Concomitant down-regulation of *SPRY1* and *SPRY2* in prostate carcinoma

S Fritzsche, M Kenzelmann[1], M J Hoffmann, M Müller, R Engers[2], H-J Gröne[1] and W A Schulz

Department of Urology, Heinrich-Heine University, Düsseldorf, Moorenstr. 5, 40225 Düsseldorf, Germany
[1]Department of Cellular and Molecular Pathology, German Cancer Research Centre, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany
[2]Department of Pathology, Heinrich-Heine University, Düsseldorf, Germany

(Requests for offprints should be addressed to W A Schulz; Email: wolfgang.schulz@uni-duesseldorf.de)

Abstract

Sprouty proteins encoded by the *SPRY* genes act as modulators and feedback inhibitors of signalling by epidermal growth factor (EGF) and fibroblast growth factor (FGF). Overactivity of EGF and FGF signalling common in prostate cancer might therefore be exacerbated by Sprouty down-regulation. Indeed, down-regulation of *SPRY1* and *SPRY2* expression has been independently reported. We found both genes modestly down-regulated by microarray expression analysis of microdissected prostate cancers and by quantitative RT-PCR in macrodissected specimens compared with benign tissues. Importantly, the decreases paralleled each other and expression levels of both genes were significantly lower in cancers that recurred within the average follow-up period of 32 months. In contrast to a previous report, no hypermethylation was found to accompany down-regulation of *SPRY2* in cancer tissues and cell lines. We additionally investigated the expression of an *SPRY1* alternative transcript presumed to be specific for fetal tissues and found its expression moderately well correlated with expression of the standard transcript through diverse tissues and cell lines. The present study confirms and extends previous reports by demonstrating concomitant down-regulation and a significant association with recurrence of *SPRY* genes.

Endocrine-Related Cancer (2006) **13** 839–849

Introduction

As in many other carcinomas, growth factor signalling through receptor tyrosine kinases is activated in prostate cancer (Djakiew 2000, Blackledge 2003, Cronauer *et al.* 2003, Kwabi-Addo *et al.* 2004*a*). Several growth factors of the epidermal growth factor (EGF) and fibroblast growth factor (FGF) families are implicated acting by autocrine and paracrine mechanisms. Intracellularly, EGF and FGF signals are transmitted prominently through the canonical MAPK (mitogen-activated protein kinase) pathway. In normal cells, growth factors initiate numerous mechanisms in parallel with MAPK activation that limit the strength and duration of pro-proliferative signals. One such mechanism comprises induction and phosphorylation of sprouty (SPRY) proteins, which modulate growth factor signalling through receptor tyrosine kinases in a complex fashion (Hanafusa *et al.* 2002, Christofori 2003).

Modulation of growth factor signalling by SPRY proteins is particularly important during fetal development in shaping tissues and organs, including those of the urinary tract (Chi *et al.* 2004). In fact, the designation 'sprouty' derives from a *Drosophila* mutant displaying deficiencies in organ development (Hacohen *et al.* 1998). In humans, four *SPRY* genes are known (*SPRY1–SPRY4*), which are expressed in a tissue-specific fashion. In many adult tissues, *SPRY1* and *SPRY2* are deemed most important. Recently, a novel alternative *SPRY1* mRNA has been reported to represent a fetal isoform (Wang *et al.* 2003). It differs from the standard transcript by an alternative non-coding exon 1 arising through alternative promoter use. We suggest the name *SPRY1b* to distinguish it from the standard transcript *SPRY1a*.

SPRY1 and SPRY2 proteins modulate signalling from tyrosine receptor kinases through RAS and MAP kinases and exert overall similar effects on FGF

1351-0088/06/13–839
DOI: 10.1677/erc.1.01190

signalling, but differ in their influence on EGF signalling (Gross *et al.* 2001, Egan *et al.* 2002, Wong *et al.* 2002, Yusoff *et al.* 2002, Li *et al.* 2004). They disrupt the interaction between FGF receptors and the adaptor protein GRB2 and block activatory phosphorylation of RAF. However, SPRY2 in particular is not a pure feedback inhibitor, but also prolongs signalling by the EGF receptor under some circumstances.

Enhanced signalling by EGF and FGF family members in cancer ought to lead to an accordingly strong induction of MAPK pathway feedback inhibitors including SPRY mRNAs and proteins. Since this induction should impede cancer growth, one would expect selection to favour *SPRY* down-regulation, thereby exacerbating the tumourigenic effects of enhanced growth factor signalling. In contrast, oncogenic mutations of RAS or BRAF activate MAPK signalling downstream of SPRY action. Accordingly, SPRY2 has been reported as becoming induced in melanoma cell lines carrying such mutations (Bloethner *et al.* 2005). In prostate and breast cancers RAS mutations are rare and, indeed, evidence for down-regulation of *SPRY* expression has been published (Kwabi-Addo *et al.* 2004*b*, Lo *et al.* 2004, McKie *et al.* 2005). However, several issues are unresolved.

SPRY1 and *SPRY2* were reported to be down-regulated in prostate cancer in two separate publications. The relation between the two genes has not been determined, i.e. are they down-regulated co-ordinately, as reported in breast cancer, or independently of each other? The degree of down-regulation reported was only moderate. Therefore, independent confirmation seems warranted. The alternative splice form of *SPRY1* mRNA (*SPRY1b*) has not been investigated in cancers. Specifically, it is not known whether down-regulation of the presumed adult mRNA form is accompanied by up-regulation of the fetal isoform. Additionally, the role of epigenetic mechanisms such as DNA hypermethylation in *SPRY* down-regulation is unclear. In breast cancer, down-regulation did not appear to be associated with altered methylation, whereas in prostate cancer partial hypermethylation of *SPRY2*, but none of *SPRY1*, was reported. These questions were therefore addressed in the present study.

Materials and methods

Laser-controlled microdissection

RNase-free glass slides were prepared by baking at 200 °C for 4 h followed by covering with a PEN membrane (P.A.L.M. GmbH, Bernried, Germany). Frozen tissues were cut into 8 μm sections, mounted onto the PEN membrane and haematoxylin and eosin stained. Laser-controlled microdissection on up to 30 000 epithelial cells each from histological hyperplasia, prostatic intraepithelial neoplasia and cancerous samples was performed using the P.A.L.M. microlaser technology (P.A.L.M. GmbH) according to the manufacturer's protocol. Total RNA was extracted from microdissected cells according to a standard method (Chomczynski & Sacchi 1987) and RNA quality was checked using the RNA6000 nanoassay on the Bioanalyzer 2100 Lab-On-A-Chip system (Agilent Technologies, Palo Alto, CA, USA). RNA yields were usually >200 ng per sample.

RNA amplification and microarray analysis

Linear T7 polymerase-based amplification of total RNA from microdissected samples was performed essentially as previously described by Kenzelmann *et al.* (2004). RNA samples were then hybridized to HG U133A GeneChips (Affymetrix, Berlin, Germany) according to the manufacturer's instructions. Statistical analysis and data mining were performed with the software package MicroArray Solution, version 1.0, from SAS (SAS Institute, Minneapolis, MN, USA). GeneChip hybridization quality was controlled by correlation analysis; normalization of data and data mining were performed using log-linear mixed models with Bonferroni corrections that were fitted for values of perfect-matches, and by mixed model ANOVA (Cui & Churchill 2003).

Tissues for RT-PCR and methylation analyses

Prostate carcinoma (PCa) specimens were obtained between 1997 and 2002 by radical prostatectomy. Cancerous and morphologically normal areas of the prostate were identified and specimens collected by a pathologist, rapidly frozen in liquid nitrogen and stored at −80 °C. Since several micrograms of high molecular weight DNA as well as sufficient amounts of RNA were prepared from the same sample, no microdissection was performed. Representative samples of 3 mm maximal diameter of tumour and tumour-free tissue specimens were collected, immediately snap frozen in liquid nitrogen and stored at −80 °C. Non-cancerous tissue samples were taken from areas of the transition zone as far away as possible from the grossly apparent tumour (i.e. in general, from the transition zone of the contra-lateral lobe). Tumour and matched tumour-free specimens were only collected first when tumours were grossly apparent in the peripheral zone and could be unequivocally identified by their characteristic yellow or orange-yellow colour and secondly when the transition zone was macroscopically free of tumour. Separation

between tumour and non-tumourous tissues was histologically verified by analysing tissue specimens immediately adjacent to the specimens collected for analysis. Tumour node metastases (TNM) classification was performed according to the guidelines of the International Union Against Cancer from 1997. Clinical data are summarized in Table 1. Of 49 prostate carcinoma tissues, 23 were staged as pT2, 24 as pT3, and 2 as pT4. Lymph node metastases were present in 10 patients. None of the patients had detectable distant metastases at the time of surgery. Fifteen tumours had Gleason scores ≤ 6, 26 tumours scores of 7, and 8 tumours scores of 8–10. Median patient age was 68 years, ranging from 55 to 76 years. The median follow-up period was 66 months (range 44–130). The study was approved by the Ethics Committee of the Heinrich Heine University medical faculty.

Table 1 Tumour sample characteristics

Sample number	Patient age	Stage	Lymph node status	Gleason score
36	72	pT3b	pN0	7
38	75	pT2b	pN0	7
50	67	pT3b	pN1	7
65	62	pT3b	pN0	7
83	76	pT3b	pN0	7
87	62	pT3a	pN0	8
89	68	pT2b	pN0	3
93	73	pT3b	pN0	7
95	74	pT3b	pN1	10
97	71	pT3a	pN0	7
99	67	pT2b	pN0	5
105	59	pT3a	pN0	5
107	59	pT3a	pN0	7
117	68	pT3b	pN0	5
121	65	pT2b	pN0	6
123	58	pT2a	pN0	5
125	69	pT2b	pN0	6
127	71	pT2b	pN0	6
133	72	pT2b	pN1	7
137	73	pT2b	pN0	8
139	65	pT3b	pN1	9
141	69	pT2b	pN0	4
145	70	pT4	pN1	7
157	58	pT2	pN0	8
161	64	pT2b	pN0	5
163	65	pT3a	pN1	5
169	72	pT3a	pN0	7
171	61	pT2b	pN0	5
175	73	pT2b	pN0	8
183	67	pT3a	pN0	6
187	68	pT2b	pN0	8
189	63	pT2b	pN0	7
191	72	pT2b	pN0	7
205	73	pT3a	pN0	7
209	71	pT3a	pN0	7
213	59	pT2a	pN0	7
215	58	pT3a	pN0	7
217	62	pT2b	pN0	8
219	64	pT4	pN0	7
225	62	pT3b	pN0	6
227	72	pT2a	pN1	7
230	68	pT2a	pN0	7
232	70	pT2b	pN1	7
236	74	pT3a	pN0	7
238	62	pT2a	pN0	6
245	66	pT3a	pN0	7
247	55	pT3b	pN1	7
253	61	pT3a	pN1	7
256	71	pT3b	pN0	7

Cell line cultivation and treatment

The prostate carcinoma cell lines 22Rv1, LNCaP, PC3 and Du145 were cultured in RPMI-1640 (Gibco Life Technologies), supplemented with 10% fetal calf serum and 100 μg/ml penicillin/streptomycin. Normal uroepithelial cells (UC) were cultured as described (Swiatkowski *et al.* 2003). For experiments using 5-aza-2′-deoxycytidine (5-aza-dC), the compound (Sigma) was used at a concentration of 2 μM every 24 h for 3 days. Treated and untreated cells, which received solvent only, were cultured in parallel until RNA extraction.

RNA isolation and RT-PCR

Total mRNA was isolated from cell cultures grown to 80% confluence, using the RNeasy Midi Kit (Qiagen). For tissues the same kit was used following guanidinium/acid phenol/chloroform extraction (peqGOLD TriFast, peqLab, Erlangen, Germany). Following photometric quantification, 2 μg mRNA was transcribed into first strand cDNA using SuperscriptII (Invitrogen) according to the manufacturer's protocol with oligo-dT primers. Real-time PCR assays were performed using a fluorescence-detecting temperature cycler (LightCycler; Roche Diagnostics, Mannheim, Germany). The amplification mixture consisted of 1× reaction mix (LightCycler-FastStart DNA Master PLUS SYBR Green I; Roche), 10 pmoles of each primer (see Table 2 for sequences and annealing temperatures) and 20 ng cDNA in a final volume of 10 μl. The generation of target amplicons for each sample was monitored between the annealing and elongation steps at 640 nm. After the final cycle, melting-point analysis of the samples was performed over the range of 69–99 °C. Turning-point values for the specific genes were related to those for β-actin or CK18.

DNA extraction and bisulphite sequencing

High molecular weight genomic DNA from tissue, cell lines and whole blood was isolated using the blood and

Table 2 Primers used

Designation	Sequence (5′→3′)	T_M (°C)	Annealing T_M (°C)	Amplicon size (bp)
SPRY1a S	GTG TGT TGG AAA TCC ACG GT	57.3	62	171 bp
SPRY1a AS	AAA GAA GGC TGC TGG ATC AC	57.3		
SPRY2 S	GAT TGC TCG GAA GTT GGT CT	57.3	64	168 bp
SPRY2 AS	GGT CAC TCC AGC AGG CTT AG	61.4		
Aktin S	TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA	69.1	65	661 bp
Aktin AS	CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG	70.9		
CK 18 S	AGT CTG TGG AGA ACG ACA TCC	67.2	61	312 bp
CK 18 AS	TGG TGC TCT CCT CAA TCT GC	65.1		
SPRY1b S	CGC TGT TAA ATG TGC CTG AA	55.1	61	162 bp
SPRY1b AS	AAA GAA GGC TGC TGG ATC AC	57.3		
GAPDH 350S	TCC CAT CAC CAT CTT CCA	53.7	59	379 bp
GAPDH 350AS	CAT CAC GCC ACA GTT TCC	56.0		
SPRY1a S bisulphite DNA	CAT CAC CAT CTT AAG GAT CAC	55.9	54	319 bp
SPRY1a AS bisulphite DNA	AAG AAC TTG TTT TCA TTC ATA AC	51.7		
SPRY2 S bisulphite DNA	GGT AGG AT(CT) GGT TTG GGG A	57.7	55	160 bp
SPRY2 AS bisulphite DNA	AAC AAT TAT TAT CCC AAA AAA TAT ATC	52.8		

cell culture DNA kit (Qiagen) with additional proteinase K treatment. Following bisulphite treatment using the CpGenome DNA Modification Kit (Q-Biogene, Eschwege, Germany), 80 ng of bisulphite-treated DNA were used for amplification by HotStar Taq polymerase (Qiagen) in a 50 µl reaction with 20 pmoles of each primer indicated in Table 2. Following 15 min initial denaturation at 95 °C, 36 cycles of 30 s at 95 °C, 30 s at the annealing temperature and 45 s at 72 °C were performed, with a final 10 min elongation. Q-solution (Qiagen) was added for *SPRY2* amplification. PCR products were separated by 2.5% agarose gel electrophoresis and cloned into the TA-vector of the TOPO TA Cloning Kit (Invitrogen). Plasmid DNA was prepared from several clones for each cell line and sequenced by standard methods.

Immunohistochemistry

Immunohistochemistry using the avidin-biotinylated enzyme complex (ABC) method was performed essentially as described by Cohen *et al.* (2002) using a rabbit polyclonal antibody against SPRY2 (07-524, Upstate/Biomol, Hamburg, Germany).

Results

SPRY1 and SPRY2 are co-ordinately but modestly down-regulated in prostate cancer

In order to study the expression patterns of SPRY1 and SPRY2 along with prostate cancer development, HG U133A Affymetrix GeneChip microarray-based analyses were performed on histopathologically confirmed laser-controlled microdissected epithelial cells of 7 human hyperplastic tissues, 6 tissues showing severe prostatic intraepithelial neoplasia (PIN III; a precancerous lesion), and 7 tissues of prostate cancer, respectively. Microarray results for *SPRY1* and *SPRY2* revealed a gradual and co-ordinated down-regulation of both mRNAs from hyperplasia to PIN and prostate cancer, with *SPRY1* being more abundantly expressed (Fig. 1). However, due to tissue-inherent individual differences in the expression intensity of both genes the down-regulation was not statistically significant ($P > 0.05$). Since the microarray oligonucleotide probe

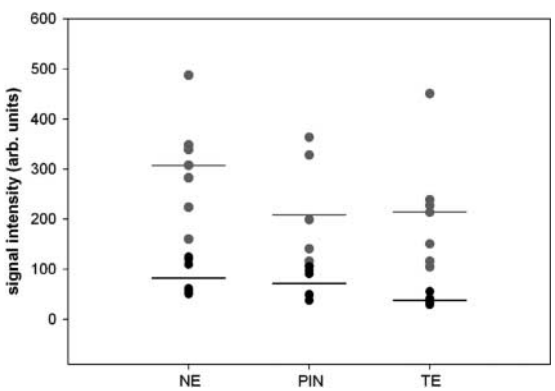

Figure 1 Analysis of *SPRY1* and *SPRY2* expression in prostate cancer development by microarray hybridisation. Distribution of the signal intensity values (arbitrary values) of *SPRY1* and *SPRY2* within microdissected human prostate tissues according to microarray analysis. Both genes are gradually and co-ordinately, but moderately down-regulated along with disease progression. Signal intensities for *SPRY1* and *SPRY2* in individual samples are shown in grey or black, respectively; mean expression values for each gene are also indicated. NE, hyperplastic (normal) epithelia; PIN, prostatic intraepithelial neoplasia; TE, tumour epithelia.

set for SPRY1 does not distinguish between the two *SPRY1* isoforms (*1a* and *1b*), it cannot be decided whether the expression signal belongs to only one isoform or to a cumulative detection of both isoforms.

Since the decreases in *SPRY* expression determined in this experiment were rather moderate, we attempted to confirm down-regulation of the genes by quantitative real-time RT-PCR in an independent, larger series of 49 macrodissected prostate cancer samples and 9 non-cancerous tissues (Fig. 2). *KRT18* (CK18) was used as a reference gene to adjust for the epithelial content in normal and cancerous prostate samples (Kwabi-Addo *et al.* 2004*b*). *SPRY1a* expression was not significantly lower in cancerous than in non-cancerous tissues, whereas the *SPRY2*/CK18 expression ratio was significantly decreased in the cancer tissues ($P=0.017$). Importantly, expression of *SPRY1a* and *SPRY2* in the carcinoma tissues was positively correlated ($r^2=0.658$, $P<0.001$) moderately strongly, i.e. cancers with low expression of *SPRY2* also displayed low expression of *SPRY1*. This indicates that down-regulation typically affects both *SPRY* genes in parallel, although in the case of *SPRY1* it is typically modest.

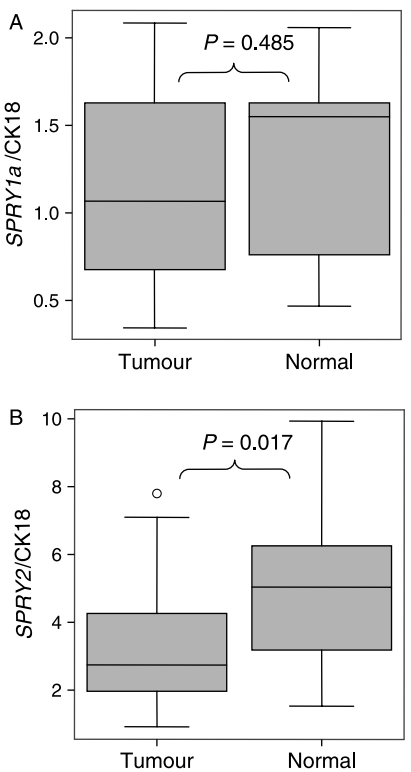

Figure 2 Real-time RT-PCR analysis of *SPRY* expression in 49 macrodissected prostate cancer tissues. Box plot representation of *SPRY1* (A) and *SPRY2* (B) mRNA expression levels adjusted to CK18 mRNA.

Mean expression of *SPRY1a* as well as *SPRY2* was significantly lower in patients experiencing biochemical recurrences during the average follow-up period of 66 months (Fig. 3). Expression of both genes tended to be lower in advanced stage cancers and in cases with lymph node metastases, although the difference did not reach statistical significance (Fig. 3). No association with pre-operative serum levels of prostate specific antigen and Gleason score (data not shown) was found.

SPRY2 is specific to epithelial cells in the prostate

To further ensure that the observed changes were specific to the epithelial component of the prostate, SPRY protein expression was investigated by immunohistochemistry. After testing commercially available antibodies in Western blot analyses we found only one antibody against SPRY2 (see Methods and materials) that was sufficiently specific, as it yielded a single band of the expected 35 kDa molecular mass (data not shown). Using this antibody, SPRY2 was found localized in prostate sections predominantly in the cytoplasm of basal cells of the glands (Fig. 4A, arrows), while secretory cells showed a weaker and more heterogeneous staining pattern and the mesenchymal compartment remained almost unstained (Fig. 4A). Prostate cancer specimens retained staining in the epithelial compartment, at a staining intensity similar to that in normal glands (Fig. 4B). This finding supports the RT-PCR data, since the moderate decreases found at the mRNA level are unlikely to result in protein changes detectable by immunohistochemistry.

All *SPRY* mRNAs are expressed in prostate cancer cell lines

The expression of *SPRY1a* and *SPRY2* and, in addition, that of the alternative splice form *SPRY1b* was also determined in prostate cancer cell lines (Fig. 5). Normal urothelial cells proliferating in primary culture were used as a control. All three messages were detectable in the prostate cancer cell lines. Whereas *SPRY1b* and *SPRY2* mRNA were detectable at similar or higher levels compared to normal cells, the level of SPRY1a mRNA was somewhat lower in three prostate carcinoma lines (except PC3) than in normal cells.

The alternative splice forms of *SPRY1* are not independently expressed

Although the ratio of the two alternative *SPRY1* mRNAs differed among the prostate carcinoma cell lines, both forms were detected. We further compared

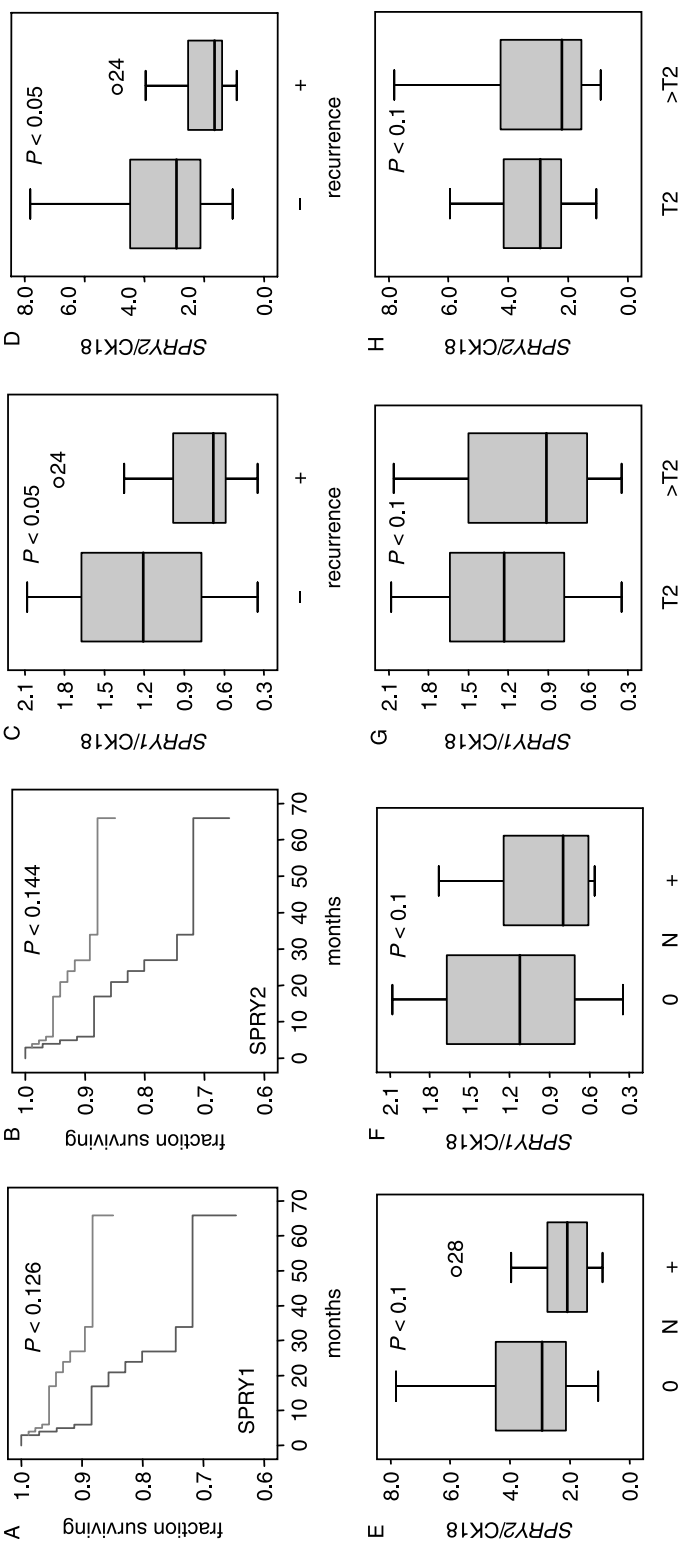

Figure 3 Relationship of *SPRY* expression to clinical parameters in prostate cancer. (A, B) Cox regression analysis of the relationship of *SPRY* expression (> vs < median) to biochemical recurrence after prostatectomy. (C, D) *SPRY* expression in cases without (−) or with (+) biochemical recurrence. (E, F) Relationship of *SPRY* expression to lymph node metastasis. (G, H) Relationship of *SPRY* expression to tumour stage.

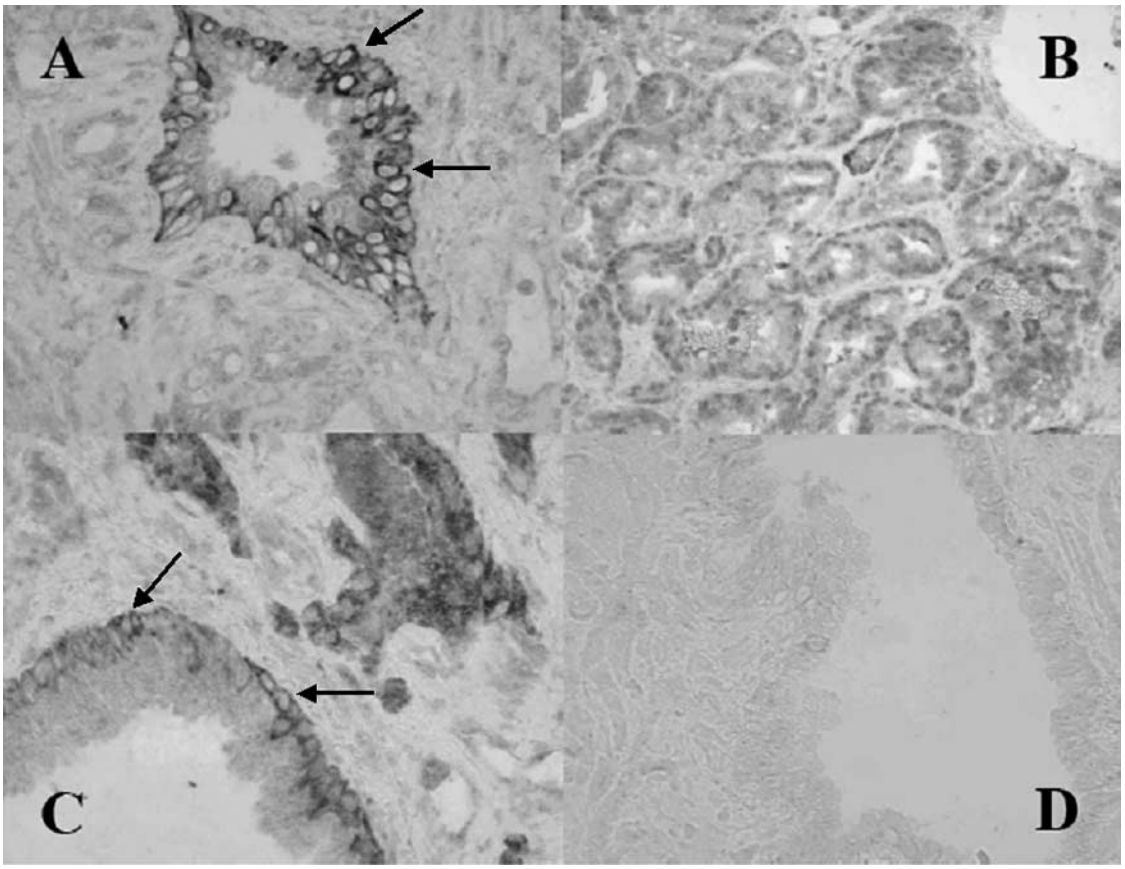

Figure 4 Immunohistochemical analysis of SPRY2 expression in prostate cancer. Double staining was used for SPRY2 (dark grey) and the basal cell marker 34β2 (black). (A) Normal gland; (B) carcinoma; (C) normal gland adjacent to carcinoma; (D) negative control. Note similar levels of expression in basal cells of normal glands (arrows) and carcinoma, and lower staining intensity in secretory cells. Stromal cells are very weakly stained.

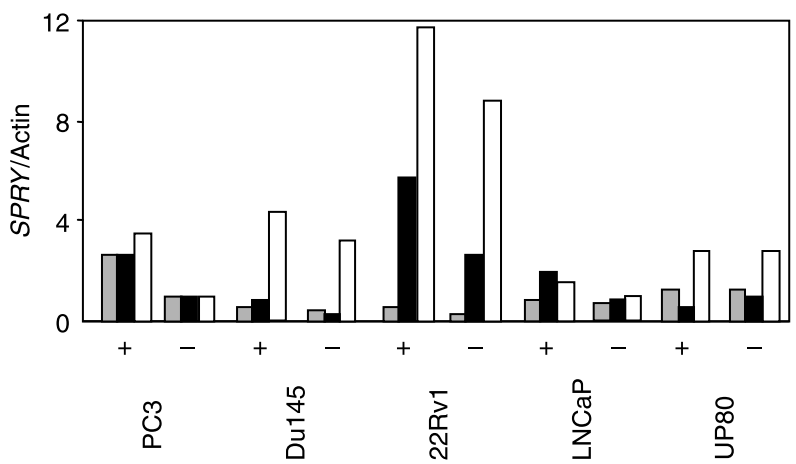

Figure 5 *SPRY* expression in prostate cancer cell lines. Expression of *SPRY1a* (grey bars), *SPRY1b* (black bars), and *SPRY2* (white bars) mRNA determined by real-time RT-PCR in four prostate carcinoma cell lines and cultured normal uroepithelial cells under normal growth conditions (−) and following 3 days of treatment with 2 μM 5-aza-dC (+).

the expression of the two *SPRY1* mRNA splice forms in various normal genitourinary tissues and cells. Their expression correlated positively and moderately well ($r^2 = 0.60$) with each other (data not shown).

SPRY promoter methylation shows few changes in prostate cancer

Since genes hypermethylated in cancer can often be induced by DNA methyltransferase inhibitors, the prostate carcinoma cell lines were treated with 5-aza-dC at an active but non-toxic concentration (Hoffmann *et al.* 2005). This treatment led only to small increases, none of them more than 2.5-fold (Fig. 5). In particular, the strongest increase in *SPRY1a* expression was observed in PC3, which had the strongest expression before treatment.

Additionally, DNA methylation was investigated by bisulphite sequencing in a region near the transcriptional start sites of *SPRY1a* and *SPRY2* each. Three prostate carcinoma cell lines were compared to normal leukocyte samples and normal prostate tissue. The *SPRY1a* promoter contains only a single CpG site located close to the transcriptional start site, but twelve sites in the first exon (Fig. 6). These were found to be largely, but not completely, methylated in normal leukocytes and prostate tissue. In three cell lines differing in expression levels (cf. Fig. 5), moderate differences in methylation were discernible. In particular, DU145 presented the lowest level of methylation, while the sequence was fully methylated in LNCaP. Intermediate levels were found in the PC3 line, which exhibits the highest mRNA level (cf. Fig. 5).

In contrast to *SPRY1*, the *SPRY2* gene contains a large CpG-island encompassing the promoter and first exon, which according to the most recent VEGA annotation comprises 1161 bp. We analysed a segment near the 5′-end of this CpG-island in which methylation differences had been reported previously (McKie *et al.* 2005). A few sites were found methylated in individual alleles of normal prostate tissue and a female leukocyte sample, whereas the sequence was entirely devoid of methylation in three prostate carcinoma cell lines investigated (Fig. 6). Surprisingly,

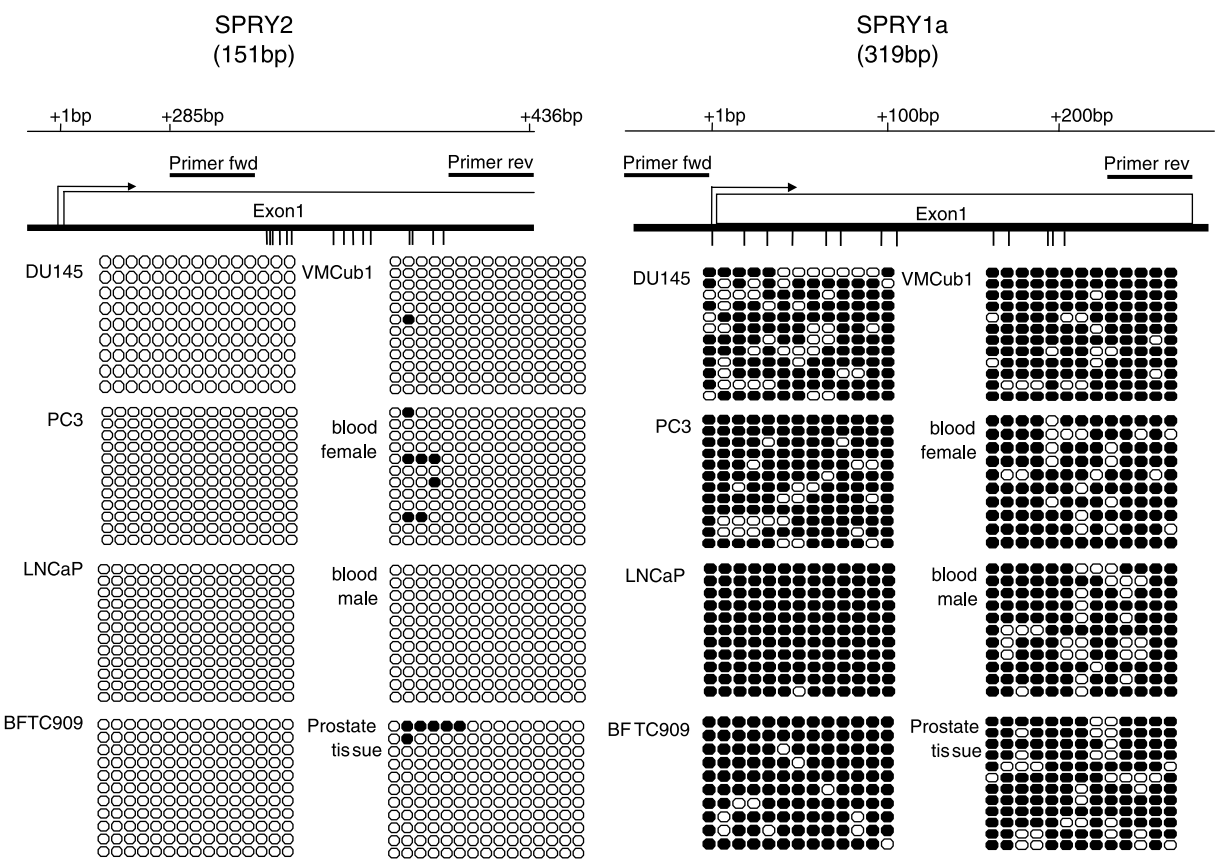

Figure 6 DNA methylation analysis of *SPRY1a* and *SPRY2*. Bisulphite sequencing analysis of *SPRY1* exon 1a including the single CpG-site in the basal promoter (left) and a CpG-rich region in *SPRY2* exon 1 (right). CpG-sites are indicated at the top; black symbols denote methylated and white symbols unmethylated sites; 9–12 alleles from each sample are shown.

a series of 5 prostate carcinoma tissues with low *SPRY2* expression levels lacked significant methylation as well (Fig. 6) Importantly, with one exception, these specimens had been observed to exhibit significant hypermethylation in *GSTP1*, *RARB2*, *APC* and *RASSF1A* (Florl *et al.* 2004).

Discussion

We observed diminished expression of both *SPRY1* and *SPRY2* mRNAs in microarray analyses of microdissected prostate carcinoma specimens and confirmed this change for *SPRY2* in a larger series of macrodissected tumours compared to normal tissues. Thus, in general, our data confirm two other reports on *SPRY1* (Kwabi-Addo *et al.* 2004*b*) and *SPRY2* (McKie *et al.* 2005) down-regulation in prostate cancer. Importantly, we found that the decrease in the expression of the two genes is typically co-ordinate. On a note of caution, the decreases were quite moderate in many cases. The previously reported difference in *SPRY2* mRNA expression between cancerous and normal prostate expression (11 normal and 31 cancer specimens) was 8-fold (McKie *et al.* 2005), whereas we found a significant, but only on average 2-fold, decrease in mRNA expression. Changes of this magnitude at the protein level are difficult to detect by immunohistochemistry, likely accounting for our results with a SPRY2 antibody. However, since SPRY expression is also regulated at the protein level (Egan *et al.* 2002), mRNA levels do not necessarily parallel protein expression. We could not confirm the decrease in SPRY1 protein expression reported previously in a tissue microarray containing 407 specimens using a rabbit antibody supplied by Upstate Biotechnology (Kwabi-Addo *et al.* 2004*b*), because we could not obtain an antibody sufficiently specific for immunohistochemistry. In fact, the 'SPRY'-antibody available from Upstate Biotechnology was raised against SPRY2. This may mean that the data in the previous publication may reflect changes in SPRY2 expression or expression of SPRY proteins overall. The decrease in SPRY1 mRNA expression reported by Kwabi-Addo *et al.* in 8 normal and 16 cancer specimens was only 2-fold on average, in line with our results showing smaller changes (1.5-fold) for *SPRY1* than for *SPRY2*. Thus, in spite of quantitative differences, the three studies, taken together, indicate that a concomitant, albeit often moderate, down-regulation of *SPRY1* and *SPRY2* takes place in many prostate cancers.

A factor potentially confounding expression analyses of *SPRY1* is the presence of the alternative splice form *SPRY1b*, which shares the coding exon 2 (Wang *et al.* 2003). It was postulated to represent a fetal isoform of the transcript, but our analyses show it to be expressed in several adult tissues and cell lines generally in parallel with the standard RNA form. In prostate carcinoma cell lines, *SPRY1b* expression also remained detectable. If the alternative mRNA is translated at the same efficiency as the standard transcript, its presence would tend to further buffer any changes in *SPRY1* expression. Since *SPRY1b* is not simply the fetal isoform of *SPRY1a*, its physiological function deserves further investigation, e.g. whether the two promoters respond differently to growth factor stimulation.

In prostate cancer, DNA hypermethylation of promoter CpG-islands is a particularly frequent mechanism responsible for the down-regulation of tumour suppressors and other genes (Florl *et al.* 2004, Kang *et al.* 2004, Yegnasubramanian *et al.* 2004). Accordingly, each of the prostate carcinoma cell lines and cancer tissues investigated here contains multiple aberrantly methylated CpG-islands in genes like *GSTP1*, *RARB2*, *APC* and *RASSF1A*. Nevertheless, no indication was obtained for a major function of DNA methylation in down-regulation of SPRY genes. *SPRY1* does not contain CpG-islands at its promoters. Several CpG-sites in exon 1a were predominantly methylated in normal tissue and leukocytes as well as in two prostate carcinoma cell lines. Interestingly, the sequence was almost completely methylated in LNCaP displaying the lowest expression level. Increased methylation in this cell line could be a consequence, rather than a cause, of decreased expression, as observed in a number of similar cases (Chen & Riggs 2005). Increased methylation of the *SPRY2* CpG-island had been reported in prostate carcinomas (McKie *et al.* 2005). In contrast, we could neither detect any methylation of this sequence in prostate carcinoma cell lines by bisulphite sequencing nor any significant induction by the DNA methylation inhibitor 5-aza-dC by quantitative RT-PCR. According to our data, down-regulation of *SPRY2* in prostate cancers occurs independently of DNA methylation, as previously reported for breast cancers (Lo *et al.* 2004). The discrepancies may relate to the tendency of techniques used for bisulphite treatment and methylation detection to yield false positive results. Moreover, most of the sites found hypermethylated by McKie *et al.* are located at the 3′-end of the CpG-island in a segment extending into intron 1.

In conclusion, the present study confirms and extends previous reports on down-regulation of *SPRY* genes demonstrating concomitant down-regulation and a significant association with recurrence underlining the

importance of these changes. However, it should be cautioned that the decreases in expression might be quite modest and appear not to be accompanied by promoter hypermethylation. Therefore, it remains to be investigated to what extent down-regulation is mediated by changes in chromatin structure, e.g. histone methylation, and which signals regulate the *SPRY* gene promoters in normal and cancerous prostate epithelial cells.

Acknowledgements

The authors wish to dedicate this paper to the memory of Dr. Manfred Hergenhahn, who initiated the collaboration between our groups. We are grateful to Dr. M.V. Cronauer for performing initial experiments and to Ms C Hader for technical assistance. This study was supported by the Deutsche Krebshilfe (70-3193 Schu I). The authors declare that there is no conflict of interest that would prejudice the impartiality of this study.

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