

# BORIS/CTCF-mediated transcriptional regulation of the *hTERT* telomerase gene in testicular and ovarian tumor cells

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## ABSTRACT

**Telomerase activity, not detectable in somatic cells but frequently activated during carcinogenesis, confers immortality to tumors. Mechanisms governing expression of the catalytic subunit *hTERT*, the limiting factor for telomerase activity, still remain unclear. We previously proposed a model in which the binding of the transcription factor CTCF to the two first exons of *hTERT* results in transcriptional inhibition in normal cells. This inhibition is abrogated, however, by methylation of CTCF binding sites in 85% of tumors. Here, we showed that *hTERT* was unmethylated in testicular and ovarian tumors and in derivative cell lines. We demonstrated that CTCF and its paralogue, BORIS/CTCF, were both present in the nucleus of the same cancer cells and bound to the first exon of *hTERT* *in vivo*. Moreover, exogenous BORIS expression in normal BORIS-negative cells was sufficient to activate *hTERT* transcription with an increasing number of cell passages. Thus, expression of BORIS was sufficient to allow *hTERT* transcription in normal cells and to counteract the inhibitory effect of CTCF in testicular and ovarian tumor cells. These results define an important contribution of BORIS to immortalization during tumorigenesis.**

## INTRODUCTION

Telomerase activity is one of the most important factors that has been linked to multiple developmental processes including cell proliferation, differentiation, aging and senescence (1,2). This complex enzyme stabilized telomeres

length by adding hexameric repeats (TTAGGG) to telomeric ends of linear chromosomes, thus compensating for the continued erosion of telomeres (3). Maintenance of telomeres is required for cells to escape from replicative senescence and proliferate indefinitely. In adult humans, telomerase activity is not detectable in most somatic cells (4). In contrast, highly proliferative cells, such as germ cells and stem cells, and 85–95% of cancers express telomerase (5). Among the various components of human telomerase, only the telomerase RNA component, *hTERC*, and the human telomerase reverse transcriptase, *hTERT*, are essential for the reconstitution of telomerase activity *in vitro* (6–8). Moreover, it has been shown that ectopic expression of *hTERT* is sufficient to restore telomerase activity in telomerase-negative cells (8–10). Therefore, *hTERT* expression is defined as the rate-limiting factor in regulating telomerase activity (11), and many studies suggest that the regulation of *hTERT* occurs primarily at the transcriptional level.

Following the characterization of the *hTERT* genomic sequence and gene organization, a minimal promoter encompassing a 283 bp region upstream of the initiation ATG codon and numerous binding sites for transcription factors have been described (12–15).

The fact that the *hTERT* promoter lies within a CpG island has led to studies of transcriptional regulation through DNA methylation. Contradictory results have been published (16–21), but apparently hypermethylation of *hTERT* is required for its expression in the majority of telomerase-positive cells (18,22). We previously showed that the proximal exonic region of *hTERT* is involved in transcriptional inhibition of the gene (23) caused by binding of CTCF to the first two exons (24). In fact, hypermethylation of the *hTERT* exon 1 region in cancer cell lines and tumors prevents the binding and the repressive effects of CTCF. In addition, a specific 110 bp region

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within the core promoter was found to be hypomethylated in *hTERT*-expressing cells thereby allowing the transcriptional effect of the minimal promoter (25). A recent study also demonstrated that this region of the *hTERT* promoter upstream of the transcriptional start site is unmethylated and linked with active chromatin in cancer cells, thus explaining *hTERT* activity in the face of hypermethylation of the 5' regulatory region (26). Moreover, Meeran *et al.* have reported that down-regulation of DNMTs in response to HDAC inhibition by treatment with potential cancer-prevention drug Sulforaphane (SFN) generates site-specific CpG demethylation primarily in the first exon of the *hTERT* gene, which, in turn, leads to the repressive recruitment of CTCF to the same sites we mapped earlier (23,24,27).

Although methylation of its promoter appears to be the most frequently observed mechanism of the *hTERT* gene regulation in tumor tissues and tumor cell lines (18,20,28), it is obvious that one or more other methylation-independent mechanisms exist for certain types of telomerase-positive cells (16,17,19,28–30) including testicular and ovarian cancers (19,28). Therefore, a mechanism that does not require *hTERT* methylation must be active in these telomerase-positive and *hTERT* unmethylated tumors to prevent the repressive effects of CTCF.

Previous studies shown that BORIS (Brother of the Regulator of Imprinted Sites), also termed CTCFL (CTCF-like), is the mammalian paralogue of a highly conserved (31,32), multi-functional chromatin factor encoded by the candidate tumor suppressor gene, *CTCF* (33–36). BORIS was found to be present mainly in testicular and has first been found to be expressed in a mutually exclusive manner with CTCF during male germ cell development (37). It has recently been shown to be critical to normal spermatogenesis in mice, by regulation of the *CTS* (Cerebroside Sulfotransferase) gene (38). In addition to its normal expression in testis, studies revealed that various tumors and cancer cell lines, also expressed BORIS (39–43). *BORIS* is also involved in epigenetic reprogramming, both in normal development and in tumorigenesis (34,37,44). In addition, it has been shown that conditional expression of BORIS induced expression of a series of Cancer Testis Antigens (CTA) genes, as *MAGE-A1*, *NY-ESO-1* (40,42) and *SPANX* (45). Although BORIS and CTCF were shown to bind to the same recognition sequences, data provided clear demonstration that BORIS is functionally different from CTCF (38). Therefore, BORIS could also bind the CTCF target site within *hTERT* exon1.

In this study, we examined the potential contribution of BORIS to *hTERT* transcriptional regulation in testicular and ovarian cancer cells. We first analyzed the methylation pattern of *hTERT* in primary tumors of these types, in the NCCIT and OVCAR-3 cell lines, and investigated the role of BORIS in the *hTERT* gene regulation. Our data indicate that expression of BORIS in tumor cells is permissive for the transcription of *hTERT* in spite of the presence of the CTCF repressor. We concluded that BORIS and CTCF have opposite effects on *hTERT* transcription. Moreover, we showed that

expression of BORIS in normal cells is sufficient to allow *hTERT* transcription and to extend their lifespan *in vitro*, revealing an important role for BORIS in immortalization.

## MATERIALS AND METHODS

### Tissue samples and cell lines

Ten testicular and five ovarian tumors were analyzed. The use of human tissue samples for this study was according to the guidelines of the ethical committee of the Medical Faculty of Lausanne. The human tumor cell lines (HeLa, cervical adenocarcinoma; NCCIT, testis teratocarcinoma, OVCAR-3, ovary carcinoma) and normal BJ fibroblast cells were obtained from the ATCC and were grown in the medium recommended by the ATCC. HeLa, NCCIT and OVCAR-3 cell lines are telomerase-positive, whereas BJ cells are telomerase-negative. The Normal Human Bronchial Epithelial cell line (NHBE) was obtained from Lonza (Basel, Switzerland) and maintained in medium without Retinoic Acid as recommended by Lonza. The mouse normal fibroblast NIH3T3 cell line was used in transient transfection experiments and was maintained in DMEM supplemented with 10% FBS.

### DNA methylation analysis

DNA was extracted from cultured cells using the DNeasy tissue kit (Qiagen, Hilden, Germany). After bisulfite modification (EpiTect Bisulfite, Qiagen, Hilden, Germany), methylation analysis of the *hTERT* promoter was done by amplification of a 224 bp fragment (–443 to –274 from the ATG translational start site) and analyzed by a methylation-sensitive single strand conformation assay (MS-SSCA) as previously described (46). For cell lines, methylation analysis of the promoter and first exon of *hTERT* (from –200 to +100) was also performed after amplification of bisulfite modified DNA with the primers 5'-CTACCCCTTCACCTTCCAA-3' and 5'-GTTAGTTT TGGGGTTTTAGG-3'. Triplicate PCR products were cloned into the TOPO TA cloning kit (Invitrogen). Plasmid DNAs were extracted from bacterial clones with the QIAprep Spin Miniprep Kit (Qiagen). Each clone was analyzed by sequencing, with the M13 forward primer (5'-GTAAAACGACGGCCAG-3') by the NIAID sequencing facility.

### Plasmid construction and siRNA sequences

*hTERT reporters.* pTERT-297 contains the *hTERT* minimal promoter (23). The pTERT-297/ex1 vector contains the *hTERT* minimal promoter and 80 bp of the first exon. To generate this vector, an *hTERT* fragment was generated by PCR and cloned into the pGL3 basic vector (Promega). The pTERT-297/ex1mut contains a mutated version of the CTCF binding site located in exon1 (24). All constructs were used in transient transfection experiments.

*BORIS and CTCF expression vectors.* In the pCMV-BORIS and pCMV-CTCF vectors, BORIS cDNA and CTCF cDNA were cloned in pCMV6-XL4 by

ORIGENE Technologies (Rockville, MD, USA). A pBIG-BORIS vector was created on a template containing the tetracycline-responsive, autoregulated, bidirectional expression vector pBIG2i (42). The original plasmid was used as a control. A pBORIS-puro vector contained the cDNA of BORIS under control of the CMV promoter and contains the puromycin gene as a selective marker. The empty vector was used as a negative control in transfection experiments.

*siRNA BORIS and CTCF sequences.* siRNAs were ordered from Sigma Life Science. An siRNA sequence targeting BORIS was: 5'-CGAGUUGAUGCCGGAAA AA[dT]-3' and a siRNA sequence targeting CTCF was: 5'-UUGGUUCGGCAUCGUCGUU[dT]-3'.

### Transient transfection and luciferase assays

Cells were seeded at a concentration of  $2 \times 10^5/3.8 \text{ cm}^2$  for NIH3T3, HeLa, NCCIT and OVCAR-3, and  $5 \times 10^4$  cells/ $3.8 \text{ cm}^2$  for BJ and cultured overnight. Transient transfections of reporter plasmids (0.75  $\mu\text{g}/\text{well}$ ) and expression vector (1  $\mu\text{g}/\text{well}$ ) were carried out in triplicate using JetPEI Cationic Polymer Transfection reagent (8  $\mu\text{l}/\text{well}$ ) (Polyplus-transfection, Illkirch, France). All experiments were performed at least twice. The pRL-tk vector (0.25  $\mu\text{g}/\text{well}$ ) (Promega, Madison, WI) was co-transfected as an internal control for transfection efficiency in transient transfection experiments. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

NHBE cells were seeded at  $3.5 \times 10^2$  cells/ $\text{cm}^2$  in 60 mm dishes. The day after, cells were transfected using FUGENE6<sup>®</sup> 3  $\mu\text{l}$  for 2  $\mu\text{g}$  of DNA (Roche, Rotkreuz, Switzerland) In co-transfection experiments, a reporter: expression vector ratio of 1:1 was used. Expression of pBIG-BORIS was induced by two different concentrations of doxycycline: a low concentration of 0.0625  $\mu\text{g}/\text{ml}$  and a high concentration of 1  $\mu\text{g}/\text{ml}$ .

Co-transfection of expression plasmids and siRNA was performed in triplicate in six-well-plates for OVCAR-3 and NCCIT cell lines using the jetPRIME<sup>™</sup> transfection reagent (Polyplus-transfection, Illkirch, France) following the manufacturer's instructions.

### Immunofluorescence

HeLa, NCCIT and OVCAR-3 cells were grown for one day on glass slides in a four-well tissue culture chamber. Cells were washed in 1 $\times$  PBS and fixed 10 min in 4% paraformaldehyde/1 $\times$  PBS solution, freshly prepared. After two washes with 1 $\times$  PBS, cells were permeabilized for 15 min in freshly prepared 1 $\times$  PBS/0.2% Triton solution. Slides were washed in 1 $\times$  PBS and then cells were blocked in a 1 $\times$  PBS/10% goat serum for 90 min. Rabbit anti-BORIS and mouse anti-CTCF antibodies (produced by D.L. *et al.*) were diluted at 1/200 and 1/400, respectively, in antibody diluent solution (DakoCytomation, Carpinteria, CA) with 0.5 M NaCl. Slides were incubated at 4°C overnight. The next day, cells were washed three times in 1 $\times$  PBS/1% milk/0.5% TritonX-100. Secondary antibodies including

FITC-anti-rabbit and Cy3-anti-mouse (Sigma-Aldrich, St. Louis, MO) were added at a 1/100 dilution in 1 $\times$  PBS and incubated for 1 h at RT in the dark. Cells were then washed three times in 1 $\times$  PBS/1% milk/0.5% TritonX-100 followed by three washes in 1 $\times$  PBS. Slides were mounted in 1 $\times$  PBS/20% glycerol. Images were analyzed on a confocal microscope (Nikon, Chiyoda-ku, Tokyo).

### RNA extraction and RT-PCR

RNA was extracted from cells using the TRIzol Reagent (Invitrogen, Basel, Switzerland). Platinum quantitative RT-PCR Thermoscript one-step system (Invitrogen) was used to amplify *hTERT* mRNA as previously described (24). RT-PCR products were analyzed on 2% agarose gels.

BORIS expression was screened with the primers RT-BORIS-FW 5'-AAGCCGCGAACGGAGACG AAG-3' and RT-BORIS-REV 5'-ACGCCTTCATCCAC TTCCTCTTT-3'. CTCF expression was screened with the primers RT-CTCF-FW 5'-GTGGCGCGGAGAATGAT TAC-3' and RT-CTCF-REV 5'-TCCACAATGGCTTCG ACTGC-3'. RT-PCR products were analyzed on 2% agarose gels.

For quantitative RT-PCR, RNA was converted to cDNA using random primers and superscript III reverse transcriptase (Invitrogen). Quantitative real-time RT-PCR (qPCR) analysis was performed using the TaqMan Universal PCR master Mix (Applied Biosystems, Foster city, CA) and using the Applied Biosystem 7900HT Real-Time PCR system. CTCF expression was determined using the following primers and probe: 5'-TGACACAGT CATAGCCCGAAAA-3' (FW), 5'-TGCCTTGCTCAAT ATAGGAATGC-3' (REV) and 6FAM-TGATTTGGGT GTCCACTTGCGAAAGC-MGB (probe). Human Glyceraldehyde-3-phosphate dehydrogenase (hGAPDH), human BORIS (hBORIS) and human Telomerase Reverse transcriptase (hTERT) primers/probe mixtures were purchased as pre-developed assays (Applied Biosystems, Foster city, CA).

### Chromatin immunoprecipitation assay

NCCIT, OVCAR-3 and HeLa cells were used for chromatin immunoprecipitation (ChIP) assays to show the *in vivo* binding of CTCF and BORIS on *hTERT* exon1. We used a ChIP Assay kit (Upstate, Charlottesville, VA, USA) and followed the manufacturers' recommendations. One ChIP reaction used 10  $\mu\text{g}$  anti-CTCF monoclonal antibodies previously described (32) or 10  $\mu\text{g}$  of anti-BORIS polyclonal antibody (Abcam, Cambridge, UK). Immunopurified DNAs were used in qPCR using SYBR green mix and the following specific primers for *hTERT* exon1: FW 5'-GCGGCGCGAGTTTCAG-3' and REV 5'-GCAGCACCTCGCGGTAGT-3'. The human Nmyc CTCF binding site was used as positive control for CTCF binding using the following primers: FW 5'-GGC TCTGTGAGGAGGCAAGGTG-3' REV and 5'-GCTCT CTATTTGGAGTGGCGGG-3'. Primers used to co-amplified immunoprecipitated DNA of *hTERT* exon1 and H19 after ChIP were previously described (25).



## RESULTS

### Testicular and ovarian tumor cells exhibited the same methylation profile as normal cells

Using the MS-SSCA approach, we analyzed methylation of 20 CpGs within the *hTERT* 5' regulatory region (between -443 and -274) in primary tumor samples obtained from five testis and five ovaries (Figure 1A). Analyses of the *hTERT* promoter showed an unmethylated pattern except for one ovarian tumor that had a hypermethylated *hTERT* gene (Figure 1A). Methylation analysis of the testicular (NCCIT) and the ovarian (OVCAR-3) tumor cell lines identified an unmethylated *hTERT* 5' regulatory region in contrast with the hypermethylation observed in the telomerase-positive HeLa cervical tumor cell line (Figure 1A). RT-PCR analyses showed that *hTERT* was expressed in all of the primary tumors and cell lines regardless of the methylation profile (Figure 1B). The methylation status of the *hTERT* CpG island in NCCIT and OVCAR-3 cell lines was examined in greater detail around the ATG within sequences previously shown to be involved in *hTERT* regulation (25). Figure 1C presents data from representative clones of the HeLa and BJ cell lines as reference methylation profiles from a cancer cell line and a normal cell line, respectively (24,46). Sequencing of bisulfite-modified DNA from NCCIT and OVCAR-3 revealed hypomethylation of these regions at levels close to that observed in the normal cell line BJ, with, respectively, 17% and 0% of methylation within the region B containing the CTCF binding site. This result contrasted with data we reported previously for the HeLa cell line. In NCCIT and OVCAR-3, the mechanism preventing CTCF binding to the first exon of *hTERT* is thus independent of DNA methylation and had to involve another mechanism.

### The repressor effect of the proximal exonic region of the *hTERT* gene is less efficient in testicular and ovarian tumor cell lines than in HeLa cells

The inhibitory effect of the first exon of *hTERT* on its minimal promoter was tested in NCCIT and OVCAR-3 and compared to HeLa and BJ fibroblasts. These four cell lines were transiently transfected with the pTERT-297 and the pTERT-297/ex1 luciferase reporter vectors containing, respectively, the *hTERT* minimal promoter alone or with *hTERT* exon 1 (Figure 2A). The transcriptional activity of the *hTERT* minimal promoter was different in these four cell lines, ranging from 16.5 to 88% of relative luciferase activity in BJ and HeLa cells respectively. Transfection of the pTERT-297/ex1 construct resulted in a 6-fold diminution of luciferase activity in HeLa cells. Interestingly, this reduction was only 3-fold and 2.3-fold in NCCIT and OVCAR-3, respectively (Figure 2B). It is interesting to note that in BJ, the low activity of the reporter vector containing the *hTERT* minimal promoter was completely inhibited by the addition of the first exon containing the CTCF binding site. Therefore, in NCCIT and OVCAR-3 cell lines, the inhibitory effect of CTCF on *hTERT* minimal promoter transcription was less efficient than in HeLa cells, showing that cellular representation of

transcription factors were distinct and might differentially influence *hTERT* expression.

### CTCF and BORIS were co-expressed in the testicular and ovarian tumor cell lines

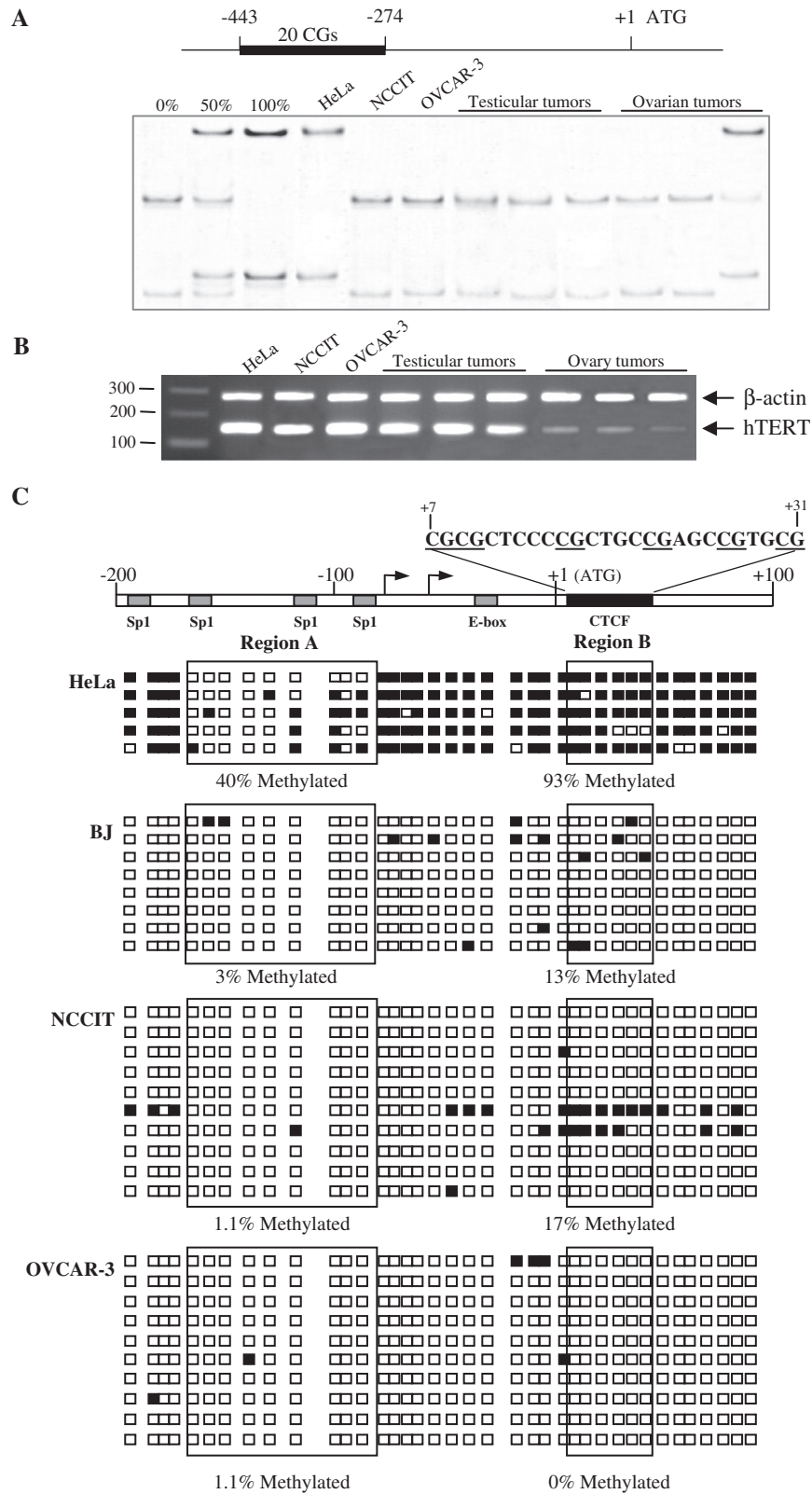
As BORIS, a paralogue of CTCF, is expressed in male germ cells, we investigated the expression of these two factors in NCCIT and OVCAR-3. RT-PCR analyses of CTCF and BORIS transcripts showed that these two factors are expressed in both lines (Figure 3A). In normal cells, BORIS and CTCF were found to be expressed in a mutually exclusive manner (37). This prompted us to investigate the expression of these two proteins by immunofluorescence in NCCIT, OVCAR-3 and HeLa. These studies showed that BORIS and CTCF were coexpressed in the nuclei of NCCIT and OVCAR-3 (Figure 3B, a-f). In HeLa cells, CTCF was localized in the nucleus, whereas BORIS could be detected only at background levels (Figure 3B, g-i). In NCCIT and OVCAR-3, the merger of CTCF and BORIS signals showed that BORIS is likely localized in the nucleus where it could compete with CTCF.

### BORIS and CTCF bound *in vivo* to the same region of the *hTERT* gene

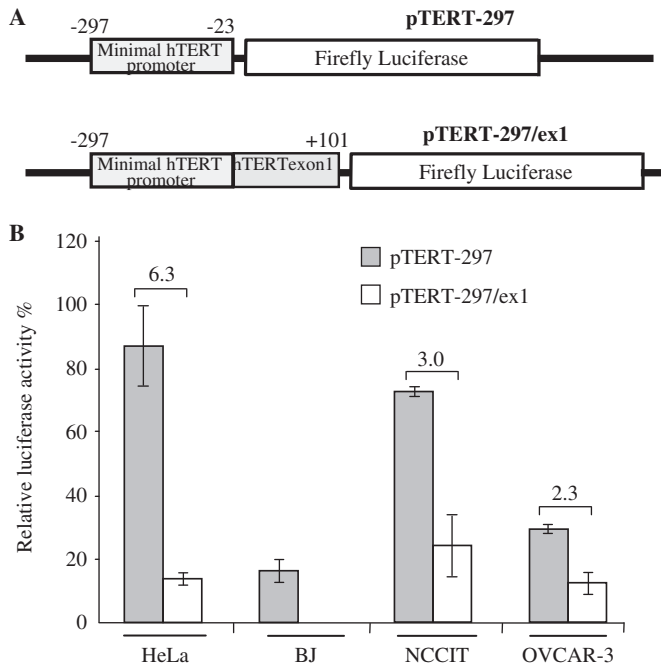
As the BORIS protein has the same 11 zinc finger domain as CTCF (34,37), we investigated the hypothesis that BORIS can replace CTCF on *hTERT* repressive site(s), thus allowing *hTERT* expression. Consequently, we studied *in vivo* binding of CTCF and BORIS to *hTERT* exon 1. Quantitative ChIP assays confirmed that CTCF did not bind the exon 1 of *hTERT* in HeLa cells *in vivo*, whereas it did bind exon 1 of *hTERT* in OVCAR-3 and NCCIT cells with a 3.6- and 2.2-fold change respectively (Figure 4A). Similarly, BORIS bound to the first exon of *hTERT* in OVCAR-3 and NCCIT cells but not in HeLa cells (Figure 4A). These results indicated that BORIS and CTCF can bind the same region of *hTERT* in the telomerase-positive cell lines OVCAR-3 and NCCIT. These results were confirmed by ChIP analyzed on agarose gels (Figure 4B and C) as described previously (24,25).

### BORIS and CTCF expression levels modulated *hTERT* transcription

The finding that CTCF and BORIS bound *hTERT* exon1 *in vivo*, prompted us to examine the effect of BORIS downregulation on *hTERT* transcription. First we co-transfected NCCIT and OVCAR3 cells with reporter plasmids containing the *hTERT* minimal promoter and the first exon with siRNA against BORIS. The data in Figure 5A showed that a decrease in BORIS expression induced a decrease of reporter activities in both cell lines. These results demonstrated that BORIS acted as an inhibitor of the *hTERT* minimal promoter in transient transfection experiments. Because the use of reporter vectors did not fully reflect *hTERT* transcription *in vivo*, we performed additional transfection experiments in OVCAR-3. Then we modulated the balance between BORIS and CTCF expression levels using specific



**Figure 1.** Expression and methylation patterns of *hTERT* in human tumors and cell lines. **(A)** Schematic representation of *hTERT* promoter sequence: +1 represents the translational start site, the black bar represents the region of promoter analysed by MS-SSCA. Controls obtained from plasmids containing *hTERT* sequences that are either unmethylated (0%), or 1:1 mixture of unmethylated and fully methylated (50%), or fully methylated (100%). The cell lines are: HeLa, cervical adenocarcinoma; NCCIT, testicular teratocarcinoma and OVCAR-3, ovarian carcinoma. **(B)** *hTERT* mRNA was detected by RT-PCR, with  $\beta$ -actin as internal control. **(C)** Genomic bisulfite sequencing of *hTERT* promoter and proximal exonic region (-200 to +100nt bases around the ATG transcriptional start site). After PCR amplification and cloning, 10 clones of NCCIT and OVCAR-3 were analyzed. As a comparison, five clones representing the methylation in HeLa cell line and 8 clones representing the methylation in BJ normal cell line are shown (25). Each square represents one CpG site. Methylated CpG sites are indicated in black and unmethylated in white. On top of the bar is displaying the entire sequence of the region B, in which CGs are underlined.



**Figure 2.** Transcriptional activity of the *hTERT* minimal promoter with or without the proximal exonic region. (A) Schematic representation of the luciferase reporter plasmids pTERT-297 and pTERT-297/ex1. (B) Luciferase reporter plasmids containing the *hTERT* minimal promoter, without (pTERT-297) or with (pTERT-297/ex1) the proximal exonic region, are transfected into HeLa, BJ, NCCIT and OVCAR-3 cell lines. For each cell line, the fold differences between the activity of pTERT-297 and pTERT-297/ex1 were calculated and added on top of the graphic bars.

siRNAs against BORIS and CTCF. The endogenous levels of *hTERT*, BORIS and CTCF were then quantified by qPCR. Transcript levels in siRNA-treated cells were normalized to the levels detected after transfection with a scrambled siRNA. A 2-fold decrease in *BORIS* transcripts was observed in cells transfected with siRNA against BORIS and levels of *CTCF* transcripts were decreased by 30%. In contrast, levels of *hTERT* transcripts were essentially unchanged. (Figure 5B). A 2.5-fold decrease in *CTCF* transcripts was observed in cells transfected with siRNA against CTCF while levels of *BORIS* and *hTERT* transcripts were unaffected. These results demonstrated that control of *BORIS* and *CTCF* transcription were closely linked such that modulation of one could affect the expression of the other.

In a second experiment, we overexpressed CTCF in OVCAR-3 cells and measured the transcript level of endogenous *hTERT* and *BORIS* using levels in unmanipulated cells referenced as 100%. Overexpression of CTCF resulted in increased *BORIS* transcription, but had no effect on the transcriptional levels of *hTERT* (Figure 5C). This suggests that the predicted repressive effect on *hTERT* of increased CTCF expression was countered by the increase in BORIS expression, thereby indicating that transcription of *hTERT* cannot be repressed by CTCF in cells that also express BORIS. Taken together, these results demonstrated that the equilibrium between the levels of CTCF and BORIS

expression have a great importance for regulating *hTERT* transcription.

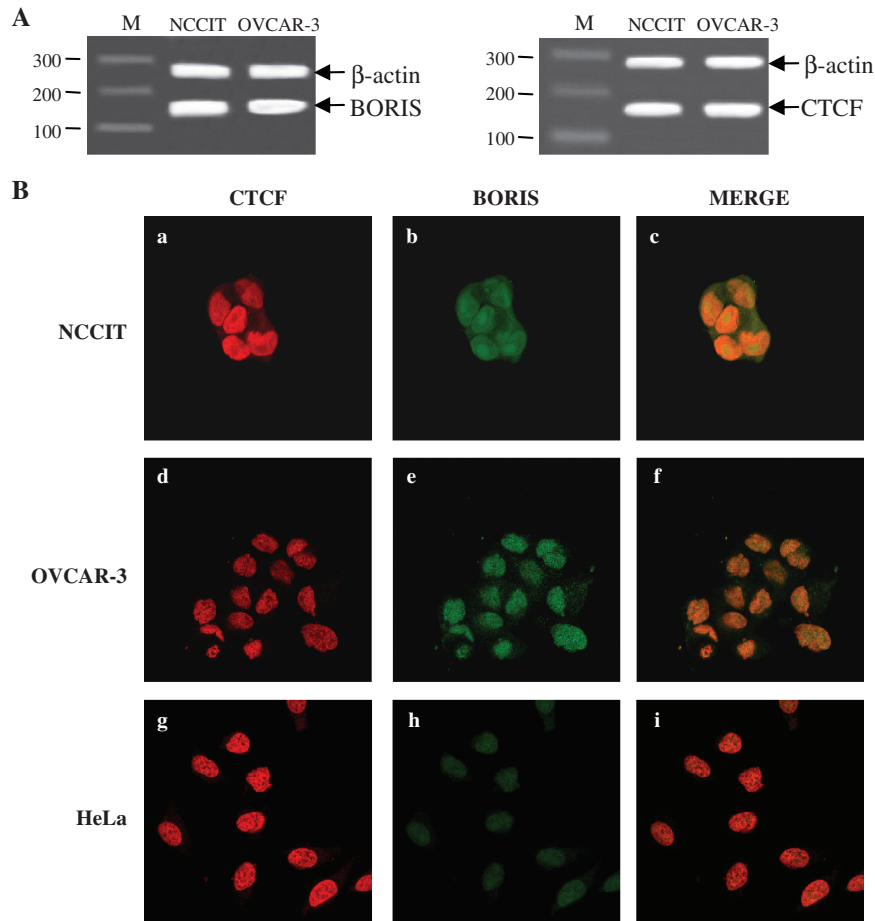
In studies of HeLa cells that do not express BORIS, a reporter vector with a mutated CTCF binding site in exon1 exhibited increased *hTERT* promoter activity. Parallel studies of BORIS-positive OVCAR-3 cells revealed that the mutation had no effect on *hTERT* promoter activity (Figure 5D). This experiment showed that in BORIS-negative cells, mutation of the CTCF binding site diminished the inhibitory effect of CTCF on *hTERT* promoter, and that in BORIS-positive cells, the presence of BORIS maintained *hTERT* promoter activity in the presence of CTCF.

#### Expression of BORIS induced *hTERT* transcription in telomerase-negative cells

To further investigate the contributions of BORIS to *hTERT* transcriptional activation, NIH3T3 cells were co-transfected with a BORIS expression vector (pBIG-BORIS) and *hTERT* reporter constructs. Expression of BORIS was induced with doxycycline and luciferase activities were measured 24 h after transfection. The level of reporter activity of pTERT-297, which contains only the *hTERT* minimal promoter, was set as the reference point of 100%. The activity of the pTERT-297/ex1 vector was 2.5-fold lower in NIH3T3 cells co-transfected with the empty vector. In the co-transfection experiments with pBIG-BORIS, the reporter activity of pTERT-297/ex1 reporter activity was only 1.5-fold lower (Figure 6A), a level similar to that observed in OVCAR-3 cells transfected with pTERT-297/ex1 (Figure 2B). To confirm the stimulating effect of BORIS on *hTERT* transcription, the pCMV-BORIS expression vector was transfected in the BJ normal human fibroblast cells that do not express either *BORIS* or *hTERT*. After transfection with the pCMV-BORIS vector, *BORIS* and *hTERT* transcripts were easily detected by RT-PCR (Figure 6B). We also performed RT-PCR assays for the full-length and the  $\alpha$ -spliced variants of *hTERT* (24). The amplified BORIS fragment obtained from BJ cells transfected with pCMV-BORIS was purified and directly sequenced, revealing the presence of only the full-length *hTERT* transcript (data not shown). In addition, qPCR analysis showed that high levels of BORIS transcripts were associated with substantial expression of *hTERT* while expression of CTCF was unchanged (Figure 6C).

#### Expression of BORIS in NHBE cells induced expression of *hTERT* and promoted cell viability

The lifespan of Normal Human Bronchial Epithelial cells (NHBE) in culture is very limited such that after 5 passages, virtually all cells detach and die. NHBE cells were transiently transfected with either a BORIS expression vector or an empty vector and BORIS expression was induced with doxycycline. After days 4, 7 and 10, the cultures were split. Half the cells were returned to culture and the other half was used for RNA extraction and qPCR to quantify *BORIS* and *hTERT* transcripts (Figure 7). Between Days 7 and 10, cells transfected with



**Figure 3.** Expression and localization of CTCF and BORIS in NCCIT, OVCAR-3 and HeLa cells. **(A)** Transcriptional expression of CTCF and BORIS were performed by RT-PCR from extracted RNA of NCCIT and OVCAR-3.  $\beta$ -actin was co-amplified as internal control **(B)** Immunofluorescence staining of CTCF and BORIS in NCCIT (a, b, c), OVCAR-3 (d, e, f) and HeLa (g, h, i) cells. Images a, d and g: CTCF immunostaining with a mouse monoclonal CTCF antibody, followed by incubation with an anti-mouse-Cy3 antibody. Images b, e and h: BORIS immunostaining with a rabbit polyclonal BORIS antibody, followed by incubation with an anti-rabbit-FITC antibody. Images c, f and i: merge of BORIS and CTCF staining revealed a coexpression within the nucleus.

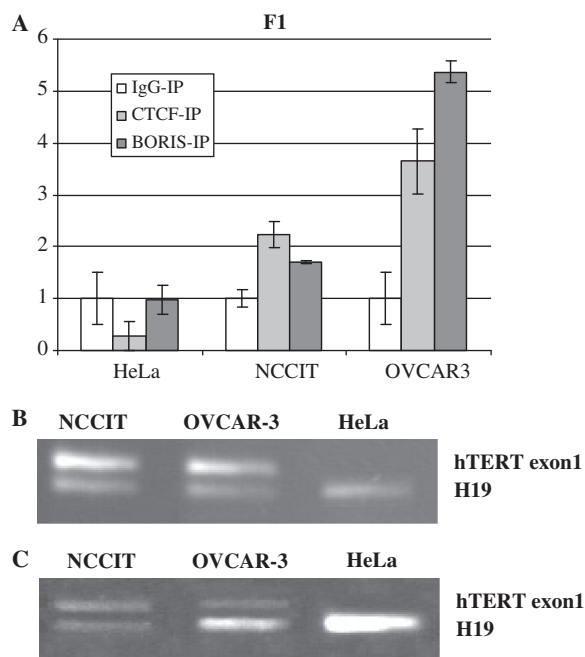
empty vector detached and died. At Day 10, cells transfected with the BORIS expression vector exhibited high levels of *BORIS* and *hTERT* transcripts. Lower levels of both *BORIS* and *hTERT* transcripts observed at Day 7 could be explained by plasmid dilution occurring with cell growth. The difference between the levels of *BORIS* transcripts at days 4 and 10 was  $\sim 6$ -fold with  $0.625 \mu\text{g/ml}$  of doxycycline and 55-fold with  $1 \mu\text{g/ml}$  doxycycline. It is interesting to note that a low or a high concentration of doxycycline did not significantly affect the levels of *BORIS* transcripts at Day 4 while the levels of *hTERT* transcripts were almost 2-fold lower with the higher concentration of the drug. These observations lead us to suspect that a high level of *BORIS* transcripts were not necessarily associated with high levels of active BORIS protein. At Day 10, as we considered that plasmids were integrated, the levels of *BORIS* transcripts were 10-fold higher with a high concentration of doxycycline than with a low concentration (Figure 7). The levels of *hTERT* transcripts detected at low concentration of doxycycline did not differ at Days 4 or 10. With a high concentration of doxycycline, however, *hTERT* transcripts were 28-fold

higher at Day 10 than at Day 4. Cells kept in continuous culture after day10 were viable for two more weeks, but then they stopped proliferating and finally detached. As we had found that low or high levels of *BORIS* transcripts affected the levels of *hTERT* transcripts after 10 days, the same experiments were performed using the non-inducible pBORIS-puro vector with similar results; the cells finally stopped proliferating and detached (data not shown).

## DISCUSSION

Our studies of *hTERT* regulation in testicular and ovarian tumors showed that the presence of unmethylated CTCF repressor sites in the gene were not inhibitory to transcription raising the question as to how these cancers, despite a normal methylation profile, can express *hTERT*. Here we describe a novel mechanism that disrupts CTCF repression of *hTERT*—occupancy of the CTCF binding sites by BORIS. Since BORIS has the same DNA binding domain as CTCF and is known to be expressed at a high level in testis (37,38), in oocytes (47), as well as in various tumors





**Figure 4.** *In vivo* binding of CTCF and BORIS on *hTERT* exon1. Chromatin Immunoprecipitation (ChIP) Assays were performed in HeLa, NCCIT and OVCAR-3 cell lines. After immunoprecipitation with serum or specific antibodies against CTCF or BORIS, DNAs were purified and amplified by PCR and quantitative PCR. (A) Quantitative PCR were performed to amplify the exon1 of *hTERT*. Bars represent the mean value of three independent experiments. (B) PCR coamplification of the *hTERT* exon1 and H19 from immunoprecipitated fractions bound by CTCF antibody in NCCIT, OVCAR-3 and HeLa cells. (C) PCR coamplification of the *hTERT* exon1 and H19 from immunoprecipitated fractions bound by BORIS antibody in NCCIT, OVCAR-3 and HeLa cells.

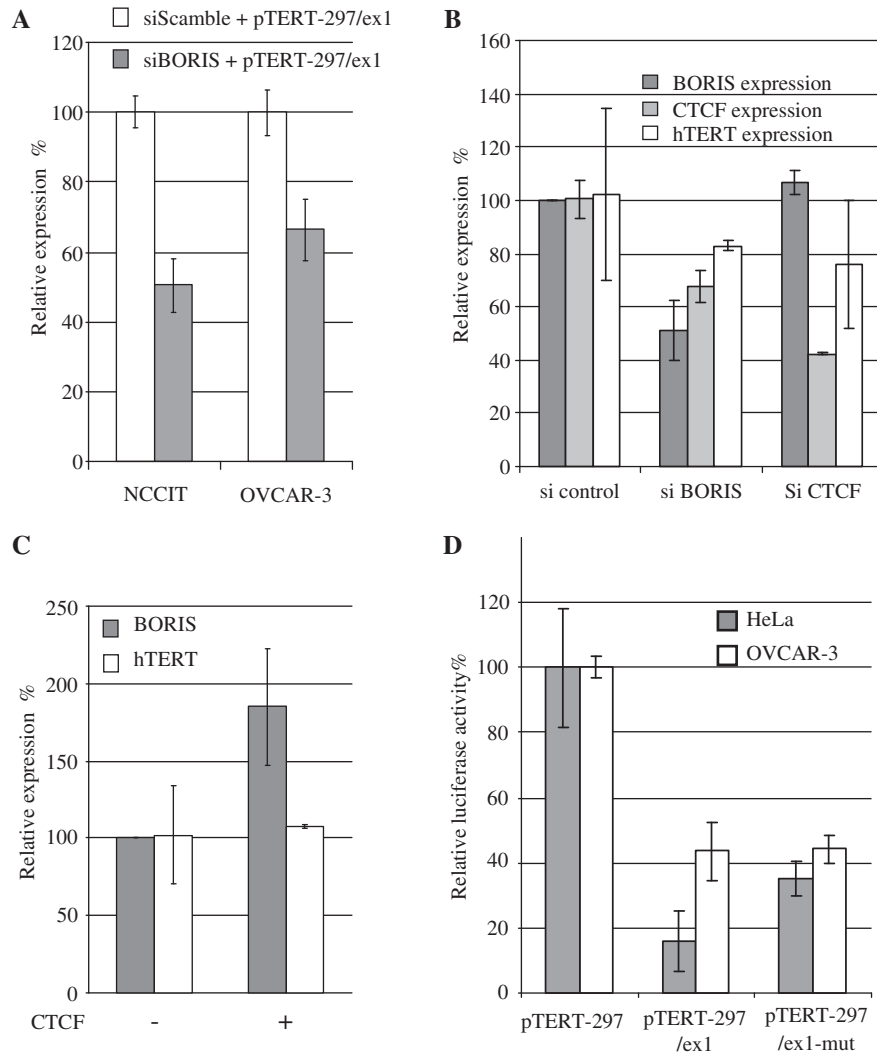
and cancer cell lines (39–43), we investigated its potential role in the regulation of *hTERT* in testicular and ovarian cancers. Analysis of testicular and ovarian cancer tissues showed, at the exception of one ovarian cancer, the *hTERT* promoter was hypomethylated, that is in correlation with what was observed in a previous study (28). Parallel studies of the same 5' regulatory region of *hTERT* in OVCAR-3 and NCCIT cell lines showed they were also hypomethylated. A more detailed bisulfite analysis of the promoter and exon 1 regions of *hTERT* in NCCIT and OVCAR-3 cell lines showed they were unmethylated as in normal cells, findings that contrast with the hypermethylated state of these sequences in the HeLa carcinoma cell line, that reflected the *hTERT* methylation profile in most cancer cells (16–18,20).

In transient transfection experiment using HeLa cells, it was shown that the activity of a reporter containing only the minimal *hTERT* promoter was 6-fold higher than the activity of a reporter containing the minimal promoter together with the first exon. In BJ cells, the reporter containing the minimal promoter and the exon 1 of *hTERT* did not exhibit any activity, most likely due to the absence of factor(s) essential for activation of the *hTERT* minimal promoter. In the same experiment, we showed that the activities of reporters containing the minimal promoter and the exon 1 were 2- to 3-fold higher in NCCIT and

OVCAR-3 than in HeLa cells. It is noteworthy that these two tumor telomerase-positive cell lines expressed relatively high level of BORIS transcripts. Therefore, the inhibitory effect of *hTERT* exon 1 in reporter assays was less pronounced in BORIS-positive cells than in HeLa or BJ cells. The levels of BORIS expression is correlated with reduced inhibitory effects of the first exon of the *hTERT* gene. Similarly, co-transfection of a BORIS expression and *hTERT* reporter vectors in NIH3T3 cells revealed a reduced inhibitory effect of the first exon on its promoter activity. Indeed, when BORIS was transiently expressed in NIH3T3 cells, the reduction in luciferase activity observed with the *hTERT* exon1 reporter was less pronounced compared to the activity resulting in co-transfection of *hTERT* exon1 reporter with the empty vector. Moreover, this activity was close to that observed in the both BORIS-positive cell lines NCCIT and OVCAR-3. We also showed that transfection of telomerase-negative normal fibroblast BJ cells with a BORIS expression vector was sufficient to induce transcription of the endogenous *hTERT* gene. These results demonstrated that BORIS has the opposite effect of CTCF on *hTERT* transcription. BORIS might counteract the repressive effect of CTCF by binding the target sequences in the *hTERT* gene thereby permitting transcription of the *hTERT* promoter. Moreover, NHBE cells transfected with BORIS exhibited active transcription of *hTERT* and the lifespan of transfected cells was prolonged.

BORIS and CTCF were found to be coexpressed in the nuclei of NCCIT and OVCAR-3 cells and both proteins bound to the first exon of *hTERT* *in vivo*. Thus in NCCIT and OVCAR-3 cells, CTCF and BORIS might compete for the same binding sites in the *hTERT* gene. We demonstrated *in vitro* that downregulation of BORIS in NCCIT or OVCAR-3 was associated with reduced *hTERT* minimal promoter activity. However, experiments measuring the endogenous levels of *hTERT* after downregulation of BORIS did not lead to the same conclusion. Indeed, the levels of *hTERT* transcripts were not significantly different. This could be explained by the facts that (i) BORIS expression was reduced by only 50% and (ii) downregulation of BORIS was also associated with downregulation of CTCF. Conversely, overexpression of CTCF was associated with increased levels of BORIS transcripts. Taken together, these results demonstrated that BORIS or CTCF expression could influence levels of each other in cancer cell lines. To further examine the effects of reduced BORIS expression on *hTERT* transcription, we performed serial siRNA transfections and observed massive cell death. Similar observations were made in recent studies showing that downregulation of BORIS by siRNA induced apoptosis in a breast cancer cell line (48). These results suggest that disturbances of the equilibrium between CTCF and BORIS in these cells might induce cell death. In order to avoid deregulation of the balance between CTCF and BORIS, we performed transient transfection of *hTERT* luciferase reporters containing or lacking exon1 of *hTERT* with or without mutation of the CTCF binding site. In HeLa cells, where BORIS levels are low, when we expressed *hTERT* with a mutant CTCF target site, we observed an increase





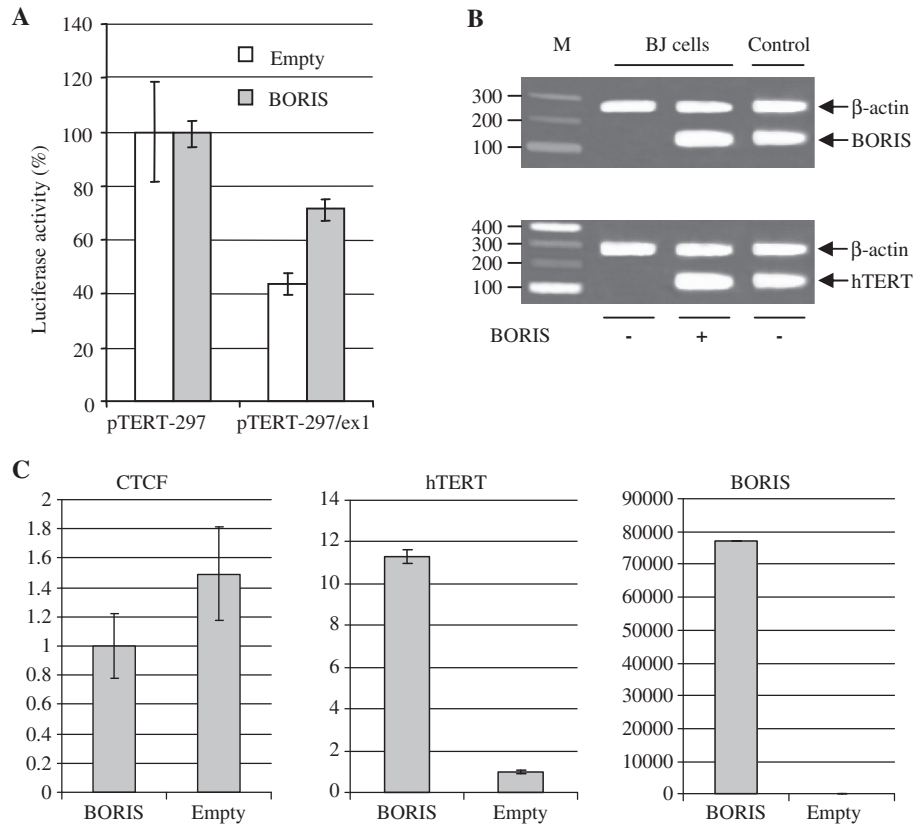
**Figure 5.** Modulation of BORIS and CTCF expression levels. (A) Co-transfection of siRNA scramble or against BORIS with luciferase reporters containing the *hTERT* minimal promoter or the *hTERT* minimal promoter + exon1, in NCCIT and OVCAR-3. Relative luciferase expression is expressed in percentage with the expression of the minimal *hTERT* promoter referred as 100%. (B) Transfection of siRNA scramble or against CTCF or BORIS in OVCAR-3. Endogenous transcriptional levels of *BORIS*, *CTCF* and *hTERT* are measured 24 h after transfection by quantitative PCR. Relative expressions were calculated with the expression of each gene after transfection with the scramble siRNA referred as 100% of transcription. (C) Transcriptional level of endogenous *BORIS* and *hTERT* in OVCAR-3 after transfection of CTCF expression vector. Relative levels were calculated taking the transcriptional levels of *BORIS* and *hTERT* in OVCAR-3 without over-expression of CTCF as 100%. (D) Transfection of *hTERT* reporters in BORIS-negative and BORIS-positive cell lines. Luciferase reporter constructs containing either the minimal promoter of *hTERT*, or the minimal promoter of *hTERT*+exon1, or the minimal promoter of *hTERT*+exon1 with CTCF mutated binding site, were transfected into HeLa and OVCAR-3 cell lines. Relative luciferase activities were calculated taking the level of the minimal promoter as 100%.

in luciferase activity, indicating that CTCF cannot bind this site, its inhibitory effect is diminished. In contrast, parallel studies of the BORIS-positive cell line OVCAR-3 revealed that luciferase activity was the same in cells bearing the normal or mutant CTCF binding site. There are multiple explanations to the observation but most feasible that BORIS isoforms that have different Zinc Finger composition can bind to minimal promoter of *hTERT* (E.Pugacheva *et al.*, submitted elsewhere). The mechanism by which BORIS counteracted the inhibitory effect of CTCF on the *hTERT* transcription resulting in activation of *hTERT* transcription in testicular and ovarian cancers has to be elucidated. However, such mechanism involving CTCF and other proteins

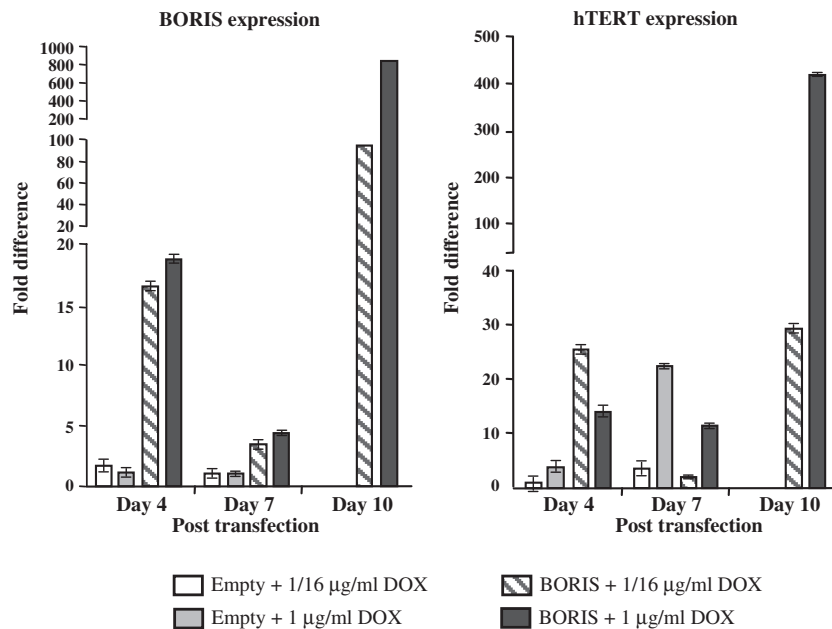
counteracting its effect on *hTERT* transcription has already been suggested recently. Indeed, in lymphoid cells, the PAX5 and CTCF factors bound simultaneously the *hTERT* exon 1. In this situation, PAX5 might antagonize the chromatin-mediated transcriptional repression by CTCF on *hTERT* promoter (30).

Since recent studies have shown that *hTERT* is an endogenous inhibitor of the mitochondrial pathway of apoptosis (49), our data might explain how BORIS acts as inhibitor of apoptosis in cancer cells by activating *hTERT* transcription.

Taken together, these findings show the importance of BORIS for transcriptional activation of *hTERT* and, possibly, to the process of immortalization during



**Figure 6.** Transient transfection of a BORIS expression vector in normal cells. (A) Firefly Luciferase activities of pTERT-297 or pTERT-297/ex1 co-transfected with pBIG-BORIS or pBIG-empty in NIH3T3 mouse normal fibroblasts. The Renilla Luciferase was used to normalize the firefly luciferase. In each experiment, the activity was calculated by setting the activity of *hTERT* minimal promoter as 100%. pTERT-297: minimal *hTERT* promoter; pTERT-297/ex1: minimal promoter and first exon of the *hTERT* gene. (B) RT-PCR of *BORIS* and *hTERT* from total RNA of BJ cells 24 h after transfection with the pCMV-BORIS expression vector. (C) Real-time RT-PCR of CTCF, *hTERT* and *BORIS* from RNA of BJ cells after transfection of pCMV-BORIS or pCMV-empty. All qRT-PCR were normalized on GAPDH levels.



**Figure 7.** pBIG-BORIS inducible expression vector or the pBIG-empty vector were transfected into NHBE cell line (Normal Human Bronchial Epithelial). BORIS expression was induced with 1/16  $\mu$ g/ml (0.0625  $\mu$ g/ml) or 1  $\mu$ g/ml of doxycycline and maintained in culture. RNAs from cells were extracted 4, 7 and 10 days after transfection. Parental cells and cells transfected with empty vector died after 7 days. The transcriptional expression of *BORIS* and *hTERT* were measured by quantitative RT-PCR and normalized on GAPDH levels.

carcinogenesis. As CTCF binds directly to SIN3A, which recruit histone deacetylase and thus prevents transcription (50), we hypothesize that BORIS might have the opposite effect and open chromatin around the transcriptional start site of *hTERT*. This opening would then allow other factors to activate the *hTERT* transcription. Moreover, recent studies showed that down-regulation of DNMT by anti-cancer drug or TSA, induced a demethylation of *hTERT* exon1 within the CTCF binding site, allowing binding of CTCF and inhibition of *hTERT* (27,51). In addition, downregulation of DNMT also induced an increase in CTCF binding on BAG-1 oncogene promoter, while an overexpression of DNMT led to increase in BORIS binding (52). This opposite behavior is further reflected by changes in H3K4Me2 and H3K9me2 ratio. Taking together these studies showed that promoters regulation by CTCF/BORIS involved epigenetic changes.

In tumors, co-expression of CTCF and BORIS could be directly responsible for epigenetic deregulation leading to cancer (34). The demonstrated utility of BORIS as a target for immunotherapy (53,54) is consistent with the importance of BORIS in carcinogenesis. The importance of BORIS for *hTERT* activation suggests that it might be an essential factor in the immortalization of testicular and ovarian cancer cells, possibly opening new ways to understanding of carcinogenesis and for potential anti-cancer therapies.

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