Specific association between the methyl-CpG-binding domain protein 2 and the hypermethylated region of the human telomerase reverse transcriptase promoter in cancer cells

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Human telomerase reverse transcriptase (hTERT) is expressed in most cancer cells. Paradoxically, its promoter is embedded in a hypermethylated CpG island. A short region escapes to this alteration, allowing a basal level of transcription. However, the methylation of adjacent regions may play a role in the maintenance of low hTERT expression. It is now well established that methyl-CpG binding domain proteins mediate the transcriptional silencing of hypermethylated genes. The potential involvement of these proteins in the control of hTERT expression was firstly investigated in HeLa cells. Chromatin immunoprecipitation assays showed that only methyl-CpG-binding domain protein 2 (MBD2) associated the hypermethylated hTERT promoter. In MBD2 knockdown HeLa cells, constitutively depleted in MBD2, neither methyl CpG binding protein 2 (MeCP2) nor MBD1 acted as substitutes for MBD2. MBD2 depletion by transient or constitutive RNA interference led to an upregulation of hTERT transcription that can be downregulated by expressing mouse Mbd2 protein. Our results indicate that MBD2 is specifically and directly involved in the transcriptional repression of hTERT in HeLa cells. This specific transcriptional repression was also observed in breast, liver and neuroblastoma cancer cell lines. Thus, MBD2 seems to be a general repressor of hTERT in hTERT-methylated telomerase-positive cells.

Introduction

An increasing body of evidences indicate that the alterations of DNA methylation patterns are a characteristic of cancer cells (1). Generally, global reduction of DNA methylation level is associated with localized hypermethylation (1). Specifically, an aberrant hypermethylation of CpG islands at the 5’ end of tumor suppressor genes, leading to transcriptional repression, has been described both in cancer cell lines and tumor tissues (2,3). In human epithelial and fibroblast cells, telomere shortening is a key event in replicative senescence. In >85% of cancer cells, telomere length is maintained through telomerase holoenzyme activity (4,5). Although germ cells and stem cells also exhibit high telomerase activity (4), in normal somatic cells, the catalytic subunit of the telomerase [human telomerase reverse transcriptase (hTERT)] is silenced, leading to a limited life span (6). The regulation of hTERT transcription has been extensively investigated and several inducers and repressors have been identified, including c-Myc, Sp1, hALP, Hif-1, Mbi-1, USF1/2, estrogen response element, p53, Mad1, myeloid-specific zinc finger protein 2, transforming growth factor-β, Wilms’ Tumor 1 and CTCF (7–9). In addition, the hTERT promoter region is embedded in a large CpG island spanning nucleotides (nt) –1100 to +1500 from the transcription start site, suggesting that transcription of the gene might be regulated by DNA methylation.

The first studies examining the methylation status of the hTERT CpG island had led to a paradox. In normal somatic cells, this CpG island was found unmethylated while the gene was transcriptionally silent. However, in most of cancer cells, this region was aberrantly methylated, whereas telomerase activities and hTERT messenger RNAs (mRNAs) were unambiguously detected (10–13). This paradox was recently solved. hTERT methylation prevents the binding of negatively acting transcription factors such as CTCF inhibitor (9), and a partial hypomethylation of the hTERT promoter region can result in some level of transcriptional activity (14). In several cancer cell lines and tumor tissues, careful analysis of hTERT methylation patterns has shown that a short region of the CpG island (positions nt –165 to nt –80) is unmethylated or slightly methylated despite highly methylated border regions (14). This unmethylated region is located in the hTERT core promoter (positions nt –279 to nt +5) (15), and chromatin immunoprecipitation (ChIP) assays have shown that active chromatin marks are associated with this unmethylated region (16). Moreover, studies using plasmid expression vectors and patch methylation techniques indicate that the hTERT core promoter does not show any activity when all CpG sites are methylated (17). In contrast, the selective demethylation of a small region upstream the transcription start site significantly activates the hTERT promoter in a reporter plasmid. Nevertheless, the activity of the promoter under these conditions is significantly lower than when using the unmethylated core promoter. In this condition, hTERT does not provide a real exception to the general model of gene silencing by promoter methylation and the hypermethylation around the unmethylated region seems to play a major role in the reduction of hTERT transcriptional activity. Indeed, in telomerase-positive cancer cells, only 0.2–6 mRNA molecules per cell can be detected (18,19), suggesting that the transcriptional activity of the hTERT promoter is limited by cellular factors since high transcription rates are induced when the unmethylated promoter is used in plasmid expression vectors (14).

Transcriptional repression mediated by CpG methylation often involves methyl-CpG-binding domain (MBD) proteins. The five MBD proteins identified to date, methyl CpG binding protein 2 (MeCP2), MBD1, MBD2, MBD3 and MBD4, share a highly conserved MBD.

with the exception of MBD4, which is primarily a thymine glycosylase involved in DNA repair (20), all MBD proteins are involved in the transcriptional repression mediated by DNA methylation. It has now been well established that MeCP2, MBD1 and MBD2 bind to methylated DNA and recruit different histone deacetylase complexes and histone methyl transferases, belonging to the chromatin remodeling complexes that control chromatin compaction and induce gene silencing (21–23). MBD3 lacks a functional MBD but is an integral subunit of the Mi2–NuRD complex that is repressed (24,25).

These data have prompted us to investigate whether MBD proteins are involved in the repression of hTERT expression when hypermethylated in telomerase-positive cells.

Materials and methods

Cell culture

Five human tumor cell lines, HeLa (cervical adenocarcinoma), MCF7 (breast adenocarcinoma), HepG2 (hepatocellular carcinoma), LAN-1 (neuroblastoma) and NCCIT (teratocarcinoma) and a normal human embryonic lung fibroblast...
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Results

hTERT hypermethylated CpG island is selectively associated with MBD2 in HeLa cells

To explore the potential involvement of MBD proteins in hTERT regulation, we have chosen cervical cancer cell line HeLa, as a first model. In these telomerase-positive tumor cells, as was shown in our earlier studies, the hTERT core promoter is also regionally hypermethylated (17) and might be a target of MBD proteins.

To address this issue, ChIP assays were performed using antibodies directed against MeCP2, MBD1 and MBD2. As a control, the fractions immunoprecipitated with a non-MBD protein-specific antibody (anti-mouse IgG) were also analyzed. The relative amounts of hTERT core promoter were determined by a dose-dependent PCR assay (supplementary Figure S1 available at Carcinogenesis Online) using a constant amount of DNA from each fractionation process: input, unbound and bound fractions. Representative data from at least three independent experiments are shown in Figure 1A. A strong enrichment in hTERT promoter was observed in the fraction immunoprecipitated by anti-MBD2 antibodies when compared with input or non-retained fractions (Figure 1A ‘input’, ‘unbound’ and ‘IgG’). Since dose-dependent PCR assays were performed with a constant amount of DNA, these data strongly suggest that MBD2 is associated with the methylated region of the hTERT promoter. In contrast, analysis of the fractions immunoprecipitated by anti-MeCP2 or anti-MBD1 antibodies showed that these fractions were depleted in hTERT DNA (Figure 1A), indicating that these two proteins are not bound to hTERT promoter in HeLa cells. Furthermore, the depletion in hTERT DNA observed in the fractions bound by anti-MeCP2 and anti-MBD1 antibodies suggests that these two proteins are probably linked to other chromatin domains in HeLa cells.

Taken together, these data strongly suggest that the methylated regions adjacent to the unmethylated region of the hTERT core promoter are selectively associated with MBD2.

Recently, we have developed high-throughput analyses of MBD2-binding pattern using a ChIP-on-chip approach (A. Chatagnon, L. Perriaud, L. Jachuer and R. Dante, in preparation). DNAs obtained from the chromatin immunoprecipitated by anti-MBD2 antibodies were hybridized to Affymetrix Human Promoter 1.0R Array. On this chip, 25 500 human promoter regions tiled at 35 bp resolution are representing. Each promoter region covers ∼7.5 kb upstream through 2.45 kb downstream of 5’ transcription start site and for 1300 cancer-associated genes, additional 2.45 kb are also represented. Data obtained from two independent experiments performed with two different anti-MBD2 antibodies indicated that MBD2 not only bound the
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ChIP assays performed in the hTERT promoter in HeLa cells. White dotted box, core promoter; black arrows, squares. The slightly methylated region (positions nt 165 to nt 80), corresponding to a part of the hTERT core promoter, was hypomethylated in the two cell lines analyzed (Figure 1B). Indeed, this region exhibits a low level of methylation (~30%) but no significant difference was observed between wild-type HeLa cells and HeLa cells depleted in MBD2. Thus, the methylation patterns of the hTERT promoter in MBD2 KD HeLa cells were not altered by the absence of MBD2.

As expected, in HeLa cells depleted in MBD2 proteins, ChIP assays indicated that MBD2 was no longer detected at the hypermethylated region of the hTERT core promoter (Figure 1C). Furthermore, this region seemed to remain free of MBD proteins since the immunoprecipitated fractions are depleted in the methylated hTERT promoter when anti-MeCP2 or anti-MBD1 antibodies were used in ChIP experiments (Figure 1C).

Thus, the hypermethylated region of the hTERT promoter is specifically targeted by MBD2 in HeLa cells and no redundancy between MBD2 and MeCP2 or MBD1 was observed at this hypermethylated region.

MBD2 depletion enhances hTERT gene transcription in HeLa cells

MBD2 depletion might alter DNA methylation patterns. Therefore, the DNA methylation patterns of the hTERT promoter were determined in MBD2 KD HeLa cells. DNA extracted from the cell lines was modified and amplified by PCR with primers specifically designed to amplify bisulfite-modified DNA sequence of the hTERT promoter and proximal exonic region. PCR fragments were cloned and sequenced. The analysis of 10 clones from wild-type HeLa cells and MBD2 KD HeLa cells indicated that over the hypermethylated hTERT promoter and proximal exonic region, a small sequence (positions nt 165 to nt 80), corresponding to a part of the hTERT core promoter, was hypomethylated in the two cell lines analyzed (Figure 1B). Indeed, this region exhibits a low level of methylation (~30%) but no significant difference was observed between wild-type HeLa cells and HeLa cells depleted in MBD2. Thus, the methylation patterns of the hTERT promoter in MBD2 KD HeLa cells were not altered by the absence of MBD2.

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First, we determined the consequence of a transient MBD2 depletion on the expression of hTERT in HeLa cells. At 24 h intervals after MBD2-specific siRNA transfection, RNA was extracted and the levels of hTERT and MBD2 transcripts determined by RT–PCR assays were compared with their levels in HeLa cells transfected with a non-specific siRNA. Quantitative competitive RT–PCR assays (27) indicated that a reduction of ~90% in MBD2 mRNA level was reached 24 h after transfection with MBD2 siRNA and maintained ~96 h after transfection (Figure 3A). Western blot analysis also showed a dramatic decrease in MBD2 proteins in these cells (data not shown). In HeLa cells, 48 h after the MBD2 siRNA transfection, an elevation of 1.2-fold of hTERT mRNA level was observed when compared with HeLa cells transfected with a non-specific siRNA. This stimulation increased to a maximum of 1.9 at 96 h after MBD2 siRNA treatment. The expression level of hTERT was determined using relative RT–PCR (Figure 3A) and identical results were obtained with real-time RT–PCR for critical points (Figure 3B). It should be noted that neither the level of MBD2 transcripts nor the level of hTERT transcripts were altered by non-specific siRNA transfection when compared with untreated wild-type HeLa cells (data not shown).

These data suggest that MBD2 actually represses hTERT expression in HeLa cells.

Stimulation of hTERT transcription in MBD2 KD HeLa cells is reversed by ectopic expression of Mbd2

The MBD2 KD HeLa cell line offers the opportunity to investigate the functional control of the specific repression of hTERT by MBD2. In this cell line, MBD2 expression can be rescued using pRev-Mbd2, a vector coding for a mouse Mbd2 RNA containing five silent point mutations and, therefore, refractory to siRNA-mediated decay (26). We also observed a 2-fold stimulation of hTERT expression in the MBD2 KD HeLa cells as compared with wild-type HeLa cells (Figure 4). In these almost undetectable in western blot experiments (26). In addition, neither the amounts of MeCP2 and MBD1 transcripts nor the amounts of the corresponding proteins were altered by MBD2 depletion (data not shown).

As MBD2 belongs to the DNA methylation machinery, a prolonged MBD2 depletion might alter DNA methylation patterns. Therefore, the DNA methylation patterns of the hTERT promoter were determined in MBD2 KD HeLa cells. DNA extracted from the cell lines was modified and amplified by PCR with primers specifically designed to amplify bisulfite-modified DNA sequence of the hTERT promoter and proximal exonic region. PCR fragments were cloned and sequenced. The analysis of 10 clones from wild-type HeLa cells and MBD2 KD HeLa cells indicated that over the hypermethylated hTERT promoter and proximal exonic region, a small sequence (positions nt 165 to nt 80), corresponding to a part of the hTERT core promoter, was hypomethylated in the two cell lines analyzed (Figure 1B). Indeed, this region exhibits a low level of methylation (~30%) but no significant difference was observed between wild-type HeLa cells and HeLa cells depleted in MBD2. Thus, the methylation patterns of the hTERT promoter in MBD2 KD HeLa cells were not altered by the absence of MBD2.

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MBD2 depletion enhances hTERT gene transcription in HeLa cells

MBD2 is a member of the MBD protein family and their methylation-dependent repressive activities are now well established (29). Therefore, we investigated the potential involvement of MBD2 in the repression of the endogenous hTERT promoter in HeLa cells.

First, we determined the consequence of a transient MBD2 depletion on the expression of hTERT in HeLa cells. At 24 h intervals after MBD2-specific siRNA transfection, RNA was extracted and the levels of hTERT and MBD2 transcripts determined by RT–PCR assays were compared with their levels in HeLa cells transfected with a non-specific siRNA. Quantitative competitive RT–PCR assays (27) indicated that a reduction of ~90% in MBD2 mRNA level was reached 24 h after transfection with MBD2 siRNA and maintained ~96 h after transfection (Figure 3A). Western blot analysis also showed a dramatic decrease in MBD2 proteins in these cells (data not shown). In HeLa cells, 48 h after the MBD2 siRNA transfection, an elevation of 1.2-fold of hTERT mRNA level was observed when compared with HeLa cells transfected with a non-specific siRNA. This stimulation increased to a maximum of 1.9 at 96 h after MBD2 siRNA treatment. The expression level of hTERT was determined using relative RT–PCR (Figure 3A) and identical results were obtained with real-time RT–PCR for critical points (Figure 3B). It should be noted that neither the level of MBD2 transcripts nor the level of hTERT transcripts were altered by non-specific siRNA transfection when compared with untreated wild-type HeLa cells (data not shown).

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cells, the ectopic expression of Mbd2 reduced hTERT transcription to a level similar to the level observed in wild-type HeLa cells (Figure 4), suggesting that MBD2 directly represses hTERT expression in HeLa cells. In wild-type HeLa cells, the abundance of MBD2 does not seem to be a limiting factor since the overexpression of MBD2 mediated by pRev-Mbd2 transfection did not modify hTERT expression level in cells containing normal amounts of MBD2 (Figure 4).

Our results demonstrate that MBD2 specifically binds to hTERT and represses its expression in HeLa cells.

MBD2 hTERT repression is specifically observed in hTERT-methylated telomerase-positive cancer cell lines and independent of the cellular types

Data obtained suggest that MBD2 might be a general repressor of hTERT transcription in hTERT-methylated telomerase-positive cells. In order to address this point, we tested whether the above-mentioned MBD2 KD-mediated hTERT stimulation in HeLa cells could be recapitulated in other hTERT-methylated cell lines. A functional study was undertaken in different cell lines exhibiting characteristic hTERT DNA methylation patterns: (i) MCF-7, LAN-1 and HepG2 cells, three telomerase-positive cancer cell lines showing an hTERT core promoter unmethylated or slightly methylated despite an hypermethylated CpG island (ii) NCCIT cells, an hTERT-unmethylated telomerase-positive teratocarcinoma cell line and (iii) MRC5 cells, an hTERT-unmethylated telomerase-negative normal embryonic cell line. The hTERT core promoter methylation status of these cell lines and the corresponding transcript levels are shown in Figure 5. Control experiments indicated that these cell lines exhibited approximately the same level of MBD2 transcripts (4.8 ± 2.7 × 10⁶ mRNA molecules per microgram of total RNA), with the exception of NCCIT (7 × 10⁴ mRNA molecules per microgram of total RNA).

As observed in HeLa cells, a significant decrease of MBD2 transcripts (60–79%) was observed in the different cell lines 96 h after MBD2 siRNA transfection. Relative to the transfection with a non-specific siRNA, a ~2-fold increase in hTERT mRNA was observed in all hTERT-methylated cell lines, LAN-1, MCF7 and HepG2 cells. In contrast, MBD2 depletion did not affect hTERT expression in hTERT-unmethylated cell lines, NCCIT and MRC5 cells (Figure 6). Since MBD2 does no bind to unmethylated DNA (24,25), these data indicate that hTERT induction due to MBD2 depletion is not mediated by an indirect effect.

Taken together, the specific transcriptional repression of hTERT by MBD2 does not seem to be restricted to a particular cancer cell line since this effect was observed in cervix, breast, liver and neuroblastoma cancer cell lines. Thus, MBD2 seems to be a general repressor of hTERT in hTERT-methylated telomerase-positive cells.

Discussion

Most (~96%) of the CpG islands are unmethylated in normal cells (30), whereas hypermethylation of these sequences is a characteristic
of cancer cells (1). These aberrant DNA methylation patterns have been correlated with the transcriptional silencing of genes undergoing such alterations at their 5' end CpG islands. The \textit{hTERT} gene has provided an interesting exception since a bimodal alteration of the DNA methylation status of its 5' end CpG island is associated with its expression in 75% of cancer cells and tumors tissues (4,5). The large CpG island, 2.6 kb in length, lying from nt 1102 to nt +1519 from the \textit{hTERT} transcription, is hypermethylated at the exception of a short region (positions nt 165 to nt +80) that is unmethylated or slightly methylated despite highly methylated border regions (16,17). This particular pattern of methylation seems crucial for establishing a specific transcriptional activity of the \textit{hTERT} gene.

Fig. 3. Transient depletion of MBD2 proteins by MBD2-specific siRNA induces time-dependent stimulation of \textit{hTERT} expression in HeLa cells. (A) HeLa cells were transiently transfected, with either MBD2 siRNA or a negative control scrambled siRNA. After 24, 48, 72 or 96 h following the transfection, RNA was extracted and the efficiency of the MBD2 siRNA treatment was determined by quantification of MBD2 mRNA as described previously (27). \textit{hTERT} mRNA levels were monitored by relative RT–PCR. The fold induction of \textit{hTERT} expression was calculated from the ratio \textit{hTERT} mRNA/PBGD mRNA in MBD2 siRNA-transfected cells versus scrambled siRNA-transfected cells. Mean values (± standard deviation) obtained from at least three independent transfection experiments are shown. Filled squares, MBD2 mRNA level; filled circles, fold induction of \textit{hTERT} mRNA. (B) Quantitative RT–PCR expression analysis of \textit{hTERT} in HeLa cells 96 h after transfection with either a negative control siRNA or MBD2 siRNA. Real-time RT–PCR was done on cell lines and \textit{hTERT} expression was normalized to \textit{b-actin}. The relative level of each mRNA was calculated on the basis of the two standard curves using the relative quantification method. At least three independent determinations of fold differences were used to calculate the average fold difference values and associated standard deviation ($P = 0.0027$, t-test).

Fig. 4. Mbd2 expression rescues the reduction of \textit{hTERT} transcript in MBD2 KD HeLa cells. The transcriptional expression of \textit{hTERT} was analyzed by quantitative RT–PCR 48 h after lipofectamine transfection of HeLa and MBD2 KD HeLa cells using pRev-Mbd2, an Mbd2 vector expressing a transcript resistant to RNAi or an empty pGL3 basic vector. The relative \textit{hTERT} mRNA in pRev-Mbd2-transfected cells or in pGL3-transfected cells or in KD HeLa cells was calculated using the same reference: the \textit{hTERT} mRNA level in untransfected HeLa cells. Mean values obtained from at least three independent transfection experiments are shown ($P = 0.0035$, t-test).

Fig. 5. DNA methylation patterns of \textit{hTERT} and the corresponding transcript levels in different cell lines. (A) Bisulfite sequencing of five telomerase-positive cancer cell lines (HeLa, MCF7, HepG2, LAN-1 and NCCIT) and one normal telomerase-negative fibroblast cell line (MRC5). Bisulfite-treated DNA was PCR amplified using a primer set spanning the end of the promoter and the proximal exonic region of \textit{hTERT}. PCR products were then directly sequenced. The analyzed 314 bp region (−206 to +108) is presented on a schematic map and results are shown for each cell lines. Filled and open squares, respectively, represent complete methylated and unmethylated CpG sites, whereas gray squares correspond to partial methylated CpG sites. (B) Quantitative expression of \textit{hTERT} in MRC5, NCCIT, HepG2, LAN-1, MCF7 and HeLa cells. Real-time RT–PCR was done on cell lines, and \textit{b-actin} was used as a reference.
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Fig. 6. Depletion of MBD2 by siRNA results in transcriptional activation of hTERT in hTERT-methylated telomerase-positive cancer cell lines. Fold change of hTERT expression between MBD2-depleted cells (MBD2 siRNA-transfected cells) and control cells (scrambled siRNA-transfected cells). hTERT expression was monitored by quantitative RT–PCR 96 h after transfection. Mean values (± standard deviation) obtained from at least three independent transfection experiments are shown.

hTERT expression at a basal level. Indeed, hypermethylation of CpG islands seems to be a crucial event in carcinogenesis. Thus, the methylation-free region in hTERT promoter may result from antagonistic pressure between the mechanisms leading to aberrant methylation and the need to keep hTERT expressed for unlimited life span of cancer cells.

A body of evidence has been accumulated concerning association between hypermethylation of CpG islands, transcriptional silencing and MBD-proteins binding (21). ChIP experiments and ChIP-on-chip analysis indicate that MBD2 associated the hypermethylated CpG island of hTERT. In this context, MBD2 seems to be a limiting factor rather than a transcriptional silencer.

Recently, a large screening of tumor suppressor gene promoters in 10 cell lines showed that human cancer cell lines tend to use a particular MBD protein (31). Furthermore, in MRC5 cells, ChIP experiments have indicated that MeCP2 and MBD2 proteins have non-overlapping binding specificities in vivo (23). These data, as well as our data are in favor of the ‘one gene—one MBD’ hypothesis, at least for some genes. Indeed, MBD2 was specifically associated with the methylated region of the hTERT CpG island, whereas MeCP2 and MBD1 were not detected at this locus. Moreover, MBD2 depletion did not induce MeCP2 or MBD1 binding at this methylated area in HeLa cells. Thus, the specificity of MBD proteins does not seem to be driven by their relative concentrations in a cell line, as it was suggested in an other study (31): a strong expression of a particular MBD is not necessarily associated with its preferential use in promoters.

Several studies have shown that transcriptional activation could be realized upon depletion of MBD proteins by RNA interference (RNAi) (26,28). A large-scale microarray analysis indicated that 15% of 6386 genes analyzed exhibited an increased expression change of 2-fold upon MBD2 depletion (28). Furthermore, the fold change observed in MBD2-deficient mouse cells is not very different from our own data. For example, it has been shown (32) that Mbd2−/− fibroblasts had 3-fold higher levels of Xist than wild-type cells. Moreover, elevated hTERT transcription upon MBD2 depletion is not limited to cervix (HeLa cells) since this effect was observed in breast, liver and neuroblastoma cancer cell lines. MBD2 RNAi experiments in cell lines exhibiting unmethylated hTERT CpG island (MRC5 and NCCIT) did not affect hTERT transcription indicating that MBD2 specifically and directly represses hTERT expression in methylation-dependent manner. Collectively, these data strongly suggest that MBD2 is a general repressor of hTERT expression in cancer cells.

A considerable number of transcription factors have been proposed as negative regulators of hTERT transcription. Nevertheless, direct evidence indicating that hTERT gene transcription is downregulated remains to be firmly established. Thus, MBD2 may represent a new factor directly involved in the negative regulation of hTERT expression. In cancer cells, MBD2 seems to play a specific role since its binding to the hypermethylated part of the hTERT promoter establishes a direct link between a common DNA alteration of tumor cells, CpG hypermethylation and a reduced level of hTERT transcription. The binding of MBD2 represents an additional layer for the control of hTERT expression, thereby underlying the importance of hTERT regulation in immortalized and cancers cells.

**Supplementary material**

Supplementary Figure S1 and Table S1 can be found at http://carcin.oxfordjournals.org/

**Funding**

Ligue Nationale contre le Cancer (Comité du Rhône and Comité de la Loire); Institut National contre le Cancer (EpiPro, CircBio, 419807-37/NG-LC); Swiss National Science Foundation (3100A0-101732, 3100A0-113505).

**Acknowledgements**

Conflict of Interest Statement: None declared.

**References**


Received August 20, 2008; revised October 3, 2008; accepted October 11, 2008