ERG upregulation and related *ETS* transcription factors in prostate cancer

KARI ROSTAD[1*], MONICA MANNELQVIST[2*], OLE JOHAN HALVORSEN[5], ANNE MARGRETE ØYAN[1,6], TROND HELLEM BØ[3], LAILA STORDRANGE[3], SUE OLSEN[1], SVEIN ANDREAS HAUKAAS[4,7], BIAOYANG LIN[9], LEROY HOOD[9], INGE JONASSEN[3,8], LARS ANDREAS AKSLEN[2,5] and KARL-HENNING KALLAND[1,6]

Sections for [1]Microbiology and Immunology, [2]Pathology, The Gade Institute, Departments of [3]Informatics, [4]Surgical Sciences, University of Bergen; Departments of [5]Pathology, [6]Microbiology and Immunology, The Gade Institute, [7]Department of Surgery, Haukeland University Hospital; [8]Computational Biology Unit, Bergen Center for Computational Science, University of Bergen, Norway; [9]Institute for Systems Biology, Seattle, WA, USA

Abstract. The aim of this study was to identify and validate differentially expressed genes in matched pairs of benign and malignant prostate tissue. Samples included 29 histologically verified primary tumors and 23 benign controls. Microarray analysis was initially performed using a sequence verified set of 40,000 human cDNA clones. Among the genes most consistently and highly upregulated in prostate cancer was the *ETS* family transcription factor *ERG* (*ETS* related gene). This finding was validated in an expanded patient series (37 tumors and 38 benign samples) using DNA oligonucleotide microarray and real-time quantitative PCR assays. *ERG* was 20- to more than 100-fold overexpressed in prostate cancer compared with benign prostate tissue in more than 50% of patients according to quantitative PCR. Surprisingly, *ERG* mRNA levels were found to be significantly higher in the endothelial cell line, HUVEC, than in the prostate cell lines PC3, DU145 and LNCaP. *In situ* hybridization of prostate cancer tissue revealed that *ERG* was abundantly expressed in both prostate cancer cells and associated endothelial cells. The consistency and magnitude of *ERG* overexpression in prostate cancer appeared unique, but several related *ETS* transcription factors were also overexpressed in matched pairs of tumor and benign samples, whereas *ETS2* was significantly underexpressed. Our findings support the hypothesis that *ERG* overexpression and related *ETS* transcription factors are important for early prostate carcinogenesis.

Correspondence to: Dr Karl-Henning Kalland, Centre for Research in Virology, The Gade Institute, Biobuilding-BBB, Jonas Liesv. 91, N-5009 Bergen, Norway
E-mail: kalland@vir.uib.no

*Contributed equally

Introduction

Recent DNA microarray based studies have revealed characteristic gene expression profiles associated with either primary prostate cancer or metastasis (1-3), progression to hormone refractory tumors (4), or patient survival (3,5). Some of these reports have identified novel and potentially important tumor markers such as α-methylacyl-CoA-racemase (6-9) and hepsin (3,10-13). The aim of our present study was to explore the expression profiles of prostate cancer with special focus on important transcription factors. Among the genes most consistently and highly upregulated was the transcription factor *ERG* (*ETS* related gene), showing 20-fold to more than 100-fold increased expression of *ERG* in 50% of prostate tumors (T) compared with benign samples (B).

ERG is one of more than 30 transcription factors belonging to the ETS family, which is defined by a conserved DNA binding domain that recognizes the promoter sequence GGA^{A}/$_{T}$ (14). *ETS* transcription factor genes have been acquired and selected for during evolution of oncogenic retroviruses (14,15), and they also appear as fusion partners in certain tumor chromosomal translocations (14). During mammalian embryogenesis, ERG is first expressed in endothelium and later in the kidney, urogenital tract and hematopoietic cells, whereas down-regulation is observed following tissue differentiation (16,17). The embryonic activation pattern of *ERG* is relevant for our present observations that *ERG* transcription is strongly increased in prostate cancer epithelial cells, and with additional *ERG* expression in prostate endothelial cells. This upregulation of *ERG* in prostate cancer may provide an example of oncogenic reactivation of an embryonic transcription factor.

The literature is not consistent regarding the exon mapping of *ERG* (18). *ERG* transcripts from benign prostate hyperplasia were therefore PCR amplified and DNA sequenced. A resulting revised exon map of *ERG* isoforms 1 and 2 is presented. An *ERG* isoform 1 specific exon was consistently overexpressed in prostate cancer samples.

ERG overexpression in prostate cancer was distinct compared with other *ETS* family transcription factors, several endothelial markers and neighbouring genes on chromo-

some 21q22. In parallel with our work, *ERG* overexpression in prostate cancer and a plausible mechanism have been published (19,20) involving a chromosomal translocation that brings *ERG* transcription under control of the androgen responsive promoter of the gene *TMPRSS2* which is highly expressed in both benign and malignant prostate tissue (21). It is likely that *ETS* transcription factors, in particular over-expressed *ERG*, and possibly underexpressed *ETS2*, are important for prostate carcinogenesis.

Materials and methods

Patient series and characteristics. During 1997-2003, fresh prostate tissues were collected from patients treated by radical prostatectomy for clinically localised and biopsy verified prostate cancer at the Department of Surgery, Section of Urology, Haukeland University Hospital, Bergen, Norway (22). Tissue samples were immediately frozen in liquid nitrogen and stored for later use at -80˚C. For the cDNA microarray study, 52 tissue samples from 33 patients (median age 60 years, range 47-69) were used, including 4 solitary benign (B) samples and 29 histologically verified and pathologically characterized primary tumors (T), for which we had matched B samples for 19 of them, as described elsewhere (22). For validation purposes this series was expanded to a total of 37 T and 38 B samples, including 27 paired prostate T and B samples.

RNA purification, enzymatic modification and fluorochrome labelling. Individual biopsies were ground to powder under liquid N_2. Total RNA was extracted according to standard protocols (23) (InVitrogen TRIzol LS protocol and Qiagen RNeasy minikit protocol). T7 RNA polymerase promoter-containing double stranded cDNA and T7 RNA polymerase amplified RNA (cRNA) were generated as previously described (22) and according to the Ambion T7 Megakit protocol. Aminoallyl-U (aminoallyl-UTP from Ambion) incorporation into cRNA followed by cross-coupling to Cy5 and Cy3 by means of reactive Cy-NHS compounds (Amersham) was used for fluorochrome labelling of nucleic acids. Cy5 and Cy3 incorporations were measured by absorption readings at 649 and 550 nm, respectively, using a PowerWave Spectrophoto-meter. Simultaneous recording of absorptions at 260 nm allowed calculation of specific labelling.

DNA microarray. The Research Genetics human 40k cDNA microarray printed at the Institute for Systems Biology, Seattle has been described previously (22). RNA derived from a pool of 18 cell lines was Cy-labeled in parallel with patient samples and served as reference RNA during cDNA micro-array hybridizations. The Agilent human 1A oligonucleotide microarray (21k) was used for validation according to the Agilent protocols except for a more stringent wash (0.X SSC at 25˚C for 10 min). The Stratagene Universal RNA was the reference RNA for Agilent microarrays. The oligonucleotide microarrays were scanned and features automatically extracted, recorded and analysed using the Agilent Microarray Scanner Bundle. Normalization, flooring or filtration of data were done as previously described (22). Genes were filtered before inclusion in the dataset, and genes were included if the signal intensities in both channels differed >2SE over background in at least 70% of samples in each class (e.g., T or B) (filtered

dataset). As filtering may exclude candidate genes expressed in subsets of samples, an alternative method, flooring, was also performed (22). In the case of low or no signal in at least one channel the log-ratio was based on intensities floored at a value of 20 and included in a second dataset (floored dataset). Data were formatted in a J-Express-file suitable for additional data mining (http://www.molmine.com/) (24).

Identification of differentially expressed genes. Following normalisation, a Cy5/Cy3 $\log_2$-ratio was calculated for each feature of the microarray. Average fold change was calculated for each gene. Fold change for gene *i* was calculated by taking the absolute difference *d* of the average log-ratio in T and B, and then taking 2^d. In addition we determined the t-score (two-sample t-test) of each gene, thus quantifying the distance of the average log-ratios between the groups compared to the spread of log-ratios within each group. A paired t-test was also used.

PCR and real-time quantitative PCR (qPCR). Single-stranded cDNA for qPCR analysis was synthesised from 50 ng/μl of total RNA using a final concentration of 5 μM random hexamer primers, pd($N)_6$ (Amersham Pharmacia Biotech) and M-MLV reverse transcriptase according to Ambion instructions. qPCR reactions were set up in a total volume of 25 μl containing 12.5 μl 2X TaqMan Universal Master Mix, including ROX-reference dye, uracil N'-glycosylase (Applied Biosystems), AmpliTaq Gold DNA polymerase (Perkin-Elmer), 900 nM of each primer and 250 nM of FAM-labelled TaqMan probe, and 5-10 ng of total RNA (as hexamer primed ss-cDNA). The mixtures were prepared in 96-well optical microtiter plates and amplified on the ABI7900HT Sequence Detection System using the following cycling parameters: 2 min at 50˚C, 10 min at 95˚C, and 40 alternate cycles of 15 sec at 95˚C and 60 sec at 60˚C. Serial dilutions of pooled prostate cDNA were used for the generation of standard curves for each TaqMan assay including the endogenous control. Each sample cDNA was tested in triplicate. The SDS2.2 software was used for analysis and relative quantification according to program manuals and the Applied Biosystems User Bulletin #2. Table I shows the TaqMan Custom made assays (Assay-by-Design) for the detection of *ERG* sequences and the ß-actin TaqMan assay used as endogenous control for normalization between samples.

qPCR in the low density array (LDA) format. Low density arrays (LDA) are customisable, 384-well microfluidic cards for real-time qPCR (Applied Biosystems). Each LDA card was configured for 95 different genes in duplicates including Celera gene ID: -hCG401221 v-ETS erythroblastosis virus E26 oncogene like (avian) (*ERG*). This TaqMan assay targets nucleotide position 330 of NM_182918 (*ERG1*) and position 444 of NM_004449 (*ERG2*). Hexamer-primed single stranded cDNA corresponding to 5 ng of prostate total RNA was diluted in TaqMan Universal buffer (Applied Biosystems) and added to each loading well. Using the above configuration each sample occupied 4 wells or one half of each card. The samples were distributed to the microwells by centrifugation for 1 min at 343 x g. The cards were sealed and placed in the ABI 7900HT Sequence Detection System using the following cycling parameters: 2 min at 50˚C, 10 min at 95˚C, and 40 alternate cycles of 15 sec at 95˚C and 60 sec at 60˚C. The

Table I. TaqMan assays used for the quantification of *ERG1* and *ERG2* mRNA isoform specific exons, *ERG* common exons and the endogenous ß-actin mRNA.[a]

Accession no. mRNA (nucleotide position)	Primer/FAM-probe	Sequence
NM_182918 *ERG1*	182918-T1F (736-756)	TCTCCACGGTTAATGCATGCT
	182918-T1R (821-796)	CTTTGCGTAGCTTCAGGATATACTGA
	FAM-182918-T1M2 (772-757)	CACCCCCTGTGTTTCT
NM_004449 *ERG2*	004449-T1F (169-193)	GCTGGCTTACTGAAGGACATGATTC
	004449-T1R (257-237)	GACTGGTCCTCACTCACAACT
	FAM-004449-T1M1 (215-236)	CTCATATCAAGGAAGCCTTATC
NM_182918 and NM_004449	ERG182918-T3F (964-990; 1006-1032)	GATCCTTATCAGATTCTTGGACCAACA
	ERG182918-T3R (1042-1022; 1084-1064)	GGAACTGCCAAAGCTGGATCT
	FAM-ERG182918-T3M2 (1019-1004; 1061-1046)	CCACTGCCTGGATTTG
AA424601 (ERG-UTR)	AA424601ER-T3F (62-84)	CAGGAGCTCTCACTAGGTAGACA
IMAGE no.: 767130	AA424601ER-T3R (139-118)	CTAGTGAATCCCAAGCCACAGT
	FAM-AA424601ER-T3M2 (93-111)	CCTGCTACATCAGAGTTAC
NM_001101 ß-actin	001101AD-T7F (1037-1061)	CCCAGCACAATGAAGATCAAGATCA
	001101AD-T7R (1118-1103)	GCGAGGCCAGGATGGA
	FAM-001101AD-T7M2 (1094-1078)	TCCACACGGAGTACTTG

[a]Accession nos. and nucleotide positions are shown for primers and probes. F, sense primer; R, antisense primer; FAM, fluorochrome label.

SDS2.2 software was used for qualitative analysis and data were exported to Excel for further exploration and visualization.

DNA sequencing of ERG1 and ERG2 isoforms. Specific cDNA was made from 2 µg total RNA in 20 µl 1X first strand buffer with 0.01 M DTT, 0.5 mM dNTPs, 1.0 µl RNaseOUT (Invitrogen), 2.0 µl Superscript III RT (Invitrogen) and 2.0 µM *ERG* reverse oligo primer. The cDNA synthesis was performed at 50°C for 90 min. cDNA (1.5 µl) was amplified using 1X HotMasterMix (Eppendorf) and 0.2 µM of each forward and reverse primer. PCR amplification was performed with an initial denaturation at 95°C for 10 min followed by 40 cycles at 94°C for 15 sec, 58°C for 20 sec and 72°C for 40 sec. PCR product (5 µl) was mixed with 2 µl Exo-SAP-IT (USB) for removal of unincorporated dNTPs and primers. The Exo-SAP clean up was done at 37°C for 15 min followed by enzyme inactivation at 80°C for 15 min. Cleaned PCR product (3.5 µl) was mixed with 1 µl BigDye Terminator v1.1 (Applied Biosystems), 2 µl 5X BigDye Terminator sequencing buffer and water to a final volume of 10 µl with 210 nM of forward and reverse primers. The reaction was run at 96°C for 1 min followed by 25 cycles at 96°C for 10 sec and 58°C for 5 sec followed by 60°C for 4 min. The sequence reaction was analysed on a 3100 Genetic Analyser (Applied Biosystems).

Cell culture and cell lines. The cell lines SaOS-2 (osteosarcoma derived), HLF (human lung fibroblasts), HUVEC (human endothelial cell line) and the prostate cancer cell lines PC3, DU145 and LNCaP were obtained from the American Type Culture Collection (ATCC, Rockwell, MD, USA). SaOS-2 monolayers were grown in McCoy medium (BioWhittaker) with 2 mM glutamine. All media contained 5-10% fetal calf serum (BioWhittaker) and 100 µg/µl gentamicin (BioWhittaker). The EGM-2 bullet kit (BioWhittaker) was used for HUVEC monolayers. Iscove's modified Dulbecco's medium (IMDM) (BioWhittaker) with 25 mM HEPES and 2 mM glutamine was used for HLF, DU145 and LNCaP monolayers. PC3 monolayers were grown in HAM's F12K (BioWhittaker) with 2 mM glutamine. The cell monolayers were harvested in TRIzol LS. RNA was extracted according to the InVitrogen TRIzol LS protocol. The cell lines CRL-2392 (GA10, Burkitt lymphoma, human) and CRL-1593.2 (U937, histiocyte human) obtained from ATCC were grown in suspension in IMDM with 25 mM HEPES and 2 mM glutamine and were harvested by centrifugation at 840 x g for 5 min. Prior to lysis in TRIzol LS the cells were gently resuspended in 500 µl of 0.9% NaCl and RNA was extracted according to the InVitrogen TRIzol LS protocol. The pellet following isopropanol precipitation was dissolved in RLT ß-mercaptoethanol buffer (Qiagen) and total RNA was extracted according to the instructions enclosed with the Qiagen RNeasy minikit.

RNA probe synthesis and in situ hybridization. T3- and T7-containing Bluescript SK⁻ ERG/Actin plasmids (Invitrogen) were used for synthesis of DIG-RNA *ERG* antisense, *ERG* sense and ß-actin antisense probes, respectively. Plasmids were cut with restriction enzymes and then sequenced to verify the specificity of the sequence. MEGAscript High Yield Transcription Kit T3/T7 (Ambion) was used for RNA synthesis. The synthesis was performed with 1 µg DNA and 1.9 mM ATP/CTP/GTP, 1.3 mM UTP, 0.7 mM DIG-UTP and buffer and enzyme mix according to the kit manual (Ambion) at 37°C for 4 h. The DIG-cRNA was next fragmented to 60-200 long nucleotides using RNA Fragmentation Reagents according to the manual (Ambion).

Slides with paraffin embedded tissue were incubated at 65°C for 1 h, deparaffinized 5 min twice in xylene and re-

Table II. Differentially upregulated genes in 29 localized prostate cancers compared with benign tissue using 40k cDNA microarrays (Research Genetics).[a]

Fold change	t-score	IMAGE no.	Description	Gene ontology term
6.7	5.9	1034473	*AMACR* (α-methylacyl-CoA racemase)	Isomerase activity
4.0	4.5	788180	*AMACR* (α-methylacyl-CoA racemase)	Isomerase activity
2.8	3.5	450049	*EST* (similar to cDS4 retroviral related polyprotein)	NA (not available)
2.7	3.4	646037	*EST* [weakly similar to protease (*H. sapiens*)]	NA
2.7	3.0	1553723	*SIM2* [single-minded homolog 2 (*Drosophila*)]	Transcription
2.6	4.9	782383	*EST*	NA
2.4	2.6	281003	*TRGV9* (T cell receptor γ variable 9)	T-cell receptor
2.3	2.7	109316	*SERPIN3* (serine proteinase inhibitor, clade A)	Proteinase inhibitor
2.2	3.9	784168	*EST*	NA
2.2	4.4	767130	*EST* [*ERG* (Ets-related gene)]	Transcription
2.2	3.1	510576	*AGR2* [anterior grad. 2 homolog (*Xenopus laevis*)]	Oncogenesis
2.2	5.2	208413	*HPN* (hepsin transmembrane protease, serine 1)	Protease
2.2	4.1	838478	*NCALD* (neurocalcin)	Phosphorylation
2.1	2.6	2119355	*EST*	NA
2.1	4.3	745283	*EST*	NA
2.0	2.0	77915	*PLA2G2A* (phospholipase A2, group IIA)	Phospholipase

[a]Genes were ranked according to average fold change between T and B based upon an unpaired t-test of a floored dataset.

hydrated 5 min twice in 100% ethanol followed by 5 min in DEPC-H$_2$O. Slides were boiled in citrate buffer for 20 min and then treated with 0.2 M HCl and 0.25% acetic anhydride for 15 min each. Fragmented DIG-labelled probes were mixed with hybridization solution (Sigma) to 20 ng/µl, added to slides and incubated 12 h at 42˚C. Post-hybridization wash was done twice in 2X SSC at 52˚C for 10 min. Unbound probe was removed using 10 µg/ml RNase at 37˚C for 10 min followed by 2X SSC/50% formamide at 42˚C for 10 min to refix. Slides were blocked with 2% horse serum for 30 min before incubation with anti-DIG-AP Fab fragments diluted 1:250 overnight at 4˚C. Staining was done with liquid permanent red chromogen (LPR) for 5-20 min (Dako) and counterstaining with hematoxylin.

Results

Differential gene expression of malignant and benign prostate samples. Both filtered and floored 40k cDNA microarray data of 52 prostate samples [29 malignant tumors (T) and 23 benign (B) samples, including 19 paired T and B samples] were examined (22). Some variation was found among gene lists based upon floored or filtered data used in either paired or unpaired t-tests and ranked according to either t-score or fold change. A disadvantage with filtered data is the possible removal of genes that are not expressed in benign tissue, or expressed in only a minor subset of the tumors. Table II was based upon a floored dataset (floored at a value of 20) and the unpaired t-test included all 29 Ts and 23 Bs. The genes were ranked according to fold change and Table II confirmed several previous publications regarding AMACR (9,10), T cell receptor γ locus (9,25), AGR2 (26,27) and hepsin (3,10-13). We were also able to confirm the overexpression of *SIM2*

in a subset of prostate cancers (28-30). Many ESTs were differentially expressed (Table II).

Validation of ERG overexpression in prostate cancer using qPCR. Two different ESTs (IMAGE nos. 767130 and 123755, Fig. 1) corresponding to *ERG* sequences according to BLAST alignments were consistently and highly upregulated in prostate cancers according to t-test analysis of DNA micro-array data. Both ESTs, however, mapped 3' to the *ERG* coding region (Fig. 1). One TaqMan Assay corresponding to GenBank Accession no. AA424601 and another TaqMan assay targeting ERG exons 12-13 were therefore designed (Table I, Fig. 1). Both confirmed the specific upregulation of *ERG* transcripts. Next an expanded patient series (22) was examined using the TaqMan assay targeting *ERG* exons 12 and 13 (Figs. 1 and 2). This series included 37 Ts and 38 Bs, including 27 paired Ts and Bs from the same patients. The median (mean) mRNA ERG expression among 37 T samples was 4.09 (4.56) and among 38 benign samples 0.18 (0.25) (P<0.001, Mann-Whitney test) (Fig. 2). The median (mean) mRNA ERG expression among 27 matched pairs was 4.28 (5.07) in the tumor samples and 0.17 (0.28) in the benign samples (P<0.001, Mann-Whitney test). More than 20-fold upregulation of prostate *ERG* expression was evident in 14 of 27 matched pairs of T and B obtained from the same gland. T/B ratios above 40 were observed for six of these patients, and T/B ratios above 100 were observed for two of these patients (TaqMan qPCR assays).

DNA sequencing and exon mapping of the ERG gene. Transcripts derived from the *ERG* gene of a sample of benign prostate hyperplasia and spanning almost 300,000 bp of genomic contig were sequenced and aligned. The resulting
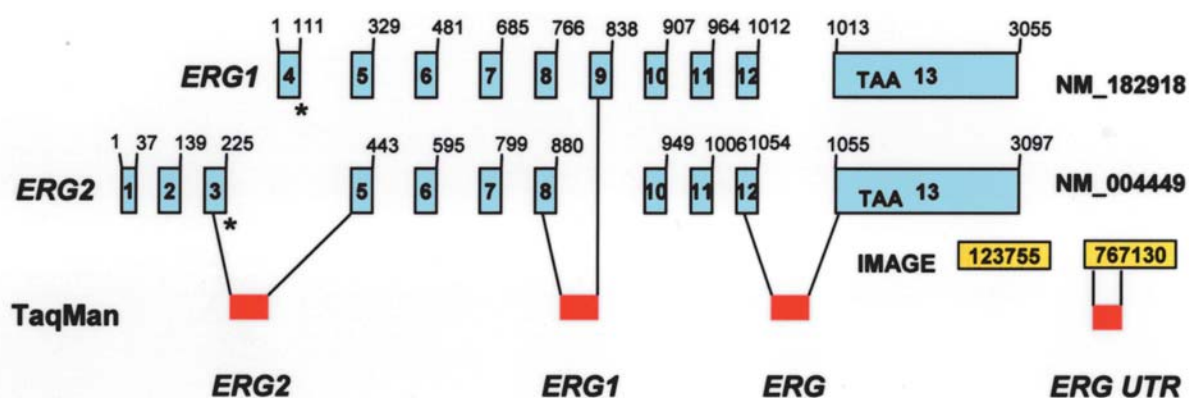
Figure 1. Revised exon organization of *ERG* isoforms 1 and 2. Nucleotide positions of exons corresponding to Genbank Accession nos. NM_182918 (isoform 1) and NM_004449 (isoform 2) are shown. The positions of the IMAGE sequence numbers 123755 and 767130 [in the *ERG* 3' UTR (untranslated region)] are indicated. Asterisks and TAA mark localization of start and stop codons, respectively. TaqMan assays were devised to be specific for *ERG1, ERG2* or common to both *ERG* transcripts as indicated. The exact sequences and nucleotide positions of the 4 TaqMan assays are displayed in Table I.
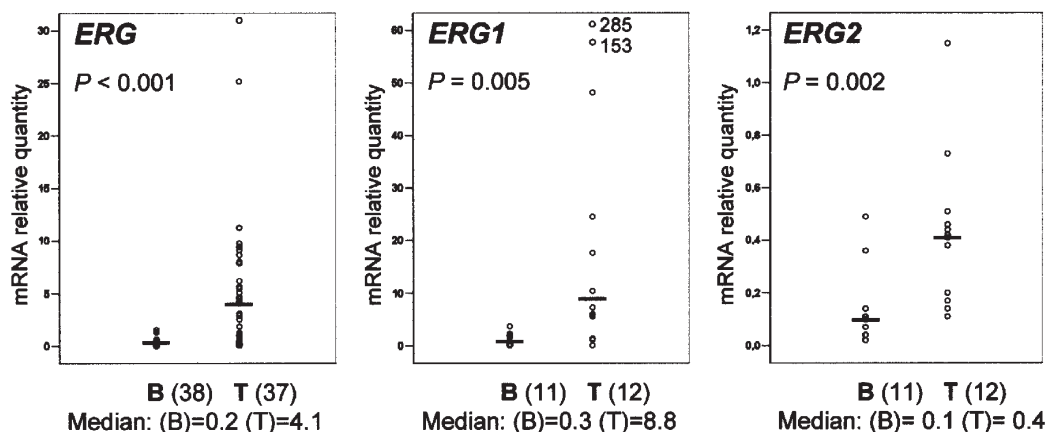

Figure 2. Total RNA was extracted from 37 prostate cancers (T) and 38 benign prostate samples (B) including 27 paired samples of T and B. mRNA expression levels were determined by real-time qPCR. Quantification of samples was performed according to the standard curve method. Data for both transcript variants (*ERG*), isoform 1 specific (*ERG1*) and isoform 2 specific (*ERG2*) exons are shown. The ß-actin normalized sample values are indicated by circles and the medians by horizontal lines. P-values: Mann-Whitney test.

revised exon map is shown in Fig. 1 and is consistent with the GenBank Accession nos. NM_182918 (*ERG* isoform 1) and NM_004449 (*ERG* isoform 2).

Expression of ERG isoform specific exons in different prostate samples. Eleven paired T/B prostate samples, supplemented with one solitary T, were examined using TaqMan qPCR assays specific for the most 3' *ERG1* specific exon (NM_182918) and a more 5' *ERG2* specific exon (NM_004449) (Fig. 1, Table I). A low level expression of both *ERG1* and *ERG2* specific exons were detectable in benign samples (Fig. 2). The median (mean) *ERG1* exon expression (normalized by ß-actin expression) among 12 Ts was 8.82 (46.7) and among 11 Bs 0.31 (1.15) (P=0.005, Mann-Whitney test). The median (mean) *ERG2* exon expression among 12 Ts was 0.42 (0.43) and among 11 Bs 0.1 (0.14) (P=0.002, Mann-Whitney test). The very high overexpression of *ERG* detected in a large subset of prostate cancers therefore included the 3' *ERG1* specific exon (exon 9) (Fig. 2, Table I). A control sample of benign prostate hyperplasia (BPH) revealed moderate expression of both *ERG1* and *ERG2* isoform specific exons, but 4-fold higher expression of *ERG1* than *ERG2* exons (Table III).

Table III. Real-time qPCR values of *ERG*, *ERG1* or *ERG2* specific exons in different cell lines and benign prostate hyperplasia relative to the SaOs-2 cell line.[a]

Sample	*ERG*	*ERG1*	*ERG2*
SaOs-2	1.0	1.0	1.0
HUVEC	1928	3900	3.2
DU145	1.4	5.0	3.0
PC3	1.2	2.0	-
LNCaP	-	-	-
HLF	0.3	0.4	ND
U937	19.5	8.0	7.0
GA10	-	-	-
BPH10	298	99	24

[a]ß-actin was used for normalization between samples. All samples were examined in triplicates.

Expression of ERG isoforms in different cell lines. A preferential endothelial expression of *ERG* has been reported (31-33). As one initial approach to define the cell type specific
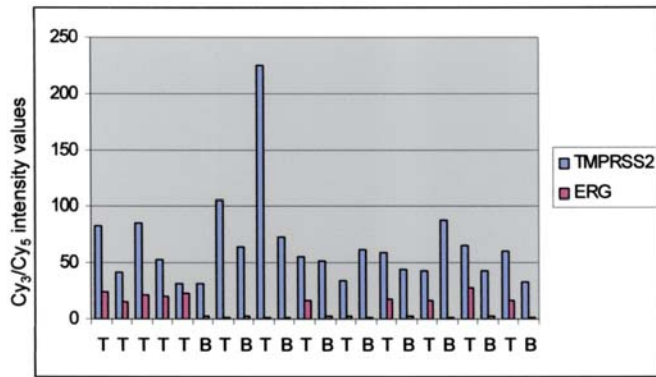
Figure 3. Cy3 T/Cy5 Stratagene U RNA and Cy3 B/Cy5 Stratagene U RNA intensity values according to the Agilent 21k human oligonucleotide micro-array. Four solitary prostate cancers (T) and nine matched prostate T/B pairs are shown. The *TMPRSS2* mRNA was abundantly expressed in both Ts and Bs relative to the Stratagene Universal RNA. The *ERG* mRNA was abundantly expressed in a large subgroup of Ts but not in Bs relative to the Stratagene Universal RNA.

Figure 4. Tumor (T) versus benign (B) expression ratios of *ERG, ELF5* and *ETS2* in 9 matched pairs of tumor and benign prostate samples according to Agilent oligonucleotide microarrays. Prostate nucleic acids were Cy3 labeled. The reference nucleic acid (Stratagene U RNA) was Cy5 labeled. *ERG* expression ratios are in blue, *ELF5* in red and *ETS2* in yellow. *ERG* expression ratios above 5 are indicated next to the relevant sample. Mean T and B values and associated P-values are in the Results.

expression of *ERG* a panel of endothelial (HUVEC), mesenchymal (HLF, SaOS-2), lymphoid (GA10, U937) and prostate cancer (PC3, DU145, LNCaP) cell lines were examined using qPCR (Table III). The *ERG1* specific exon was over 1,000-fold more abundant than the *ERG2* specific exon in HUVEC cells. SaOS-2, DU145 and U937 contained much lower, but detectable levels of both *ERG1* and *ERG2* specific exons. In PC3 cells low level expression of only the *ERG1* exon was found. Low level expression of exon 1 was also found in HLF. In GA10 and the androgen responsive prostate cancer derived cell line LNCaP neither *ERG1* nor

ERG2 exons were detectable (Table III). *ERG* mRNA was not detected in LNCaP cells stimulated with the synthetic androgen R1881 for up to 48 h using any *ERG* specific TaqMan assay (results not shown).

Correlation between gene expression of ERG and selected vascular markers. Twenty-two randomly selected prostate samples (4 unpaired prostate cancers and 9 paired T and B
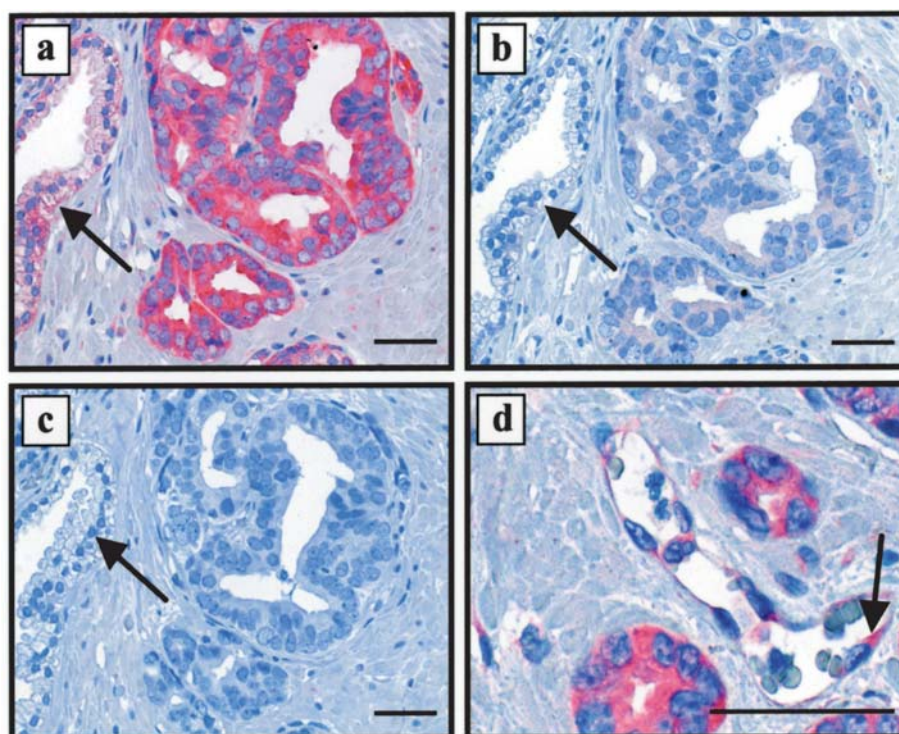
Figure 5. ERG mRNA expression shown in prostate cancer and benign glands (arrows) by *in situ* hybridization. Scale bars 100 μm. a, The *ERG* antisense RNA probe reveals strong expression of *ERG* in the cytoplasm of tumor cells and a much weaker expression in benign cells. b, The *ERG* sense probe was used as a negative control. c, Additional negative control with omission of the DIG-labelled probe. d, Expression of *ERG* in the endothelial cells (arrow) of a vessel. Scale bar 100 μm.

Table IV. Agilent 21k oligonucleotide microarray paired T/B ratios (columns) of selected vascular markers (rows) and *ERG*. The Agilent microarray values shown are Cy3 T/Cy5 B intensity values.

Gene	T/B 1.3P29	T/B 2.4P54	T/B 2.2P33	T/B 4.4P41	T/B 4.8P85	T/B 4.6P90	T/B 5.1P46	T/B 5.3P98	T/B 4.2P78
PECAM1	0.47	0.37	1.52	1.21	0.90	2.05	1.24	2.04	0.99
TEM5	0.38	0.40	0.97	0.90	0.48	0.47	0.51	0.92	0.72
FLT1	0.84	0.43	0.76	0.56	27.39	1.21	0.37	1.13	0.56
CD34	0.52	0.58	0.63	0.31	0.78	1.36	0.62	0.46	0.64
TEM7R	0.68	0.71	1.55	1.33	0.73	0.77	0.79	1.44	1.13
TEM7	0.38	0.55	0.87	0.45	1.00	2.30	3.23	1.16	5.73
PODLX2	2.83	2.62	0.89	1.32	1.84	2.53	12.95	1.37	1.82
TEM1	0.37	1.12	1.13	0.79	0.90	0.92	1.31	0.81	1.06
VWF	0.38	0.49	0.74	0.85	1.64	0.93	2.14	2.17	2.04
FLT4	0.32	0.62	0.84	0.94	0.74	0.44	0.71	0.80	0.78
PECAM1	0.36	11.46	0.84	0.96	0.87	2.04	16.04	0.90	1.39
ERG	10.27	0.29	1.23	7.75	1.60	6.54	9.60	12.23	14.03

samples) were examined using the Agilent human oligo-nucleotide 1A (v1.0) microarrays (Fig. 3). A paired t-test including all 9 T/B pairs shown in Fig. 4 revealed that *ERG* was significantly overexpressed in T compared with B (mean *ERG* T=13.6, mean *ERG* B=1.9, P=0.007). The same statistically significant relationship was present in the unpaired t-test including 13 Ts and 9 Bs. Selected endothelial cell markers were analysed (Table IV). In neither case did T/B ratios of *ENG* (endoglin/CD105), *PECAM1* (CD31), *PODLX2* (podocalyxin-like, CD34 family), *VWF* (von Willebrand factor) or several *TEM* factors correlate with the T/B ratios of *ERG* (Table V). In contrast, the Spearman test revealed several significant correlations between T/B ratios of different endothelial markers (Table V). In addition, altogether 10 of the prostate T/B pairs previously analysed by microarrays were validated using the Applied Biosystems LDA qPCR format for *ENG, PECAM1, PODLX2* and *VWF* (Table VI). Again, no significant relationship was evident between *ERG* over-expression and expression of the vascular marker genes in prostate cancer.

In situ hybridization detects ERG mRNA mainly in epithelial prostate cancer cells but also in endothelial cells. The detection of *ERG*-specific mRNA using DIG-labelled antisense ribo-probes and *in situ* hybridization of prostate cancer tissue is shown in Fig. 5. The corresponding prostate cancer tissue expressed abundant *ERG* mRNA according to microarray and real-time qPCR analysis. A ß-actin riboprobe control showed extensive staining of all cell types of the sections (data not shown). The malignant prostate glands contained abundant *ERG* mRNA in the epithelial cytoplasm (Fig. 5) while the benign glands were negative or only weakly positive. In addition *ERG* mRNA was clearly detected in endothelial cells (Fig. 5).

Correlation between microarray expression data of ERG and other ETS family transcription factors. Agilent microarray expression data were available for 20 different *ETS* trans-cription factors (Table VII). No case of overexpression in

prostate cancers (high T/B among the 9 pairs) was evident for 11 of the 20 *ETS* factors. The 3 out of 9 T/B pairs lacking increased *ERG* instead overexpressed alternative *ETS* trans-cription factors (Table VII). *ELF5* exhibited elevated T/B expression ratios in patients 2.4P54, 2.2P33 and 4.8P85, res-pectively (Fig. 4, Tables VI and VII). *ETV1* T/B was elevated in 4.8P85, and *ETS1* T/B was elevated in 2.4P54 (Table VII). A Spearman test was conducted for the correlation of T/B ratios between the 9 paired Ts and Bs of different *ETS* family transcription factors (Table VII). The results (Table VIII) showed a negative correlation between *ELF5* T/B and *ERG* T/B ratios (Spearman correlation coefficient = -0.72, P=0.03), between *ETV1* T/B and *ERG* T/B ratios (Spearman correlation coefficient = -0.61, P=0.08) and also between *ETS1* T/B and *ERG* T/B ratios (Spearman correlation coefficient = -0.72, P=0.03). The Spearman test revealed that *ERG* T/B was not positively correlated at a significant level to any other *ETS* transcription factor T/B ratios. The *ELF5* oligonucleotide microarray data were validated using LDA qPCR and high T/B ratios of 13.6, 2.6 and 6.6 were found for 2.4P54, 2.2P33 and 4.8P85, respectively. Moderately high T/B ratios of *ELF3* were also verified using LDA qPCR (Table VI). *ERG* oligo-nucleotide microarray T/B ratios corresponded well to LDA qPCR T/B ratios taking into consideration that especially high ratios are compressed in DNA microarrays compared with qPCR analysis (Table VI) (34,35).

Down-regulation of ETS2 in matched prostate T/B pairs according to microarray data. *ETS2*, which also belongs to the *ETS* family of transcription factors and is the closest mapped gene upstream of the *ERG* transcription initiation site, is note-worthy. *ERG* and *ETS2* are transcribed in opposite directions and both have promoters within the same region of less than 150,000 bp of chromosome 21q22. Seven of nine T/B pairs, including all with high *ERG* T/B ratios, displayed negative to very negative *ETS2* T/B ratios (Fig. 4). A paired t-test including all 9 T/B pairs shown in Fig. 4 revealed that *ETS2* was significantly underexpressed in T compared to B (mean T=1.41, mean B=3.13, P=0.029). The same relationship

Table V. Spearman rank correlation between *ERG* and vascular markers (data in Table IV).

Spearman's rho	PECAM1	TEM5	FLT1	CD34	TEM7R	Correlations TEM7	PODLX2	TEM1	VWF	FLT4	PECAM12	ERG
PECAM1												
Correl. coeff.	1.000	0.567	0.276	0.183	0.667[a]	0.467	0.367	0.150	0.517	0.300	0.100	0.167
Sig. (2-tailed)	-	0.112	0.472	0.637	0.050	0.205	0.332	0.700	0.154	0.433	0.798	0.668
N	9	9	9	9	9	9	9	9	9	9	9	9
TEM5												
Correl. coeff.	0.567	1.000	-0.059	-0.183	0.983[b]	0.250	-0.817[b]	0.200	0.467	0.917[b]	-0.150	0.150
Sig. (2-tailed)	0.112	-	0.881	0.637	0.000	0.516	0.007	0.606	0.205	0.001	0.700	0.700
N	9	9	9	9	9	9	9	9	9	9	9	9
FLT1												
Correl. coeff.	0.276	-0.059	1.000	0.368	-0.075	-0.059	-0.234	-0.519	0.059	-0.109	-0.577	-0.017
Sig. (2-tailed)	0.472	0.881	-	0.330	0.847	0.881	0.544	0.152	0.881	0.781	0.104	0.966
N	9	9	9	9	9	9	9	9	9	9	9	9
CD34												
Correl. coeff.	0.183	-0.183	0.368	1.000	-0.167	0.567	0.133	0.433	0.133	-0.317	0.150	-0.233
Sig. (2-tailed)	0.637	0.637	0.330	-	0.668	0.112	0.732	0.244	0.732	0.406	0.700	0.546
N	9	9	9	9	9	9	9	9	9	9	9	9
TEM7R												
Correl. coeff.	0.667[a]	0.983[b]	-0.075	-0.167	1.000	0.283	-0.800[b]	0.217	0.450	0.867[b]	-0.083	0.167
Sig. (2-tailed)	0.050	0.000	0.847	0.668	-	0.460	0.010	0.576	0.224	0.002	0.831	0.668
N	9	9	9	9	9	9	9	9	9	9	9	9
TEM7												
Correl. coeff.	0.467	0.250	-0.059	0.567	0.283	1.000	0.083	0.533	0.800[b]	0.033	0.533	0.383
Sig. (2-tailed)	0.205	0.516	0.881	0.112	0.460	-	0.831	0.139	0.010	0.932	0.139	0.308
N	9	9	9	9	9	9	9	9	9	9	9	9
PODLX2												
Correl. coeff.	-0.367	-0.817[b]	-0.234	0.133	-0.800[b]	0.083	1.000	0.133	-0.083	-0.867[b]	0.417	0.050
Sig. (2-tailed)	0.332	0.007	0.544	0.732	0.010	0.831	-	0.732	0.831	0.002	0.265	0.898
N	9	9	9	9	9	9	9	9	9	9	9	9
TEM1												
Correl. coeff.	0.150	0.200	-0.519	0.433	0.217	0.533	0.133	1.000	0.200	0.033	0.567	-0.350
Sig. (2-tailed)	0.700	0.606	0.152	0.244	0.576	0.139	0.732	-	0.606	0.932	0.112	0.356
N	9	9	9	9	9	9	9	9	9	9	9	9
VWF												
Correl. coeff.	0.517	0.467	0.059	0.133	0.450	0.800[b]	-0.083	0.200	1.000	0.317	0.350	0.517
Sig. (2-tailed)	0.154	0.205	0.881	0.732	0.224	0.010	0.831	0.606	-	0.406	0.356	0.154
N	9	9	9	9	9	9	9	9	9	9	9	9
FLT4												
Correl. coeff.	0.300	0.917[b]	-0.109	-0.317	0.867[b]	0.033	-0.867[b]	0.033	0.317	1.000	-0.167	0.050
Sig. (2-tailed)	0.433	0.001	0.781	0.406	0.002	0.932	0.002	0.932	0.406	-	0.668	0.898
N	9	9	9	9	9	9	9	9	9	9	9	9
PECAM12												
Correl. coeff.	0.100	-0.150	-0.577	0.150	-0.083	0.533	0.417	0.567	0.350	-0.167	1.000	-0.067
Sig. (2-tailed)	0.798	0.700	0.104	0.700	0.831	0.139	0.265	0.112	0.356	0.668	-	0.865
N	9	9	9	9	9	9	9	9	9	9	9	9
ERG												
Correl. coeff.	0.167	0.150	-0.017	-0.233	0.167	0.383	0.050	-0.350	0.517	0.050	-0.067	1.000
Sig. (2-tailed)	0.668	0.700	0.966	0.546	0.668	0.308	0.898	0.356	0.154	0.898	0.865	-
N	9	9	9	9	9	9	9	9	9	9	9	9

[a]Correlation is significant at the 0.05 level (2-tailed); [b]Correlation is significant at the 0.01 level (2-tailed). Correl. coeff., correlation coefficient.

was present in the unpaired t-test including all 13 Ts and all 9 Bs of the Agilent oligoarrays (mean T=1.68, mean B=3.13, P=0.04). There was, however, no significant correlation between the T/B ratios of *ERG* and *ETS2* (Table IX).

Correlation between gene expression of ERG and neighbouring genes on chromosome 21. The Agilent oligonucleotide array was used to examine gene expression of the nine paired Ts and Bs in the 5 megabase physical map surrounding *ERG*

on chromosome 21q22 (http://www.dsi.univ-paris5.fr/genatlas/ fiche.php?n=3837). Altogether 16 genes in this region were present in the J-Express file (Table X). Among the genes transcribed in the opposite direction of *ERG*, only *SIM2* displayed a comparable proportion of high T/B ratios. Other correlations according to the Spearman test are shown in Table IX and do not indicate positive coregulation with *ERG*. In addition, *TMPRSS2*, present in the same region of chromosome 21q22.3 as *ERG*, was abundantly expressed in both B

Table VI. A, Comparison of Agilent 21k microarray T/B and LDA qPCR T/B ratios for selected ETS family transcription factors and selected vascular markers.[a]

Assay	Gene	2.4P54	2.2P33	4.8P85	4.6P90	5.1P46	5.3P98	4.2P78	1.3P29	4.4P41	1.1P92	1.6P52	6.2P82
Agilent	ERG	0.29	1.23	1.60	6.54	9.60	12.23	14.03	10.27	7.75	ND	ND	ND
LDA	ERG	0.39	1.06	3.33	118.10	170.58	38.47	151.33	ND	ND	64.24	12.48	0.82
Agilent	ELF3	0.80	2.91	1.01	0.84	2.12	1.15	5.30	1.97	4.42	ND	ND	ND
LDA	ELF3	2.28	3.68	1.92	2.47	3.07	2.94	6.26	ND	ND	2.04	1.15	2.66
Agilent	ELF5	4.66	3.87	1.51	0.51	1.15	1.17	0.99	0.41	1.40	ND	ND	ND
LDA	ELF5	13.62	2.61	6.61	0.18	1.01	2.29	1.02	ND	ND	0.09	3.97	0.01
Agilent	EHF	0.72	2.51	0.72	0.96	1.11	0.60	0.66	0.61	0.74	ND	ND	ND
LDA	EHF	5.65	0.91	2.03	2.50	1.39	1.64	1.17	ND	ND	1.26	0.90	0.50
Agilent	ENG	0.62	0.83	53.95	0.91	5.61	1.46	1.26	0.43	0.96	ND	ND	ND
LDA	ENG	3.00	0.78	0.90	2.05	2.08	4.12	2.22	ND	ND	0.36	2.63	1.26
Agilent	PECAM1	0.37	1.52	0.90	2.05	1.24	2.04	0.99	0.47	1.21	ND	ND	ND
LDA	PECAM1	1.00	0.67	1.07	2.49	1.12	3.02	1.09	ND	ND	0.85	1.85	0.52
Agilent	PODLX2	2.62	0.89	1.84	2.53	12.95	1.37	1.82	2.83	1.32	ND	ND	ND
LDA	PODLX2	6.11	1.14	5.58	6.06	0.98	3.61	1.33	ND	ND	13.69	1.02	3.60
Agilent	VWF	0.49	0.74	1.64	0.93	2.14	2.17	2.04	0.38	0.85	ND	ND	ND
LDA	VWF	1.74	0.25	4.27	2.41	0.96	1.28	0.72	ND	ND	1.21	2.85	0.49

[a]Agilent, Agilent human 1A 21k oligonucleotide microarray. LDA, applied biosystems real-time qPCR low density array. ND, not done. The Agilent microarray values are Cy3 T/Cy5 B intensity values and not the $\log_2$ transformed T/B values.

B, Expression of ERG exons in malignant (T) versus benign (B) samples (T/B ratios) using different methods.[b]

Assay/sample	qPCR ERG1 T/B	qPCR ERG2 T/B	qPCR ERG T/B	LDA ERG T/B	Agilent ERG T/B
4.2P78	295.6	22.7	116.8	151.3	14.0
5.1P46	141.8	4.9	80.5	170.6	9.6
5.3P98	66.9	8.4	33.8	38.5	12.2
4.4P41	56.1	1.6	31.5	ND	7.7
1.1P92	44.0	3.9	38.5	64.2	ND
4.6P90	42.4	3.0	71.8	118.1	6.5
6.7P12	29.4	4.7	26.0	ND	ND
1.6P52	8.9	3.0	6.3	12.5	ND
5.8P23	4.7	0.8	7.5	ND	ND
4.8P85	2.8	1.1	2.1	3.3	1.6
2.4P54	0.3	2.6	0.8	0.4	0.3
6.4P82	ND	ND	1.4	0.8	ND
2.2P33	ND	ND	0.7	1.1	1.2

[b]Comparison of ERG T/B ratios according to different qPCR assays and Agilent 21k oligonucleotide microarrays for 13 of the 27 paired prostate samples of the extended patient series. Amplification and detection was designed for isoform specific exons (ERG1 and ERG2) or for an isoform common exon (ERG) (Fig. 1). LDA, qPCR low density array. Agilent, 21k human DNA oligonucleotide array.

and T samples compared with the Stratagene U cell pool RNA (Fig. 3).

Discussion

ERG was among the most consistently and highly expressed transcription factors in prostate cancer compared with benign tissue, and very high ERG T/B expression ratios (above 20) were found by qPCR in 14 of 27 patients for which paired tumor (T) and benign tissue (B) samples were available. Recently, one independent work has reported ERG over-expression in prostate cancer (20). Overexpression of ERG has further been reported in acute myelogenous leukemia (AML) (36) and in megalokaryoblastic leukemia (37). A plausible

Table VII. Agilent 21k oligonucleotide microarray paired prostate T/B ratios (columns) of *ETS* transcription factors (rows). The Agilent microarray values shown are Cy3 T/Cy5 B intensity values.

Gene	T/B 1.3P29	T/B 2.4P54	T/B 2.2P33	T/B 4.4P41	T/B 4.8P85	T/B 4.6P90	T/B 5.1P46	T/B 5.3P98	T/B 4.2P78
ETV1	0.37	1.18	0.93	1.46	9.77	0.54	0.86	0.46	0.86
TEL2	1.12	0.88	0.93	1.24	1.40	0.95	1.04	1.20	0.73
ELK3	0.70	0.57	0.90	1.24	0.89	0.82	2.52	1.15	1.14
ELF2	1.21	1.01	1.24	1.01	1.57	0.88	0.49	1.44	0.92
ELK1	1.11	1.47	1.02	1.25	1.10	3.18	0.05	0.24	0.98
ETV6	0.78	0.73	1.14	1.24	0.82	0.65	4.48	1.08	0.80
ETS2	0.48	0.09	1.34	0.77	0.60	0.60	0.53	0.15	0.61
ERF	0.95	0.32	1.70	1.06	1.44	0.68	1.59	0.79	0.77
ETS1	0.78	7.22	1.12	1.62	1.35	0.85	1.10	0.52	0.95
ETV5	0.81	0.60	1.14	0.49	1.10	0.97	0.41	0.11	9.60
FLI1	0.67	2.37	1.11	0.93	0.83	0.83	2.62	0.41	14.22
ELF3	1.97	0.80	2.91	4.42	1.01	0.84	2.12	1.15	5.30
ELF5	0.41	4.66	3.87	1.40	1.51	0.51	1.15	1.17	0.99
ELF4	1.79	2.77	0.54	0.38	1.75	0.97	0.70	0.56	7.19
SPIB	1.00	0.23	1.18	0.88	1.01	0.91	22.34	0.97	1.02
EHF	0.61	0.72	2.51	0.74	0.72	0.96	1.11	0.60	0.66
HSRNAFEV	1.97	2.76	0.80	0.40	1.65	3.48	0.04	1.89	1.18
SPI1	1.08	0.63	1.66	1.35	0.62	0.65	1.86	0.62	1.16
ELK4	1.27	1.37	1.38	1.07	0.87	1.41	0.46	0.09	0.44
ERG	10.27	0.29	1.23	7.75	1.60	6.54	9.60	12.23	14.03

mechanism of ERG upregulation in prostate cancer has been found, involving a translocation that brings *ERG* under control of the androgen responsive promoter of a highly expressed prostate protease gene, *TMPRSS2* (19). In Ewing's sarcoma, *ERG* and *FLI-1* DNA-binding domains are involved in defining translocations with the activation domains of RNA-binding proteins like EWS and FUS (38,39). The *ERG-FUS* fusion has also been found in some cases of AML (40-42).

ERG has been described as an endothelial transcription factor (30-32) and is expressed during vascular embryogenesis in mice (17). In our study, *ERG* mRNA was more abundantly expressed in endothelial (HUVEC) cells than in three tested prostate cancer derived cell lines, including androgen stimulated LNCaP cells. Since gene expression analysis of tissues can be biased by the proportion of different cell types present (43), both DNA microarray data and real-time qPCR were used to analyse possible coexpression of *ERG* and various vascular markers, such as *PECAM1 (CD31)*, *ENG* (endoglin, *CD105*), *PODLX2* (podocalyxin-like) and von Willebrand factor (*VWF*), but no significant covariation was found. Using *in situ* hybridization the present work clearly identified the epithelial prostate cancer cells as the main source of *ERG* mRNA, in accordance with one previous study (20). The present study in addition shows that *ERG* mRNA was also expressed by prostate endothelial cells. The pronounced cytoplasmic staining of *ERG* mRNA indicates the production of functional mRNA. In both prostate cancer tissue and in HUVEC cells an *ERG* isoform 1 specific exon was over-expressed. Future work will address the different roles of ERG expression in prostate cancer epithelial cells versus endothelial cells.

More than 30 ETS family transcription factors have been identified (14). Oligonucleotide microarray expression values were available for 20 of these factors including *ERG*. Most *ETS* factors were not elevated in prostate cancer compared with matched benign tissue, but 3 of 9 patients who did not overexpress *ERG* showed increased expression of *ELF5* with T/B ratios between 2.6 and 13.6 as validated by real-time qPCR. Also, *ETV1* and *ETS1* were abundantly expressed in some cases lacking *ERG* overexpression. According to the Spearman test *ELF5, ETV1* and *ETS1* T/B ratios were inversely related to *ERG* T/B ratios suggesting that these *ETS* transcription factors might substitute for *ERG* in prostate cancer. Prostate cancer *TMPRSS2-ETV1* fusions as an alternative to *TMPRSS2-ERG* fusions were recently reported (19), and also *ETS1* overexpression has been reported in prostate cancer (44). Very high expression ratios of most *ETS* transcription factors were found only occasionally and did not match the consistency and magnitude of *ERG* levels. This observation supports the possibility that *ERG* is of particular significance in prostate carcinogenesis.

Following the recent publication of Tomlins *et al* (19), we re-examined our data and found *TMPRSS2* to be abundantly overexpressed in all our T and B samples compared with the cell pool control RNA. Of interest, *ETS2* and *ERG* have their promoters within a common region of less than 150,000 base-pairs and are transcribed in opposite directions on chromosome 21q22. Although both are ETS family transcription

Table VIII. Spearman rank correlation between *ERG* and *ETS* transcription factors (data in Table VII).

Spearman's rho		ETV1	TEL2	ELK3	ELF2	ELK1	ETV6	ETS2	ERF	ETS1	ETV5	FLI1	ELF3	ELF5	ELF4	SPIB	EHF	HSRNAFEV	SPI1	ELK4	ERG
ETV1	Correl. coeff.	1.000	0.184	0.075	0.193	0.184	0.276	0.378	0.259	0.912[b]	0.209	0.378	0.025	0.762[a]	-0.142	-0.100	0.395	-0.360	0.017	0.092	-0.611
	Sig. (2-tailed)	-	0.635	0.847	0.618	0.635	0.472	0.316	0.500	0.001	0.589	0.316	0.949	0.017	0.715	0.797	0.293	0.342	0.966	0.814	0.081
	N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
TEL2	Correl. coeff.	0.184	1.000	0.267	0.502	-0.033	0.367	-0.025	0.400	-0.033	-0.433	-0.661	-0.100	0.000	-0.483	-0.117	-0.176	-0.167	-0.301	-0.250	0.050
	Sig. (2-tailed)	0.635	-	0.488	0.168	0.932	0.332	0.949	0.286	0.932	0.244	0.053	0.798	1.000	0.187	0.765	0.651	0.668	0.431	0.516	0.898
	N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
ELK3	Correl. coeff.	0.075	0.267	1.000	-0.209	-0.683[a]	0.867[b]	0.360	0.517	-0.083	-0.367	0.226	0.650	-0.050	-0.583	0.433	0.209	-0.833[b]	0.494	-0.617	0.467
	Sig. (2-tailed)	0.847	0.488	-	0.589	0.042	0.002	0.342	0.154	0.831	0.332	0.559	0.058	0.898	0.099	0.244	0.589	0.005	0.177	0.077	0.205
	N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
ELF2	Correl. coeff.	0.193	0.502	-0.209	1.000	-0.075	0.050	-0.034	0.259	-0.008	0.075	-0.597	-0.159	0.410	-0.159	-0.059	-0.391	0.117	-0.563	-0.134	-0.167
	Sig. (2-tailed)	0.618	0.168	0.589	-	0.847	0.898	0.932	0.500	0.983	0.847	0.090	0.683	0.273	0.683	0.881	0.298	0.764	0.114	0.731	0.667
	N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
ELK1	Correl. coeff.	0.184	-0.033	-0.683[a]	-0.075	1.000	-0.667[a]	-0.017	-0.517	0.333	0.167	-0.201	-0.483	0.050	0.133	-0.767[a]	0.092	0.633	-0.276	0.767[a]	-0.533
	Sig. (2-tailed)	0.635	0.932	0.042	0.847	-	0.050	0.966	0.154	0.381	0.668	0.604	0.187	0.898	0.732	0.016	0.814	0.067	0.472	0.016	0.139
	N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
ETV6	Correl. coeff.	0.276	0.367	0.867[b]	0.050	-0.667[a]	1.000	0.368	0.800[b]	0.167	-0.350	0.201	0.600	0.250	-0.683[a]	0.500	0.343	-0.933[b]	0.561	-0.450	0.150
	Sig. (2-tailed)	0.472	0.332	0.002	0.898	0.050	-	0.330	0.010	0.668	0.356	0.604	0.088	0.516	0.042	0.170	0.366	0.000	0.116	0.224	0.700
	N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
ETS2	Correl. coeff.	0.378	-0.025	0.360	-0.034	-0.017	0.368	1.000	0.510	0.218	0.577	0.294	0.678[a]	0.059	-0.410	0.360	0.550	-0.536	0.542	0.192	-0.059
	Sig. (2-tailed)	0.316	0.949	0.342	0.932	0.966	0.330	-	0.160	0.574	0.104	0.442	0.045	0.881	0.273	0.342	0.125	0.137	0.132	0.620	0.881
	N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
ERF	Correl. coeff.	0.259	0.400	0.517	0.259	-0.517	0.800[b]	0.510	1.000	0.117	0.033	0.042	0.467	0.183	-0.583	0.700[a]	0.485	-0.767[a]	0.527	-0.100	-0.083
	Sig. (2-tailed)	0.500	0.286	0.154	0.500	0.154	0.010	0.160	-	0.765	0.932	0.915	0.205	0.637	0.099	0.036	0.185	0.016	0.145	0.798	0.831
	N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
ETS1	Correl. coeff.	0.912[b]	-0.033	-0.083	-0.008	0.333	0.167	0.218	0.117	1.000	0.117	0.502	-0.050	0.767[a]	-0.050	-0.233	0.452	-0.267	0.151	0.283	-0.717[a]
	Sig. (2-tailed)	0.001	0.932	0.831	0.983	0.381	0.668	0.574	0.765	-	0.765	0.168	0.898	0.016	0.898	0.546	0.222	0.488	0.699	0.460	0.030
	N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
ETV5	Correl. coeff.	0.209	-0.433	-0.367	0.075	0.167	-0.350	0.577	0.033	0.117	1.000	0.326	0.233	-0.017	0.417	0.317	0.176	0.083	0.084	0.317	-0.150
	Sig. (2-tailed)	0.589	0.244	0.332	0.847	0.668	0.356	0.104	0.932	0.765	-	0.391	0.546	0.966	0.265	0.406	0.651	0.831	0.831	0.406	0.700
	N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
FLI1	Correl. coeff.	0.378	-0.661	0.226	-0.597	-0.201	0.201	0.294	0.042	0.502	0.326	1.000	0.410	0.226	0.276	0.343	0.450	-0.469	0.601	-0.042	-0.092
	Sig. (2-tailed)	0.316	0.053	0.559	0.090	0.604	0.604	0.442	0.915	0.168	0.391	-	0.273	0.559	0.472	0.366	0.225	0.203	0.087	0.915	0.814
	N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
ELF3	Correl. coeff.	0.025	-0.100	0.650	-0.159	-0.483	0.600	0.678[a]	0.467	-0.050	0.233	0.410	1.000	-0.183	-0.250	0.500	0.117	-0.767[a]	0.695[a]	-0.367	0.517
	Sig. (2-tailed)	0.949	0.798	0.058	0.683	0.187	0.088	0.045	0.205	0.898	0.546	0.273	-	0.637	0.516	0.170	0.764	0.016	0.038	0.332	0.154
	N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
ELF5	Correl. coeff.	0.762[a]	0.000	-0.050	0.410	0.050	0.250	0.059	0.183	0.767[a]	-0.017	0.226	-0.183	1.000	-0.233	-0.167	0.285	-0.183	-0.117	0.117	-0.717[a]
	Sig. (2-tailed)	0.017	1.000	0.898	0.273	0.898	0.516	0.881	0.637	0.016	0.966	0.559	0.637	-	0.546	0.668	0.458	0.637	0.764	0.765	0.030
	N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
ELF4	Correl. coeff.	-0.142	-0.483	-0.583	-0.159	0.133	-0.683[a]	-0.410	-0.583	-0.050	0.417	0.276	-0.250	-0.233	1.000	-0.033	-0.452	0.483	-0.326	-0.067	0.133
	Sig. (2-tailed)	0.715	0.187	0.099	0.683	0.732	0.042	0.273	0.099	0.898	0.265	0.472	0.516	0.546	-	0.932	0.222	0.187	0.391	0.865	0.732
	N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
SPIB	Correl. coeff.	-0.100	-0.117	0.433	-0.059	-0.767[a]	0.500	0.360	0.700[a]	-0.233	0.317	0.343	0.500	-0.167	-0.033	1.000	0.310	-0.600	0.519	-0.300	0.267
	Sig. (2-tailed)	0.797	0.765	0.244	0.881	0.016	0.170	0.342	0.036	0.546	0.406	0.366	0.170	0.668	0.932	-	0.417	0.088	0.152	0.433	0.488
	N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
EHF	Correl. coeff.	0.395	-0.176	0.209	-0.391	0.092	0.343	0.550	0.485	0.452	0.176	0.450	0.117	0.285	-0.452	0.310	1.000	-0.410	0.651	0.544	-0.569
	Sig. (2-tailed)	0.293	0.651	0.589	0.298	0.814	0.366	0.125	0.185	0.222	0.651	0.225	0.764	0.458	0.222	0.417	-	0.273	0.057	0.130	0.110
	N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
HSRNAFEV	Correl. coeff.	-0.360	-0.167	-0.833[b]	0.117	0.633	-0.933[b]	-0.536	-0.767[a]	-0.267	0.083	-0.469	-0.767[a]	-0.183	0.483	-0.600	-0.410	1.000	-0.711[a]	0.417	-0.183
	Sig. (2-tailed)	0.342	0.668	0.005	0.764	0.067	0.000	0.137	0.016	0.488	0.831	0.203	0.016	0.637	0.187	0.088	0.273	-	0.032	0.265	0.637
	N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
SPI1	Correl. coeff.	0.017	-0.301	0.494	-0.563	-0.276	0.561	0.542	0.527	0.151	0.084	0.601	0.695[a]	-0.117	-0.326	0.519	0.651	-0.711[a]	1.000	0.126	0.075
	Sig. (2-tailed)	0.966	0.431	0.177	0.114	0.472	0.116	0.132	0.145	0.699	0.831	0.087	0.038	0.764	0.391	0.152	0.057	0.032	-	0.748	0.847
	N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
ELK4	Correl. coeff.	0.092	-0.250	-0.617	-0.134	0.767[a]	-0.450	0.192	-0.100	0.283	0.317	-0.042	-0.367	0.117	-0.067	-0.300	0.544	0.417	0.126	1.000	-0.717[a]
	Sig. (2-tailed)	0.814	0.516	0.077	0.731	0.016	0.224	0.620	0.798	0.460	0.406	0.915	0.332	0.765	0.865	0.433	0.130	0.265	0.748	-	0.030
	N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
ERG	Correl. coeff.	-0.611	0.050	0.467	-0.167	-0.533	0.150	-0.059	-0.083	-0.717[a]	-0.150	-0.092	0.517	-0.717[a]	0.133	0.267	-0.569	-0.183	0.075	-0.717[a]	1.000
	Sig. (2-tailed)	0.081	0.898	0.205	0.667	0.139	0.700	0.881	0.831	0.030	0.700	0.814	0.154	0.030	0.732	0.488	0.110	0.637	0.847	0.030	-
	N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9

[a]Correlation is significant at the 0.05 level (2-tailed); [b]Correlation is significant at the 0.01 level (2-tailed). Correl. coeff., correlation coefficient.

Table IX. Spearman rank correlation between ERG and gene expression in DSCR of chromosome 21 (data in Table X).

Spearman's rho	Correlations															
	CHAF1B	DSCAM	WRB	ETS2	SH3BGR	CLDN14	PCP4	DSCR5	KCNJ6	SIM2	DSCR8	DSCR2	DYRK1A	KCNJ15	DSCR4	ERG
CHAF1B																
Correl. coeff.	1.000	0.924[b]	0.285	-0.176	-0.349	-0.619	-0.611	-0.092	-0.452	-0.075	0.301	-0.134	0.527	0.059	0.075	-0.385
Sig. (2-tailed)	-	0.000	0.458	0.650	0.357	0.075	0.081	0.814	0.222	0.847	0.431	0.730	0.145	0.881	0.847	0.306
N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
DSCAM																
Correl. coeff.	0.924[b]	1.000	0.435	-0.319	-0.340	-0.720[a]	-0.678[a]	0.084	-0.218	-0.008	0.460	-0.004	0.711[a]	-0.259	0.025	-0.510
Sig. (2-tailed)	0.000	-	0.242	0.402	0.370	0.029	0.045	0.831	0.574	0.983	0.213	0.991	0.032	0.500	0.949	0.160
N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
WRB																
Correl. coeff.	0.285	0.435	1.000	-0.385	-0.271	-0.100	-0.817[b]	0.267	0.167	0.367	0.550	0.326	0.083	-0.033	0.400	-0.383
Sig. (2-tailed)	0.458	0.242	-	0.306	0.480	0.798	0.007	0.488	0.668	0.332	0.125	0.391	0.831	0.932	0.286	0.308
N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
ETS2																
Correl. coeff.	-0.176	-0.319	-0.385	1.000	0.417	0.067	0.452	-0.444	-0.025	-0.485	-0.059	0.458	-0.502	0.276	0.142	-0.059
Sig. (2-tailed)	0.650	0.402	0.306	-	0.264	0.864	0.222	0.232	0.949	0.185	0.881	0.215	0.168	0.472	0.715	0.881
N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
SH3BGR																
Correl. coeff.	-0.349	-0.340	-0.271	0.417	1.000	0.203	0.509	0.034	0.119	-0.525	-0.136	0.085	-0.322	-0.034	-0.644	0.119
Sig. (2-tailed)	0.357	0.370	0.480	0.264	-	0.600	0.162	0.931	0.761	0.146	0.728	0.828	0.398	0.931	0.061	0.761
N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
CLDN14																
Correl. coeff.	-0.619	-0.720[a]	-0.100	0.067	0.203	1.000	0.117	-0.467	-0.117	0.417	-0.650	0.042	-0.783[a]	0.433	-0.100	0.717[a]
Sig. (2-tailed)	0.075	0.029	0.798	0.864	0.600	-	0.765	0.205	0.765	0.265	0.058	0.915	0.013	0.244	0.798	0.030
N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
PCP4																
Correl. coeff.	-0.611	-0.678[a]	-0.817[b]	0.452	0.509	0.117	1.000	0.067	0.300	-0.550	-0.350	-0.209	-0.200	0.017	-0.333	0.250
Sig. (2-tailed)	0.081	0.045	0.007	0.222	0.162	0.765	-	0.865	0.433	0.125	0.356	0.589	0.606	0.966	0.381	0.516
N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
DSCR5																
Correl. coeff.	-0.092	0.084	0.267	-0.444	0.034	-0.467	0.067	1.000	0.350	-0.067	0.667[a]	-0.301	0.450	-0.383	-0.050	-0.550
Sig. (2-tailed)	0.814	0.831	0.488	0.232	0.931	0.205	0.865	-	0.356	0.865	0.050	0.431	0.224	0.308	0.898	0.125
N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
KCNJ6																
Correl. coeff.	-0.452	-0.218	0.167	-0.025	0.119	-0.117	0.300	0.350	1.000	-0.267	0.133	0.218	0.167	-0.283	0.067	-0.117
Sig. (2-tailed)	0.222	0.574	0.668	0.949	0.761	0.765	0.433	0.356	-	0.488	0.732	0.574	0.668	0.460	0.865	0.765
N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
SIM2																
Correl. coeff.	-0.075	-0.008	0.367	-0.485	-0.525	0.417	-0.550	-0.067	-0.267	1.000	0.050	-0.100	-0.017	0.050	0.133	0.017
Sig. (2-tailed)	0.847	0.983	0.332	0.185	0.146	0.265	0.125	0.865	0.488	-	0.898	0.797	0.966	0.898	0.732	0.966
N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
DSCR8																
Correl. coeff.	0.301	0.460	0.550	-0.059	-0.136	-0.650	-0.350	0.667[a]	0.133	0.050	1.000	0.134	0.433	-0.283	0.267	-0.950[b]
Sig. (2-tailed)	0.431	0.213	0.125	0.881	0.728	0.058	0.356	0.050	0.732	0.898	-	0.731	0.244	0.460	0.488	0.000
N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
DSCR2																
Correl. coeff.	-0.134	-0.004	0.326	0.458	0.085	0.042	-0.209	-0.301	0.218	-0.100	0.134	1.000	-0.351	-0.318	0.460	-0.025
Sig. (2-tailed)	0.730	0.991	0.391	0.215	0.828	0.915	0.589	0.431	0.574	0.797	0.731	-	0.354	0.404	0.213	0.949
N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
DYRK1A																
Correl. coeff.	0.527	0.711[a]	0.083	0.502	-0.322	-0.783[a]	-0.200	0.450	0.167	-0.017	0.433	-0.351	1.000	-0.517	-0.233	-0.533
Sig. (2-tailed)	0.145	0.032	0.831	0.168	0.398	0.013	0.606	0.224	0.668	0.966	0.244	0.354	-	0.154	0.546	0.139
N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
KCNJ15																
Correl. coeff.	0.059	-0.259	-0.033	0.276	-0.034	0.433	0.017	-0.383	-0.283	0.050	-0.283	-0.318	-0.517	1.000	0.167	0.200
Sig. (2-tailed)	0.881	0.500	0.932	0.472	0.931	0.244	0.966	0.308	0.460	0.898	0.460	0.404	0.154	-	0.668	0.606
N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
DSCR4																
Correl. coeff.	0.075	0.025	0.400	0.142	-0.644	-0.100	-0.333	-0.050	0.067	0.133	0.267	0.460	-0.233	0.167	1.000	-0.150
Sig. (2-tailed)	0.847	0.949	0.286	0.715	0.061	0.798	0.381	0.898	0.865	0.732	0.488	0.213	0.546	0.668	-	0.700
N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
ERG																
Correl. coeff.	-0.385	-0.510	-0.383	-0.059	0.119	0.717[a]	0.250	-0.550	-0.117	0.017	-0.950[b]	-0.025	-0.533	0.200	-0.150	1.000
Sig. (2-tailed)	0.306	0.160	0.308	0.881	0.761	0.030	0.516	0.125	0.765	0.966	0.000	0.949	0.139	0.606	0.700	-
N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9

[a]Correlation is significant at the 0.05 level (2-tailed); [b]Correlation is significant at the 0.01 level (2-tailed). Correl. coeff., correlation coefficient.

Table X. Agilent 21k oligonucleotide array paired T/B ratios (columns) of gene expression (rows) in the DSCR of chromosome 21. The Agilent microarray values shown are Cy3 T/Cy5 B intensity values.

Gene	T/B 1.3P29	T/B 2.4P54	T/B 2.2P33	T/B 4.4P41	T/B 4.8P85	T/B 4.6P90	T/B 5.1P46	T/B 5.3P98	T/B 4.2P78
CHAF1B	1.31	1.31	1.25	1.23	0.87	0.92	2.43	0.77	0.86
DSCAM	1.47	1.83	1.07	0.98	1.04	1.01	4.82	0.67	0.67
WRB	0.90	1.04	0.71	0.77	0.99	0.89	0.78	0.41	0.83
ETS2	0.48	0.09	1.34	0.77	0.60	0.60	0.53	0.15	0.61
SH3BGR	0.60	0.25	0.60	1.05	1.37	0.39	0.45	0.60	0.49
CLDN14	1.72	0.80	1.02	1.11	1.09	1.14	0.18	1.48	5.77
PCP4	0.12	0.15	0.51	0.52	0.43	0.19	0.26	0.80	0.48
DSCR5	0.77	2.40	0.83	1.03	1.59	0.94	0.84	1.05	0.62
KCNJ6	0.68	3.24	0.77	0.72	17.92	0.69	0.75	2.06	3.28
SIM2	15.09	10.73	2.03	0.76	1.50	56.18	0.97	6.80	2.23
DSCR8	0.84	5.76	1.10	1.07	2.73	2.33	0.89	0.80	0.62
DSCR2	1.00	0.66	1.10	0.79	2.46	1.31	1.28	0.63	1.31
DYRK1A	1.02	1.32	1.10	0.86	1.04	0.99	1.29	1.09	0.85
KCNJ15	2.06	1.91	1.21	3.69	0.53	1.17	0.57	0.59	3.13
DSCR4	0.94	2.60	1.01	1.71	1.14	11.17	2.04	0.11	5.62
ERG	10.27	0.29	1.23	7.75	1.60	6.54	9.60	12.23	14.03

factors, *ETS2* in general was moderately to strongly down-regulated in most prostate cancers. Overexpression of *ERG* and reduced expression of *ETS2* in prostate cancer compared with benign prostate tissue is also evident in the data of another microarray study of 17 untreated prostate cancers (30). It is a curious observation that the *ETS2* gene lies between *ERG* and *TMPRSS2* within a rather narrow region of chromosome 21q22.3. One possibility therefore is that *ETS2* gene expression might be compromised as a result of the fusion of the *TMPRSS2* promoter to the *ERG* sequences. Antagonism between ERG and ETS2 at the level of transcriptional activation in cell lines has been published, and ERG and ETS2 seem to compete in a transcriptional complex including FOS and JUN (45,46).

In conclusion, we have found a very high overexpression of *ERG* in a large subset of prostate cancers compared with matched benign prostate tissues. The epithelial cancer cells were the main source of *ERG* overexpression. In addition *ERG* was abundantly expressed in prostate cancer endothelial cells. In a smaller proportion of cases, other *ETS* transcription factors were overexpressed in prostate cancer, such as *ETV1* and *ELF5*. In contrast, *ETS2* levels were lower in prostate cancer than in paired benign samples. Pronounced ERG expression in the mesenchyme of the kidney, the urogenital tract and the genital tubercle, but not in the Wolfian duct, has been found during mouse embryogenesis. Following tissue differentiation, *ERG* was down-regulated (17). It is therefore possible that the presently observed activation of *ERG*, or related *ETS* transcription factors, reactivates an embryonic proliferation program in prostate tissue.

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