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Differential Recruitment of Methyl CpG-Binding Domain Factors and DNA Methyltransferases by the Orphan Receptor Germ Cell Nuclear Factor Initiates the Repression and Silencing of Oct4

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ABSTRACT

The pluripotency gene Oct4 encodes a key transcription factor that maintains self-renewal of embryonic stem cell (ESC) and is downregulated upon differentiation of ESCs and silenced in somatic cells. A combination of cis elements, transcription factors, and epigenetic modifications, such as DNA methylation, mediates Oct4 gene expression. Here, we show that the orphan nuclear receptor germ cell nuclear factor (GCNF) initiates Oct4 repression and DNA methylation by the differential recruitment of methyl-CpG binding domain (MBD) and DNA methyltransferases (Dnmts) to the Oct4 promoter. When compared with wild-type ESCs and gastrulating embryos, Oct4 repression is lost and its proximal promoter is significantly hypomethylated in retinoic acid (RA)-differentiated GCNF−/− ESCs and GCNF−/− embryos. Efforts to characterize mediators of GCNF’s repressive function and DNA methylation of the Oct4 promoter identified MBD3, MBD2, and de novo Dnmts as GCNF interacting factors. Upon differentiation, endogenous GCNF binds to the Oct4 proximal promoter and differentially recruits MBD3 and MBD2 as well as Dnmt3A. In differentiated GCNF−/− ESCs, recruitment of MBD3 and MBD2 as well as Dnmt3A to Oct4 promoter is lost and subsequently Oct4 repression and DNA methylation failed to occur. Hypomethylation of the Oct4 promoter is also observed in RA-differentiated MBD3−/− and Dnmt3A−/− ESCs, but not in MBD2−/− and Dnmt3B−/− ESCs. Thus, recruitment of MBD3, MBD2, and Dnmt3A by GCNF links two events: gene-specific repression and DNA methylation, which occur differentially at the Oct4 promoter. GCNF initiates the repression and epigenetic modification of Oct4 gene during ESC differentiation.

Key Words. Germ cell nuclear factor • Oct4 • DNA methylation • Methylated CpG binding domain • DNA methyltransferases • Embryonic stem cell

INTRODUCTION

Self-renewal of Embryonic stem cell (ESC) is maintained by a core set of transcription factors: Oct4, Sox2, and Nanog, which through transcriptional regulatory loops maintain pluripotency gene expression [1, 2]. Specific mechanisms are required to disrupt this regulatory loop upon ESC differentiation to inhibit the pluripotency phenotype and allow the acquisition of a differentiated cell fate. One of the key questions in the regulation of mammalian transcription is how to regulate silencing of pluripotency genes. The Oct4 gene is an excellent transcriptional model for understanding the regulation of pluripotency gene expression because its expression and cis-regulation have been well-defined in both the mouse and ESCs [2–4]. Oct4 is essential for regulation of pluripotency gene expression during early embryonic development and ESC renewal [3, 5–7]. Expression of the Oct4 gene is maintained in the blastocyst and epiblast, after which it is restricted to primordial germ cells and silenced in all somatic cell lineages [8–10]. Oct4 is also expressed in ESCs and its expression is rapidly downregulated during differentiation of these cells [3, 4, 8, 11, 12].

The orphan nuclear receptor germ cell nuclear factor (GCNF) has been shown to play a central role in the repression of the Oct4 gene upon differentiation of ESCs by binding to an evolutionarily conserved direct repeat with a zero base pair spacing (DR0) cis-element located in the Oct4 promoter.

Disclosure of potential conflicts of interest is found at the end of this article.
of Oct4 [16]. Inactivation of the GCNF gene in mice results in embryonic lethality [8, 16, 17]. Loss of GCNF causes sustained expression of Oct4 in the early neuroectoderm and blocks the differentiation of primitive to definitive neural stem cells in vitro [18]. In ESCs, GCNF is transiently induced during early stages of retinoic acid (RA)-induced differentiation [8, 13, 19]. GCNF+/− ESCs fail to repress Oct4 expression upon differentiation and maintain pluripotent gene expression during RA treatment [13].

Methylation of Oct4 cis-regulatory regions and histone modifications have been reported to contribute to the silencing of the Oct4 gene during mouse and human ESC differentiation [20–25]. DNA methylation occurs subsequent to Oct4 repression, as loss of DNA methylation and chromatin remodeling have no effects on the initial repression of Oct4 [20]. The regulation of Oct4 DNA methylation is currently not well understood. The DNA methylation machinery consists of all DNA methyltransferases (Dnmts) (including Dnmt1, Dnmt2, Dnmt3A, Dnmt3B, and Dnmt3L), and a family of methyl-DNA binding domain (MBD) proteins [26–29]. Two MBD proteins, MBD2 and MBD3, are closely related to each other in their primary structure and belong to the MeCP1 and methyl-DNA binding domain (MBD) proteins [26–29]. Two family of DNA methyltransferases (Dnmts) (including Dnmt1, Dnmt3A and -3B) have no effects on the initial repression of Oct4 cis expression during RA treatment [13].

To address the question of what links Oct4 sequence-specific repression with covalent epigenetic modifications that lead to gene silencing, we investigated the molecular mechanism of Oct4 silencing by GCNF to identify mediators of its repression function. Our results demonstrate that the interaction of GCNF with MBD2, MBD3, and Dnmt3A during differentiation initiates the repression of the Oct4 gene and DNA methylation by means of sequential recruitment of these novel nuclear receptor corepressors.

**Materials and Methods**

### P19 and ESC Lines

GCNF+/− and MBDB2+/− ESCs were established from mutant embryos at Embryonic 3.5-day by blastocyst outgrowth on mouse embryonic fibroblasts (MEFs). The ESCs were passaged three times off MEFs for genotyping. MBDB2−/−, Dnmt1−/−, Dnmt3A−/−, Dnmt3B−/−, and Dnmt3A−/−; as well as Dnmt3B−/− ESCs were kindly provided by Dr. Brian Hendrich [34], Dr. Ruediger Haenisch [38], and Dr. En Li [39], respectively. Wild-type (wt) and mutant ESCs were maintained with leukemia inhibitory factor (LIF) (Chemicon, Temecula, PA, www.chemicon.com) on gelatinized tissue culture dishes in ESC media [13]. For the differentiation of ESCs, monolayer cultured ESCs were treated with 1 μM of all-trans-RA (Sigma, St. Louis, MO, http://www.sigmaaldrich.com) daily in ESC media. P19 cells were maintained in the Dulbecco’s modified Eagle’s media (DMEM) supplemented with 10% fetal calf serum (FCS) and 100 units of penicillin and streptomycin.

### Bisulfite Genomic Sequencing

ESC genomic DNA was extracted with Qiagen DNAeasy kit (Valencia, CA, www.qiagen.com). Genotyping of embryos was performed as reported previously [16]. Bisulfite-modified DNA with EZ DNA methylation kit (Zymo Research, Orange, CA, http://www.zymoresearch.com) was purified and used as template for nested polymerase chain reaction (PCR). Second round PCR products were subcloned into TOPO vector cloning (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) and clones were randomly picked for DNA sequencing. The sequence of the primers is listed in Supporting Information Table 1. Statistical analysis of the data used the Student’s t test.

### Yeast Two-Hybrid Screen and Assays

DNA extracted from an amplified mouse E7 embryonic cDNA library in the yeast vector pACT2 (Clontech, Cat# 638844, Mountainview, CA, http://www.clontech.com) was cotransfected with GCNF bait plasmid pGBK7-GCNF (ligand binding domain [LBD]) into yeast AH109 cells according to the manufacturer’s protocols. First round selection was performed with 7.5 mM 3-amino-1,2,4 triazole (3-AT) and second round selection with 25 mM 3-AT. The interaction was confirmed by colony-lift and liquid β-galactosidase assays according to Clontech’s protocols.

### Antibodies, GST-Pull Down, and Co-IP Assays

Anti-GCNF and anti-LHR-1 antibodies were produced by our laboratory [13, 42]. Anti-Myc, -Haemagglutinin tag (HA), - Oct4, and -MBD3 antibodies were from Santa Cruz (Santa Cruz, CA, http://www.scbt.com/). Anti-Flag and -β-actin antibodies were from Sigma. Anti-MBD2 antibody for chromatin immunoprecipitation (ChIP) was from Upstate Biotechnologies (Lake Placid, NY, http://www.millipore.com) and for Westerns from Santa Cruz. Anti-Dnmt3A and -Dnmt3B monoclonal antibodies were from Imagenex (San Diego, CA, http://wwwimagenex.com). Glutathione-S-transferase (GST), GST-GCNF, or GST-MBD3 proteins were expressed in E. coli BL21 (DE3) and purified with glutathione-agarose beads (Amersham Bioscience, Littlechalfont, England, http://www.gelifesiences.com). In vitro translated proteins were labeled with S35-methionine (iCN Pharmaceuticals, Costa Mesa, CA, http://www.icnpharm.com) using TNT T7 in vitro translation kit (Promega, Madison, WI, http://www.promega.com). GST-pulldown and coimmunoprecipitation (Co-IP) were performed in TBST buffer. Transfected CV-1 (simian) in Origin, and carrying the SV40 genetic material (CO-1) cell total proteins and RA-differentiated P19 cell nuclear proteins were extracted in Buffer D (25 mM Hepes pH 7.9, 150 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), Protease inhibitors, and 5% glycerol). TrueBlot beads from eBioscience (San Diego, CA, http://www.ebioscience.com) were used in the co-IPs to remove the IgG, the heavy-chain of which runs very close to GCNF and MBD2.

### ChIP Assays

Undifferentiated or RA-differentiated ESCs were cross-linked with 1% formaldehyde (Sigma) and soluble chromatin was extracted and sonicated following a protocol provided by Upstate Biotechnology. Sonicated chromatin proteins were incubated with antibodies or normal IgGs and immunoprecipitated.
with protein A/G agarose beads (Santa Cruz). The bound DNA was eluted with sodium dodecasulphate (SDS)-proteinase K solution overnight at 65°C and extracted with phenol/CHCl₃.

P19 Nuclear Extract Fractionation and Gel Mobility Shift Assays

P19 cell nuclear extract was prepared following the procedure described before [8] and separated through a Superose six column (HR 16/50, Pharmacia, Stockholm, Sweden, http://www.sigmaaldrich.com) by chromatography. Each 1.5 ml fraction was collected and analyzed by gel mobility shift assays [13] and western blot. The running buffer for the fast protein liquid chromatography (FPLC) was Buffer D.

Whole-Mount In Situ hybridizations and RT-PCR

Embryos from timed matings between GCNF heterozygous mice were harvested between E8.5 and E8.75 and fixed in 4% paraformaldehyde. Whole-mount in situ hybridizations were carried out as described [13, 42]. Total RNA of ESCs was extracted with Trizole reagents (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) and reverse transcribed with Advantage RT for PCR with Taq DNA polymerase (Clontech, Mountain View, CA).

RESULTS

Hypomethylation of Oct4 Promoter in RA-Treated GCNF⁻/⁻ ESCs

DNA methylation is required for Oct4 gene silencing during RA-induced embryocarcinoma cells (EC) and ESC differentiation [21, 24]. Thus, we analyzed the DNA methylation profile of the Oct4 promoter during RA-induced differentiation in GCNF⁻/⁻ ESCs when compared with wt. Repression of Oct4 is lost in RA-differentiated GCNF⁻/⁻ ESCs (Fig. 1A) [13]. The methylation status of 16 CpG sites in the Oct4 promoter was scanned at different time points of RA treatment (Fig. 1B). In undifferentiated wt ESCs and GCNF⁻/⁻ ESCs, the CpG sites were unmethylated. The number of somites for each embryo is indicated in each panel. Abbreviations: DR0, direct repeat with zero base pair spacing; GCNF, germ cell nuclear factor; LRH-1, liver receptor homolog-1; mu, mutant; RA, retinoic acid; RT-PCR, reverse transcriptase-polymerase chain reaction. so, somites; wt, wild-type.
methylation was detected when wt ESCs were treated with RA for 3 days. After 4 days, more than 50% of the CpGs were methylated, and the methylation status increased between 4 and 6 days. Interestingly, on day 3 of RA treatment, CpG methylation was not initiated in the GCNF DR0−/− ESCs and even at day 6 less than 10% of CpG dinucleotides were methylated (Fig. 1C). The DNA methylation analysis clearly showed that the Oct4 promoter was hypomethylated in GCNF DR0−/− ESCs. When we compared the GCNF expression profile (Fig. 1A) and previous DNA binding studies [13] with CpG methylation profile (Fig. 1C), it was obvious that methylation of the Oct4 promoter occurred concomitantly with the period of elevated GCNF expression and DNA binding activity between days 1 and 3 of RA treatment in wt ESCs. Although Oct4 repression was detectable as early as day 2 of RA treatment (Fig. 1A), there was no RA treatment in wt ESCs. Although Oct4 repression was detectable as early as day 2 of RA treatment (Fig. 1A), there was no significant (p < 0.1) difference in the percentage of methylated CpG dinucleotides between the wt and GCNF DR0−/− ESCs. After 3 days of differentiation, the percentage of methylation in GCNF DR0−/− ESCs was significantly lower (p < .01) than that observed in wt cells. At later time points of differentiation greater differences were observed (p < .0001) between wt and GCNF DR0−/− ESCs.

Hypomethylation of the Oct4 Promoter in GCNF DR0−/− Embryos

Previous studies demonstrated that DNA methylation of the Oct4 gene took place by 6.5 dpc in normal mouse embryos [24, 43]. There was loss of repression of the Oct4 gene in many of the somatic cells in 8.5–8.75 dpc GCNF DR0−/− embryos [8]. Thus, the DNA methylation status of the Oct4 promoter was analyzed in GCNF DR0−/− embryos. As expected, the Oct4 promoter was heavily methylated (40% overall) in wt embryos and DNA methylation was also observed in the GCNF DR0−/− embryos (over all 20% at 8.5 dpc and 30% at 9.5 dpc) (Fig. 1D, 1E). However, when the Oct4 promoter was divided into two parts: the region proximal to the GCNF DR0 element (from the first CpG to seventh CpG) and the region distal to the DR0 (from the eighth to 1sixth CpG), significant differences in DNA methylation levels were observed in the proximal region close to the DR0 between wt and GCNF DR0−/− embryos at both 8.5 dpc and 9.5 dpc (Fig. 1E). In contrast, there was no difference in the region distal to the DR0 between 9.5 dpc of wt and GCNF DR0−/− embryos. These results demonstrated that hypomethylation of the Oct4 promoter close to the GCNF binding site also occurred in GCNF DR0−/− embryos.

Whole-mount in situ analysis showed that the Oct4 gene is silenced in somatic cells of wt embryos (Fig. 1F, panels a and b) and there is widespread loss of repression in somatic cells of the GCNF DR0−/− embryos. Careful analysis of the expression of Oct4 in GCNF DR0−/− embryos at multiple time points indeed showed that some of the Oct4 expression observed at the five somite stage, compared to the two somite stage, was due to re-expression of Oct4 in the neurepithelium (Fig. 1F, panel c and d, arrowheads) as opposed to loss of repression that is observed in other regions of the embryo. Re-expression of the Oct4 gene in the neurepithelium spatially matched the expression pattern of liver receptor homolog-1 (LRH-1), which was previously shown to regulate Oct4 expression (Fig. 1F, panel e and f) [42]. These results support the contention that loss of GCNF function leads to not only loss of repression of Oct4 but also loss of DNA methylation, allowing for a transcriptional activator, like LRH-1, to reverse the Oct4 repression and reactivate the gene.

Identification of GCNF Interacting Factors

The hypomethylation of the Oct4 promoter in GCNF DR0−/− ESCs and embryos suggests that GCNF-mediated repression is directly or indirectly linked to DNA methylation of the promoter; thus, we set out to identify mediators of GCNF repression. A yeast two-hybrid screen was performed to identify GCNF interacting partners. More than 3 × 10^6 independent mouse E7.0 embryo cDNA clones were screened with a Gal4 activation domain (AD)-GCNF LBD fusion protein as a bait. The screen identified several groups of positive colonies. One group of cDNAs shared identical sequences and encoded the short form of mouse MBD protein MBD3b. Another positive colony was identical to a region of Dmnt1 cDNA (which encodes the C-terminal of Dmnt domain). We also identified a colony encoding sequences for the corepressor NCoR, which was expected based on previous reports [8, 44, 45]. In the liquid assays, Gal4-GCNF dependent activation of β-gal reporter activity by MBD3b or Dmnt1 cotransfection was augmented to levels comparable to that observed with nuclear receptor co-repressor (NCoR) (Fig. 2A), which confirmed the interaction between GCNF, MBD3b, and Dmnt1. Considering the high conservation within the MBD family methyl-CpG binding domain (MBD) [28] and the Dmnt family in the C-terminal Dmnt catalytic domain [29], other DNA methylation components were tested for interaction with GCNF. The results demonstrated a specific interaction between MBD2, MBD3, Dmnt1, Dmnt3A, Dmnt3B, and the LBD of GCNF in vitro (Fig. 2C). In contrast, MBD1 and MBD4 yielded unreliable interaction results in these two assays. As a positive control, in vitro translated NCoR was also pulled down by GST-GCNF. As expected, in vitro translated retinoid X receptor (RXR) did not show any interaction with GST-GCNF, establishing a negative control.

GCNF Interacts with a Subset of the MBD Family Via the MBD Domain

Association of MBD3 and MBD2 with GCNF was further corroborated in a mammalian system using Co-IP assays (Fig. 2D). When Myc-tagged GCNF and Flag-tagged MBD3a (full-length) or MBD3b were coexpressed in CV-1 (simian) cells, anti-Myc antibody coimmunoprecipitated flag-MBD3a or flag-MBD3b (lanes 5–6 in Fig. 2D, Co-IP-GCNF). Similarly, anti-Flag antibody coimmunoprecipitated Myc-GCNF (lanes 5 and 6, Co-IP-MBD3). In the untransfected (lane one) or singly transfected COS-1 cells (lanes 2–4), no Co-IP signal was observed even though the expression of the transfected protein was detected in the input and single immunoprecipitate. These results confirm that in mammalian cells GCNF can associate with MBD3. The interaction of GCNF with MBD2 was further verified by Co-IP in mammalian cells too (Fig. 2G). In cotransfected COS-1 cells, anti-HA antibodies (specific for HA-GCNF) coimmunoprecipitated Myc-MBD2, and vice versa. Thus, GCNF can interact with a subset of MBD family members, specifically MBD3 and MBD2 via the MBD domain. However, GCNF is not expressed in COS-1 cells; rather it is transiently expressed in differentiating P19 and ESCs [8, 13]. Therefore, interaction of endogenous GCNF and MBD3 was assayed in differentiating P19 cells (Fig. 2E, 2F). First, we fractionated P19 nuclear extracts by Superose 6 gel filtration chromatography (Fig. 2E) and found that there were GCNF peaks: fraction numbers 12–14 corresponded to the transiently retinoid induced factor (TRIF) complex in gel mobility shift assays [13], and fraction numbers 18–21 were a major GCNF peak but had no DNA binding activity. MBD3a and MBD3b are both expressed in P19 cells and appeared as a major peak between fraction numbers 10 and 11. The tail of the MBD3 peak overlapped with the GCNF-TRIF complex but little MBD3 was coimmunoprecipitated with the second GCNF
peak. The gel filtration results suggested partial cofractionation of MBD3 with the GCNF-TRIF complex. Co-IP assays of P19 nuclear extracts also demonstrated that a small fraction of MBD3a and MBD3b were associated with GCNF and could be coimmunoprecipitated with anti-GCNF antibody (Fig. 2F, lanes 7 and 8). Under the same Co-IP conditions, no signal was detected when IgG was used. Thus, gel filtration and Co-IP results confirmed that endogenous GCNF and MBD3 interact in differentiating P19 cells.

In vitro experiments to this point confirmed interaction between GCNF’s LBD and MBD3 and the catalytic domain of Dnmt3A, Dnmt3B, and Dmnt3B in vitro. (A): Putative GCNF interaction partners identified in a Yeast two-hybrid screen. Gal4-nuclear receptor corepressor (NCoR) was used as a positive control and Gal4 DBD and Gal4 AD empty vectors were used as negative controls. (B): Interaction of GCNF and other DNA methylation machinery components was measured by liquid β-gal assay in yeast two-hybrid system. (C): Interaction of GCNF with MBDs and Dnmts was detected by GST-pulldown assay. In vitro translated NCoR was used as a positive control and RXR as a negative control. (D): Detection of interaction of coexpressed recombinant GCNF and MBD3 in COS-1 cells using coimmunoprecipitation (Co-IP) assays. Myc-GCNF signal is indicated with an arrow and the IgG heavy-chain is indicated with a star. (E): Fractions of P19 nuclear extract separated by FPLC were analyzed by gel mobility shift assays (Gelshift) and western blots with anti-GCNF and -MBD3 antibodies. (F): Detection of the interaction of endogenous GCNF and MBD3 in differentiated P19 cell nuclear extracts. (G): Interaction of GCNF with MBD2 in cotransfected COS-1 cell lysates was detected by Co-IP. (H): Illustration of the MBD3 deletions generated and analyzed. The number of amino acids was labeled. The black box denotes the MBD and the open box denotes for the polyglutamic acid motif (poly E). (I): Analysis of interaction of GCNF and different MBD3 deletions by GST-pulldown assay. (J): Comparison of amino acid sequence of MBDs. Abbreviations: Co-IP, coimmunoprecipitation; COS-1, CV-1 (simian) in Origin, and carrying the SV40 genetic material; DBD DNA binding domain; Dnmt, DNA methyltransferases; FPLC fast protein liquid chromatography;GCNF, Germ cell nuclear factor; GST, glutathione S transferase; IP, immunoprepitation; IR, interaction; LBD, ligand binding domain; MBD, methyl CpG binding domain; MD, MegaDaltons; NCoR, nuclear receptor corepressors; RXR, retinoid X receptor; SMRT, silencing mediator of retinoic acid and thyroid hormone receptors; TRIF, transiently retinoid induced factor.

Figure 2. Interaction of germ cell nuclear factor (GCNF) with MBD3, MBD2, DNA methyltransferases 3A (Dnmt3A), and Dnmt3B in vitro. (A): Putative GCNF interaction partners identified in a Yeast two-hybrid screen. Gal4-nuclear receptor corepressor (NCoR) was used as a positive control and Gal4 DBD and Gal4 AD empty vectors were used as negative controls. (B): Interaction of GCNF and other DNA methylation machinery components was measured by liquid β-gal assay in yeast two-hybrid system. (C): Interaction of GCNF with MBDs and Dnmts was detected by GST-pulldown assay. In vitro translated NCoR was used as a positive control and RXR as a negative control. (D): Detection of interaction of coexpressed recombinant GCNF and MBD3 in COS-1 cells using coimmunoprecipitation (Co-IP) assays. Myc-GCNF signal is indicated with an arrow and the IgG heavy-chain is indicated with a star. (E): Fractions of P19 nuclear extract separated by FPLC were analyzed by gel mobility shift assays (Gelshift) and western blots with anti-GCNF and -MBD3 antibodies. (F): Detection of the interaction of endogenous GCNF and MBD3 in differentiated P19 cell nuclear extracts. (G): Interaction of GCNF with MBD2 in cotransfected COS-1 cell lysates was detected by Co-IP. (H): Illustration of the MBD3 deletions generated and analyzed. The number of amino acids was labeled. The black box denotes the MBD and the open box denotes for the polyglutamic acid motif (poly E). (I): Analysis of interaction of GCNF and different MBD3 deletions by GST-pulldown assay. (J): Comparison of amino acid sequence of MBDs. Abbreviations: Co-IP, coimmunoprecipitation; COS-1, CV-1 (simian) in Origin, and carrying the SV40 genetic material; DBD DNA binding domain; Dnmt, DNA methyltransferases; FPLC fast protein liquid chromatography;GCNF, Germ cell nuclear factor; GST, glutathione S transferase; IP, immunoprepitation; IR, interaction; LBD, ligand binding domain; MBD, methyl CpG binding domain; MD, MegaDaltons; NCoR, nuclear receptor corepressors; RXR, retinoid X receptor; SMRT, silencing mediator of retinoic acid and thyroid hormone receptors; TRIF, transiently retinoid induced factor.

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GCNF-Dependent Recruitment of MBD2, MBD3, and Dnmt3A to the Oct4 Promoter During ESC Differentiation

Direct interaction between GCNF and MBD2, MBD3, Dnmt3A, and Dnmt3B raised the question of whether GCNF recruits these factors to the Oct4 promoter. Wild-type and GCNF−/− ESCs were cultured to compare the binding of GCNF, MBD2, and MBD3 to the Oct4 promoter in vivo. Expression of MBD2, MBD3a, and MBD3b at the RNA and protein levels was not significantly altered upon RA treatment in either wt or GCNF−/− ESCs (Fig. 3A). As expected, GCNF expression was upregulated (ca. 24-fold) based on quantitation of protein signals and Oct4 expression was downregulated (90%) after RA treatment; however, in GCNF−/− ESCs, the repression of Oct4 was lost and expression was maintained at high levels.

Direct binding of MBD2 and MBD3 to the Oct4 promoter in wt and GCNF−/− ESCs was analyzed by ChIP (Fig. 3B). To quantitatively estimate the binding signal of GCNF, MBD2, and MBD3 in wt and GCNF−/− ESCs, ChIP signals were analyzed by densitometry and the results were plotted (Fig. 3C). To validate the ChIP samples prepared from GCNF−/− ESCs, LRH-1 was used as a positive control to investigate binding to Oct4 promoter [42]. Binding of LRH-1 to the Oct4 promoter was clearly detected in GCNF−/− ESCs, similar to wt cells. In undifferentiated ESCs no GCNF, MBD2, or MBD3 binding was detected, as expected. Upon differentiation, GCNF and MBD3 binding was enriched at the Oct4 promoter after 40 hours of RA treatment in wt ESCs (sevenfold and fivefold increases in DNA binding were detected, respectively). At the 72-hour RA differentiation time point, GCNF protein was still detected (Fig. 3A, lane 9) and bound to the Oct4 promoter (Fig. 3B, lane 3); meanwhile, MBD2 replaced MBD3 at the Oct4 promoter, displaying a nine-fold increase in DNA binding. As expected, loss of GCNF binding at the Oct4 proximal promoter resulted in the loss of recruitment of both MBD2 and MBD3 (Fig. 3B). Thus, GCNF is essential for the recruitment of MBD2 and MBD3 to the Oct4 promoter during ESC differentiation.

We investigated recruitment of the de novo Dnmts, Dnmt3A and Dnmt3B, to the Oct4 promoter during ESC differentiation. We observed that the expression pattern of Dnmt3A and Dnmt3B was distinctively different from each other during ESC differentiation (Fig. 3D). In wt ESCs, Dnmt3A was induced upon RA treatment. In contrast, Dnmt3B was quickly repressed. In GCNF−/− ESCs, the expression pattern of Dnmt3A was similar to wt ESCs; however, the repression of Dnmt3B was slower. Thus, Dnmt3A exhibits a similar expression pattern to GCNF, whereas Dnmt3B exhibits an expression pattern similar to Oct4. The differential expression

Figure 3. Germ cell nuclear factor (GCNF) recruits MBD2, MBD3, and DNA methyltransferases 3A (Dnmt3A) to the Oct4 promoter in embryonic stem cells (ESCs). (A): Expression of GCNF, Oct4, MBD2, and MBD3 was detected in differentiated wild-type (wt) and GCNF−/− ESCs by RT-PCR and Western blot. (B): Binding of GCNF, MBD2, and MBD3 to Oct4 promoter in wt and GCNF−/− ESCs was detected by chromatin immunoprecipitation (ChIP) assay. (C): Quantitation of PCR signals in (B). The strength of GCNF, MBD2, and MBD3 binding signals at the undifferentiated time point was set as one. The bound LRH-1 signal at 72-hour retinoic acid-differentiation was set as 1. (D): Expression of Oct4, Dnmt3A, and Dnmt3B in wt ESC and GCNF−/− ESCs was detected by Western blot. (E): Binding of Dnmt3A to Oct4 promoter in wt ESC was detected by ChIP assay. (F): Binding of Dnmt3B to major satellite repeat sequence in wt and GCNF−/− ESCs was detected by ChIP assay. Abbreviations: ChIP, chromatin immunoprecipitation; Dnmt, DNA methyltransferase; GCNF, Germ cell nuclear factor; LRH-1, liver receptor homolog-1; MBD, methyl CpG binding domain; RA, retinoic acid; RT-PCR, reverse transcriptase-polymerase chain reaction.
DNA methylation at early stages of ESC differentiation. Dnmt3A rather than Dnmt3B to the Oct4 (Fig. 3E). Our data suggests that GCNF preferentially recruits MBD3 during ESC differentiation. We observed that Dnmt3A was preferentially recruited to the Oct4 proximal promoter to initiate DNA methylation at early stages of ESC differentiation.

**Figure 4.** Comparison of Oct4 expression and Oct4 promoter methylation status in MBD3−/− and MBD2−/− embryonic stem cells (ESCs) with wild-type (wt) ESCs and germ cell nuclear factor−/− (GCNF−/−) ESCs. Oct4 and GCNF mRNA level (A) and protein level (B) were detected during retinoic acid (RA)-induced differentiated wt and MBD3−/− ESCs by RT-PCR (A) and Western blot (B). Oct4 and GCNF mRNA (C) and protein expression (D) were detected during RA-induced differentiated wt and MBD2−/− ESCs by RT-PCR (C) and Western blot (D). (E): Comparison of re-expression of Oct4 in RA-treated ESCs with LIF reactivation in wt ESCs, GCNF−/−, MBD3−/−, and MBD2−/− ESCs. (F): Methylation status of Oct4 promoter in different time point RA treatment and LIF reactivated wt ESCs, MBD3−/−, and MBD2−/− ESCs. (G): Comparison of percentage of methylated CpG sites of 16 CpGs in the Oct4 proximal promoter. Student’s t test was used for the statistic analyses. *p < .05, **p < .01, ***p < .0001. Abbreviations: ESC, embryonic stem cell; GCNF, germ cell nuclear factor; LIF, leukemia inhibitory factor; MBD, methyl CpG binding domain; RA, retinoic acid; wt, wild-type; RT-PCR, reverse transcriptase-polymerase chain reaction.

**DNA Methylation of the Oct4 Promoter is Defective in MBD3−/− and MBD2−/− ESCs**

To further investigate the role of the interaction of GCNF with MBD2 and MBD3 in ESCs, the expression of GCNF and Oct4 was compared in wt ESCs to MBD3−/− (Fig. 4A, 4B) and MBD2−/− ESCs (Fig. 4C, 4D). Oct4 expression was maintained at a low level in the MBD3−/− ESCs during RA-treatment, which implied that it was not properly silenced. This pattern was different from that of the GCNF−/− ESCs, where Oct4 repression is lost and expression is maintained at comparable levels to undifferentiated cells (Figs. 1A, 3D). However, another pluripotency factor, Nanog, exhibited the same repression pattern in MBD2−/− ESCs as in wt ESCs (Fig. 4B). Surprisingly, GCNF was induced in MBD3−/− ESCs and maintained at later stages of RA treatment (days 3–6). These observations support our hypothesis that loss of MBD3 results in loss of Oct4 silencing during RA-induced ESC differentiation. In MBD2−/− ESCs, we observed that Oct4, Nanog, and GCNF showed the same expression profile at the mRNA and protein levels as in wt cells (Fig. 4C, 4D). Thus, we were unable to determine the role of MBD2 recruitment to the Oct4 promoter. To further understand the different functions of MBD2 and MBD3 in the regulation of Oct4 expression in ESCs, we designed an RA-differentiation and LIF rescue experiment to study silencing and methylation status of the Oct4 promoter in MBD3−/− and MBD2−/− ESCs. As expected, Oct4 expression in wt ESCs is sufficiently silenced because there was no reactivation of Oct4, and methylation of the Oct4 promoter was maintained (60%, Fig. 4F, 4G) with re-addition of LIF after 6 days RA-treatment in wt ESC.
Surprisingly, although Oct4 repression was observed in MBD3−/− and MBD2−/− ESCs, readministration of LIF restored Oct4 expression in both mutant ESC lines (Fig. 4E). Loss of MBD3 causes deficiency of DNA methylation of the Oct4 promoter in RA-differentiated ESCs (18% in MBD3−/− vs. 60% in wt at 6-day RA treatment), despite the fact that GCNF exhibited higher expression in MBD3−/− ESCs (Fig. 4B) at this time point. The hypomethylation defect observed in MBD3−/− ESCs was reiterated when LIF was added back after RA treatment. Deletion of MBD2 did not affect the DNA methylation status of the Oct4 promoter during RA-induced differentiation. Surprisingly, methylation of the Oct4 promoter was dramatically reduced from 55% to 20% in MBD2−/− ESCs with readadministration of LIF, indicating that Oct4 silencing was affected. The DNA methylation profiles of the Oct4 promoter in MBD3−/− and MBD2−/− ESCs (Fig. 4F, 4G) reinforced the separate functions of MBD3 and MBD2 in the repression and silencing of Oct4. These data suggest that MBD2 plays a role in maintenance of Oct4 silencing, while MBD3 is essential for initiating DNA methylation and Oct4 silencing. Importantly, both factors function in a GCNF-dependent manner.

**Defective DNA Methylation of the Oct4 Promoter in Different Dnmt Mutant ESCs**

In light of the direct interaction between GCNF and Dnmt3A in ESCs (Fig. 3E), we further compared the expression of Oct4 and the DNA methylation status of its promoter in different Dnmt mutant ESC lines. GCNF induction and Oct4 repression were not altered in all Dnmt mutant cell lines, including Dnmt1−/−, Dnmt3A−/−, and Dnmt3B−/−, as well as Dnmt3A−/− and Dnmt3B−/− (Fig. 5A). Their DNA methylation status are depicted in Figure 5B and quantitated in Figure 5C. Dnmt1 disruption leads to a reduction of methylated CpG percentage to approximately half that of wt ESCs at 6 days of treatment, and is consistent with the role of Dnmt1 in mediating DNA methylation during DNA replication. Interestingly, disruption of Dnmt3A alone resulted in almost complete loss of DNA methylation of Oct4 promoter, whereas deletion of Dnmt3B did not affect DNA methylation of Oct4 promoter (19% vs. 20% at day 3 and 52% vs. 59% in wt ESCs at day 6). As previously reported, double KO of Dnmt3A and Dnmt3B caused a complete loss of DNA methylation of Oct4 promoter [40]. The DNA methylation profiles support our previous finding that GCNF preferentially interacts with Dnmt3A rather than Dnmt3B in differentiated ESCs.

**Recruitment of MBD3 is Independent of De Novo DNA Methylation**

To address whether the differential recruitment of MBD3 and MBD2 is dependent on DNA methylation, we analyzed Oct4 repression and DNA methylation in de novo DNA methylation deficient ESCs. Although DNA methylation is lost in Dnmt3A−/− and Dnmt3B−/− ESCs (Fig. 5B), the initial repression of Oct4 exhibits the same temporal pattern as wt ESCs.
was observed in the Oct4 recruitment of MBD3 to the Oct4 promoter with a DR0 and unmethylated CpG sites is activated by LRH-1 under the control of LIF. At the beginning of retinoic acid (RA) induction (1.5 days), induced expression of GCNF hexamer replaces LRH-1 binding at the DR0 site. GCNF recruits MBD3 complex to unmethylated CpG sites and Oct4 repression is initiated. Once de novo DNA methylation is triggered through direct recruitment of Dmnt3A to the Oct4 promoter by GCNF-MBD3 and MBD2/3 complexes recruited to CpG sites and silencing of Oct4 gene occurs (days 1.5–3.0). At late stages of RA-induced differentiation (days 3–6), the expression of GCNF is downregulated and the MBD2 and MBD3 complexes are no longer bound to the Oct4 promoter but DNA methylation is maintained and Oct4 gene is completely silenced (day 6). HP1 complex maybe replaced by GCNF/DNA methylation complex to bind to methylated Oct4 promoter region. Abbreviations: Dmnt, DNA methyltransferase; DR0, direct repeat with zero base pair spacing; GCNF, germ cell nuclear factor; HP1, heterochromatin protein 1; LIF, leukemia inhibitory factor; LRH-1, liver receptor homolog-1; MBD3, methyl CpG binding domain 3; RA, retinoic acid.

**Figure 6.** Model of Oct4 gene repression and silencing initiated by germ cell nuclear factor (GCNF)-dependent recruitment of MBD2, MBD3, and DNA methyltransferases 3A (Dmnt3A). The Oct4 promoter with a DR0 and unmethylated CpG sites is activated by LRH-1 under the control of LIF. At the beginning of retinoic acid (RA) induction (1.5 days), induced expression of GCNF hexamer replaces LRH-1 binding at the DR0 site. GCNF recruits MBD3 complex to unmethylated CpG sites and Oct4 repression is initiated. Once de novo DNA methylation is triggered through direct recruitment of Dmnt3A to the Oct4 promoter by GCNF-MBD3 and MBD2/3 complexes recruited to CpG sites and silencing of Oct4 gene occurs (days 1.5–3.0). At late stages of RA-induced differentiation (days 3–6), the expression of GCNF is downregulated and the MBD2 and MBD3 complexes are no longer bound to the Oct4 promoter but DNA methylation is maintained and Oct4 gene is completely silenced (day 6). HP1 complex maybe replaced by GCNF/DNA methylation complex to bind to methylated Oct4 promoter region. Abbreviations: Dmnt, DNA methyltransferase; DR0, direct repeat with zero base pair spacing; GCNF, germ cell nuclear factor; HP1, heterochromatin protein 1; LIF, leukemia inhibitory factor; LRH-1, liver receptor homolog-1; MBD3, methyl CpG binding domain 3; RA, retinoic acid.

(Fig. 5A). Western blot analysis confirmed the induction of GCNF and maintenance of Oct4 repression. Furthermore, MBD3 and MBD2 expression is maintained in the Dmnt3A−/− and Dmnt3B−/− ESCs (Fig. 5C). The recruitment of MBD2 and MBD3 to the Oct4 promoter was detected in Dmnt3A−/− and Dmnt3B−/− double KO ESCs (Fig. 5E) and quantitated in Figure 5F. Binding of GCNF, MBD2, and MBD3 to the Oct4 promoter was confirmed between 0 and 3 days of RA-treatment; interestingly, neither MBD2 nor MBD3 is bound to the Oct4 promoter at day 6. GCNF binding to the Oct4 promoter was observed in the Dmnt3A−/− and Dmnt3B−/− ESCs in a pattern similar to wt cells. Recruitment of MBD2 to the Oct4 promoter was lost in the Dmnt3A−/− and Dmnt3B−/− ESCs; however, the GCNF-dependent binding of MBD3 was maintained in Dmnt3A−/− and Dmnt3B−/− ESCs. These results demonstrate that the recruitment of MBD3 is independent of de novo DNA methylation, but requires GCNF. In contrast, MBD2 recruitment is dependent on both CpG methylation and the binding of GCNF. Finally, it is clear that disruption of Dmnt3A alone causes hypomethylation of Oct4 promoter; however, this defect has no bearing on the GCNF-dependent recruitment of MBD3 to the Oct4 promoter.

**DISCUSSION**

This study demonstrates that repression of Oct4 is mediated by recruitment of novel NCoRs CpG binding proteins MBD3 and MBD2 and the de novo Dmnt, Dmnt3A, to the Oct4 promoter via direct interactions with the orphan nuclear receptor GCNF during RA-induced differentiation of mouse ESCs. The time course of differential recruitment of MBD2 and MBD3 to the Oct4 promoter correlates with the DNA methylation status of the promoter (Fig. 1). Although MBD2 and MBD3 are structurally closely related to each other, KO mouse models for each gene clearly establish that they are not functionally redundant. MBD2 was shown to bind methylated DNA and interact with MBD3 in a NuRD repression complex [30, 48] or with Sin3A in a Sin3A repression complex [49]. The MBD3 KO and MBD3−/− ESCs demonstrate an important role for the MBD3-NuRD complex during the early embryonic development and ESC differentiation, respectively [34, 37]. The dependence of MBD2 and independence of MBD3 recruitment to the Oct4 promoter on DNA methylation underscores the mechanistic differences between these factors and their complexes. The differential dependence of MBD3 and MBD2 on DNA methylation may provide a rationale for the significant differences observed in the phenotypes of the two KO models. One could speculate that MBD3 being important for the repression of genes such as Oct4 would display dramatic defects upon inactivation due to loss of repression of target genes. In contrast, inactivation of MBD2 would have less acute effects, because it appears to be involved in maintaining gene silencing rather than repression. Our findings clearly define a role for MBD3 at the molecular level in the repression of Oct4 expression during ESC differentiation. The time-delay between Oct4 repression and DNA methylation also indicates that Oct4 repression occurs at the unmethylated stage, in which GCNF binding and interaction with MBD3 take place. Oligomerization of GCNF in differentiated P19 and ESCs likely accelerates the recruitment of MBD3 and spreading of the repression complex throughout the Oct4 promoter [50]. Oligomerization of GCNF may also facilitate the simultaneous recruitment of de novo Dmnt, Dmnt3A, to CpG sites to initiate methylation of the promoter. Additionally, promoter occupation by GCNF and MBD3 may protect from transactivator binding (e.g., LRH-1) and facilitate subsequent histone deacetylation, histone methylation, DNA methylation, and recruitment of MBD2, leading ultimately to gene silencing [20]. Thus, direct recruitment of MBD2 and MBD3 represents a novel repression model for nuclear receptors and may link epigenetic modification to gene-specific repression by nuclear receptors. Our results are also consistent with the recent observations that showed MBD2 and MBD3 belong to distinct NuRD repression complexes [51].

It has been reported that GCNF interacts with Dmnt3A and Dmnt3B via the proline tryptophan tryptophan proline (PWWP) domain and the catalytic C-terminus in vitro [46]. We also found that GCNF can directly interact with the C-terminus of Dmnt family members, Dmnt1, Dmnt3A, and Dmnt3B in vitro; however, in vivo GCNF preferentially recruits Dmnt3A to Oct4 promoter (Fig. 3). It is consistent with a previous study using the Dmnt KO ESCs, which showed that although both Dmnt3A and Dmnt3B play a role
in the repression of Oct4. Dnmt3B plays the dominant role [40]. Dnmt3B protein is rapidly downregulated with RA-treatment, thus there is little overlap between the periods of expression of Dnmt3B and GCNF. Dnmt3A induction parallels GCNF expression and overlap with each other from 1.5 to 4 days of RA-treatment, which provides a temporal window of overlap that allows for interaction (Fig. 5).

Repression and silencing of Oct4 is a multistep process requiring various epigenetic covalent modifications (DNA and histones) [20, 22-25, 52, 53]. Each modification requires specific factors and complexes be brought to the promoter to facilitate efficient repression. Loss of any one of these factors or failure to recruit a repressor complex will lead to loss of proper silencing; however, repression of Oct4 can still be observed [20, 52]. Case in point are Dnmt 3A and Dnmt3B, which are required for the de novo methylation of the Oct4 gene and its silencing (Fig. 6) [20, 52]; however, Oct4 expression is still repressed when the double KO ESCs are treated with RA. The same phenomenon occurs in G9a KO [20], MBD3 KO (Fig. 4), and Dnmt3A KO ESCs (Fig. 5). Repression of Oct4 still occurs in these mutant ESCs most likely because GCNF is still expressed and expression of activators, like LRH-1, are repressed [42]. The binding of GCNF to the Oct4 promoter is sufficient to displace activators, like LRH-1, and trigger a transition from activation and to repression. Thus, inactivation of individual corepressors such as MBD2, MBD3, Dnmt3A, and G9a that mediate repression and silencing of Oct4 does not lead to loss of repression, rather they result in hypomethylation of the Oct4 promoter and loss of silencing of Oct4 expression (Figs. 4, 5). Only when the initiator and coordinator of Oct4 repression, GCNF, is inactivated, both Oct4 repression and DNA methylation are lost and Oct4 expression is maintained at higher levels than in the mutant corepressor ESC lines (Figs. 3-5). Recently, it was found that G9a can recruit Dnmt3a and Dnmt3b to the Oct4 promoter through direct interaction between the G9a ankyrin (ANK) domain and the Dnmt MTD domain [54]. We found abnormal Dnmt3 expression in G9a KO ESCs. It will be interesting to further understand the relationship between GCNF-dependent repression and histone modification by G9a and DNA methylation by Dnmt3a/Dnmt3b in different genetically deficient ESCs.

**SUMMARY**

Our results have established GCNF as an important initiator of Oct4 gene repression and silencing, and we propose the following model as the important key steps in Oct4 repression in ESCs (Fig. 6). In undifferentiated and early differentiating ESCs transactivators, such as LRH-1 bind to the DR0 site in the Oct4 promoter and sites in the proximal enhancer to maintain Oct4 gene expression [42]. Upon induction with RA, GCNF hexamer displaces LRH-1 from the DR0, causing passive loss of activation. Concomitant with binding to the DR0, GCNF recruits MBD3 and likely the Mi-2/NuRD complex, which binds to unmethylated CpGs to initiate active repression of Oct4. Subsequently, de novo DNA methylation occurs with the recruitment of Dnmt3A. Later events, recruitment of Dnmt3A and MBD2 by GCNF to bind to methylated CpG dinucleotides, lead to an increase in DNA methylation. Corepression of MBD3, MBD2, and Dnmt3A to the Oct4 promoter can be detected in ESCs between 36 and 72 hours. The apparent corecruitment could reflect asynchrony in the ESC cultures or may be because of direct interactions between MBD2 and MBD3 [30], which cannot be ruled out. Alternatively, simple intermediate complexes containing both MBD2 and MBD3 may be present simultaneously on the Oct4 promoter as depicted in Figure 6. In addition, the differentiating ESCs undergo proliferation thus Dnmt1 is required to maintain the fully methylated status of CpG dinucleotides after DNA replication. Subsequently, GCNF expression itself is repressed but Oct4 DNA methylation is maintained, reflective of true gene silencing as MBD2 complexes and/or other repression complexes, such as the HP1 complex are docked on the Oct4 promoter [55]. This model is the first to define the initiation steps of Oct4 gene repression and DNA methylation mediated by GCNF and proposes differential functions for MBD3 before de novo DNA methylation and MBD2 after DNA methylation.

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**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicate no potential conflicts of interest.

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