

Distinct functions of Dnmt3a and Dnmt3b de novo DNA methyltransferases in ES cell proliferation and differentiation

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ABSTRACT

Two de novo DNA methyltransferases, Dnmt3a and Dnmt3b, have been identified in humans and mice to contribute to the methylation of unmodified DNA. We recently showed a transition of de novo DNA methyltransferase expression from Dnmt3b to Dnmt3a during mouse embryogenesis and in tissue-specific stem cells, suggesting distinct functions of Dnmt3a and Dnmt3b during these processes. In this study, to characterize the functions of Dnmt3a and Dnmt3b in pluripotent stem cells, we exogenously transfected ES cells with Dnmt3a and Dnmt3b cDNAs linked to an internal ribosome entry site-green fluorescent protein gene, and then analyzed the effects of expression of these de novo DNA methyltransferases on ES cell growth and differentiation. ES cells expressing Dnmt3b showed specific downregulation of pluripotency marker genes such as Nanog and Oct 3/4. In addition, Dnmt3a-transfected ES cells showed a specific increase in mitotic index, while Dnmt3b-transfected ES cells showed a decrease in mitotic index. These results suggest that Dnmt3b has important physiological roles in the initial process of stem cell differentiation and that Dnmt3a has a function in stem cell proliferation.

Keywords: DNA Methylation; Dnmt3a; Dnmt3b; Stem Cells; ES Cells; Differentiation; Proliferation

1. INTRODUCTION

DNA methylation is an epigenetic modification that plays crucial roles in multiple biological processes, such

as transcriptional repression of genes, genomic imprinting, X chromosome inactivation, and transposon silencing [1-3]. In addition, aberrant DNA methylation patterns have been reported in many tumor and diseases in humans [4]. In the mammalian genome, cytosine residues of CpG dinucleotide sequences are modified post-replication by DNA methylation; about 70% - 80% of CpG dinucleotides are methylated [5]. During mammalian development, the levels of methylation of genomic DNA undergo dynamic changes. De novo DNA methylation starts after implantation, following genome-wide demethylation after fertilization [6]. Three active DNA methyltransferases, Dnmt1, Dnmt3a and Dnmt3b, have been identified in humans and mice [7-10]. Dnmt1 preferentially acts on hemimethylated CpG dinucleotides and is necessary for the maintenance of specific methylation patterns during DNA replication, while Dnmt3a and Dnmt3b contribute to the methylation of unmodified DNA. These enzymes are essential for normal mouse development. Inactivation of Dnmt1 in mice leads to global genome DNA demethylation and embryonic lethality [11]. Dnmt3a null mice die 4 weeks after birth, while Dnmt3b null mice die in the post-gestation stage as a result of multiple developmental defects, including growth impairment and rostral neural tube defects [12]. Furthermore, conditional knockout of Dnmt3a in germ cells resulted in a lack of methylation at imprinted loci, impaired spermatogenesis and embryonic death of offspring from Dnmt3a conditional mutant females. However, disruption of Dnmt3b in germ cells did not result in any apparent phenotype [13].

Recently, we study the expression patterns of de novo methyltransferases during mouse embryogenesis and stem cell differentiation, and show that Dnmt3b is specifically expressed in pluripotent embryonic cells such as epiblasts and embryonic ectoderm, while Dnmt3a is significantly and ubiquitously expressed in embryos after

E10.5 [14]. Dnm3b is also expressed in CD34-positive hematopoietic progenitor cells in fetal liver and dorsal aorta, type A spermatogonia cells in adult testis, and neural progenitor cells in the spinal cord, retina and olfactory epithelium cells. Furthermore, we observe the transition of expression of these *de novo* methyltransferases from Dnm3b to Dnm3a during the differentiation of stem/progenitor cells in these tissues [15,16]. These results imply important roles for Dnm3b in stem/progenitor cell development and the transition of *de novo* methyltransferase expression from Dnm3b to Dnm3a suggests the existence of distinct functions of Dnm3b and Dnm3a during embryogenesis and ontogenesis. Dnm3b but not Dnm3a is highly expressed in the nuclei of ES cells [14,16-18]. During *in vitro* neural differentiation of ES cells, Dnm3a is transiently expressed in differentiating immature neural cells, but not in undifferentiated ES cells or differentiated neurons [16]. Thus, the transition of the predominant *de novo* methyltransferase from Dnm3b to Dnm3a is also reproduced during *in vitro* differentiation of ES cells.

In this study, to explore the functions of Dnm3a and Dnm3b *de novo* methyltransferases, we exogenously transfect ES cells with Dnm3b and Dnm3a cDNAs linked to an internal ribosome entry site (IRES)—green fluorescent protein (GFP) gene, and then analyze the functions of these *de novo* DNA methyltransferases in the pluripotent stem cells. Exogenous Dnm3b progress ES cell differentiation through the downregulation of Oct 3/4 and Nanog genes, while Dnm3a specifically promote cell proliferation.

2. MATERIALS AND METHODS

2.1. ES Cell Culture

The ES cell line (R1) was grown on a 0.1% gelatin-coated cell culture dish with KnockOut™ D-MEM (GIBCO) supplemented with 15% KnockOut™ Serum Replacement (GIBCO), 0.1 mM non-essential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol, L-glutamine, and 1000 U/ml leukemia inhibitory factor (LIF; Nacalai Tesque). Cells were routinely propagated by trypsinization and re-plating every two days, with a split ratio of 1 in 10.

2.2. Plasmids Constructs and DNA Transfection

Dnm3a or Dnm3b full-length cDNAs and an IRES linked to the enhanced GFP gene sequence were cloned into pcDNA3 vector using the EcoRV cloning site. Plasmids were purified using an EndoFree Plasmid Maxi Kit (QIAGEN). Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Forty-eight hours after transfection,

the ES cells were fixed with 4% paraformaldehyde/ PBS and then immunohistochemically stained. The numbers of anti-GFP-, Oct 3/4-, Nanog- and phosphohistone H3-positive mitotic cells were counted in images acquired with a laser scanning confocal microscope (Carl Zeiss, LSM 510 Meta). Means \pm standard error (SE) for three independent experiments were calculated.

2.3. Immunohistochemistry

After fixation with 4% paraformaldehyde/PBS, the cells were reacted with rabbit polyclonal anti-Dnm3a (1:2000, a gift from Dr. S. Tajima), mouse monoclonal anti-Dnm3b (1:800, IMGEX), rabbit polyclonal anti-Oct 3/4 (1:1000, MBL), rabbit polyclonal anti-Nanog (1:5000, COSMO BIO), rabbit polyclonal anti-GFP (1:10,000, MBL), rat monoclonal anti-GFP (1:2000, Nacalai Tesque), or rabbit polyclonal anti-phosphohistone H3 antibody (1:5000, Abcam). They were then incubated with goat anti-mouse IgG conjugated with Alexa Fluor 568, goat anti-rabbit IgG conjugated with Alexa Fluor 488, goat anti-rabbit IgG conjugated with Alexa Fluor 568 (1:2000, Molecular Probes), or donkey anti-rat IgG conjugated with DyLight 488 as a secondary antibody (1:2000, Jackson ImmunoResearch Laboratories). Antibodies were diluted with Signal Enhancer HIKARI (Nacalai Tesque). The samples were counterstained with Hoechst 33258. Images were acquired using a laser scanning confocal microscope (Carl Zeiss, LSM 510 Meta).

3. RESULTS AND DISCUSSION

3.1. Specific Expression of Dnm3a and Dnm3b in GFP-Positive ES Cells

To elucidate the functions of Dnm3a and Dnm3b, we exogenously transfected ES cells with cDNA vectors encoding Dnm3a or Dnm3b by lipofection. To identify cells expressing Dnm3a and Dnm3b, each cDNA was subcloned upstream of the IRES-GFP gene. The vectors express bicistronic transcripts including Dnm3a or Dnm3b and EGFP under the control of the CMV promoter (**Figure 1(A)**). Forty-eight hours after transfection, expression of Dnm3a and Dnm3b was confirmed by immunohistochemistry. Dnm3a was specifically expressed in the nuclei of GFP-positive ES cells transfected with Dnm3a-IRES-GFP, but not in GFP-negative ES cells, or in control IRES-GFP- and Dnm3b-IRES-GFP transfected ES cells (**Figures 1(B)-(D)**). Dnm3b is ubiquitously expressed in the nuclei of untransfected ES cells, and was specifically upregulated in GFP-positive ES cells transfected with Dnm3b-IRES-GFP, but not in IRES-GFP- or Dnm3a-IRES-GFP transfected ES cells (**Figures 1(E)-(G)**). Thus, Dnm3a and Dnm3b were specifically upregulated in the nuclei of ES cells identified by GFP expression.

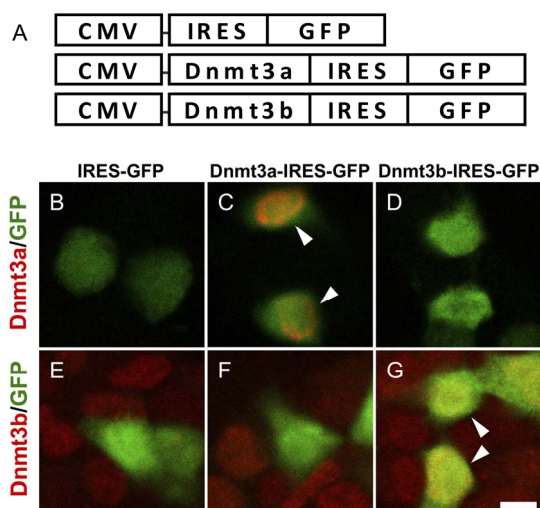


Figure 1. Dnmt3a and Dnmt3b expression in ES cells transfected with Dnmt3a-IRES-GFP and Dnmt3b-IRES-GFP. Schematic representations of Dnmt-IRES-GFP vectors (A); Dnmt3a-IRES-GFP (C, F); Dnmt3b-IRES-GFP (D, G) and control IRES-GFP (B, E) vectors were transfected into ES cells. After transfection, the ES cells were double-stained with anti-Dnmt3a (B, C, D; red) or anti-Dnmt3b (E, F, G; red) and GFP (green) antibodies. Dnmt3a and Dnmt3b protein levels were specifically enhanced in GFP-positive ES cells (C, G arrowheads). Scale bar: 10 μ m.

3.2. Dnmt3b Expression Downregulates Nanog and Oct3/4 Expression in ES Cells

Nanog and Oct3/4 are the two most important genes for the self-renewal and maintenance of ES cell pluripotency, and are downregulated following the initiation of ES cell differentiation. To investigate the physiological functions of Dnmt3a and Dnmt3b in the maintenance of pluripotency or the differentiation of ES cells, we first examined the levels of Nanog and Oct 3/4 proteins in ES cells exogenously transfected with Dnmt3a-IRES-GFP or Dnmt3b-IRES-GFP (**Figures 2(A)-(F)**). Among GFP-positive ES cells expressing the Dnmt3b gene, the proportions of Nanog- and Oct 3/4-positive cells were both specifically reduced to 47.7% and 78.4%, respectively, compared with control IRES-GFP transfected ES cells which showed 56.2% and 88.3% positivities for Nanog and Oct 3/4, respectively. Dnmt3a-IRES-GFP transfected ES cells showed 60.0% and 89.4% positivities, respectively, almost equal to the levels in IRES-GFP transfected ES cells. Conversely, the numbers of Nanog- and Oct 3/4-negative cells were increased in Dnmt3b-IRES-GFP transfected ES cells to 52.3% and 21.6%, respectively, compared with 43.8% and 11.7%, respectively, in control IRES-GFP transfected ES cells. Thus, the transient upregulation of Dnmt3b expression in ES cells specifically downregulates Oct 3/4 and Nanog expression

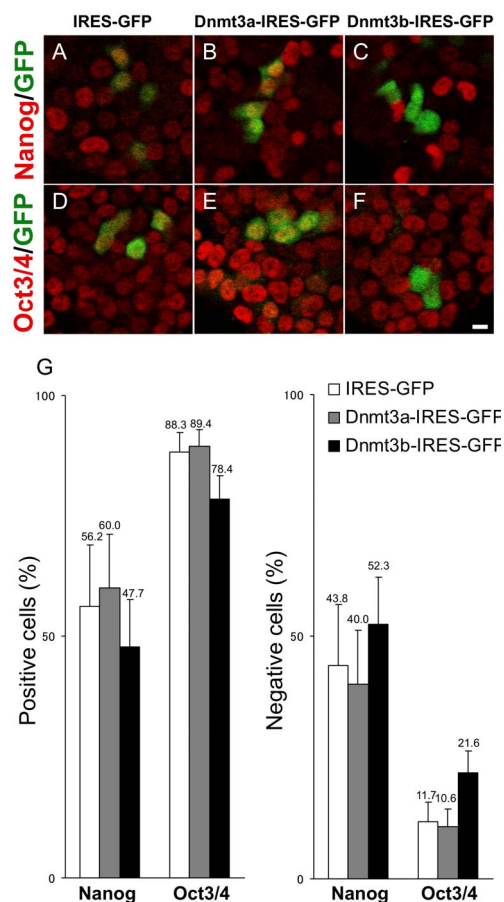


Figure 2. Nanog and Oct 3/4 expression in ES cells transfected with Dnmt3a-IRES-GFP or Dnmt3b-IRES-GFP. ES cells transfected with Dnmt3a-IRES-GFP (B, E), Dnmt3b-IRES-GFP (C, F) and control IRES-GFP (A, D) are double-stained with anti-GFP (green) and anti-Nanog (A-C, red) or anti-Oct 3/4 (D-F, red) antibodies. Percentages of Nanog- and Oct 3/4-positive or -negative cells in Dnmt3a- or Dnmt3b-expressing GFP-positive ES cells. Error bars represent standard error (n = 3). Scale bar: 10 μ m.

and promotes ES cell differentiation.

These results are consistent with a former report that Dnmt3b knockout ES cells failed to downregulate Oct3/4 and Nanog expression when differentiation was induced [19]. Dnmt3b may function to control ES cell differentiation through the de novo methylation of transcription factors required for the maintenance of pluripotency.

By immunohistochemical staining, Dnmt3b was heterogeneously expressed in ES cells and some ES cells express very low levels of Dnmt3b; however, Dnmt3b was transiently upregulated in ES cells in which differentiation was initiated by LIF withdraw or retinoic acid treatment, and then continuously downregulated during the differentiation process [14,16,18, data not shown].

Furthermore, a greater than 10-fold induction of

Dnmt3b mRNA was reported in ES cells that in which differentiation had been induced [20]. During mouse embryogenesis, Dnmt3b is heterogeneously expressed in inner cell mass (ICM) cells, but is most abundantly expressed in epiblast cells [14,21]. These results strongly suggest that the differentiation of pluripotent stem cells is regulated by the transient upregulation of Dnmt3b that induces the downregulation of Oct 3/4 and Nanog through the de novo methylation of promoter sequences. Dnmt3b is specifically expressed in stem/progenitor cells and has very important roles in the initial steps of stem/progenitor cell differentiation rather than in the maintenance of pluripotency.

3.3. Distinct Functions of Dnmt3a and Dnmt3b in ES Cell Proliferation

In Dnmt3a-IRES-GFP transfected ES cells, we observed a relatively higher number of clustered GFP-positive cells that proliferated from one transfected progenitor cell, compared with the number that proliferated from a single IRES-GFP- or Dnmt3b-IRES-GFP transfected ES cell (**Figures 3(A)-(C)**). We then analyzed cell proliferation activity by counting the numbers of clustered GFP-positive cells expressing the Dnmt3a-IRES-GFP or Dnmt3b-IRES-GFP gene at 48 hours post-transfection. Compared with IRES-GFP transfected ES cells, Dnmt3a-IRES-GFP transfected ES cells showed a greater average number of clustered GFP-positive cells (3.7 vs 2.2). Dnmt3b-IRES-GFP transfected ES cells produced an average of 2.4 clustered GFP-positive cells, almost equal to the number in IRES-GFP transfected ES cells (**Figure 3(D)**). To examine the possibility of a direct relationship between de novo methyltransferase expression and cell cycle progression in each ES cell, we next analyzed the mitotic index in Dnmt3b-IRES-GFP- and Dnmt3a-IRES-GFP transfected ES cells. For this purpose, we double-stained cells with anti-phospho-histone H3 (a mitotic marker) and an anti-GFP antibody (**Figures 4(A)-(C)**). We then analyzed the mitotic index (phospho-histone H3-positive cells/GFP-positive cells) at 48 hours post-transfection. As expected, the mitotic index was also specifically increased in Dnmt3a-IRES-GFP transfected ES cells, which had a mitotic index of 6.5% compared with 5.6% in IRES-GFP transfected ES cells (**Figure 4(D)**). Thus, the expression of Dnmt3a specifically promotes the proliferation of ES cells as confirmed by the upregulation of the mitotic index and the GFP-positive cell number after transfection.

In adult mouse testis, Dnmt3a is specifically expressed in type B spermatogonia cells, which show high proliferation activities compared with type A spermatogonia cells [15]. In addition, Dnmt3a-deficient knockout mice show impaired mitotic proliferation of spermatogonia

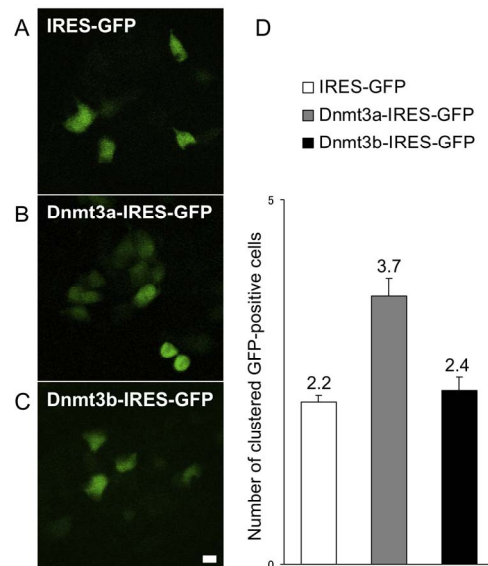


Figure 3. Cell proliferation activities in Dnmt3a- and Dnmt3b-transfected ES cells. Dnmt3a-IRES-GFP (B), Dnmt3b-IRES-GFP (C) and control IRES-GFP (A) vectors were transfected into ES cells, and the average numbers of GFP-positive colonies were counted after 48 hours (D). Error bars represent standard error (n = 3).

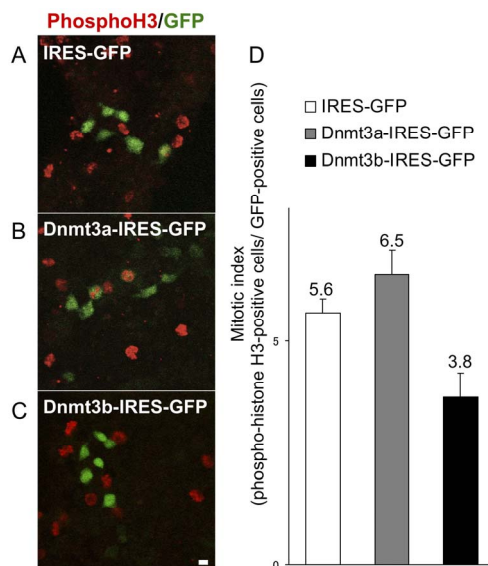


Figure 4. Mitotic index of Dnmt3a-IRES-GFP or Dnmt3b-IRES-GFP transfected ES cells. Dnmt3a-IRES-GFP (B), Dnmt3b-IRES-GFP (C) and control IRES-GFP (A) vectors were transfected into ES cells, and the mitotic index (phospho-histone H3-positive cells/GFP-positive cells) was assessed (D). Error bars represent standard error (n = 3).

cells [13]. Thus, Dnmt3a seemed to have a general function in stem/progenitor cell proliferation. Furthermore, Dnmt3a begins to be expressed in the nuclei of all tissues from E10.5 mouse embryos, but its expression is de-

creased during postnatal mouse development, with almost undetectable levels in adult tissues except for some epithelial tissues and stem/progenitor cells including neural progenitor cells and type B spermatogonia cells [14-16]. Thus, Dnmt3a expression levels are well correlated with cell proliferation activities in many tissues after birth. During postnatal development, cell proliferation may be directly or indirectly regulated by the expression of Dnmt3a. Dnmt3a may have an important function in promoting cell proliferation, not only in pluripotent stem cells, but also in other somatic cells during embryogenesis or organogenesis via the inactivation of some genes that inhibit cell proliferation through de novo methylations of their promoter sequences.

In the present study, we detected significant downregulation of the mitotic index in Dnmt3b-transfected ES cells. Dnmt3b-transfected ES cells had a mitotic index of 3.8%, while IRES-GFP transfected ES cells had an index of 5.6% (**Figure 4(D)**). In contrast, the numbers of Dnmt3b-transfected ES cells did not differ from the numbers of IRES-GFP transfected ES cells (**Figure 3(D)**). It is possible that there is a delay in the Dnmt3b-dependent suppression of cell proliferation, and that GFP-positive cells may proliferate before Dnmt3b activation. The mitotic index directly represents cell cycle progression in ES cells. Thus, the transient expression of Dnmt3b suppresses pluripotent stem cell proliferation. These results are consistent with former results and suggest novel functions of Dnmt3b that induce the initiation of stem/progenitor cell differentiation via downregulation of ES cell proliferation coordinated with the downregulation of Oct3/4 and Nanog genes.

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