

***PRKC- ζ -b* expression promotes the aggressive phenotype of human prostate cancer cells and is a novel target for therapeutic intervention**

Sheng Yao¹, Alix Bee¹, Daniel Brewer², Andrew Dodson¹, Carol Beesley¹, Youqiang Ke¹, Laurence Ambroisine³, Gabrielle Fisher³, Heinrich Møller⁴, Tim Dickinson¹, Patricia Gerard¹, Lu-Yu Lian⁵, Janet Risk⁶, Brian Lane⁷, Victor Reuter⁸, Daniel Berney⁹, Christine Gosden¹, Peter Scardino⁸, Jack Cuzick³, Mustafa Djamgoz¹⁰, Colin Cooper², Christopher Foster¹

¹Division of Cellular Pathology and Molecular Genetics, University of Liverpool, L69 3GA, UK

²Institute of Cancer Research, Sutton, Surrey, SM2 5NG, UK. ³Cancer Research UK Centre for Epidemiology, Mathematics and Statistics, Queen Mary University of London, EC1M 6BQ, UK. ⁴King's College London, Thames Cancer Registry, SE1 3QD, UK. ⁵School of Biological Sciences, University of Liverpool, L69 7ZB, UK. ⁶Molecular Genetics and Oncology Group, University of Liverpool, L69 3GN, UK. ⁷Liverpool Biomedical Research Center, University of Liverpool, L69 7ZB, UK. ⁸Departments of Pathology and Urology, Memorial Sloan Kettering Cancer Center, NY 10021, USA. ⁹The Orchid Tissue Laboratory, Barts and The London School of Medicine and Dentistry, E1 2AD, UK. ¹⁰Division of Cell & Molecular Biology, Imperial College London, South Kensington Campus, SW7 2AZ, UK

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Address: Division of Cellular Pathology and Molecular Genetics
School of Cancer Studies
6th Floor, Duncan Building
University of Liverpool
Daulby Street
Liverpool
L69 3GA, U.K.
Telephone: 0151 706 4484
Fax: 0151 706 5883
email: csfoster@liv.ac.uk

Abstract

We show Protein Kinase C-zeta variant “b” (PKC- ζ_b) to be a novel predictive biomarker for survival from prostate cancer ($p < 0.001$). We also confirm that transcription of the *PRKC- ζ* gene is crucial to the malignant phenotype of human prostate cancer. Following siRNA silencing of *PRKC- ζ* in PC3-M prostate cancer cells, stable transfectant cell-line si-*PRKC- ζ_b* .PC3-M_{TI-6} is phenotypically non-malignant *in-vitro* and *in-vivo*. Genome-wide expression analysis identified 373 genes to be differentially expressed in the knockdown cells and four key gene-networks to be significantly perturbed during phenotype modulation. Functional interconnection between some of the modulated genes is revealed, although these may be within different regulatory pathways, emphasizing the complexity of their mutual interdependence. Genes with altered expression following *PRKC- ζ* knockdown include *HSPB1*, *RAD51* and *IDI* that we have previously described to be critically important in prostatic malignancy. Since expression of *PRKC- ζ_b* is functionally involved in promoting the malignant phenotype, we propose PKC- ζ_b as a novel and biologically-relevant target for therapeutic intervention in prostate cancer.

Introduction

Prostate cancer is the second most common cancer to affect men worldwide. In the USA, in 2008, 186,320 new cases of prostate cancer and 28,660 deaths from this disease were reported ¹. Equivalent figures from the United Kingdom reveal that in 2006, 35,515 new cases were diagnosed and that 10,239 men died of this disease in 2007 ². Unlike some human malignancies, prostate cancer is not associated with a specific high penetrance susceptibility gene ³ but with multiple gene loci ^{4,5} each independently conferring a low but cumulative risk. The clinical potential of an individual prostate cancer may range from relative indolence to highly aggressive with progression occurring rapidly. Hence, there is an urgent requirement to develop reliable biomarkers that accurately stratify prostate cancer at diagnosis and segregate men with aggressive cancers requiring urgent treatment from those who may be managed conservatively ⁶. While extent of disease is more useful than clinical stage at diagnosis, and retains a low level of significance in multivariate analysis, neither parameters is of clinical utility being significantly inferior to Gleason score or PSA ⁷.

A potential functional relationship between PKC- ζ , encoded by the gene *PRKCZ* located on chromosome 1 at p36.33-p36.2, and prostate cancer was first suggested by our study that showed expression of PKC- ζ to be enhanced in prostatic malignancy when compared to morphologically non-malignant prostatic epithelium ⁸. These findings were subsequently validated by other studies in cancers of breast ⁹, lung ^{10, 11} and pancreas ^{12, 13}. Reduction in PKC- ζ inhibits cell migration and invasion, confirming the protein to be functionally important to the malignant phenotype ^{10, 11}. While our original data suggested that strong PKC- ζ expression might identify a subset of aggressive prostate cancers, the cohort was heterogeneous and too few to provide reliable statistics. This study has assessed PKC- ζ expression in the largest group of well-characterized and conservatively managed patients yet to be assembled world-wide ⁷.

Protein Kinase C (PKC) isoenzymes together comprise a complex super-family of 14 serine-threonine kinases characterized by structural similarities in certain gene-elements and functional peptide domains ¹⁴. Broadly considered as regulators of cellular homeostasis and behavioral phenotype, these enzymes are increasingly identified to be polyfunctional. The genes for all of these enzymes characteristically encode multiple splice variants that are differentially expressed between tissues in a wide range of animal species during tissue morphogenesis ¹⁵, especially between malignant tissues and their benign histogenic counterparts ^{8, 12}. Different PKC isoforms exhibit opposing cell-regulatory actions ^{16, 17}. In prostate cancer, preliminary studies demonstrated only the expression of PRKC- ζ isoform “b” in PC3-M cells ¹⁸.

Examples of genes selectively modulated in prostate cancer are now relatively frequent ¹⁹. Some of these, either up- or down-regulated, have been proposed as robust biomarkers able to segregate different cancer phenotypes according to parameters that include behavior and response to therapy ²⁰. While altered expression does not confirm, *ipso facto*, a functional role in malignancy, genes that we have demonstrated to be functionally relevant to the aggressive phenotype of prostate cancer include *cFABP* ²¹, *RPL19* ²², *ID1* ²³, *RAD51* ²⁴ and *HSPB1* ²⁵. Studies reported from this laboratory ²⁶ and elsewhere ^{27, 28} have also established a functional relationship between PKC- ζ voltage-gated ion

channels ¹⁶ and the invasive phenotype of prostate cancer cells ²⁹. Similarly, the behavior of malignant cells is influenced by expression of cell-surface complex glycoconjugates, particularly sialic acids ^{30, 31}. Therefore, the objectives of this study were: (i) to confirm the predictive biomarker potential of PKC- ζ _b protein expression in a large cohort of untreated human prostate cancers (ii) to test the hypothesis that translation and expression of *PRKC- ζ* is functionally important in promoting and/or maintaining the aggressive phenotype of prostate cancer, and hence a potential drug target, and (iii) to elucidate the genes and their associated networks modulated by *PRKC- ζ* .

Results

PKC- ζ protein expression in prostate tissues and clinical correlations

Information on PKC- ζ staining intensity was available for 2879 cores from 783 patients. Morphological appearances of the distribution of cytoplasmic PKC- ζ expression ranged from benign through malignant (Figure 1). For the 667 patients with positive staining (i.e. ≥ 1), their demographics and tumor characteristics are presented in Table 1. Figures 2a & 2b show the distribution of PKC- ζ staining and intensity in the 901 morphologically unremarkable cores (10%), 798 hyperplastic cores (36%), 38 PIN cores (76%) and 1142 cancer cores (90%) analyzed in the arrays. The relationship of PKC- ζ expression (binary variables: staining intensity ≤ 2 and $= 3$) with demographics and tumor characteristics is presented in Table 2. At both levels, PKC- ζ expression was associated with Gleason score ($p < 0.001$), clinical stage ($p < 0.001$) and amount of cancer in each specimen ($p < 0.001$). No association was found with age or baseline PSA. At high intensity (i.e. $= 3+$), expression of PKC- ζ was associated with worse survival from prostate cancer (Figure 2c) and poor overall survival (Figure 2d). Univariate Cox model analysis (Table 2) revealed PKC- ζ expression to be a significant prognostic factor of cause-specific survival ($\chi^2 = 20.53$, $p < 0.001$) and overall survival ($\chi^2=8.54$, $p = 0.002$). However, when added to a model with Gleason score, PKC- ζ expression became a weakly significant prognostic factor for cause-specific survival (HR = 1.44, 95% CI = 1.00-2.07, $p = 0.05$). Similarly, when added to a multivariate model comprising Gleason score, extent of disease, baseline PSA and age at diagnosis, PKC- ζ expression was of borderline significance (HR = 1.41, 95% CI = 0.98-2.02, $p = 0.06$) In each multivariate of these models, there was no association with overall survival. We conclude from these analyses that PKC- ζ expression is highly correlated with existing prognostic markers including Gleason score and clinical stage but does not, alone, represent an independent prognostic marker. However, within poor-prognosis prostate cancers, it might define a phenotypic subset amenable to therapeutic manipulation of *PRKC- ζ_b* .

PRKC- ζ_b mRNA expression

Previous Western blotting studies indicated that *PRKC- ζ_b* was the only isoform expressed in prostate cancer cell-lines and most strongly expressed in PC3-M cells (Figure 3a) that we selected for use as our model system. qRT-PCR (Figure 3b) revealed *PRKC- ζ_b* mRNA expression in PC3-M (androgen-independent) cells at ~ 5 -fold ($p < 0.01$), DU145 at ~ 5 -fold ($p < 0.01$) and LNCaP (androgen-dependent) at ~ 3 -fold ($p < 0.01$) higher than that in PNT-2 cells (Figure 3c). We used *PRKC- ζ_b* siRNA expressed in pSilencerTM 4.1-CMVneo (Ambion) to create nine PC3-M transfectant cloned cell-lines (si-*PRKC- ζ_b* -PC3-M_{T1-1} - T1-6 and si-*PRKC- ζ_b* -PC3-M_{T2-1} - T2-3). All nine clones displayed a significant loss of substrate adhesion when compared to un-transfected PC3-M_{control} cells. The transfected cells appeared disparate, non-adhesive and contained multinucleate forms easily identifiable microscopically (Figure 3d), suggesting failure to complete mitosis. mRNA analysis (Figure 3e) demonstrated the most consistent effective reduction in colony si-*PRKC- ζ_b* -PC3-M_{T1-6}, in which *PRKC- ζ_b* was reduced to 37% \pm 17% of the PC3-M_{control} cells relative to β -actin ($p < 0.005$). Other cell lines exhibited less reduction,

ranging from $43\% \pm 4\%$ to $54\% \pm 8\%$ ($p < 0.01$) or were less consistent in their level of gene knockdown. Cells transfected with empty vector and PC3-M_{scramble} cells exhibited no significant reduction in their level of *PRKC-ζ_b* mRNA ($p > 0.5$).

Growth characteristics of si-PRKC-ζ_b-PC3-M_{TI-6} cells in-vitro

Proliferation assay revealed the rate of cell division by the si-*PRKC-ζ_b*-PC3-M_{TI-6} cells was significantly reduced ($p < 0.05$) when compared to PC3-M_{control} and PC3-M_{scramble} (Figure 4a). Apoptosis quantified by flow cytometry, was not significantly affected in the transfected si-*PRKC-ζ_b*-PC3-M_{TI-6} cells (Figure 4b). The levels of endogenous apoptosis identified within the control cells were similar to those obtained during comparable studies of the *RPL19* gene³². In the soft agar tumorigenesis assays (Figure 4c), cell colonies formed after three weeks. PNT-2 cells yielded no colonies while PC3-M_{control} cells and all transfectant cells formed colonies (Figure 4d). Whereas parental PC3-M_{control} cells generated 499 ± 22 colonies and PC3-M_{scramble} cells generated 541 ± 10 colonies, the si-*PRKC-ζ_b*-PC3-M_{TI-6} cells generated 120 ± 11 colonies ($p < 0.005$). In the Matrigel invasion assay, all knockdown cell-lines revealed reduced cell migration ($p < 0.01$) when compared to the parental PC3-M_{control} cells, (1775 ± 65 cells). The si-*PRKC-ζ_b*-PC3-M_{TI-1} cells scored 106 ± 26 cells migrating while si-*PRKC-ζ_b*-PC3-M_{TI-2} cells scored 129 ± 26 cells (Figure 4e,f). Thus, the effect of selectively inhibiting *PRKC-ζ_b* was to severely reduce the ability of all transfected cells to invade a semi-solid collagen-based stroma.

Tumorigenic behavior of si-PRKC-ζ_b-PC3-M_{TI-6} cells in-vivo

Statistically significant reductions in tumor volume and weight occurred for *PRKC-ζ_b*-PC3-M_{TI-6} cells. Comparison of mean tumor volumes (Figure 5a) revealed: PC3-M_{control} (272 ± 213 mm³), PC3-M_{scramble} (81 ± 70 mm³), si-*PRKC-ζ_b*-PC3-M_{TI-2} (9 ± 8 mm³) and si-*PRKC-ζ_b*-PC3-M_{TI-6} (36 ± 38 mm³). Significant differences were observed between the PC3-M_{scramble} group and si-*PRKC-ζ_b*-PC3-M_{TI-6} ($p < 0.005$, Mann-Witney U Test). At autopsy (Figure 5b), the average tumor weight generated by PC3-M_{control} cells (337 ± 249 mg) was 4.7-fold and 21-fold, respectively, higher than that of the siRNA transfectant cells si-*PRKC-ζ_b*-PC3-M_{TI-2} (16 ± 11 mg) and si-*PRKC-ζ_b*-PC3-M_{TI-6} (71 ± 80 mg). The mean tumor weight generated by PC3-M_{scramble} cells was 7.5 fold higher than that in the group si-*PRKC-ζ_b*-PC3-M_{TI-2} ($p < 0.005$) and in si-*PRKC-ζ_b*-PC3-M_{TI-6}, although no significant was observed between PC3-M_{scramble} and si-*PRKC-ζ_b*-PC3-M_{TI-6}. However, the trend in tumor weight between the control groups and the transfectant gene knockdown groups decreased dramatically (Figure 5c). Although the mean weight of the si-*PRKC-ζ_b*-PC3-M_{TI-6} tumors was higher than that in the si-*PRKC-ζ_b*-PC3-M_{TI-2} group, the difference was not significant.

Hsp-27 expression and phosphorylation status

PKC-ζ and Hsp-27 are known to be involved in the regulation of cell locomotion and metastasis, particularly through actin filament organization²⁵, although no direct relationship has been reported between these two proteins. Western blotting confirmed the level of Hsp-27 expression identified by the generic antibody to be lower in the PNT-2_{control} cells than in the PC3-M_{control}, PC3-M_{scramble} and the si-

PRKC-ζ_b-PC3-M_{T1-6} lines, where expression was comparable (Figure 6a). While no differences were apparent in the site-specific phosphorylation of PC3-M_{control} and PC3-M_{scramble}, phosphorylation of Ser⁸² was significantly enhanced in the si-*PRKC-ζ_b*-PC3-M_{T1-6} cells. Phosphorylation at Ser¹⁵ and Ser⁷⁸ was similarly low in PC3-M_{control} and PC3-M_{scramble} when compared to the benign PNT-2_{controls}. Thus, unlike the effect of knocking-down the genes *FABP5* or *RPL19*, reducing *PRKC-ζ_b* expression did not alter the overall level of Hsp-27 protein but selectively increased Hsp-27 phosphorylation at Ser⁸² although not at Ser¹⁵ or Ser⁷⁸.

Glycoconjugate expression

Western blotting to detect sialylated glycoproteins revealed subtle differences in binding-patterns between the control and knockdown cells, particularly gain of Neu5Acα2,6Gal- structures identified by *Sambuccus* (Figure 6b) indicating significant changes to specific proteins. These might be caused by modulated levels of the particular proteins or changes in the structure of their oligosaccharides consequent upon alterations to the level of certain glycosyltransferases. Intact PC3-M_{control} and si-*PRKC-ζ_b*-PC3-M_{T1-6} cells expressed identical patterns of complex oligosaccharides identified by the five lectins (Table 3). The expression of sialic acids linked either Neu5Acα2,6Gal- (*Sambuccus*) or Neu5Acα2,3Gal- (*Maackia*) was confirmed following neuraminidase digestion of the intact cell preparations but revealed no gross differences (Figure 6c). The biotinylated lectins from *Ulex europaeus*, *Lotus tetragonolobus* and *Aleuria aurantia* were employed to detect subtle differences in terminal fucosyl linkages recognized to modulate metastatic behavior of a range of malignancies especially prostate cancer cell trafficking³³. No differences were revealed in terminal fucose linked L-Fucα1,6GlcNAc-/L-Fucα1,2Glc- (*Lotus*) or L-Fucα1,6GlcNAc-/L-Fucα1,3NLac- (*Aleuria*) although L-Fucα1,2Galβ1- (*Ulex*) was not identified in either the control or knockdown cells.

Comparative DNA oligonucleotide expression profiling of si-PRKC-ζ_b-PC3-M_{T1-6} cells

No statistically significant (adjusted $p \geq 0.05$) differences were found between PC3-M_{control} and PC3-M_{scramble} cell-lines, confirming that the transfection technique was not responsible for off-target effects to bias the experimental data. The hybridisation data revealed 549 DNA sequences representing 373 genes to be differentially expressed ($p \leq 0.05$, Benjamini and Hochberg multiple testing correction applied) following RNAi knockdown of *PRKC-ζ_b*. Of these, 85 genes were enhanced and 288 down-regulated, 188 different genes being modulated at least four-fold, 31 up-regulated (Supplemental Table 1) and 157 down-regulated (Supplemental Table 2). Frequently, multiple sequences for an individual gene were ranked in close proximity, confirming the quality of this dataset. For three up-regulated genes (*PLAT*, *CDKN2C* and *HSPB1*) and one down-regulated gene (*FOXA2*) the alterations were validated in qRT-PCR experiments (data not shown). According to current databases (Gene Cards <http://www.genecards.org/> and AceView NCBI <http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/>) these genes reflected a wide range of biological functions, with Gene ontology (GO) term enrichment analysis identifying some 40 biological process and 13 molecular function GO terms (Supplemental Tables 3,4) to be significantly associated ($p < 0.001$) different. A heat map generated from the 100

genes most affected (Figure 7) showed the knockdown phenotype to be distinct and further shifted from the parental PC3-M cells than the non-malignant PNT-2 cells, thus confirming modulation of the malignant phenotype through down-regulation of *PRKCZ-ζ_b*. Ingenuity pathway analysis identified the top four ranked interlinked pathways and biological networks (Supplemental Table 5) containing genes expressed differentially following *PRKC-ζ* knockdown to be highly significant ($p \leq 10^{-28}$) and to include two networks with genes regulated by NFκB, networks centralised around p53 and networks around *KDMSB*, *MCM6* and *DDIT3* (Figure 8). This analysis further revealed that some of the genes modulated by down-regulated expression of *PRKC-ζ_b* are not discrete or independent but are functionally interconnected. This high level of cross-talk between apparently different biological processes provides the possibility for strategic manipulation of a single gene (demonstrated by the modulation of *PRKCZ-ζ_b*) to profoundly affect the phenotype of target cancer cells.

Glycosyltransferase genes modulated in si-PRKC-ζ_b-PC3-M_{TL-6} cells

Interrogation of the gene expression array data confirmed down-regulation of glycosyltransferase enzyme genes *GALNTL4* (~1.5-fold $p < 0.01$) and *GCNT3* (~1.5-fold $p < 0.01$). Potentially, their change in expression would significantly alter the terminal antennary structure of glycoprotein oligosaccharides thus affecting the substrates available for subsequent fucosyl- and sialyl-transferase activity³⁴. This possible mechanism is supported by the elevated expression (~1.6-fold $p < 0.01$) of the sialyl-transferase gene *ST6GALNAC2* (N-acetyl-GalNAc2,6sialyltransferase) responsible for the Neu5Acα2,6Gal- structure identified by *Sambucus* and found to be increased in the *PRKCZ-ζ_b* knockdown cells (Figure 6b).

Ion channel genes modulated in si-PRKC-ζ_b-PC3-M_{TL-6} cells

The oligonucleotide expression arrays were interrogated to determine whether genes encoding ion channels were modulated following *PRKC-ζ_b* knockdown. Two groups affected were voltage-gated K⁺ channel genes *KCNN4* (up-regulated ~2.4-fold, $p < 0.005$), *KCNK10* (down-regulated ~1.8-fold, $p < 0.01$) and *KCNMA1* (down-regulated ~1.5-fold, $p < 0.01$) together with the Na⁺/K⁺ transporting ATPase's β1 and β3 polypeptide genes *ATP1B1* (down-regulated ~1.6-fold, $p < 0.01$) and *ATP1B3* (down-regulated ~1.5-fold, $p < 0.01$). Although the specific functions of these channels is complex, they are all involved in cellular homeostasis of various types, including maintenance of cell polarity. These expression array data reveal a possible functional interaction between the glycosyltransferase genes and the ion channel genes such that they together contribute to modulating the parental PC3-M cells from a malignant towards a benign phenotype.

Discussion

At diagnosis, PKC- ζ protein expression is confirmed as a powerful predictive biomarker for survival from prostate cancer ($p < 0.001$) and for overall survival ($p < 0.001$). Furthermore, PKC- ζ expression accurately segregates men with poor prognosis prostate cancer that requires active treatment from those with relatively indolent disease that should be managed conservatively. PKC- ζ expression also correlates strongly with Gleason grade ($p = 0.001$) but not with baseline PSA values ($p > 0.2$) despite PSA being an independent predictive variable in the original cohort of patients⁷. In multivariate analysis, diminished significance ($p < 0.05$) of this variable suggests that it defines a biologically distinct group within the overall population of aggressive prostate cancers. Although most benign tissues did not express PKC- ζ , a small proportion of hyperplasias and PIN (Figure 2a,b) were stained, indicating that early up-regulation of the protein may be a biomarker of epithelial pre-neoplasia *in-situ*, one of the precursors of invasive prostate cancer³⁵.

These RNAi studies, the first to be reported in human prostate cancer (Supplemental Table 6), support the original hypothesis by establishing functionality of *PRKC- ζ_b* in the malignant phenotype of human prostate cancer cells. Knockdown of the gene results in a significant reduction in malignancy, confirmed both *in-vitro* and *in-vivo*. The subsequent array data also show that *PRKC- ζ_b* is pleiotropic and regulates a variety of genes involved in several distinct but interactive networks. Hence, there is no *single* mechanism by which *PRKC- ζ_b* knock-down ameliorates the malignant phenotype. Nevertheless, one of the critical mechanisms revealed by this study is consistent with modulation of the genes for ion channels *KCNK10* and *KCNMA1*. Cellular proliferation of prostate cancer cells is controlled by a variety of K⁺ channels, including voltage-, Ca²⁺-, ATP-gated and two-pore K⁺ and maxi-K_{Ca} channels³⁶ encoded by *KCNK10* and *KCNMA1* through in a regulatory network with *PRKC- ζ_b* . Conversely, down-regulation of voltage-gated K⁺ channel activity can be associated with invasive behavior³⁶. Such behavior could be subserved by *KCNN4* coding for small/intermediate-conductance K_{Ca} channels found earlier in the metastatic PC-3 but not in non-metastatic LNCaP cells³⁷. These data together with the finding that enhanced *KCNMA1* expression is functionally involved in breast cancer invasion and metastasis to brain³⁸, not only exemplify the biological relevance of these channels but supports a reciprocal interaction between ion channels, alterations in glycosyltransferase expression and malignancy. The cellular distribution and role of the ion channels modulated following *PRKC- ζ_b* -knockdown is directly dependent upon their appropriate patterns of N-linked glycosylation such that any alteration inhibits their functionality causing impaired cell polarization^{39, 40}. Similarly, changes in ion channels disturb intracellular pH gradients with the consequence that certain proteins undergo alternative patterns of glycosylation, as evidenced by the glycoconjugate expression data (Figure 6) potentially resulting in their modulated distribution and/or functionality⁴¹.

The importance of *PRKC- ζ_b* in prostate cancer cell motility is supported by reduced colony formation in soft agar and impaired tumor cell invasion through a collagen matrix (Figure 4c-f) following gene knock-down. A similar role for PKC- ζ has been proposed in pancreatic cancer cells¹³ after inhibition of *PRKC- ζ* either chemically⁴² or genetically⁴³. To develop the mechanical forces necessary for cell migration, as well as interacting with actin^{44, 45}, direct phosphorylation of myosin II-

B protein by PKC- ζ occurs in TSU-pr1 prostatic adenocarcinoma cells in an EGF-dependant manner when complexed with p21-activated kinase-1 (PAK-1). In this pathway, PKC- ζ is phosphorylated and activated by PAK-1, promoting phosphorylation of myosin II-B ⁴⁶.

In addition to a functionally-competent locomotor assembly, maintenance of cell polarity and loss of cell-cell adhesion are essential for cell migration ⁴⁷. For a cell to migrate, generation of a forward-rear polarity axis occurs principally through the PAR-PKC- ζ system ⁴⁸. A complex established between PAR6-Akt2 and PKC- ζ is activated by Cdc42 ⁴⁹. Upstream, the interaction between PKC- ζ and Akt2 is regulated directly by EGF ⁵⁰ and indirectly by TGFbeta controlling the integrity of intracellular tight junctions through the interaction of PAR6 with TGFbeta receptors ⁵¹. During locomotion, stabilization of lamellipodia required for cell migration and invasion ⁵² involves formation of the Cdc42/PAR6/Akt2/Rac1 polarity complex that, following activation by PKC- ζ , recruits Smurf1 to regulate the local level of RhoA ⁴⁵. Within these lamellipodia, phosphorylation of Hsp-27 simultaneously modulates transformation of G \rightarrow F actin ⁵³ in the presence of fascin to generate the mechanical forces required for cell motility to occur through organization of cytoskeletal components ⁵⁴ that we have previously shown to characterize the aggressive phenotype of human prostate cancer ²⁵. Our current studies clearly demonstrate enhanced site-specific phosphorylation of Hsp-27 Ser⁸² in si-*PRKC- ζ_b* -PC3-M_{T1-6} cells when PKC- ζ_b is reduced (Figure 6a). Thus, human prostate cancer cells depend upon the presence and integrity of both PKC- ζ_b and Hsp-27 to develop a malignant phenotype that is biologically competent to invade and metastasize. Presently, these are regarded as simultaneous and independent processes because there is no evidence of that Hsp-27 Ser⁸² is a substrate for PKC- ζ_b phosphorylation or that *PRKC- ζ_b* transactively regulates transcription of *HSPB1* and hence the level of Hsp-27, despite increasing evidence that PKC- ζ is an essential transcriptional regulator for other genes ^{10, 55, 56}.

Identification of gene interaction networks is a powerful approach to segregating prostatic malignancies into biologically-defined groups amenable to different types of therapeutic intervention ⁵⁷. ⁵⁸. However, there is a caveat that application of the types of data reported herein depends upon the integrity and expression of key genes that determine the genotype of each individual prostatic malignancy. In PC3-M prostate cancer cells, the gene *TP53* is non-mutated, characteristic of approximately 60% of primary prostate cancers, supporting the proposition that down-regulation of *PRKC- ζ* would be effective in this group. Such cells are potentially able to regulate DNA replication through GINS complex members ⁵⁹, an essential component of the human replisome. In the current study, down-regulation of *GINS2* (~7-fold, $p < 0.05$) was associated with a concomitant reduction in *PCNA* (~8-fold, $p < 0.01$), *CDC2* (~7-fold, $p < 0.05$), *CCDC74B* (~8-fold, $p < 0.01$) and *CDC45L* (~10-fold, $p < 0.05$) indicating altered regulation of mitosis during initiation as well as S-phase and G₂-M progression ⁶⁰ to occur as a consequence of *PRKC- ζ_b* knockdown. This method has revealed the members of several different networks to be reduced following knockdown of *PRKC- ζ_b* (Supplemental Table 2). These included the gene *IDI* (~8-fold, $p < 0.05$) and *RAD51* (~10-fold, $p < 0.01$) that we have already shown to be associated ($p < 0.001$) with aggressive prostate cancer ^{23, 24}. In this study, members of the NF κ B pathway were principally reduced, particularly *NFKBIA* (~10-fold, $p < 0.01$). The NF κ B

complex is recognized to be inhibited by I κ B proteins (NF κ BIA or NF κ BIB) that trap and inactivate NF κ B⁶¹. Phosphorylation of serine residues within the I κ B proteins by a range of kinases marks them for destruction following ubiquitination, thereby allowing activation of the NF κ B complex. Simultaneous up-regulation of phosphoenolpyruvate carboxykinase 2 (*PKC2*, ~4-fold, $p < 0.05$, Supplemental Table 1) provides evidence of a more complex pathway modulated by *PRKC- ζ_b* knockdown. Other genes down-regulated in this extensive network include *BEX1* (~15-fold, $p < 0.05$), *CLX1* (~70-fold, $p < 0.01$) and *CLX2* (~20-fold, $p < 0.05$). *BEX1* knockdown accelerates cell differentiation and potentiates NF κ B in response to NGF while reduction of *CXCL1* and *CXCL2* expression is NF κ B-dependent, requiring intact I κ B.

This study has revealed how suppression of a single key pleiotropic gene in an established cancer cell can perturb the interactive balance between members of the cell's genome so that a shift occurs from a malignant to a benign phenotype. Interpolation of the current data suggests that such an effect may be the converse of the network imbalance now believed to be generated by the combined small effects of a number of different genes acting together to promote emergence of the malignant phenotype^{3, 62}. Although RNAi is effective *in-vivo* against established prostate cancers⁶³, small molecule inhibitors would provide a more flexible therapeutic approach. Novel protein kinase C inhibitors⁶⁴, already exhibit both tissue- and tumor-specificity against different PKC isoforms⁶⁵. Presently, the most effective agents are the PKC- β inhibitors briostatins and enzastaurin, the latter being trialed against human lung cancer⁶⁶ while in human NSCLC, aurothiomalate and aurothioglucose are selective and effective inhibitors of PKC- α , the homologue of PKC- ζ ⁶⁷. Recently, spheciosterol sulfates extracted from Philippine sponges⁶⁸ and a novel class of small molecules (the 3-hydroxy-2-(3-hydroxyphenyl)-4H-1-benzopyran-4-ones) have been described as isoform-selective PKC- ζ inhibitors⁶⁹. Therapeutically employed for psychostimulant abuse, the potential value of these latter agents has yet to be assessed in treating epithelial malignancies. With reference to their inhibitory effects on prostate cancer metastases, bisphosphonates (e.g. pamidronate) specifically inhibit PKC- α and PKC- ζ , possibly through a network effect on urokinase-type plasminogen activator⁷⁰. It is anticipated that the emerging PKC isoform-selectivity of new small molecule inhibitors⁷¹ together with the identification of tissue-specific PKC variants^{18, 72} will provide a high level of tumor-specificity for therapeutic intervention in the management of malignant diseases.

Herein, we provide robust evidence that PKC- ζ is not only functionally involved in promoting and maintaining prostate cancer but that the gene *PRKC- ζ_b* is a biologically effective target for control of this malignancy. Furthermore, the consequences of reducing PKC- ζ through *PRKC- ζ_b* gene knockdown, particularly identification of other potential targets such as the voltage-gated K⁺ channels⁷³, allows development of a comprehensive therapeutic strategy based upon detailed knowledge of biological pathways active in subsets of human prostate cancer. Understanding the potential impact of such approaches is not possible without a robust and validated database of molecular interactions. For cancer therapeutics, there are two corollaries: First, is that mathematical modeling of genes expressed by an individual cancer will allow accurate prediction of susceptibilities and weaknesses that might be exploited to treat each malignant disease in a biologically-appropriate manner⁷⁴. Second, is the concept

of phenotypic modulation to treat cancers ⁷⁵. Through phenotypic modulation, prevention of metastases while maintaining the general health of a patient with early cancer would provide additional time to introduce second-line therapies to target specific networks such as NFκB ^{76, 77} in the knowledge that these would be biologically appropriate to accommodate the phenotypic changes induced in malignant cells by a first-line therapy.

Materials and Methods

Patient Cohort and Tissue Micro-Arrays (TMAs) for PKC- ζ analysis

The study analysed a panel of TMAs constructed from a retrospective cohort of >2000 men with prostate cancer assembled from the records held by UK cancer registries and managed only conservatively^{7, 78}.

Ethical approval

National approval for the collection of the cohort was obtained from the Northern Multi-Research Ethics Committee followed by local ethics committee approval at each of the collaborating hospital trusts. This work was approved by the Clinical Research and Ethics Committee at the Royal Marsden Hospital and The Institute of Cancer Research.

PKC- ζ immunohistochemistry

Mouse monoclonal antibody sc-216 to PKC- ζ (Insight Biotechnology Ltd. Wembley, Middlesex, UK) recognizes a unique 20-peptide sequence located in the –COOH terminal domain of variant “b” (NM_002774) encoded by exon 90 (AceView nomenclature) of the *PRKCZ* gene¹⁸. TMA sections were processed, stained and analyzed^{8, 25}.

Analysis of PKC- ζ expression

Specimens were considered positive only when at least 5% of the epithelial cells (either normal or malignant) unequivocally expressed PKC- ζ staining⁷⁹. This cut-off was the same as that used to distinguish positive and negative immunohistochemical staining in our previous studies^{8, 25}. Staining was assessed as negative, weakly positive or only focally positive (low-level expression), or strongly positive (high-level expression) and scored as 0, 1, 2 or 3, respectively.

Statistical analysis of TMA immunohistochemistry

The primary endpoint for this study was time to death from prostate cancer. Time to death from any cause was the secondary endpoint. Univariate and multivariate analysis were performed by proportional hazard (Cox) regression analysis^{25, 80}. Correlation of PKC- ζ staining (3+ vs 2+ or less) with other known prognostic factors was done by 2-sample trend tests.

Cell lines

Human prostate cell-lines PNT-2 (benign) and PC3-M_{control} (highly malignant) were grown as monolayer cultures in RPMI 1640 (Invitrogen, Paisley, UK) supplemented with 10% (v/v) foetal calf serum (FCS, Invitrogen), penicillin (1000units/ml), streptomycin (100 μ g/ml), and L-glutamine (2mM). Media for the culture of all subsequent transfected cell-lines were also supplemented with 1 μ g/ μ l Geneticin (Sigma).

RNAi molecule designed to knock down PRKC- ζ_b

A unique 21nt sequence (GTGAGAGACATGTGTCGTCTT) located at the 5' end of *PRKC- ζ_b* was selected as the target site beginning with AA dinucleotide downstream of the start codon according to the siRNA user guide (<http://www.rockefeller.edu/labheads/tuschl/project.html>) using siRNA Target Finder http://www.ambion.com/techlib/misc/siRNA_finder.html. The target site was a unique sequence within an exonic coding region of the human *PRKCZ* gene and not recognized elsewhere in the human genome. Of the 40 potential *PRKCZ* splice variants, BLAST search against NCBI Human Nucleotides Database, GenBank, RefSeq and PDB confirmed this sequence to be contained within *PRKCZ* variants “a”, “h”, “l”, “v-l” and “v-f” in addition to variant “b”, the wild-type variant (NM_002774) recognized to occur in human prostate cancer. These are either not expressed in prostatic carcinoma cells PC3-M, Du145 or LNCaP or are truncated and considered to be functionally ineffective.

Top Strand:

5'-GATCCGTGAGAGACATGTGTCGTCTTCAAGAGAGACGACACATGTCTCTCACTTA-3'

Bottom Strand:

5'-CTAGGCACTCTCTGTACACATCAGAAGTTCTCTCTGCTGTGTACAGAGAGTGAAT -3'

The default Ambion loop sequence, TTCAAGAGA, was used to complete the hairpin structure. The siRNA expression vector kit used was p*Silencer*TM 4.1-CMV neo (Ambion). Top and bottom strands of the siRNA hairpin oligonucleotide were diluted to 1 μ g/ μ l in TE buffer and annealed in 50 μ l solution according to the manufacturer's instructions. The annealed siRNA template was ligated into the p*Silencer* 4.1-CMV vector using T4 DNA ligase (5U/ μ l) and the products cloned into DH5 α cells (Invitrogen). Transformed cells were grown for 16 hours on LB plates containing 100 μ g/ml ampicillin at 37°C. A negative control of non-transformed competent cells was also included. Clones were picked and the DNA plasmid isolated using a Qiaprep spin Miniprep Kit (Qiagen, Crawley, UK). Isolated plasmids were digested with *Bam*HI and *Hind*III (New England Biolabs, Hitchin, UK) and the presence of the siRNA 55bp insert was confirmed by sequencing prior to the siRNA expression vector being used to transfect recipient prostate cancer cell-lines. Orientation of the insert was confirmed by DNA sequencing (Lark Technologies, Essex, UK) using internal sequence primers.

Transfection of siRNA PRKC- ζ_b silencing construct

1.5x10⁵ PC3-M cells were transfected with p*Silencer* 4.1 CMV *PRKC- ζ_b* siRNA (1 μ g) or p*Silencer* 4.1 CMV-scramble-insert (1 μ g) using *SiPORT XP-1* (3 μ l) reagent (Ambion, Warrington, UK) in 6-well-plates (35mm diameter). 24 hours after transfection, 500ng/ml of G418 was added to medium RPMI1640 for selection. After 9-10 days selection, individual colonies from single cells containing stable clones were isolated using ring cloning and transferred into 24-well plates with medium containing G418 at 500ng/ml.

RNA extraction and cDNA synthesis

Total RNA was extracted with RNeasy Mini Kits (Qiagen). Total RNA concentration was measured using a NanoDrop (Labtech, Ringmer, UK) and RNA integrity assessed with a 2100 Bioanalyser (Agilent, Santa Clare, USA). The RNA integrity number (RIN) for all RNA used exceeded 9.0. First strand cDNA was synthesized from 0.5µg total RNA using AffinityScript™ Multiple Temperature cDNA synthesis kits (Stratagene, La Jolla, USA) according to the manufacturer's protocol.

Quantitative Real-Time PCR (qPCR)

PRKC-ζ_b mRNA expression levels were quantified by qPCR and normalized relative to human β-actin mRNA expression. Primer sequences employed are shown in Table 4. Reaction volumes were in 25 µl comprising 12.5 µl Stratagene's Brilliant® SYBR® Green Master Mix (2X), 0.5µM of both forward and reverse primers, 1µl cDNA and 11.5 µl water. Primers for qPCR were designed to span exon/exon boundaries within the mRNA to avoid amplification of genomic DNA.

Proliferation assay of controls and siRNA transfectants

An assay to identify the effect of *PRKC-ζ_b* suppression on cellular proliferation assessed cell-lines PNT-2, PC3-M_{control}, PC3-M_{scramble} and two si-*PRKC-ζ_b*-PC3-M clones. Exponentially-growing cells were seeded in triplicate sets at a density of 1 x 10³ cells/ml/well in 24 well plates. Over 6 days at 24 hour intervals, cell proliferation was calculated by measuring the increase in cell numbers in each replicate using a conventional MTT assay ⁸¹.

Apoptotic properties of controls and siRNA transfectants

Apoptosis was quantified using flow cytometry. Cells were seeded at 2x10⁵ cells/ml in 75cm² tissue culture flasks and the assay conducted during their linear growth-phase prior to reaching confluence. Cells were harvested by trypsinization, washed twice with PBS and re-suspended in buffer from the BioVision Annexin V-FITC kit in a 5ml flow cytometry tube. AnnexinV-FITC (5µl) and propidium iodide (10ng in 5µl aqueous solution) were added and the tubes incubated for 10 minutes in darkness at 4°C. Quantitative analyses of apoptotic cell levels were performed using an Epics Flow Cytometer (Beckman Coulter). The procedure was performed three times using biological replicates.

Soft agar tumorigenicity assay

Cells were grown under standard environmental conditions as suspension cultures at a concentration of 2.5 x 10³ cells/ml in 0.3% (w/v) agarose at 37°C, 100% humidity and 5% CO₂ in air. After 3 weeks incubation, cell colonies were counted and photographed using a GelCount™ (Oxford Optronix, Oxford, UK) instrument. Any colonies of diameter <30µm or >100µm were excluded, the latter probably signifying that these were not derived from single cells.

In-vitro invasion

Invasiveness of the si-*PRKC-ζ_b* transfectants was assessed *in-vitro*⁸². At 24-hour intervals, following fixation and staining with Crystal Violet (Sigma-Aldrich, St Louis, USA), invasion was measured by counting the number of cells transmigrating the membrane to its under-surface.

In-vivo growth

Tumorigenicity and metastatic ability *in-vivo* were measured⁸³. A deposit of 2×10^6 cells in 200µl PBS of each cell-line was injected into a single subcutaneous site in the right shoulder of 8 week-old male Nu/nu mice (Harlan Ltd., Oxon, UK). Four groups of cells were assessed: PC3-M_{control}, PC3-M_{scramble}, si-*PRKC-ζ_b*-PC3-M_{T1-6} and si-*PRKC-ζ_b*-PC3-M_{T1-3}. Tumor growth was monitored twice-weekly and the volume calculated⁸⁴. When any tumor reached the maximum size allowed under the conditions of the Home Office Project Licence PPL 40/2270, all mice were sacrificed. Each animal was autopsied. All tissues were processed, embedded in paraffin wax and histological sections cut at 4µm before staining with Gill's hematoxylin. All animal experiments were conducted under UKCCR guidelines.

Hsp-27 expression and phosphorylation status

Differential phosphorylation of regulatory protein Hsp-27 at Ser¹⁵, Ser⁷⁸ and Ser⁸² was assessed by Western blotting the proteins extracted from $\sim 1 \times 10^7$ cells from each line. Cell pellets were suspended in 1 ml of CellLytic-M lysis buffer (Sigma C2978) containing 10µl protease inhibitor cocktail (Sigma P8340), 10µl PMSF (0.1mg/ml), Na₃VO₄ (1mM) and NaF (1mM). Protein concentrations were determined by Bradford assay (BioRad kit 500-0006). Aliquots containing $\sim 10\mu\text{g}$ cell lysate proteins were separated electrophoretically in 12.5% (w/v) polyacrylamide Protogel quick-cast separating gels (National Diagnostics, EC-895). After separation, proteins were transferred onto PVDF membranes (GE Healthcare, RPN303F), blocked with a suspension of powdered dried milk in PBS (100mM, pH 7.6) and incubated overnight with monoclonal antibodies to generic Hsp-27 or to each of the phosphorylated serine sites (Upstate Cell Signaling Solutions 06-478, 07-388, 04-447 and 04-448, respectively). After washing and incubation with HRP-labeled secondary antibodies, membranes were incubated in ECL-Plus reagent (GE Healthcare, RPN 2133) before exposure to Amersham Hyperfilm (GE Healthcare, 28906839).

Glycoconjugate expression

To determine whether *PRKC-ζ* knockdown affected oligosaccharide profiles, lectin binding was performed using intact cells and their protein extracts. Cell blocks were prepared from cell-lines PNT-2, PC3-M_{control}, PC3-M_{scramble}, si-*PRKC-ζ_b*-PC3-M_{T1-6}⁸⁵. Sections cut at 5 µm were stained for Neu5Acα2→3Gal- and Neu5Acα2→6Gal- using the biotinylated lectins (Vector Laboratories, Peterborough, UK) from *Sambucus nigra* and *Maackia amurensis* respectively⁸⁶. Negative controls included the absence of staining when the lectins were not included in the staining protocol and removal of sialic acids with subsequent abolition of staining following pre-treatment of the slides with neuraminidase⁸⁷ prior to incubation with either of these lectins. Lectin-binding was confirmed using

avidin-fluorochrome conjugates visualized with an Olympus BX61 fluorescent microscope and x100 objective. To identify global changes in glycoprotein expression following siRNA knockdown of *PRKC-ζ_b*, Western blotting of cell lysates using each lectin was performed as described for the detection of Hsp-27 (above) with the exception that non-specific binding of protein to the PVDF membranes was blocked with BSA (1.5%, w/v) rather than milk. Before use, the optimal dilution of each lectin in PBS (100mM, pH 7.6) containing BSA (1.5%, w/v) was determined by titration against separated PC3-M_{control} cell-lysates.

Gene microarray and expression analysis

The effect of suppressing *PRKC-ζ_b* by gene knockdown on whole genome expression profiles was investigated using two-color Agilent Human genome 44k microarrays. Each hybridization was a distinct biological replicate. The design incorporated five cell-lines treated as fixed biological factors: PNT-2, PC3-M_{control}, PC3-M_{scramble}, PC3-M_{pool} and si-*PRKC-ζ_b*-PC3-M_{TI-6} (*PRKC-ζ_b* knockdown). The PC3-M_{scramble} cells were the common control comparator and so expression differences between these cells and the other cell-types were used as contrasts in the gene expression analysis. Hybridizations and data acquisition were performed according to the Agilent Human Genome Microarray 44K protocol. Spatial representations of the hybridisation signals were examined to ensure that there were no artefacts. The distribution of background and foreground signals and pre-normalisation MA plots were examined to measure the quality of the hybridisation. Low quality spots identified by the Agilent image processing software were not used in the subsequent analyses. Expression signal estimates were derived from the red (cy3) and green (cy5) Agilent Processed Signal data by normalizing using the LOESS algorithm and background correction using a fitted convolution of normal and exponential distributions^{88, 89}. An Aquantile normalisation between arrays was also performed. Expression analysis of log₂ transformed normalised data was performed in the R statistical programming language (R v 2.8.0) using the BioConductor framework⁹⁰. Gene expression was modeled with a fixed effects linear model with a term representing residual dye-effects and four cell-line specific contrasts in which the gene expression values of the control PC3-M_{scramble} cell lines were subtracted from the expression values of the PNT-2, PC3-M_{control}, PC3-M_{pool} and si-*PRKC-ζ_b*-PC3-M_{TI-6} cell lines. A channel-specific design matrix was constructed using the PC3-M_{scramble} cells as a common reference and this was incorporated into a linear model using BioConductor limma⁹¹. For each cell-line contrast, a moderated *t*-statistic was computed for each probe with the resulting *p*-values adjusted for multiple testing using Benjamini and Hochberg's method to control the false discovery rate⁹². Those sequences with an adjusted *p*-value < 0.05 were considered significantly differentially expressed between the two groups being compared. Gene ontology (GO) term enrichment analysis was performed separately with lists of significantly up- and down-regulated genes to find significant functional terms (FatiGO <http://babelomics.bioinfo.cipf.es/>). GO terms and KEGG networks that were significantly associated with the genes expressed differentially between si-*PRKC-ζ_b*-PC3-M_{TI-6} and PC3-M_{control} cell lines were assessed using hypergeometric tests (*p* < 0.001)⁹³. The list of genes expressed differentially between si-*PRKC-ζ_b*-PC3-M_{TI-6} and PC3-M_{control} cell lines was uploaded into the Ingenuity pathway analysis application

(Ingenuity® Systems, www.ingenuity.com). A score was computed for each network according to the fit of the original set of significant genes. This score reflects the negative logarithm of the *p*-value, which indicates the likelihood of the focus genes in a network being found together as a result of random chance.

Microarray validation

In addition to *NFKB1A*, *TNFSR6*, *MMP3* and *MMP10*³², gene profiles were validated in the si-*PRKC-ζ*-*b*-PC3-M_{T1-6} cells using qPCR to confirm the expression of *PLAT*, *HSPB1*, *CDKN2C* and *FOXA2* with respect to PC3-M_{scramble} cells, when normalized against human β-actin.

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

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Figure Legends

Figure 1 - Immunohistochemical expression of PKC- ζ in TMA cores of prostatic tissues.

Location of the high magnification regions shown below each of the cores is indicated by a corresponding rectangular field. Stromal expression was not identified in any of the malignant tissues examined. No expression of PKC- ζ was identified in the nuclei although the amounts may be below the level of immunohistochemical detection.

- i., ii, iii.) Normal, hyperplastic and mildly dysplastic prostatic tissue in which neither luminal nor basal epithelial cells express PKC- ζ either within the cytoplasm or in the nuclei.
- iv.) Dysplastic intra-glandular epithelium of cribriforming type strongly expressing PKC- ζ within the cytoplasm.
- v.) Moderately-differentiated prostatic adenocarcinoma (Gleason 3+3) weakly expressing PKC- ζ in the majority of malignant cells.
- vi.) Prostatic adenocarcinoma of pseudoglandular morphology (Gleason 4+3) strongly expressing PKC- ζ throughout the cytoplasm of all malignant cells.
- vii.) Moderately-differentiated prostatic adenocarcinoma (Gleason 3+3) morphologically similar to that in v.) but negative for PKC- ζ expression.
- viii.) Poorly-differentiated prostatic adenocarcinoma (Gleason 5+5) strongly expressing PKC- ζ in the cytoplasm of the majority of the malignant cells.

Magnification: All cores are magnified at x60. The detailed fields within each of the cores are magnified at x200.

Figure 2 - Expression of *PRKC- ζ_b* in prostate cell-lines and following RNAi.

PKC- ζ expression assessed between normal, hyperplastic, PIN and cancer cores. Information on PKC- ζ staining intensity was available for 2879 cores (783 patients). The figure presents the distribution of PKC- ζ staining intensity in the 901 normal cores, 798 hyperplastic cores, 38 PIN cores and 1142 cancer cores. **(B)** The maximum staining intensity in the 1142 cancer cores was subsequently calculated for each patient. The distribution of this variable is shown in the histogram. **(C)** Clinical outcome of patients with respect to death from prostate cancer ($p < 0.001$) according to intensity of PKC- ζ expression (≤ 2 vs $= 3$). **(D)** Clinical outcome of patients with respect to death from all causes ($p < 0.002$) according to intensity of PKC- ζ expression (≤ 2 vs $= 3$).

Figure 3

Expression of *PRKC- ζ_b* in prostate cell-lines and following RNAi: **(A)** Western blotting of control cell-lines confirming high expression of PKC- ζ in PC3-M cells and virtually undetectable levels in the non-malignant PNT-2 cell-line. Detection of β -actin protein was used as the standard. **(B & C)** Northern blot detection of *PRKC- ζ_b* mRNA in four prostate cell-lines following standard two-step RT-PCR.

Expression of β -actin was used as the reference gene. **(D)** Following stable knockdown of *PRKC- ζ_b* using vector-based si-RNA, altered cell morphologies were observed in the subsequent cell-clones. Comparison of the low power views of the parental (i) and si-*PRKC- ζ_b* -PC3-M_{T1-6} cells (ii) revealed significant loss of adhesion by the genetically modified cells. On high power, whereas the original (iii) cells were unicellular and compact, the transfected cells (iv) were disparate, non-adhesive and contained many multinucleate forms. **(E)** Using qPCR, reduced gene expression was identified in all nine cloned knockdown cell-lines when compared to the parental control cells. The data shown are the mean (\pm S.D.) of three separate experiments. The level of gene expression in the PNT-2 cells was set at unity.

Figure 4

Growth and migration of cells *in-vitro*: **(A)** Comparison of cell proliferation in monolayer cultures. *PRKC- ζ_b* gene knockdown reduced proliferation of the transfectant clones, particularly the si-*PRKC- ζ_b* -PC3-M_{T1-6} cells. The effect of transfecting scrambled RNA was not statistically significant. **(B)** Apoptosis was marginally enhanced in the knockdown si-*PRKC- ζ_b* -PC3-M_{T1-6} cells when compared to the controls, transfected scramble RNA and to comparator clone si-*PRKC- ζ_b* -PC3-M_{T1-2} cells. **(C & D)** Down-regulation of *PRKC- ζ_b* gene expression impaired the ability of cells to form colonies in oft agar. Cells from clones T1-6 and T1-2, scramble control, PC3-M and PNT-2 controls were cultured 5 days. Measurements were taken each day. Significant differences were found between different cell clones with the most pronounced down-regulation of colony formation occurring in the si-*PRKC- ζ_b* -PC3-M_{T1-6} cells. Quantitative assessment of tumorigenesis of different clones was determined by counting the number of colonies. The data shown are the mean (\pm S.D.) of three separate experiments. **(E & F)** RNAi-induced down-regulation of *PRKC- ζ_b* impaired the ability of PC3-M transfectant cells to migrate in a Matrigel invasion assay. After five days in culture, invasion probabilities in T1-6, T1-2 groups were calculated as the ratio of number of cells invading through the Matrigel insert membrane relative to the mean number of PC3-M_{control} cells.

Figure 5

Growth of cells *in-vivo*: **(A)** Inoculation of 2×10^6 cells was performed subcutaneously into the shoulder fat pad of each animal in 4 groups of 8 male immuno-compromised CD/1 nude mice. The groups comprised the transfectant clones si-*PRKC- ζ_{vb}* -PC3-M_{T1-6} and si-*PRKC- ζ_{vb}* -PC3-M_{T1-2}, PC3-M_{control} and PC3-M_{scramble} cells. Tumor size was measured every 3 days for 15 days after the inoculation. Tumor volumes were calculated using the formula $V=L \times H \times W \times 0.5237$. The data are the mean tumor volumes (\pm S.D.) of eight animals in each group. **(B & C)** The histogram shows the occurrence and weight of tumor present in each animal on day 15 when all animals were sacrificed. When the data in each group were combined, the histogram shows the mean tumor volume (\pm S.D.) of the eight animals in each group.

Figure 6

Effect of *PRKC-ζ-b* knockdown on Hsp-27 and glycoconjugate expression: (A) Western blotting of Hsp-27 protein and of phosphorylation-specific sites P-Ser15, P-Ser78 and P-Ser82 following si-RNA reduction of *PRKC-ζ-b* in the si-*PRKC-ζ-b*-PC3-MT1-6 cells did not affect expression of the generic protein, although this was reduced in comparable experiments to knock down genes *FABP5* and *RPL19*. Conversely, phosphorylation of P-Ser82 was specifically enhanced when compared to the PC3 and PC3-Mcontrol cells and to the *FABP5* and *RPL19* knock-down cells. (B) Western blotting using *Sambucus nigra* and *Maackia amurensis* revealed differences in sialylated glycoproteins expressed by the si-*PRKC-ζ-b*-PC3-MT1-6 cells when compared to the PC3-Mcontrol and PC3-Mscramble cells confirming specificity of the role of the gene *PRKC-ζ-b* in modulating expression of the proteins, or the level of their sialylation, at 56kD and 49kD. (C) Control cell-line PC3-M and gene knockdown cell-line si-*PRKC-ζ-b*-PC3-MT1-6 expressed terminal fucose residues identified by *Lotus* and *Aleuria* lectins but not by *Ulex*. The later was not affected by removal of sialic acid residues using neuraminidase. Sialic acids linked 2→3Gal- and 2→6Gal- were identified using the lectins from *Sambucus nigra* and *Maackia amurensis*, respectively. These were differentially expressed by the control and knockdown cell-lines since staining by *Maackia* was abrogated by neuraminidase. However, no global difference in expression of these glycoconjugates was identified following *PRKC-ζ-b* knockdown.

Figure 7

Differential expression of genes following si-*PRKCZ-ζ-b* knockdown: Heat map of gene expression profiles from mRNA expressed by the top 50 up-regulated and top 50 down-regulated genes (Supplemental Tables 2 & 3) in the cloned prostate cell-line si-*PRKC-ζ-b*.PC3-M_{TI-6} when compared to non-malignant PNT-2 cells and to PC3-Mscramble cells. Hierarchical clustering is shown. Green indicates genes over-expressed and red indicates genes down-regulated in each sample when compared to scramble transfected cells.

Figure 8 - Gene Ontology (GO) enrichment pathway analysis.

Analysis of genes modulated following *PRKCZ-ζ-b* knockdown identified four pathways principally affected: (a. & b.) genes known to be regulated by *NFKB* and almost universally down-regulated (c.) (c.) gene networks linked to *KDMSB*, *MCM6* and *DDIT3* and (d.) This analysis revealed that some of the genes modulated by down-regulated expression of *PRKC-ζ-b* are interconnected, emphasizing the numerous pathways for cross-talk between apparently distinct biological processes.