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13. ABSTRACT (Maximum 200 Words) Our hypothesis that the human PPC-1 prostate tumor cell lines with experimentally altered Ets transcription factor function, which show a reduction in the transformed phenotype, do so because of altered expression patterns in important genes downstream of Ets factors. We proposed to analyze global differences in gene expression between these cell lines, and assess the functional significance of changes in gene expression. Altered Ets function was found to delay xenograft tumor onset, and tumors from Ets2 overexpressing cells had dramatically reduced tumor microvasculature. Expanded microarray expression analysis has now identified over 65 potential Ets target genes in PPC-1 cells, including genes whose products can contribute to the observed changes in motility, invasiveness, survival, tumorigenicity, and tumor angiogenesis. Regulation of Ets target gene expression in prostate tumor cells was quite different than in other cell types, and prostate Ets family expression relative to other tissues was characterized. Follow-up analysis of Ets target genes has implicated IL-8 in cell motility, PKC delta in survival, CC3 in tumor angiogenesis, and MT-MMP-1 in invasiveness. The ongoing characterization of Ets factor signaling and targets in prostate cell transformation should provide important insights on the molecular basis of aggressive prostate tumor cell behavior.				
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INTRODUCTION

The proposed studies will broadly characterize the changes in gene expression that take place in a cell line model system for androgen-independent tumor progression, and assess their functional consequences. Differences in gene expression resulting from experimentally altered Ets transcription factor function, which reduces the tumor cells cancerous behavior, will be identified in gene microarray experiments, and correlated with their altered phenotype. Observed alterations in gene expression will be followed up to confirm regulation and assess potential functional consequences. Identifying gene products whose altered expression is involved in resistance to cell death, increased invasiveness, as well as tumor growth and angiogenic signaling by prostate tumor cells can identify important new therapeutic targets in androgen-independent prostate cancer.

BODY

1. Characterize differences in gene expression between PPC-1 prostate tumor cells and the Ets-construct altered PPC-1 lines with altered phenotypes.

In initial experiments, to assess the role of Ets transcription factors in the transformed characteristics of the PPC-1 prostate tumor cell line, we established sublines which stably expressed either a dominant inhibitor of Ets family function, the Ets2 DNA binding domain (Ets2DBD) or full-length Ets2, a prototypical transcriptional activator in the 27 member Ets transcription factor family. These cell lines with altered Ets activity(hereafter referred to as PPC1-Ets2 and PPC1-E2DBD) had lost many of their *in vitro* transformed characteristics, including substantial reductions in anchorage-independent growth, motility, invasiveness, and survival. We previously reported results from initial microarray and quantitative PCR (q-PCR) analysis, where 21 potential Ets target genes in PPC-1 cells were identified. This analysis was performed with probe made from RNA from PPC-1 cells as the reference standard, and probe made from PPC1-E2DBD or PPC1-Ets2 cells. The analysis was performed on arrays representing approximately 5,000 genes. To more fully characterize Ets-mediated changes in gene expression that led to loss of the transformed phenotype, we have utilized microarrays with oligonucleotide probes for over 20,000 known genes. The results of this analysis are summarized in Figure 1. Applying the cutoffs of a minimum signal and significant upregulation of over 2-fold, or downregulation to less than 0.6 fold in the majority of replicates (criteria we determined to leads to >80% validation by extensive q-PCR analysis), the expression of sixty-five different genes was found to be altered in either PPC1-E2DBD or PPC1-Ets2 cells.

Interestingly, expression of only 15 of these Ets target genes was found altered in both lines. Additionally, only five of these common target genes exhibited reciprocal activation of expression by Ets2 and repression by Ets2DBD, the pattern anticipated from standard reporter gene assays. Analysis of the direction of regulation of target genes in PPC1-E2DBD cells revealed that the Ets2DBD "dominant inhibitor" actually activated the expression of more genes than it repressed (Fig. 1). This suggests a unique balance of Ets family members in prostate tumor cells, with Ets family repressors predominating and being displaced by the Ets2DBD, rather than displacing the activating Ets family members which predominant in most cell types. This stimulated the comparison of Ets family member expression in normal prostate, prostate tumors, and prostate tumor cell lines described in aim 4. A further unexpected result was that expression of full-length Ets2 in PPC1-Ets2 cells caused an equal number of genes to be upregulated or downregulated (Fig. 1). Despite the unanticipated directions of Ets target gene

expression, the finding that only 8/65 Ets target genes were changed in the same direction in PPC1-Ets2 and PPC1-E2DBD cells, supports our hypothesis that the similar but not identical reversal of multiple transformed features in these two lines likely results from distinct sets of altered target genes. The potential role of the identified Ets target genes in the reversal of specific aspects of the transformed phenotype, and the follow-up analysis, is described in aim 6.

2. Generate PPC-1 cell lines with inducible Ets2DBD or Ets2 expression and determine their *in vitro* phenotype.

As stated in the previous report, generation and analysis of PPC-1 cells with inducible Ets2 or Ets2DBD constructs became a low priority. In a separate project, breast tumor cell lines with inducible Ets2 or Ets2DBD constructs exhibited the same *in vitro* and *in vivo* phenotypes (loss of transformed characteristics) as stably expressed constructs. Thus, it appears in another tumor cell context, that the observed reversal of transformation by these Ets2 constructs is not an artifact of clonal selection. In addition, for target gene analysis, inducible changes in Ets target genes suggests a direct role for Ets factors, but promoter-based analysis is still required to demonstrate this.

3. In vivo analysis of the tumorigenic , angiogenic, and metastatic potential of the altered PPC-1 lines.

We previously reported the results of initial tumor analysis, where palpable xenograft tumor onset from either PPC1-E2DBD or PPC1-Ets2 cells was significantly delayed relative to the parental PPC-1 cells ($p = 0.0008$ and 0.0003 , respectively). Due to the large fluctuation in xenograft tumor size and growth, the clear 2-fold average reduction in tumor size from the PPC1-E2DBD or PPC1-Ets2 cells was not found to be statistically significant. Immunohistochemistry was used to compare the tumor microvasculature in the PPC1-E2DBD or PPC1-Ets2 cells to tumors from the parental PPC-1 cells. Size-matched tumors (rather than age-matched) generated from these three cell lines were sectioned and immunostained with an antibody to CD31, an endothelial cell marker, and counterstained with hematoxylin. Figure 2 shows representative fields of such analysis. There was a dramatic reduction of the brown stained blood vessels specifically in the PPC1-Ets2 cells. This reduced vasculature was particularly apparent towards the centers of the tumors, which also appeared necrotic. The inhibition of signaling to tumor angiogenesis by Ets2 in prostate carcinoma cells represents a novel phenotypic role for Ets signaling, and two biologically relevant Ets target genes whose expression pattern correlates to the specific inhibition of tumor angiogenesis have been identified (aim 6). Assessment of the metastatic potential of the PPC1-E2DBD and PPC1-Ets2 cells using the tail vein injection assay is complicated by the reduced survival and growth rate of these cells, and their strongly impaired ability to form tumors and grow *in vivo*. Thus, it has not been undertaken.

4. Expand the analysis of altered gene expression in prostate cells to PC-3 lines that show reduced or increased invasiveness, and to non-malignant prostate tissue.

The PPC1-E2DBD and PPC1-Ets2 cells exhibited very different invasiveness in the Matrigel assays, with PPC1-Ets2 cells exhibiting the highly invasive phenotype of the parental cells, whereas invasiveness was almost entirely lost in the PPC1-E2DBD cells. We have used these genetically nearly identical cell lines to focus on the Ets target genes mediating invasiveness in

prostate tumor cells, and two proinvasive candidate mediators, MT-MMP1/MMP14 and MMP3 were identified as specifically exhibiting downregulation in the PPC1-E2DBD line.

The primary reason that the Ets2DBD alters the expression of many genes not regulated by Ets2 overexpression, is that it acts as a broad inhibitor of Ets family function by displacing Ets factors from promoter binding sites. However, the expression status of most of the 27 Ets family members in prostate tumors or tissue is unknown. To understand how Ets targets are regulated in prostate tumor cell lines and in non-malignant prostate tissue, we determined the mRNA expression profiles for the entire Ets family in these contexts, and compared it to other types of cells. Figure 3 shows the results of this analysis, with the expression of each Ets factor in normal prostate, the hormone-dependent and less aggressive LNCaP prostate tumor line, and several more invasive prostate tumor cell lines. In addition, in collaboration with Dr. Robert Abraham, we also assessed the impact of hypoxia, a condition found in advanced tumors, on Ets family expression in PC-3 cells. Relative to the mixed cell line "Universal RNA" standard, a variety of Ets factors are more abundant in prostate tissue. This included the epithelial-specific family of Ets/PSE, as well as Ets2. A number of immune-specific Ets factors were also increased, likely due blood in the prostate tissue. The prostate tumor cell lines, unlike the complex prostate tissue composed of many cell types, expressed lower levels of many Ets factors. However, in PPC-1 cells, 16 different Ets factors were still expressed at >20% of mixed tissue, demonstrating unexpected complexity in which Ets family members are mediating transformation. It is likely that some of the differences between normal prostate tissue and the tumor cells are not tumor-specific but rather cell-type specific, and we are obtaining normal prostate epithelial cell RNA to compare with the tumors. Strong expression of several Ets family repressors was observed, but they were not elevated sufficiently at the level of mRNA to explain the unique repressive state of Ets-mediated gene expression in PPC-1 cells. Overall, the pattern of Ets factor expression was quite similar in the PPC-1, PC-3, and DU-145 cells, with contrasting results in the LNCaP, which are now being correlated to altered target gene expression.

An example of the ongoing analysis of Ets target gene expression in normal prostate tissue and various prostate tumor cell lines is shown in figure 4. Q-PCR was used to determine the expression of several genes, relative to expression of the housekeeping gene Cph/PPIA in the same cDNA sample. The data are expressed as percent expression of the same gene in normal prostate tissue. Expression of NK3.1 is often lost in advanced tumors, and its expression was reduced in LNCaP cells, and absent in the more invasive tumor cell lines. Expression of MMP3 and MT-MMP1, Ets target genes that we have implicated in PPC-1 cell invasiveness, were specifically downregulated in the PPC1-E2DBD line, relative to the parental cells and DU145 cells. In contrast, TFPI2, a potential anti-angiogenic factor, was specifically upregulated in the PPC1-Ets2 cells, which exhibit reduced tumor angiogenesis. The constant signal from the GAPDH housekeeping gene for all of the cell lines shows that normalization to Cph was appropriate. Overall, this ongoing approach is extending the correlation of identified Ets target gene expression to normal and tumor cells, further indicating a biological role for these targets in features such as invasiveness and angiogenesis.

5 Bioinformatic analysis of altered gene expression

Several kinds of comparisons with our data set have been performed. These include matching functional annotation (e.g. GO terms and functional descriptions) of identified targets with

observed changes in phenotype. This has led to a list of targets that "make sense". While it might seem circular to look for targets with known roles, many of these functions are from other tumor types, or inferred from protein sequence. Thus, they represent novel targets in prostate tumors. A second kind of analysis involved comparison of the direction of change in Ets target genes in PPC-1 cells relative to what has been reported in the literature and our array analysis of breast tumor cells. While genes downregulated in PPC1E2DBD cells corresponded well to other results, genes upregulated in these cells (e.g. MMP1 or KLKB1) were the opposite of other findings. Similarly, Ets targets were also often regulated in the opposite direction in the PPC1Ets2 cells relative to other cells overexpressing Ets2 (e.g. TFPI2, IL-8, THBS1, S100A4). These results highlight the value of assessing Ets target genes in prostate tumor cells to identify important and potentially unique regulators of transformation. The proposed global informatic analysis and establishment of a resource for this information has not yet been accomplished, but enhanced bioinformatic support from our new Information and Data Management Shared Resource should allow this to be accomplished.

6 Determine functional significance of observed changes in prostate tumor cell line gene expression.

We previously reported follow-up analysis on one target gene that we identified, IL-8. Protein levels for IL-8, a interleukin implicated in several functions including cell motility, were substantially reduced in the PPC1E2DBD cells. These cells displayed strongly reduced motility relative to the parental cells, and addition of exogenous IL-8 to the motility assay significantly enhanced their motility, but did not alter motility of the parental cells. This indicates that IL-8 functionally contributes to the motility of PPC-1 prostate tumor cells, and highlights a new role for IL-8 in prostate tumors beyond tumor angiogenesis.

A second target gene that has been followed up is PKC delta. Expression of PKC delta mRNA and of protein levels (fig. 5) were strongly upregulated in PPC1-E2DBD cells. PKCs have a wide role in cellular responses, and upregulation of PKC delta is associated with impaired cell survival, consistent with the increased apoptosis observed in PPC1-E2DBD cells. Addition of rottlerin, a selective inhibitor for PKC delta activity, enhanced PPC1-E2DBD cell attachment and growth, and changed their rounded morphology back to that of the parental PPC-1 cells. This suggests that PKC delta is a functional Ets target in PPC-1 cells, and that modulating Ets signaling and PKC expression could be used sensitize tumor cells to killing by other agents. Other targets may mediate the impaired growth and apoptotic phenotype of the PPC1-Ets2 line, including upregulation of the cell cycle inhibitor p16INK4A, downregulation of the anti-apoptotic v-myb homolog MYBBL2, and upregulation of DUSP5, a dual specificity phosphatase that inhibits MAP kinase signaling.

Ets targets that are candidates for the specific downregulation of PPC1-Ets2 cell xenograft tumor angiogenesis are CC3/TIP30, a recently characterized anti-angiogenic factor, and the strongly upregulated TFPI2, an inhibitor of MMPs, which are required for blood vessel invasion into the tumor. The role of CC3 in the observed phenotype is currently under investigation.

An Ets target gene whose upregulated expression may contribute to the reduced growth rate of both PPC1-E2DBD and PPC1-Ets2 cells is MAP2K6, which activates p38 signaling leading to

stress induced cell cycle arrest. The S100A4 calcium binding protein is also a common Ets target in the two cell lines, and its strong downregulation in both cell lines and its function in cell motility likely contribute to reduced motility in these cell lines. Finally, several identified Ets targets are candidates for mediators of PPC-1 invasiveness, based on their reduced expression in the less invasive PPC1-E2DBD cells. Specific downregulation of MMP3, MMP7, and MT-MMP-1 were seen in this cell line. In addition, production of HGF, an autocrine and motility factor for prostate tumor cells, is also downregulated in PPC1-E2DBD cells. The biological significance and generality of these findings is under investigation.

KEY ACCOMPLISHMENTS

Microarray analysis has identified 65 Ets target genes whose expression is altered in PPC-1 prostate tumor cells that have lost many hallmarks of oncogenic transformation.

In vivo xenograft tumor analysis has revealed a new role for Ets2 signaling in prostate tumors – an anti-angiogenic function. Candidate Ets target genes with anti-angiogenic activity (e.g. CC3 and TFPI2) have been identified.

PKC delta has been identified as an Ets target gene whose altered expression impacts on tumor cell survival, and other candidates (e.g. p16INK4A, MYBBL2, DUSP5) mediating survival have been identified.

Candidates for mediating PPC-1 cell invasiveness were identified by differential expression in the cell lines, including MMP3, MMP7, and MT-MMP-1.

Expression analysis in normal prostate and other prostate tumor cell lines has demonstrated the significance and generality of some identified Ets target genes.

Analysis of the expression of the entire Ets family in normal prostate and multiple prostate tumor cell lines has revealed unexpected complexity in the number of Ets factors present, and has highlighted which Ets factors are more abundant in prostate tissues and tumor cells. These are important data for interpreting how Ets2DBD targets may be regulated and modulated.

REPORTABLE OUTCOMES

Foos G, Hauser CA (2004) The role of Ets transcription factors in mediating cellular transformation. In: Handbook of Experimental Pharmacology, Vol. 166: Transcription Factors. Springer, Heidelberg and New York, pp 259-275 (in press).

Two manuscripts are in preparation – one describing the array results and follow-up, the other the expression of Ets family members in prostate-related tissues.

The phenotypically characterized PPC-1 prostate tumor cells with altered Ets functions are distributed on request.

Results from these studies were the basis for part of an NIH RO1 application proposing a detailed analysis of how Ets factors mediate transformation.

CONCLUSIONS

This study is based on the hypothesis that PPC-1 prostate tumor cell lines with experimentally altered Ets transcription factor function, which show a reduction in the transformed phenotype, do so because of altered expression patterns in important downstream genes. Thus, we proposed to further validate this cell model in vivo, analyze global differences in gene expression between these cell lines, and assess the functional significance of the observed changes. We have accomplished many of these goals. The completed tumorigenicity studies demonstrate that the Ets2DBD and Ets2Full expressing cell lines exhibit a highly significant delay in tumor formation. In addition, a strong inhibition of angiogenesis was specifically observed in xenograft tumors from the cells overexpressing Ets2. These data extend the in vitro results, and make this a more compelling model system for understanding the genes whose altered expression cause reduced tumorigenicity. Indeed, a new signaling pathway which one can modulate to attack tumor angiogenesis has exciting therapeutic implications, and we are following up on Ets targets implicated in this signaling, including CC3 and TFPI2.

The broad microarray analysis of altered gene expression has identified 65 Ets targets in PPC-1 cells, many of which have been confirmed by quantitative PCR. These target genes are associated with the regulation of many important aspects of cancer cell behavior, and many of the changes in are unique to prostate cancer. We now have identified promising Ets targets which can modulate PPC-1 cell survival, growth, motility, invasiveness, and the above-mentioned tumor angiogenesis. Analysis of altered protein expression and functional assays have now been completed for several target genes, including IL-8 and PKC delta. Real-time PCR analysis was used to analyze the expression of the entire 27 member Ets family in normal prostate tissue and various prostate tumor cell lines, and to compare this to other cell types. Fairly striking differences were observed in the expression of several Ets factors, and these differences are now being correlated to differences in the expression of identified Ets target genes in these same contexts.

While it is premature for such analysis to lead to new drugs or gene therapy, the identification of Ets target genes that modulate the cancerous phenotype promises to yield new therapeutic targets for androgen-independent tumors (e.g. upregulating anti-angiogenic regulators). Approaches to identify important changes that take place in prostate tumors based on cell lines have both advantages and disadvantages. In our system, we are looking at events that occur in the reversal of cancerous behavior. The advantage we hypothesized and our results demonstrate, is that a fairly defined number of changes can and have been identified in these nearly genetically identical cells. The characterization of how these changes in gene expression act to reverse prostate cell transformation, will provide important new knowledge on the molecular basis of aggressive prostate tumor cell behavior.

REFERENCES CITED none

Figure 1. Genes whose expression was significantly changed in PPC-cells with altered Ets activity.

Downregulated in Ets2DBD

Gene	Accession #
MYADM	AK027693
MYADML	AK057470
EIF1A	NM_001412
EIF2S3	NM_001415
EIF4A2	NM_001967
RBBP7	NM_002893
DUSP5	NM_004419
HGF	NM_000601
MMP7	NM_002423
MT-MMP1/MMP14	NM_004995
IL-7R	NM_002185
MGCA	NM_139321
S100A4	NM_002961
HLA-DRg	NM_019111
THBS1	NM_003246
MMP3	NM_002422

Upregulated in Ets2DBD

SHD	AK056268
EPHB1	AL133099
CSE1L	NM_001316
EEF1A2	NM_001958
EIF5	NM_001969
FGF7	NM_002009
GAB1	NM_002039
MMP1	NM_002421
NFIX	NM_002501
MAP2K6	NM_002758
VEGFC	NM_005429
IPO7	NM_006391
PIAS1	NM_016166
P38IP	NM_017569
KLK12	NM_019598
RASL10B	NM_033315
KLKB1	NM_000892
CD73	NM_006410
ENPP2/ATX	NM_006209
Bcl2L1	NM_138578
NCOA/SRC1	NM_147223
PKRCD	NM_006254

Upregulated in Ets2

Gene	Accession #
UCHL1	AK055249
CDK6	AK055922
HSPCA	AK056446
HLA-C	NM_002117
CDKN2A/p16INK4A	NM_000077
ARHGDIB/Rho GDI 2	NM_001175
CCNA2	NM_001237
MAP2K6	NM_002758
DUSP5	NM_004419
TFPI2	NM_006528
ATX/ENPP2	NM_006209
KLKB1	NM_000892
CD73/NT5E	NM_002526
IL-8	NM_000584
HXB/TNC	NM_002160
CC3/HTATIP2	NM_006410
Bcl2L1	NM_138578
IL-7R	NM_002185
MT-MMP1/MMP14	NM_138578
HGF	NM_000601
ITGB3	NM_000212
NCOA/SRC1	NM_147223
TIMP3	NM_000362

Downregulated in Ets2

HLA-DMB	NM_002118
TNC	NM_002160
MMP1	NM_002421
MYBL2	NM_002466
NFIX	NM_002501
PRSS11	NM_002775
TFF1	NM_003225
TFF3	NM_003226
IKBKG	NM_003639
NRP1	NM_003873
ELF3	NM_004433
ITGBL1	NM_004791
GFRA1	NM_005264
TRIP	NM_005879
SKP2	NM_005983
IPO7	NM_006391
PIK3CD	NM_005026
MGCA	NM_139321
THBS	NM_003246
HLA-DRg	NM_019111
S100A4	NM_002961

Combined data from replicate microarray analysis (5K and 20K human arrays), with many of these target genes confirmed by quantitative PCR analysis. Genes are grouped by regulatory pattern, with significant upregulation a ≥ 2.0 fold average increase, or downregulation to ≤ 0.6 fold less in the indicated PPC-1 lines (stably expressing Ets2DBD or Ets2) relative to expression of the same gene in the parental PPC-1 prostate tumor cells. There are a total of 65 different genes, with 15 significantly altered in both the Ets2 and Ets2DBD cell lines.

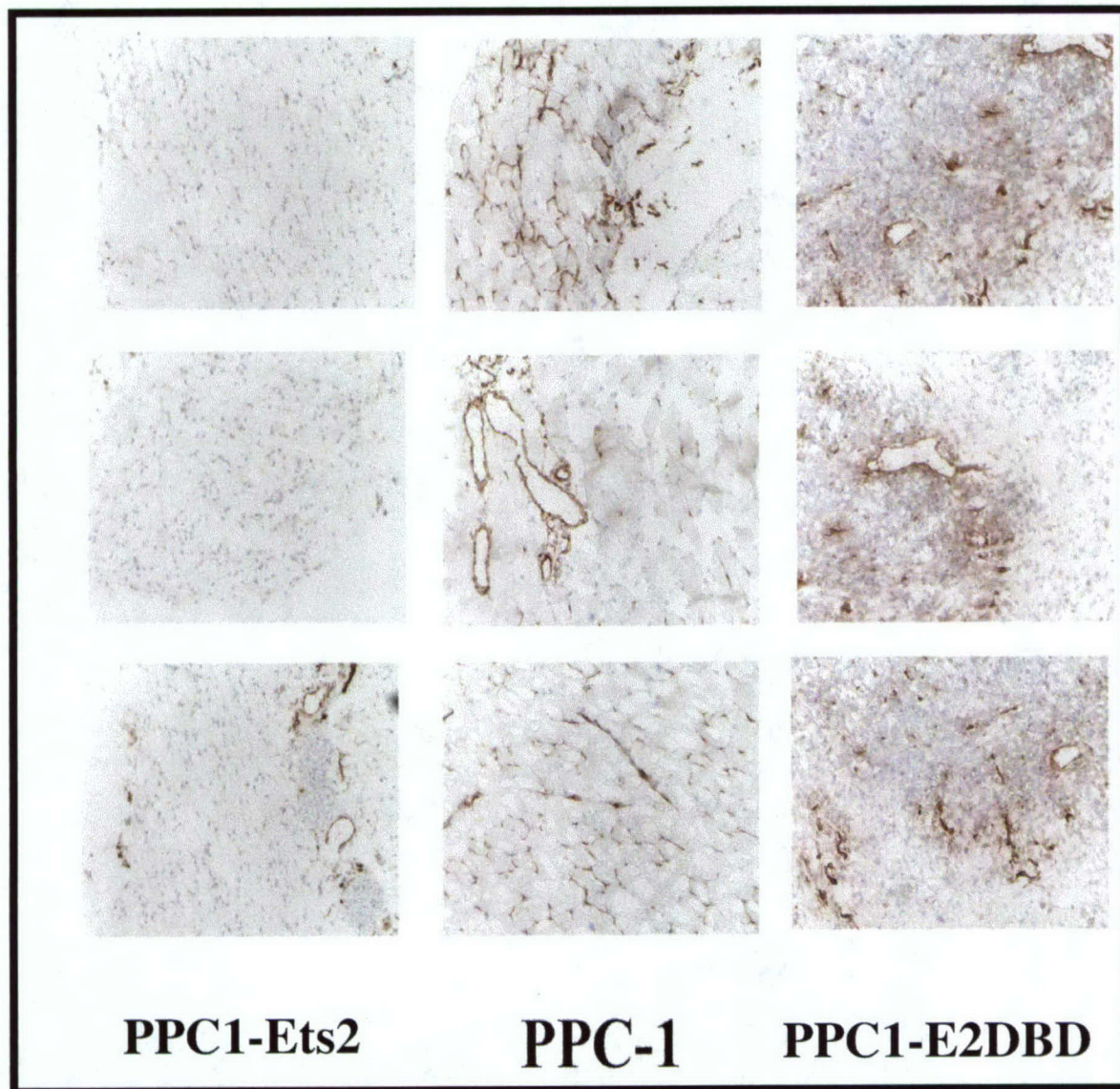


Figure 2. Representative CD31 stained frozen sections from xenograft tumors derived from the indicated cell line

Ets Factor	Prostate tissue	LNCaP	PPC-1	Normal PC-3	Hypoxic PC-3	DU-145
Ets2	1,068	2	65	47	45	153
Ets1	171	0	24	210	154	37
ER71	311	281	58	167	7	44
GABPa	121	5	56	115	69	44
PEA3	17	0	108	166	88	23
ER81	144	0	144	88	63	1
ERM	90	3	39	15	12	3,936
Fli	243	0	0	27	13	0
ERG	649	0	0	0	1	0
ERF	216	0	81	56	14	0
PE1	155	2	0	73	30	5
Elk1	289	66	214	134	104	48
Elk3	184	1	119	153	80	44
Elk4	107	12	65	42	41	109
Elf1	289	1	31	66	39	8
Elf2	254	0	14	36	17	0
Elf4	182	3	90	309	80	29
Ese1	857	41	98	80	23	254
Ese2	2,086	34	0	0	0	0
Ese3	27,897	2,315	452	868	936	6
PSE	2,203	173	218	97	20	1
TEL1	438	3	38	72	64	41
TEL2	2,928	5	0	185	20	245
Spi1	262	0	0	7	2	5
SpiB	341	1	0	34	16	0
SpiC	46	3	0	0	0	0
FEV	3,300	1	0	16	8	1

Figure 3. Expression of the entire Ets family in normal human prostate tissue and human prostate tumor cell lines. Expression was quantitated by q-PCR, and normalized to Cph/PPIA levels. Data shown are the percent expression for each Ets factor, relative to its expression in a mixture of RNAs from 10 diverse cell lines (Stratagene Universal RNA). Red and green denote 2-fold up- or down-regulation, respectively

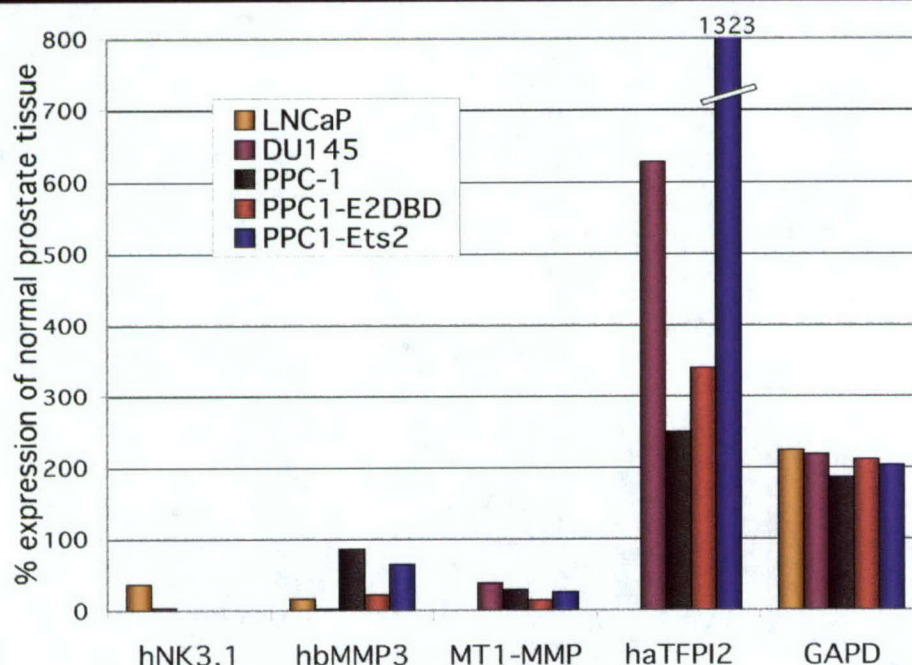


Figure 4. Regulation of Ets target genes in human prostate tumor cell lines, relative to normal prostate tissue. Gene expression was determined by q-PCR and normalized to Cph/PPIA mRNA levels in each sample.

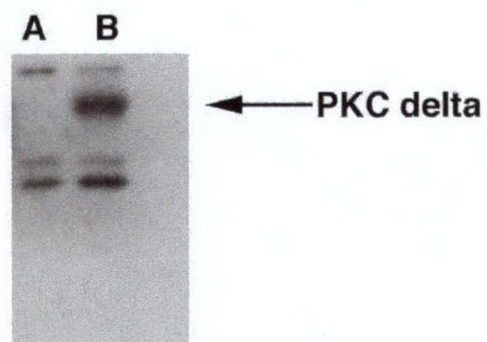


Figure 5. Immunoblot analysis of PKC delta protein expression. Whole cell extract from PPC-1 cells (lane A) and PPC1-E2DBD (lane B).