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PRDM14 maintains pluripotency of embryonic stem cells through TET-mediated active DNA demethylation

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ABSTRACT

Pluripotency and self-renewal of mouse embryonic stem cells (ESCs) depend on a network of transcription factors maintained by exogenous leukaemia inhibitory factor (LIF). PR-domain containing transcriptional regulator 14 (PRDM14), is essential for maintenance of ESC self-renewal when the cells are cultured in serum plus LIF, but not in 2i medium plus LIF. Here, we show that pluripotency of ESCs is maintained by enforced expression of PRDM14 at a high level, as observed in ESCs in 2i plus LIF and developing primordial germ cells in the absence of LIF. Constitutive expression of PRDM14 represses *de novo* DNA methylation in pluripotency-associated genes, resulting in the maintenance of gene expression after withdrawal of LIF, while also repressing the upregulation of differentiation markers. Further, knockdown of *Tet1*/*Tet2* and administration of base excision repair (BER) pathway inhibitors impairs the PRDM14-induced resistance of ESCs to differentiation. We conclude that, in the absence of LIF, PRDM14 governs the retention of pluripotency-associated genes through the regulation of TET functions in the BER-mediated active demethylation pathway, while acting to exert TET-independent transcriptional repressive activity of several differentiation markers.

1. Introduction

DNA methylation is formed by the *de novo* DNA methyltransferases DNMT3A/DNMT3B, and is maintained by the UHRF1/DNMT1 complex during DNA replication [1–3]. Inhibition of the UHRF1/DNMT1 complex triggers passive dilution of DNA methylation, whereby less 5mC is incorporated into the genome after each subsequent cell division. Recently, it has been shown that oxidation of 5mC by ten-eleven translocation (TET) proteins produces 5-hydroxymethylcytosine (5hmC) [4]. 5hmC is further oxidized to 5-formylcytosine (5fC) and 5-carboxycytosine (5caC), both of which can be repaired by the base excision repair (BER) pathway to produce unmodified cytosine [5,6]. Importantly, the level of DNA methylation in the regulatory elements of the pluripotency-associated genes is closely linked to their role in ESC self-renewal and differentiation [7,8]. In undifferentiated ESCs, the balance between 5mC to 5hmC/5fC/5caC in these genes is shifted toward 5hmC/5fC/5caC, promoting both passive demethylation and BER-dependent active demethylation and also sustained hypomethylation in the cells [9–12].

Recently, we observed high levels of PR-domain containing transcriptional regulator 14 (PRDM14) in developing primordial germ cells (PGCs) [13] and provided evidence indicating that PRDM14 promotes TET-BER-dependent active DNA demethylation in pluripotency-associated genes, germ cell-specific genes, and imprinted loci in ESCs [14]. Furthermore, PRDM14 represses the expression of *de novo* DNA methyltransferase *Dnmt3a*/*b*/*l* and induces rapid proliferation, which propagates passive DNA demethylation in ESCs cultured with 2i medium plus LIF [11–13]. However, the complete biological function of PRDM14-induced TET-BER-dependent active demethylation is largely unknown.

In this study, we sought to elucidate the biological role of PRDM14 during TET-BER-dependent active demethylation. Moreover, we investigated the effects of PRDM14 overexpression on ESC fate in the absence of LIF. In doing so, we revealed that enforced expression of PRDM14 could maintain ESC pluripotency, even in the absence of LIF, through TET-BER-mediated active demethylation.

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2. Materials and methods

2.1. Cell culture

E14tg2a ESCs were cultured in feeder-free conditions in GMEM (Wako Pure Chemical Industries Ltd.) supplemented with 10% FCS (Invitrogen, Life Technologies), 1 mM glutamine (Wako Pure Chemical Industries Ltd.), nonessential amino acids (Wako Pure Chemical Industries Ltd.), and 0.1 mM 2-mercaptoethanol (Wako Pure Chemical Industries Ltd.). The ESCs were also supplemented with LIF (Wako Pure Chemical Industries Ltd.).

ESCs stably expressing PRDM14 were established from single E14tg2a mouse ESC colonies by transduction of the pCAG–FHH–*Prdm14*-IRES-Puro vector. Puromycin selection (2 μg/mL puromycin; Sigma–Aldrich) was applied 48 h after transfection and PRDM14 expression levels were confirmed by qRT-PCR and western blotting.

Inducible PRDM14 ESCs and Tet KD ESCs were established as shown previously [14].

2.2. Long culture assay

ESCs (2×10^5) were plated on 6-cm culture dishes in ESC culture medium without LIF. Three days after plating, these cells were treated with 0.05% Trypsin (Gibco, Life Technologies), dissociated from the culture dish, and then re-plated at 2×10^5 cells per dish in ESC medium. This passage culture was continued for up to 30 d.

2.3. Alkaline phosphatase staining

Cultured ESCs were fixed with 4% paraformaldehyde for 20 min. The cells were then washed with cold phosphate buffered saline (PBS) and stained for AP activity as described previously [15].

2.4. qRT-PCR

Total RNA was extracted from the cells using TRIzol (Invitrogen). The ReverTra Ace qPCR RT kit (Toyobo) was used for cDNA synthesis, according to the manufacturer's instructions. Subsequently, the synthesized cDNA was used as a template for qPCR with the Thermal Cycler Dice® Real Time System (TaKaRa) and Thunderbird SYBR qPCR Mix (Toyobo) using gene-specific primers (Supplementary Table S1).

2.5. Western blotting

Cells were lysed by boiling in sodium dodecyl sulphate (SDS) sample buffer (Wako Pure Chemical Industries Ltd.). 2-mercaptoethanol was added to denature the proteins prior to being separated on polyacrylamide-SDS gels. The protein was then transferred onto polyvinylidene fluoride membranes and probed using the following primary antibodies: α-Histone H3 (Abcam; ab1791), α-PRDM14, α-FLAG (Sigma–Aldrich; F1804), α-OCT3/4 (Santa Cruz Biotechnology, Inc.; sc-8628), α-NANOG (eBioscience; 14-5761-80), α-KLF2 (Santa Cruz Biotechnology, Inc.; sc-28675), α-TET1 (Millipore; 09–872), and α-TET2 (Santa Cruz Biotechnology, Inc.; sc-136926). Following the primary antibody reaction, the membranes were incubated with secondary horseradish peroxidase-coupled antibodies. Detection was performed using Luminata Forte Western HRP Substrate (Millipore).

2.6. Immunofluorescence staining

ESCs were fixed with 4% paraformaldehyde for 20 min. After being washed with cold PBS, the ESCs were permeabilized for 5 min with cold PBS containing 0.5% Triton X-100. Permeabilized cells were then washed with cold PBS and treated using the primary antibodies α-OCT3/4 and α-NANOG. After 1 h, the cells were washed with cold PBS and treated using fluorescently tagged secondary antibodies and DAPI or Hoechst dye for 45 min. Cells were once again washed with cold PBS and mounted with Vectashield (Vector Laboratories, Ltd.). Images were acquired using confocal microscope system A1 (Nikon).

2.7. Teratoma formation and histological analysis

Approximately 5×10^6 ESCs were injected subcutaneously into both flanks of female nude mice. After 30 d, the tumours were excised and embedded in paraffin, and the sectioned into 4-μm-thick slices. Hematoxylin & eosin staining was done according to the standard protocol. Expression of markers for the three germ layers were monitored with immunofluorescence with antibodies: α-AFP (Proteintech Group, Inc.; 14550-1-AP), α-SMA (Abcam; ab5694), and α-β-Tubulin (Cell Signalling; #2146).

2.8. GlucMS-qPCR analysis

Genomic DNA (2350 ng) was treated with T4 phage β-glucosyltransferase (T4-BGT, NEB M0357S), according to the manufacturer's instructions. Glycosylated genomic DNA (750 ng) was digested with 40 U of either *Hpa*II, *Msp*I, or no enzyme at 37 °C overnight, followed by enzyme inactivation with proteinase K. The *Hpa*II- or *Msp*I-resistant fractions were quantified by qPCR using primers designed around one *Hpa*II/*Msp*I site, which was normalized to a region lacking *Hpa*II/*Msp*I sites. Resistance to *Msp*I directly translated to the percentage of 5hmC, whereas the percentage of 5mC was calculated by subtracting the 5hmC contribution from the total *Hpa*II resistance. The primers used in this experiment are presented in Supplementary Table S1.

2.9. Inhibition of the BER pathway

ESCs were treated with 1 μg/mL Dox to induce PRDM14 expression. In order to inhibit the BER pathway, one of two small molecule inhibitors, 3-AB (5 mM; Sigma–Aldrich) or CRT0044876 (100 μM; Calbiochem) in 0.1% DMSO, was added to the medium.

3. Results

3.1. High expression of PRDM14 promotes maintenance of ESC capacity in a LIF-independent manner

We previously showed that overexpression of *Prdm14*, as observed in developing PGCs, promotes TET-BER-dependent active demethylation in ESCs [14]. To investigate the function of TET-BER-dependent demethylation by PRDM14 in the spontaneous differentiation of ESCs induced by LIF-withdrawal, we established PRDM14-overexpressing ESCs (PRDM14-OE ESCs), which mirror the high expression levels observed in ESCs with 2i medium plus LIF and developing PGCs (Fig. 1A). We firstly detected AP activity, representing the pluripotency, in the control and PRDM14-OE ESCs with LIF and 6 d after the withdrawal of LIF (Fig. 1B). The control ESCs spontaneously differentiated, resulting in the loss of AP activity, while PRDM14-OE ESCs showed sustained AP activity after withdrawal of LIF. Next, we measured the downstream effects of PRDM14 expression on pluripotency-associated genes and differentiation-associated genes in these cells. Here, the expression of Socs3, which is a direct target of LIF-stimulated STAT3, was rapidly and significantly reduced in both the control and PRDM14-OE ESCs after removal of LIF (Fig. 1C). The expression of *Oct3/4*,

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