Methylated DNA Binding Domain Protein 2 (MBD2) Coordinately Silences Gene Expression through Activation of the MicroRNA hsa-mir-496 Promoter in Breast Cancer Cell Line

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Abstract

Methylated DNA binding protein 2 (MBD2) binds methylated promoters and suppresses transcription in cis through recruitment of a chromatin modification repressor complex. We show here a new mechanism of action for MBD2: suppression of gene expression indirectly through activation of microRNA hsa-mir-496. Overexpression of MBD2 in breast epithelial cell line MCF-10A results in induced expression and demethylation of hsa-mir-496 while depletion of MBD2 in a human breast cancer cell lines MCF-7 and MDA-MB-231 results in suppression of hsa-mir-496. Activation of hsa-mir-496 by MBD2 is associated with silencing of several of its target genes while depletion of MBD2 leads to induction of hsa-mir-496 target genes. Depletion of hsa-mir-496 by locked nucleic acid (LNA) antisense oligonucleotide leads to activation of these target genes in MBD2 overexpressing cells supporting that hsa-mir-496 is mediating in part the effects of MBD2 on gene expression. We demonstrate that MBD2 binds the promoter of hsa-mir-496 in MCF-10A, MCF-7 and MDA-MB-231 cells and that it activates an in vitro methylated hsa-mir-496 promoter driving a CG-less luciferase reporter in a transient transfection assay. The activation of hsa-mir-496 is associated with reduced methylation of the promoter. Taken together these results describe a novel cascade for gene regulation by DNA methylation whereby activation of a methylated microRNA by MBD2 that is associated with loss of methylation triggers repression of downstream targets.

Introduction

Covalent modification of cytosine in CpG dinucleotides in 5′ regulatory regions of genes by methylation has been shown to regulate gene function in cis by suppressing transcription of the juxtaposed gene [1]. However, genome wide analyses of RNA transcription have repeatedly shown only partial correlation between methylation states of promoters and transcription. Most commonly seen are unmethylated promoters that are nevertheless inactive [2]. Remarkably, even pharmacological DNA demethylation does not result uniformly in gene induction but a fraction of the transcriptome is suppressed [3,4]. This suggests that DNA methylation states are not limited to cis-suppression but might be controlling downstream cascades of gene regulatory events. An accepted hypothesis is that the DNA methylation signal is read by methylated DNA binding proteins that recruit repressive complexes and suppress gene expression in cis [5,6]. Methylated DNA binding protein 2 (MBD2) binds methylated DNA and has been shown to partner with the NuRD complex a multisubunit containing histone deacetylase activities (HDAC) [7], thus promoting gene silencing through inactivation of chromatin configuration [8]. There is a wide body of research that has established the role of MBD2 in methylation dependent cis-gene suppression. However, it was also proposed that MBD2 could act as an activator of gene expression either through recruitment of histone acetyl transferases (HAT) and other transcriptional activators such as TACC3 [9] the HTLV-1 TAX1 activator [10] or through promoting DNA demethylation [11,12] although the DNA demethylation biochemical activity of MBD2 has been contested by several studies [7,8]. We have previously shown that MBD2 is required for activation and maintaining a demethylated state of prometastatic genes in breast cancer [13] prostate cancer [14] and liver cancer [15] and that inhibition of MBD2 in colorectal and lung cancer cell lines reverses tumor growth as explants in vivo [16] [17] and overexpression of MBD2 leads to activation of Methylated TypeII Hexokinase Gene in hepatocytes [18]. A conserved sequence required for demethylation of cytokines in mature Th2 cells CNS-1 is bound to MBD2 when these cells undergo demethylation [19]. mbd2−/− mice exhibit hypermethylation of certain tumor suppressor genes which is partial in mbd2−/-/+ mice [20]. In this paper, we examined the hypothesis that MBD2 could cause
coordinate gene repression through activation of repressive pathways of gene regulation.

Highly-networked candidate repressors in the cell are microRNAs. microRNAs are small 17–22 nt non coding RNAs expressed in several eukaryotic organisms that regulate the stability and processing of target mRNA through direct binding to 3'UTRs [21,22]. The average microRNA has the potential to bind up to 100 different targets in the cell positioning them as nodal global regulators of disease where transcriptional programs involving multiple genes change drastically [23]. MicroRNAs have been shown to play a role in cell proliferation, differentiation, apoptosis and development causing small reductions in the level of hundreds of target mRNAs for each microRNA [21,22]. We tested, therefore, the possibility that MBD2 could affect gene repression of network of genes through activation of a methylated microRNA. Although microRNAs have been demonstrated by several studies to be regulated by DNA methylation [24–27] chromatin modification [28] and transcriptional regulators [29], the possibility that methylated DNA binding proteins could control expression of a group of genes through changing the methylation state of a microRNA has not been considered. We show here that MBD2 could cause gene repression of a group of genes through activating a microRNA that silences these genes.

### Materials and Methods

#### Plasmid Promoter Constructs and *in vitro* methylation of *mir-496*

A PCR amplified fragment (Using primers in Table 1) containing the *mir-496* promoter 5' regulatory region (−315→+161 relative to the TSS) was cloned into PCR2.1 and sub-cloned using HindIII and BamHI restriction sites into the CpG-free pCpGl luciferase reporter [30] in sense and antisense directions. Methylation of promoter constructs was carried out with 2 rounds of methylation with the CG specific SssI Methyltransferase and the methyl donor S-adenosylmethionine as recommended by the manufacturer’s guidelines (NEB, Cat# M0226L).

#### Luciferase Assay

For luciferase assays; HEK293 cells were plated at a density of 1×10^5 cells per well in 6 well plates. MBD2b and mtMBD2b (with a deletion of the methylated DNA binding domain) were subcloned into the pEF6 (Invitrogen) expression vector from the previously described pCDNA3.1-His-MBD2 [11]. To generate the mutant MBD deleted plasmid, pCDNA3.1-His-MBD2 was digested (KpnI) to remove the MBD domain (nucleotide 601–812) and blunted by Klenow (Roche). Subsequently, the pCDNA3.1 plasmid was digested (NotI) and the released fragment was ligated into

| Name | Sequence |
|------|----------|
| Outer bisulfite mir-496 forward | TGGGTGGTGTGTTGttATTTT |
| Outer bisulfite mir-496 reverse | TCGGTAGATGTGTTGTTG |
| Nested bisulfite mir-496 forward | TGGATGGTGTGTTGTTGTTG |
| Nested bisulfite mir-496 reverse | TCGGTAGATGTGTTGTTG |
| Nested bisulfite mir-496-pcpGL forward | AAAAAAAAAATAACCAATATAATCTCATCAT |
| Nested bisulfite mir-496-pcpGL reverse | AAAAAAAAAATAACCAATATAATCTCATCAT |
| Pyrosequencing mir-496-pcpgl forward | AGTAGGGGATGAG |
| Pyrosequencing mir-496-pcpgl reverse | TGTTTGTATTTTTGTATTTT |
| ChIP mir-496 −11→+264 for | GAAGCGCAGCAACCAAGT |
| ChIP mir-496 −11→+264 rev | CATGCAGCTAACGCCGAGCA |
| ChIP mir-496 −334→−550 for | GGGCTGCCAGCAGGTTG |
| ChIP mir-496 −334→−550 rev | AAGCTTCCTTTCCTCCTCC |
| mir-496-pcpgl ChIP forward | GGAAGCGCAGCAACCAAGT |
| mir-496-pcpgl ChIP reverse | TGCCATCTTCAGAGGAG |
| pcpgl ChIP forward | GCAGGTGGTGCTCAGAG |
| pcpgl ChIP reverse | TCCCCGAAGTGGTGGAG |
| qPCR MBD2 forward | CAAAGTCACAAATCTCCTAGT |
| qPCR MBD2 reverse | TATAATTTGCTTGTACACACATT |
| qPCR CTSH forward | TACTGTCGGTTGGGTATTTG |
| qPCR CTSH reverse | CGAAGAGGAGGACCCCCTC |
| qPCR POU2F3 forward | ACTCCTAAGCAGCAGT |
| qPCR POU2F3 reverse | CCGTCCTCAGTCCGAG |
| qPCR PTGS1 forward | CGTAGGCAGAGAGAGAG |
| qPCR PTGS1 reverse | AGAAGCGCAGGAGAG |

Expression of targets and MBD2.

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pEF6 (Invitrogen). Co-transfections for luciferase assays were performed with CaCl2 precipitation as described previously [31] with a total of 2 μg of plasmid transfected using a fixed 200 ng of luciferase promoter and a ratio of 1:3 of expression construct (MBD2/mRMBD2 to empty backbone, pCMV9). Constructs were transfected into HEK293 cells, harvested at 72 hours and assayed with the Luciferase Assay System as recommended by the manufacturer's guidelines (Promega, Cat.# E1483). Luciferase activity per condition was normalized to total protein concentration.

Cell culture and transfections

Human non-invasive breast cancer cells MCF-7, invasive breast cancer cells MDA-MB-231 and nontransformed immortalized breast epithelial cells MCF-10A were purchased from American Type Culture Collection (ATCC, Manassas, Virginia). MCF-7 cells were cultured in minimum Eagle's medium with 10 μg/ml of insulin (Invitrogen, Carlsbad, California). MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen). All media were supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

MCF-10A cells were cultured in Mammary Epithelial Basal Media (MEGM#; Lonza/Clonetics Corporation, CC-3150) supplemented with the BulletKit® provided by the manufacturer. The GA-1000 (gentamycin-amphotericin B mix) from the BulletKit® was not added to the media as recommended by ATCC. Transient transfections were carried out using lipofectin (Invitrogen, Cat. #18292-037) as described previously [32] with minor modifications, siRNAs were selected from one of the siGENOME SMARTpool™ sequences provided by Dharmacon, (Lafayette, Colorado). This concentration was optimized in preliminary experiments using transient knockdowns at several concentrations from 20 to 70 nM. SiRNAs were selected at a final concentration of 70 nM. SiRNAs were selected from one of the siGENOME SMARTpool™ sequences provided by Dharmacon, (Lafayette, Colorado). This concentration was optimized in preliminary experiments using transient knockdowns at several concentrations from 20 to 70 nM. SiRNAs were selected at a final concentration of 70 nM. SiRNAs were selected from one of the siGENOME SMARTpool™ sequences provided by Dharmacon, (Lafayette, Colorado). This concentration was optimized in preliminary experiments using transient knockdowns at several concentrations from 20 to 70 nM. SiRNAs were selected at a final concentration of 70 nM. SiRNAs were selected from one of the siGENOME SMARTpool™ sequences provided by Dharmacon, (Lafayette, Colorado). This concentration was optimized in preliminary experiments using transient knockdowns at several concentrations from 20 to 70 nM. SiRNAs were selected at a final concentration of 70 nM.

Expression arrays and validation

RNA was extracted using Trizol according to manufacturer’s instructions (Invitrogen, 15596-026). For transcriptome analysis, 1 μg of RNA from MCF-10A cells infected with MBD2 lentivirus or control empty virus and were subjected to microarray analysis. DNA methyltransferase expression analysis using Affymetrix Human Genome U133_Plus 2.0 (Array hybridization was performed at the Génome Québec Innovation Centre, Montréal, Canada). Biological replicates were chosen to be those with greater than 1.5-fold increase or less than 0.5-fold decrease in each sample as compared to untreated control. For expression array validation of the microarray results a total of 3 μg of RNA was used for RT-PCR cDNA conversion with AMV reverse transcriptase (Roche, Life Technologies) and random hexamers. Primers were designed to extend across exon boundaries to rule out genomic DNA contamination (Autoprime) and expression was normalized relative to GAPDH expression (primers in Table 1). Quantitative PCR (qPCR) was carried out using the SYBR Green method (Roche, Life Technologies) on LightCycler 480. MicroRNA cDNA conversion was carried out using the Mirscript PCR System (Qiagen,Cat. #218160). Expression of the mature mir-496 was measured using miScript primer assays (Qiagen, MS0007707) that spanned the entire mature miRNA and calculated its levels relative to RNU1A1 noncoding housekeeping RNA control. This control was suggested by the manufacturer to be stable across different conditions. qPCR was carried out using miScript SYBR Green PCR kit (Qiagen, Cat. # 218073). All data was Analyzed using the Absolute Relative Quantification LightCycler 480 software.

MBD2 Chromatin Immunoprecipitation

MCF-10A cells were cultured 24 hours before transfected with lipofectin to a final concentration of 200 nM. Scrambled control was used as control (target sequence provided by Dharmacon, (Lafayette, Colorado). This siRNA was transfected at a final concentration of 70 nM. Transfections were carried out using lipofectin (Invitrogen, Cat. #18292-037) as described previously [32] with minor modifications, siRNAs were selected from one of the siGENOME SMARTpool™ sequences provided by Dharmacon, (Lafayette, Colorado). This concentration was optimized in preliminary experiments using transient knockdowns at several concentrations from 20 to 70 nM. SiRNAs were selected at a final concentration of 70 nM. SiRNAs were selected from one of the siGENOME SMARTpool™ sequences provided by Dharmacon, (Lafayette, Colorado). This concentration was optimized in preliminary experiments using transient knockdowns at several concentrations from 20 to 70 nM. SiRNAs were selected at a final concentration of 70 nM. SiRNAs were selected from one of the siGENOME SMARTpool™ sequences provided by Dharmacon, (Lafayette, Colorado). This concentration was optimized in preliminary experiments using transient knockdowns at several concentrations from 20 to 70 nM. SiRNAs were selected at a final concentration of 70 nM. SiRNAs were selected from one of the siGENOME SMARTpool™ sequences provided by Dharmacon, (Lafayette, Colorado). This concentration was optimized in preliminary experiments using transient knockdowns at several concentrations from 20 to 70 nM. SiRNAs were selected at a final concentration of 70 nM. SiRNAs were selected from one of the siGENOME SMARTpool™ sequences provided by Dharmacon, (Lafayette, Colorado). This concentration was optimized in preliminary experiments using transient knockdowns at several concentrations from 20 to 70 nM. SiRNAs were selected at a final concentration of 70 nM.
Bisulfite mapping

Bisulfite mapping of DNA was carried out as described previously [35] on a total of 3 ug of DNA. In MCF-10A, MCF-7 and MDA-231, The genomic mir-496 locus was amplified with a nested PCR using primers in Table 1 (n = 50 clones). PCR products were then cloned into PCR2.1 using a TOPO TA cloning kit (Invitrogen, Cat. # K4500-02) and sequenced with a Beckman Coulter CEQ sequencer. Bisulfite mapping of the transiently transfected hsa-mir-496-pCpGl Luciferase expression plasmid was carried out using pyrosequencing with a first round of outer PCR primers located within the pCpGl construct (to differentiate between the exogenous transfected promoter and the endogenous promoter) and a second round of inner PCR within the cloned mir-496 sequence with biotinylated primers in Table 1. Pyrosequencing was carried out directly on the PCR product using a Biotage Q24 Pyrosequencer according to the manufacturer’s guidelines. Data was analyzed with Pyromark Q24 Software.

Statistical Analysis

Statistical analysis for bisulfite mapping and qPCR data was performed using unpaired \( t \)-tests. Each value represents the mean ± S.E.M. of three independent experiments. The results were considered statistically significant when \( *P<0.05, **P<0.001, ***P<0.0001 \).

Results

Over expression of MBD2 triggers demethylation and activation of hsa-mir-496

MCF-10A, MCF-7 and MDA-MB-231 mammary cell lines were used in our study since they express varying levels of endogenous MBD2 (Fig. 1A Fig. 1C) ranging from low (MCF-10A) to high, (MDA-MB-231). Deregulation of MBD2 expression was previously observed in tumors. For example Stefanska et al reported that MBD2 expression is highly elevated in hepatocel-
lular carcinoma biopsies relative to control normal liver tissue. Similarly Billard et al. reported that in invasive breast ductal carcinoma the level of MBD2 expression is significantly associated with tumor size [36]. We then chose the cell line with the lowest level of MBD2 (MCF-10A) (Fig. 1A) as a model system to determine the impact of increased MBD2 expression level on the methylome by overexpressing ectopic MBD2 in these cells (Fig. 1C). This cell line was then subject to methylated DNA immunoprecipitation and array hybridization (and mRNA expression array analysis). From our meDIP array we identified a panel of demethylated microRNAs in response to MBD2 overexpression (Table 2). We focused our study in this paper on hsa-mir-496 which showed robust demethylation by ectopic expression of MBD2 (Fig. 1 B,D). It is clear however that levels of MBD2 per se are not exclusively determining the steady state levels of hsa-mir-496 since MCF-7 cells express lower levels of hsa-mir-496 (Fig. 1D) than MCF-10A cells despite their higher levels of endogenous MBD2 (Fig. 1C). Nevertheless, endogenous MBD2 is required for expression of hsa-mir-496 cells in both MCF-7 and MDA-MB-231 cells since depletion of MBD2 results in concomitant reduction of hsa-mir-496 expression (Fig. 1D).

| microRNA | Differential Methylation | p-value |
|----------|--------------------------|---------|
| hsa-mir-369/hsa-mir-409/hsa-mir-410/hsa-mir-412 | −1.84488999 | 0.003090962 |
| hsa-mir-LET7D | −1.705679801 | 0.002119279 |
| hsa-mir-369/hsa-mir-409/hsa-mir-410/hsa-mir-412 | −1.467069246 | 0.00059518 |
| hsa-mir-496 | −1.108922459 | 0.002357951 |
| hsa-mir-7-1 | −1.029150729 | 0.005266684 |
| hsa-mir-554 | −1.008874183 | 0.000642931 |
| hsa-mir-487A | −0.939064743 | 0.005950832 |
| hsa-mir-195/hsa-mir-497 | −0.873277823 | 0.004407324 |
| hsa-mir-556 | −0.85831035 | 0.003958848 |
| hsa-mir-138-1 | −0.826516597 | 0.003580814 |
| hsa-mir-299/hsa-mir-411 | −0.79663597 | 0.005423464 |
| hsa-mir-1258 | −0.664067098 | 0.005363877 |
| hsa-mir-30B | 0.829194578 | 0.005779723 |
| hsa-mir-1197/hsa-mir-323/hsa-mir-329-1/hsa-mir-380/hsa-mir-758 | 0.884900076 | 0.005776804 |
| hsa-mir-105-1 | 0.902827667 | 0.002761197 |
| hsa-mir-145 | 0.929830403 | 0.004033838 |
| hsa-mir-181A1/hsa-mir-181B1 | 0.952147603 | 0.002793798 |
| hsa-mir-376C/hsa-mir-654 | 0.962272074 | 0.000988923 |
| hsa-mir-LET7G | 0.976118085 | 0.004514429 |
| hsa-mir-376C/hsa-mir-654 | 0.993443213 | 0.001503151 |
| hsa-mir-95 | 1.018465615 | 0.005226545 |
| hsa-mir-218-2 | 1.195031708 | 0.001063377 |
| hsa-mir-122 | 1.245473815 | 0.002277151 |
| hsa-mir-197 | 1.26353292 | 0.003891208 |
| hsa-mir-299/hsa-mir-379/hsa-mir-411 | 1.264605864 | 0.001428673 |
| hsa-mir-218-2 | 1.407366769 | 0.000159035 |
| hsa-mir-376C/hsa-mir-654 | 1.415468586 | 0.005711929 |
| hsa-mir-376C/hsa-mir-654 | 1.437384831 | 0.004117179 |
| hsa-mir-145 | 1.927577721 | 0.001394248 |
| hsa-mir-609 | 3.152198973 | 0.002154375 |

We then validated the demethylation of hsa-mir-496 in response to expression of MBD2 as predicted by the DNA methylation array (Fig. 1B). The microRNA hsa-mir-496 5′ region upstream to the transcription start site (TSS) (Fig. 2A) is highly methylated in control MCF-10A and MCF-7 cells and is hypomethylated in MDA-MB-231 which express higher levels of hsa-mir-496 (Fig. 2B) as determined by DNA methylation mapping analysis using bisulfite converted DNA. We show that expression of ectopic MBD2 in MCF-10A cells results in almost complete demethylation of the hsa-mir-496 promoter (results of bisulfite analysis in Fig. 2D).

To determine whether MBD2 directly interacts with hsa-mir-496 promoter in the human breast cancer cell line examined, we performed a chromatin immunoprecipitation with an MBD2 antibody as previously described and amplification with primers covering the transcriptional start site (TSS) [15]. Our assay demonstrates binding of MBD2 within the (TSS) (−211 to +246) of hsa-mir-496 that correlates with MBD2 expression in the different cell lines (Fig. 2C). MBD2 binding to the hypomethylated hsa-mir-496 promoter in MDA-MB-231 (Fig. 2C) is higher than its binding to the methylated promoter in MCF-10A and MCF-7 cells, consistent with a role for MBD2 in interacting with an active and
demethylated hsa-mir-496 (Fig. 2C). Ectopic expression of MBD2 results in dramatically increased binding of MBD2 to the region proximal to the TSS (Fig. 2C) and loss of DNA methylation of the same region (specifically CpGs at $-17$, $-15$, and $-6$ are demethylated in MCF-10A cells transfected with MBD2) (Fig. 2D).

To determine whether endogenous MBD2 plays a role in hsa-mir-496 DNA methylation we measured the effects of depletion of MBD2 mRNA in two breast cancer cell lines MCF-7 and MDA-MB-231 that express significant levels of MBD2 (Fig. 1C). Hsa-mir-496 is highly methylated in MCF-10A cells and we reasoned that depletion of MBD2 in these cells would have very little further impact on DNA methylation. SiRNA knock down achieved significant MBD2 depletion of 80% in MCF-7 and 60% in MDA-MB-231 (Fig. 1C for qPCR) and depletion of MBD2 binding to the TSS of hsa-mir-496 in both cell lines (Fig. 2C). Following MBD2 knockdown in MDA-MB-231 cells methylation at the hsa-mir-496 promoter is significantly increased at $-40$ while remaining sites are relatively hypomethylated following depletion of MBD2 (Fig. 2F). In MCF-7 cells, which express lower levels of MBD2 than MDA-MB-231 cells the hsa-mir-496 promoter is heavily
methylated except at site −103 which is completely hypomethylated. This site is completely methylated in response to MBD2 depletion (Fig. 2E) while sites at +40 and +16, which are fully methylated in these cells, are partially demethylated following MBD2 depletion (Fig. 2E). In summary, endogenous MBD2 depletion results in alteration of the DNA methylation state of hsa-mir-496 with increased methylation of specific sites as well as demethylation of other sites. Interestingly, different sites are impacted in response to MBD2 depletion in different breast cancer cell lines. However, in both cases MBD2 depletion resulted in concomitant partial reduction in hsa-mir-496 expression (Fig. 1D; 1.76-fold and 3-fold decrease in MCF-7 cells and MDA-MB-231 respectively). It is unclear whether these partial changes in DNA methylation are involved in the inhibition of MBD2 expression or whether MBD2 has an impact on hsa-mir-496 expression that is independent of DNA methylation.

MBD2 activates methylated hsa-mir-496 promoter activity in a luciferase reporter transient transfection assay

Our data derived from forced expression of MBD2 in MCF10A cells suggested that MBD2 expression could activate and lead to demethylation of hsa-mir-496 (Fig. 1 and 2). To directly test the hypothesis that MBD2 could bind and activate the hsa-mir-496 promoter and alter its state of methylation we used a transient transfection reporter assay. The hsa-mir-496 region overlapping the predicted TSS, whose state of methylation was examined by bisulfite mapping in Fig. 2B–F, was cloned into CpG-free pCpGl – luciferase reporter (hsa-mir-496-pCpGl) (Fig. 3A). This construct has CG DNA methylation target sequences only in the hsa-mir-496 promoter region and the assay is therefore not confounded by vector DNA methylation and measures directly the effects of DNA methylation and MBD2 on the transcriptional activity of this region as well its state of methylation. We first demonstrate that this region directs transcriptional activity as indicated by the fact that the region cloned in the sense orientation directed 45-fold higher luciferase activity than in the antisense direction (Fig. 3B).
methyltransferase which recapitulates the situation in MCF-10A cells reduced luciferase activity 59-fold relative to the unmethylated control supporting the conclusion that methylation of CG sites in the hsa-mir-496 promoter region silenced its activity (Fig. 3B). The transcriptional activity of methylated hsa-mir-496-pCpGl increases when the methylated reporter is co-transfected with MBD2 expression vector as compared with an empty backbone (pEF6) (1.8 fold) and does not increase with an MBD2 methylated DNA binding domain (mtMBD2) (Fig. 3C). A ChIP assay with an anti-MBD2 antibody demonstrated that MBD2 directly interacts with the ectopic hsa-mir-496-promoter region (our primer set selectively amplifies ectopic hsa-mir-496 (see description in materials and methods) but not to a distal region in the vector, which served as a negative control (Fig. 3D).

We then tested whether the binding of MBD2 to ectopic hsa-mir-496 promoter results in a change in its state of methylation. Any demethylation detected must be active since the plasmid is transiently transfected and does not contain an origin of replication. We captured the ectopic hsa-mir-496 promoter DNA molecules that were interacting with MBD2 (in both empty vector transfection where the transfected hsa-mir-496 was interacting with endogenous MBD2 and ectopic MBD2 transfectants which had an excess of ectopically transfected MBD2) by ChIP using anti MBD2 antibody and treated the captured DNA with sodium bisulfite. The bisulfite converted ectopic hsa-mir-496 promoter was amplified with specific primers (that don’t amplify the endogenous gene, see Table 1) and was subjected to pyrosequencing. The results presented in Fig. 3E show that sites −44, −40, −17, −15, −6 and +16 in methylated-hsa-mir-496-pCpGl were demethylated in endogenous MBD2 bound DNA (Fig. 3E, empty and dark box) when compared to naked methylated DNA (grey box). Ectopic expression of MBD2 increased demethylation at −44, −40, −17, −15, and −6 and +16 suggesting that increasing levels of MBD2 over endogenous levels enhanced the extent of demethylation of transiently transfected hsa-mir-496 promoter bound to MBD2 (Fig. 3E dark box). Since our assay measures demethylation in molecules that are physically bound to the MBD2 protein, these results show that binding of MBD2 to the hsa-mir-496 promoter is associated with site specific demethylation.

**Figure 4. Repressed targets of MBD2 in MBD2 overexpressing cells are putative targets of hsa-mir-496.** (A) CTSH expression in MBD2 overexpressing MCF-10A [+] and siMBD2 depleted MCF-7 [−] and MDA-231 cell lines and controls [C]. (B) POU2F3 expression in MBD2 overexpressing MCF-10A [+] and in response to transient depletion of MBD2 [−] in MCF-7 and MDA-231 and controls [C]. (C) PTGS1 expression in MBD2 overexpressing MCF-10A [+] and in response to transient depletion of MBD2 [−] in MCF-7 and MDA-231 and controls [C]. (D) hsa-mir-496 expression as determined by QPCR analysis in LNA treated MCF-10A, MCF-7 and MDA-MB-231 cells. (E) CTSH expression in response to transient knockdown of hsa-mir-496 in MCF-10A overexpressing MBD2, MCF-7 and MDA-231 and controls. (F) POU2F3 expression in a transient knockdown of hsa-mir-496 in MCF-10A overexpressing MBD2, MCF-7 and MDA-231 and controls. (G) PTGS1 expression in a transient knockdown of hsa-mir-496 in MCF-10A overexpressing MBD2, MCF-7 and MDA-231 and controls.

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Genes silenced in response to MBD2 overexpression are targets of hsa-mir-496

We tested the hypothesis that MBD2 would repress specific genes through activation of microRNA, in silico scanning using mirANDA identified a set of predicted targets of hsa-mir-496. We then tested whether some of these genes were silenced in MCF-10A cells that overexpressed MBD2 (Table S2) by examining an Affymetrix gene expression array of control and MBD2 overexpressing MCF-10A cells. In a preliminary scan we examined by qPCR 20 mRNAs. We then tested whether these 20 target mRNAs would also be affected by changes in MBD2 in other cell lines. Three of these 20 genes that are in silico targets of hsa-mir-496 that were found to be silenced in response to MBD2 overexpression were also regulated by MBD2 in other cell lines: Cathespin H (CTSH), POU domain class 2 transcription factor 3 (POU2F3) and prostaglandin-endoperoxide synthase 1 (PTGS1). CTSH, POU2F3 and PTGS1 were all down regulated in MCF-10A cells in response to

Figure 5. Ingenuity pathway analysis of putative targets of the MBD2-hsa-mir-496 pathway in MCF-10A cells overexpressing MBD2. (A) A list of genes repressed by MBD2 overexpression in MCF-10A cells was compared to a computed list of hsa-mir-496 targets (miRANDA) and subjected to Ingenuity pathway analysis. (B) Associated network functions identified a network with a role in cell migration and haptotaxis. Down regulated mRNA and putative hsa-mir-496 targets are highlighted in bold and light blue outline. Data were analyzed through the use of IPA (Ingenuity Systems, www.ingenuity.com). doi:10.1371/journal.pone.0074009.g005
MBD2 overexpression (3.4-fold, 10.7-fold and 2.13-fold respectively Fig. 1 A–C). Upon endogenous MBD2 depletion CTSH and POU2F3 were upregulated in MCF-7 (1.61-fold and 1.56-fold, respectively) and MDA-MB-231 cells (1.93-fold, and 1.91-fold respectively) (Fig. 4A–C). This supports the conclusion that endogenous MBD2 is indeed involved in silencing of these genes.

We then determined whether the effect of up or down regulation of MBD2 on expression of these genes was mediated by hsa-mir-496. We depleted hsa-mir-496 with a locked nucleotide antisense oligonucleotide targeting hsa-mir-496 (using a scrambled LNA as a control) in MCF-10A that overexpress ectopic MBD2 as well as MCF-7 and MDA-MB-231 cells which express endogenous MBD2 (Fig. 4D). Following hsa-mir-496 knockdown, CTSH, POU2F3 were induced in all three cell lines as expected if these genes are downregulated by hsa-mir-496 (Fig. 4C,E) while PTGS1 was induced in MCF-10A cells overexpressing MBD2 and MDA-MB-231 cells expressing high level of MBD2 but not in MCF-7 cells (Fig. 4G). These data are consistent with the hypothesis that CTSH and POU2F3 suppression by MBD2 is mediated by hsa-mir-496 while PTGS1 regulation by MBD2 seems to involve other factors in MCF-7 cells.

Down regulated mRNAs with MBD2 overexpression identify networks of putative targets of hsa-mir-496

Expression analysis of mRNA in MCF-10A stably overexpressing MBD2 identified 5129 genes that were significantly (p < 0.005) repressed (<0.9 ratio fold change) in comparison with control MCF-10A cells (Table S1). Cross-referencing this list with a computed list of putative hsa-mir-496 targets (using miRanda) identified a dataset of 141 (Table S2) genes repressed by MBD2 that are putative targets of hsa-mir-496. We used Ingenuity Pathways Analysis (IPA) suite to delineate the gene networks that the genes in the list fall into. IPA identified networks with a unique role in cell movement, antigen presentation, cell cycle and cell death (Fig. 5A). Associated network functions identified down regulated mRNAs integral to pathways that promote migration (p = 6.79E-5 - 9.1E-5) and haptotaxis (p = 8.62E-5 - 2.94E-2) (Fig. 5B). Interestingly, down regulation of MBD2 was previously shown to reverse invasiveness and metastasis in breast cancer [37] and prostate cancer cell lines [14].

Discussion

The cis silencing of gene expression by DNA methylation is very well documented. Methylated DNA binding proteins including MBD2 bind methylated CGs and recruit chromatin repressor complexes that silence in cis gene expression [7]. The data presented here delineates a potential novel pathway of gene regulation by MBD2 that amplifies a DNA methylation signal by affecting downstream genes through mechanisms that don't necessarily require cis-DNA methylation in the affected genes.

We provide here several lines of evidence that in combination provide strong support for this hypothesis. We show first that ectopic MBD2 expression in untransformed epithelial cells results in upregulation of a microRNA hsa-mir-496 (Fig. 1D). Second, expression of hsa-mir-496 is partially dependent on endogenous MBD2 in two breast cancer cell lines; partial depletion of MBD2 results in reduction of hsa-mir-496 expression (Fig. 1D). Third, overexpression of MBD2 in MCF-10A cells triggers demethylation of the promoter of hsa-mir-496 (Fig. 2D). Fourth, MBD2 interacts with chromatin at the hsa-mir-496 promoter as determined by a ChIP assay, which is consistent with the hypothesis that MBD2 activates hsa-mir-496 directly in cis rather than the alternative hypothesis that MBD2 activation of hsa-mir-496 is mediated through MBD2 repressive activity on a putative trans acting repressor gene (Fig. 2B). Fifth, a luciferase reporter assay demonstrates that the hsa-mir-496 promoter region per se is silenced by DNA methylation and that it is activated with ectopic expression of MBD2 (Fig. 3 B and C). Sixth, MBD2 targets and binds an ectopic hsa-mir-496 promoter (Fig. 3D). This provides further evidence for direct action of MBD2 on the hsa-mir-496 promoter as an MBD2 antibody pulls down the hsa-mir-496 DNA region and not other regions on the vector. Seventh, the physically MBD2 bound ectopic hsa-mir-496 promoter molecules are partially demethylated delineating a tight relationship between MBD2 binding and DNA demethylation of the hsa-mir-496 promoter (Fig. 3E). Taken together these results are consistent with the hypothesis that MBD2 could regulate hsa-mir-496 promoter activity and its DNA methylation state.

Although our data shows that interaction of ectopic MBD2 with the hsa-mir-496 promoter results in demethylation, our data does not directly demonstrate that MBD2 is demethylating hsa-mir-496 promoters in cells nor does it claim that MBD2 is involved in its demethylation. Our data is also consistent with the alternative possibility that MBD2 interaction with this promoter results indirectly in recruitment of other mechanisms that were recently proposed to cause DNA demethylation such as complexes including TET enzymes and base excision repair activity (BER) [38] or hydroxymethylation mediated [39] or direct demethylation by DNMTs [40]. Although we have no evidence for these indirect mechanism in the case of hsa-mir-496 promoter, further experiments are required to test this hypothesis that are beyond the scope of this paper.

We also show in two breast cancer cell lines that endogenous MBD2 is required for expression of hsa-mir-496 since depletion of MBD2 results in reduction of hsa-mir-496 expression (Fig. 1D). However, not surprisingly the expression of hsa-mir-496 in these cells is not determined exclusively by the levels of MBD2. MCF10A cells express higher levels of hsa-mir-496 than MCF-7, which have higher steady-state levels of MBD2 (Fig. 1 A, C, D). Genes are known to be regulated by networks of factors that differ from cell type to cell type, nevertheless in both cell lines expression of MBD2 is required for expression.

In contrast to over expression of MBD2 in MCF-10A cells which results in a dramatic hypomethylation, depletion of MBD2 in MCF-7 and MDA-MB-231 cells results in very limited hypermethylation in spite of a significant decrease in expression of hsa-mir-496. This is consistent with the conclusion that MBD2 is required for hsa-mir-496 activity independently of the state of methylation. However, it is also possible that our assay wasn’t sensitive enough to detect partial changes in DNA methylation in response to partial depletion of MBD2. The fact that MBD2 depletion results in both increase and decrease in DNA methylation of different sites suggests that MBD2 has a complex effect on the state of methylation.

A single microRNA has several hundred putative targets in the transcriptome. This allows for amplification and coordination of gene regulation events in the cell. Thus, activation of one microRNA could result in suppression of several RNAs. It has been previously shown that DNA methylation regulates expression of several microRNAs and that hypermethylation of microRNA silences them particularly in cancer leading to gene activation [41–43]. However, a microRNA dependent pathway of suppression of gene expression by activation of a microRNA by a methylated DNA binding protein has not been described before. The presence of such a pathway can explain the paradoxical observation that treating cells with DNA methylation inhibitors could result in not only in gene induction but gene silencing as well.
While here we present a case for the activation of a microRNA through binding of a methylated DNA binding protein that involves promoter demethylation, it most probably involves several other mechanisms including recruitment of transcriptional machineries [46,47] and histone complexes [48]. Further experiments are required to unravel these additional events regulating hsa-mir-496 expression in different cell types.

The genes that were validated here as targets of hsa-mir-496 are known to be involved in different aspects of cancer progression. CTSH (Cathespin H) is a cysteine protease whose activity is often upregulated during cancer metastasis [49]. It is surprising therefore that this gene is downregulated by MBD2 and hsa-mir-496 in highly invasive breast cancer cells MDA-MB-231. It is clear however that what defines the metastatic state is not a select list of genes but a complex network and the overall output of the network. On the other hand downregulation of CTSH has been observed during osteolyis in highly metastatic breast cancers which is consistent with a role in promoting metastasis [50]. POU2F3 is a transcription factor that has been largely silenced in cervical cancer [51] and has been highlighted as a tumor suppressor gating the transformation of primary cell lines to metastatic melanomas [52]. Its silencing by overexpression of MBD2 through hsa-mir-496 is consistent with a role in cancer. These data suggest a different mechanism for suppression of tumor suppressors in cancer than the known mechanism of suppression by cis DNA methylation; long-range suppression through demethylation of regulatory microRNA. PTGS1 is involved in prostaglandin synthesis and is deregulated in pancreatic cancer [53].

A limitation of our studies is that we only used mir-496 antagonists in the current study. Follow up studies should focus on other assays to strengthen this relationship (3’UTR assays, exogenous mir-496, etc.).

In addition to experimentally validating several targets of hsa-mir-496, by cross-referencing of hsa-mir-496 in silico targets with down regulated mRNAs in MBD2 overexpressing MCF-10A cells we derived a list of 141 genes whose repression is potentially downstream to the MBD2-hsa-mir-496 pathway. Ingenuity Pathway analysis of this list revealed highly significant functional gene networks involved in cellular movement, cell cycle, cell death and antigen presentation (Fig. 5A). These are molecular pathways that are potentially involved at different stages of cancer progression. Within this subset we looked directly at the pathways of down regulated mRNAs and putative targets of hsa-mir-496 to identify a possible role in migration and haptotaxis. Future studies need to test the hypothesis that this is a mechanism for a coordinated repression of important gene networks in cancer by DNA methylation regulators such as MBD2.

In summary, our data points to the intricate ways by which DNA methylation and its binding proteins could regulate gene expression. Several genome-wide studies have tried to correlate overall gene expression patterns and cis-DNA methylation states. Invariably, these are not perfect correlations. Although these inconsistencies could easily be explained by DNA methylation independent mechanisms, our data shows that bona fide DNA methylation regulators such as MBD2 could trigger a sequence of gene expression events downstream from the initial cis acting DNA methylation signals (Fig. 6 for model). The data illustrates how a DNA methylation signal in a single region could be amplified and affect multiple downstream targets without necessarily altering their state of methylation. If the targets fall into discrete functional pathways (Fig. 5) this mechanism could coordinate responses to single DNA methylation regulators such as MBD2. Similarly, pharmacological demethylation as well as global hypomethylation in cancer and other diseases must not result in gene induction exclusively as is commonly thought, but could result.
in gene repression as well through pathways such as the one delineated here. Future computations of the impact of DNA methylation in genome-wide and transcriptome wide studies need to take into account these hierarchies of gene expression-repression that could be triggered by single demethylation events.

Supporting Information

Table S1 Repressed transcripts following MBD2 over-expression in MCF-10A.

Table S2 Repressed transcripts following MBD2 over-expression in MCF-10A that overlap with putative targets of hsa-mir-496.

Author Contributions

Conceived and designed the experiments: SA M. Szyf. Performed the experiments: SA JW. Analyzed the data: SA M. Suderman. Contributed reagents/materials/analysis tools: SAA. Wrote the paper: M. Szyf SA.

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