Studies from our laboratory and others have established that PKCδ generally acts as a negative regulator of proliferation and/or mediates apoptotic responses [5-6]. On the contrary, PKCε is a prosurvival and mitogenic kinase [7-8]. In prostate cancer cells PKCε mediates survival through Bad-dependent and Bad-independent mechanisms and is involved in the transition to androgen-independence [9-10]. PKCε up-regulation is observed in >95% of human prostate specimens [11-12].

Notably, PKCε emerged as a potential oncogene and is up-regulated not only in prostate cancer but also in several other epithelial cancers including lung, breast, and thyroid cancer [12-14]. Up-regulated PKCε levels have also been reported in prostate tumors from TRAMP mice [12]. We recently demonstrated that transgenic overexpression of PKCε in the mouse prostate causes preneoplastic lesions with elevated NF-κB levels [15-16]. 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. Furthermore, we demonstrated that PKCε facilitates the formation of the TNFR-I complex, thereby mediating the activation of NF-κB in prostate cancer [16].There is ample evidence that the NF-κB pathway is significantly dysregulated in metastatic prostate cancer and play important roles in the regulation of several cellular processes including survival, proliferation, inflammation, migration, and invasion [17-18].

The main goal of our research supported by DOD is to determine if PKCε mediates the activation of COX-2, a well-known NF-κB responsive gene, to drive essential responses relevant to prostate cancer progression. We also aim to elucidate if PKCε is a mediator of TNFα signaling in prostate cancer.

BODY

1. **Role of PKCε in TNFα-triggered TNF receptor phosphorylation.** The p60TNFR contains numerous potential phosphorylation sites in the cytoplasmic region [19]. We first determine if TNFα triggers phosphorylation on serine, threonine, and/or tyrosine residues of the receptor. LNCaP cells were stimulate with TNFα (25 ng/ml) for 5 min and subjected to IP with an anti-TNFR-I antibody. Western blots were probed with phosphoamino acid antibodies. It was observed that TNFα treatment mediates the phosphorylation on serine and threonine residues but not on tyrosine residues of p60TNFR **(Fig. 1A-1C)**. Next, we intended to determine the involvement of PKCε in TNFα-induced receptor

phosphorylation. For this set of experiment, we first inhibited PKCε in LNCaP cells using the specific PKCε antagonist εV1-2. Cells were then stimulated with TNFα (25 ng/ml) and receptor was immunoprecipitated after 5 min. Notably, PKCε inhibition significantly decreased the TNF-α-induced serine phosphorylation of the TNFR, with marginal effects on threonine phosphorylation **(Fig. 2A-B)**.

2. **Mass spectrometry to identify sites in TNFR-I that undergoes phosphorylation.** To our knowledge there are no commercial phospho-TNFR antibodies available, hence we next intended to identify phosphorylation sites in TNFR-I using mass spectrometry. TNFR-I immunoprecipitated from LNCaP cells and immunoprecipitation was confirmed by Western blot. However, we could not detect a corresponding band on the Coomassie Blue gel, indicating very low yield of the immunoprecipitated TNFR-I (**Fig. 3A-B**). We then modify our approach of immunoprecipitation by covalently coupling the TNFR-I antibody onto an amine-reactive resin in the presence of sodium cyanoborohydride solution. This marginally increased the yield (**Fig. 3C-D**) as detected on a Coomassie Blue-stained gel. The TNFR-I band was excised from the gel and subjected to trypsin digestion. The peptides were enriched using immobilized metal affinity chromatography, and spotted on MALDI plate. The peptide masses obtained by MALDI analysis were used to search protein databases with search engines Mascot and Sequest. This analysis identified only 5-6 peptides corresponding to TNFR-I but none identified as a phosphoresidue bearing peptide (**Fig. 3E**). Our repeated attempts for analyzing phospho-residues by mass spectrometry were not successful because of the low yield of the immunoprecipitated TNFR-I. The possible reasons for this low yield could be the very low abundance of the endogenous receptors in cells [20]. In fact, most of the adaptor proteins that bind to the receptor were identified under the conditions of overexpression utilized in yeast two-hybrid systems or by affinity purification using recombinant proteins [20-21].

3. **Cyclooxygenase-2 (COX-2) is overexpressed in human prostate cancer cell lines akin to PKCε and NF-κB.** We first measure the protein expression of COX-2 across different prostate cell lines. As seen in **Fig. 4A**. prostate cancer cells express higher levels of COX-2 as compared to the normal prostate (RWPE-1) cells. Furthermore, this correlated with the mRNA levels as determined by qPCR (**Fig. 4B**). These results are in conjunction with the upregulated levels of PKCε and NF-κB observed in prostate cancer cell lines compared to RWPE-1 cells [16].

4. **PKCε regulates LPS- or TNFα-stimulated COX-2 levels in human prostate cancer cell lines**. In the first set of experiment, LNCaP cells were transfected with either control or PKCε siRNA duplexes and stimulated 48 h later with LPS (5 μg/ml) or TNFα (20 ng/ml). RNA was extracted 4 h later. Notably, silencing PKCε with two different RNAi duplexes significantly attenuated LPS- and TNFα-induced COX-2 mRNA levels **(Fig. 5)**. As an alternative approach, we overexpressed PKCε in LNCaP cells using an adenoviral approach. A LacZ adenovirus (AdV) was used as a negative control. Notably, ectopic expression of PKCε potentiates LPS or TNFα-induced COX-2 mRNA levels **(Fig. 6)**.

5. **PKCε regulates constitutive COX-2 levels in human prostate cancer cell lines**. PC3 cells were transfected with either control or PKCε siRNA duplexes. After 48 h, COX-2 mRNA levels were determined by qPCR. We observed that silencing PKCε in PC3 cells using two different RNAi duplexes

led to a significant reduction in COX-2 mRNA levels **(Fig. 7)**. Therefore, PKCε mediates constitutive activation of COX-2 in androgen-independent prostate cancer cells.

6. **PKCε regulates PGE2 levels in human prostate cancer cell lines.** COX is the rate limiting enzyme involved in the biosynthesis of prostaglandins and exists in two forms: COX-1 (constitutively expressed) and COX-2 (regulated isoform) [22]. Importantly, increased prostaglandins synthesis is associated with poor disease prognosis in humans [23]. PKCε-depleted PC3 cells show significant decrease in PGE2 production **(Fig. 8A)**. To further establish the requirement of PKCε in this response, we used a pharmacological approach. The cell-permeable, Tat-fused peptide εV1–2 selectively blocks the translocation and activation of PKCε but not of other PKCs [16, 24]. As shown in **Fig. 8B**, εV1–2 significantly inhibited PGE2 levels in PC3 cells compared with the Tat control peptide. Overall, these results strongly advocate for a role of PKCε in the COX-2 and PGE2 activation in prostate cancer cells.

7. **Overexpression of PKCε in "normal" prostate epithelial RWPE-1 cells increases COX-2 and PGE2 levels**: We overexpressed PKCε in RWPE-1 cells, a model used as "normal" non-transformed epithelial prostate cells. PKCε cDNA was subcloned into the pLenti6/V5-DEST lentiviral vector (Invitrogen). Lentiviral particles were generated using HEK293T cells and the Virapower packaging System (Invitrogen). RWPE-1 cells stably transduced with the PKCε lentivirus and selected with blasticidin (0.3 μg/ml) showed elevated PKCε expression relative to the vector control (**Fig. 9A**). RWPE-PKCε and parental RWPE1 cells were treated with LPS (5 μg/ml) for 4 h and RNA was isolated. RWPE-PKCε display increased COX-2 mRNA levels in response to LPS stimulation **(Fig. 9B)**. Likewise, we observe significantly enhanced PGE2 levels in LPS-treated RWPE-PKCε cells compared to parental cells **(Fig. 9C)**.

KEY RESEARCH ACOMPLISHMENTS

(i) We successfully demonstrated the role of PKCε in TNFα-induced serine phosphorylation of TNFR-I in PC3 prostate cancer cells.

(ii) We successfully knocked down PKCε in LNCaP and PC3 cells.

(iii) We successfully overexpressed PKCε in RWPE-1 cells.

(iv) We determined that COX-2 is overexpressed in human prostate cancer cells.

(v) We demonstrated that PKCε regulates stimulated and constitutive activation of COX-2 in prostate cancer cells.

(vi) We demonstrated that PKCε regulates PGE2 levels in prostate cancer cells.

(vii) We found a marked induction of COX-2 and PGE2 levels in normal prostate epithelial cells upon PKCε overexpression.

CONCLUSION

The main conclusions from the research carried out during the first year of DOD funding are as follows: (i) PKCε mediate TNFα-induced phosphorylation of TNF receptor at serine residues. (ii) PKCε mediates the activation of COX-2, a well-known NF-κB responsive gene 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. We will continue with our studies to address the role of PKCε overexpression in normal prostate epithelium and how it leads to COX-2 activation. We would also put our efforts in determining if COX-2 mediates PKCε responses in prostate cancer, particularly in the context of Pten loss, a common genetic alteration found in prostate cancer.

REPORTABLE OUTCOMES

Garg, R., Benedetti, L., Abera, M., Wang, H., and Kazanietz, M.G. PROTEIN KINASE C AND CANCER: WHAT WE KNOW AND WHAT WE DON'T. Oncogene. Accepted, In Press (2013).

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APPENDICES

FIGURE LEGENDS:

Figure 1: TNF-α treatment mediates TNFR phosphorylation on serine and threonine residues. LNCaP cells were treated with either vehicle or TNFα (25 ng/ml) and subject to IP with an anti-TNFR-I antibody. Total

and immunoprecipitated TNFR-I levels were determined by Western blot. IgG was used as a control for the immunoprecipitation. TNFR-I immunoprecipitates were analyzed for phosphorylation by Western blot using (A) anti-phosphotyrosine (PY20), (B) anti-phosphoserine, or (C) anti-phosphothreonine antibodies.

Figure 2: Effect of PKCε on TNFα-triggered TNFR phosphorylation. LNCaP cells were treated with thePKCε specific inhibitor εV1-2 or control Tat carrier peptide (1 μM, 1 h) and then stimulated with TNFα (25 ng/ml) or vehicle. After 5 min of TNFα stimulation, TNFR-I was immunoprecipitated with an anti-TNFR-I antibody. The effect of PKCε inhibition on receptor phosphorylation was determined by Western blot in TNFR-I immunoprecipitates using (A) anti-phosphoserine and (B) anti-phosphothreonine antibodies.

Figure 3: Mass spectrometry to identify phosphorylation sites in TNFR-I. LNCaP cells (10 $x10^6$) were treated with TNFα (25 ng/ml) or vehicle for 5 min. Cleared cell lysates were immunoprecipitated with an anti-TNFR-I antibody, washed, and resolved by SDS/PAGE. IgG was used as a control. (A) Western Blot to confirm TNFR-I immunoprecipitation. (B) Coomassie blue stained gel picture. Molecular weight markers in kDa are indicated. TNFα-stimulated LNCaP cell lysates were immunoprecipitated using a TNFR-I antibody that is coupled to an amine-reactive resin in the presence of sodium cyanoborohydride solution. (C) Western blot to confirm the TNFR-I immunoprecipitation. (D) Coomassie blue stained gel picture. The arrow indicates the position of the band that was excised for enzymatic digestion by trypsin and subsequent mass spectrometric analysis. (E) List of some of the molecules identified from the in-gel digest. Number of tryptic peptides observed by MALDI analysis that matched tryptic peptides derived from a given protein in the database is shown. Arrow indicates the identification of TNFR-I. List has been curtailed from a total of 66 proteins identified.

Figure 4: COX-2 is overexpressed in human prostate cancer cell lines. (A) Protein and (B) mRNA expression of COX-2 in different prostate cell lines. *, $p<0.05$ *vs.* RWPE-1 cells (n=3).

Figure 5: Silencing PKCε reduces LPS- or TNFα-stimulated COX-2 levels in human prostate cancer cell lines. LNCaP cells were transfected with two different PKCε RNAi duplexes (ε 1 or ε 2) or a non-target control RNAi duplex (*NTC*). After 48 h, cells were stimulated with LPS (5 μg/ml) or TNFα (20 ng/ml) or vehicle control for 4 h and RNA was isolated. PKCε depletion was confirmed by qPCR. Results were normalized to endogenous 18 S.*, $p<0.05$ *vs.* control (n=3).

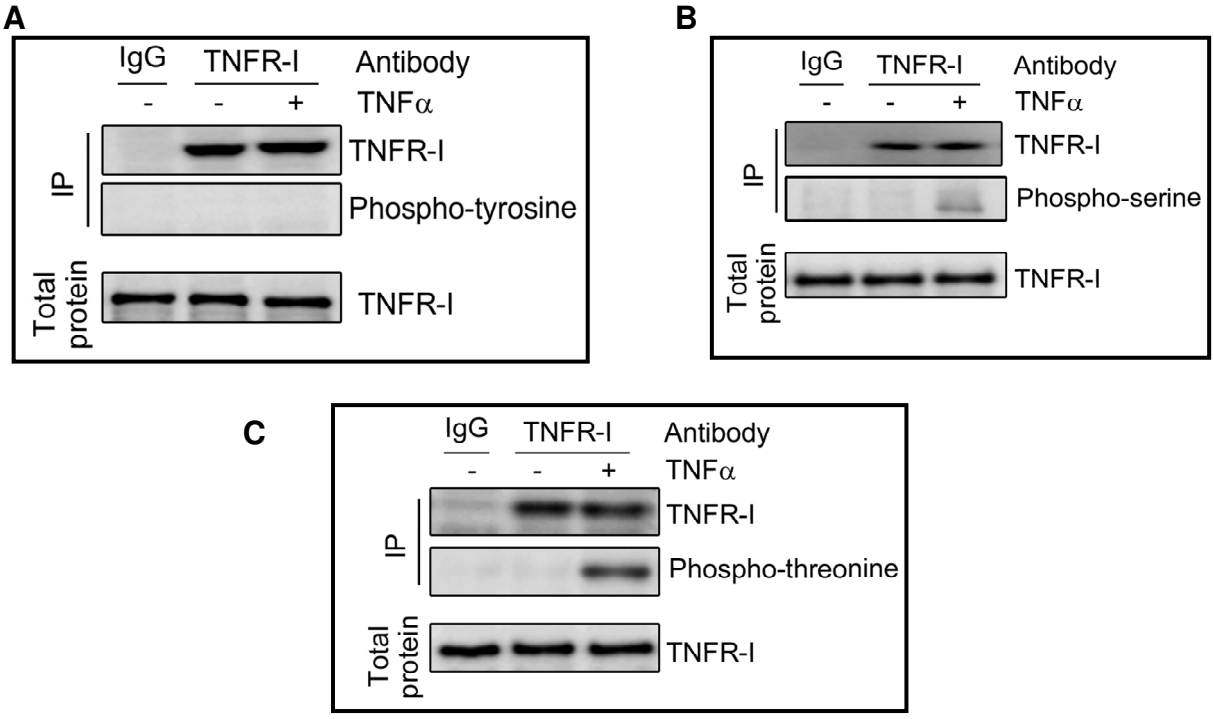
Figure 6: Overexpression of PKCε potentiates LPS- or TNFα-stimulated COX-2 levels in human prostate cancer cell lines. LNCaP cells were infected with PKCε AdV or LacZ control (10 MOI). After 48 h, cells were stimulated with LPS (5 μg/ml) or TNFα (20 ng/ml) or vehicle control for 4 h and RNA was isolated. PKCε overexpression was confirmed by qPCR. Results were normalized to endogenous 18 S.*, $p<0.05$ *vs.* control (n=3).

Figure 7: PKCε regulates constitutive COX-2 levels in human prostate cancer cell lines. PC3 cells were transfected with either control or PKCε siRNA duplexes. After 48 h, COX-2 mRNA levels were determined by qPCR. Results were normalized to endogenous 18 S.*, $p<0.05$ *vs.* control (n=3).

Figure 8: PKCε regulates PGE2 levels in human prostate cancer cell lines. PC3 cells were transfected with either control or PKCε siRNA duplexes. After 48 h, PGE2 levels (normalized to total protein), were measured in the culture media using an EIA kit. *, $p<0.05$ *vs.* control (n=3).

Figure 9: Overexpression of PKCε in "normal" prostate epithelial RWPE-1 cells increases COX-2 and PGE2 levels. (A) RWPE-1 cells were infected with ViraPower lentiviruses containing a human PKCε construct cloned into pLenti6/V-5 DESTRWPE-1. Expression of PKCε was analyzed by Western blot using an anti-PKCε antibody. Vinculin was used as control. RWPE-1 cells overexpressing or parental cells were stimulated with LPS (5 μg/ml) or vehicle control for 4 h and RNA was isolated. (B) COX-2 mRNA levels were determined by qPCR. (C) PGE2 levels (normalized to total protein), were measured in the culture media using an EIA kit. *, $p<0.05$ *vs.* control (n=3).

Figure 1



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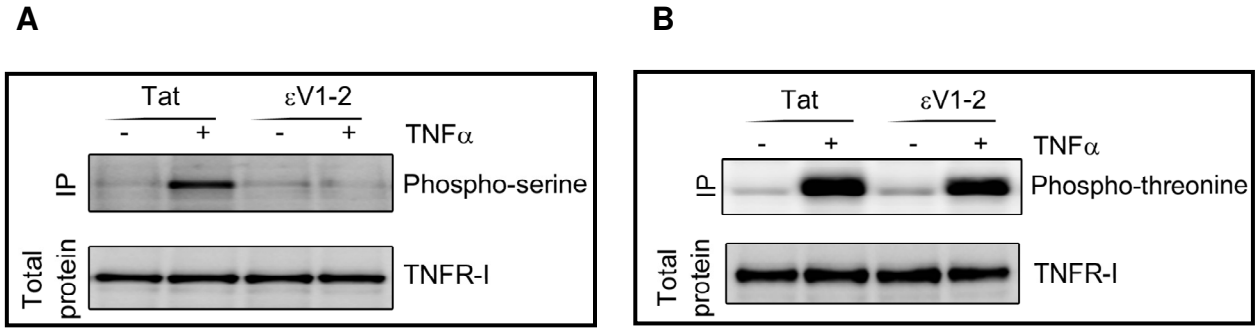

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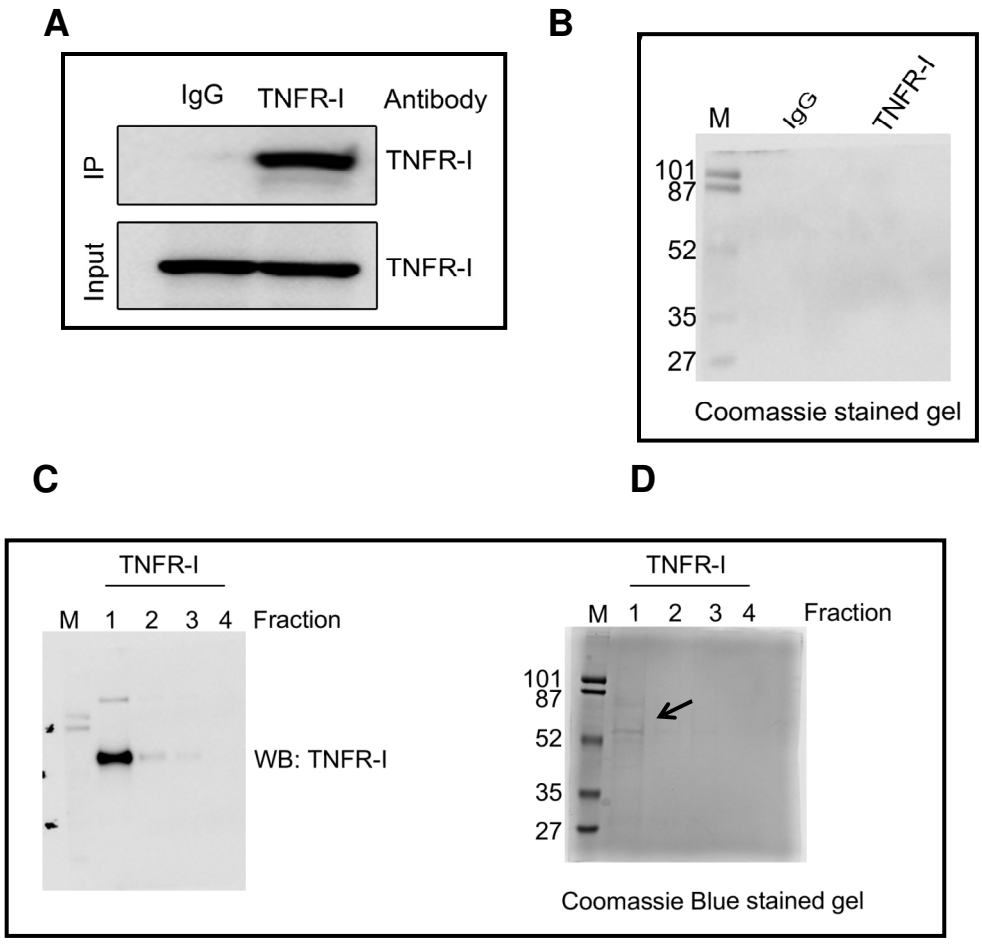

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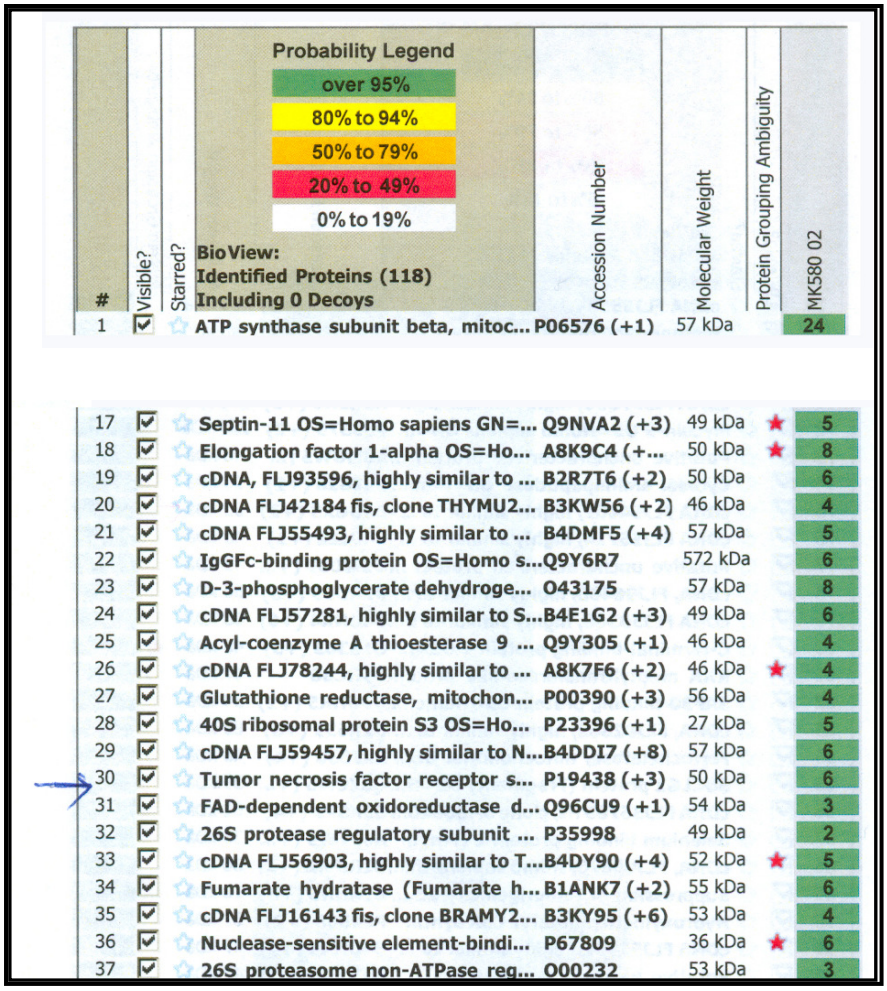
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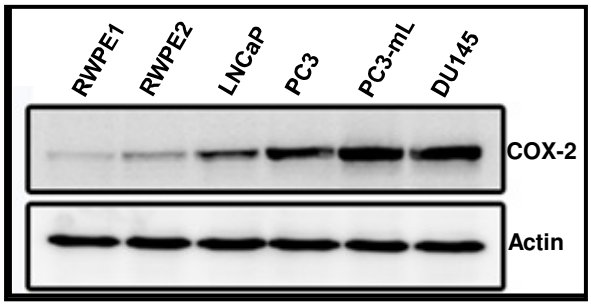
Figure 4

A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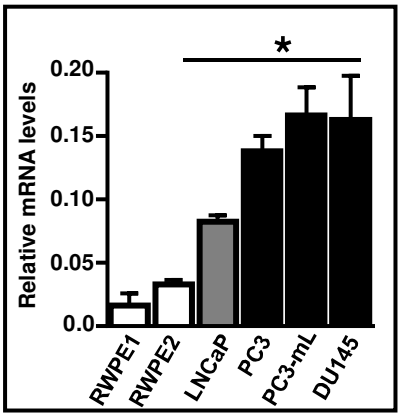
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Figure 5

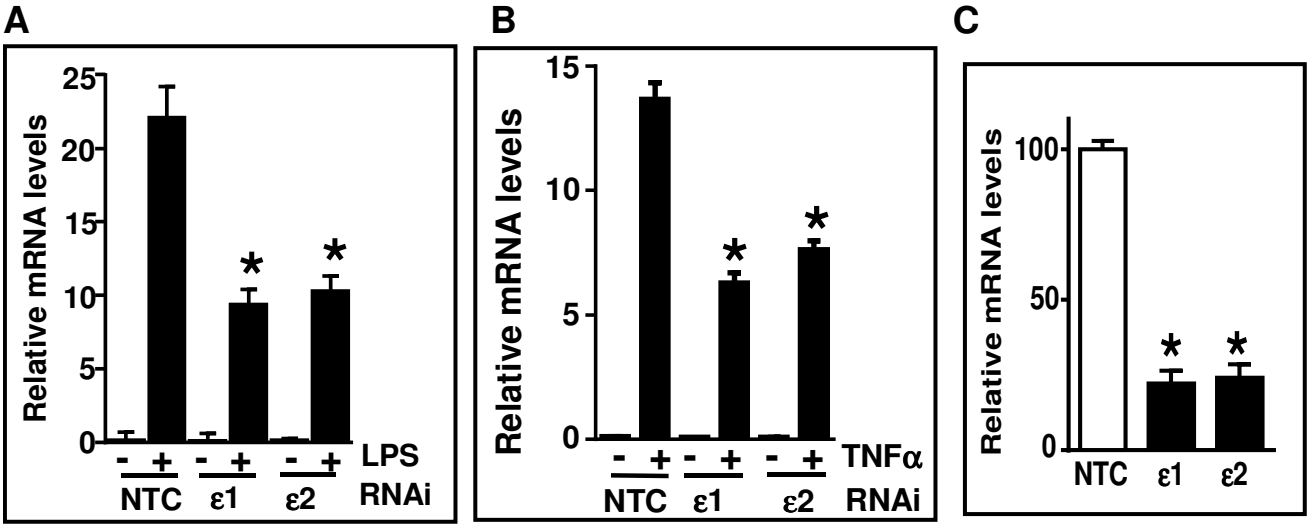


Figure 6

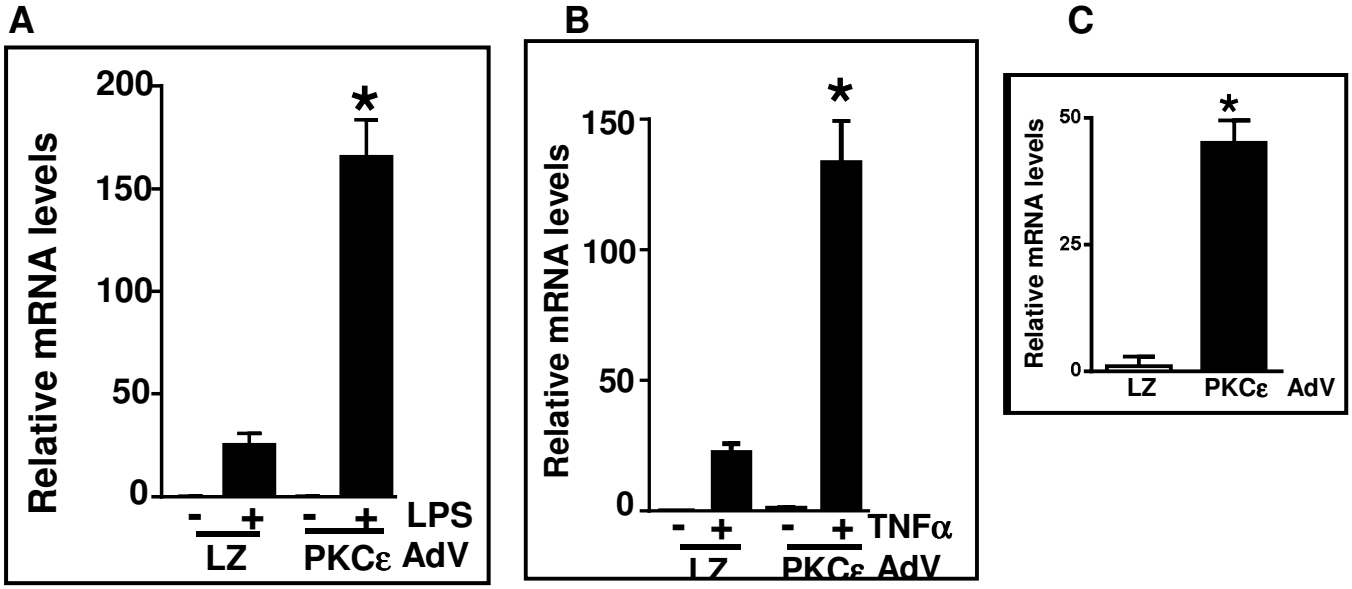


Figure 7

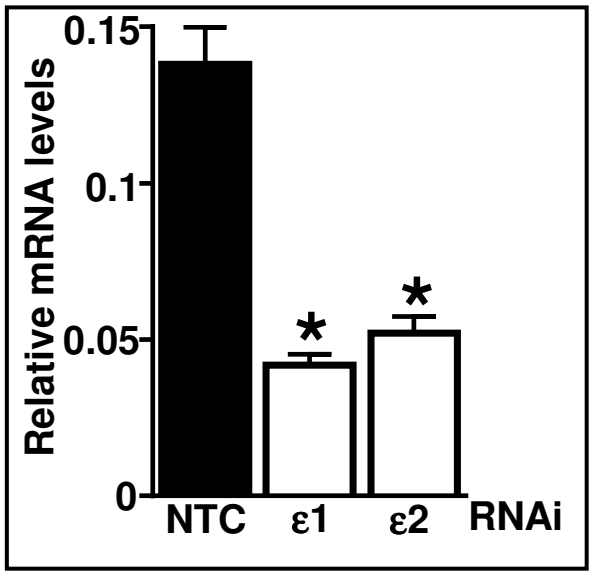
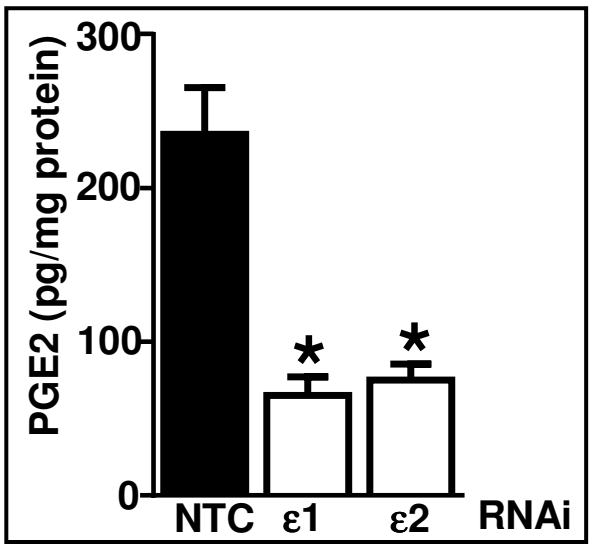


Figure 8

A



B

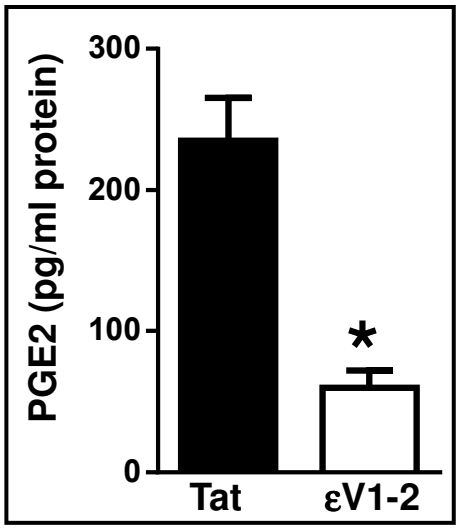
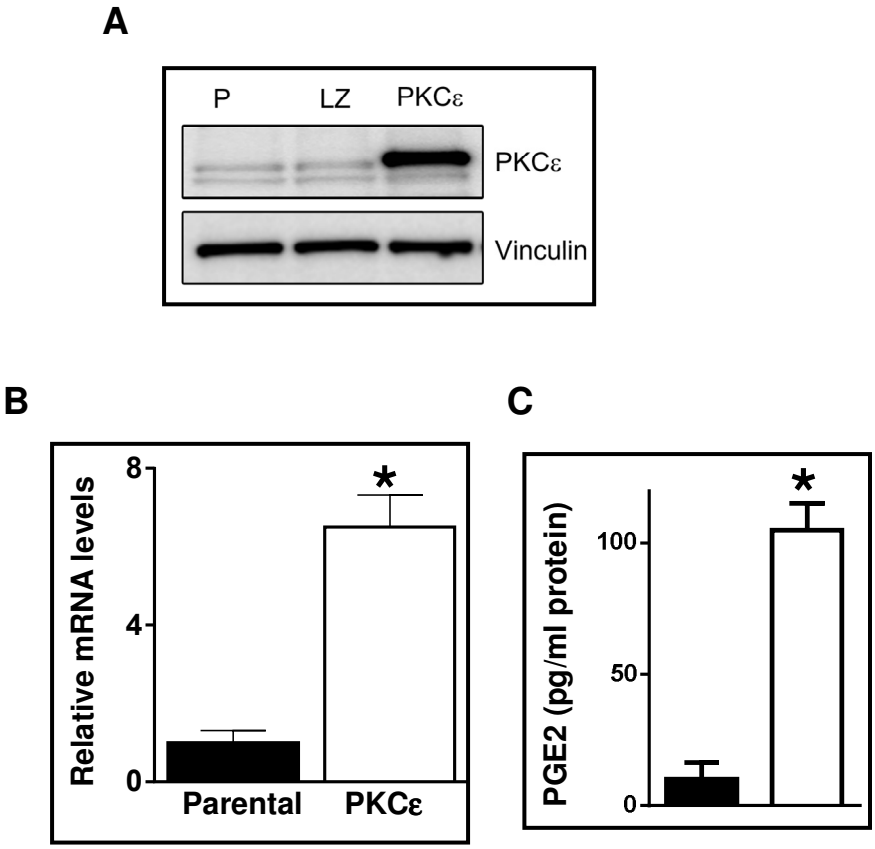


Figure 9


A
P
LZ
PKCε
PKCε
Vinculin
B
Relative mRNA levels
8
4
0
*
Parental
PKCε
C
PGE2 (pg/ml protein)
100
50
0
*