**Mbd3**, a Component of NuRD/Mi-2 Complex, Helps Maintain Pluripotency of Mouse Embryonic Stem Cells by Repressing Trophoderm Differentiation

Dongmei Zhu\(^1,2\), Junshun Fang\(^1\), Yanxin Li\(^1\), Jian Zhang\(^1\)

\(^1\) Laboratory of Molecular and Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China, \(^2\) Graduate School of the Chinese Academy of Sciences, Beijing, China

### Abstract

Embryonic stem (ES) cells can differentiate into cells derived from all three germ layers and extraembryonic tissues. While transcription factors such as Oct4 and Nanog are well known for their requirements for undifferentiated ES cell growth, mechanisms of epigenetic repression of germ layer specific differentiation in ES cells are not well understood. Here, we investigate functions of Mbd3, a component of nucleosome remodeling and histone deacetylation complex (NuRD/Mi-2) in mouse ES cells. We find that compared to wild type ES cells, Mbd3 knockdown cells showed elevated RNA expression of trophoderm markers, including Cdx2, Eomesodermin, and Hand1. In parallel, these cells show an increased acetylation level of histone 3 in promoters of the respective genes, suggesting Mbd3 plays a role in repression of these genes in undifferentiated ES cells. However, these changes are not sufficient for definitive differentiation to trophoderm (TE) in chimeric embryos. When further cultured in ES medium without LIF or in trophoblast stem (TS) cell medium, Mbd3 knockdown cells differentiate into TE cells, which express Cdx2 and, at later stages, trophoblast lineage specific marker Cadherin 3. These results suggest that Mbd3 helps restrict ES cells from differentiating towards the trophoderm lineage and is an important epigenetic player in maintaining full pluripotency of mouse ES cells.

### Citation

Zhu D, Fang J, Li Y, Zhang J (2009) Mbd3, a Component of NuRD/Mi-2 Complex, Helps Maintain Pluripotency of Mouse Embryonic Stem Cells by Repressing Trophoderm Differentiation. PLoS ONE 4(11): e7684. doi:10.1371/journal.pone.0007684

### Competing Interests

The authors have declared that no competing interests exist.

### Funding

This work was supported by a project grant from Ministry of Science and Technology China (grant number: 2006CB701504) and grants from National Natural Science Foundation of China (grant numbers: 30500063; 30425013). J.Z. is a Bai-Ren-Ji-Hua investigator of Chinese Academy of Sciences. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

### Introduction

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of growing blastocysts. They maintain an undifferentiated state in defined culture conditions, but can also be induced to differentiate into diverse cell types representative of all germ layers both in vitro and in vivo [1]. ES cells are powerful tools for expanding our knowledge in mammalian early development and are thought to hold great promise for regenerative medicine [2]. ES cells share many characteristics of ICM cells at the level of transcriptional regulation. For example, they both express pluripotent cell specific transcription factors, such as Oct4 and Nanog [3–5]. In mouse, loss of Oct4 expression by targeted gene deletion causes ES cells to develop into trophoderm [6,7], while deletion of Nanog causes ES cells to differentiate into primitive endoderm [5] and to compromise PGC maturation [8]. Considerable efforts have been devoted to elucidate transcriptional networks of these and other transcription factors and their associated cofactors [9,10]. These transcription factors have been implicated in cooperatively activating or repressing a broad range of downstream target genes [11]. However, less attention has been paid to epigenetic regulation of these lineage specific transcription factors. Recent studies have shown that the ES cell pluripotent state is critically maintained by Polycomb group (PcG) complexes that mediate suppression of key differentiation genes [12–14]. Other epigenetic studies point to similar lineage restriction schemes to govern ES cell pluripotency (reviewed in [15]). Despite these studies, detailed mechanisms of how global epigenetic control is achieved, especially how lineage specific transcription programs are suppressed in ES cells, remain to be fully elucidated (reviewed in [15,16]).

Major epigenetic modifications include DNA methylation, histone acetylation and methylation which are often closely coupled [17]. DNA methylation at the dinucleotide CpG in regulatory regions is a hallmark of stable transcriptional silencing [18]. Recruitment of specific binding proteins to methylated CpG islands is believed to repress target gene transcription [19]. On the other hand, acetylation of histone tails is critical for nucleosome structure alterations that facilitate DNA accessibility to regulatory factors [20–22].

Purification of nucleosome remodeling and histone deacetylation complex (NuRD, also known as Mi-2, NURD, or NRD) links together two epigenetic modifications: DNA methylation and histone deacetylation [23–25]. Several components of the NuRD complex have been shown to be necessary for early embryonic development. Methylated DNA-linked chromosomal remodeling and gene silencing are thought to be mediated by methyl-CpG binding (MBD) proteins [19,26]. Unlike other mammalian MBD protein, Mbd3 does not bind to methyl-CpG biochemically. Instead, Mbd3 is directly associated with Chd4 protein as core
subunits of the NuRD complex. Study of Mbd3 null mice indicates that it is essential for early embryogenesis while Mbd2 is dispensable for viability [29]. Since dynamic epigenetic regulations occur during ICM formation and differentiation of primary germ layers, early embryonic lethality caused by Mbd3 deletion may be attributed to abnormal epigenetic modifications, and therefore dysregulation of gene expression in early embryos [30,31]. Mbd3 function was reported to be dispensable for ES cell growth in culture, but essential for their commitment to a full spectrum of embryonic lineages when aggregated with wild type embryos, indicating pluripotency of these cells is indeed affected [15,32]. A detailed mechanism for restricted differentiation of the Mbd3-deficient cells remains to be elucidated. Interestingly, when cultured in vitro to promote embryonic stem cell outgrowth, Mbd3-deficient ICMS fail to generate pluripotent cells [33]. This difference may be attributed to different sets of molecular factors that are required for the derivation and maintenance of the pluripotent state [13,33].

Specification of trophectoderm is the first sign of differentiation of early mouse embryos. Studies of molecules required for the specification of the trophectoderm have led to identification of Oct4 as a negative regulator while Cdx2 as a positive transcription factor in the process. Conditional deletion of Oct4 in mouse ES cells leads to trophectoderm differentiation and increased expression of trophectoderm-specific markers [6]. Trophoblast stem (TS) cells can be derived when these cells are cultured under conditions that promote trophectoderm proliferation [34]. On the other hand, Cdx2 is specifically expressed in outer cells of the blastocyst, which are destined to form trophectoderm [35]. Without functions of Cdx2, transcription of Oct4 and Nanog are not downregulated in these outer cells, thus resulting in the implantation failure of the mutant embryos [35].

Interestingly, though Cdx2 is essential for TS cell self-renewal, it is dispensable for trophectoderm differentiation induced by Oct4 repression [34]. Since Cdx2 and Oct4 form a complex in early embryos, reciprocal inhibition of their respective target genes was proposed to be important in achieving the correct segregation of the ICM and trophectoderm lineages [34,36].

Although studies using Mbd3−/− ES cells have greatly helped us to understand the roles of NuRD complex in maintaining full ES pluripotency, the underlying molecular mechanism remains obscure. In the present study, we selectively reduced expression of Mbd3 in mouse ES cells by RNA interference to address why Mbd3 is required for maintain mouse ES cell pluripotency. We find that reduction of Mbd3 compromises the full differentiation potential of ES cells. Moreover, with reduced Mbd3 expression, mouse ES cells are set at an intermediate state and are more prone to differentiate into trophectoderm. Our results suggest that Mbd3 is involved in maintaining pluripotency of mouse ES cells by repressing trophectoderm differentiation.

Materials and Methods

Plasmids

Mbd3 and its control short hairpin RNA (shRNA) plasmids were all placed into pSuper.retro.puro vector (Oligo Engine Inc.). RNA interference (RNAi) target sequences for Mbd3 were selected using Ambion siRNA converter online software (http://www.ambion.com/techlib/misc/siRNA_finder.html). The target sequences are as follows:

Mbd3 shRNA1: 5'-GATGAAATAAGAGTCGCCAG-3'
Mbd3 shRNA2: 5'-AGGCTTCATGGTGACAGAT-3'
Mbd3 control shRNA: 5'-GGGAATGCTATTGTTGGG-3'.

Oligonucleotides were annealed and inserted into Bgl II/HindIII sites of pSuper.retro.puro vector. EGFP fragment from pEGFP-N1 (Clontech) was subcloned into the AccI site of the RNAi plasmids to visualize transfected cells.

Mouse Mbd3 cDNA and full-length human Mbd3 cDNA were cloned into XbaI and HindIII sites in pRK5-thkneo vector (Genentech, South San Francisco, Calif. [37]).

Cell culture, plasmid transfection and cell proliferation assay

Mouse ES cell line CGR8 (kindly provided by Dr. Austin Smith) [6] was maintained in GMEM (Sigma G5154) supplemented with 10% fetal bovine serum (PAA, pre-tested for ES cells, A15-080), 1 mM sodium pyruvate (Sigma S8636), 2 mM L-glutamine (HyClone SH30034), 0.1 mM non-essential amino acids (HyClone SH30238), 0.1 mM 2-mercaptoethanol (Sigma M7522), 50 µg/ml penicillin/streptomycin (HyClone SV30010), 10⁻⁷ Units/ml leukemia inhibitory factor (LIF, Chemicon ESG1107). CGR8 cells were cultured in plates coated with 0.1% gelatin (Sigma G9391) without feeder layer cells. NIH3T3 and mouse primary fibroblast cells were maintained in DMEM (HyClone SH30022) supplemented with 10% fetal bovine serum (HyClone SH30088), 50 µg/ml penicillin/streptomycin, and 2 mM L-glutamine.

Plasmids were transfected into cells with PolyFect (Qiagen 301105) or Lipofectamin 2000 (Invitrogen 11668) according to the manufacturers’ instructions. All antibiotic selections were started at 24 hours after transfections. Mouse CGR8 ES cells were selected with 1 µg/ml puromycin (Sigma P8833) and/or 300 µg/ml G418 (Invitrogen 11811-023).

For cell proliferation assays, transfected cells were maintained in medium without antibiotic selection. Equal numbers of EGFP positive cells were seeded in triplicate in 12-well plates one day after transfection. Green fluorescent cells were counted in the following days. Each experiment was repeated at least three times.

Lentivirus construction, package and infection

For generation of ES cell lines stably overexpressing shRNA, the oligonucleotides used for Mbd3 shRNA1 were cloned into pLentiLox3.7 vector. For lentivirus production, pLentiLox3.7 plasmids were co-transfected with packaging vectors into 293T cells, and the supernatant was harvested after 48 hours. After centrifugation and filtration, the supernatant was added into ES cells, and the supernatant was harvested after 48 hours. After centrifugation and filtration, the supernatant was added into ES cell suspension for infection. Single colonies were then picked and propagated.

Western blotting

Cells were collected after trypsinization and washed twice with cold PBS. Cell lysate was extracted with five times volume of cold EBC buffer (120 mM NaCl, 50 mM Tris-CI pH 8.0, 0.5% Nonidet P-40) containing protease inhibition cocktails (Roche 1697498) and 1 mM PMSF (Ameresco 0754). Protocols used for protein fractionation in SDS-PAGE, blotting and antibody incubation were essentially the same as those described in Molecular Cloning [36]. Anti-MBD3 (C-18) (sc-9402) was purchased from Santa Cruz and anti-α-Tubulin (T5168) antibodies was from Sigma. The protein signals were detected using Pierce SuperSignal kit (Pierce 34095) and chemiluminescent images were captured using a cold CCD camera (UVG BioImaging Systems).

Real-time RT-PCR

Total RNAs were extracted with Trizol reagent (Invitrogen 15596-026) followed by DNase I (Roche 11994020) treatment. Reverse transcription reactions from 2 µg RNA were carried out.
with MMLV reverse transcriptase (Invitrogen 28025-015). cDNAs from 25 ng of RNA were used as templates for quantitative PCR amplification using SYBR PCR Master Mix (ABI 4367639) in ABI Prism 7900 HT sequence detection system (Applied Biosystems). Data were analyzed by SDS2.2 software. Reactions were set up in triplicate for each sample. Gene expressions were normalized to β-actin expression. Data are shown as fold inductions relative to control. Primers are shown in Table S1.

**Chromatin immunoprecipitation (ChIP)**

About 10⁵ cells for ChIP were treated with shRNA for six days while selected with puromycin for five days. ChIP assays with an acetylated histone 3 (AcH3) antibody (Upstate, Catalog # 17-245) were carried out following the manufacturer’s protocol. Briefly, cells were cross-linked with 1% formaldehyde for 10 min at 37°C. Cell lysates were sonicated at 100 w for 10 s with ultrasonic apparatus (Scientz JY92-2D). The sonication step was repeated four times with 30 s intervals. Chromatin extracts containing DNA fragments with an average size of 500 bp were immunoprecipitated using 5 μg AcH3 antibody. Quantitative PCR was carried out as described in real-time RT-PCR section. Primers are shown in Table S2.

**Embryoid body (EB) formation and chimeric embryo production**

For EB formation assays, 2×10⁵ cells were seeded into 35 mm low attachment sterile cell plate (Ai Si Jin Co., China) in 2 ml ES cell medium without LIF. Fresh medium was exchanged every two days.

For chimera production, eight-cell stage embryos were collected from ICR female mice. Embryos were treated with acidified Tyrode’s solution (Sigma T7188) for 10 sec. to remove the zona pellucida. Naked embryos were washed through four droplets of M2 medium (Sigma M7167) and subsequently cultured in the “well-in-well” of 50 μl KSOM-AA medium (Chemicon MR-106-D) individually to maintain the developmental competence and embryonic integration. One small cluster of Mbd3 knockdown stable cells (10–20 cells) was gently put into the culture droplet which contained the naked embryo. After 24 h, either aggregated morula or blastocyst stage embryos were selected. Morula-stage embryos were further cultured in 50μl droplet of fresh KSOM medium until the blastocyst stage [39].

**Trophoblast stem (TS) cell derivation and cell immunostaining**

CGR8 cells were treated with Mbd3 shRNAs for at least three days then cultured in TS cell culture condition. TS cells were derived and maintained in 30% fresh TS medium [GMEM (Sigma G3154) supplemented with 20% (v/v) of FBS (Hyclone SH30396), 1 mM sodium pyruvate (Sigma S8636), 2 mM L-glutamine (Hyclone SH30034), 0.1 mM 2-mercaptoethanol (Sigma M7522), 50μg/ml penicillin/streptomycin (Hyclone SV30010), 1μg/ml of sodium heparin (Sigma H3149), and 25 ng/ml of recombinant FGF4 (Sigma F8424)] and 70% (v/v) of the MEF-conditioned TS medium [40]. MEF-conditioned medium was collected from mitomycin C-treated MEF cells cultured in TS medium for 3 days.

For immunostaining, cells were fixed in 4% paraformaldehyde at room temperature for 10 min. After rinsing twice with PBS, cells were blocked with blocking buffer (PBS+0.1% Gelatin+1% BSA+0.2% NaN3+0.4% TritonX-100) for 30 min at room temperature (for Cdh3 staining, withdrawal of TritonX-100 from blocking buffer). Primary antibodies anti-Cdh3 (Neomarkers, MS-1741) and anti-Cdx2 (BioGenex, MU392-UC) were diluted at 1:100 and 1:50 in blocking buffer, respectively. Secondary antibody detecting mouse IgG conjugated with TRITC or FITC (ZhongShanJinQiao, ZF-0313, 0312) were diluted at 1:100 in blocking buffer. Fixed cells were stained with primary and secondary antibodies for 1 hour, respectively. Hoechst (Sigma B2261) were used for cell nuclei staining. Images were captured with a fluorescence microscope (Nikon Eclipse TE2000-U) or Zeiss confocal microscope (LSM510META).

**Results**

**Mbd3 is required in mouse ES cells for suppressing expression of trophectoderm specific genes**

Much progress has been made in defining requirements for maintenance and differentiation of ES cells, but only limited information is available as to how lineage restriction is achieved epigenetically [41]. One of the major epigenetic regulations is modification of histone acetylation levels at genes required for certain biological processes. The NuRD complex uniquely processes both nucleosome remodeling and histone deacetylation activities and functions primarily in transcriptional repression [42]. To better understand the functions of the NuRD complex in maintaining pluripotency of mouse ES cells, we investigated its functional components. We chose to inhibit expression of Chd4 (will be reported elsewhere) and Mbd3 which is essential for very early development as demonstrated in mouse Mbd3 knock-out experiments [29]. More recent studies by Kaji and colleagues strongly suggest that Mbd3 is critically required for mouse embryonic stem cells both in vitro and in vivo [32,33]. However, the underlying mechanisms for the requirement remain to be fully elucidated.

Two RNA interference plasmids were made against mouse Mbd3. Transfection of either shRNA plasmids can efficiently reduce both Mbd3 RNA expression and its protein expression in CGR8 cells (Figure 1A, B). We first asked if Mbd3 shRNAs treatment influences ES cell proliferation. Mbd3 shRNAs transfected cells (thereafter referred as Mbd3 shRNA cells) showed only a slightly lower proliferation rate compared with wild type ES cells (Figure 1C). However, the majority of the Mbd3 shRNA cells displayed marked differentiated morphology when compared to control shRNA cells. In contrast to tightly packed ES cell colonies with smooth edges, Mbd3 shRNA cells showed morphological changes ranging from a fibroblast-like shape to loosely associated cell aggregations (Figure 1D, compare a,a’ to b,b’ and c,c’). Minor cell proliferation changes after Mbd3 shRNA transfection may be partially explained by ES cell differentiation into other cell types. These results indicate that Mbd3 may be essential for maintaining mouse ES cells in an undifferentiated state.

Although mouse ES cells can give rise to all types of cells in an embryo, they can only differentiate directly into three cell lineages: trophectoderm, primitive endoderm and primitive ectoderm (reviewed in [41]). The observed morphological changes after knockdown of Mbd3 in ES cells did not clearly indicate into which lineages they may have differentiated. Therefore, we analyzed specific molecular markers for the three cell lineages in ES cells after Mbd3 knockdown. Quantitative RT-PCR analysis confirmed that Mbd3 RNA levels were decreased to about twenty five percent of controls. Noticeably, the trophectoderm markers Cdx2, Eomes and Hand1 were upregulated dramatically in Mbd3 shRNA cells (Figure 2A). Transcription of primitive endoderm markers, Gata4 and Hhex (Figure 2A), Sox7, Tpf1 and AFP (data not shown) did not show obvious changes. Expression of Gata6, which has been used as a marker for primitive endoderm, increased at least
four fold (Figure 2A). It should be noticed, however, that Gata6 is also expressed in early trophectoderm [43]. There were no meaningful changes at the RNA level of primitive ectoderm marker Fgf5 and its derivative mesoderm marker T. It appears that knockdown of Mbd3 promotes ES cells to differentiate towards trophectoderm based on these molecular studies. The differentiation of ES cells into trophectoderm cells is usually accompanied by downregulation of pluripotent genes, such as Oct4 and Nanog. However, we did not observe transcriptional changes for Oct4, Nanog and Esrrb (Figure 2A), suggesting the observed upregulation of trophectoderm genes may not be a sufficient indicator of a full commitment to the trophectoderm lineage.

To test the specificity of the Mbd3 RNA interference effect on ES cells, we co-transfected human Mbd3 and mouse Mbd3 shRNA plasmids into ES cells and attempted to rescue the observed upregulation of trophectoderm markers. Human Mbd3 and mouse Mbd3 exhibit 95.8% identity at the amino acid level based on protein alignment. Human Mbd3 cannot be targeted by either mouse Mbd3 shRNAs as judged by significant DNA sequence divergence. Quantitative RT-PCR results indicated that human Mbd3 clearly rescued expression of the trophectoderm markers to control levels (Figure 2B). High levels of histone 3 acetylation in a promoter are usually correlated with active gene transcription [44,45]. Thus the ChIP results are consistent with the upregulated transcription of these genes. Together, our data suggest that Mbd3 expression is critical to suppress TE lineage specific gene expression in mouse ES cells.

Suppression of Mbd3 expression is not sufficient for ES cell differentiation to TE lineage

Since the NuRD complex is involved in histone deacetylation, we further examined the histone acetylation status of those genes that showed upregulation upon Mbd3 knockdown. By scanning promoter regions of Cdx2, Eomes and Gata6 using chromatin immunoprecipitation (ChIP) coupled with quantitative PCR analysis, we uncovered specific regions in these promoters that show significantly higher acetylated histone 3 modification in Mbd3 shRNA cells compared with the control (Figure 2C). High levels of histone 3 acetylation in a promoter are usually correlated with active gene transcription [44,45]. Thus the ChIP results are consistent with the upregulated transcription of these genes. Since the NuRD complex is involved in histone deacetylation, we further examined the histone acetylation status of those genes that showed upregulation upon Mbd3 knockdown. By scanning promoter regions of Cdx2, Eomes and Gata6 using chromatin immunoprecipitation (ChIP) coupled with quantitative PCR analysis, we uncovered specific regions in these promoters that show significantly higher acetylated histone 3 modification in Mbd3 shRNA cells compared with the control (Figure 2C). High levels of histone 3 acetylation in a promoter are usually correlated with active gene transcription [44,45]. Thus the ChIP results are consistent with the upregulated transcription of these genes. Together, our data suggest that Mbd3 expression is critical to suppress TE lineage specific gene expression in mouse ES cells.
Transient transfection might result in loss of plasmids during the chimera development, and thus could make it difficult to interpret the differentiation potential of labeled Mbd3 shRNA cells. To circumvent this drawback of transient transfection for in vivo assays, we made stable Mbd3 knockdown ES cell lines by lentivirus infection. These cell lines show consistent and stable Mbd3 mRNA knockdown during the course of the study (Figure 3A), thus avoiding any significant phenotypic variations possible with transient cell knockdowns. Among the Mbd3 knockdown stable lines, the upregulation of Cdx2 is inversely correlated with Mbd3 RNA level. Consistent with the previous transient knockdown result, the expression level of pluripotency marker Oct4 shows no obvious change among the stable lines (Figure 3A). We also observed that these cells showed fibroblast-like morphology with loose cell-cell contacts (Figure 3B–n, o, p). We aggregated cells from three independent Mbd3 knockdown stable lines (ESL D8, E8 and G11) and wild type mouse embryos to form chimeras. We examined which cell lineage the Mbd3 stable cells can associate with. Mbd3 cells, including those cell lines with obvious differentiated morphology (ESL G11), were shown to integrate...
into the ICM of chimeric embryos in almost all cases (Figure 3B). At a minimum, this observation suggests that Mbd3 shRNA cells and ICM cells share similar cell surface molecules essential for cell sorting. In previous studies [32], Oct4 and Nanog expression changes little in Mbd3 shRNA cells, which may partially explain why these cells remain associated with the ICM in the chimera embryos (Figure 3B). Moreover, we failed to detect Cdx2 protein expression in these cells (Figure 4b, c), although Cdx2 mRNA levels were consistently upregulated many fold (Figure 3A), suggesting that either there is not sufficient mRNA transcription or that translational regulation plays a role in these cells. Taken together, these results indicate that reduction of Mbd3 expression is not sufficient for fully committed TE lineage differentiation.

**Mbd3 is required for maintaining full differentiation potential of ES cells**

Although the Mbd3 knockdown ES cells retain the ability to remain associated with the ICM, whether they retain ES cell like differentiation capability is not known. Previously, Mbd3-/- cells were shown to retain self-renewal capability upon withdraw of LIF
and also to show defective differentiation potential [32]. However, which specific early lineages are compromised is unclear for \textit{Mbd3} cells.

If \textit{Mbd3} shRNA cells show upregulated trophodermal markers, an indication of greater potential for re-specification towards the trophodermal lineage, we reasoned their capability to form primitive ectoderm, precursor of three germ layers, might be compromised. To investigate this possibility, we tried to induce \textit{Mbd3} shRNA-transduced cells to differentiate by adding retinoic acid (RA, all-trans), an agent which causes wild type ES cells to convert into cells comprising all three germ layers [48]. Compared with control shRNA cells, \textit{Mbd3} knockdown cells showed reduced induction of \textit{fgf5} (ectoderm) and \textit{gata4} (endoderm) (Figure 5A). This result indicates \textit{Mbd3} indeed is required for full differentiation potential of primitive ectoderm layers.

We also used embryoid body (EB) formation by suspension culture of ES cells to examine their differentiation capability. Successfully differentiated EB is a three-dimensional spheroid structure that mimics post-implantation embryos and contains three germ layers [49]. Since \textit{Mbd3} seems to regulate ES cell differentiation, it is possible that reduction of \textit{Mbd3} expression in ES cells may also cause abnormal differentiation in EB. We used three separate \textit{Mbd3} knockdown cell lines (ESL D8, E8 and G11) in the assay. All cell lines except ESL G11 form aggregates by day five (Figure 5B–e, f, g). By day twelve, in contrast to control cells which form heterogeneous spheroids in the aggregates, ESL D8 and ESL E8 cells only show aggregated solid ‘cell balls’ similar to those at day five, suggesting the lack of robust differentiation shown by control ES cells (Figure 5B, i–q). Moreover, even the ESL G11 cell line with the most severe differentiation morphology failed to generate obvious EB by day five, suggesting severely compromised differentiation capability (Figure 5B–h). Before day twelve, the remaining small ESL G11 cell aggregates are all disintegrated (data not shown). It is clear that \textit{Mbd3} is required for the formation of normal EB, another indication of full differentiation potential. Together, these results strongly suggest that \textit{Mbd3} is essential for mouse ES cells to maintain full pluripotency.
Mbd3 reduced ES cells are prone to differentiate into trophoderm lineage

*Mbd3* knockdown cells showed substantial increased mRNA expression of trophoderm lineage markers, a strong indication of trophoderm differentiation. However, we did not detect Cdx2 protein expression in *Mbd3* knockdown cells (Figure 4b, c). These cells were shown to integrate into the ICM of chimera embryos (Figure 3) which indicates no definitive differentiation to trophoderm lineage at least in the chimeras. Although we cannot rule out that the *Mbd3* knockdown cells have reverted to a more pluripotent state by in vivo factors when placed in the milieu of a developing embryo, it is also possible that reduction of *Mbd3* in ES cells may reduce the intrinsic threshold for these cells to differentiate towards TE lineage.

Leukemia inhibitory factor (LIF) is one of the key components in suppression of spontaneous differentiation for mouse ES cells.

**Figure 5. Full differentiation potential of mouse ES cells is compromised when *Mbd3* expression is reduced.** A) ES cells cultured with or without retinoic acid for three days were subjected to real-time RT-PCR analysis. For differentiations, 1μM retinoic acid (RA) was used or LIF was withdrawn from the medium. ES cells were transfected with respective plasmids for three days before RA addition and selected with antibiotics for five days. Error bars represent standard deviation from three technical repeats. B) Embryoid body (EB) formation of control or *Mbd3* knockdown stable cells. Stable cells (2 x 10⁵) were suspension cultured in ES cell medium without LIF in 35 mm cell dishes. Scale bars: k, 200μm (also applies to a to j); q, 50μm (also applies to l to p).

doi:10.1371 journal.pone.0007684.g005
Withdrawal of LIF from the culture medium allows ES cells to differentiate randomly into multiple lineages [50]. However, mouse ES cells rarely differentiate into trophectoderm in various culture conditions. Alterations of Cdx2 and Eomes were reported to trigger trophectoderm differentiation of mouse ES cells [34,51]. We attempted to test whether Mbd3 knockdown cells have a tendency to differentiate into trophectoderm in the absence of LIF. After removal of LIF from the culture medium for 4 days, we detected Cdx2 protein expression in the nuclei of Mbd3 shRNA cells, but not in the control cells (Figure 4). These results indicate that Mbd3 knockdown cells seem to be biased to the trophectoderm lineage.

Previously, overexpression of Cdx2 in mouse ES cells was shown to induce trophectoderm differentiation, likely by directly inhibiting Oct4 functions [34,52]. When ES cells are induced to differentiate toward trophectoderm, trophoblast stem (TS) cells can be derived from these cells in appropriate culture conditions [34,47]. To further confirm that Mbd3 knockdown cells are indeed biased towards trophectoderm differentiation, we cultured control and Mbd3 shRNA stable cells under TS culture conditions containing FGF4 and MEF-conditioned medium [40] for 6 days. Although we did not observe representative TS colonies, the Cdx2 proteins were detected in Mbd3 shRNA stable cells (Figure 4a, o). Moreover, with prolonged culture of Mbd3 knockdown cells three days after shRNAs transfection in TS cell medium, we observed formation of flattened TS-like colonies by passage two (Figure 6a, f) which expressed trophectoderm cell surface marker Cadherin3 (Cdh3, also known as placenta Cadherin) (Figure 6d, i). In contrast, there were no Cadherin3 positive cells in control shRNA cells under the same culture condition (Figure 6a). It is likely that both TS culture condition and withdrawal of LIF further promote Mbd3 shRNA cells towards a trophectoderm lineage. These results suggest Mbd3 helps mouse ES cells maintain pluripotency by partial suppression of the trophectoderm lineage.

**Discussion**

The NuRD complex exists as a co-repressor of gene expression in a broad range of different cells [23–27; 30–31]. In this study, we selectively reduced the expression of Mbd3 and further investigated its epigenetic functions in mouse ES cells. We find that reduction of Mbd3 expression lowers the threshold of ES cells to differentiate towards the trophectoderm lineage. Our results thus indicate that Mbd3 is essential to maintain full mouse ES cell pluripotency by helping repress the trophectoderm specific differentiation program.

Trophectoderm specification is the very first cellular differentiation of early mouse embryos. The upregulation of Cdx2 expression has been shown to be important for the formation of trophectoderm [35]. In addition, Oct4 is normally downregulated in trophectoderm cells in vivo and in vitro. Cdx2 and Oct4 also antagonize each other at the transcription level [34,53]. However, knockdown of Mbd3 in mouse ES cells upregulates Cdx2 RNA while Oct4 RNA remains unchanged (Figure 2A, 3A). Similarly, Oct4 expression was previously shown to be unchanged in Mbd3−/− ES cells [32]. Lack of Cdx2 expression data in Mbd3−/− ES cells precludes direct comparison of Cdx2 expression in the two studies. Several observations may be used to explain why Oct4 remains constant in Mbd3 compromised cells. First, Oct4-null ES cells with constitutive expression of transfected Oct4 and overexpression of Cdx2 were differentiated into TE lineage, suggesting expression of Oct4 alone is not sufficient for blocking trophectoderm differentiation triggered by Cdx2 overexpression [34]. Second, Oct4 downregulation during normal trophectoderm differentiation may require epigenetic regulations, which may be positively influenced by the NuRD complex. In fact, Gu and colleagues recently found that Mbd3 was recruited by GCNF to the Oct4 promoter to repress its expression through DNA methylation in the process of ES cell differentiation [53]. Thus loss of Mbd3 in ES cells may lead to deregulation of Oct4 transcription during ES cell differentiation. Furthermore, in the Mbd3 knockdown cells, Cdx2 protein was not appreciably increased, indicating that alteration of Mbd3 mRNA level may not significantly alter Cdx2 translation.

The morphological change and expression of trophectoderm genes in Mbd3 knockdown cells (Figure 1D, 2, 3A, and 3B m–p) are considered as early indicators of TE differentiation, but further chimera analysis (Figure 3B a–l) suggest that these cells have properties that resemble those of ICM. However, further
subjection of Mbd3 knockdown cells to differentiation challenges, such as withdrawal of LIF or TS culture medium resulted in Ceb2 protein expression, indicating that Mbd3 knockdown cells are biased to differentiation. In contrast, Mbd3−/− ES cells could be maintained in the absence of LIF. The discrepancy between these two studies might be significant, but it should be emphasized that in both cases disruption of Mbd3 function did not affect cell proliferation and only affected some differentiation potential. We showed that in the absence of LIF, Mbd3 shRNA cells express Ceb2 protein, which might explain why trophoblast markers Tpbpa and Pl-1 were observed in Mbd3−/− ES cells ([32] and Figure 2A). It would be interesting to test whether Mbd3−/− ES cells are also biased towards TE lineage. It is surprising that Ceb2 protein did not shown increased expression upon Mbd3 knockdown despite strong upregulation of its mRNA. It is possible that translation efficiency of elevated Ceb2 mRNA is negatively controlled by unknown mechanisms, such as micro RNAs. Future experiments may help us elucidate the discrepancy between mRNA and protein expression.

In the embryoid body formation assay, the Mbd3−/− ES cells showed restricted differentiation potential, and this was correlated with upregulation of trophoblast markers Tpbpa and Pl-1 and primitive ectoderm marker Fgf5. The embryoid body formation result using Mbd3 knockdown cells is consistent with that of Mbd3−/− ES cells. Mbd3 shRNA cells are also partially resistant to retinoic acid induced differentiation towards embryonic ectoderm, mesoderm and endoderm cells (Figure 4A). We reason that Mbd3 shRNA cells may have undergone undergone necessary, albeit not fully sufficient, changes towards trophectoderm lineage even though their morphological changes are obvious. This may also explain why Mbd3−/− ES cells and Mbd3 shRNA cells fail to differentiate normally upon RA addition or in EB formation ([32], Figure 5). Therefore, it would be interesting to examine expression of early trophectoderm markers in Mbd3−/− ES cells in addition to markers of mature trophectoderm. Our results also raise an interesting point in that morphological change alone, in certain ES cell lines, cannot be used to judge their differentiation potentials.

The reason why Mbd3 reduction introduces differentiation bias towards trophectoderm lineage, but not definite commitment is worthy of further investigation.

Investigation of how the NuRD complex regulates target genes in ES cells should help elucidate epigenetic mechanisms in maintaining ES cells pluripotency. Understanding what and how NuRD complex components are assembled in ES cells and how they function during lineage specific differentiation will be helpful in understanding epigenetic controls in general.

Supporting Information

Table S1 Primer sequences used in quantitative PCR

Table S2 Primer sequences used in ChIP

Acknowledgments

We thank members of our laboratory for valuable discussions during this study. We are grateful to Dr. Qing-Yuan Sun for assisting in whole-mount immunostaining, Drs. Austin Smith and Yin Jin for CGR8 cell line, Drs. Ye-Guang Chen, Duan-Qing Pei, Masahide Takahashi, Yi Zhang, Jian-Wu Dai for plasmds and antibodies.

Author Contributions

Conceived and designed the experiments: DZ, JZ. Performed the experiments: DZ, JF, JY, GC, YL, JZ. Analyzed the data: DZ, JF, JZ. Contributed reagents/materials/analysis tools: DZ, JF, JZ. Wrote the paper: DZ, JZ.

Reference

1. Keller G (2005) Embryonic stem cell differentiation: emergence of a new era in biology and medicine. Genes Dev 19: 1275–1299.
2. Wolbus AM, Boehler KR (2005) Embryonic stem cells: prospects for developmental biology and cell therapy. Physiol Rev 85: 635–678.
3. Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klever-Nebenius D, et al. (1998) Formation of pluripotent stem cells in the mammalian embryo depends on the Pou5f1 transcription factor Oct4. Cell 95: 379–391.
4. Chambers I, Colby D, Robertson M, Nichols J, Lee S, et al. (2002) Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 111: 643–655.
5. Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, et al. (2003) The homeoprotein Nanog is required for maintenance of pluripotency in mouse embryonic stem cells. Cell 113: 631–642.
6. Niwa H, Miyazaki J, Smith AG (2000) Quantitative expression of Oct3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet 24: 372–376.
7. Velley JM, O'Shea KS (2003) Oct4 RNA interference induces trophectoderm differentiation in mouse embryonic stem cells. Genesis 37: 10–14.
8. Chambers I, Silva J, Colby D, Nichols J, Nieuwenhuizen B, et al. (2007) Nanog safeguards pluripotency and mediates germline development. Nature 450: 1230–1234.
9. Ivanova N, Dobrin R, Lu R, Kotenko I, Levorse J, et al. (2006) Dissecting self-renewal in stem cells with RNA interference. Nature 442: 533–538.
10. Wang J, Rao S, Chen J, Shen X, Levasseur DN, et al. (2006) A protein interaction network for pluripotency of embryonic stem cells. Nature 444: 364–368.
11. Loh YH, Wu Q, Chew JL, Vega VB, Zhang W, et al. (2006) The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat Genet 38: 431–440.
12. Bernstein BE, Mikkelsen TS, Xie Z, Ngo Q, Zhang W, et al. (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125: 315–326.
13. Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, et al. (2006) Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature 441: 349–353.
14. Lee TI, Jeon JG, Boyer LA, Guenther MG, Levine SS, et al. (2006) Control of developmental regulators by Polycomb in human embryonic stem cells. Cell 125: 301–313.
15. Surani MA, Hayashi K, Hajkova P (2007) Genetic and epigenetic regulators of pluripotency. Cell 129: 747–762.
16. Spitzak M, Fisher AG (2007) Epigenetic signatures of stem-cell identity. Nature Rev Genet 8: 263–271.
17. Li E (2002) Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet 3: 662–673.
18. Bird AP, Wolffe AP (1999) Methylation-induced repression—beats, braces, and chromatin. Cell 99: 451–454.
19. Hendrich B, Bird A (2000) Mammalian methyltransferases and methyl-CpG-binding domains: proteins involved in DNA methylation. Curr Top Microbiol Immunol 249: 55–74.
20. Turner BM (1991) Histone acetylation and control of gene expression. J Cell Sci 99 (Pt 1): 13–20.
21. Grinstein M (1997) Histone acetylation in chromatin structure and transcription. Nature 389: 349–352.
22. Struhl K (1998) Histone acetylation and transcriptional regulatory mechanisms. Genes Dev 12: 599–606.
23. Tong JK, Hassig CA, Schützler GR, Kingston RE, Schreiber SL (1998) Chromatin deacetylase by an AT-rich-dependent nucleosome remodelling complex. Nature 395: 917–923.
24. Wade PA, Jones PL, Vermaak D, Wolffe AP (1998) A multiple subunit Mi-2 histone deacetylase from Xenopus laevis cofractionates with an associated Srf2 superfamily ATPase. Curr Biol 8: 843–846.
25. Xie Y, Wong L, Moreno GT, Young MK, Qie J, et al. (1998) NURD, a novel complex with both AT-rich-dependent chromatin-remodeling and histone deacetylase activities. Mol Cell 2: 851–861.
26. Zhang Y, LeRoy G, Seelig HP, Lane WS, Reinberg D (1998) The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodelling activities. Cell 95: 279–289.
27. Zhang Y, Ng HH, Erdjument-Bromage H, Tempst P, Bird A, et al. (1999) Analysis of the NURD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. Genes Dev 13: 1924–1935.
28. Hendrich B, Bird A (1998) Identification and characterization of a family of mammalian methyl-CpG binding proteins. Mol Cell Biol 18: 6538–6547.

PLoS ONE | www.plosone.org 10 November 2009 | Volume 4 | Issue 11 | e7684
29. Hendrich B, Guy J, Ramasahoye B, Wilson VA, Bird A (2001) Closely related proteins MBD2 and MBD3 play distinctive but interacting roles in mouse development. Genes Dev 15: 710–723.

30. Santos F, Hendrich B, Reik W, Dean W (2002) Dynamic reprogramming of DNA methylation in the early mouse embryo. Dev Biol 241: 172–192.

31. Hattori N, Nishino K, Ko YG, Hattori N, Ohgane J, et al. (2004) Epigenetic control of mouse Oct-4 gene expression in embryonic stem cells and trophoblast stem cells. J Biol Chem 279: 17693–17699.

32. Kaji K, Cavallerio IM, MacLeod R, Nichols J, Wilson VA, et al. (2006) The NuRD component Mbd3 is required for pluripotency of embryonic stem cells. Nat Cell Biol 8: 283–292.

33. Kaji K, Nichols J, Hendrich B (2007) Mbd3, a component of the NuRD co-repressor complex, is required for development of pluripotent cells. Development 134: 1123–1132.

34. Niwa H, Toyooka Y, Shimosato D, Strumpf D, Takahashi K, et al. (2005) Interaction between Oct3/4 and Cdx2 determines trophoderm differentiation. Cell 123: 917–929.

35. Strumpf D, Mao CA, Yamanaka Y, Ralston A, Chawengsaksophak K, et al. (2005) Cdx2 is required for correct cell fate specification and differentiation of trophoblast in the mouse blastocyst. Development 132: 2093–2102.

36. Chew JL, Loh YH, Zhang W, Chen X, Tam WL, et al. (2005) Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. Mol Cell Biol 25: 6031–6046.

37. Schall TJ, Lewis M, Koller KJ, Lee A, Rice GC, et al. (1990) Molecular cloning and expression of a receptor for human tumor necrosis factor. Cell 61: 361–370.

38. Sambrook J, Russell DW (2001) Molecular Cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press.

39. Hogan B, Costantini F, Lacy E (1996) Manipulating the Mouse Embryo. New York: Cold Spring Harbor Laboratory Press.

40. Tanaka S, Kunath T, Hadjantonakis AK, Nagy A, Rossant J (1998) Promotion of trophoblast stem cell proliferation by FGF4. Science 282: 2072–2073.

41. Niwa H (2007) How is pluripotency determined and maintained? Development 134: 635–646.

42. Li J, Lin Q, Wang W, Wade P, Wong J (2002) Specific targeting and constitutive association of histone deacetylase complexes during transcriptional repression. Genes Dev 16: 687–692.

43. Koutsourakis M, Langeveld A, Patient R, Bedington R, Grosvold F (1999) The transcription factor GATA6 is essential for early extraembryonic development. Development 126: 723–732.

44. Kouzarides T (1999) Histone acetylases and deacetylases in cell proliferation. Curr Opin Genet Dev 9: 40–49.

45. Nightingale KP, O’Neill LP, Turner BM (2006) Histone modifications: signalling receptors and potential elements of a heritable epigenetic code. Curr Opin Genet Dev 16: 125–136.

46. Saburi S, Azuma S, Sato E, Toyoda Y, Tachi C (1997) Developmental fate of single embryonic stem cells microinjected into 8-cell-stage mouse embryos. Differentiation 62: 1–11.

47. Zhang J, Tan WL, Tong GQ, Wu Q, Chan HY, et al. (2006) Salt4 modulates embryonic stem cell pluripotency and early embryonic development by the transcriptional regulation of Pou5f1. Nat Cell Biol 8: 1114–1123.

48. Soprano DR, Teets BW, Soprano KJ (2007) Role of retinoic Acid in the differentiation of embryonal carcinoma and embryonic stem cells. Vitam Horm 75: 69–95.

49. Keller GM (1995) In vitro differentiation of embryonic stem cells. Curr Opin Cell Biol 7: 962–969.

50. Smith AG, Heath JK, Donaldson DD, Wong GG, Moreau J, et al. (1988) Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature 336: 680–690.

51. Beddington RS, Robertson EJ (1989) An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. Development 105: 733–737.

52. Tolknova E, Cavaleri F, Eckardt S, Reinbold R, Christenson LK, et al. (2006) The caudal-related protein Cdx2 promotes trophoblast differentiation of mouse embryonic stem cells. Stem Cells 24: 139–144.

53. Gu P, Le Menaert D, Chung AG, Chung AC, Cooney AJ (2006) Differential recruitment of methylated CpG binding domains by the orphan receptor GCGF imitates the repression and silencing of Oct4 expression. Mol Cell Biol 26: 9471–9483.

54. Wang X, Seed B (2003) A PCR primer bank for quantitative gene expression analysis. Nuclear Acids Res 31: e154.