ABNORMAL EXPRESSION OF THE ERG TRANSCRIPTION FACTOR IN PROSTATE CANCER CELLS ACTIVATES OSTEOPONTIN

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ERG regulates osteopontin in prostate cancer cells

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ABBREVIATIONS
PCA, prostate cancer; ChIP, chromatin immunoprecipitation; EMSA, electromobility shift assay

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ABSTRACT

Osteopontin (OPN) is an extracellular matrix glycoprophosphoprotein that plays a key role in the metastasis of a wide variety of cancers. The high level of OPN expression in prostate cells is associated with malignancy and reduced survival of the patient. Recent studies on prostate cancer tissue have revealed recurrent genomic rearrangements involving the fusion of the 5’ untranslated region of a prostate-specific androgen-responsive gene with a gene coding for transcription factors from the ETS family. The most frequently identified fusion gene is TMPRSS2:ERG, which causes ERG protein overexpression in prostate cancer cells. ERG is a transcription factor linked to skeletogenesis. This study was designed to test whether ERG and the product of the TMPRSS2:ERG fusion gene modulate OPN gene expression in prostate cancer cells. To characterize ERG and TMPRSS2:ERG transcriptional activity of OPN, we focused on ETS binding sites (EBS) localized in conserved regions of the promoter. Using in vitro and in vivo molecular assays, we demonstrate that ERG increases OPN expression and binds to an EBS (nt -115 to -118) in the OPN promoter. Moreover, stable transfection of prostate tumor cell lines by TMPRSS2:ERG up-regulates endogenous OPN expression. Finally, in human prostate tumor samples, detection of the TMPRSS2:ERG fusion gene was significantly associated with OPN overexpression. Taken together, these data suggest that OPN is an ERG-target gene in prostate cancer where the abnormal expression of the transcription factor ERG, due to the TMPRSS2:ERG fusion, disturbs the expression of genes that play an important role in prostate cancer cells and associated metastases.

INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed non-skin cancer in men and continues to be one of leading causes of male cancer-related death among the elderly. PCa has high metastatic capacity and extensively metastasizes to bone, lymph nodes, and visceral organs, such as the liver and lungs. Research over the last few decades on metastasis has revealed detailed steps of this mechanistic cascade (1): some primary tumor cells detach and escape from the original tumor sites, intravasate into circulation, extravasate out into the secondary tissues in which they begin to proliferate (2). Chemotaxis plays an important role in the metastasis of cancer cells, directing the motility of metastatic cells through gradients of growth factors or chemoattractants.

Osteopontin (OPN), also called secreted phosphoprotein 1 (SPP1), is a glycoprophosphoprotein cytokine that is expressed by numerous cells and secreted into body fluids. This extracellular matrix protein is an active player in many physiological and pathological processes including bone mineralization, defense against infectious agents, blood vessel formation, arteriosclerosis, disruption of the growth of calcium oxalate crystals and nitric oxide production, and tumorigenesis (3, 4). Substantial evidence associates OPN expression with tumor invasion and metastasis (5) in a number of
cancers such as breast (6), prostate (7), lung (8), stomach (9) colon (10), brain (11) and other cancers (12).

Transcriptional regulation of \textit{OPN} is complex and involves multiple signal transductions (13). Identifying transcriptional regulators that contribute to the modulation of \textit{OPN} expression is of interest for therapy that aims to control the \textit{OPN}-mediated metastatic phenotype. Several known cis-acting transcription factors have been described and most of them have been localized to a conserved region at 250 bp upstream of the proximal promoter. Potential binding sites for transcriptional regulators, such as AP-1, Myc, Oct-1, USF, v-Src, TGF-beta/BMPs/Smad/Hox, Wnt/b-catenin/APC/GSK-3b/Tcf-4, Ras/RRF, TF53, Runx2 and ETS family members, have been identified (14-21).

In the past few years, the \textit{ERG} (ETS-related gene) gene, a member of the ETS transcriptional factor family, has been shown to be highly overexpressed in most PCa (60 to 80% according to the studies) (22, 23). Thus, its presence in prostate cells perturbs normal gene expression. ERG overexpression (and to a lesser extent the overexpression of some other ETS family members) is the result of chromosomal translocations between the androgen-regulated \textit{Trans-membrane protease serine 2} (\textit{TMPRSS2}) gene promoter and the coding sequence of the \textit{ERG} gene (24). The \textit{TMPRSS2:ERG} fusion is associated with aggressiveness and recurrence of PCa (25), metastasis (26) and PCa-specific death (27, 28).

Two members of the ETS family, PEA3 and Ets-1, have been demonstrated to be partially responsible for the overexpression of \textit{OPN} in human breast cancer and in murine colorectal cancer cells, respectively (29, 30). In this study, we investigated whether \textit{ERG} overexpression stimulates \textit{OPN} expression in PCa cells. \textit{OPN} promoter-luciferase deletion constructs in transient transfection experiments demonstrate \textit{OPN} transactivation by the \textit{ERG} factor. After localizing ETS binding site(s) (EBS) in sequences conserved across several species, we characterized the \textit{OPN} promoter-\textit{ERG} transcription factor complex using an electromobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP). Due to the expression of the \textit{TMPRSS2:ERG} fusion gene in prostate tumor cells, the overexpression of \textit{ERG} in prostate cancer cells increased \textit{OPN} expression. The coincident expression and colocalization of \textit{TMPRSS2:ERG} and \textit{OPN} in primary prostate tumor tissue samples suggest that \textit{ERG} may be implicated in the dysregulation of \textit{OPN} in prostate cancer.

**MATERIALS AND METHODS**

**Patient information and tissue selection**—All prostate cancer patients included in this study had undergone radical prostatectomy in the Lille University Hospitals. Clinical data and patient consent were provided by the referring physician. Immunohistochemical studies were conducted on formalin-fixed and paraffin-embedded prostate specimens. Frozen tissues used for RT-PCR involved primary tumors from 35 male patients (mean age: 63 years, with Gleason score >5) and were obtained
from the urological collection of the local tumor tissue bank (Tumorothèque CRRC/Canceropole Nord-Ouest, Lille, France) after approval by the internal review board.

**Plasmid constructs**—We used the expression plasmids for ERG (pSG5-ERGp55 and pcDNA3.1-ERG), described in (31). The TMPRSS2(exon 1):ERG(exon 4) fusion transcript was amplified and subcloned into pcDNA3.1(+) (Invitrogen) using patient cDNA samples and primers: TMPRSS2 forward primer 5'-CGCGAGCTAAGCAGGAGGC-3' and ERG reverse primer 5'-CCTCCGCCAGGTCTTTAGTA-3'. OPN promoter luciferase reporters were constructed by PCR with the following primers: OPN-116/+77 (-116 to +77), OPN-136/+77 (-136 to +77), OPN-341/+77 (-341 to +77), OPN-1441/+77 (-1441 to +77). The fragments were subsequently cloned into a pGL3-basic luciferase reporter plasmid. Consensus ERG binding site mutations corresponding to nt -121 to -114 (5'-GGAGGAAG-3' to 5'-GGTAAAAG-3') and to nt -112 to -106 (5'-GTAGGAG-3' to 5'-GTCGGAG-3') were constructed using the OPN-136/+77 luciferase wild-type fragment as the template. Oligonucleotides encoding for small hairpin RNAs (shRNA) against ERG mRNA (targeted sequence described in (32)) was synthesized with appropriate loop and cohesive ends sequences according to the plasmid provider instructions and was cloned into pSilencer 2.1-U6 (Ambion Inc., Austin, TX). All constructs were verified by sequencing (Genoscreen, Lille) and restriction enzyme digestion.

**Cell Culture**—PC3c (a PC3 subclone provided by E. Bonnelye) and HeLa cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum, 1% gentamycin and 1% glutamin (Invitrogen). Cultures were maintained at 37°C under 5% CO2.

**Transient transfection and activity assay**—One day prior to transfection, cells were plated in 12-well plates to be 50-60% confluent on the day of transfection. Each well was transfected using polyethylenimine (PEI, Eurogente, Illkirch, France) according to the manufacturer’s instructions, with a DNA mixture including 500 ng of the firefly luciferase reporter gene containing the OPN promoter, 25 ng of a control plasmid tk-luciferase (tk-renilla; Promega) and the indicated concentration of pSG5-ERG. In the RNA silencing analysis, 300 ng of pSilencer ERG or psilencer control were co-transfected. The total amount of transfected DNA was adjusted to 1 µg per well. After 48 h of transfection, cells were harvested and luciferase activities were assayed using the Dual-Luciferase Reporter Assay System (Promega) using a luminometer (Berthold Biolumat centro LB960). Firefly luciferase values were normalized to those of the control renilla luciferase values. All experiments were repeated twice. Results are presented as the mean ± SEM.

**Generation of PC3c cell subclones**—PC3c cells stably expressing ERG or TMPRSS2:ERG were obtained by transfecting them with pcDNA3-ERG or pcDNA3-TMPRSS2:ERG plasmids. Resistant clones were selected based on antibiotic resistance over 15-21 days in DMEM containing 300 µg/mL G418. Selected colonies were isolated, allowed to proliferate and characterized for ERG and TMPRSS2-ERG expression using RT-PCR and Western blot analysis.
EMSA and supershift assays- The assays were performed as described in ref. (33) using purified ERG protein (expressed and purified using the T7-Impact™ System (New England Biolabs®) as described previously (34)) and ERG transfected-HeLa nuclear extracts. An oligonucleotide (Eurogentec) probe nt -123 to -101 (5'-CCAGAGGAGGAAGTGTAGGAGCAGGT) was prepared by end-labeling double-stranded oligonucleotides with [32P]ATP (2500 Ci/mmol) using T4 polynucleotide kinase (Promega) followed by G-50 column purification (GE-Healthcare). Radiolabeled oligonucleotide probes (100,000 cpm) were incubated with protein or nuclear extracts in a total volume of 20 µl for 30 min at 20°C. Binding buffer consisted of 100 ng/ml of poly(dI-dC)-poly(dI-dC), 20 mM Tris HCl (pH 7.9), 50 mM KCl, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.02% NP-40 and 10% glycerol. DNA-protein complexes were resolved by electrophoresis for 1 h at 200 V on a 5% non-denaturing polyacrylamide gel. The gels were dried, and signals were detected by exposure to autoradiography film. Nuclear extract was used as the negative control. In competitive binding assays, unlabeled oligonucleotides were added in 100 M excess. Oligonucleotides with mutated ETS binding sites were designed as follows: mutant 1 5’-CCAGAGGTAAAAGTGTAGGAGCAGGT, mutant 2 5’-CCAGAGGAGGAAGTGAAAAAGCAGGT and mutant 1+2 5’-CCAGAGGTAAAAGTGAAAAAGCAGGT. Supershift assays were performed by adding 1 µl of polyclonal antibodies directed against ERG (sc-353 and sc-354, Santa Cruz Biotechnology).

Chromatin immunoprecipitation (ChIP)- ChIP assays were performed as previously described (35) by using anti-ERG (sc-353, Santa Cruz Biotechnology). Briefly, formaldehyde cross-linked chromatin was sonicated and immunoprecipitated with either IgG (control) or the indicated Ab. ChIP analyses were performed in at least triplicate using distinct DNA preparations. Immunoprecipitated DNA was purified and quantified by PCR. The targeted OPN promoter sequence (-269;-75) was amplified using the following primers: 5’-CATGGGATCCCTAAGTGCTC-3’, 5’-TGAGGTTTTCTGCCACTGCCC-3’. The target irrelevant sequence was amplified using primers: a: 5’-TGAGAGCAATGAGCATTCCGATG-3’ and B: 5’-CAGGGAGTTTCCATGAAAGC-3’.

RNA preparation and RT-PCR- Total RNA was extracted using the Nucleospin RNA II kit according to the manufacturer’s protocol (Macherey-Nagel). One microgram of total RNA was reverse-transcribed using Superscript II RT (Invitrogen), random hexamers (Roche), and dNTPs at 42°C for 1 h. OPN fragments were then amplified using the High Fidelity PCR Master kit (Roche) and the appropriate oligonucleotides (A: 5’-TGAGAGCAATGAGCATTCCGATG-3’, B: 5’-CAGGGAGTCTCATGAAAGC-3’) using the following cycling parameters: initial denaturation at 94°C (5 min), then 30 cycles of 94°C (30 s), annealing at 60°C (30 s) and extension at 72°C (30 s), followed by a final extension at 72°C (7 min). The TMPRSS2-ERG fusion transcripts were amplified with the primers: TMPRSS2 forward primer 5’-CGCGAGCTAAGCAGGAGGC-3’ and ERG reverse primer 5’-GTAGGCACACTCAAACACGACTGG-3’ described in (24). As an internal control, hypoxanthine–guanine phosphoribosyltransferase (HPRT) fragments were also amplified under the
same conditions (forward primer: 5’-GCTGGTGAAAAAGGACCTCT-3’ and reverse primer: 5’-AAGTAGATGGCCACAGGACT-3’). PCR products were analyzed by electrophoresis in a 2% agarose gel.

**Cellular extract preparation and Western blot analysis**- Cellular extract preparation and Western blot analysis were carried out as previously described (35, 36). Immunodetections were performed using a polyclonal anti-ERG antibody (sc-353, Santa Cruz Biotechnology), a monoclonal anti-OPN antibody (sc-21742, Santa Cruz Biotechnology) and an anti-actin monoclonal antibody (Sigma).

**Immunohistochemistry**- Immunohistochemistry was performed on an automated immunostainer (Benchmark XT, Ventana, Strasbourg, France) with the XT ultraview diaminobenzidine kit. The primary antibodies were directed against ERG (sc-353, Santa Cruz Biotechnology, dilution 1/50) or OPN (sc-21742, Santa Cruz Biotechnology, dilution 1/100). Antigen retrieval was performed in Tris-EDTA buffer (pH 9) for 30 minutes at 95°C for ERG and in citrate buffer (pH 6) for 60 minutes at 95°C. Negative controls were realized by omitting primary antibodies. Counterstaining was done with hematoxylin and bluing reagent.

**Statistical Analysis**- Statistical analysis was performed using GraphPad Quick calcs (GraphPad Software). To analyze gene expression and the correlation between the expression of TMPRSS2:ERG and OPN, we used a 2x2 correlation table and Fisher’s exact test, and Spearman's correlation coefficient. We considered results statistically significant at \( P \)-value <0.05.

**RESULTS**

**Identification of ERG binding sites in OPN promoter sequences conserved across species and transactivation of the OPN promoter by ERG proteins**- To investigate the possibility of ERG-mediated OPN transactivation, we undertook a comparative genomic bioinformatics analysis to identify regions conserved between species and to define ETS binding sites (EBS). Using the ECR browser, the 5’ flanking region of the human OPN start site was compared to mouse, dog and rhesus macaque genomes (supplementary Fig. S1). This analysis shows that the OPN proximal promoter is well-conserved in all species analyzed, and two domains were defined: one from nt -289 bp to +6 bp, the other from nt -1100 kb to -920 kb. These two conserved domains were scanned using Transfac to find potential EBS composed of the 5’ consensus sequence -GGA/W-3’ (W:A or T). We found only one putative conserved EBS upstream of the OPN gene at nt -115 to -118 (EBS1). Another potential inverted EBS was identified at nt -108 to -111 (EBS2), but was found only in the mouse and rhesus macaque genomes. The computer search for transcription factor binding sites (Transfac
analysis) also disclosed Runx2, SBF-1, C/EBP and AP-1 sites, which have already been reported (30).

To test ERG transactivation, a series of sequential 5’-deletion mutant constructs were prepared from the region of the promoter sequence that confers efficient transcription of the *OPN* gene (37). The 5’-deletion sequences were cloned into the pGL3 reporter plasmid (Fig. 1A) and assayed for ERG responsiveness via luciferase activity in transient transfected HeLa cells (Fig. 1B). Moreover, since ETS-1 factors have been shown to regulate OPN promoter (ref), we performed luciferase assays using the same constructs (Supplementary Figure S2A and B). As expected, ETS-1 regulate the OPN promoter. Since ERG protein overexpression have been associated with more than 50% of prostate cancers, we performed transfection assays in a biologically relevant cell type for studying *OPN* in prostate cancer: PC3c cells that derived from the human prostate carcinoma PC3. Similar results were obtained compared to HeLa cell experiments (Fig. 1C). As shown in Fig. 1B (and supplementary Fig. S2A, assay using the plasmid pcDNA3-ETS-1), the deletion of nt -136 bp to -116 bp resulted in a dramatic loss of *OPN* promoter activity after ERG (or ETS-1) overexpression while OPN expression was not affected after the deletions including nucleotides downstream from -136 bp. Luciferase activity increased in a dose-dependent manner with the plasmid pSG5-ERG (Fig. 1D) or with the plasmid pcDNA3-ETS-1 (supplementary Fig. S2B). As expected OPN expression was dramatically decreased after shRNA-ERG cotransfections compared to the shRNA control. These results demonstrate that ERG transactivates the *OPN* promoter and this region, nt -136 bp to -116 bp, contains one or several essential cis-regulatory sites for ERG.

**Interaction of ERG with the OPN promoter**—To investigate whether these EBS were direct ERG targets, we performed an electromobility shift assay (EMSA) using a 32P-labeled WT nt -126 to -101 oligonucleotide substrate and purified ERG protein (Fig. 2A). Banding patterns revealed ERG affinity for these binding sites (designated as S in Fig. 2A) compared to the probe alone. In the presence of ERG antibodies (sc-353 Ab and sc-354 Ab), there were fewer S complexes and specific antibody-supershift bands SS’ and SS” were formed. No supershift was observed using the IgG isotype. Similar banding patterns were obtained when EMSA assays were performed in the presence of nuclear extracts from HeLa cells (supplementary Fig. S3) and PC3c (Fig. 2B) transfected with pSG5 or with pSG5-ERG. A supershift SS’ was only detected with ERG specific antibody in pSG5-ERG transfected cells.

In accordance with these results, the shift assay revealed ERG binding on the nt -126 to -101 sequence of the *OPN* promoter. ChIP assays were then performed with transfected HeLa cells to confirm that the EBS identified by EMSA in this portion of the *OPN* promoter is able to recruit ERG *in vivo* (Fig. 2C). As expected, ERG proteins were tethered to the endogenous *OPN* promter. No precipitation was detected with ERG antibodies in an irrelevant region (IR), validating the specificity of binding ERG to EBS. Together, these experiments clearly demonstrate that ERG binds to the *OPN* promoter.
**Identification of a functional cis-regulatory element** - To further characterize which EBS are involved in ERG binding within the WT nt -126 to -101 oligonucleotide region, we used site-directed mutant probes and constructs (Fig. 3A) and analyzed them using EMSA (Fig. 3B and 3C) and luciferase-promoter assays (Fig. 3D). A gel-shift complex (S) was present with the wt probe itself and the mutant 2 probe, but not with the mutant 1 or mutant 1+2 probes (Fig. 3B). In contrast, this complex was absent in 100-fold excess of unlabeled wt and mutant 2 probes, but persisted with mutant 1 probe (Fig. 3C). Similar patterns were obtained with nuclear extracts of transfected HeLa cells (data not shown).

Next, to assess the functionality of the EBS, we investigated the effect of ERG overexpression using a luciferase reporter construct driven by mutated EBS. The $\text{OPN}$ promoter fragment -136/+77, with ERG-responsive transcriptional activity similar to the intact 1518 bp promoter (Fig. 1B), was used as a template to introduce site-directed mutations in predicted EBS1 and EBS2 sequences, named $\text{OPN}$ -136/+77 mutant1 and $\text{OPN}$ -136/+77 mutant2, respectively, and transfected into HeLa cells (Fig. 3D). A mutation in nt -119 to -115 from GGAGGAAG to GGTTAAAA resulted in the suppression of ERG transactivation. In contrast, luciferase activity was not affected by mutations in nt -112 to -106 from GTAGGAAG to GTCGGAAG. Thus, these experiments suggest that ERG may play a direct role in $\text{OPN}$ promoter activation through a very specific EBS located at nt -118 to -115.

**Up-regulation of endogenous $\text{OPN}$ expression in prostate cancer cells with stable expression of ERG and TMPRSS2:ERG** - A high incidence recurrent fusion of TMPRSS2 with ERG has recently been highlighted in prostate cancer. This fusion results in aberrant androgen-regulated ERG in prostate cells. We therefore examined endogenous $\text{OPN}$ expression in a prostate cell line, PC3c, that we stably transfected with ERG and TMPRSS2:ERG (Fig. 4). Efficiencies of ERG and TMPRSS2:ERG overexpression were characterized in several subclones and compared with non-transfected cells and empty pcDNA3 vector-transfected cells by RT-PCR (Fig. 4A) and Western blotting (Fig. 4B). The molecular weight of ERG was expected to be 55 kD whereas TMPRSS2:ERG was expected to be 49 kD, consistent with the deletion of 32 amino acids from the N-terminus (Fig. 4B). Results obtained with representative subclones were similar for several subclones (data not shown). Expression analysis showed that, compared to the non-transfected PC3c and empty pcDNA3 vector-transfected PC3c, $\text{OPN}$ expression was greater in clones that overexpress ERG following transfection of ERG and TMPRSS2:ERG. Interestingly, ChIP assays revealed that ERG transcriptional factors were associated with the $\text{OPN}$ promoter in clones of PC3c cells expressing ERG and TMPRSS2:ERG in which $\text{OPN}$ was up-regulated but not in empty pcDNA3 clones (Fig. 4C). Together, these data suggest that ERG and TMPRSS2:ERG gene fusion products bind to the cis-regulatory domain of the endogenous $\text{OPN}$ promoter in PC3c cells and transactivate this gene.

**TMPRSS2:ERG status and osteopontin/TMPRSS2:ERG expression in human primary prostate cancers** - Samples of human primary prostate cancers were assessed for $\text{OPN}$ and TMPRSS2:ERG transcript expression using RT-PCR on total isolated RNA (Table 1A):
TMPRSS2:ERG fusions were detected in 71.4% (25/35) of the prostate tumors. This frequency of TMPRSS2:ERG fusions is in the range reported from studies conducted in Western countries (38, 39). Among these TMPRSS2:ERG+ tumors, 96% were also positive for OPN expression. Using a two-tailed, cross-tabulated Fisher’s exact test showed a significant association (P <0.01) between TMPRSS2:ERG and OPN expression (Table 1B).

Next, we examined the expression of ERG and OPN from human prostate tumors (Fig. 5A). Using immunohistochemistry, we observed the co-expression of ERG and OPN in areas of prostatic adenocarcinoma. Staining against ERG was nuclear with a moderate to strong signal in adenocarcinomatous glands as shown in Fig. 5. Osteopontin was detected in the cytoplasm of adenocarcinomatous glands. As a control, in normal prostatic glands, we did not observe ERG expression except in endothelial cells of vessels (Supplementary Figure S4), whereas, as previously described (51), none or weakly stained OPN expression was observed in the prostatic stroma. Moreover, no increase of the OPN expression level was observed in higher Gleason Score samples (Supplementary Figure S4 and data not shown) (51).

Among 16 samples analyzed immunohistochemically, there were 12 (75%) cases with OPN positive cells and 9 (56%) cases with TMPRSS2:ERG fusion positive cells (Table 2). Spearman's correlation coefficient was used to evaluate the correlation between protein expression levels (refered as % of cells stained) of ERG and OPN in IHC (Fig. 5B). Our data showed that there was a significantly positive correlation between expression levels of ERG and OPN ($r = 0.531; P < 0.05$).

**DISCUSSION**

OPN is a secreted phosphoprotein that has been found to facilitate anchorage of normal bone or cancer cells to mineralize tissue surfaces (40). This protein can be considered as one of the critical determinants in bone formation and tumor progression. The malignant and metastatic phenotype of human cancers has been correlated with elevated OPN expression, both at mRNA and protein levels (7). Uncovering transcriptional regulators of the OPN promoter has been stimulated by research seeking potential molecular targets that could modulate the metastatic phenotype and thereby constitute an effective therapeutic treatment. Several signaling pathways/transcription factors have already been proposed (for review, see ref. (41)). In this study, we demonstrate that ERG, a transcription factor associated with pre-cartilaginous condensation and chondrogenesis preceding bone formation (42, 43), binds to the OPN promoter and transactivates this gene. Through EMSA experiments, a functional and conserved cis-regulated enhancer site was localized at nt -118 to -115. A mutation in this consensus binding site abolished ERG transactivation. In agreement with EMSA, ChIP assays revealed that ERG directly binds to the OPN promoter in cells. These results suggest that
ERG is a critical transactivator of the OPN promoter. However, as noted (supplementary Fig. S1), many transcription factors have DNA binding sites adjacent to the EBS sequence in the OPN promoter. Transcriptional activity of the ERG factor, as in other ETS members, is modulated by the cooperation with other key transcription factors such as AP-1, CBP-P300, Runx2, NFkB, Gata1, etc. (44-46). The combinatorial control of OPN regulation by the ERG factor and its partners is a fertile ground for ongoing and future investigations on ERG expression in the cell.

Recently, ERG has been discovered to be highly overexpressed in the majority of human prostate tumors, due to the fusion of TMPRSS2:ERG through chromosomal rearrangements. The consequences of the TMPRSS2:ERG gene fusion in prostate tumorigenesis initiation remains controversial (47) and studies on its prognostic value have produced contradictory results (28, 48-50). Furthermore, prostate cancer is characterized by its high propensity — occurring in more than 80% of patients — to spread to skeletal tissue. However, the mechanisms underlying this preferential homing of prostate cells to bone tissues are only beginning to be understood. The high level of OPN expression is closely linked to the degree of malignancy and with the reduced survival of patients (51).

OPN has been demonstrated, along with other skeletal and matrix-associated proteins, to be expressed in cancer cells as major soluble factors stimulating the migration, survival, anchorage-independent growth and invasion of prostate cancer cells (52, 53). In this context, we expected that the abnormal expression of ERG protein in prostate tumor cells via the TMPRSS2:ERG rearrangement would induce ERG overexpression and thereby affect the expression of ERG-target genes. Among these deregulated genes, we hypothesized that the OPN gene would increase through ERG overexpression. In this study, we show that, in PC3c cells stably transfected by ERG or TMPRSS2(exon 1):ERG(exon 4) (the most frequent fusion in PCa), OPN transcription is enhanced and that ERG binds to the OPN promoter in PC3c DNA. Proteins encoded by this TMPRSS2(exon 1):ERG(exon 4) fusion transcript are truncated, lacking 32 amino acids from the N-terminus. Despite this truncation in the N-terminal region, whose functional role remains unknown (54), both ERG and TMPRSS2(exon1):ERG(exon4) similarly regulate OPN expression. However, in normal prostate cells, endogenous ETS factors may regulate a set of genes. Therefore, in prostate cancer cells, which exhibit the TMPRSS2-ERG fusion, the ERG proteins are strongly over-expressed causing a perturbation of transcriptional gene regulation. These changes could affect newly genes or ETS targets already regulated by endogenous ETS factors. For example, since ETS-1 is able to directly activate the OPN promoter, a competition could exist between ETS-1 and the overexpressed ERG factors for the OPN promoter binding and activation.

To test the biological relevance of our findings suggesting that TMPRSS2:ERG may increase OPN expression levels in prostate tumors, co-expression of OPN and ERG were assessed using RT-PCR and immunohistochemical analyses in specimens from patients diagnosed with prostate cancer (mainly biopsies from prostatectomy with Gleason scores evaluated at 7). Despite the biological heterogeneity that characterizes prostate cancer specimens, which can complicate molecular studies, a significant association was observed between TMPRSS2(exon 1):ERG(exon 4) fusion and OPN
expression. Moreover, tumor specimens showed similar immunostaining localization for ERG transcription and OPN. In this study, we only focused on the involvement of the most common fusion variant TMPRSS2(exon1):ERG(exon 4) reported in OPN up-regulation. However, this does not exclude the possibility that other fusion variants between androgen-dependent gene promoters and ETS family members are present in prostate tumor samples where TMPRSS2(exon1):ERG(exon4) has not been detected.

OPN has multi-functional properties that promote cell survival, cell adhesion and cell migration (41, 55). By binding to αvβ3 integrins, OPN protects cells from apoptosis through the activation and phosphorylation of the PI3-K/AKT pathway (56, 57). OPN activates various matrix-degrading proteases, such as matrix metalloproteinases (MMPs) and the urokinase plasminogen activator (PLAU) that contribute to malignancies (58, 59). MMP3, MMP9, a disintegrin and metalloproteinease 19 (ADAM19) and PLAU were recently reported to be increased in benign prostate cell lines RWPE and PrEC (primary benign prostate epithelial cells) transfected with overexpressed ERG factors (60). They were identified as direct targets of ETS transcription factors. Based on our results, we can also suggest that the activation of the OPN by TMPRSS2:ERG mediates the matrix metalloproteinase pathway and thereby prostate cancer progression.

In summary, the results presented here demonstrate that OPN is a target gene of ERG. The expression of the extracellular matrix protein osteopontin, which plays a crucial role in tissue remodeling, inflammation, tumor growth, angiogenesis and metastasis, is associated with TMPRSS2:ERG expression in prostate tumors cells and thereby participates in metastasis progression and aggressiveness. Small molecules that can prevent the interaction of TMPRSS2:ERG with the promotor of ERG-target genes in prostate cancer cells may be an interesting avenue of research for developing metastasis prevention strategies.

REFERENCES

FIGURE LEGENDS

Figure 1. ERG transactivates the OPN promoter as determined by transient transfection with OPN promoter-luciferase constructs
(A) 5’-deletion series of the OPN promoter constructs. The names of the deletion constructs indicate the location of the OPN promoter that was cloned in the upstream of the pGL3 luciferase gene (Luc). Boxes indicate EBS. (B) 5’-deletion analysis of the OPN promoter with ERG overexpression. HeLa cells were co-transfected with the OPN promoter-luciferase constructs (500 ng) with or without 200 ng of pSG5-ERG, as indicated. (C) Luciferase activity from -136/+77 and -1441/+77 OPN promoter constructs (500 ng) with or without 200 ng of pSG5-ERG transfected in PC3c cells. (D) Luciferase activity of OPN -136/+77 luciferase constructs (500 ng) in the presence of increasing quantities of ERG expression plasmids (0 ng, 50 ng, 100 ng, 150 ng, 200 ng), with or without shRNA targeting ERG (300 ng). In (B and C), empty pSG5 vector was added to adjust the total amount of DNA to 1 µg in all transfections. Activities were assayed after 48 h of transfection. The values obtained were normalized to the co-transfection of renilla luciferase (control). Data bars represent the mean of triplicate (or more) experiments, the error bars represent standard errors.

Figure 2. ERG interaction with the OPN promoter
(A and B). EMSA validation of ERG-DNA binding to the OPN promoter’s EBS sites with purified ERG protein (A) and nuclear extracts (NE) from pSG5 or pSG5-ERG transfected PC3c cells (B). EMSA were performed with purified ERG protein or nuclear extracts from transfected PC3c incubated with 32P-labeled probe (nt -126 to -101) in the presence of antibodies (Ab) directed against ERG (sc-353 Ab) or an IgG control Ab (IgG). The reaction was resolved on a non-denaturing polyacrylamide gel. The DNA-protein complexes resulting from sequence-specific transcription factor binding are designated with an S, supershift complexes are indicated by short arrowheads denoted SS’ and SS”. (C) ChIP analysis of ERG binding. ChIP analyses were performed as described in Materials and methods with pSG5 or pSG5-ERG transfected HeLa cells using 10 µg of anti-ERG Ab or the corresponding IgG isotype as a control. The input fraction corresponds to 5% of the chromatin solution before immunoprecipitation. Immunoprecipitated OPN promoter region (-371;-75) and an irrelevant region (IR) were analyzed on agarose gels by semi-quantitative OPN-specific PCR primers. Input chromatin (Input) used as PCR control and IgG are shown.

Figure 3. Identification of functional EBS in vitro and in vivo
(A) Sequences of the wild-type and mutant probes used in EMSA experiments. (B and C) EMSA performed with purified ERG protein. Doubled-stranded oligonucleotides were radiolabeled and incubated without (B) or with (C) a 100-fold excess of the unlabeled-competitor. (D) Luciferase activity from HeLa cells transfected by OPN (-136/+77) promoter constructs containing EBS mutations subcloned into pGL3 vector and normalized to renilla-luciferase activity. The values are expressed as the mean +/- S.D. of three triplicate experiments.

Figure 4. OPN expression in stable ERG-expression prostate cancer PC3c cells
(A) RT-PCR analysis of ERG and OPN transcript expression in stably transfected PC3c cells. M, DNA marker; -, negative control (no cDNA); NT, non transfected PC3c cells. (B) Representative Western blot analysis of whole cell lysates for ERG and OPN expression in stably transfected PC3c cells. Actin was also revealed to control protein loading. (C) ChIP analysis of ERG binding. ChIPs were performed in stably pcDNA3-, P55 ERG- and TMPRSS2:ERG- transfected clones of PC3c cells. PCR product band intensities relative to input in each stably transfected PC3c cells were determined with quantitative PCR analysis ((specific antibody ChIP – control Ig ChIP)/input DNA). Values were indicated underneath each gel results and summarized in a schematic diagram.

Figure 5. Immunohistochemical staining for ERG and OPN in human prostate cancer tissues
(A) OPN and ERG immunohistochemistry of consecutive prostate cancer tissue sections. The sections were counterstained with hematoxylin (blue). Arrows indicate positive staining (brown). Original magnification, x100 in top panels and x 400 in bottom panels. (B) Correlation between expression levels of ERG and OPN protein in 16 samples of prostate cancer tissue sections (table 2).
Spearman’s correlation coefficient was used to evaluate the correlation between mRNA and protein expression levels of WAVE2 in IHC. Our data showed that there was a significantly positive correlation between expression levels of WAVE2 mRNA and protein in HCC ($r = 0.531; P < 0.05$).

Table 1. **TMPRSS2:ERG and OPN RNA expression in human primary prostate tumor of patients**

Results from **TMPRSS2:ERG and OPN** expression in 35 samples of patients who had given their consent (mean age, 63 years, and Gleason score >7) diagnosed with prostate cancer (A) were analyzed in a 2x2 table using Fisher’s exact test, $P = 0.0040$ (B). (ND, not determined)

Table 2. **ERG and OPN protein expression in human primary prostate tumor of patients**

Expression status of ERG and OPN in 16 random samples of prostate cancer tissue sections using immunohistochemical experiments. The OPN signal was cytoplasmic with a weak to moderate intensity whereas ERG signal was nuclear with a moderate to strong intensity. The results were expressed as the percentage of cells showing staining.

Supplementary figure S1. **Promoter sequence of OPN**

(A) Schematic illustration of the **OPN** (Spp1) promoter region. Genomic organization of **OPN** (chr4: 89,114,320-89,115,830) is shown together the plots of pairwise genomic nucleotide conservation between human and mouse, rat, and chicken. Genomic alignment plots were constructed using ECR BROWSER (http://ecrbrowser.dcode.org). Based on genomic alignments, nucleotide conservation was calculated on a 100 bp window; conservation percentage is given. (B) Alignment of the **OPN** proximal promoter region and consensus binding sites of known transcription factors. Nucleotides sequences (positions indicated above the sequence) from the mouse and human **OPN** gene promoter were aligned. Locations of binding sites (obtained using Transfac) are indicated for ETS (ETS), upstream stimulating factor (USF), C:EBP, spliceosome-binding factor-1 (SBF-1), Runx2, AP-1. Sequences corresponding to oligonucleotide probes used for EMSA analysis (underlined) and sequences corresponding to PCR primers used to amplify the promoter in ChIP assays (in italic letters) are indicated.

Supplementary figure S2. **ETS-1 transactivates the OPN promoter**

(A) 5’-deletion analysis of the **OPN** promoter with ETS-1 overexpression. HeLa cells were co-transfected with the **OPN** promoter-luciferase constructs (500 ng) (fig.1A) with or without 200 ng of pcDNA3-ETS-1, as indicated. (B) Luciferase activity of **OPN** -136/+77 luciferase constructs (500 ng) in the presence of increasing quantities of ETS-1 expression plasmids (0 ng, 50 ng, 100 ng, 150 ng, 200 ng). Empty pcDNA3 vector was added to adjust the total amount of DNA to 1 µg in all transfections. Activities were assayed after 48 h of transfection. The values obtained were normalized to the co-transfection of renilla luciferase (control). Data bars represent the mean of triplicate (or more) experiments, the error bars represent standard errors.

Supplementary figure S3. **ERG interaction with the OPN promoter**

EMSA were performed with nuclear extracts (NE) from pSG5- or pSG5-ERG-transfected HeLa cells incubated with 32P-labeled probe (nt -126 to -101) in the presence of antibodies (Ab) directed against ERG (sc-353 Ab) or an IgG control Ab, as indicated. The reaction was resolved on a non-denaturing polyacrylamide gel. The DNA-protein complexes resulting from sequence-specific transcription factor binding are designated with an S, supershift complexes are indicated by short arrowheads denoted SS'.

Supplementary figure S4. **Immunohistochemical staining of human prostate tissues located at the periphery of the tumor for ERG and OPN**

(A, B and C) Immunohistochemical staining of ERG and OPN protein (A) in normal human prostate tissues located at the periphery of the tumor. Left panel: ERG-stained endothelial cells in small vessels (arrow) are a positive control for endogenous ERG expression for TMPRSS2:ERG fusion-negative prostate gland. Right panel: Weak OPN staining (arrow) was detected in stromal elements of normal...
prostate glands. The sections were counterstained with hematoxylin (blue). (*) normal prostatic gland with in the lumen, corporea amylacea showing a non specific staining (brown). Original Magnification: x 400. (B) Prostate cancer tissue (Gleason score 4+5), negative for the TMPRSS2:ERG fusion, ERG-stained endothelial cells in small vessels (arrow, left panel), no OPN staining (right panel). (C) Prostate cancer tissue (Gleason score 2+3), positive for the TMPRSS2:ERG fusion, ERG-stained cancer cells (arrow, left panel), OPN –stained cancer cells (arrow, right panel).
Figure 2

A. Purified ERG protein

B. pSG5 PC3c

C. pSG5 HeLa cells

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 3

A. Probes:

WT -126 to -101: 5'-CCAGAGGAGGAAGTGTAGGAGCAGGT-3'

MUTANT 1: 5'-CCAGAGGAGGAAGTGTAGGAGCAGGT-3'

MUTANT 2: 5'-CCAGAGGAGGAAGTGTAGGAGCAGGT-3'

MUTANT 1+2: 5'-CCAGAGGAGGAAGTGTAGGAGCAGGT-3'

B. Purified ERG protein

[^32P] labeled probe

Wt  Mutant 1  Mutant 2  Mutant 1+2

C. Purified ERG protein

Unlabeled-competitor

D. Fold induction

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Figure 4

A.

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Statistical Analysis

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2 x 2 Correlation Table

OPN expression vs. TMPRSS2-ERG expression

Fisher's Exact Test: The two-tailed P value < 0.01
A.

![ERG and OPN images](image)

B.

Spearman correlation $= 0.53136$, $P = 0.034$, $n = 16$
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ABNORMAL EXPRESSION OF THE ERG TRANSCRIPTION FACTOR IN PROSTATE CANCER CELLS ACTIVATES OSTEOPONTIN

Sébastien Flajollet, Tian V Tian, Anne Flourens, et al.

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