Comprehensive gene expression microarray analysis of Ets-1 blockade in PC3 prostate cancer cells and correlations with prostate cancer tissues: Insights into genes involved in the metastatic cascade

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Abstract. Ets-1 is the prototype of the ETS family of transcription factors and is suggested to play an important role in the malignant progression of prostatic carcinomas. Therefore, in this study we investigated the effect of blocking Ets-1 in PC3 prostate cancer cells on genes involved in the metastatic cascade, and correlated these findings with prostate cancer tissues. Two stable PC3 cell cultures were established by transfection with either an Ets-1 inverse antisense expression vector or a mock control vector. The effect of blocking Ets-1 on genes involved in the metastatic cascade was assessed by a comprehensive gene expression microarray analysis of Ets-1 inverse and mock control cells. Correlating the sets of genes found in the PC3 microarray data with prostate cancer tissues was performed by verifying the genes in a comprehensive gene expression microarray analysis of RNA extracted from laser microdissected normal prostate glands and from carcinoma glands taken from prostate cancer patients. Western blot analysis confirmed the presence of Ets-1 in mock cells and its absence in Ets-1 inverse cells. In the Ets-1 blockade microarray, many differentially expressed genes were found; however, only genes with a greater than 10-fold up- or down-regulation between the Ets-1 blockade and mock control were considered significant. The genes were placed into four groups that play a role in the so-called metastatic cascade based on their known functions in proliferation, apoptosis, migration and angiogenesis. The genes found in the Ets-1 blockade microarray analysis were verified for their presence in the microarray analysis of prostate cancer tissues. Genes found in the microarray analysis of prostate cancer tissues with an >2-fold change and a p-value <0.01 were considered significant. We identified sets of genes that are involved in the metastatic cascade and are known to be implicated in prostate cancer to show changes in the expression patterns due to the Ets-1 blockade in PC3 cells. Correlating these sets of genes with the findings in prostate cancer tissues, we identified 16 genes that are up- or down-regulated in healthy compared to tumor prostate glands. Further investigation revealed that 4 out of the 16 genes have been reported to be regulated by members of the ETS family. These findings provide *in vitro* and *in vivo* evidence for the importance of Ets-1 in the development and progression of prostate cancer.

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Introduction

The development and progression of tumors involves different steps of the metastatic cascade, such as proliferation, apoptosis, migration, invasion and angiogenesis (1,2). A family of transcription factors called the ETS family is known to play important roles for such processes in both normal and neoplastic cells of different tissues (3-7).

The ETS family is characterized by an evolutionary highly conserved DNA-binding domain, termed the Ets domain, which consists of 80 amino acids with 4 tryptophan repeats (8). Several of the Ets genes are known to be rearranged to produce chimeric oncoproteins and are often found to be expressed in various types of human malignant tumors (9). In prostate cancer, unique chromosomal rearrangements are found in which the TMPRSS2 gene, encoding an androgen-regulated prostate-specific serine protease, is fused with several genes of the ETS family, most commonly ERG (7,10,11). Such fusions increase the expression of rearranged Ets-factors as a response to androgens, resulting in an androgen-stimulated early stage prostate cancer progression (7).

Among these family members, Ets-1 is the prototype, which is expressed in various cell types and is reported to be involved in tumor progression by different mechanisms. Ets-1 is implicated in tumor angiogenesis and contributes to tumor proliferation and invasion by acting within both neoplastic

cells and fibroblasts of the tumor stroma (12-18). It has been reported that Ets-1 is overexpressed in latent and clinically manifest prostatic carcinomas and that strong expression of Ets-1 is associated with poor tumor differentiation (14). In our previous studies, we reported that Ets-1 promotes proliferation, migration and invasion in various neoplastic cells such as melanoma, HeLa and glioma cells (5,6,19).

Therefore, in this study we specifically blocked Ets-1 in PC3 prostate cancer cells and examined the effect of the blockade on genes involved in the metastatic cascade using a comprehensive gene expression microarray. Furthermore, we correlated our Ets-1 blockade gene expression microarray findings with the results of a second microarray analysis that we have performed on RNA extracted from laser microdissected healthy glands of the prostate proper (or the peripheral zone) and of prostate carcinomas.

We report that blocking Ets-1 in PC3 prostate cancer cells affects the expression of genes that may be involved in various aspects of the metastatic cascade, such as proliferation, apoptosis, migration and angiogenesis. Some of the genes affected are known to be involved in prostate cancer (20-38), while others have not yet been implicated. Correlating these genes with the findings of the microarray analysis of prostate cancer tissues, we identified a set of 16 genes that are up- or down-regulated in healthy compared to cancerous prostate glands. Further analysis revealed that 4 out of the 16 genes have been reported to be regulated by members of the ETS family. Our present work provides *in vivo* and *in vitro* evidence for different roles of Ets-1 in prostate cancer.

Materials and methods

Cell culture. PC3 cells were grown in F-12K media with L-glutamine (Invitrogen, USA) supplemented with penicillin, streptomycin (Invitrogen) and 10% heat-inactivated fetal calf serum (Invitrogen). In addition, G418 (4.3 mg/ml) (PAA, Austria) was added in the media of transfected cells.

Experimental blocking of Ets-1 in PC3 cells. Plasmids pcDNA3.1*h-ets-1* inverse and pcDNA3.1(-) (mock control), respectively, were transfected into the PC3 cells by the calcium phosphate method as previously described (39). At 24 h after transfection, selection was started using 4.3 mg/ml G418 (PAA). The cells were then lysed by freezing and thawing at room temperature. A total of 20 μg of protein was analyzed by 10% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose filters (Bio-Rad, USA). The filters were then blocked with 5% dry milk in TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.5) for 2 h at room temperature. The filters were then incubated in TBST and with anti-Ets-1 mouse monoclonal antibodies (1:5000; Transduction Laboratories), then with anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (1:5000; Amersham Biosciences, USA). Antibodies were detected using ECL reagents (Amersham Biosciences). The housekeeping protein β-actin was used as an internal control and was detected using mouse monoclonal anti-β-actin antibody (1:5000; Sigma).

Microarray-analysis of mRNA expression of PC3 cells. RNA was isolated from cells with the RNeasy Mini kit (Qiagen, Germany) as described by the manufacturer. The recommended DNase digestion was carried out with the RNase-free DNase set (Qiagen). The amount of isolated RNA was measured with the Nanodrop ND-1000 (Thermo Fisher Scientific, USA). The RNA was sent to Miltenyi Biotec (Germany), for microarray analysis and bioinformatical interpretation. The RNA was labelled with Cy3 and hybridized on the Whole Human Genome Oligo Microarray 4x44K (Agilent Technologies, USA) as described by the manufacturer. The microarray data were validated by qRT-PCR of a subset of genes.

Processing of human prostatectomy specimens. Fresh tissue samples from 5 moderately differentiated (Gleason-scores 6 and 7a), 4 poorly differentiated (Gleason-scores 8 and 9) prostate carcinomas as well as from 10 normal peripheral zones (prostate proper, sample sizes around 0.5x0.5x0.3 cm) were taken immediately after radical prostatectomy and shock-frozen in liquid nitrogen with ice-cold isopentane. The age of the patients ranged from 45 to 83 (average 67.1) years. Frozen sections (6 μm) were cut from the samples using a cryotome (Leica, Germany) and mounted on membrane-coated slides (MembraneSlides, 1 mm PEN) (Zeiss, Germany) for subsequent laser microdissection. One section was mounted on conventional slides and stained with hematoxylin and eosin (H&E) for diagnostic evaluation by an experienced pathologist. Laser microdissection was carried out according to our previously published method (40-42). Frozen sections were dried for 2 min in the cryotome, washed for 2 min with 70% ethanol in DEPC-treated water and stained for 30 sec in 1% Cresyl Violet diluted in 50% ethanol-DEPC-treated water. Slides were then briefly washed in 70 and 100% ethanol, dried for 10 min and stored at -80˚C until use for laser microdissection.

Quality control. One frozen section from each sample was used for control of RNA integrity prior to laser-capture microdissection (LCM) using a Lasercapture Microscope (Axio Observer.Z1, Zeiss, Germany). The section was washed with 600 μl DTT (2 μM) in RLT buffer (RNeasy Mini kit, Qiagen) and vortexed for 30 sec. RNA-extraction was performed as described by the manufacturer. The recommended DNase digestion was carried out with an RNase-free DNase set (Qiagen). RNA integrity was measured with the Agilent Bioanalyzer 2100 (Agilent Technologies). Samples with a RIN factor >6 were used for LCM.

LCM. LCM of Cresyl Violet-stained sections was performed using the Axio Observer.Z1 Microscope (Zeiss) with the integrated Palm MicroBeam software (Zeiss). LCM was carried out under a 10x objective. Isolated normal or cancerous glands were collected in 200 μl AdhesiveCap tubes (Zeiss).

Microarray analysis of RNA from prostate samples. Total RNA was isolated from samples with the RNeasy Micro kit (Qiagen) as described by the manufacturer. The recommend DNase digestion was carried out with the RNase-free DNase set (Qiagen). The amount of the isolated RNA was determined using the Nanodrop ND-1000 (Thermo Fisher Scientific). Thereafter, an equal amount of RNA from normal peripheral glands on the one hand and from poorly and moderately differentiated prostate carcinoma glands on the other, were

Table I. Cell proliferation genes up-regulated over 10-fold in Ets-1 blocked PC3 cells compared to mock control.

Gene	Full name
IGFBP3	Insulin-like growth factor binding protein 3
CD40	CD40 molecule TNF receptor superfamily member 5
FOXG1	Forkhead box G1
EPGN	Epithelial mitogen homolog (mouse)
STAT6	Signal transducer and activator of transcription 6, interleukin-4 induced
ARNT2	Aryl-hydrocarbon receptor nuclear translocator 2
SPOCK1	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 1
FES	Feline sarcoma oncogene
RBP4	Retinol binding protein 4, plasma
KDR	Kinase insert domain receptor (a type III receptor tyrosine kinase)
COL4A3	Collagen, type IV, α 3 (Goodpasture antigen)
PLG	Plasminogen; lipoprotein, Lp(a)-like 2 pseudogene; solute carrier family 22 (extraneuronal monoamine transporter), member 3

Table II. Cell proliferation genes down-regulated over 10-fold in Ets-1 blocked PC3 cells compared to mock control.

Gene	Full name
AR	Androgen receptor
IFI16	Interferon, γ-inducible protein 16
CCL2	Chemokine (C-C motif) ligand 2
DLC1	Deleted in liver cancer 1; proline-rich nuclear receptor coactivator 2
PRRX2	Paired related homeobox 2
GCG	Glucagon
PRKCQ	Protein kinase C, θ
P2RX7	Purinergic receptor P2X, ligand-gated ion channel, 7
EGR4	Early growth response 4
BST2	Bone marrow stromal cell antigen 2
PBX1	Pre-B-cell leukemia homeobox 1
FABP4	Fatty acid binding protein 4, adipocyte
HCLS1	Hematopoietic cell-specific Lyn substrate 1

pooled to a final concentration of 300 ng of RNA. These pools were sent to Miltenyi Biotec for microarray analysis and bioinformatical interpretation. The RNA was labelled with Cy3 and hybridized on the Whole Human Genome Oligo Microarray 4x44K (Agilent Technologies) according to the manufacturer's instructions. The microarray data were validated by qRT-PCR of a subset of genes.

Transcriptional Regulatory Element Database (TRED). We used the TRED database (43) in order to find out whether Ets-transcription factors are known to participate in the transcriptional regulation of 16 genes which we have found to be differentially expressed between PC3 cells with an Ets-1 blockade and PC3 mock control cells. For this purpose we entered the name of each gene in the TRED database and retrieved the list of reported regulators.

Results

Ets-1 can be experimentally blocked in PC3 cells. In order to experimentally block Ets-1, two stable PC3 cell cultures were established by transfection with either an Ets-1 inverse antisense expression vector or a mock control vector. Western blot analysis showed that PC3 cells expressing the mock control have detectable amounts of Ets-1, whereas PC3 cells expressing the Ets-1 inverse lacked detectable amounts of Ets-1 over a period of 121 days (44).

Gene expression analysis of Ets-1 blockade compared to mock control in PC3 cells using Whole Human Genome Oligo Microarrays. To study the effect of Ets-1 in PC3 prostate cancer cells upon expression of genes that are involved in the metastatic cascade, we analyzed the gene expression

Table III. Apoptosis genes up-regulated over 10-fold in Ets-1 blocked PC3 cells compared to mock control.

Gene	Full name
IGFBP3	Insulin-like growth factor binding protein 3
BEX2	Brain expressed X-linked 2
ITGA1	Integrin, α 1
NME5	Non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)
VAV3	Vav 3 guanine nucleotide exchange factor
BARHL1	BarH-like homeobox 1
ARNT2	Aryl-hydrocarbon receptor nuclear translocator 2
PYCARD	PYD and CARD domain containing
PTH	Parathyroid hormone
C5	Complement component 5
SNCA	Synuclein, α (non A4 component of amyloid precursor)
MMD2	Monocyte to macrophage differentiation-associated 2
PRKCE	Protein kinase C, ε
COL4A3	Collagen, type IV, α 3 (Goodpasture antigen)
PLG	Plasminogen; lipoprotein, Lp(a)-like 2 pseudogene; solute carrier family 22 (extraneuronal monoamine transporter), member 3
PARM1	Prostate androgen-regulated mucin-like protein 1

Table IV. Apoptosis genes down-regulated over 10-fold in Ets-1 blocked PC3 cells compared to mock control.

Gene	Full name
AR	Androgen receptor
IFI16	Interferon, γ-inducible protein 16
CCL2	Chemokine (C-C motif) ligand 2
DLC1	Deleted in liver cancer 1; proline-rich nuclear receptor coactivator 2
FOXL2	Forkhead box L2
NLRP2	NLR family, pyrin domain containing 2
P2RX7	Purinergic receptor P2X, ligand-gated ion channel, 7
UNC5B	Unc-5 homolog B (*C. elegans*)
GAS2	Growth arrest-specific 2; Fanconi anemia, complementation group F
XAF1	XIAP associated factor 1
LOC100137724	Fanconi anemia, complementation group F; growth arrest-specific 2
ENST00000367248	Spectrin repeat containing, nuclear envelope 1
TLR4	Toll-like receptor 4
KCNH8	Potassium voltage-gated channel, subfamily H (eag-related), member 8

profile in PC3 cells transfected with either an Ets-1 inverse antisense expression vector or a mock control vector. Genes whose expression was up- or down-regulated >10-fold, were considered significant and were selected for further analysis. A clustering analysis based on gene function placed different genes into four main processes: proliferation, apoptosis, migration and angiogenesis.

Genes involved in proliferation. Analysis of gene expression based on gene function identified genes with an >10-fold up- or down-regulation that may be involved in cell proliferation (Tables I and II). Among these genes, AR, IGFBP3, CD40, STAT6, SPOCK1, IFI16 and CCL2 have been reported to be involved in prostate cancer (20-27).

Genes involved in apoptosis. Genes with an >10-fold up- or down-regulation that may be involved in apoptosis are shown in Tables III and IV. Of these, AR, IGFBP3, VAV3, PTH, PRKCE, IFI16, CCL2, DLC1, UNC5B, GAS2, XAF1 and TLR4 have been reported to be involved in prostate cancer (20,24,28-35).

Genes involved in cell migration. Among the genes that were identified to be >10-fold up- or down-regulated and that may

Table V. Cell migration genes up-regulated over 10-fold in Ets-1 blocked PC3 cells compared to mock control.

Gene	Full name
IGFBP3	Insulin-like growth factor binding protein 3
ITGA1	Integrin, α 1
VAV3	Vav 3 guanine nucleotide exchange factor
FOXG1	Forkhead box G1
OTX2	Orthodenticle homeobox 2
WNT5B	Wingless-type MMTV integration site family, member 5B
DCLK1	Doublecortin-like kinase 1
SPOCK1	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 1
RELN	Reelin
ISL1	ISL LIM homeobox 1
C5	Complement component 5
KDR	Kinase insert domain receptor (a type III receptor tyrosine kinase)
PLG	Plasminogen; lipoprotein, Lp(a)-like 2 pseudogene; solute carrier family 22 (extraneuronal monoamine transporter), member 3

Table VI. Cell migration genes down-regulated over 10-fold in Ets-1 blocked PC3 cells compared to mock control.

Gene	Full name
CCL2	Chemokine (C-C motif) ligand 2
DLC1	Deleted in liver cancer 1; proline-rich nuclear receptor coactivator 2
AMOT	Angiomotin
UNC5B	Unc-5 homolog B (*C. elegans*)
DRD5	Dopamine receptor D5
TIE1	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1
MTSS1	Metastasis suppressor 1

be involved in cell migration, IGFBP3, VAV3, SPOCK1, CCL2, DLC1 and MTSS1 are known to be implicated in prostate cancer (22-24,27-29,32,33,36) (Tables V and VI).

Genes involved in angiogenesis. Genes that may be involved in angiogenesis and were found to be >10-fold up or down-regulated are listed in Tables VII and VIII. Among these genes, VAV3, KDR and TIE1 are known to be implicated in prostate cancer (32,33,37,38).

Genes identified in prostate cancer tissues by Whole Human Genome Oligo Microarray. The genes from Tables I-VIII that we found to be up- or down-regulated in the gene expression microarray analysis of poorly and moderately differentiated human prostate cancers compared to healthy glands of the peripheral zone with a fold-change of >2 and a p-value <0.01 are summarized in Tables IX and X. Among these genes, CD40, IGFBP3, FES and TLR4 are reported to be regulated by members of the ETS family according to the TRED database. Furthermore, we found Ets-1 to be up-regulated 2.6-fold in prostate carcinomas compared to healthy glands. Previous studies have also shown that Ets-1 is overexpressed in latent and clinically manifest prostatic carcinomas and strong expression of Ets-1 is associated with poor tumor differentiation (14).

Discussion

Tumor development and progression is characterized by changes in gene expression patterns involving various processes responsible for different steps of the so-called metastatic cascade (such as proliferation, apoptosis, migration and angiogenesis) (1,2). In our previous studies, we reported that the Ets-1 transcription factor plays a role in various of these steps such as proliferation, migration and invasion in different neoplastic cells including melanoma, HeLa and glioma cells (5,6,19).

In this study, we report that blocking Ets-1 in PC3 prostate cancer cells likewise affects the expression of genes that may be involved in the metastatic cascade. In a recent study, we have examined the effect of blocking Ets-1 in PC3 cells, and we have found that Ets-1 promotes cell migration, but not invasion (44), while others have shown that inhibition of Ets-1 blocks cell proliferation (45). Furthermore, we have correlated these microarray findings with those of microarray analysis of prostate cancer tissues. Several of the genes affected are

Table VII. Angiogenesis genes up-regulated over 10-fold in Ets-1 blocked PC3 cells compared to mock control.

Gene	Full name
ITGA1	Integrin, α 1
VAV3	Vav 3 guanine nucleotide exchange factor
EPGN	Epithelial mitogen homolog (mouse)
EMCN	Endomucin
BAI3	Brain-specific angiogenesis inhibitor 3
NPY1R	Neuropeptide Y receptor Y1
GUCY1A3	Guanylate cyclase 1, soluble, α 3
KDR	Kinase insert domain receptor (a type III receptor tyrosine kinase)
COL4A3	Collagen, type IV, α 3 (Goodpasture antigen)
PLG	Plasminogen; lipoprotein, Lp(a)-like 2 pseudogene; solute carrier family 22 (extraneuronal monoamine transporter), member 3

Table VIII. Angiogenesis genes down-regulated over 10-fold in Ets-1 blocked PC3 cells compared to mock control.

Gene	Full name
PRRX2	Paired related homeobox 2
PRKCQ	Protein kinase C, θ
AMOT	Angiomotin
DRD5	Dopamine receptor D5
TIE1	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1
ADRA2B	Adrenergic, α-2B-, receptor

already known to be involved in prostate cancer, while others have not yet been implicated.

Cell proliferation. Among the genes regulated by Ets-1 in PC3 cells and involved in cell proliferation, we identified AR, IFI16, CCL2, IGFBP3, CD40, STAT6, and SPOCK1 which are known to be implicated in prostate cancer (20-27) (Tables I and II).

AR, IFI16 and CCL2 were found to be more than 10-fold down-regulated in Ets-1 blocked PC3 cells compared to mock control cells, whereas IGFBP3, CD40, EPGN, STAT6 and SPOCK1 were found to be more than a 10-fold up-regulated. This means that Ets-1 induces the gene expression of AR, IFI16 and CCL2. In contrast, Ets-1 inhibits the expression of IGFBP3, CD40, EPGN, STAT6 and SPOCK1.

Briefly, among the genes that are induced by Ets-1 in PC3 cells and known to be involved in prostate cancer are the androgen receptor (AR), IFI16 and CCL2. Both normal and neoplastic growth of the prostate gland is dependent on the expression and function of the AR (20). In prostate cancer, the activity of the AR is critical for cell proliferation and progression of the disease (46,47). Increased expression of IFI16 in normal human prostate epithelial cells is associated with cellular senescence-associated cell growth arrest, whereas transcriptional silencing of IFI16 in such cells contributes to the development of prostate cancer (21). It has been suggested that CCL2 promotes PC3 prostate cancer cell survival (22), as well as prostate cancer tumorigenesis and metastasis (23).

On the other hand, genes that are inhibited by Ets-1 in PC3 cells and known to be involved in prostate cancer include IGFBP3, CD40, STAT6 and SPOCK1. Briefly, IGFBP3 has been shown to inhibit proliferation, adhesion, migration, and invasion processes of prostate tumor cells (24). Studies that investigated CD40 expression in tissues from patients with prostate cancer have concluded that invasive prostate cancer is a CD40-negative tumor (25). STAT6 has been suggested to be a survival factor in prostate cancer and to be involved in prostate cancer progression (26). Finally, SPOCK1 has been reported to be up-regulated in prostate cancer (27).

Apoptosis. Among the genes affected by Ets-1 in PC3 cells and involved in apoptosis, we analyzed AR, IGFBP3, VAV3, PTH, PRKCE, IFI16, CCL2, DLC1, XAF1 and TLR4 which are known to be involved in prostate cancer (20,24,28-35) (Tables III and IV). AR, IFI16, CCL2, DLC1, XAF1 and TLR4 were found to be more than 10-fold down-regulated in Ets-1 blocked PC3 cells compared to mock control cells, whereas IGFBP3, VAV3, PRKCE, and PTH were shown to be more than a 10-fold up-regulated. This demonstrates that Ets-1 induces the gene expression of AR, IFI16, CCL2, DLC1, XAF1 and TLR4. In contrast, Ets-1 inhibits the expression of the IGFBP3, VAV3, PRKCE, and PTH genes.

Briefly, among the genes that are induced by Ets-1 in PC3 cells and are known to be involved in prostate cancer are the AR, IFI16, CCL2, DLC1, XAF1 and TLR4. The involvement

Table IX. Genes from Tables I-VIII that were found to be down-regulated in human prostate carcinomas compared to healthy glands of the peripheral zone.

Gene	Full name
VAV3	Vav 3 guanine nucleotide exchange factor
FES	Feline sarcoma oncogene
PYCARD	PYD and CARD domain containing
TLR4	Toll-like receptor 4
IGFBP3	Insulin-like growth factor binding protein 3
CD40	CD40 molecule, TNF receptor superfamily member 5
GAS2	Growth arrest-specific 2; Fanconi anemia, complementation group F
SNCA	Synuclein, α (non A4 component of amyloid precursor)
AMOT	Angiomotin
NPY1R	Neuropeptide Y receptor Y1

Candidate genes with a fold-change of >2 and a p-value <0.01 are summarized in this Table.

Table X. Genes from Tables I-VIII that were found to be up-regulated in human prostate carcinomas compared to healthy glands of the peripheral zone.

Gene	Full name
PRRX2	Paired related homeobox 2
ISL1	ISL LIM homeobox 1
NLRP2	NLR family, pyrin domain containing 2
BST2	Bone marrow stromal cell antigen 2
FOXL2	Forkhead box L2
EGR4	Early growth response 4

Candidate genes with a fold-change of >2 and a p-value <0.01 are summarized in this Table.

of the AR, IFI16 and CCL2 in prostate cancer has been discussed above. Studies have indicated that down-regulation of DLC-1 is associated with a variety of cancer types including prostate cancer (28,29). XAF1 has been suggested to contribute to prostate cancer pathogenesis by disrupting the balance of the apoptosis machinery (30). TLR4 is implicated in tumor cell invasion, survival, and metastasis in a variety of cancers (31,48,49). Furthermore, knockdown of TLR4 in PC3 cells has been reported to result in a reduction of cell migration and invasion (31).

On the other hand, genes that are inhibited by Ets-1 in PC3 cells and known to be implicated in prostate cancer include IGFBP3, VAV3, PRKCE, and PTH. The role of IGFBP3 in prostate cancer has been discussed above. VAV3 has been shown to be overexpressed in human prostate cancer, to activate the AR and to stimulate the growth of prostate cancer cells (32,33). PRKCE has been implicated in the promotion of human prostate cancer cell survival (34). Increased PTH concentrations have been detected in patients with advanced prostate cancer (35).

Cell migration. In a set of genes regulated by Ets-1 in PC3 cells and involved in cell migration, we analyzed IGFBP3, VAV3, SPOCK1, CCL2, DLC1 and MTSS1 which are known to be implicated in prostate cancer (22-24,27-29,32,33,36) (Tables V and VI). CCL2, DLC1 and MTSS1 are shown to be more than a 10-fold down-regulated in Ets-1 blocked PC3 cells compared to mock control cells, whereas IGFBP3, VAV3 and SPOCK1 are shown to be more than 10-fold up-regulated. This shows that Ets-1 induces the gene expression of CCL2, DLC1 and MTSS1. In contrast, Ets-1 inhibits the expression of the IGFBP3, VAV3 and SPOCK1 genes.

Briefly, the roles of CCL2 and DLC1 which are induced by Ets-1 in PC3 cells and shown to be more than 10-fold down-regulated have been discussed. It has been reported that reduction of MTSS1 gene expression in prostate cancer may contribute to tumor growth, development, and metastasis (36). On the other hand, genes that are inhibited by Ets-1 in PC3 cells and have more than 10-fold up-regulation such as IGFBP3, VAV3 and SPOCK1 have been discussed above.

Angiogenesis. Genes involved in angiogenesis with more than a 10-fold up- and down-regulation due to blocking of Ets-1 in PC3 cells with known roles in prostate cancer include VAV3, KDR and TIE1 (32,33,37,38) (Tables VII and VIII).

Briefly, TIE1 is down-regulated, whereas VAV3 and KDR are up-regulated after Ets-1 blockade. This means that Ets-1 induces the expression of the TIE1 gene, and in contrast, inhibits the expression of the VAV3 and KDR genes. TIE1 has been found to be expressed at a higher level in cancer patients compared to healthy donors (37). The role of the up-regulated gene KDR in prostate cancer has been discussed above. Finally, KDR has been shown to be expressed highly in prostate adenocarcinomas (38).

Correlations with prostate cancer tissues. The identified sets of genes shown above were verified for their presence in the microarray analysis of laser microdissected normal peripheral zone glands and a series of human prostate carcinomas. A set of 16 genes was found to be up- or down-regulated in prostate

carcinomas compared to healthy glands of the peripheral zone with an >2-fold-change and a $p<0.01$ (Tables IX and X). Among these 16 genes, CD40, IGFBP3, FES and TLR4 were found to be down-regulated in tumor tissues (Table IX) and reported to be regulated by members of the ETS family SPI-1, FLI-1, SPI-1 and SPI-1/ETS-1, respectively (50-53). Furthermore, we found Ets-1 to be up-regulated 2.6-fold in prostate carcinomas compared to healthy glands.

It is worth noting that CD40, IGFBP3 and FES which were reported to be regulated by ETS family members (43), were found to be up-regulated in the PC3 Ets-1 blockade, while down-regulated in the tumor prostate tissues along with Ets-1 up-regulation. These observations support a possible role of Ets-1 in regulating these genes in prostate cancer and may provide more insights into events of the metastatic cascade.

In conclusion, our present work provides *in vitro* and *in vivo* evidence for roles of the Ets-1 transcription factor in the biology of prostate cancer. It shows changes in gene expression patterns due to Ets-1 blockade in PC3 cells, as well as correlations with human prostate cancer tissues. These patterns may involve various steps of the metastatic cascade (such as proliferation, apoptosis, migration and angiogenesis). The findings seem important with respect to frequent translocations of different ETS family members in prostate carcinoma (7,10,11), since Ets-1 is the prototype of this family. Thus, the differentially expressed genes and pathways provide a more comprehensive view into the broad roles that Ets-1 might play in the development and progression of prostate cancer.

Additional studies such as DNA binding- and co-transfection assays are needed in order to gain additional knowledge about the exact molecular mechanisms by which Ets-1 regulates the expression of these sets of genes. Particular candidates may prove useful as diagnostic or prognostic markers or even novel therapeutic targets.

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