Ets-1 is implicated in the regulation of androgen co-regulator FHL2 and reveals specificity for migration, but not invasion, of PC3 prostate cancer cells

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Abstract. Different members of the Ets-family of transcription factors are involved in TMPRSS-2-Ets translocations frequently found in human prostate cancers. We previously reported that Ets-1, which is the prototype of Ets-family members, promotes both migration and invasion of melanoma, HeLa and glioma cells. Here, we examined whether Ets-1 has a similar effect upon migration and invasion of PC3 prostate cancer cells, and whether it is implicated in the regulation of the androgen co-regulator four and a half LIM only protein-2 (FHL2). 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 confirmed presence of Ets-1 in mock and absence in Ets-1 inverse cells. Microarray and qRT-PCR revealed an up-regulation of FHL2 in Ets-1 blocked cells, compared to mock. To examine the effects of Ets-1 upon cell migration, a wound assay was performed, and demonstrated that wounds were completely colonized by mock compared to Ets-1 blocked cells after 55 h. Evaluation of the effect upon invasion was examined using the Boyden chamber, which revealed no significant difference between mock and Ets-1 blocked cells. In conclusion, our study demonstrated for the first time that Ets-1 is implicated in the regulation of the androgen co-regulator FHL2, and reveals specificity of action for migration, but not invasion of PC3 prostate cancer cells.

Introduction

According to current models, tumour development and progression is a result of changes in gene expression patterns involving pathways responsible for different steps of the so-called metastatic cascade (such as proliferation, migration, invasion and tumour angiogenesis (1,2). Particular genes involved in these pathways are classified as oncogenes, tumour suppressor genes, apoptosis-relevant genes or genes involved in DNA-repair according to their functions (2-5). The expression of these genes is tightly regulated through different combinations of transcription factors (in cooperation with co-activators and co-suppressors), which play key roles in activating or repressing transcription by binding to regulatory regions in their target genes (6).

The ETS family of transcription factors is characterized by an evolutionary highly conserved DNA-binding domain, the Ets domain, which consists of 80 amino acids with 4 tryptophane repeats (7). ETS family members have been shown to play important roles in different cell types (such as B cells, endothelial cells and fibroblasts as well as in neoplastic cells) for processes including development, differentiation, proliferation, apoptosis, migration, tissue remodelling, invasion and angiogenesis (8-12).

In prostate cancer unique chromosomal rearrangements have been found that fuse the TMPRSS2 gene, an androgen-regulated prostate-specific serine protease, with several members of the ETS family, most commonly ERG (12-14). Such fusions increase expression of rearranged Ets-factors as a response to androgens, leading to an androgen-stimulated early stage prostate cancer progression (12).

Among the ETS family members, Ets-1 is the prototype that has been studied most extensively, and found to be expressed in different cell types. It has been shown to play a role in tumour progression by acting upon both stromal and neoplastic cells. Ets-1 participates in tumour angiogenesis and tumour invasion by inducing matrix-degrading proteases in both endothelial cells and fibroblasts of the tumour stroma (15-21). In neoplastic cells such as melanoma, HeLa and glioma cells, Ets-1 promotes proliferation, migration and invasion (10,11,22). Ets-1 has been also found to be expressed in prostate cancer cells.

As a continuation of our efforts into understanding the implications of Ets-1 expression in neoplastic cells, we examined in this study the effects of a blockade of Ets-1 upon migration and invasion of PC3 prostate cancer cells. We used an Ets-1 antisense vector for blockade. Further-

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more, as prostate cancer progression involves the over-activation of the androgen receptor (AR) and several of its co-activators (23-26), we decided to investigate whether Ets-1 is implicated in the regulation of the androgen co-regulator four and a half LIM only protein-2 (FHL2) in PC3 prostate cancer cells. FHL2 enhances the activity of the AR and has been found in different tissues including prostate glands (27). In prostate cancer, FHL2 has been shown to be over-expressed and correlated with poor prognosis (25,26,28).

Here, we demonstrate for the first time that Ets-1 is implicated in the regulation of the androgen co-regulator FHL2, and reveals specificity of action for migration, but not invasion of PC3 prostate cancer cells.

Materials and methods

Cell culture. PC3 cells were grown in F12K media with L-Glutamine (Invitrogen, USA) supplemented with penicillin, streptomycin (Invitrogen) and 10% heat-inactivated fetal calf serum (Invitrogen). Into media of transfected cells, 4.3 mg/ml G418 (PAA, Austria), was added.

Cytoplasmic and nuclear extracts. Cytoplasmic and nuclear extracts were prepared from $5x10^6$ cells as described (29). Briefly, the pelleted cells were resuspended in 300 μl of buffer A containing 10 mM Hepes, pH 7.5, 10 mM KCl, 3 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol, protease and phosphatase inhibitors. The resuspended cells in buffer A were then incubated on ice for 15 min. Following incubation, 0.05 volumes of 10% Nonidet P-40 were added, and cells were vortexed for 10 sec. The sample was then centrifuged at 500 x g for 10 min at 4˚C. The supernatants were collected and labeled as cytoplasmic extract. The remaining pellet was washed in 200 μl of buffer A and centrifuged as described above. The pelleted nuclei was then resuspended in 50 μl of buffer B containing 20 mM Hepes, pH 7.5, 25% glycerol, 0.8 M KCl, 1 mM $MgCl_2$, 1% Nonidet P-40, 0.5 mM EDTA, 2 mM dithiothreitol, protease and phosphatase inhibitors. The pelleted nuclei in buffer B were incubated on ice for 20 min and centrifuged at 14000 x g for 15 min at 4˚C. The supernatant recovered thereafter was labeled as nuclear extract. Protein concentration was then determined using ND1000 Nanodrop (Peqlab, USA). The presence of Ets-1 in the cytoplasmic and nuclear fractions was determined by Western blot analysis using anti-Ets-1 mouse polyclonal antibodies (Transduction Laboratories).

Ets-1 blockade. PC3 cells were cultured in F12K media (Invitrogen) supplemented with antibiotics and 10% heat-inactivated fetal calf serum. Plasmids pcDNA3.1*h-ets*-1 inverse and pcDNA3.1 (-) (mock control), respectively, were transfected into cells by the calcium phosphate method as described (30). At 24 h after transfection, selection was started using 4.3 mg/ml G418 (PAA). The cells were then lysed by freeze and thaw 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). 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 polyclonal antibodies (1:5000; Transduction Laboratories), with anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (1:5000; Amersham Biosciences). Antibodies were detected using ECL reagents (Amersham Biosciences). The house-keeping protein ß-actin was used as an internal control and was detected using mouse monoclonal anti-ß-actin antibody (1:5000; Sigma).

Wound assay. The wound-induced migration assay was performed as described (31). Briefly, PC3 Ets-1 inverse and mock control cells were suspended in F12K media with 10% FCS. Cells/well ($2x10^5$) were plated onto 6-well plates and incubated for 24 h. The monolayers of the cells were then scraped with a plastic pipette tip and washed with PBS, and the medium was then replaced with F12K media with 10% FCS. The cells were photographed at 0, 24, 48 and 55 h after scraping.

Invasion assay. Boyden chamber invasion assay was performed as described (32) using 24-well Matrigel invasion chambers with 8-μm polycarbonate filters coated with Matrigel (BD Bioscience, Bedford, MA). A fixed number of PC3 inverse or mock cells ($1x10^5$ cells) in routine medium containing 2% FCS were placed in the Matrigel pre-coated upper chamber inserts (BD Bioscience). The lower chamber contained routine medium with 10% FCS. Cells were incubated for 24 h, and then the non-invading cells on the upper surface of the filter, were removed with cotton swab. The membranes were removed from the insert with a scalpel, and stained with hematoxylin (Fluka, Germany). The invading cells were counted in 4 different random fields. T-test was performed to determine the statistical significance of the data.

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. A total RNA of 38 and 17 μg was recovered from PC3 Ets-1 invers cells and mock control cells, respectively. The recommended DNase digestion was carried out with the RNase-Free DNase Set (Qiagen). The amount of the isolated RNA was measured with the ND1000 Nanodrop (Peqlab). The RNA was sent to Miltenyi Biotec, Germany, for Microarray analysis and bio-informatical interpretation. The RNA was labeled with Cy3 and hybridized on the Whole Human Genome Oligo Microarray 4x44K (Agilent, USA) as described by the manufacturer.

Quantitative RT-PCR. The reverse transcription was performed on RNA using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen) as described by the manufacturer. In the real-time PCR SYBR-Green ER qPCR SuperMix (Invitrogen) reaction, a volume of 10 μl was used. The oligonucleotides and their sequences were as follows: the housekeeping gene rPL13a: (forward), TACGCTGTGA AGGCATCAAC, rPL13a (reverse), CACCATCCGCTTTT TCTTGT. FHL2, (forward), CGAGTAAGGCACACCCA AAT, FHL2: (reverse), TCTGAATAACTTTATTGCAAG TCACA (each sample was tested in triplicate). The PCR
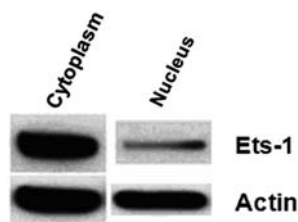
Figure 1. Western blot analysis showing Ets-1 expression in PC3 cells. Cytoplasmic and nuclear proteins from PC3 prostate cancer cells were separated on a 10% SDS-PAGE, transferred to PVDF membranes and blotted with Ets-1 specific antibodies.
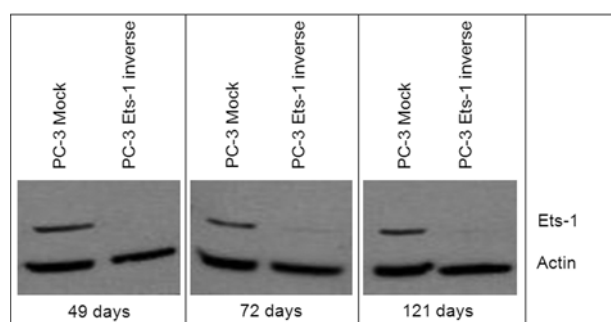

Figure 2. Stable transfection of Ets-1 in PC3 cells. Western blot analysis showing stable transfection over a period of 121 days of PC3 cells with empty plasmid (Mock) and plasmid carrying Ets-1 inverse.

Table I. Microarray analysis of FHL2 expression in PC3 cells.

	Fold change
PC3 Mock	0.00
PC3 Ets-1 inverse	2.36

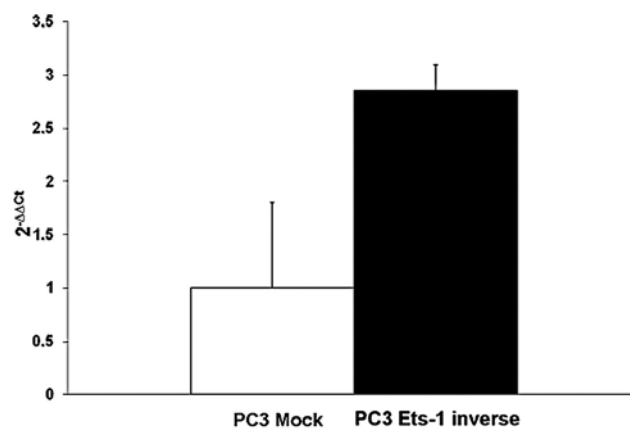The values are the result of the ratio PC3 Ets-1 inverse to PC3 Mock.

Figure 3. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis of FHL2 in PC3 cells. FHL2 mRNA levels in PC3 Mock and PC3 Ets-1 inverse cells determined with qRT-PCR.
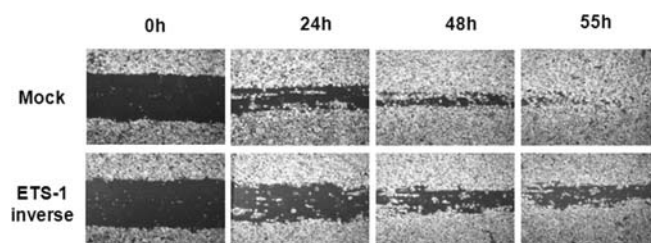

Figure 4. Wound assay: effects of Ets-1 on cell migration. Monolayers of confluent cultures were lightly scratched with a pipette tip and phase contrast images of cultures were taken immediately after wounding (0 h) and after 24, 48 and 55 h.

conditions in the Applied Biosystems 7900HT instrument were as follows: initially 2 min 50˚C, 10 min 95˚C, 40 cycles 15 sec 95˚C and 60˚C for 1 min. Thereafter, a melting point analysis was performed with 15 sec 95˚C, 15 sec 60˚C and 15 sec 95˚C. The data were collected after the 60˚C step at every cycle and after the melting point analysis.

Data processing. Absolute Ct-values were assessed with SDS 2.2 Software (Applied Biosystems) and transferred to Microsoft Excel for further analysis. The Ct-Values were normalized with the reference to the rPL13a as the housekeeping gene. After that the $2^{-\Delta\Delta Ct}$ was calculated with the values of the mock control in cell culture as a second reference. SEM is shown in the error bar.

Results

Ets-1 is expressed in PC3 prostate cancer cells and can be experimentally blocked. Ets-1 is expressed in both the cytoplasm and the nucleus in PC3 prostate cancer cells as shown by Western blot analysis (Fig. 1). Therefore, two stable PC3 cell cultures were established by transfection with either an Ets-1 invers antisense expression vector or a mock control vector. Using Western blot analysis, we found that PC3 cells expressing the mock control showed detectable amounts of Ets-1, whereas PC3 cells expressing the Ets-1 invers lacked detectable amounts of Ets-1 over a period of 121 days (Fig. 2).

Up-regulation of FHL2 in Ets-1 blocked PC3 prostate cancer cells. Based on our microarray data analysis, we observed a 2.36-fold up-regulation of FHL2 in Ets-1 blocked PC3 cells compared to the mock control (Table I). To further verify these findings, we determined the relative expression of FHL2 in the mock control and Ets-1 blocked PC3 cells by qRT-PCR. We found that FHL2 is expressed at a higher level in the Ets-1 blocked cells compared to the mock control cells (Fig. 3).

Ets-1 plays a role in PC3 prostate cancer cell migration. In order to examine the effect of Ets-1 on cell migration we used the wound assay. Wounds were almost completely colonized by PC3 mock control cells in comparison to the PC3 Ets-1 blocked cells after 55 h (Fig. 4).
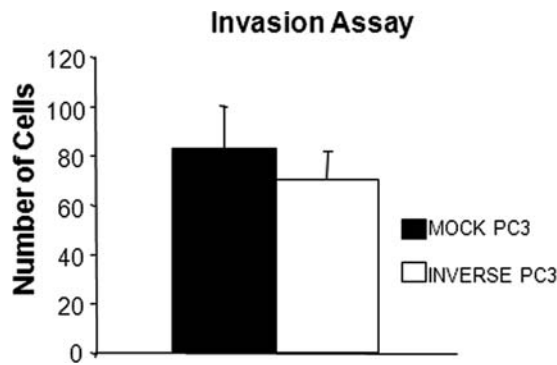
Invasion Assay

Figure 5. Boyden chamber invasion assays: effect of Ets-1 on cell invasion. Cells were placed into matrigel coated Boyden chambers, and the invading cells were fixed, stained and counted in four different areas after 24 h. Statistical analysis using the T-test revealed no significant differences between mock and inverse PC3 cells.

Ets-1 does not promote invasion of PC3 prostate cancer cells. We investigated the effect of Ets-1 on cell invasion of PC3 prostate cancer cells using the Boyden-chamber assay. The invading cells of the Ets-1 inverse and mock were counted and the T-test revealed that the difference between Ets-1 inverse and mock is not statistically significant (Fig. 5).

Discussion

Cancer cell migration is an important step of tumour progression towards metastasis and requires coordinated adhesion and detachment of tumour cells (33). We reported previously that the Ets-1 transcription factor promotes proliferation, migration and invasion in different neoplastic cells such as melanoma, HeLa and glioma cells (10,11,22). In HeLa cells, Ets-1 promotes cell invasiveness and migration, whereas, down-regulation of Ets-1 reduces cell attachment (11).

In the present study, we first investigated whether Ets-1 has a similar effect upon migration and invasion of PC3 prostate cancer cells. Our wound assay showed a decrease in migration in the Ets-1 blocked PC3 cells, indicating a direct role of Ets-1 in this event. However, evaluation of the effect upon invasion was examined using the Boyden chamber, which revealed no significant difference between mock and Ets-1 blocked cells. These findings suggest that Ets-1 possesses a specificity of action for migration, but not invasion of PC3 prostate cancer cells. Furthermore, it suggests that the role of Ets-1 in migration of PC3 cells can not be fulfilled by other member of the ETS family which can replace each other under certain conditions (34).

In prostate cancer, the activity of the androgen receptor (AR) is critical for cell proliferation and progression of the disease. Activity of the AR is tightly regulated by different factors and FHL2 (four and a half LIM only protein-2) functions as a nuclear receptor co-regulator that enhances the AR activity and is found in different tissues including the prostate gland (26,27). Additionally, FHL2 is over-expressed and correlated with poor prognosis in prostate cancer (25,26,28). The role of FHL2 in cancer is worth

unraveling, as it can function as an oncoprotein or as a tumor suppressor in a tissue-dependent manner, as well as a repressor or an activator of transcription depending on the cell type (35). We therefore, examined whether Ets-1 is implicated in the regulation of FHL2 in PC3 prostate cancer cells. Our results show that FHL2 is up-regulated in the Ets-1 blocked PC3 cells. These findings suggest that Ets-1 is implicated in the regulation of FHL2 and may play a role in a pathway that inhibits the expression of FHL2 in PC3 cells.

In summary, we demonstrate for the first time that Ets-1 is implicated in the regulation of the androgen co-regulator FHL2, and reveals specificity of action for migration, but not invasion of PC3 prostate cancer cells. These findings underline the significance of the role Ets-1 plays in tumor development and suggest that the Ets-1 gene may become a target of novel therapies in the future.

Acknowledgements

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