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Epigenetic control of CTCFL/BORIS and OCT4 expression in urogenital malignancies

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5-aza-dC, 5-aza-2′-deoxycytidine
SAHA, suberoylanilide hydroxamic acid
TSA, trichostatin A
UEC, normal uroepithelial cells

ABSTRACT

Aberrant hypomethylation in many cancers reactivates retrotransposons and selected single-copy genes such as cancer-testis antigens. Genes reactivated in this manner have recently been postulated to include *CTCFL/BORIS*, a presumptive testis-specific chromatin regulator, and *OCT4/POU5F1*, a transcriptional activator in pluripotent cells. We found both genes expressed at high levels in testis and at much lower levels in normal prostate tissue. In prostate and bladder carcinoma cell lines and cancer tissues expression remained largely unchanged, but individual prostate carcinomas showed modestly increased *CTCFL* expression compared to normal tissues. *OCT4* expression was significantly decreased in cancer tissues. Promoter methylation in both genes paralleled expression levels. *CTCFL*, but not *OCT4* was dramatically induced in cancer cell lines by 5-aza-2′-deoxycytidine, but neither gene by the histone deacetylase inhibitor suberoylanilide hydroxamic acid. Thus, *CTCFL* and *OCT4* resemble cancer-testis antigens in being selectively hypomethylated and expressed in male germ cells but differ in lacking significant reexpression and hypomethylation in prostate carcinomas. DNA methylation appears the crucial mechanism in the control of *CTCFL* transcription, but less decisive in that of *OCT4*. These findings imply that inhibitors of DNA methylation used for cancer treatment may induce *CTCFL* expression. Immunohistochemistry demonstrated nuclear localization of CTCFL in developing spermatocytes, and cytoplasmatic localization in spermatogonia, Leydig cells, and epithelial prostate cells. Teratocarcinoma cell lines showed nuclear, and 5-aza-2′-deoxycytidine-treated prostate cancer lines nuclear or cytoplasmatic localization. These different localizations might indicate additional control of CTCFL function via intracellular compartmentation.

1. Introduction

Somatic cells are distinguished from germ line and embryonic pluripotent cells by epigenetic mechanisms, most obviously by different DNA methylation patterns. In particular, several genes expressed selectively in developing germ cells are silenced in somatic cells. The mechanisms involved in silencing are not fully characterized, but are likely to include DNA methylation of regulatory sequences and altered chromatin structure.

Cancers cells are characterized by aberrant DNA methylation patterns and chromatin structure [1]. For instance, retrotransposon sequences densely methylated in somatic cells become hypomethylated and re-expressed. In a similar fashion, a number of single-copy genes whose expression is restricted to the testis in adults, become hypomethylated and re-expressed in some cancers [2,3]. Best characterized among these ‘cancer-testis antigens’ are MAGE proteins [4–6]. In developing germ cells, they participate in cell survival and

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stress responses. While it is unclear whether they possess similar functions in cancer cells, they are certainly recognized as antigens by the immune system.

The observation that reactivation of *MAGE* genes is associated with promoter hypomethylation raises the question whether DNA hypomethylation reactivates further germ cell specific genes. Since some of these are responsible for proliferation, pluripotency, and immortality, their reexpression in somatic cells could contribute to a more stem cell-like phenotype of cancer cells thereby promoting progression. This was recently proposed for the presumed chromatin regulator CTCFL, also called BORIS (brother of regulator of imprinted sites) [7,8] and for OCT4 (also called OCT3/4 or POU5F1) [9–11].

CTCFL contains a DNA binding domain similar to that of *CTCF*, an established regulator of chromatin structure [7]. CTCF may have multiple functions, of which the regulation of chromatin boundaries at imprinted genes is best characterized. *CTCF* is ubiquitously expressed in somatic cells. Expression of *Ctcfl* is well studied in the mouse where it appears to be restricted to male germ cells, especially primary spermatocytes [7]. Ctcfl is thought to permit the reprogramming of gene expression during male germ cell development, including the establishment of novel germ-cell specific methylation and expression patterns. CTCF function is disturbed in some cancers, due to decreased expression and more rarely to point mutations [7,12,13]. CTCFL presumably acts as a CTCF antagonist in germ cells, but also in human cancers [7], including prostate cancer, and is therefore considered a potential cancer-testis antigen [6,7,14,15]. Re-expression of *CTCFL* has even been speculated to be responsible for the hypomethylation of retrotransposons and other DNA sequences in cancer [16]. There are indications that *CTCFL* is controlled by DNA methylation, but more detailed investigations are required.

The restriction of *OCT4* expression to cells of the germ line and the early embryo may also be mediated by DNA methylation. The mouse ortholog *Oct4* is more strongly methylated in differentiated cells than in embryonic stem cells [17] and the human gene has been shown to become more densely methylated upon differentiation of a teratocarcinoma cell line [18]. However the regulation of the gene is known to differ between species [19], and *OCT4* methylation in human tissues has not been studied. Expression of *OCT4* is characteristic of human testicular cancers, including the undifferentiated component of teratocarcinoma [20]. Likely OCT4 is required for growth of these cancers and their immortal phenotype. A survey of cancers from somatic tissues using immunohistochemistry has suggested that expression of *OCT4* is restricted to testicular cancers. In contrast, RT-PCR analyses have indicated detectable levels in normal human tissues [11] and suggested increased expression in cancers of the breast, pancreas and colon [9].

Our group is interested in DNA methylation alterations in urological cancers, and especially in the causes and consequences of hypomethylation [3]. As outlined above, genome-wide hypomethylation in cancer cells could permit the reexpression of genes like *CTCFL* and *OCT4* that are normally restricted to germ and embryonic cells and become silenced by DNA methylation at later developmental stages. We have therefore investigated the function of DNA methylation in the control of *CTCFL* and *OCT4* and whether re-expression of these genes takes place in prostate and bladder cancer cell lines and prostate carcinoma tissues that display, often substantial, genome-wide hypomethylation.

2. Materials and methods

2.1. Cell line cultivation and treatment

Prostate carcinoma cell lines 22Rv1, LNCaP, PC-3 and DU145 were cultured in RPMI-1640 (Gibco Life Technologies, Karlsruhe, Germany), supplemented with 10% fetal calf serum and 100 μg/ml penicillin/streptomycin. Teratocarcinoma cell lines TERA-1, TERA-2, NT2, and NCCIT kindly provided by Dr. R. Löwer, Langen, Germany, were maintained in McCoy's 5A Medium (Gibco Life Technologies, Karlsruhe, Germany), supplemented as above. Bladder carcinoma cell lines and normal uroepithelial cells (UEC) were cultured as described [21]. In induction experiments 5-aza-2′-deoxycytidine (Sigma, Taufkirchen, Germany) was supplied at 2 μM every 24 h for 3 days and suberoylanilide hydroxamic acid (Biomol, Hamburg, Germany) at 2 μM for 2 days.

2.2. Tissues

Normal and cancerous prostate tissues were obtained and dissected as described [22]. Clinical data are listed in Table 1. Normal and cancer testicular tissue samples were from a previous study [23]. The study was approved by the ethics committee of the Heinrich Heine University medical faculty.

2.3. DNA extraction

High molecular weight genomic DNA from tissue, cell lines, and whole blood was isolated as described [22].

2.4. DNA methylation analyses

The LINE-1 hypomethylation data in Table 1 are taken from a previous study [22]. To study promoter methylation, DNA was bisulfite treated using the CpGenome™ DNA Modification Kit (Q-Biogene, Heidelberg, Germany) followed by PCR amplification using primer sequences and conditions in Table 2. PCR products were separated by agarose gel electrophoresis and cloned into the TA-vector pCR4-TOPO (Invitrogen, Karlsruhe, Germany). Several clones of each sample were sequenced by standard methodology.

2.5. RNA isolation and RT-PCR

Total mRNA was isolated from cell cultures using the RNeasy® Mini Kit (Qiagen, Hilden, Germany). For tissues the same kit was used following guanidinium/acid phenol/chloroform extraction (peqGOLD TriFast, peqLab, Erlangen, Germany). cDNA was prepared using SuperscriptII (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol with oligo-dT primers. Conventional PCR reactions were carried out as described using primers and conditions described in Table 2 [24]. Real-time PCR assays were performed using the

Table 1 – LINE-1 hypomethylation and clinical data for prostate cancer specimens used

Number	Tumor stage			Gleason sum	% LINE-1 hypomethylation[a]
22	pT3b	pN1	M0	9	0
36	pT3b	pN0	M0	7	1
38	pT2b	pN0	M0	7	4
50	pT3b	pN1	M0	7	10
65	pT3b	pN0	M0	7	9
83	pT3b	pN0	M0	7	6
85	pT3a	pN0	M0	5	13
87	pT3a	pN0	M0	8	6
89	pT2b	pN0	M0	3	5
93	pT3b	pN0	M0	7	4
95	pT3b	pN1	M0	10	7
97	pT3a	pN0	M0	7	2
99	pT2b	pN0	M0	5	0
101	pT3a	pN0	M0	8	0
105	pT3a	pN0	M0	5	1
107	pT3a	pN0	M0	7	3
117	pT3b	pN0	M0	5	3
119	pT3b	pN1	M0	9	1
121	pT2b	pN0	M0	6	7
123	pT2a	pN0	M0	5	1
125	pT2b	pN0	M0	6	1
127	pT2b	pN0	M0	6	2
129	pT2b	pN0	M0	7	0
133	pT2b	pN1	M0	7	0
135	pT2a	pN0	M0	5	0
137	pT2b	pN0	M0	8	1
139	pT3b	pN1	M0	9	1
141	pT2b	pN0	M0	4	2
145	pT4	pN1	M0	7	11
157	pT2a	pN0	M0	8	2
161	pT2b	pN0	M0	5	3
163	pT3a	pN1	M0	5	2
169	pT3a	pN0	M0	7	3
171	pT2b	pN0	M0	5	8
175	pT2b	pN0	M0	8	11
183	pT3a	pN0	M0	6	19
187	pT2b	pN0	M0	8	1
189	pT2b	pN0	M0	7	0
191	pT2b	pN0	M0	7	0
195	pT3a	pN1	M0	8	17
205	pT3a	pN0	M0	7	5
209	pT3a	pN0	M0	7	8
213	pT2a	pN0	M0	7	8
215	pT3a	pN0	M0	7	9
217	pT2b	pN0	M0	8	5
219	pT4	pN1	M0	7	21
223	pT2b	pN0	M0	7	4
225	pT3b	pN0	M0	6	4
227	pT2a	pN1	M0	7	3
230	pT2a	pN0	M0	7	0
232	pT2b	pN1	M0	7	13
236	pT3a	pN0	M0	7	4
238	pT2a	pN0	M0	6	0
240	pT2b	pN0	M0	7	0
245	pT3a	pN0	M0	7	4
247	pT3b	pN1	M0	7	3
253	pT3a	pN1	M0	7	n.d.
256	pT3b	pN0	M0	7	n.d.

[a] According to Ref. [22].

LightCycler apparatus (Roche, Mannheim, Germany). For real-time RT-PCR of *CTCFL* the same primers as above were used with a specific FAM/TAMRA-labeled hybridization probe (see Table 2). The reaction mixture contained LightCycler-FastStart DNA Master PLUS Hybridization Probes (Roche, Mannheim, Germany). Reaction mixtures for β-actin, *CK18*, *CTCF*, *OCT4* and $p21^{CIP1}$ mRNAs employed the LightCycler-FastStart DNA Master PLUS SYBR Green I (Roche, Mannheim, Germany).

Table 2 – PCR primer sequences and conditions

	Annealing (°C)	Cycles
Bisulfite sequencing		
CTCFL prom forward: 5′-GAG GAG AGT AGG TGG GTT TGA-3′ CTCFL prom reverse: 5′-CAC TAC CAC CCT CCA CTC TC-3′	58	30
OCT4 prom forward: 5′-AAG TTT TTG TGG GGG ATT TGT AT-3′ OCT4 prom reverse: 5′-CCA CCC ACT AAC CTT AAC CTC TA-3′	58	40
Qualitative RT-PCR		
CTCFL forward: 5′-CAG GCC CTA CAA GTG TAA CGA CTG CAA-3′ CTCFL reverse: 5′-GCA TTC GTA AGG CTT CTC ACC TGA GTG-3′	57	35
CTCF forward: 5′-TCG CAA GTG GAC ACC CAA ATC-3′ CTCF reverse: 5′-GAA CCC ATT CAG GGG AAA AGC-3′	56	30
GAPDH forward: 5′-TCC CAT CAC CAT CTT CCA-3′ GAPDH reverse: 5′-CAT CAC GCC ACA GTT TCC-3′	54	30
Quantitative RT-PCR		
CTCFL forward: 5′-CAG GCC CTA CAA GTG TAA CGA CTG CAA-3′ CTCFL reverse: 5′-GCA TTC GTA AGG CTT CTC ACC TGA GTG-3′ CTCFL probe FAM/TAMRA: 5′-AGT AAA TTG AAG CGC CAT GTC CGA TC-3′	66	45
CTCF forward: 5′-TCG CAA GTG GAC ACC CAA ATC-3′ CTCF reverse: 5′-GAA CCC ATT CAG GGG AAA AGC-3′	61	45
OCT 4 forward: 5′-GGT ATT CAG CCA AAC GAC CAT-3′ OCT4 reverse: 5′-TGG GAG AGC CCA GAG TGG TGA C-3′	66	40
β-Actin forward: 5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3′ β-Actin reverse: 5′-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3′	65	40
CK18 forward: 5′-AGT CTG TGG AGA ACG ACA TCC-3′ CK18 reverse: 5′-TGG TGC TCT CCT CAA TCT GC-3′	61	40
p21 Forward: 5′-GTG AGC GAT GGA ACT TCG AC-3′ p21 Reverse: 5′-TTC CTC TTG GAG AAG ATC AGC-3′	63	40
Promoter transfection		
CTCFL transf forward: 5′-CCA GTA TCT CAG TGC CTC CTG-3′ CTCFL transf reverse: 5′-CTG CCT CGT GCA CCG CGT G-3′	63	35

2.6. Plasmids

For promoter experiments, the 5′-end of the *CTCFL* gene was amplified by PCR (see Table 2), cloned into the pCR 2.1-TOPO TA-vector (Invitrogen, Karlsruhe, Germany), and recloned into the pGL3 basic-vector (Promega, Mannheim, Germany). The p850 construct containing a LINE-1 promoter has been described [25].

2.7. Transfection assays

Cells were grown on six-well plates. The CTCFL/pGL3-promoter construct, the empty pGL3 vector, or the p850 vector were mixed with the pRL-tk-LUC Renilla vector (Promega, Mannheim, Germany) to correct for transfection efficiency. Cells were transfected using FuGene6 (Roche Diagnostics, Basel, Switzerland). After 48 h, promoter activity was measured using the Dual-Luciferase Assay System (Promega, Mannheim, Germany). For in vitro methylation, 1 μg plasmid DNA was treated using *Hpa*II or *Sss*I methylase (NEB, Bad Homburg, Germany). Complete DNA methylation was verified by *Hpa*II restriction digestion.

2.8. Immunohistochemistry and cytochemistry

Immunohistochemistry was performed according to standard protocols with sodium citrate antigen retrieval. CTCFL/BORIS protein was detected with a specific rabbit anti-human polyclonal antibody (Abcam, Cambridge, UK). For immunocytochemistry, cells were cultured and treated with 5-aza-dC as described above on cover slips. Cells were fixed in methanol, treated with 0.5% saponin and 0.1% sodium boron hydride in TBS. After washing with TBS, cells were blocked with goat serum, washed and incubated with the same antibody overnight. Following washing steps a FITC-conjugated secondary antibody goat-anti-rabbit IgG (Jackson ImmunoResearch, West Grove, USA) was applied in PBS with 0.01% Evans blue for 1 h at room temperature. After further washing the samples were covered with Vectashield mounting medium (Vector Laboratories, Burlingame, USA) and stored at −20 °C.

3. Results

Expression of *CTCFL*, *CTCF*, and *OCT4* mRNAs was initially determined in cell lines from prostate and bladder cancers, and in several teratocarcinoma cell lines. According to conventional RT-PCR, *CTCFL* expression was generally low in cancer cell lines and in normal urothelial cells compared to testis or testicular cancers. In contrast, *CTCF* was strongly expressed at similar levels throughout all cell lines (data not

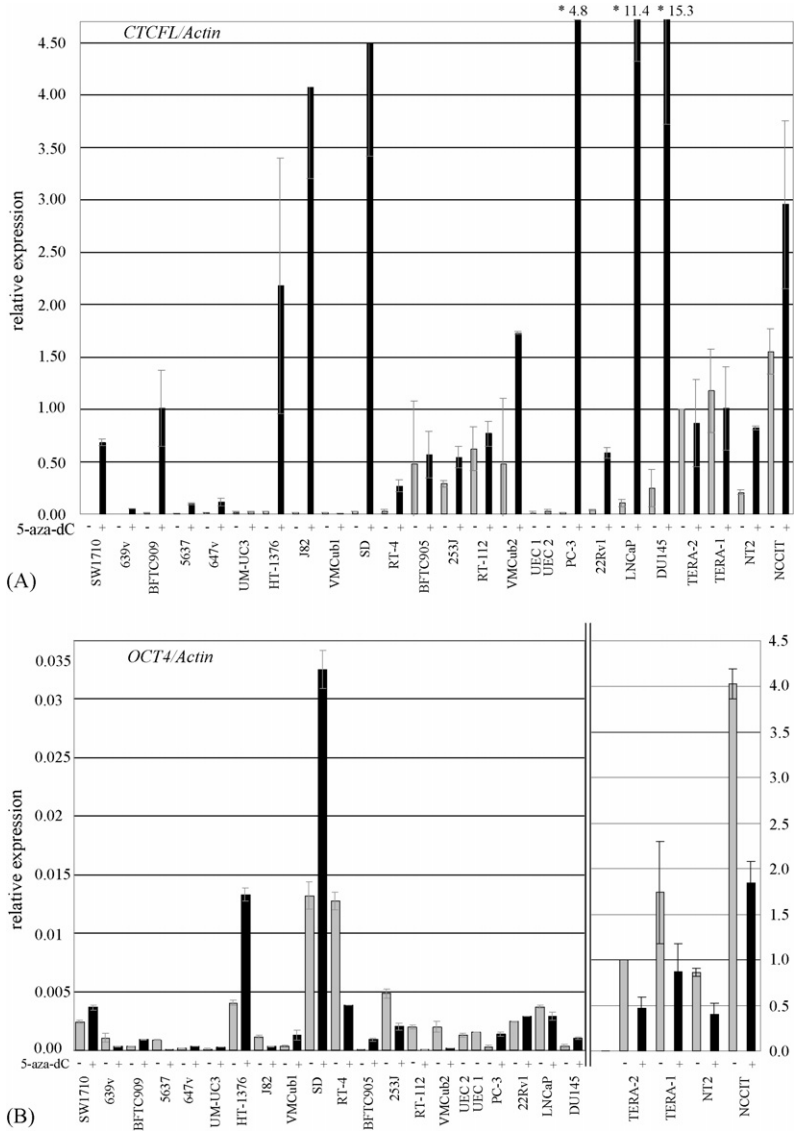

Fig. 1 – Expression of *CTCFL* and *OCT4* mRNAs in cancer cell lines and effect of 5-aza-dC. Expression of *CTCFL* (A) and *OCT4* (B) relative to β-actin by real time quantitative RT-PCR. Expression in TERA-2 was set as 1, note the differences in scales. Grey bars show basal expression levels, black bars levels after 5-aza-dC treatment. The cell lines used were as follows: SW1710 through VMCub2 are bladder cancer cell lines, UEC are normal urothelial cells, PC-3 through DU145 are prostate cancer cell lines. TERA-2 through NCCIT are teratocarcinoma cell lines.

shown). The *CTCFL* findings were confirmed by real-time quantitative PCR (Fig. 1A). In teratocarcinoma cell lines *CTCFL* expression was modestly increased compared to most bladder and prostate carcinoma lines, whereas *OCT4* expression was higher by two orders of magnitude (Fig. 1B). In normal testicular versus prostate tissues, *CTCFL* expression was on average 180-fold higher and *OCT4* expression 45-fold higher.

In almost all cell lines *CTCFL* expression increased dramatically following treatment with the DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-aza-dC), while *CTCF* mRNA expression changed little (Fig. 1A). According to real-time PCR, *CTCFL* expression was more than 100-fold induced in some carcinoma lines (Fig. 1A). Induction in the teratocarcinoma cell lines was more modest and no induction was observed in normal uroepithelial cells (Fig. 1A). In contrast, *OCT4* expression was only slightly affected by 5-aza-dC throughout all cell lines, and in general tended to decrease upon treatment with the inhibitor (Fig. 1B).

The striking effect of 5-aza-dC treatment suggests that *CTCFL* expression is predominantly controlled by DNA methylation. Therefore, the promoter region containing a CpG-island was cloned into a luciferase reporter vector and transfected into cell lines of different tissue origin (Fig. 2A). In all cell types tested, the *CTCFL* 5′-region proved a moderately strong promoter, compared to the strong L1.2B retrotransposon promoter [25]. Methylation in vitro by *Hpa*II methylase which modifies inner cytosines in the sequence CCGG or *Sss*I methylase which modifies all CpGs (cf. Fig. 2A) repressed *CTCFL* promoter activity in all cell lines tested (Fig. 2A). As expected, the *Sss*I methylase exerted a stronger effect than the

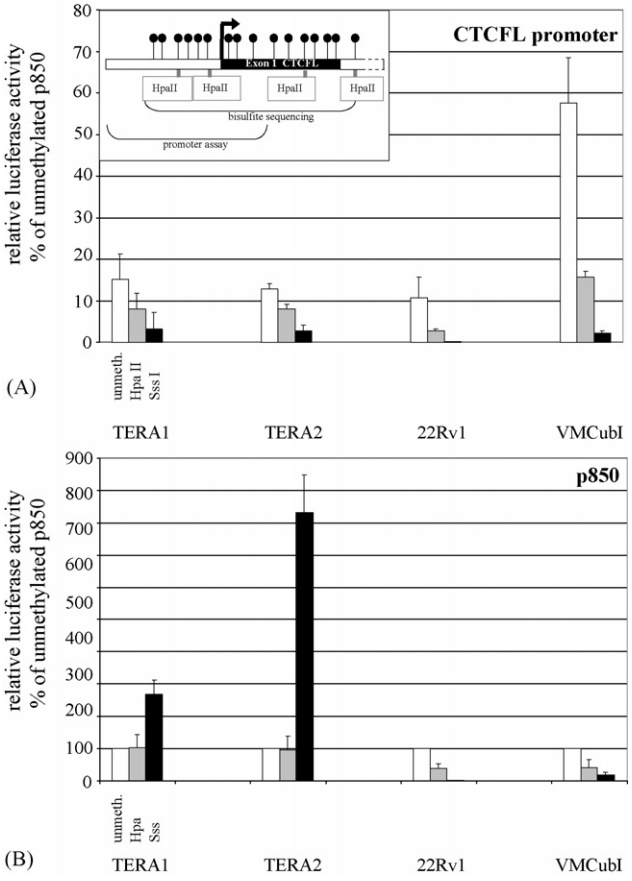

Fig. 2 – Activity and methylation sensitivity of the *CTCFL* promoter (see inset figure). (A) Activity of the promoter in transfection experiments measured by luciferase reporter activity relative to p850 set as 100% (white bars) in different cell lines (VMCubI: bladder cancer; 22Rv1: prostate cancer; TERA1/2: teratocarcinoma) and effect of in vitro methylation by *Hpa*II (grey bars) or *Sss*I (black bars) methylases. (B) Sensitivity of p850 to in vitro methylation by *Hpa*II (grey bars) or *Sss*I (black bars) methylases. (C) Bisulfite sequencing of the *CTCFL* promoter in tissues and cell lines. Several alleles are depicted for each sample, grey circles denote unmethylated and black circles methylated sites. Left column (from top to bottom): two normal leukocyte samples, three normal sperm samples, two paired testicular cancer and normal tissues, TERA cell lines. Center column: normal bladder tissue, normal cultured urothelial cells (UEC), eight bladder cancer cell lines, three also treated with 5-aza-dC (aza). Right column: cancer-free prostate tissue, seven prostate carcinoma specimens covering the range of *CTCFL* expression, four prostate carcinoma cell lines before and following 5-aza-dC (aza) treatment.

*Hpa*II methylase. To control the specificity of the methylation effect, the p850 retrotransposon promoter construct was treated in the same fashion. As expected [25], this promoter was repressed by either *Sss*I or *Hpa*II methylation in the prostate and bladder cell lines, but, surprisingly, not in the two teratocarcinoma cell lines. In fact, SssI methylation even increased promoter activity (Fig. 2B).

Methylation of the *CTCFL* promoter CpG-island was analyzed by bisulfite sequencing in multiple tissue samples and cell lines (Fig. 2C). Overall, the sequence was strongly methylated in somatic cells from blood, prostate, and bladder and remained so in cancer cell lines and cancer tissues, even though all cancer cell lines and some prostate cancer tissues display substantial hypomethylation of LINE-1 retrotransposons [22,26]. More hypomethylated *CTCFL* alleles were detected in normal and cancerous testicular tissues, while sperm DNA was completely unmethylated. Interestingly, the *CTCFL* CpG-island was densely methylated in teratocarcinoma cell lines. As expected, methylation decreased in cells treated with 5-aza-dC, particularly in the 5′-region of the sequence that corresponds to the basal promoter of the gene. Of note, this decrease was weak in some cell lines, e.g. J82 and DU145.

Bisulfite sequencing was also performed for a segment of the *OCT4* promoter previously described as becoming hypermethylated during differentiation of NT-2 teratocarcinoma cells (Fig. 3) [18]. The *OCT4* promoter was densely methylated in leukocyte DNA. Slightly lower methylation was found in normal prostate and prostate cancer cell lines and even more hypomethylated alleles were encountered in testis. Different from *CTCFL*, the *OCT4* promoter was strongly methylated in sperm, but completely unmethylated in teratocarcinoma lines, in accord with their strong *OCT4* mRNA expression.

We additionally investigated the effect of the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) on the expression of *OCT4* and *CTCFL* using concentrations that induced *CDKN1A*/$p21^{CIP1}$ mRNA (data not shown). In the teratocarcinoma cell line TERA-2 *CTCFL* expression could be induced by SAHA treatment. In another teratocarcinoma cell line (NCCIT), prostate cancer cell lines (DU145, PC-3, 22Rv1), and bladder cancer cell lines (J82, VMCub1), SAHA rather diminished the effect of 5-aza-dC (data not shown). Expression of *OCT4* was not significantly affected by SAHA alone or in combination with 5-aza-dC (data not shown).

Further, we investigated *CTCFL* and *OCT4* expression in prostate cancer and non-cancerous prostate tissues previously characterized for DNA methylation alterations [22]. Approximately half of these specimens exhibit hypomethylation of LINE-1 retrotransposons. From this series, high quality RNA was available from 58 carcinomas (Table 1) and 11 paired normal prostate specimens. Expression of *CTCFL* and *OCT4* mRNAs was determined by quantitative real-time PCR relative to Cytokeratin 18 (*CK18*) to correct for different proportions of epithelial cells (Fig. 4).

The expression of *CTCFL* mRNA showed no significant difference between normal and cancerous tissues overall and no relationship was seen to clinical parameters. Instead, selected cancer samples presented moderately increased transcript levels outside the range of normal tissues (Fig. 4A). These cancers showed no significant differences towards the others with regard to clinical parameters or histology. The *CTCFL* CpG-island in these samples remained densely methylated (Fig. 2C). *OCT4* mRNA expression was slightly decreased in cancerous compared to normal tissues (t-test: $p < 0.044$) (Fig. 4B), but no significant relationship to clinical parameters was observed. Expression of neither *CTCFL* nor *OCT4* mRNAs was related to LINE-1 retrotransposon methylation (Table 1).

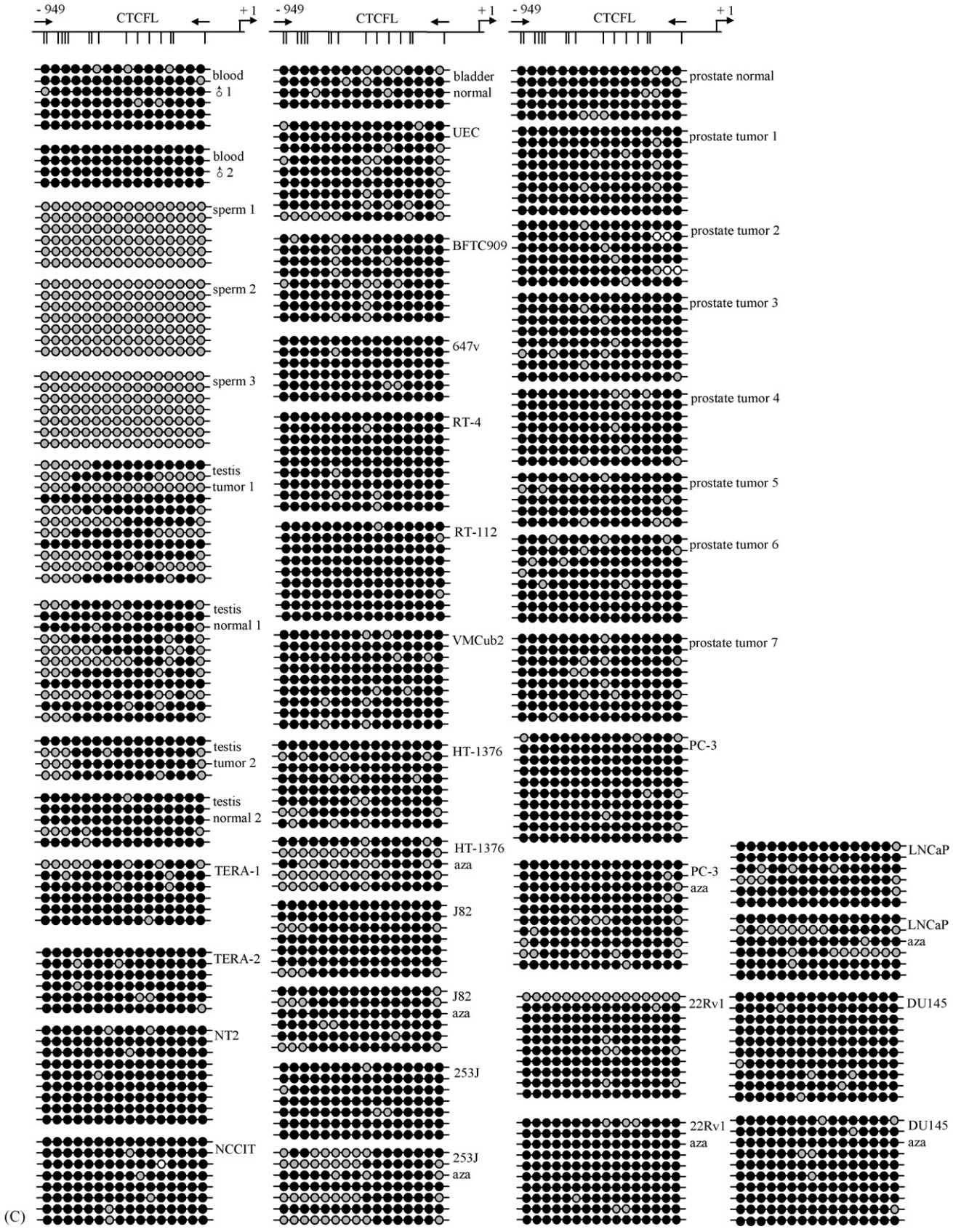

Fig. 2. (Continued).

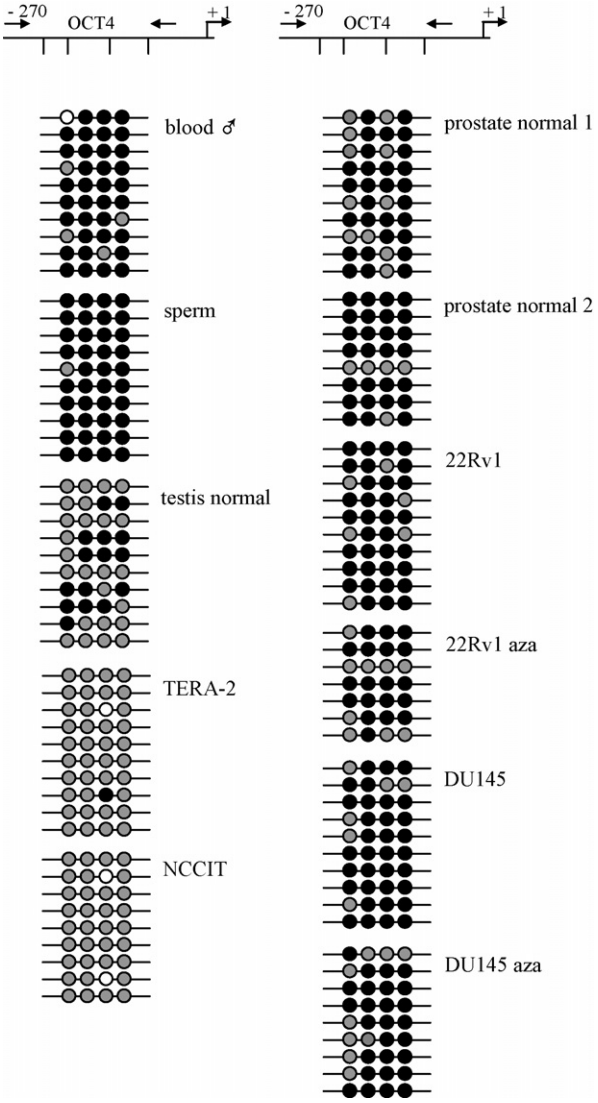

Fig. 3 – Bisulfite sequencing of the *OCT4* promoter region in selected tissues and cell lines. Bisulfite sequencing of a segment of the *OCT4* promoter representative of overall promoter methylation [18] in tissues and cell lines. Several alleles are depicted for each sample, grey circles denote unmethylated and black circles methylated sites. Left column (from top to bottom): a normal leukocyte samples, a normal sperm sample, normal testicular tissues, two teratocarcinoma cell lines. Right column: two normal prostate tissues, and two prostate cancer cell lines before and after 5-aza-dC (aza) treatment.

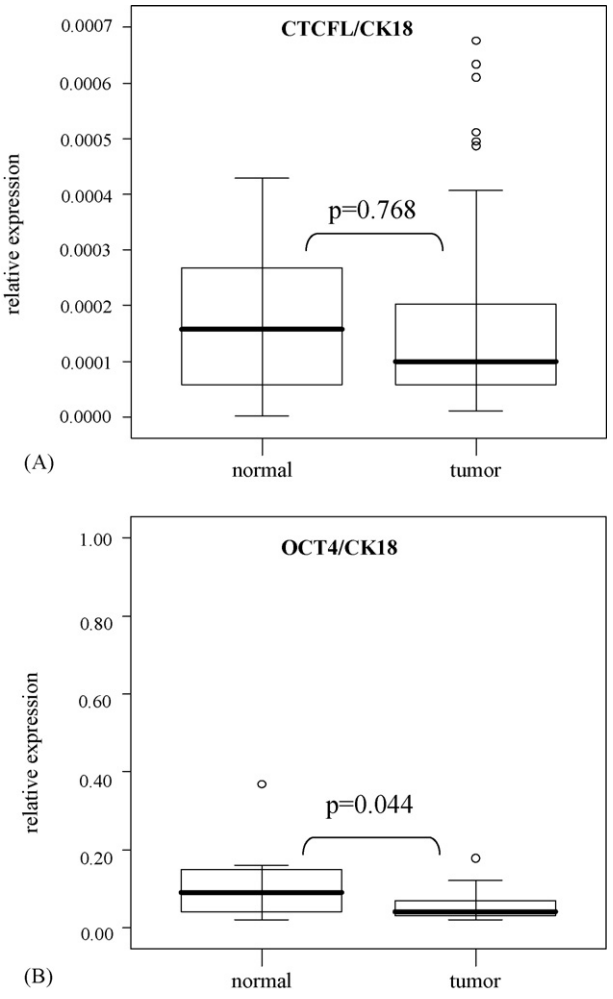

Fig. 4 – Expression of *CTCFL* and *OCT4* mRNAs in prostate cancer. Box plots showing *CTCFL* (A) or *OCT4* (B) mRNA expression in prostate cancer (tumor) and non-cancerous (normal) tissues measured by quantitative real-time PCR relative to the epithelial cell marker *CK18*. Circles indicate values of individual samples outside the normal range. *p*-Values from Student's *t*-test.

As CTCFL protein expression is not well studied in human tissues, expression in testis and prostate tissue was investigated by immunohistochemistry (Fig. 5). Strong expression was found in normal testis tissue throughout all stages of spermatogenesis, but also in Leydig cells (Fig. 5A). Importantly, expression was exclusively cytoplasmatic in spermatogonia and Leydig cells, whereas more mature germ cells including primary spermatocytes also showed nuclear staining (Fig. 5A and B). Normal prostate tissue displayed overall fainter, but specific staining of the glandular epithelium, while the mesenchyme lacked expression (Fig. 5D). Basal cells showed cytoplasmatic CTCFL expression, whereas the protein appeared associated with the inner face of the plasma membrane in secretory epithelial cells (Fig. 5D and E). Nuclear staining was observed neither in normal nor in cancerous glands in which CTCFL was restricted to the cytoplasm (Fig. 5C and F). A comparison of normal glands and cancer areas in the same section (Fig. 5C) confirmed the result from RT-PCR that no major increase in CTCFL expression occurs in prostate cancers. In teratocarcinoma cell lines, CTCFL was localized to the nucleus (Fig. 5G) in a punctuate pattern. Following 5-aza-dC treatment, CTCFL was detected in a comparable pattern, albeit more weakly, in the nucleus of LNCaP prostate carcinoma cells (Fig. 5I and J), but in PC-3 cells the protein was localized in the cytoplasm or under the cell membrane (Fig. 5K and L) as in prostate tissues.

4. Discussion

The present study demonstrates that tissue-specific expression of *CTCFL* is predominantly controlled by DNA methylation and extends previous indications [10,17] that DNA methylation contributes to *OCT4* regulation. Both gene promoters were less methylated in testis than in normal somatic tissues like prostate and mRNA expression levels differ by approximately two orders of magnitude between germ cells and somatic tissue. Apart from this general similarity, several intriguing differences were discovered.

First, *OCT4* remained expressed in teratocarcinoma cell lines, in line with previous reports proposing that high *OCT4* expression in testicular cancers increases their malignant potential [20]. In contrast, *CTCFL* was expressed in teratocarcinoma cell lines at levels not dramatically surpassing those in bladder and prostate carcinoma cell lines. This relatively low expression level raises the surprising possibility that *CTCFL* may be weakly expressed in certain testicular cancers, in spite of its postulated pro-proliferative role in germ cells [7,8]. Conceivably, low expression may reflect the derivation of germ-cell cancers from a stage of development prior to maximal CTCFL expression in primary spermatocytes.

Secondly, accordingly, DNA methylation in the *CTCFL* promoter region was as dense in teratocarcinoma as in somatic carcinoma lines, whereas the *OCT4* promoter displayed complete hypomethylation in all teratocarcinoma cells lines. The latter finding is in keeping with a previous study on differentiation of NT-2 teratocarcinoma cells [18]. Conversely, we found the *OCT4* promoter completely methylated in sperm, fitting the notion that *OCT4* expression is restricted to type A spermatogonia [27], whereas the *CTCFL* promoter was completely unmethylated in sperm suggesting that it does not become remethylated subsequent to maximum expression in spermatocytes [8].

Thirdly, the DNA methylation inhibitor 5-aza-dC had strikingly different effects on the two genes. It induced *CTCFL* in almost all cell lines, often dramatically, although only partial demethylation of the promoter took place. A histone deacetylase inhibitor was inefficacious, and, if anything, inhibited induction by 5-aza-dC. These results indicate that DNA methylation as such is limiting *CTCFL* transcription. In contrast, *OCT4* is differentially methylated in expressing and non-expressing cells, but DNA methylation is clearly not the only mechanism involved in its repression.

The transfection experiments demonstrated that the region of the *CTCFL* gene investigated indeed represents a moderately strong promoter, which is sensitive to partial or complete DNA methylation. In principle, methylation-dependent repression can be achieved by two different mechanisms, i.e. inhibition of the binding of specific transcriptional activators or represssion mediated through binding of chromatin remodelling complexes containing the methylcytosine-binding proteins MBD2 or MeCP2. In fact, methylation-dependent repression of the LINE-1 promoter used as a control in this study has been shown to be mediated by binding of MBD2 and MeCP2 in diverse cell types [25,28]. However, while the *CTCFL* promoter was repressed by partial or complete methylation in teratocarcinoma cells, the activity of a LINE-1 promoter was not affected in these particular cell lines. This indicates that the TERA cell lines may be deficient in the function of MBD2 and MeCP2 or their associated repressor complexes. Consequentially, it is unlikely that these complexes are responsible for methylation-dependent repression of the *CTCFL* promoter in TERA cells and this repression is caused by inhibition of binding of specific transcription factors. A direct effect on binding of crucial transcription factors would also be compatible with the dramatic effect of methylation inhibition on *CTCFL* transcription.

While promoter methylation evidently limits *CTCFL* transcription, a certain level of transcription can apparently be obtained without demethylation, as evidenced by a few prostate cancer samples and cell lines with moderately increased activity, and by the presence of the protein in prostate glands at levels detectable by immunohistochemistry (Fig. 5). Demethylation of the region, however, is associated with a much higher expression in germ cells and strongly increased expression after 5-aza-dC treatment (Fig. 1A). This suggests a stepwise regulation for *CTCFL*, whereby maximum activity of the gene requires promoter demethylation. Promoter demethylation allows an increase of roughly two orders of magnitude, while smaller changes can be affected without methylation changes.

Our data imply that inhibitors of DNA methylation used in cancer treatment may induce *CTCFL* in vivo. Interestingly, however, *CTCFL* was poorly induced by 5-aza-dC in normal urothelial cells. Accordingly, Karpf et al. [29] have observed that 5-aza-dC induced a significantly lower number of genes in normal than in tumor cell lines and, in particular, did not induce the cancer-testis antigen MAGE-1 in normal cells.

Indeed, two recent studies demonstrated that CTCFL/BORIS specifically regulate several cancer testis antigens, particularly *MAGE-A1* [30] and *NY-ESO1* [31]. Interestingly, *OCT4* was also identified as a potential CTCFL target. Obviously, the results fit those in our study quite well. First, rapid induction of *CTCFL* through partial demethylation by 5-aza-dC was also found. Secondly, expression of cancer-testis antigens like *MAGE-A1* and *NY-ESO-1* in primary prostate cancers is rare, in accord with the rare increases in *CTCFL*. On a note of caution, it seems unlikely that CTCFL is the only regulator of these cancer-testis antigens, since their expression during spermatogenesis precedes the peak of CTCFL expression [15]. Thirdly, the CTCFL focal nuclear staining in teratocarcinoma and prostate cell lines reported by us is better compatible with a function in the transcription of specific genes such as cancer-testis antigens rather as a general chromatin regulator postulated originally [7,8,16]. An apparent discrepancy to our results is that Vatolin et al. [30] report overexpression of *CTCFL* mRNA in various human cancers including prostate carcinoma. However, these results were obtained by qualitative RT-PCR in a small number of unspecified samples and could reflect the moderate variation we observed using real-time quantitative RT-PCR. Indeed, quantitative RT-PCR applied by Hong et al. [31] showed expression of *CTCFL* at a level similar to testis in only a single lung cancer cell line, while all others exhibited at least 100-fold lower levels, in good accord with our measurements. These authors also found induction by 5-aza-dC in lung cancer cell lines, which was in many cases augmented by the histone deacetylase inhibitor depsipeptide FK228. The latter difference

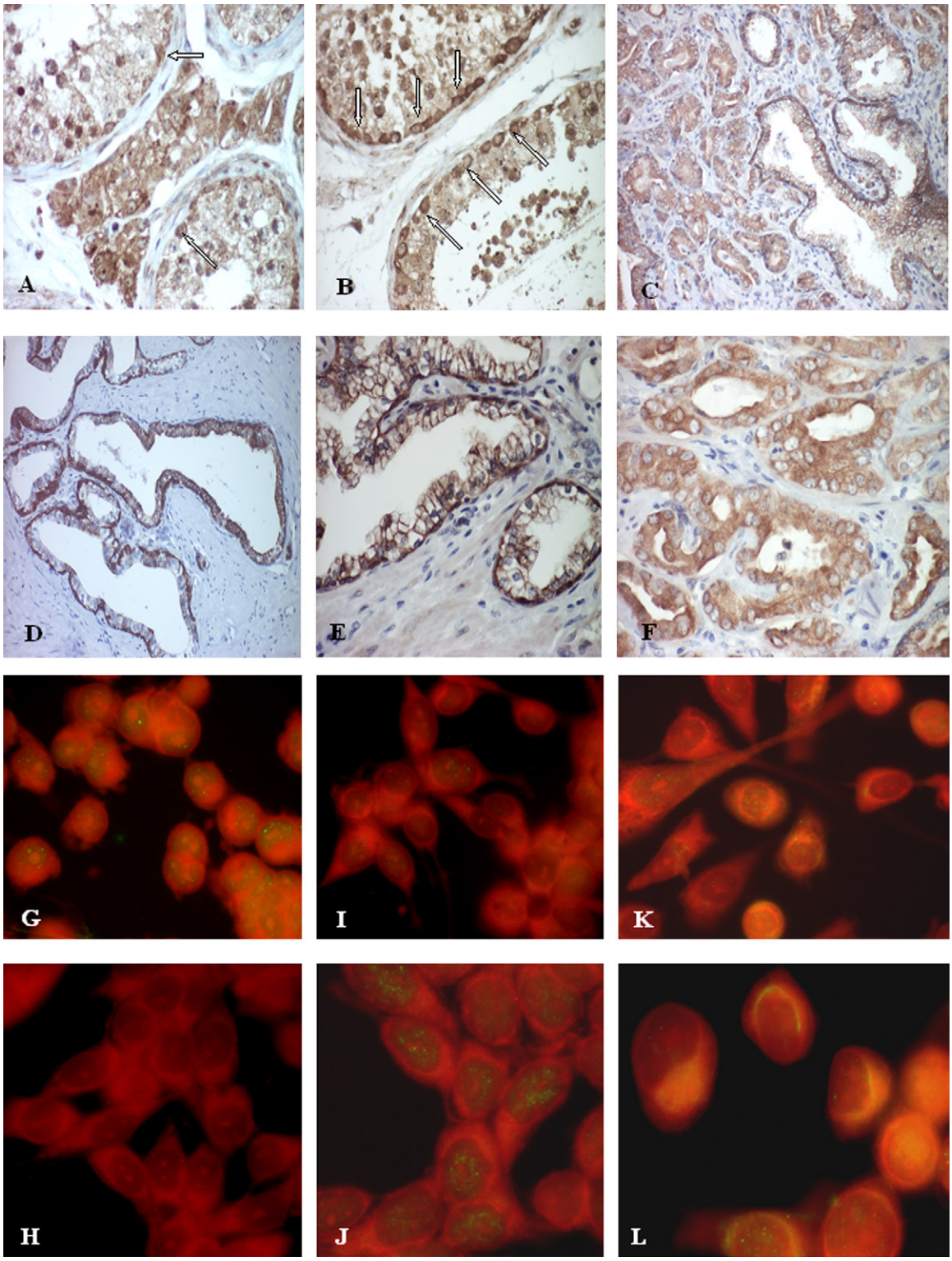

Fig. 5 – CTCFL protein expression analysis. (A) Heterogeneous expression of CTCFL in human testis, showing strong nuclear localization of CTCFL in spermatocytes (left upper part) and cytoplasmatic localization in spermatogonia (arrows) and Leydig cells (center). (B) Strong cytoplasmatic CTCFL expression in spermatogonia (arrows). (C) Representative human prostate tissues showing CTCFL expression in both prostate cancer (left part) and in adjacent normal prostate glands (right part). (D) CTCFL expression in normal prostate glands, showing cytoplasmatic expression in basal cells and heterogeneous cytoplasmatic and membrane-associated expression in normal secretory epithelial cells. (E) Higher magnification showing cytoplasmatic localization of CTCFL in basal cells and a mainly membrane-associated CTCFL expression in normal secretory epithelial cells. (F) CTCFL expression in prostate cancer of the same patient in cytoplasm. Original magnifications: A, B, E, F, 400×; C, D, 200×; G–L, fluorescence immunocytochemistry of CTCFL (green fluorescence) in cell lines. Red

may point to differences in specificities between SAHA and FK228 or between cell types. Indications for such differences have indeed emerged from clinical trials of these histone deacetylase inhibitors [32,33].

If CTCFL functions as a chromatin regulator [8,16], or transcription factor [30,31] the protein should be localized in the nucleus. However, we observed a nuclear localization only at specific stages of spermatogenesis, in teratocarcinoma cells and in some, but not all prostate cancer cell lines treated with 5-aza-dC. Protein expression was widespread in prostate epithelia and other testis cells, but localization was distinctly cytoplasmatic. Cytoplasmatic staining was also seen in one prostate cancer cell line. Indeed, the initial report on CTCFL likewise described nuclear as well as cytoplasmatic localization in mouse testes [8]. These observations suggest that CTCFL function might also be regulated through its intracellular localization, in addition to transcriptional control mediated by DNA methylation. This would not come as a surprise, since differential nuclear transport is frequently employed as a mechanism of regulation during spermatogenesis [34]. In particular, OCT4 is most strongly expressed in spermatogonia and localized in the nucleus, but is transported into the cytoplasm at later stages of spermatogenesis [35].

OCT4 expression was reported in several adult tissues and cancer cells [9,11,36], although another study suggested expression to be restricted to testicular tissue and cancers [20]. By a sensitive and quantitative RT-PCR method, we could detect *OCT4* mRNA in normal prostate tissues, but at low levels compared to testicular tissues. This observation likely accounts for the discrepant reports. However, while we confirmed expression in testicular cancer tissue and cell lines, we found no indication of increased *OCT4* expression in prostate and bladder cancer lines or prostate carcinoma tissue. The function of OCT4 in embryonic cells is crucially dependent on its expression level [37] suggesting that the overall levels in somatic tissues are unlikely to be significant. Obviously, we cannot fully exclude increased expression in a more stem-cell like fraction of normal or cancerous prostate tissues or cell lines, as a consequence of reactivation or persistent expression in rare tissue stem-cells [11]. Clearly, however, our data do not support a generalized reactivation of *OCT4* in human cancers. With the same caveats, our results neither yielded evidence for a major reactivation of *CTCFL* expression in prostate or bladder carcinoma.

In summary, thus, our findings suggest that *CTCFL/BORIS* and *OCT4* share properties with cancer-testis antigens [7,14–16] in being strongly methylated in somatic tissue and hypomethylated in testis, with according changes in expression. However, we could not find evidence for any major reactivation and hypomethylation in prostate cancers and bladder cancer cell lines. Interestingly, many prostate and bladder cancers display pronounced hypomethylation of LINE-1 sequences and parallel hypomethylation of certain single-copy sequences such as the *XIST* gene promoter [38]. The observation that no according hypomethylation was found in the *CTCFL* promoter adds to the accumulating evidence [3] that 'genome-wide' hypomethylation in cancers is not a random process.

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fluorescence represents the Evans blue counterstain. (G) Nuclear expression of CTCFL in the teratocarcinoma cell line NCCIT. Note the predominant punctuate pattern and the lack of nucleolar staining. (H) Negative control: LNCaP cells after omitting the primary antibody. (I) Untreated LNCaP prostate carcinoma cells showing weak nuclear CTCFL expression. (J) Expression in LNCaP treated with 5-aza-dC. (K) Untreated PC-3 prostate carcinoma cells with very weak staining. (L) PC-3 cells treated with 5-aza-dC. CTCFL signals are located around the nucleus in some cells or underlie the plasma membrane in others (compare with D).

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