SOX4 Expression in Bladder Carcinoma: Clinical Aspects and *In vitro* Functional Characterization

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Abstract

The human transcription factor SOX4 was 5-fold up-regulated in bladder tumors compared with normal tissue based on whole-genome expression profiling of 166 clinical bladder tumor samples and 27 normal urothelium samples. Using a SOX4-specific antibody, we found that the cancer cells expressed the SOX4 protein and, thus, did an evaluation of SOX4 protein expression in 2,360 bladder tumors using a tissue microarray with clinical annotation. We found a correlation ($P < 0.05$) between strong SOX4 expression and increased patient survival. When overexpressed in the bladder cell line HU609, SOX4 strongly impaired cell viability and promoted apoptosis. To characterize downstream target genes and SOX4-induced pathways, we used a time-course global expression study of the overexpressed SOX4. Analysis of the microarray data showed 130 novel SOX4-related genes, some involved in signal transduction (*MAP2K5*), angiogenesis (*NRP2*), and cell cycle arrest (*PIK3R3*) and others with unknown functions (*CGI-62*). Among the genes regulated by SOX4, 25 contained at least one SOX4-binding motif in the promoter sequence, suggesting a direct binding of SOX4. The gene set identified *in vitro* was analyzed in the clinical bladder material and a small subset of the genes showed a high correlation to SOX4 expression. The present data suggest a role of SOX4 in the bladder cancer disease. (Cancer Res 2006; 66(7): 3434-42)

Introduction

Cancer of the urinary bladder is among the five most common malignancies worldwide (1). In recent years, much effort has been made in identifying new genes involved in urinary bladder cancer development and progression (2–4). We have used an approach in which we screen with expression microarrays on a large number of clinical samples to identify deregulated transcripts in cancer. One molecule with a clearly increased expression in most bladder cancers is SOX4. SOX4 is a member of the SOX family of transcription regulators that share homology in their DNA binding domain, the high mobility group (5). The *SOX4* gene has been shown to be involved in a range of developmental processes. The SOX4−/− phenotype in mouse results in death during embryogenesis due to lack of normal heart development, which results in circulatory failure (6, 7). Furthermore, other studies have shown that SOX4 is expressed in hormone-responsive tissues of the reproductive system in mouse, as well as in brain and T- and B-cell lymphocytes (8, 9), and may play a role in osteoblastic differentiation (10).

SOX4 binds to the 7-bp DNA-motif AACAAAG through its high mobility group domain and thereby transcriptionally activates target genes (9, 11). An alternative SOX4 motif (AACAATA) in the human *CD2* gene exists and, although it is considerably different, a specific SOX4-binding was observed *in vitro* (12). At present, the knowledge of putative complex partners and genes under control of SOX4 is still limited. However, the *src* family tyrosine kinase, $p56^{lck}$, is transcriptionally activated by SOX4 in the presence of ETS1 transcription factor (13). Examples of factors that on the other hand induce SOX4 itself are cytokines and hormones. In particular, the cytokine interleukin (IL)-5 stimulates SOX4 expression in B cells through its membrane-bound receptor (IL-5Rα) that directly binds the SOX4-interaction partner, syntenin, intracellularly (14). SOX4 has been shown to be strongly induced by prostaglandins (15), progestins (16), and by heat shock protein 70 overexpression (15). Furthermore, studies have suggested that hormone-induced SOX4 expression is directly mediated through progesterone receptor binding to the *SOX4* gene (16, 17).

It is still unknown what role is played by SOX4 *in vivo* and during tumorigenesis. Overexpression of SOX4 by retroviral insertional mutagenesis in genetically modified mouse strains results in a high incidence of myeloid leukemias and B- and T-cell lymphomas (18, 19). High levels of SOX4 expression have been reported in hepatic cancer cells (15) and a variety of human cancers, such as breast cancer (16), brain (20), lung (21), pancreatic (20), salivary gland (22), and ovarian cancers (20). However, others have shown that SOX4 expression in cancer cells effectively is driving cells into apoptosis (23, 24).

In this study, we did a functional characterization of SOX4 expression in bladder carcinomas. Initially, we found high levels of *SOX4* transcript in tumors compared with normal urothelium using microarray analysis. We confirmed the presence of the protein by immunostaining. Later, we monitored gene expression profiles of SOX4-transfected bladder cells. This allowed us to relate SOX4 to cell death and to identify novel target genes of SOX4. Their encoded proteins are involved in signal transduction, angiogenesis, and cell cycle arrest. Further, we provide evidence that SOX4 protein expression is correlated to patient survival using tissue microarrays with biopsies from >2,000 patients.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Tissue material. A total of 166 urinary bladder tumor specimens and 27 normal bladder biopsies were obtained by surgery and stored at −80°C in a preserving solution of guanidinium thiocyanate solution. Informed consent was obtained in all cases and protocols were approved by the local scientific ethical committee.

Expression constructs. The human *SOX4* gene was cloned into the pCR3.1 vector (Invitrogen, Carlsbad, CA) and the insert sequence was verified by sequencing. A mock control vector containing no insert was constructed by blunt-end ligation of the pCR3.1 vector itself.

Cell culture. HU609 cells were grown in DMEM medium (10% FCS, 1% penicillin and streptomycin) purchased from Invitrogen at 37°C in a humidified environment with 5% CO_2. Cells were tested negative for *Mycoplasma* infection by MycoSensor PCR Assay set (Stratagene, La Jolla, CA).

Transfection. A nucleofector (Amaxa, Cologne, Germany) was used for cell transfection. In brief, the transfection program T-23 was applied for transfecting 6 μg vector per 10^6 cells resuspended in 100 μL of solution R (Amaxa). Cells were transfected in parallel with green fluorescent protein (GFP) vector (pmaxGFP; Amaxa) and the efficiency was >50% in all cases. Total RNA was purified from cells after 6, 12, or 24 hours of transfection using the GenElute mRNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO) according to the instructions of the manufacturer.

Cell proliferation and viability assay. Transfected cells (SOX4, mock, or GFP vector) were transferred to 24-well plates (30,000 per well) where they were allowed to grow for 24, 48, or 72 hours. Cell numbers were measured using a Bürker-Türk cell counting chamber (in quadruple). After 48 hours, the percentage of viable, apoptotic, and necrotic cells was determined by incubating with DNA intercalating dyes, propidium iodide (10 μg/mL) and Hoechst 342 (20 μg/mL), for 15 minutes (Invitrogen). Viable cells with intact nuclei stained blue (Hoechst 342) whereas necrotic/apoptotic cells stained red as they were permeable to propidium iodide as previously described in ref. 25.

DNA microarray analysis. Preparation of labeled cRNA, microarray hybridization, washing, and scanning were done according to the instructions of the manufacturer as previously described (4).

Quantitative reverse transcription-PCR. First-Strand cDNA was synthesized from 1 μg DNase I–treated total RNA. The *SOX4* gene was amplified with specific primers, SOX4 qPCR F (5′-GTGAGCGAGATGATCTCGGG-3′) and SOX4 qPCR R (5′-CAGGTTGGAGATGCTGGACTC-3′), in 25-μL reactions. Primers used for normalization were GAPDH qPCR F (5′-TGCCAAATATGATGACATCAAGAA-3′) and GAPDH qPCR R (5′-GGAGTGGGTGTCGCTGTTG-3′). The procedure was accentuated as previously described in ref. 26.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections (5 μm) were deparaffinized in ethanol and rehydrated by water rinses. Endogenous peroxidase activity was blocked (0.35% H_2O_2 in TBS buffer), antigens were retrieved by microwaving (350 W), and nonspecific binding was blocked by 1% bovine serum albumin in TBS buffer. Sections were stained with SOX4 antibody (diluted 1:100; S7318, Sigma-Aldrich) and visualized with secondary antibody (Envision, DakoCytomation, Glostrup, Denmark). Slides were then incubated with 3,3′-diaminobenzidine chromogen (DakoCytomation), counterstained in Meyer's hematoxylin, and mounted with Aquatex (Merck, Darmstadt, Germany).

Tissue microarray. We used a bladder cancer tissue microarray containing 2,360 formalin-fixed, paraffin-embedded tissues as previously described (27). All core biopsies have been reviewed by the same pathologist (G.S.) within the last 6 years. Tumor stage and grade were defined according to International Union Against Cancer (28) and WHO (29). Patients with unknown cause of death were excluded from analyses of tumor-specific survival. Five-micrometer sections of the tissue microarray block were transferred to glass slides and stained with the SOX4 antibody as described above. SOX4 expression was qualitative evaluated by only evaluating cancer cells, which were scored in respect to the most intense staining observed. To avoid misleading border staining, we excluded border areas from evaluation. Cytoplasmic expression was scored as 0, negative; 1, weak; or 2, strong; nucleic expression was scored as 0, negative; 1, <50%; or 2, >50%. A number of core biopsies were excluded from analysis because of incompleteness (385), lack of cancer cells (115), or other reasons (306). The evaluation was done independently by two persons (K.B.D and M.A.) on 1,554 core biopsies in total and only unanimous scorings were used for the later analysis.

Bioinformatics. Microarray data for *SOX4* expression profiling were either normalized using the GC-RMA procedure (ref. 30; HG-U133A) or by procedures previously described in (refs. 31, 32; Eos Hu03). The Bonferroni correction for multiple comparisons was applied (33). Probe sets regulated by SOX4 were identified by pairwise comparisons between SOX4-transfected and mock-transfected cells; *SOX4* expression value divided by mock expression value. For this purpose, it was required that for a probe set to be up-regulated, the *SOX4* expression value should be above the lowest 25% of the data, and for a probe set to be down-regulated, the mock expression value should be above the lowest 25% of the data. All probe sets with a 4-fold change [up (+) or down (−)] were reported as differentially expressed. Using a binomial test, it was tested whether the number of up-regulated genes differed significantly from the number of down-regulated genes. The false discovery rate of up-regulated genes was calculated as the ratio of down-regulated probe sets to the sum of up-regulated and down-regulated probe sets. For promoter motif analysis, see Supplementary data 1. Differences in survival time between patients with (*a*) a strong or no/weak cytoplasmic SOX4 staining or (*b*) a high (>50%) or low (<50%) frequency of SOX4-positive nuclei were assessed using Kaplan-Meier survival analysis; the statistical significance was assessed by the log-rank test for equality of survivor functions. $P < 0.05$ was considered statistically significant.

Pathway analysis by EASE. Overrepresented gene categories, annotated within GO biological process branch, were identified using EASE software (34). All significant ($P < 0.05$) gene categories were determined by EASE score, corrected for multiplicity with the global false-discovery rate function using 1,000 iteration trials.

Chromosomal copy number analysis. The procedure described in Zieger et al. (35) was used to calculate chromosomal copy numbers. We selected two individual SNPs (rs717512 and rs952578) that are located on both sides of the *SOX4* gene to represent the chromosomal region of 6p22.

Results

The *SOX4* gene transcript is up-regulated in bladder carcinomas. We did a gene expression profiling analysis using microarrays (HG-U133A). Among many other genes, the expression of SOX4 was found to be changed in bladder cancer compared with normal bladder (Fig. 1*A*). The expression of *SOX4* was found to be significantly up-regulated (5.1-fold; *t* test, $P = 3.6 \times 10^{-6}$) in bladder tumors, compared with normal bladder biopsies, at all stages of bladder cancer (Fig. 1*A*). To validate the expression profile of SOX4, we analyzed the gene expression in a set of independent samples using a customized Affymetrix array (EOS Biotechnology, Eos Hu03; Fig. 1*B*). In agreement with the first microarray study, we repeatedly found the *SOX4* transcript to be highly significantly up-regulated (5.8-fold; *t* test, $P = 2.0 \times 10^{-19}$) in tumors compared with normal bladder.

Expression of SOX4 protein. Staining of paraffin-embedded tissue sections from bladder tumors with polyclonal SOX4 antibody allowed us to locate SOX4 protein expression. SOX4 protein was found to be expressed in cancer cells of urothelial origin in both T_a and T_1 tumors (Fig. 2*A-D*). Expression of SOX4 protein was found to be strong in both the cytoplasmic and nuclear cell compartments. Variable, and relatively weak, SOX4 expression was observed in cells in the stroma, possibly due to the presence of infiltrating lymphocytes. In agreement with this, B and T cells have previously been reported to express SOX4 transcript (9).

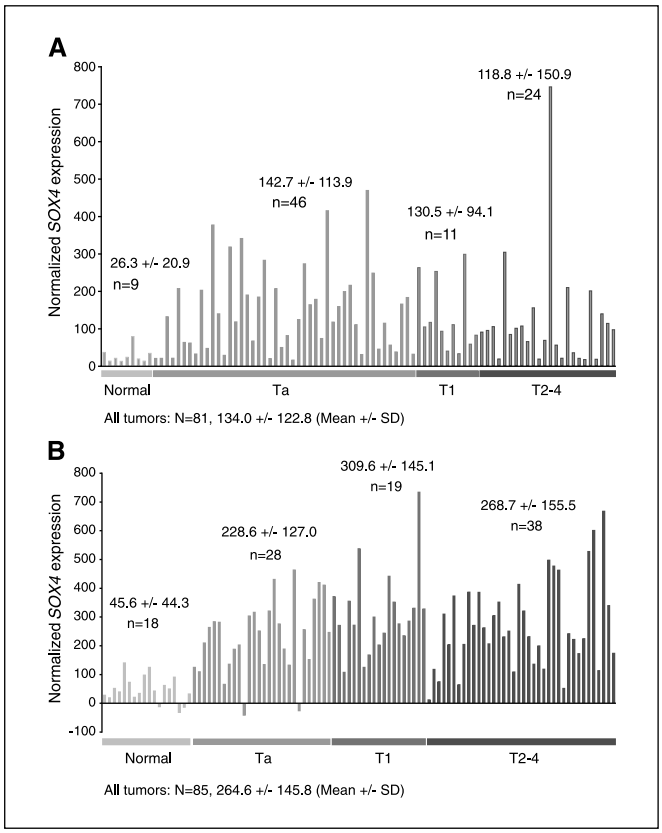

Figure 1. Microarray analysis of *SOX4* gene expression. *A,* Affymetrix microarrays (HG-U133A, $N = 90$): 9 normal bladder biopsies, 46 T_a, 11 T_1, and 24 T_{2-4} tumors. *B, SOX4* profile validation using customized Affymetrix microarrays (Eos Hu03, $N = 103$): 18 normal bladder biopsies, 28 T_a, 19 T_1, and 38 T_{2-4} tumors.

Tissue microarray analysis of SOX4 protein expression in bladder carcinomas. To assess the biological significance of SOX4 expression in bladder tumors, we evaluated its expression in a tissue microarray containing 2,360 samples from bladder cancer patients, with full clinical annotation. Core biopsies were either evaluated with respect to cytoplasmic staining (negative, weak, or strong) or frequency of nucleic expression (no nuclei stained, <50%, or >50%). For analysis, we combined negative or weakly SOX4 positive samples into one group and compared this to strongly SOX4 positive samples. The tissue microarray was evaluated independently by two persons (K.B.D. and M.A.) with a high degree of concordance, 91.3% (cytoplasmic expression) and 91.4% (nucleic expression). In case of disagreement, the tissue spot was excluded from further analysis. Among 1,419 interpretable tumors, 1,242 (87.5%) were considered SOX4 negative or weakly positive and 177 (12.5%) strongly positive for cytoplasmic staining (Supplementary Table S1; Fig. 2*E-G*). When we evaluated tumors for nucleic SOX4 expression, we found 1,216 (85.6%) tumors having no or only few SOX4-positive nuclei and 204 (14.4%) with a high number of SOX4-positive nuclei (Supplementary Table S1). A significant correlation was found between low grade of atypia and a high number of SOX4-positive nuclei when comparing grade 1 and grade 3 tumors ($P = 0.0003$). When comparing stage T_a and T_1 tumors, we found significantly more T_1 tumors being strongly SOX4 positive compared with T_a tumors ($P = 0.0376$), but no difference was found between T_a and T_{2-4} tumors (Supplementary Table S1). We did not find a significant correlation between SOX4 expression and clinical variables such as recurrence and progression. However, there was a significant correlation between SOX4 expression and tumor-specific survival. Using Kaplan-Meier survival analysis, we found that bladder cancer patients having tumors with strong cytoplasmic SOX4 expression had increased survival compared with patients with no or weak SOX4 expression ($P = 0.001$). Similarly, bladder cancer patients with tumors containing >50% SOX4-positive nuclei had also increased survival compared with those patients with tumors with no or <50% SOX4-positive nuclei ($P = 0.001$; Fig. 3*A* and *B*).

Microarray screening for SOX4 target genes. To identify genes that were directly under SOX4 transcriptional control, we compared expression profiles of human bladder carcinoma HU609 cells before and after overexpression of the *SOX4* gene. Cells were transiently transfected with SOX4 vector, mock vector, or GFP vector (for measuring transfection efficiencies). By reverse transcription-PCR (RT-PCR), high levels of *SOX4* transcript were detected in cells transfected with SOX4 vector at 6 hours: 52.25 ± 4.37 (SOX4)/0.13 ± 0.09 (mock), at 12 hours: 34.14 ± 2.12 (SOX4)/0.10 ± 0.02 (mock), and at 24 hours: 24.7 ± 2.25 (SOX4)/0.06 ± 0.02 (mock). The same samples were now analyzed on HG-U133A microarrays and a pairwise comparison analysis was done (SOX4/mock). The probe sets changing >4-fold were identified. In summary, we found 43 probe sets changed after 6 hours, 57 after 12 hours, and 97 after 24 hours (false discovery rate, $P < 1 \times 10^{-7}$). In contrast, only few probe sets were down-regulated by SOX4; 4 after 6 hours, 2 after 12 hours, and 12 after 24 hours (false discovery rate, $P < 1 \times 10^{-7}$). In total, these probe sets represented 130 different genes changing >4-fold (up or down); see Supplementary Figure S1. Comparing all three lists of probe sets, we identified 21 probe sets (19 genes) consistently changed at all time points (Table 1). They represented

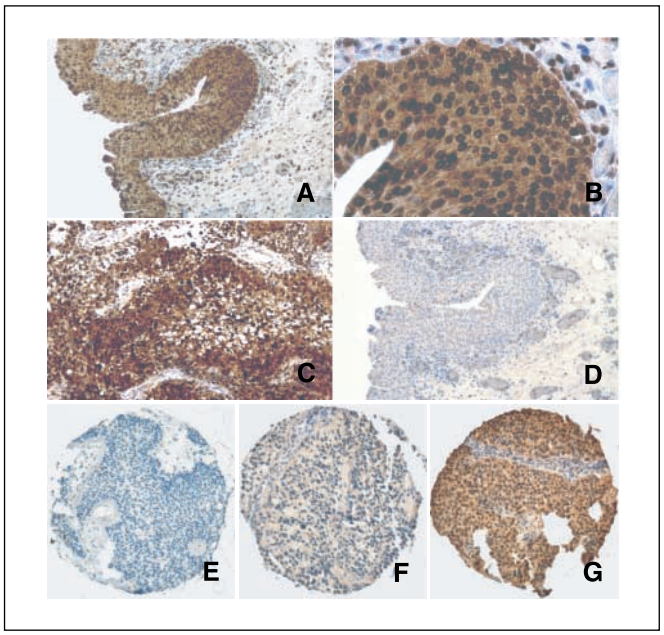

Figure 2. Immunohistochemical analysis of SOX4 expression in T_a grade 2 bladder tumor (962-10; *A* and *B*) and T_1 grade 3 bladder tumor (1191-1; *C*). *D,* negative control stain without primary antibody. Original magnification, ×10 (*A, C,* and *D*), ×40 (*B*). Tissue microarray cores with no (*E*), weak (*F*), and strong SOX4 cytoplasmic expression (*G*). Nucleic staining: none (*E* and *F*), high number (>50%) of positive nuclei (*G*).

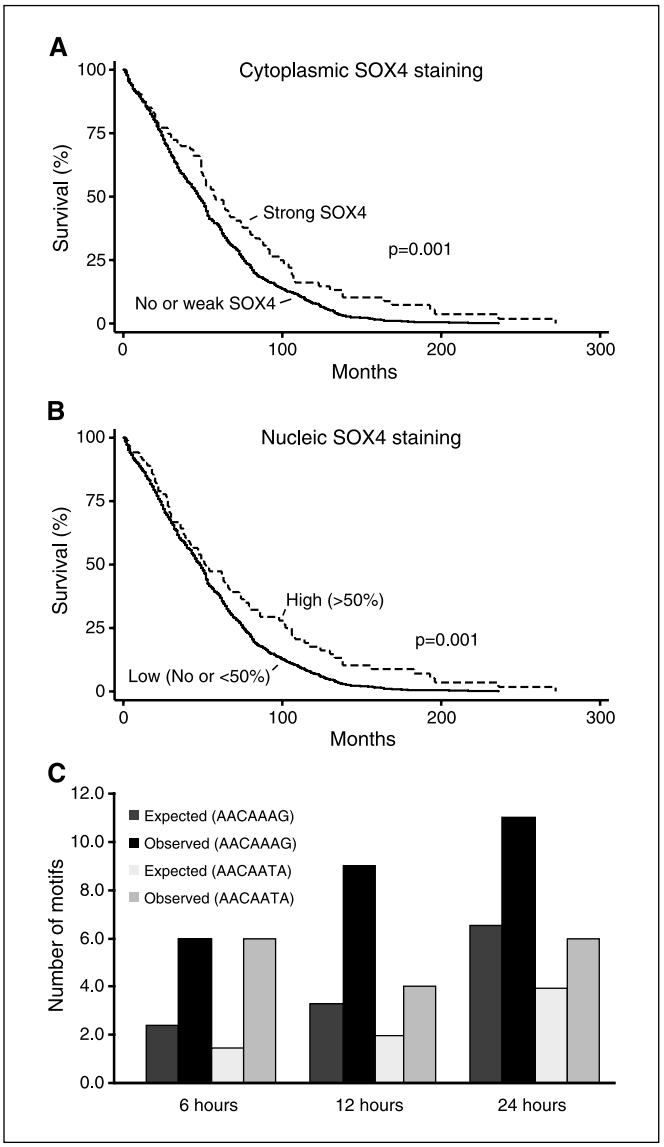

Figure 3. Clinically relevance of SOX4 protein expression (analyzed by tissue microarray). Kaplan-Meier survival analysis of SOX4 protein expression in bladder cancer patients: *A,* survival estimates of patients with tumors having either a strong cytoplasmic SOX4 staining or no/weak cytoplasmic SOX4 staining ($P = 0.001$). *B,* survival estimates of patients with tumors displaying either a high or low frequency of SOX4-positive nuclei ($P = 0.001$). *C,* the abundance of the two SOX4 promoter-binding motifs (AACAAAG and AACAATA) among SOX4 target genes. The observed motif frequency was compared with the expected frequency calculated by analysis of 200 randomly selected promoter sequences; 0.075 (AACAAAG) and 0.045 (AACAATA).

a number of functional categories, including transcription, signaling pathways, cell growth, and apoptosis. Among others, phosphoinositide-3-kinase regulatory subunit polypeptide 3, p55-γ (*PIK3R3*), which promotes cell cycle arrest (36), was strongly induced by SOX4 expression. Others were mitogen-activated protein kinase kinase 5 (*MAP2K5*), tribbles homologue 2 (*TRB2*), and MADS box transcription enhancer factor 2 polypeptide C (*MEF2C*). A previous report has shown that MEF2C is transcriptionally activated by MAP2K5 expression (37). In this study, *MAP2K5* was induced >40-fold after 6 hours and *MEF2C* was increased 19.1-fold at 12 hours. Supplementary Table S2*A* to *C* provides an overview of all probe sets changed >4-fold (up or down).

Identification of SOX4 target genes in bladder carcinomas. To estimate if candidate target genes correlated to *SOX4* transcript expression in clinical samples, Pearson correlation coefficients between *SOX4* and each of the 130 genes (represented by 145 probe sets) were calculated in the clinical data set. Among the probe sets being up-regulated by SOX4, we found 27 of 127 (21.3%) to be positively correlated to *SOX4* expression in 90 clinical samples ($\rho > 0.3197$, $P < 0.001$). As expected, we did not find a significant correlation between *SOX4* expression and the housekeeping gene *β-actin* ($\rho = 0.07$). However, the number of genes having a positive

Table 1. Identification of 19 genes consistently induced by SOX4 at all measurements (FC > +/−4)

Functional category	Gene	Fold change		
		6 h	12 h	24 h
Transcription				
MEF2C	MADS box transcription enhancer factor 2, polypeptide C (myocyte enhancer factor 2C)	4.3	19.1	15.9
BAZ2B	bromodomain adjacent to zinc finger domain, 2B	13.1	10.3	7.6
LOC114977	hypothetical protein BC014148	5.5	5.4	7.4
ZNF6	zinc finger protein 6 (CMPX1)	10.4	9.3	6.8
Signaling pathway				
MAP2K5	mitogen-activated protein kinase kinase 5	40.2	43.2	19.4
FLJ20275	hypothetical protein FLJ20275	4.1	6.6	6.0
ERG-1	estrogen regulated gene 1	5.0	7.4	13.3
PELI1	pellino homolog 1 (*Drosophila*)	9.7	8.6	13.4
PLXNB1	plexin B1	4.0	4.7	5.4
STK38L	serine/threonine kinase 38 like	4.1	9.9	12.8
TRB2	tribbles homologue 2	33.4	19.3	20.3
Cell Growth				
NEDD9	neural precursor cell expressed, developmentally down-regulated 9	6.3	6.7	16.2
DOCK4	dedicator of cytokinesis 4	4.9	15.0	5.0
Apoptosis				
PIK3R3	phosphoinositide-3-kinase, regulatory subunit, polypeptide 3 (p55, γ)	8.6	18.9	25.8
Miscellaneous				
HIST1H3E	histone 1, H3e	4.9	11.8	6.9
HCA127	hepatocellular carcinoma-associated antigen 127	4.8	7.2	6.4
DOC1	downregulated in ovarian cancer 1	4.5	5.0	8.6
SRP19	signal recognition particle 19ΔkDa	6.2	4.4	6.0
Unknown				
FLJ10260	hypothetical protein FLJ10260	4.8	5.4	4.3

Table 2. SOX4 target genes with SOX4-binding motif containing promoters

	Gene	AACAAAG motif	AACAATA motif	Correlation* (Pearson correlation coefficient/ experimental change)
Induced by SOX4 *in vitro*	*C5orf13*	Position 190	Position 990	
	BAZ2B	Position 268		0.39/6Δh: 13.1, 12Δh: 40.3. 24Δh: 7.6
	EFNA4	Position 402		0.52/6Δh: 5.6
	ISL1	Position 322		
	PELI1	Position 1384		
	ERG-1	Position 172		
	CGI-62	Position 776	Position 14	0.50/12Δh: 4.5
	SRP19	Position 190	Position 990	
	USP9Y	Position 605		
	NR4A2	Position 978		
	PELI1	Position 1384		
	FLJ11183	Position 1843		
	IL6	Position 50, 907		
	INHBA	Position 912	Position 584	
	SFPQ	Position 404		0.35/24Δh: 4.5
	COLEC11	Position 639		
	CCNG2		Position 1017	0.45/6Δh: 4.5, 12Δh: 4.2
	LOC65243		Position 555	
	STK38L		Position 311	
	HCA127		Position 59	
	MGC39325		Position 25	
	ADAM28		Position 806	
	IL1A		Position 1722	
	STARD5		Position 75	
Repressed by SOX4 *in vitro*	*NRP2*	Position 18, 360		−0.57/24Δh: 0.20
	FLJ12687		Position 446	

*Pearson correlation coefficients were calculated between each of the genes and *SOX4* expression in 90 clinical samples analyzed.

correlation with *SOX4* did not vary significantly from what would be expected by chance, as 4,060 of 22,216 probe sets (18.3%) present on the HG-U133A array also did show a positive correlation with *SOX4* in those samples. Similarly, we did not find an overrepresentation of negative correlating genes among the genes being down-regulated by SOX4 *in vitro* (data not shown). However, when looking at individual genes, we identified a set of very interesting genes with high correlation. Those were genes being up-regulated *in vitro* and displaying a high positive correlation *in vivo* or, alternatively, genes being down-regulated *in vitro* and with a high negative correlation with SOX4 *in vivo*. Prominent genes were *ZNF195* (ρ = 0.54), *KIAA1117* (ρ = 0.52), *EFNA4* (ρ = 0.52), *IHPK2* (ρ = 0.50), *FLJ20275* (ρ = 0.50), and *CGI-62* (ρ = 0.50), all with FC > 4, and *NRP2* (ρ = −0.57) with FC < 4.

Biological processes activated by SOX4 induction *in vitro*. An analysis of gene category distribution allowed us to identify overrepresented gene categories among the genes changing >4-fold with SOX4 induction. This was done using the EASE software (34). Six hours after transfection, we found a significant number of genes (false discovery rate, $P < 0.05$) involved in "signal transduction" and "phosphorylation" (Supplementary Table S3). No significantly overrepresented gene categories were identified 12 hours after transfection. However, we found genes related to "cell cycle arrest" and "regulation of cell cycle" to be significantly overrepresented among genes altered after 24 hours of transfection (Supplementary Table S3). We interpret this as a "signaling phase" at 6 hours and an "effector phase" at 24 hours, whereas a mixture of these occurs at 12 hours. Other categories were "angiogenesis" and "blood vessel development," which further support the role of SOX4 in blood vessel development as previously reported (6, 7). Examples of genes represented in those latter categories were *IL-8, CTGF, JAG1*, and *NRP2*.

SOX4-binding motifs in putative SOX4 target genes. SOX4 has previously been shown to transactivate transcription through the promoter motif AACAAAG (9) and the alternative motif AACAATA (12). To further investigate whether the genes that seemed regulated by SOX4 in this study could be direct targets of SOX4, we scanned the promoter regions of these for the two motifs. The genes harboring the AACAAAG motif were significantly overrepresented among the genes changed after 6 hours (6 observed/2.4 expected) and 12 hours (9 observed/3.3 expected; $P < 0.05$; Fig. 3*B*). In contrast, we found no significant difference between the number of observed and expected motifs after 24 hours. We repeated the analysis for the second motif (AACAATA). Again, we found more genes harboring this motif than expected among the genes regulated after 6 hours (6 observed/1.4 expected). However, the motif was not overrepresented among the genes regulated after 12 and 24 hours of transfection. We calculated the expected number of motifs that one would obtain by chance by measuring the motif frequency in 200 randomly retrieved promoter sequences and we obtained the following frequencies for the two motifs; 15/200 = 0.075 (AACAAAG) and 9/200 = 0.045 (AACAATA).

One would expect that primary SOX4 target genes gradually would be outnumbered by the increasing number of genes indirectly activated by SOX4. In agreement with this, the ratio between the number of observed and expected motifs decreased with time; for AACAAAG, the ratios were 2.5 (6 hours), 2.7 (12 hours), and 1.7 (24 hours); for AACAATA, the ratios were 4.2 (6 hours), 2.0 (12 hours), and 1.5 (24 hours). As shown in Table 2, the motifs were usually represented only once per gene, except for the genes *IL-6* and *NRP2*. Interestingly, some genes actually contained both SOX4-binding motifs; those genes were *C5orf13, CGI-62, SRP19*, and *INHBA*. Not all of the genes previously found to correlate with SOX4 did contain a SOX4-binding motif; however, the genes that did were *BAZ2B, EFNA4, CGI-62, SFPQ, CCNG2*, and *NRP2*.

SOX4 is proapoptotic in bladder cancer cells. Previously, SOX4 has been shown to induce apoptosis in a number of human cell lines [e.g., human embryonic kidney 293 cells (24) and hepatocarcinoma Hep3B and HepG2 cells (15)]. We transiently transfected HU609 cells with SOX4 vector to monitor the effect of SOX4 induction on human bladder cells, with no endogenous SOX4 expressed. Measuring the cell number 24, 48, and 72 hours after transfection allowed us to determine the SOX4-mediated effect on cell viability. Expression of SOX4 strongly impaired cell viability as the cell number decreased to ~17% after 72 hours compared with control cells transfected with mock vector. We observed no difference in cell viability between cells transfected with mock vector and the GFP transfection control cells (data not shown). As seen in Fig. 4*A*, the SOX4-induced effect on cell viability was not obvious before 48 hours after transfection. No difference was observed 24 hours after transfection. Transfected cells were stained with the two fluorescent dyes, propidium iodide and Hoechst 342, to estimate the percentage of viable versus apoptotic and necrotic cells. Propidium iodide dye is only able to penetrate nuclear membranes and thereby bind to DNA of apoptotic and necrotic cells. In agreement with previous findings, we found an increased proportion of apoptotic and necrotic cells among the SOX4-expressing cells (Fig. 4*B*). After 48 hours, the percentage of cells having permeable nucleic membranes increased ~3-fold, from 24.0 ± 2.7% (in control cells) to 70.0 ± 8.8% in SOX4-expressing cells ($P < 0.05$). The measurements were done in triplicates.

No correlation between chromosomal amplification and SOX4 expression. Previously, several independent studies have reported chromosomal amplification of the region 6p22, which contains the *SOX4* gene (38–42). Hence, we therefore speculated whether SOX4 overexpression was caused by this amplification. Works both by Oeggerli et al. (41) and Feber et al. (42) on the *E2F3* gene have shown that amplification of 6p22 positively correlates with *E2F3* overexpression. The *E2F3* gene also resides within the chromosomal region of 6p22. When we combined chromosomal copy number data and gene expression data previously obtained from 13 individual bladder cancer patients (4, 43), we were able to confirm a positive correlation between amplification and overexpression of the *E2F3* gene. Our data revealed that 30.8% of the patients had chromosomal amplification of the region. The mean *E2F3* expression of amplified tumors was 1.8-fold increased compared with not amplified tumors (Student's *t* test, $P = 0.034$). However, we did not find a significant correlation between chromosomal amplification and *SOX4* gene overexpression. We then extended the study to include the tissue microarray data previously obtained. Using the fluorescence *in situ* hybridization (FISH) *E2F3* gene copy number data from Oeggerli et al. (41), we compared SOX4 protein expression with chromosomal amplification of the 6p22 region. However, we repeatedly did not find a significant correlation between chromosomal amplification and SOX4 protein overexpression.

Discussion

Using DNA microarrays, we first identified the human transcription factor SOX4 as being up-regulated ~5-fold in bladder carcinomas. Immunohistochemistry showed the SOX4 protein to be expressed and located to both nuclei and cytoplasm. A tissue

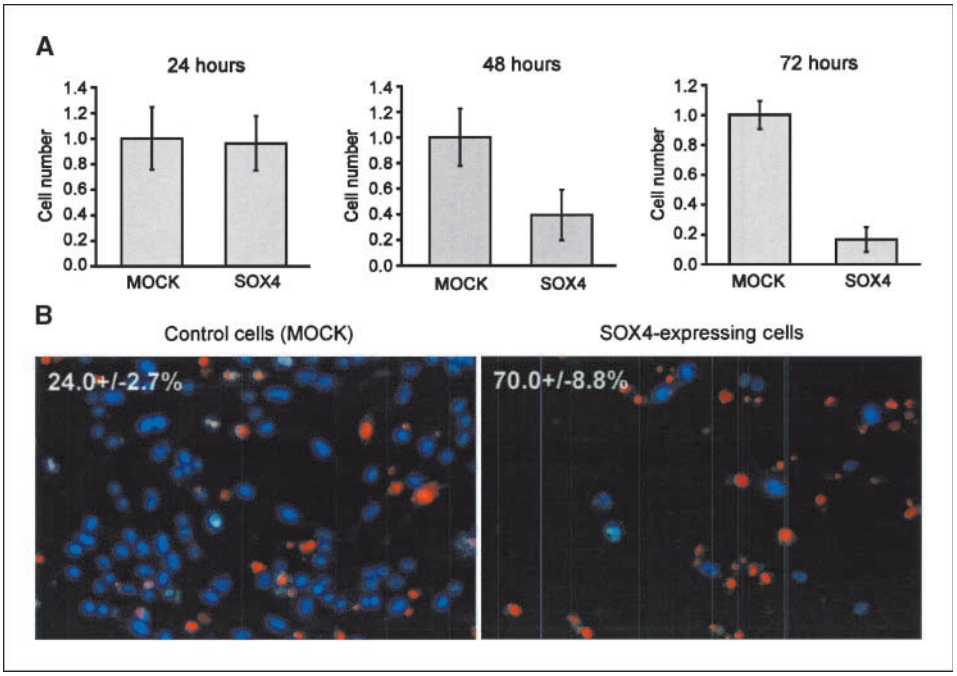

Figure 4. *A,* cell proliferation assay: estimation of cell number after 24, 48, and 72 hours of transfection. *B,* cell viability assay: visualization of apoptotic/necrotic cells using propidium iodide (*red/yellow-red*) and viable cells using Hoechst 342 (*blue*). Percentage of apoptotic/necrotic cells (after 48 hours): 70 ± 8.8% (SOX4) and 24.0 ± 2.7% (mock).

microarray with elements from >2,000 bladder tumors showed a relation between high histologic grade and low SOX4 expression and, in concert with that, a better survival in patients with a high SOX4 expression. These findings prompted us to analyze the functional effects of SOX4 *in vitro* by transient transfection of SOX4 into bladder tumor cell lines with a constitutively low SOX4 expression. Two sets of genes were identified by a time-course microarray expression approach. The first set seemed to reflect a signaling phase and the second set an effector phase. Finally, we analyzed the presence of the two SOX4 motifs in the promoter region of the genes differentially expressed by SOX4 transfection and found these to be overrepresented. The effect of SOX4 overexpression was a significant death of cells, in accord with the better survival of patients with SOX4-expressing cells. The SOX4-related genes detected *in vitro* were also, in one third of the cases, shown to correlate with SOX4 in the clinical bladder samples. Thus, we concluded that SOX4 seems to play a role in bladder cancer.

As the study was conducted using tissue biopsies instead of microdissected cancer cells, some variation in SOX4 expression was seen. Part or most of this variation could be due to differences in the numbers of cancer cells compared with other cell types within individual biopsies. However, immunostaining revealed a most intense staining in the cancer cells. Some scattered cells in the stroma also gave a signal that may originate from infiltrating B or T cells, which previously have been shown to express the SOX4 transcript (9). As no previous reports have described SOX4 protein expression, we are not aware of any similar findings from other tissues. We evaluated SOX4 protein expression in multiple bladder tumors by the use of a tissue microarray to assess the role of SOX4 in relation to tumor stage and grade, as well as clinical variables, such as recurrence, progression, and tumor-specific survival. Strong cytoplasmic SOX4 expression could be detected in 12.5% of the tumors; by Kaplan-Meier survival analysis, we found that patients having tumors with strong cytoplasmic SOX4 expression had increased survival ($P = 0.001$). High frequency of nucleic SOX4 staining also positively correlated with tumor-specific survival ($P = 0.001$). Recurrence and progression incidence did not correlate with SOX4 expression.

This study is the first to address the clinically relevance of SOX4 expression and to dissect the possible functional role SOX4 may have *in vivo*. Our data suggest that SOX4 may act as a tumor suppressor. In good agreement with this hypothesis is that SOX4, when induced in bladder cells, strongly impaired cell viability and promoted apoptosis. Previous reports have shown a similar role of SOX4 in other cell types (15, 24). More important, the impaired cell viability induced by SOX4 was a late event as it occurred after 48 and 72 hours of transfection. The pathway by which SOX4 is promoting apoptosis is still not fully understood. It has been shown that SOX4 may induce apoptosis independently of caspase-3 and without poly(ADP-ribose) polymerase cleavage (24). Others have suggested that SOX4 acts through caspase-1 (23). Lately, work by Kim et al. (44) suggests that SOX4 is involved in Δ12-prostaglandin J2–induced apoptosis through a cytochrome *c*–dependent, but AIP-independent, pathway.

Both the *E2F3* and *SOX4* genes are located on chromosome 6p22; however, in contrast to *E2F3*, expression of *SOX4* did not correlate with chromosomal amplification. In this study, we were able to confirm that *E2F3* gene overexpression did positively correlate with chromosomal amplification, as previously described (41, 42). However, we did not find correlation between *SOX4* overexpression and chromosomal amplification. This was analyzed using chromosomal copy number data combined with gene expression data, as well as tissue microarray FISH amplification data and SOX4 protein expression data. These findings altogether suggest that the mechanism responsible for *SOX4* gene up-regulation is independent of gene locus amplification.

Overexpression of a transcription factor, such as SOX4, will presumably lead to a deregulation of its target genes. Identification of those genes is therefore essential for understanding the role of SOX4 in bladder cancer cells. To do this, we did a time-course study where we transfected human bladder HU609 cells with SOX4 vector and measured the genome-wide gene expression of those cells after 6, 12, and 24 hours. Our data revealed that when induced, SOX4 was a very potent transcription factor, which switched on a considerable downstream transcriptional cascade. In contrast, SOX4 did only down-regulate a few genes. A group of consistently deregulated genes (19) could be gathered in functional categories, such as cell growth, apoptosis, transcription, and signaling pathway. We hypothesize that *PIK3R3* may be the link between SOX4 and cell cycle arrest, as this gene was strongly induced in parallel with SOX4 expression. Previously, PIK3R3 has been shown to directly bind the tumor suppressor protein Rb and to promote cell cycle arrest when induced in cells (36). Work by Kato et al. (37) shows that MEF2C is induced by MAP2K5 expression. We found MEF2C expression to be simultaneously induced in parallel with MAP2K5.

We analyzed the genes regulated by SOX4 *in vitro* for correlation to SOX4 expression in clinical samples. Although the actual number of correlating genes did not exceed what would be expected by chance, we identified an interesting subset of genes with high correlation. For us, it was rather unexpected to find genes that display such a high correlation to SOX4 *in vivo*, as those biopsy samples are composed of a mixture of different cells. We believe that this is supporting evidence that illustrates the action of SOX4 *in vivo*; however, it has to be documented in other studies as well.

Among the most prominent genes were genes that were induced by SOX4 *in vitro* and showed a positive correlation to *SOX4*. Those were *ZNF195* ($\rho = 0.54$), *EFNA4* ($\rho = 0.52$), and *CGI-62* ($\rho = 0.50$). The *CGI-62* and *EFNA4* genes contain copies of the SOX4 promoter binding motifs, suggesting that SOX4 might bind directly to the promoter sequence of these genes. In contrast, our data also reveal that SOX4 may act in a different manner, as a transcriptional repressor. The *NRP2* gene is being repressed by SOX4 *in vitro* and the NRP2 expression is inversely proportional to SOX4 *in vivo*. The presence of copies of SOX4 promoter binding motifs in the *NRP2* promoter sequence suggests an alternative and repressive role of the SOX4 protein. However, further studies are needed to characterize the mechanism(s) by which SOX4 is regulating transcription of its target genes.

Using EASE software, we did an analysis of the genes deregulated by SOX4 to identify possibly overrepresented gene categories. After 6 hours, we found genes involved in cell signaling and phosphorylation to be overrepresented, suggesting that the early or primary target genes of SOX4 may take part in signal transduction and cell communication pathways. In agreement with the latter observed SOX4-induced cell cycle arrest after 48 and 72 hours, genes related to cell cycle arrest and regulation of cell cycle were overrepresented after 24 hours. These findings indicate that these cells transfected with SOX4 are actually preparing to enter cell cycle arrest ~24 hours before the phenotypic response can be observed. Other overrepresented gene categories were cell proliferation, angiogenesis, and blood vessel development. This

finding agrees with the proposed role of SOX4 in development of the blood vessel system including the heart (6). Based on the pathway analysis results, we interpret this as if the cells enter specific phases during the course of SOX4 expression. One way of presenting it is that these cells are in a signaling phase at 6 hours, and then later progress to an effector phase after 24 hours, whereas a mixture of these occurs at 12 hours.

To identify possible primary SOX4 target genes that are directly bound and activated by SOX4, we did a promoter search for two known SOX4-promoter binding motifs (AACAAAG and AACAATA) and found 25 genes harboring at least one of those motifs. A significant overrepresentation of genes harboring SOX4-promoter binding motifs was found among the genes deregulated early after SOX4 initiation. The AACAAAG motif was significantly overrepresented at all measurements whereas AACAATA was not overrepresented beyond 6 hours of SOX4 transfection. The genes *IL-6* and *NRP2* both contained two AACAAAG motifs whereas the genes *C5orf13, CGI-62, SRP19*, and *INHBA* contained both motifs, which makes them good candidates as primary target genes of SOX4.

Thus far, only few SOX4 target genes have been described, such as the *src* family tyrosine kinase, $p56^{lck}$ that contains the AACAAAG motif (13), and the human *CD2* enhancer gene, which contains the AACAATA motif (12). However, we did not observe these genes in our cell studies.

Unfortunately, the SOX4-specific antibody was not suitable for immunocytology or Western blot analysis; therefore, it has not been possible to directly confirm the presence of SOX4 protein in the cell lines. However, others have been able to do this while observing an increased cell death as shown in this study (23, 24). We were able to detect massive induction of SOX4 transcript by RT-PCR and our microarray data also indicate specific SOX4 protein action, as the SOX4-binding motifs were overrepresented in promoter sequences of genes being up-regulated. In agreement with previous studies (24), we also observed an ~3-fold increased cell death in cells transfected with SOX4 vector, again suggesting the presence of SOX4 protein. Furthermore, indirect evidence is provided by the significant distribution of SOX4 target genes involved in angiogenesis and development of the blood system, as one would expect for SOX4 target genes. These data strongly support that SOX4 was the triggering molecule of the effects we report.

In conclusion, we have provided evidence for the existence of numerous downstream target genes of SOX4. We have shown that the *SOX4* transcript is highly up-regulated in bladder carcinomas and the encoded protein is expressed in a cancer cell–specific manner. We provide evidence that strong SOX4 protein expression is correlated with increased patient survival and that its action *in vivo* may be due to its involvement in promoting apoptosis, as evidenced by induction of cell death *in vitro*.

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