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Methyl Binding Domain Protein 2 (MBD2) dependent proliferation and survival of breast cancer cells

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Abstract

Methyl Cytosine Binding Domain Protein 2 (MBD2) has been shown to bind to and mediate repression of methylated tumor suppressor genes in cancer cells, where re-patterning of CpG methylation and associated gene silencing is common. We have investigated the role of MBD2 in breast cancer cell growth and tumor suppressor gene expression. We show that stable shRNA mediated knockdown of MBD2 leads to growth suppression of cultured human mammary epithelial cancer lines, SK-BR-3, MDA-MB-231, and MDA-MB-435. The peak anti-proliferative occurs only after sustained, stable MBD2 knockdown. Once established, the growth inhibition persists over time and leads to a markedly decreased propensity for aggressive breast cancer cell lines to form *in vivo* xenograft tumors in BALB/C nu/nu mice. The growth effects of MBD2 knockdown are accompanied by de-repression of tumor suppressor genes including DAPK1 and KLK10. Chromatin immunoprecipitation assays and bisulfite sequencing demonstrate MBD2 binding directly to the hyper-methylated and CpG-rich promoters of both DAPK1 and KLK10. Remarkably, the promoter CpG-island associated methylation of these genes remained stable despite robust transcriptional activation in MBD2 knockdown cells. Expression of a shRNA-resistant MBD2 protein resulted in restoration of growth and re-silencing of the MBD2 dependent tumor suppressor genes. Our data suggest that uncoupling CpG-methylation from repressive chromatin remodeling and histone modifications by removing MBD2 is sufficient to initiate and maintain tumor suppressor gene transcription and suppress neoplastic cell growth. These results demonstrate a role for MBD2 in cancer progression and provide support for the prospect of targeting MBD2 therapeutically in aggressive breast cancers.

Keywords

Breast; Cancer; MBD2; DNA; Methylation

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Introduction

Global hypomethylation of chromosomal DNA along with selective hypermethylation of specific gene promoters lead respectively to chromosomal instability and down regulation of tumor suppressor genes, both of which can promote cancer development and progression (1–3). The observation that CpG islands at the promoters of tumor suppressor genes are often methylated provides a mechanistic rationale for the clinical application of DNA methylation inhibitors such as decitabine and azacytidine (4, 5). The clinical utility of these agents has been limited somewhat by the potential for carcinogenicity, toxicity, and opposing off-target effects associated with non-specific global demethylation (6–8). Alternate strategies for mitigating the effects of abnormal DNA methylation in tumors would create attractive new approaches for targeted therapy.

The repressive effects of DNA methylation on gene expression are mediated in large part by Methyl-CpG Binding Proteins (MCBPs) (9–12). There has been interest in exploiting the function of MCBPs to selectively interrupt DNA methylation dependent changes in gene expression in tumors. In order to validate their utility as therapeutic targets, further research to adequately characterize the function of MCBPs in human neoplasia is required.

One member of the MCBP family, Methyl Cytosine Binding Domain Protein 2 (MBD2), has emerged in the context of cancer for several reasons. MBD2 binds densely methylated DNA with higher affinity than other known MCBP's and has been shown to act upon numerous tumor suppressor gene targets (13–15). In addition, this protein has been shown to exacerbate mouse models of intestinal carcinogenesis by increasing tumor size and number (16). Importantly, complete loss of MBD2 in the mouse does not appear to generate any major deleterious effect (17). The latter finding suggests that MBD2 is not required for survival either before or after development in normal tissues and therefore could be a selective target for therapy in tumors, where MBD2 may be playing an acquired pathologic role (18).

While abnormal DNA methylation has been reported in virtually all human cancers, breast cancer is of distinct interest for several reasons. It is now well established that CpG island hypermethylation is an early event in mammary epithelial cell transformation (19–21). At such early time points, prevention is the main therapeutic goal and arresting progression of the primary lesion remains a viable strategy. Furthermore, genetic testing and improved screening methods for breast cancer have dramatically increased the probability of successfully implementing an early intervention strategy. Finally, aggressive forms of breast cancer (e.g., triple-negative breast cancer) are often poorly responsive to conventional treatment and biologically targeted ancillary approaches for treatment are needed (19, 22). The primary rationale behind the body of work reported here was to explore the role that MBD2 plays in mammary tumor growth and progression and thus its potential as a therapeutic target.

Materials and Methods

Lentivirus cloning and infection

Lentiviral vector design and packaging was accomplished as previously described (23). Briefly, the lentivirus system was used to mediate the efficient delivery, integration and stable expression of shRNA that was rapidly FACS selectable. The bicistronic pLV-TM vector allows for the simultaneous expression of a transgene and GFP marker to facilitate tracking of transduced cells. Vectors were engineered to contain the shRNA expression cassette flanked by cis-acting elements necessary for its encapsidation, reverse transcription and integration. The packaging method used minimizes the risk of emergence of replication

competent recombinants and avoids problems linked to promoter interference (23, 24). In order to insert shRNA hairpin forming oligos the pLVTHM vector was digested with MluI and ClaI and double-stranded shRNA oligonucleotides were designed with the appropriate complementary ends.

MTT Growth Assay

The MTT Assay was performed as described previously (25). Briefly, cells were trypsinized and diluted to between 50,000 and 100,000 cells/ml. 100ul of cells were plated in each well of a 96 well culture dish (5000 to 10000 cells). At the time of assay analysis, 20ul of a 5mg/ml filtered solution of Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma Aldrich) was added to each well. Dishes were incubated for 2–4 hours and media was removed. Cells and formazan crystals were solubilized in acid isopropanol, 150ul per well (4mM HCl, 0.1% NP-40 in isopropyl alcohol). Absorbance was read on a 96 well plate reader at 595nm with a reference read at 620nm. All absorbances were normalized to a standard dilution curve and reported as relative cell densities.

SYBR Green fluorescence based quantitative real-time PCR

Gene specific mRNA levels and enrichment by ChIP were both measured using quantitative PCR essentially as described previously (26, 27). Gene quantification primers were designed either manually, using the PrimerQuest tool (Integrated DNA Technologies), or auto-generated using the qPrimerDepot tool (<http://primerdepot.nci.nih.gov/>) (28). All primers were verified through a 4 log range using serial dilutions of reference cDNA or gDNA followed by standard curve analysis. A list of qPCR primers is given in online supplemental data. All amplifications were performed using 40 cycles of 'standard' two step PCR plus a dissociation curve on either an ABI 7300 or ABI 7900 HT instrument (Applied Biosystems). All reactions were performed in 96 or 384 well microtiter plates using SYBR green chemistry. See supplemental methods for quantitative PCR data analysis.

Xenograft growth and *in vivo* bioimaging of nude mice

Human tumor xenograft growth was assayed essentially as described previously (29, 30). For xenografts, 8 week old female Balb/C nu/nu mice were injected with 5×10^5 or 1×10^6 cells (GFP positive, SC and KD FACS purified MDA-MB-231 and MDA-MB-435). Cells were suspended in a 1:1 DMEM:lrBME mixture (Gibco, Carlsbad, CA and Cultrex Inc.) and a 50ul bolus was injected with a 25½ gauge needle and 1cc syringe into the subcutaneous space overlying the dorsolateral aspects of both hind flanks. Each mouse acted as its own control, receiving scramble (left) and knockdown (right) cells in opposite flanks. Mice were examined 2–3 times per week until the appearance of a palpable mass. Upon the detection of a palpable tumor, caliper measurements and bioimaging using GFP fluorescence filters (IVIS 200, Xenogen Corp. Alameda, CA) were performed every second day. Standard IVIS 200 fluorescence acquisition settings were used including an exposure time between 4–10 seconds, background subtraction, medium binning, and a 1.5cm focal height. A constant plane of anesthesia was maintained by metered nose cone delivery of aerosolized isoflurane in 1–2 liters/min oxygen and verified at regular intervals by respiratory monitoring. Approval for all animal work was obtained from the Institutional Animal Care and Use Committee and all procedures were conducted according to VCU DAR guidelines.

Chromatin Immunoprecipitation Assay

Chromatin Immunoprecipitation assays were performed essentially as described previously (27, 31). Briefly, cross-linked chromatin was sheared (Immersion sonicator, Diagenode, Sparta, NJ) according conditions optimized to produce fragments between 200 and 500 base pairs. Precipitations were performed using chromatin from approximately 1×10^6 cells at 4

degrees C, overnight using 4µg of affinity purified ChIP grade antibody (sc-12444, Santa Cruz Biotechnology, Inc., CA). In our experience, background and non-specific genomic DNA contamination was reduced by using non-porous magnetic protein-G coated beads (Active Motif, Carlsbad, CA). SYBR green based qRT-PCR was used to measure target sequence enrichment (~200–500bp resolution promoter proximal CpG rich regions) in column-purified, immunoprecipitated DNA.

Cloning of a mutant MBD2 expression construct

A full length MBD2 clone was obtained from the IMAGE Consortium and the coding sequence was subcloned into the pCDNA 4.0 plasmid using PCR amplification to introduce restriction complementary ends and followed by ligation into a TA cloning vector, amplification, BamH1-HindIII restriction product purification, and ligation. Site directed mutagenesis was performed to generate a shRNA immune mutant using the QuickChange II kit (Stratagene, La Jolla, CA). A single, amino acid neutral third base mutation was introduced into the shRNA target sequence of the cDNA vector to allow expression in a stable shRNA expressing background. The resulting MBD2 protein was wild type with respect to amino acid sequence. Transfection using Lipofectamine Plus was followed by Zeocin selection of pooled clones (Invitrogen, Carlsbad, CA). Expression near endogenous levels in knockdown cells was confirmed by immunoblotting as described above.

Results

MBD2 knockdown leads to growth inhibition of breast cancer cells

Previous studies have shown the ability of transient transfection with MBD2 siRNA to relieve silencing of individual methylated tumor suppressor genes without a major effect on tumor growth (13, 32). We hypothesized that a rapid and sustained reactivation of tumor suppressor genes might be required for growth effects. To test this hypothesis, a lentiviral shRNA transduction assay which allowed rapid FACS based selection of stable knockdown populations was employed. This method resulted in populations with greater than 95% GFP positivity (Fig. 1A) and greater than 70% knockdown of MBD2 RNA and protein (Fig. 1, B and C). MBD2 knockdown led to significant growth inhibition relative to control virus transduced cells. MCF-7 cells were least sensitive to MBD2 knockdown, proliferating at approximately half the rate of controls (49%) as measured by MTT assay as well as serial coulter counting. Other breast cancer cell lines with more aggressive growth characteristics were significantly more sensitive to MBD2 deficiency; anti proliferative effects ranged from 77% for MDA-MB-231; 92% inhibition of SK-BR-3; and 94% inhibition of MDA-MB-435 cells (Fig. 1, D and E). Interestingly, anti-proliferative effects required between 5–7 days of sustained MBD2 protein deficiency to manifest, and the peak effect took 10–14 days to develop. The delayed kinetics of growth inhibition and the predominantly anti-proliferative as opposed to cytotoxic effect of MBD2 knockdown rendered clonogenic survival assays impractical to perform. Serial coulter counting and MTT assays of subconfluent populations were used to quantitatively assay delayed cell proliferation effects. Apoptosis and senescence assays were also performed; specifically PARP cleavage, AnnexinV Flow cytometry and beta-galactosidase staining were assayed with no significant increase in either apoptosis or senescence markers observed (data not shown).

In addition to decreased proliferation rates, MBD2 knockdown cells differed from control cells in their microscopic appearance. Knockdown cells assumed a morphology more closely resembling non-transformed human mammary epithelial cells, becoming less fusiform with a more consistent adherent border relative to control tumor lines (Figure S1, A). To determine whether MBD2 was promoting tumor associated epithelial dedifferentiation, the 3-dimensional morphology of knockdown MCF-7 cells was assayed in

3D culture using laminin-rich extracellular matrix extracts. MBD2 knockdown led to an increase in the proportion of clones which formed organized epithelial structures in 3D cultures. Stable MBD2 knockdown MCF-7 cells formed epithelialized 'mamospheres,' at a significantly increased frequency of 70% ([CI=0.55–0.83]), while untransfected and scrambled shRNA transfected controls formed fewer such structures, 46% ([CI=0.39–0.53], $p \leq 0.038$) and 37% ([CI=0.29–0.45], $p \leq 0.012$), respectively. (Figure S1, B, C, and D).

MBD2 knockdown inhibits xenograft tumor growth in BALB/c nu/nu mice

To determine whether MBD2 knockdown had any effect on the ability of aggressive breast cancer lines to form tumors in animals, MBD2 knockdown human breast cancer cells and scrambled shRNA control transformed lines were introduced subcutaneously into opposite flanks of 8 week old female BALB/c nu/nu nude mice. Tumor growth was subsequently assayed using standard caliper measurements as well as *in vivo* bio-fluorescent imaging of the GFP-positive xenograft tumors. MBD2 knockdown led to a striking decrease in tumor formation and growth of aggressively tumorigenic lines (MDA-MB-435 and MDA-MB-231) in nude mice. Mean GFP fluorescence and median tumor volume calculated from caliper measurements were significantly greater in control lines compared to MBD2 knockdown tumors for all time points following the initial appearance of a palpable tumor (Fig. 2, A–G).

MBD2 is required for selective tumor suppressor silencing in growth inhibited breast cancer cell lines

To explore the mechanism underlying MBD2 dependent growth inhibition and to establish the extent to which MBD2 is required for silencing of specific tumor suppressor genes in breast cancer cells, gene expression assays, MBD2 promoter occupancy, and promoter methylation status in MBD2 knockdown and control breast cancer cells was determined. Using high-throughput quantitative PCR, the expression of 48 tumor suppressor genes previously shown to be epigenetically silenced in breast cancer was assayed and a number of these genes were found to be significantly induced in MBD2 knockdown MDA-MB-231 and MDA-MB-435 cells. Among the most significantly de-repressed genes in knockdown cells were the Death Associated Protein Kinase (DAPK1) and Kallikrein 10 (KLK10) genes, Fig. 3A. Bisulfite conversion and sequencing demonstrated that the promoter CpG islands of both genes were heavily methylated in these breast cancer cell lines (Figure 3, C and D). The MBD2 knockdown breast cancer cells offered a unique system for interrogating the temporal dynamics of promoter methylation before and after transcriptional activation. Interestingly, the methylation status of these promoter-associated CpG rich regions remained remarkably stable, with no appreciable change even after robust transcriptional activation by MBD2 knockdown (Figure 3, C and D). Chromatin immunoprecipitation (ChIP) assays designed to quantify MBD2 occupancy at the promoter CpG islands of DAPK1 and KLK10 demonstrated MBD2 bound at the methylated promoter associated CpG-islands of both genes (Figure 3B). Binding of MBD2 to DAPK1 and KLK10 promoters was evident in scramble shRNA control (SC) cells; this binding was reduced to background levels in MBD2 knockdown (KD) cells as expected. A control gene (GAPD) was included to verify the absence of non-specific enrichment or genomic DNA contamination. By demonstrating MBD2 resident at the hyper-methylated promoter-associated CpG-islands of de-repressed targets, these assays support a model of direct transcriptional inhibition of selected CpG rich target genes by MBD2.

MBD2 knockdown reversibly and independently relieves silencing of a subset of tumor suppressor genes distinct from those induced by 5-aza-2-deoxycytidine

Pooled stable clones expressing shRNA against MBD2 in the human mammary epithelial carcinoma line, MCF-7 were selected and compared against identically derived control

shRNA expressing lines using a high throughput quantitative PCR assay that measures expression of multiple genes quantitatively and simultaneously. Once again, changes in the mRNA levels of 48 tumor suppressor genes previously shown to be epigenetically inactivated in breast tumors were assayed. For comparison additional samples were included to ascertain how MBD2 dependant changes compared to those induced by the DNA methyltransferase inhibitor, 5-aza-2-deoxy-cytidine (5-aza-dC). Quantitative PCR results were clustered into groups using self-organizing map analysis (Fig. 4A). Discrete clusters of tumor suppressor genes were induced by MBD2 knockdown primarily (n=7, 15%), 5-aza-dC primarily (4uM, n=30, 67%), or both in additive fashion (n=11, 24%, Fig. 4, A). The cluster of genes induced primarily by MBD2 included FHIT, HOXA5, DAPK1, GPC3, ADAM23, MASPIN, and THRB (fold induction ranged from 8.0 [GPC3] to 446.4 [DAPK1], $p < 0.0001$).

To further explore the role of MBD2 in silencing this discrete group of tumor suppressor genes, siRNA oligonucleotides targeted against each gene in the MBD2 selectively de-repressed cluster were transfected into pooled stable MBD2 knockdown MCF-7 clones. Specific and isolated knockdown of each individual MBD2 target gene was observed and in each case did not affect expression of any other gene in the qPCR assay panel (Figure 4B). These results suggest that genes in the MBD2 dependent cluster are regulated in parallel, non-overlapping pathways downstream of MBD2; that is, the genes are downstream targets of MBD2 and not of one another.

An shRNA-resistant MBD2 expression construct was generated by introducing a silent point mutation into the shRNA binding site of the MBD2 cDNA. The shRNA-resistant construct allowed restoration of functional levels of MBD2 protein in stable MBD2 shRNA expressing clones (Figure 4C). Restoration of MBD2 protein in knockdown clones led to resuppression of MBD2 dependent tumor suppressor gene expression (Figure 4D). This finding is consistent with the observed stability of promoter CpG methylation following MBD2 knockdown and further supports the hypothesis that MBD2 is acting as an upstream node with respect to a network of tumor suppressor gene targets in breast cancer.

Re-expression of MBD2 restores *in vitro* growth potential of breast cancer cells

To determine whether restoration of MBD2 protein led to a reversal of the growth inhibition observed in MBD2 knockdown lines, pooled stable shRNA-resistant MBD2 and vector control MDA-MB-435 clones were selected and subsequently infected with an shMBD2 targeting lentivirus as described above. Once again, the resistant MBD2 protein was expressed at significant levels in shMBD2 expressing cells (Figure 5A) while MBD2 levels remained low in shMBD2 transduced vector controls. The resistant MBD2 expressing cells were refractory to the growth inhibition imposed by shMBD2 lentivirus (Figure 5, B and C), demonstrating the effect of MBD2 knockdown on growth was both specific and reversible.

Discussion

Breast cancer cells like many tumor types accumulate epigenetic abnormalities during the progression from normal epithelium to frank carcinoma. A ubiquitous and early change in breast cancer is the abnormal hypermethylation of tumor suppressor associated CpG islands (3, 19, 33–35). Therapies that target global DNA methylation are under study but are incapable of discriminating between normal and pathologic DNA methylation and could therefore have untoward effects, especially during early stages of tumor progression. We have explored a more selective approach for reactivating transcription of hypermethylated gene targets that is specific, effective, and safe in non-neoplastic tissues. MBD2 has been shown to mediate silencing of methylated genes through targeted recruitment of a p66 α , Mi-2, and histone deacetylase containing Nuclear Remodeling and Deacetylase (NuRD) co-

repressor complex (36–38). Inhibition of MBD2 function provides an alternate, targeted approach for reactivating epigenetically silenced tumor suppressor genes in cancer.

Double stranded shRNA mediated knockdown of MBD2 was used to study the function of this protein in breast cancer cells and revealed that depletion of MBD2 led to changes in tumor suppressor gene expression and cell growth inhibition consistent with the hypothesis that breast cancer cell growth is dependent on this protein. The present studies of the gene expression changes induced by MBD2 knockdown in cultured breast cancer lines reveal insight into the function of this pleiotropic protein and provide a mechanistic framework for phenotypic changes. We now posit that MBD2 knockdown offers an alternative and more targeted approach to methylated tumor suppressor gene reactivation than global DNA methyltransferase inhibition. In support of this proposition, we found that only a subset of the tumor suppressor genes examined were transcriptionally reactivated in MBD2 knockdown breast cancer cells. Furthermore, patterns of gene expression in MBD2 knockdown cells differed from those induced by 5-aza-dC treatment. The finding that MBD2 knockdown and 5-aza-dC induce non-overlapping gene sets is further consistent with the notion that layered epigenetic mechanisms are responsible for maintaining tumor suppressor silencing and lends support to the idea that treatment with 5-aza-dC alone may be insufficient for global methylated gene reactivation, particularly at developmentally regulated and tissue specific genes marked by multivalent epigenetic modifications (39, 40). Additionally, it is reasonable to infer from these data that the function of other MBDs is not entirely redundant with that of MBD2 at least at a consequential subset of tumor suppressor genes in breast cancer. This is not surprising given published data which demonstrate that other MBDs are capable of binding to specific methylated and silent tumor suppressor genes (18, 41). Recent studies of the structure of MBD2 bound to a methylated gene target sequence suggest that sequence context may direct this preferential binding of MBD2 to certain CpG rich regions (42).

As expected, the promoter associated CpG islands of genes induced by MBD2 knockdown were heavily methylated, consistent with the proposed mechanism of action of this protein. Moreover, ChIP experiments confirm direct binding of MBD2 at the CpG-rich promoters of hypermethylated and silent tumor suppressor genes, once again implying CpG island-associated genes are being reactivated in MBD2 knockdown cell populations through depletion of promoter bound MBD2. Notably, the MBD2 knockdown model offers a powerful system for studying the temporal dynamics of methylation and transcription. Somewhat surprisingly, the methylation status of MBD2 bound regions did not change following depletion of MBD2 with ensuing transcriptional activation. This observation suggests either that passive demethylation is not a direct consequence of gene transcription or that it can be overwhelmed by active maintenance of CpG methylation at certain CpG rich sites.

Remarkably, MBD2 knockdown produced profound and sustained growth inhibition in highly aggressive human breast cancer cell lines in culture. In addition, the ability of aggressive breast cancer cell lines, including triple negative and Ras mutant lines, to form tumors in nude mice was significantly impaired by MBD2 knockdown. In the case of the MDA-MB435 line, growth was completely suppressed for at least one month. The antiproliferative effects in these breast cancer cells required a significant depletion (70%) but not complete ablation of MBD2. This is in agreement with the observation of Sansom and colleagues that knocking out a single allele of MBD2 reduced adenoma burden in *Apc^{Min}* mice (16).

A critical observation is the absolute requirement of persistent MBD2 knockdown to reproducibly inhibit the growth of breast cancer cells. In preliminary experiments in which

we transiently transfected siRNA, we achieved a rapid knockdown of MBD2 but the depletion was short lived and induced only a mild and transient effect on cell growth (Data not shown).

An integrating lentivirus-based stable shRNA delivery system proved to rapidly yield stable knockdown populations and was therefore used to determine the effect of MBD2 depletion growth. Interestingly, the maximum effect on growth that occurred after FACS purification of transduced cells took between 1 and 2 weeks to develop. We postulate that effects on downstream targets leading to the activation of cytostatic pathways required this lag period to reach peak activity although the exact basis for this lag remains unknown. It is interesting to speculate whether a gradual epigenetic reprogramming is occurring in MBD2 knockdown tumor cells, following a delayed onset model analogous to the reprogramming of iPS cells (43, 44).

The observation that sustained depletion of MBD2 for 7–14 days is required to suppress tumor growth provides a framework for revisiting previous studies using transient transfection methods which demonstrated MBD2 dependent tumor suppressor silencing without a corresponding major effect on tumor growth over a few days of observation (13, 41, 45, 46). Interestingly, recent and ongoing trials with low dose regimens of 5-aza-dC are yielding promising clinical results, suggesting that extended treatments leading to a delayed but durable response may provide a valuable model for effective application of epigenetic therapies in the future (47, 48).

Significant variation was observed among breast cancer cell lines with respect to the degree of growth suppression produced by MBD2 knockdown. These variations paralleled differences in patterns of CpG island methylation as well as differences in the spectrum of gene expression changes observed among MBD2 knockdown lines. Undoubtedly the full spectrum of phenotypic changes in a given cell line depends not only on the function of MBD2 but on variable cytosine methylation levels, differential expression of other epigenetic modifiers, and on coexisting genetic lesions that fundamentally cripple particular biochemical pathways (e.g., cell cycle arrest and apoptosis). Our results also suggest that the greatest effect of MBD2 knockdown on cell growth occurs in the most aggressive breast cancer lines including those with so-called triple negative and basal-like phenotypes. The dependence of MBD2 induced growth on patterns of methylation, specific biochemical pathways, or specific modifying factors in breast cancer warrants further study to determine whether these tumors might ultimately respond to MBD2 inhibition in patients.

MBD2 knockdown resulted in distinct changes in the morphology of breast cancer cells, which began to more closely resemble their normal counterparts in culture. Moreover, MBD2 knockdown clones of MCF-7 demonstrated a change in 3D morphology reminiscent of normal glandular epithelial architecture. This observation suggests that changes in morphology and proliferation are likely to be mechanistically linked under the influence of epigenetic forces. It is conceivable that the net function of MBD2 knockdown is to restore elements of normal epithelial differentiation in mammary derived tumors. Consistent with this notion is the acknowledged role of DNA methylation in tissue specific gene expression patterning during development (49, 50). This normal pattern of gene expression is fundamentally dysregulated in tumors, at least in part through epigenetic modifications. Of importance in determining whether MBD2 inhibition is a viable treatment strategy for human tumors is the need to account for the effects of MBD2 on normal tissues, for example normal epithelium and bone marrow. Such normal, mitotically-active tissues are known sites of off-target effects that limit the potential tolerability of cytotoxic therapy. There is good reason to believe MBD2 inhibition will not have major adverse effects on normal tissues based on the minimum phenotype of MBD2 null mice (9, 14, 51–53).

In summary, the work presented here strongly supports a pathological role for MBD2 in the growth and *in vivo* survival of breast cancer through its function in binding methyl-CpG and mediating the transcriptional inactivation of tumor suppressor genes. We find that stable MBD2 depletion by targeted shRNA leads to re-expression of selected tumor suppressor genes, a number of which appear to be directly regulated, suggesting a lack of redundancy in the inhibitory function of MCBP's on transcription of specific targets. Restored tumor suppressor expression is associated with growth inhibition, and a more organized pattern of growth in 3D culture models in addition to tumor clearance in animals. Based on the studies reported here, we conclude that targeting MBD2 may be a viable strategy for inhibiting the proliferation of human breast cancer. Given the striking effect in triple negative breast cancer cell lines, this approach may lead to much needed targeted therapy for these aggressive tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Stable shRNA mediated knockdown of MBD2 leads to growth inhibition of breast cancer cells in vitro

(A) Phase contrast LM (top) and GFP fluorescent micrographs (bottom) for MDA-MB-231, MDA-MB-435, and SK-BR-3 lines following transduction with GFP expressing MBD2 shRNA lentivirus. (B) Anti-MBD2 (α -MBD2) immunoblots, Coomassie stained PAGE gels shown as loading controls. Empty pLV-THM lentivirus packaging construct [Vector], scrambled shRNA lentivirus [shScramble], MBD2 targeting shRNA virus [shMBD2]. (C) Quantitative RT-PCR expression of MBD2, MBD2 knockdown (shMBD2) normalized to controls (shScramble). Solid bars: mean values normalized to a GAPD internal reference; Error bars: 95% confidence intervals based on 3 replicate qPCR experiments. (D) Cell proliferation MTT assay. Mean and 95% CI of relative absorbance, $n=3$ biological replicates. Vector controls normalized to 100% growth. (E) Growth curves, serial Coulter counts for 5 days, beginning 10 days post lentivirus infection (designated the 0 time point). Data points represent mean count from three replicate cultures. Error bars = 95% CI.

Figure 2. MBD2 Knockdown inhibits tumor growth in vivo

(A) MDA-MB-435 xenografts in BALBc nu/nu mice, single challenge of 5×10^5 MDA-MB-435 cells. Scramble (left flank) and Knockdown (right flank). Left to right: *in vivo* biofluorescence at 5 day intervals. (B) *In vivo* biofluorescent images at day 14 after seeding with 1×10^6 MDA-MB-231, left flank = Scramble control; right flank = MBD2 Knockdown. (E) *In vivo* biofluorescent images at day 14 after seeding with 1×10^6 MDA-MB-435, left flank = Scramble control; right flank = MBD2 Knockdown. (C and F) Tumor growth: Serial caliper measurements were made of palpable tumors and used to calculate the mean and standard error of the mean (SEM) of tumor area for Scramble (SC) and Knockdown (KD) tumors for MDA-MB-231 and MDA-MB-435 tumors. (D and G) Tumor volume at 28 days, Scramble controls (SC) and MBD2 Knockdowns (KD), significance calculated by paired T-test. $n=3$ mice per cell line (*MDA-MB-231, $p \leq 0.017$; **MDA-MB-435, $p \leq 0.005$).

Figure 3. MBD2 binds to hypermethylated tumor suppressor gene promoters and knockdown of MBD2 leads to restored gene expression

(A) Heat Map: Quantitative RT-PCR based tumor suppressor gene expression in MBD2 knockdown MDA-MB-321 and MDA-MB-435 breast cancer cell lines [red, high expression; blue, low expression]. Paired columns = duplicate experiments. Rows = Individual genes. KLK10 and DAPK1 (red arrows); MBD2 (blue arrow); internal control genes, CyclophilinA and GAPD. (B) ChIP: Quantitative RT-PCR used to probe anti-MBD2 or normal IgG control immunoprecipitated chromatin (ChIP) from Scramble (SC) and shMBD2 knockdown (KD) MCF-7 cells. Mean and SEM (error bars) are shown for 3 replicates per ChIP assay. (C and D) Bisulfite conversion and sequencing: Gene maps show the location of CpG islands, individual CpGs (vertical lines), and bisulfite primers. 'Lollipop' methylation maps show methylated CpGs (filled black circles) and unmethylated sites (white circles) at the promoters of KLK10, and DAPK1 in shScramble control and shMBD2 knockdown breast cancer cell lines. Individual clones are shown on separate rows.

Figure 4. MBD2 knockdown reversibly silences a specific subset of tumor suppressor genes in MCF-7 distinct from those induced by 5-aza-2-deoxycytidine
(A) qRT-PCR based expression cluster/heat map [red, high expression; blue, low expression] in MCF7 cells. [Arrows indicate KLK10 and DAPK1 (red arrows) and MBD2 (blue arrow)] Paired columns correspond to duplicate experiments using cells stably transformed with the indicated shRNA and/or 2'-deoxy-5-azacytidine [5aza-dC] treatment as indicated above the heat map figure. Rows = Individual genes. Gene clusters: (1) 5aza-dC induced genes, (2) genes induced by MBD2 knockdown and 5aza-dC in additive fashion, (3) genes induced specifically MBD2 knockdown and (4) the genes found down-regulated in knockdown cells. **(B)** Transfection of stable MBD2 knockdown lines with short double stranded RNA oligonucleotides (siRNA). A negative (non-targeted) siRNA oligonucleotide was included as a control (siNEG). **(C)** Resistant MBD2 expression: Western blot analysis of *shRNA site mutant MBD2 expression restored in shScramble and shMBD2 (knockdown) MCF-7 cells. Expression of wild type MBD2 (pCMV-MBD2) and Off-target mutant MBD2 shown for comparison. **(D)** Gene expression analysis of MBD2 target genes in control (Scrambled shRNA) and knockdown (MBD2 shRNA) MCF-7 cells: shRNA immune MBD2 mutant (left half of heat map, + pCMV-MBD2*-shRNA-Mut) leads to resuppression of MBD2 targets.

Figure 5. Re-expression of MBD2 restores in vitro growth potential of breast cancer cells

(A) MBD2 Immunoblot: shRNA immune mutant MDA-MB-435 clones selected for stable expression of resistant MBD2 protein (pCDNA-MBD2*) or vector only control (pCDNA-Vector Only) then transduced with either scramble shRNA lentivirus or shMBD2 lentivirus. MBD2 antibody (top) and beta actin loading control (bottom) shown. (B) Mutant MBD2 expressing knockdown cells (KD+MBD2* [Purple]) are refractory to growth inhibition imposed by shMBD2 lentivirus, similar in growth to shScramble controls (SC [Blue], SC+MBD2* [Green]). MBD2 targeted shRNA expressing cells (KD, [Red]) remain growth inhibited. (C) MTT proliferation assay: Scramble Control (SC), Scramble control + resistant MBD2 (SC+MBD2*), MBD2 knockdown cells (KD), and Resistant MBD2 (KD+MBD2*). MTT assay bars represent the mean of 3 biological replicates. All points were normalized to the respective shScramble control samples (SC, SC+MBD2*) and error bars represent the corresponding 95% CI.

Figure 6. Summary and Model

MBD2 depletion by targeted shRNA leads to reexpression of methylated tumor suppressor genes by interference with MBD2 dependant recruitment of the Mi-2/nucleosome remodeling and deacetylase (NuRD) complex to methylated tumor suppressor promoters. Restored gene expression in turn leads to growth arrest of breast cancer cells and tumor clearance in animals.