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MBD2-Mediated Transcriptional Repression of the *p14*^{ARF} Tumor Suppressor Gene in Human Colon Cancer Cells

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Key Words
DNA methylation · Methyl-CpG-binding domain protein · Gene regulation

Abstract
Objective: The *p14*^{ARF} and *p16*^{INK4A} tumor suppressor genes are commonly inactivated by aberrant methylation of their promoter regions in human colon cancer. The methyl-CpG-binding domain protein MBD2 is physically associated with the methylated promoters of the *p14*^{ARF} and *p16*^{INK4A} genes in specific tumor cell lines. Moreover, deficiency of MBD2 strongly inhibits intestinal tumorigenesis in the Min mouse, raising the possibility that the protein might be involved in transcriptional repression of methylated tumor suppressor genes. The aim of this study was to evaluate the role of MBD2 in the silencing of *p14*^{ARF} and *p16*^{INK4A} in cancer. ***Methods:*** The MBD2 protein was stably knocked down by RNA interference in RKO, a colon cancer cell line in which both *p14*^{ARF} and *p16*^{INK4A} are silenced by methylation. ***Results:*** We demonstrate here that MBD2 associates with the methylated promoter of the *p14*^{ARF} gene in the RKO colon cancer cell line. Depletion of MBD2 by RNAi leads to selective upregulation of the *p14*^{ARF} but not the *p16*^{INK4A} gene transcript. In addition, p14^{ARF} repression can be restored by expressing mouse MBD2 protein in MBD2-deficient RKO cells. ***Conclusion:*** These findings implicate MBD2 in transcriptional repression of the methylated *p14*^{ARF} tumor suppressor gene and suggest that repression by MBD2 selectively affects a subset of methylated promoters.

Introduction

Many tumor suppressor genes (TSGs) have their promoter situated in a CpG island and are silenced by aberrant methylation in cancer, which appears to be a significant pathway for TSG inactivation in neoplasia [1]. The molecular mechanisms that repress the transcription of abnormally methylated TSGs are, however, largely unknown. Two generic silencing mechanisms can be envisaged: direct or indirect. The direct mechanism involves transcription factors that can bind specifically to unmethylated promoters, but not to their methylated counterparts. The indirect mechanism utilizes methyl-CpG-binding proteins such as MeCP2, MBD1, MBD2, MBD4 and Kaiso, which have a specific affinity for methylated DNA sequences. Once bound to methylated promoters, the methyl-CpG-binding proteins are able to recruit transcriptional repression complexes at methylated promoters [2, 3]. The human *p14*^{ARF} and *p16*^{INK4A} TSGs are aberrantly methylated and their cell cycle control function is suppressed in a large number of human malignan-

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cies [4]. The two genes share exons 2 and 3, but have distinct promoter/exon 1 regions separated by about 20 kb [5]. In spite of their proximity, the *p14*^{ARF} and *p16*^{INK4A} genes are expressed independently in human primary tumors, suggesting independent regulation of their promoters [6]. Thus far, there is no reported methyl-CpG-sensitive transcription factor that could be responsible for *p14*^{ARF} and/or *p16*^{INK4A} gene inactivation in cancer cells. An indirect mechanism involving methyl-CpG-binding domain proteins is supported, however, by evidence that both *p14*^{ARF} and *p16*^{INK4A} gene promoters, when methylated, are physically associated with MBD2 and MeCP2 proteins and with histone deacetylase activity in colon cancer cells [7, 8]. In human breast cancer cells, transcriptional repression of the *Glutathione S-transferase P1 (GSTP1)* and the *Near BRCA1–2 (NBR2)* genes depends upon MBD2 [9, 10]. In several other cancer cell models, knocking down MBD2 expression by means of antisense inhibitors or small interfering RNAs (siRNAs) reduces tumor cell invasiveness, proliferation and tumorigenesis [11–13]. Finally, MBD2 deficiency greatly suppresses intestinal tumorigenesis in the ApcMin mouse model [14], a fact that would well accord with involvement of MBD2 in the repression of methylated TSGs.

In the present study, we stably knocked down the expression of MBD2 in colon cancer cells by means of RNA interference (RNAi), with the aim to examine the role of this protein in the transcriptional repression of TSGs such as *p14*^{ARF} and *p16*^{INK4A}.

Materials and Methods

Hairpin siRNA Vector Constructs

We used the pSUPER hairpin siRNA expression vector system [15], slightly modified to introduce puromycin resistance (pSUPER-puro). Two DNA fragments containing 19-bp regions of the *MBD2* transcript were subcloned into the pSUPER-puro vector. The obtained constructs were pSUPER-puro-MBD2-A, containing the 5′-GAAGGAGGAAGTGATCCGA sequence, and pSUPER-puro-MBD2-B, containing the 5′-GAGCGATGTCTACTACTTC sequence.

Cell Culture

The RKO human colon carcinoma cell line, obtained from the American Tissue Culture Collection (Atlanta, Ga., USA), was chosen because it contains several TSGs fully silenced by aberrant promoter methylation, among which are *p14*^{ARF}, *p16*^{INK4A}, *hMLH1* and *TIMP3* [16]. RKO cells were cultured under standard conditions in RPMI 1640 medium (Gibco, Grand Island, N.Y., USA) containing 10% fetal calf serum and antibiotics. They were transfected with the pSUPER-puro-MBD2-A and pSUPER-puro-MBD2-B constructs using lipofectamin (Invitrogen, Carlsbad, Calif., USA). Stable transfectants were obtained by puromycin selection. To obtain DNA demethylation, RKO cells were grown for 72 h with 1 mM 5-aza-2′-deoxycytidine (Sigma, St. Louis, Mo., USA). Cell cycle analyses were performed by means of the bromodeoxyuridine (BrdU) integration method. RKO cell derivates were grown on coverslips in 6-well dishes. BrdU (Sigma) was added to the cell culture medium at a final concentration of 10 μM and the cells were incubated at 37°C for 60 min, fixed in 4% paraformaldehyde for 20 min at room temperature, washed twice with phosphate-buffered saline (PBS) at pH 7.5 and permeabilized in 0.5% Triton X-100 for 10 min. After these pretreatments, the cells were incubated for 60 min with Alexa Fluor 594-conjugated mouse monoclonal anti-BrdU antibody (Molecular Probes, Eugene, Oreg., USA) diluted 1:40 in PBS. The coverslips were mounted onto slides using DAPI-Vectashield mounting medium (Vector, Burlingame, Calif., USA). The proportion of BrdU-positive cells was evaluated by counting a total of 600 cells on a Zeiss Axioplan 2 fluorescence microscope.

Expression Analyses

The expression of *MBD2*, *MBD1*, *MeCP2*, *p14*^{ARF}, *p16*^{INK4A}, *hMLH1*, *TIMP3* and *GAPDH* mRNA was evaluated by duplex semiquantitative RT-PCR [17], with 20–25 amplification cycles, which enables 'linear' conditions. The following primers were used: MBD2-A: 5′-CCTGCTGTTTGGCTTAACACA-3′, MBD2-B: 5′-GGCTTCATCTCCACTGTCCAT-3′, MBD1-A: 5′-GGCTACTGCTGCTTCCTGTG-3′, MBD1-B: 5′-GCTTCTTTCGCTTCTTGCTG-3′, MeCP2-A: 5′-CCGCTCTGCTGGGAAGTATGA-3′, MeCP2-B: 5′-GAGATTTGGGCTTCTTAGGTG-3′, p14-A: 5′-GCCTGCGGGGCGGAGAT-3′, p14-B: 5′-AGCACCACCAGCGTGTCC-3′, p16-A: 5′-GGTGCGGGCGCTGCTGGA-3′, p16-B: 5′-AGCACCACCAGCGTGTCC-3′, hMLH1-A: 5′-CCTTGCCATGCTTGCCTTAG-3′, hMLH1-B: 5′-GGGCACATAGTTGTCAATCAG-3′, TIMP3-A: 5′-GCTGTGCAACTTCGTGGAGAGG-3′, TIMP3-B: 5′-CTCGGTACCAGCTGCAGTAGCC-3′, GAPDH-A: 5′-ATGGGGAAGGTGAAGGTCG-3′ and GAPDH-B: 5′-AGGGGCCATCCACAGTCTT-3′. All expression analyses were performed in triplicate. The expression of MBD2 protein was assessed by standard Western blot on nuclear extracts [18], using the S923 sheep anti-MBD2 polyclonal antibody [19]. Anti-α-tubulin monoclonal antibody (Novus Biologicals, Littleton, Colo., USA) served as control.

Chromatin Immunoprecipitation

Formaldehyde cross-linked chromatin from RKO cells was precipitated using the R593 rabbit anti-MBD2 polyclonal antibody [19] as described [7]. The immunoprecipitated fraction was amplified by PCR using primers specific for the *p14*^{ARF} (p14ch-A: 5′-GTGGCCCTCGTGCTGATG-3′ and p14ch-B: 5′-TGGGCTAGAGACGAATTATC-3′), *p16*^{INK4A} (p16ch-A: 5′-GGGAGCAGCATGGAGCCG-3′ and p16ch-B: 5′-CTGGATCGGCCTCCGACCGTA-3′), *hMLH1* (MLH1ch-A: 5′-TACGATGAGGCGGCGACA-3′ and MLH1ch-B: 5′-CTTGTGGCCTCCCGCAGAA-3′), *TIMP3* (TIMP3ch-A: 5′-GCGGCAGCAGCGGCAATG-3′ and TIMP3ch-B: 5′-CACCAGGAGCGCTTACCGA-3′) and *p15*^{INK4B} (p15ch-A: 5′-TTCCCAGAAGCAATCCAG-3′ and p15ch-B: 5′-CCTGGGCTCAGCTTCATTA-3′) genes. The promoter of *p15*^{INK4B} is unmethylated in RKO cells [16]. A dilution of the input chromatin sample was also amplified as a positive control. Normal rabbit serum was used as a control antibody.

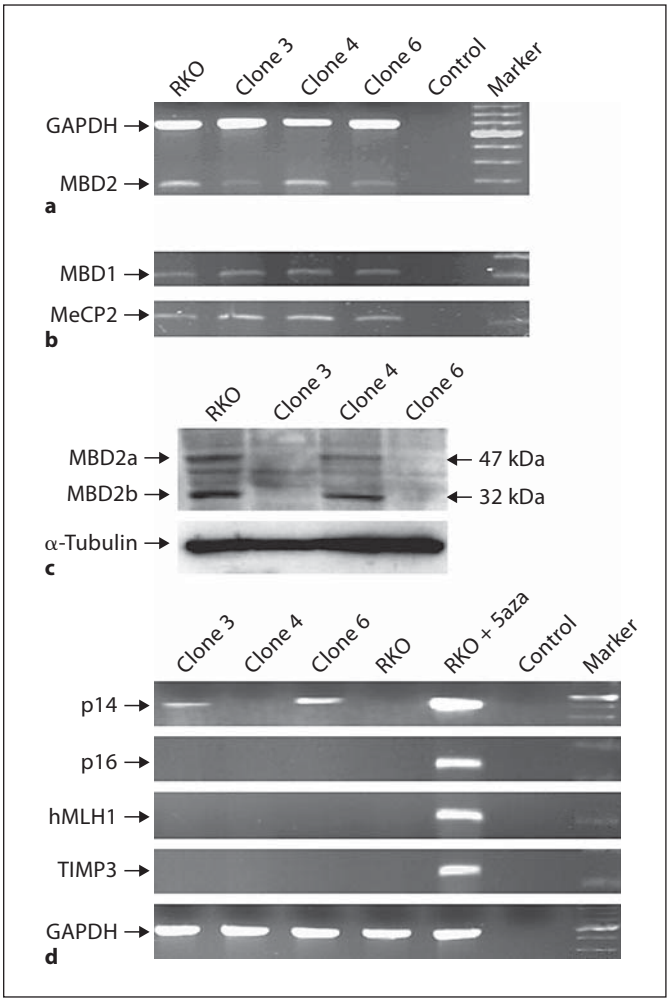

Fig. 1. Depletion of MBD2 by RNAi in the RKO colon cancer cell line results in selective derepression of $p14^{ARF}$. Three clones (clones 3, 4 and 6) corresponding to RKO cells stably transfected with the pSUPER-puro-MBD2-A construct are analyzed. **a** *MBD2* and *GAPDH* mRNA expression analyzed by duplex semiquantitative RT-PCR. Clones 3 and 6 have significantly lower levels of *MBD2* mRNA than untransfected RKO cells and clone 4. There is no reduction of *GAPDH* mRNA in clones 3 and 6. **b** Semiquantitative RT-PCR analysis of *MBD1* and *MeCP2* mRNA expression. The transcripts are not affected by hairpin RNA expression in comparison with native RKO cells. **c** Western blot analysis of MBD2 protein expression in RKO cell nuclear extracts. Native RKO cells and clone 4 express both isoforms of the MBD2 protein, i.e. MBD2a (47 kDa) and MBD2b (32 kDa), which are undetectable in clones 3 and 6. MBD2a always resolves as a doublet band for unknown reasons [19]. Anti-α-tubulin monoclonal antibody is used as a positive control. **d** Expression of $p14^{ARF}$, $p16^{INK4A}$, *hMLH1*, *TIMP3* and *GAPDH* gene transcripts analyzed by RT-PCR. $p14^{ARF}$ mRNA is undetectable in native RKO cells (RKO) and in clone 4 which contain normal levels of MBD2 protein. By contrast, expression of $p14^{ARF}$ mRNA is induced in MBD2-deficient clones 3 and 6. Strong induction of $p14^{ARF}$ mRNA is seen after treatment of RKO cells for 72 h with 1 mM 5-aza-2′-deoxycytidine (RKO + 5aza). There is no detectable $p16^{INK4A}$, *hMLH1* and *TIMP3* mRNA expression in native RKO cells or in MBD2-deficient clones 3 and 6. *GAPDH* mRNA serves as a loading control. Control = No reverse transcriptase. The marker is a 100-bp ladder.

Bisulfite Genomic Sequencing

Genomic DNA was chemically modified by sodium bisulfite [6], a treatment which changes the unmethylated but not the methylated cytosines into uracil. Bisulfite-treated DNA was subjected to PCR amplification using primers designed to recognize both methylated and unmethylated forms of $p14^{ARF}$ promoter region: p14-bis-A: 5′-GTGGGTTTTAGTTTGTAGTT-3′ and p14-bis-B: 5′-AATCACCAAAAACCTAC-3′. The amplified fragment contains 5 CpG dinucleotides at positions +33, +36, +41, +48 and +54 of the $p14^{ARF}$ promoter. Position +1 corresponds to the transcription start site of $p14^{ARF}$ [5].

Retroviral MBD2 Expression

A flag M2-tagged version of mouse MBD2 cDNA was subcloned into the pBMN-ZIN bicistronic retroviral vector. Virus was produced in Phoenix cells after transfecting either the pBMN-ZIN-flag-MBD2 construct or empty pBMN-ZIN (mock). Native and modified RKO colon cancer cells were infected with virus-containing medium, as described (see http://www.stanford.edu/group/nolan/protocols/pro_helper_dep.html). Infected cell lines were maintained under G418 selection to ensure strong mouse MBD2 expression in the vast majority of the cells. The efficiency of infection was verified by indirect immunofluorescence using the anti-Flag M2 monoclonal antibody (Sigma) and DAPI for nuclei counterstaining.

Results

MBD2 Knockdown by RNAi

Two pSUPER-puro constructs, pSUPER-puro-MBD2-A and pSUPER-puro-MBD2-B, designed to generate hairpins specific for 19-bp regions of the *MBD2* gene transcript, were stably expressed in the RKO cell line. For each construct, six stable transfectant cell lines (MBD2-A clones 1–6 and MBD2-B clones 1–6) were selected and tested for *MBD2* gene expression by semiquantitative RT-PCR. Of the 12 tested clones, two (clones 3 and 6) expressed significantly lower levels of *MBD2* mRNA than untransfected native RKO cells (fig. 1a) and were selected for further experiments. One of the clones in which

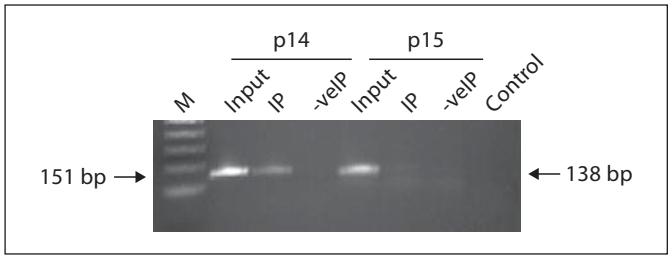

Fig. 2. MBD2 binds in vivo to the *p14*^{ARF} gene promoter in the RKO colon cancer cell line. Chromatin immunoprecipitation using the R593 anti-MBD2 antibody and primers specific for the *p14*^{ARF} and *p15*^{INK4B} promoter regions. MBD2 occupies the methylated promoter region of *p14*^{ARF} but not the unmethylated promoter region of *p15*^{INK4B}. IP = Immunoprecipitated fraction; -veIP = no-antibody control; input = dilution of the input chromatin sample; M = 100-bp ladder; control = no polymerase.

MBD2 mRNA expression remained unaffected was chosen as a negative control (clone 4). In contrast to *MBD2*, the levels of *MBD1*, *MeCP2* and *GAPDH* mRNA in clones 3 and 6 were not affected in comparison with clone 4 and native RKO cells (fig. 1a, b). MBD2 knockdown was then verified by Western blot in nuclear extracts from clones 3, 4 and 6 as well as from native RKO cells. Both isoforms of the MBD2 protein, MBD2a (47 kDa) and MBD2b (32 kDa), were expressed in clone 4 and native RKO cells, but nearly undetectable in clones 3 and 6 (fig. 1c).

TSG Expression in MBD2 Knockdown Cells

Expression of *p14*^{ARF}, *p16*^{INK4A}, *hMLH1* and *TIMP3* gene transcripts was analyzed by semiquantitative RT-PCR in clones 3, 4 and 6 as well as in native RKO cells. There was no detectable *p14*^{ARF} mRNA in native RKO cells and in clone 4. By contrast, expression of *p14*^{ARF} mRNA was slightly induced in MBD2-deficient clones 3 and 6 (fig. 1d). For comparison, we evaluated the effect of the demethylating agent 5-aza-2′-deoxycytidine on native RKO cells. After 72 h treatment with 5-aza-2′-deoxycytidine, induction of *p14*^{ARF} mRNA corresponding to 10-fold the levels observed in clones 3 and 6 was observed (fig. 1d). There was no detectable *p16*^{INK4A}, *hMLH1* and *TIMP3* mRNA expression in native RKO cells, in clone 4 or in MBD2-deficient clones 3 and 6.

Binding of MBD2 to the p14^{ARF} *Promoter Region in RKO Cells*

Occupancy of the promoter region of *p14*^{ARF} by the MBD2 protein in RKO cells was tested by ChIP. As illustrated in figure 2, the MBD2 protein was found to be associated with the methylated promoter of *p14*^{ARF}, but not with the promoter of *p15*^{INK4B}, which is unmethylated in RKO cells [16]. There was no evidence of MBD2 binding to *p16*^{INK4A}, *hMLH1* and *TIMP3* gene promoter regions (data not shown).

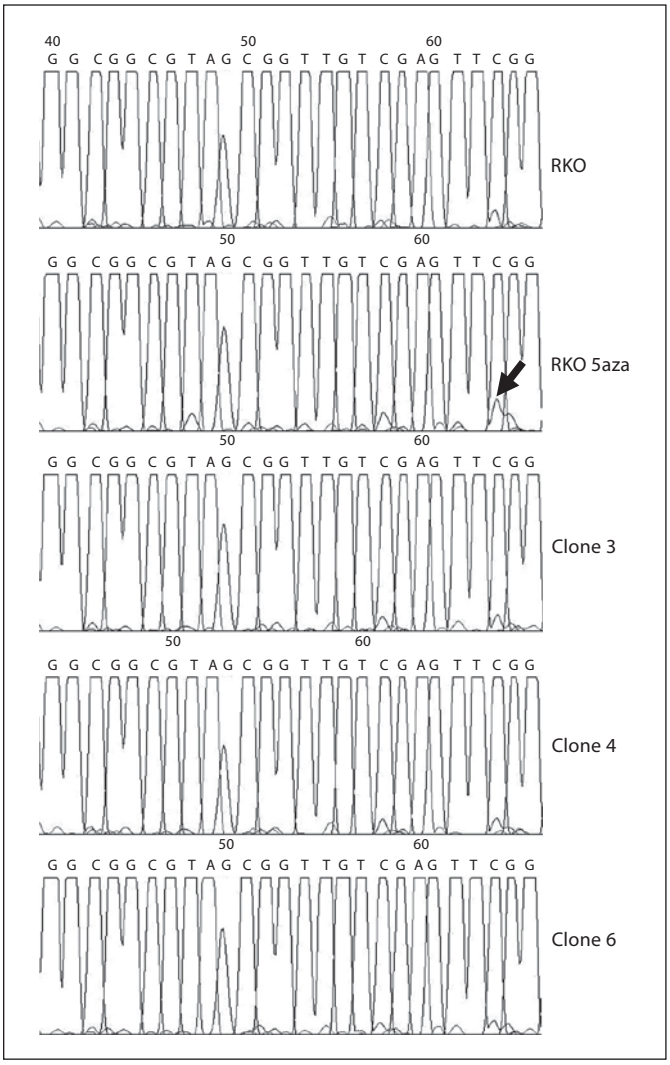

Fig. 3. Induction of *p14*^{ARF} mRNA expression in clones 3 and 6 is not due to promoter demethylation. Bisulfite genomic sequencing analysis of the *p14*^{ARF} promoter region. There is no significant T peak in clones 3, 4 and 6, indicating absence of demethylation. DNA from native RKO cells (RKO) is fully methylated and indistinguishable in this respect from clones 3 and 6.

Methylation Status of the p14^{ARF} *Gene following MBD2 Knockdown*

The methylation status of the *p14*^{ARF} gene promoter region was analyzed by direct bisulfite genomic sequencing in clones 3, 4 and 6 as well as in native RKO cells. As illustrated in figure 3, there was no conversion of cytosine

residues to thymine following bisulfite treatment, indicating fully methylated state.

Restoration of MBD2 Expression in MBD2-Deficient RKO Cells

A mouse MBD2-Flag fusion protein was exogenously expressed in MBD2-deficient RKO cells (clones 3 and 6) as well as in native RKO cells using retrovirus technology. This allowed us to obtain mouse MBD2-Flag expression in more than 90% of the cells of clone 6 and of the native RKO cell line (fig. 4a). The exogenously expressed fusion protein was localized in the nucleoplasm, excluding the nucleoli (fig. 4a). By semiquantitative RT-PCR, *MBD2* mRNA levels in clone 6 were found to be restored to approximately wild-type levels (fig. 4b). This coincided with reproducible but incomplete downregulation of the $p14^{ARF}$ transcript (fig. 4c).

Effects of MBD2 Knockdown and MBD2 Restoration on the Cell Cycle

The consequences of MBD2 knockdown and MBD2 rescue on the cell cycle were evaluated by comparing BrdU integration in MBD2-deficient RKO cells (clone 6), control cells (clone 4) and MBD2-deficient RKO cells expressing mouse MBD2-Flag fusion protein (clone 6 + MBD2). Of a total of 1,200 cells, 250 (20.8%) were positive for BrdU in clone 6, 238 (19.8%) in clone 4 and 244 (20.3%) in clone 6 + MBD2 (data not shown). The differences were statistically not significant (χ^2 test).

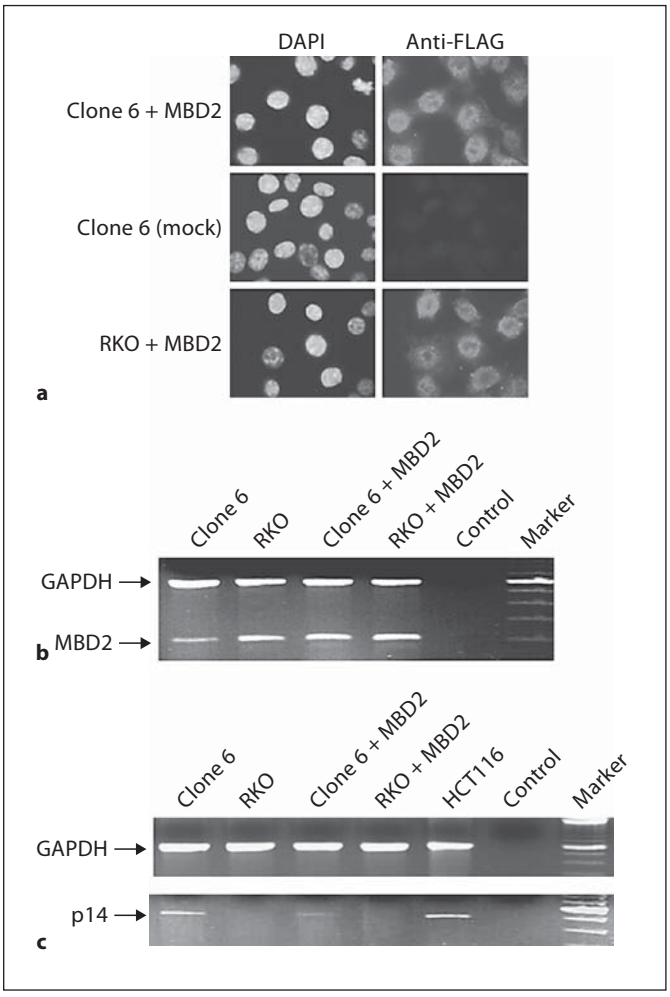

Fig. 4. Retroviral exogenous expression of mouse MBD2 protein partially restores transcriptional repression of $p14^{ARF}$ in MBD2-deficient RKO cells (clone 6). **a** Indirect immunofluorescence using anti-Flag M2 monoclonal antibody. Flag-mouse-MBD2 fusion protein is expressed in nearly 100% of the cells, but the expression level is quite low. The signal is nucleoplasmic (excluding nucleoli). Nuclei are visualized by DAPI staining. **b** Semiquantitative duplex RT-PCR analysis comparing *MBD2* and *GAPDH* mRNA expression. The level of *MBD2* mRNA is lower in clone 6 (lane 1) than in native RKO cells (lane 2). After exogenous expression of mouse MBD2 protein, the level of *MBD2* mRNA is restored in clone 6 + MBD2 to approximately initial levels (RKO, lane 2). The overexpressed mouse MBD2 transcript is hardly visible in RKO + MBD2 (lane 4), when compared to native RKO cells (lane 2) and clone 6 + MBD2 (lane 3). This can be explained by the fact that tumor cells such as RKO express huge levels of endogenous MBD2 and that the expression of exogenous MBD2 is, by contrast, quite low with our retroviral system. **c** Semiquantitative RT-PCR analysis of $p14^{ARF}$ mRNA expression before and after expression of mouse MBD2. Exogenous expression of mouse MBD2 partially restores $p14^{ARF}$ repression in clone 6 (clone 6 + MBD2). cDNA from HCT116 cells, which constitutively express $p14^{ARF}$, serves as a positive control. Control = No polymerase; marker = 100-bp ladder.

Discussion

In the present study, we used RNAi technology to stably knock down expression of the methyl-CpG-binding protein MBD2 in the human RKO colon cancer cell line, with the aim to evaluate the role of MBD2 in the transcriptional repression of methylated TSGs. The RKO tumor cell line was chosen because it contains four TSGs fully silenced by homozygous promoter methylation, which are $p14^{ARF}$, $p16^{INK4A}$, *hMLH1* and *TIMP3* [16]. RNAi was first performed using synthetic siRNAs directed against the MBD2 transcript. Due to low transfection rates of siRNAs in RKO cells, however, we did not obtain satisfactory MBD2 depletion (data not shown). In addition, MBD2 knockdown obtained with siRNAs was not sufficiently stable to reach significant effects. We therefore decided to switch to the pSUPER hairpin siRNA expression vector system [15]. The pSUPER vector was modified to introduce puromycin resistance, allowing us

to generate two RKO cell clones (3 and 6) that displayed strong, specific and stable MBD2 depletion at both mRNA and protein levels (fig. 1a–c).

We then evaluated the effects of MBD2 knockdown on the expression $p14^{ARF}$, $p16^{INK4A}$, *hMLH1* and *TIMP3*, four TSGs which are fully repressed by promoter methylation in the native RKO cell line. Using semiquantitative RT-PCR, we detected low levels of $p14^{ARF}$ transcript in the MBD2-deficient clones 3 and 6, which were not present in the control (clone 4) and native RKO cells. The levels of $p14^{ARF}$ mRNA detected in clones 3 and 6 were at least 10-fold lower than those observed in RKO cells treated with the demethylating agent 5-aza-2′-deoxycytidine. This suggests that MBD2 is necessary for leakproof silencing of the $p14^{ARF}$ gene. A formal hypothesis to explain $p14^{ARF}$ upregulation in clones 3 and 6 is that the $p14^{ARF}$ promoter undergoes demethylation and that loss of MBD2 itself plays a secondary role. To exclude this possibility, we demonstrated by bisulfite genomic sequencing that the levels of CpG methylation in the $p14^{ARF}$ CpG island were consistently high in the RKO cells, irrespective of RNAi treatment (fig. 3). The present observations accord with several previous studies that demonstrated a central role for MBD2 in developing and/or maintaining cancer cell characteristics such as tumorigenicity, uncontrolled proliferation, invasiveness and metastatic potential [11–13]. Interestingly, MBD2 seems to act by silencing methylated tumor-suppressing genes such as the *14-3-3σ* cell cycle regulator gene [12], as well as also by demethylating and activating tumor-promoting genes such as *urokinase-type plasminogen activator (uPA)*, *matrix metalloproteinase-2 (MMP-2)* [13] or *MAGE* [20].

Unexpectedly, $p14^{ARF}$ was the only TSG found to be upregulated in MBD2-deficient RKO cells (fig. 1d), and the $p14^{ARF}$ promoter was the only one to be associated with MBD2 in our ChIP analyses. No induction of the $p16^{INK4A}$, *hMLH1* and *TIMP3* gene transcripts could be detected, and in contrast to previous studies, we did not demonstrate any binding of MBD2 to these genes. In the HCT15 colon cancer cell line, Magdinier and Wolffe [7] found that MBD2 was associated with the methylated promoters of both $p14^{ARF}$ and $p16^{INK4A}$, and in the DLD1 colon cancer cell line, Kondo et al. [21] demonstrated that the hypermethylated promoters of $p16^{INK4A}$ and *hMLH1* were preferentially bound by methyl-CpG-binding domain protein 4 (MBD4), which was able to repress transcription. The lack of detectable interaction between MBD2 and the methylated promoter of $p16^{INK4A}$ in our ChIP experiment might be explained by use of a more specific anti-MBD2 antibody, which does not cross-react with MBD4. Given that MBD2, which belongs to the MeCP1 complex, requires 15 consecutive methylated CpGs for binding [22], and that its binding affinity is affected by local methyl-CpG spacing [23], the observed discrepancy might also be due to different methylation patterns at the $p16^{INK4A}$ promoter regions from RKO and other colon cancer cell lines. In breast cancer cells, MBD2 was found to be associated with the methylated bidirectional promoter shared by *BRCA1* and *NBR2*, but interestingly, only *NBR2* was upregulated by MBD2 deficiency [9]. Recent studies demonstrated highly selective MBD2 occupancy of methylated reporter plasmids in vitro and of genomic DNA sites in vivo, further supporting the idea that different methylated DNA sequences are not equivalent with respect to MBD2 binding [24, 25]. Together with our observations, these findings suggest locus-specific function of MBD2, due to chromosomal targeting of the protein or to context-dependent activity. It is possible that MBD2 partners, for example the recently reported RET finger protein [26] or the MBD2-interacting zinc finger protein MIZF [27], provide additional DNA sequence specificity that targets MBD2. Arginine methylation in the MBD2 protein is another mechanism that could potentially influence MBD2-methyl-DNA interactions at specific gene sequences [28].

If reducing the level of MBD2 protein is sufficient to partially release the transcriptional repression of $p14^{ARF}$, as suggested by the above experiments, then expression of exogenous MBD2 protein in MBD2-deficient RKO cells should restore repression. We used a retroviral expression vector system to exogenously express a Flag-tagged mouse MBD2 protein in the MBD2-deficient RKO clones. Following infection, more than 90% of the cells slightly expressed the MBD2-Flag fusion protein in the nucleoplasm (fig. 4a), suggesting that the tagged protein was functional. In addition, *MBD2* mRNA in clone 6 was restored to approximately wild-type levels (fig. 4b). This coincided with reproducible downregulation of the $p14^{ARF}$ transcript (fig. 4c), although repression remained incomplete. These findings support the conclusion that $p14^{ARF}$ reexpression in clones 3 and 6 is specifically due to deficiency of MBD2 protein. Since $p14^{ARF}$, which is a major cell cycle regulator, can be induced by knocking down and rerepressed by rescuing MBD2 expression in RKO cells, one would expect corresponding changes in cell proliferation. Such changes, however, were not observed by comparing BrdU integration in clones 6 and 4, suggesting that the degree of $p14^{ARF}$ activation was insufficient to induce cell cycle arrest in G1.

Together, our observations support a role for endogenous MBD2 in silencing a methylated tumor suppressor

gene in the RKO cancer cell line. MBD2 binds to the methylated promoter of *p14*^{ARF} and depletion of either DNA methylation or MBD2 itself causes reexpression. Restoration of exogenous MBD2 to depleted cells permits partial recovery of *p14*^{ARF} repression. Given the incomplete activation of *p14*^{ARF} mRNA by MBD2 depletion, it is evident that other factors participate in *p14*^{ARF} repression in cancer cells. Leakproof *p14*^{ARF} gene silencing, however, which may contribute to the cancer phenotype, depends upon MBD2. Our data, together with previous reports [9–13], implicate MBD2 as a significant interpreter of the repressive DNA methylation signal in cancer. Given the demonstrated importance of MBD2 for tumorigenesis in a mouse model of human familial colorectal cancer [14], it is probable that agents that interfere with MBD2 function will become therapeutically important.

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