Histone methyltransferase *Smyd3* regulates early embryonic lineage commitment in mice

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Abstract

SET and MYND domain-containing protein 3 (*Smyd3*) is a histone H3 lysine 4 (H3K4) di- and tri-methyltransferase that forms a transcriptional complex with RNA polymerase II and activates the transcription of oncogenes and cell cycle genes in human cancer cells. However, the study of *Smyd3* in mammalian early embryonic development has not yet been addressed. In the present study, we investigated the expression pattern of *Smyd3* in mouse preimplantation embryos and the effects of RNA interference (RNAi)-mediated *Smyd3* repression on the development of mouse embryos. We showed that *Smyd3* mRNA levels increased after the two-cell stage, peaked at the four-cell stage, and gradually decreased thereafter. Moreover, in two-cell to eight-cell embryos, SMYD3 staining was more intense in the nuclei than it was in the cytoplasm. In *Smyd3*-knockdown embryos, the percentage of inner cell mass (ICM)-derived colony formation and trophoblast (TE)-derived cell attachment were significantly decreased, which resulted in a reduction in the number of viable offspring. Furthermore, the expression of *Oct4* and *Cdx2* during mid-preimplantation gene activation was significantly decreased in *Smyd3*-knockdown embryos. In addition, the transcription levels of ICM and epiblast markers, such as *Oct4*, *Nanog*, and *Sox2*, the transcription levels of primitive endoderm markers, such as *Gata6*, and the transcription levels of TE markers, such as *Cdx2* and *Eomes*, were significantly decreased in *Smyd3*-knockdown blastocysts. These findings indicate that SMYD3 plays an important role in early embryonic lineage commitment and peri-implantation development through the activation of lineage-specific genes.

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Introduction

Embryonic development in mammals is characterized by an initial preimplantation phase that serves to prepare the embryo for implantation. Transcription from the newly formed zygotic genome, known as zygotic gene activation (ZGA), begins after fertilization between the late one-cell stage and the two-cell stage (Schultz & Worrall 1995, Latham & Schultz 2001, Li *et al.* 2010). Subsequently, mid-preimplantation gene activation (MGA) occurs during the four- to eight-cell stages (Hamatani *et al.* 2004). Both ZGA and MGA consist of new gene expression from the embryonic genome, and both steps require proper lineage commitment and differentiation. The first lineage differentiation gives rise to the inner cell mass (ICM) and trophoblast (TE). The pluripotency of the ICM lineage is regulated by the transcription factors *Oct4* (also known as *Pou5f1*), *Nanog*, and *Sox2* (Nichols *et al.* 1998, Avilion *et al.* 2003, Mitsui *et al.* 2003),

and the specification and differentiation of the TE lineage is regulated by the transcription factors *Cdx2* and *Eomes* (Russ *et al.* 2000, Strumpf *et al.* 2005). Before implantation, the ICM gives rise to the epiblast (EPI), which predominantly expresses *Nanog*, and the primitive endoderm (PE), which predominantly expresses *Gata6* (Rossant 2004, Chazaud *et al.* 2006). The EPI eventually gives rise to the fetus, whereas the PE develops into the visceral and parietal endoderm of the yolk sacs and the TE becomes the fetal placenta.

In general, gene expression is regulated through the transition of several epigenetic factors, including transcription factors, chromatin-remodeling factors, and some enzymes. Examples of the epigenetic changes that take place during preimplantation development include DNA methylation, histone post-translational modifications, and histone variant exchange (Sarmiento *et al.* 2004, Santos *et al.* 2005, Hirasawa *et al.* 2008, Akiyama *et al.* 2011). Drastic changes in many varieties

of histone post-translational modifications occur during ZGA. Histone post-translational modifications are introduced in a variety of ways. Several enzymes contribute to histone methylation (Zhang & Reinberg 2001), acetylation (Stern & Berger 2000), phosphorylation (Nowak & Corces 2004), and ubiquitination (Shilatifard 2006). With respect to methylation, modifications of lysines 4, 36, and 79 of histone H3 (referred to as H3K4, H3K36, and H3K79 respectively) are associated with transcriptional activation, whereas modifications of lysines 9 and 27 of histone H3 and lysine 20 of histone H4 (referred to as H3K9, H3K27, and H4K20 respectively) are associated with transcriptional repression (Lepikhov & Walter 2004, Sarmiento *et al.* 2004). Except for the H3K79 methyltransferase, which is known as DOT1L, histone methyltransferases (HMTase) include a conserved catalytic domain called the SET domain (Zhang & Reinberg 2001, Feng *et al.* 2002). SET and MYND domain containing protein 3 (*Smyd3*) encodes a protein comprising 428 amino acids and containing a SET domain, a MYND-type zinc-finger domain, and a SET-N region. SMYD3 has been reported to be capable of methylating both H3K4 and H4K5 (Hamamoto *et al.* 2004, Van Aller *et al.* 2012). Evidence has accumulated that suggests that SMYD3 recruits RNA polymerase II through an RNA helicase to form a transcription complex and that it elicits its oncogenic effects by activating the transcription of downstream target genes (Hamamoto *et al.* 2004, 2006, Liu *et al.* 2007, 2013). Previous reports have demonstrated that enhanced expression of SMYD3 is essential for the growth of human cancer cells (Hamamoto *et al.* 2004, 2006), whereas the suppression of SMYD3 expression leads to apoptosis and the inhibition of cell growth, migration, and invasion (Chen *et al.* 2007, Zou *et al.* 2009). Recent studies have determined that *Smyd3* plays an important role in the development of heart and skeletal muscle during zebrafish embryogenesis (Fujii *et al.* 2011). However, the role of SMYD3 in mammalian early embryonic development has not been previously addressed.

In the present study, we examined the expression pattern of *Smyd3* mRNA and the localization of SMYD3 protein in mouse preimplantation embryos, and we found that siRNA-mediated knockdown of *Smyd3* during the early stages of embryonic development suppressed *Oct4* and *Cdx2* at MGA. Additionally, *Smyd3* knockdown early in development reduced the blastocyst-stage expression of ICM/EPI markers (e.g., *Oct4*, *Nanog*, and *Sox2*), PE markers (e.g., *Gata6*), and TE markers (e.g., *Cdx2* and *Eomes*). However, the number of apoptotic cells was not increased until day 4 after the outgrowth experiments (E7.5) in *Smyd3*-knockdown embryos. From these results, we propose that SMYD3 plays an important role in early embryonic lineage commitment through the activation of lineage-specific genes.

Materials and methods

IVF and embryo culture

IVF was performed as previously described (Suzuki *et al.* 2015). Three hours after insemination, fertilized eggs were washed and cultured in K-modified simplex optimized medium (KSOM) containing 4 mg/ml BSA under mineral oil (Sigma–Aldrich) at 37 °C, in an atmosphere of 5% CO₂ in air until embryonic day 5.5 (E5.5) or used for microinjection (Tsukamoto *et al.* 2013) and cultured until embryonic day 5.5 (E5.5).

RNA extraction and quantitative RT-PCR

Metaphase II oocytes, and one-cell, two-cell, four-cell, eight-cell, morula, and blastocyst stage embryos were collected at 14, 28, 50, 62, 69, 90 and 122 h after the hCG injection respectively. RNA extraction and quantitative RT-PCR (qRT-PCR) were performed as previously described (Suzuki *et al.* 2013). Total RNA from 30 embryos at each stage was extracted using the TRIzol reagent (Invitrogen). Transcription levels were determined using three different sets of 30 embryos per stage and normalized to *H2afz*, which is known as a stable reference gene for the normalization of gene expression in mouse preimplantation embryos (Jeong *et al.* 2005, Mamo *et al.* 2007), or *Gapdh*; relative gene expression was analyzed using the 2^{-ΔΔC_T} method (Livak & Schmittgen 2001). All primers used for PCR are listed in Supplementary Table 1, see section on supplementary data given at the end of this article.

Microinjection of siRNA

Fertilized embryos transferred to KSOM were microinjected into the cytoplasm with 5–10 pl of 100 μM *Smyd3* siRNA (si*Smyd3*-1: 5'-GCAGGGUUAUCGUCAAGCUGA-3'; si*Smyd3*-2: 5'-GUCGUGGCGUAGUCUGUGAUC-3'; RNAi, Inc., Tokyo, Japan) between 3 and 4 h after insemination. The same amount of negative control siRNA (siControl; RNAi, Inc.), which contained scrambled sequences from the si*Smyd3*-1 or si*Smyd3*-2 construct, was also microinjected as a control. To examine the developmental competency and hatching ability, embryos were observed at 50 (E1.5), 74 (E2.5), 98 (E3.5), 122 (E4.5) and 146 h (E5.5) after the hCG injection. After the siRNA injection, embryos were harvested at either the four- and eight-cell stages (62 and 69 h after the hCG injection respectively) for qRT-PCR and immunostaining, or at the morula/blastocyst stage (98 h after the hCG injection) for outgrowth experiments, or at the blastocyst stage (122 h after the hCG injection) for qRT-PCR, immunostaining, and immunoblotting.

Outgrowth experiment and embryo transfer

Outgrowth experiments were performed using morula or blastocyst stage embryos collected 98 h after the hCG injection as previously described (Yamada *et al.* 2010). After culture for 4 days, the percentage of blastocysts that underwent outgrowth was calculated and photographed. A portion of the embryos that reached the two-cell stage after microinjection was transferred into the oviducts of 0.5 dpc pseudo-pregnant ICR

female mice. These females were killed on day 19, and the pups were counted. The experiment was repeated four times.

Immunostaining

Embryos for immunostaining were collected as described earlier in the Materials and methods. For SMYD3, trimethylated H3K4 (H3K4me3), and EOMES staining, embryos were fixed in 4% paraformaldehyde in PBS for 20 min at 4 °C after the removal of the zona pellucidae with acidic Tyrode's solution (pH 2.5). After being washed three times in PBS containing 0.3% polyvinylpyrrolidone (PVP K-30, Nacalai Tesque, Kyoto, Japan) (PBS/PVP), fixed embryos were treated with 0.5% Triton X-100 (Sigma–Aldrich) in PBS for 40 min at room temperature (RT) and were blocked in PBS containing 1% BSA for 1 h at RT (for SMYD3 and EOMES) or 3% BSA overnight at 4 °C (for H3K4me3). Next, embryos were either incubated overnight at 4 °C with a rabbit anti-SMYD3 antibody (1:100 dilution, 10 µg/ml; ab16027, Abcam Ltd, Cambridge, UK) or a rabbit anti-EOMES antibody (1:500 dilution, 0.4 µg/ml; ab23345, Abcam Ltd) or incubated for 1 h at RT with a rabbit anti-H3K4me3 antibody (1:200 dilution, 2.5 µg/ml; ab8580, Abcam Ltd) in antibody dilution buffer (PBS containing 1% BSA). Embryos were washed three times in antibody dilution buffer and then incubated with the appropriate secondary antibody diluted at 1:500 (Alexa Fluor 488-conjugated goat anti-rabbit IgG or Alexa Fluor 594-conjugated goat anti-rabbit IgG, Invitrogen) for 1 h at RT. Immunostaining with normal rabbit IgG (sc-2027, Santa Cruz Biotechnology) was performed as a negative control for the specificity of the anti-SMYD3 antibody. For NANOG, SOX2, and GATA6 staining, embryos were fixed, permeabilized, and blocked as previously described (Ralston & Rossant 2008). Next, embryos were incubated overnight at 4 °C with a rabbit anti-NANOG antibody (1:1000 dilution, 1 µg/ml; ab5731, Millipore, Bedford, MA, USA), a goat anti-SOX2 antibody (1:100 dilution, 2 µg/ml; sc-17320, Santa Cruz Biotechnology), or a goat anti-GATA6 antibody (1:1000 dilution, 0.2 µg/ml; AF1700, R&D Systems, Inc., Minneapolis, MN, USA) in blocking solution overnight at 4 °C. Embryos were washed three times in blocking solution and then incubated with the appropriate secondary antibody diluted at 1:750 (Alexa Fluor 594-conjugated goat anti-rabbit IgG, Invitrogen) or 1:500 or 1:750 (Alexa Fluor 594-conjugated rabbit anti-goat IgG, Invitrogen) for 1 h at RT. After staining, the samples were washed three times in antibody dilution buffer or blocking solution for 15 min, and nuclei were stained in PBS containing 10 µg/ml Hoechst 33342 (Sigma–Aldrich) for 10 min at RT. Immunofluorescent staining for OCT4 and CDX2 was performed as previously described (Isaji *et al.* 2013). After staining, embryos were mounted on slides in 50% glycerol/PBS, and SMYD3-related signals were observed using a fluorescence microscope (BZ-X700, Keyence, Osaka, Japan) equipped with structured illumination microscopy (Gustafsson 2005, Hosny *et al.* 2013). Fluorescein signals related to OCT4, CDX2, H3K4me3, NANOG, SOX2, GATA6, and EOMES were detected using fluorescence microscopy (BX50, Olympus). At least 20 samples were examined in each group. The numbers of ICM and TE cells were determined by counting OCT4- and

CDX2-positive cells respectively. In order to count the number of cells and observe the localization of OCT4, CDX2, NANOG, SOX2, GATA6, and EOMES in *Smyd3*-knockdown embryos, exposure time was extended as compared to that of control embryos. The total embryonic cell numbers were obtained by adding the numbers determined for the ICM and TE cells.

Immunoblotting

Immunoblotting was performed as previously described (Suzuki *et al.* 2013). Total proteins from 100 embryos were extracted using SDS sample buffer/lysis buffer (1:1). Primary antibody was used; a mouse anti- α -TUBULIN antibody (1:5000 dilution, 1.16 µg/ml; T9026; Sigma–Aldrich), a rabbit anti-SMYD3 antibody (1:100 dilution, 10 µg/ml), a rabbit anti-H3K4me3 antibody (1:200 dilution, 2.5 µg/ml), a rabbit anti-OCT4 antibody (1:500 dilution, 400 ng/ml; C-10; sc-5279, Santa Cruz Biotechnology), or a mouse anti-CDX2 antibody (1:500 dilution, 100 µg/ml; CDX-88; BioGenex, San Ramon, CA, USA). The following secondary antibodies were used: an HRP-conjugated anti-mouse secondary antibody (1:2000 or 1:10 000 dilution; GE Healthcare UK Ltd, Little Chalfont, UK) or an HRP-conjugated anti-rabbit secondary antibody (1:1000 or 1:2000 dilution; GE Healthcare UK Ltd) in TBS-T for 1 h at RT. The membrane was extensively washed three times with TBS-T, and then bound antibodies were detected using the ECL system (GE Healthcare UK Ltd). α -TUBULIN was used as an internal control.

TUNEL assay

Apoptotic cells in embryos at E3.5, E4.5, and E5.5 and outgrowth embryos at E6.5 and E7.5 were identified with the *In Situ* Cell Death Detection kit (Roche Diagnostics Corp.) using the protocol recommended by the manufacturer.

Statistical analysis

All data are expressed as means $\pm$ S.E.M. Statistical analysis of the data was performed by ANOVA with Student's *t*-test for comparing two groups.

Ethical approval for the use of animals

All animal experiments were approved by the Animal Research Committee of Kyoto University (Permit no. 24-17) and were performed in accordance with the committee's guidelines.

Results

The expression of the *Smyd3* mRNA and protein in mouse preimplantation embryos

First, we revealed the expression pattern of *Smyd3* mRNA and the localization of SMYD3 protein in mouse preimplantation embryos. qRT-PCR analysis of *Smyd3* mRNA in preimplantation embryos indicated that the

expression levels increased after the one-cell stage, peaked at the four-cell stage, and then slightly decreased until the blastocyst stage (Fig. 1A). Furthermore, immunostaining showed that SMYD3 dominantly localized to the nuclei from the two-cell to the eight-cell stage (Fig. 1B).

Effects of *Smyd3* knockdown on the development of mouse embryos

In order to investigate the role of *Smyd3* in early embryonic development, we knocked down the expression of *Smyd3* in mouse preimplantation embryos. Embryos injected with siRNA that targeted *Smyd3* (si*Smyd3*-1 and si*Smyd3*-2) were cultured until E5.5. qRT-PCR and immunoblotting showed that the reduction

Figure 1 *Smyd3* expression and the localization of SMYD3 in preimplantation embryos. (A) qRT-PCR analysis of *Smyd3* expression in preimplantation embryos. The expression levels at each developmental stage were normalized using *H2afz* as an internal control. Data are expressed as means $\pm$ s.e.m. ($n=3$). (B) Localization of *Smyd3* in mouse preimplantation embryos. SMYD3 (red) is detected by immunofluorescence and nuclei (blue) are stained with Hoechst dye.

in the expression of *Smyd3* mRNA and protein was observed at the blastocyst stage (Fig. 2A and B; Supplementary Figure 1A, see section on supplementary data given at the end of this article). Additionally, immunostaining showed that SMYD3 protein levels were also reduced beginning in the one-cell stage and continuing through the blastocyst stage (Fig. 2C). Nevertheless, no differences between *Smyd3*-knockdown and control embryos were noted with respect to morphology or the percentage of embryonic development up to E5.5 (Fig. 3A and B; Supplementary Figure 1B and C). Cell numbers in E4.5 blastocysts were counted after OCT4 (ICM) and CDX2 (TE) staining. The data demonstrated that *Smyd3*-knockdown blastocysts had normal numbers of cells in both populations (Fig. 3C). To examine the pluripotency of *Smyd3*-knockdown embryos, outgrowth experiments were performed on E3.5 embryos. In control embryos, the percentages of successful attachment and ICM-derived colony formation were 88.9 and 81.1% respectively, whereas in *Smyd3*-knockdown embryos, the percentages were 71.4 and 45.8% respectively (Fig. 3D and E). In addition, to test the viability of *Smyd3*-knockdown embryos *in vivo*, si*Smyd3*-1-injected embryos were transferred into the oviducts of pseudo-pregnant mice. The percentage of offspring derived from *Smyd3*-knockdown embryos was significantly reduced as compared to controls (Table 1).

Knockdown of *Smyd3* leads to the down-regulation of lineage-specific genes

In order to analyze the reasons for the defect of preimplantation embryonic development, we performed qRT-PCR on four- and eight-cell stage embryos for *Smyd3* and early differentiation markers, such as *Oct4* and *Cdx2*. In addition, we performed immunostaining to confirm the levels of H3K4me3 in *Smyd3*-knockdown embryos at the four- and eight-cell stages. The results demonstrated that the expression of *Smyd3* mRNA was significantly decreased in *Smyd3*-knockdown embryos (Fig. 4A); however, H3K4me3 levels remained unchanged (Fig. 4B). Interestingly, the transcription of *Oct4* at the four-cell and eight-cell stages and that of *Cdx2* at the eight-cell stage was significantly decreased in *Smyd3*-knockdown embryos (Fig. 4C and D). To further investigate the influence of the reduced expression of *Smyd3*, *Oct4*, and *Cdx2* at the four- and eight-cell stages, we performed qRT-PCR on E4.5 blastocyst stage embryos for *H2afz* – an internal control – and lineage-specific genes, such as the ICM/EPI markers *Oct4*, *Nanog*, and *Sox2*; the PE marker *Gata6*; and the TE markers *Cdx2* and *Eomes*. The results demonstrated that the transcription levels of *Oct4*, *Nanog*, *Sox2*, *Gata6*, *Cdx2*, and *Eomes* were all significantly decreased in *Smyd3*-knockdown embryos at the blastocyst stage, whereas *H2afz* transcript

Figure 2 Reduction in the expression of *Smyd3* mRNA and protein at the blastocyst stage. (A) qRT-PCR analysis of *Smyd3* mRNA at the blastocyst stage in *Smyd3*-knockdown and control embryos (* $P < 0.05$). The expression levels were normalized using *Gapdh* as an internal control. Data are expressed as means $\pm$ S.E.M. ($n = 3$). (B) Immunoblot analysis of SMYD3 and α -TUBULIN in *Smyd3*-knockdown and control blastocyst embryos. (C) Immunostaining of SMYD3 protein in *Smyd3*-knockdown and control blastocyst embryos (red, SMYD3; blue, chromatin).

levels remained unchanged (Fig. 5A; Supplementary Figure 2A). Furthermore, the protein levels of OCT4, CDX2, NANOG, SOX2, GATA6, and EOMES were confirmed on E4.5 blastocysts. The results demonstrated that the levels of OCT4, CDX2, NANOG, SOX2, GATA6, and EOMES were significantly reduced in

Smyd3-knockdown embryos at the blastocyst stage (Fig. 5B; Supplementary Figures 2B and 3A, see section on supplementary data given at the end of this article). Additionally, global H3K4me3 levels also remained unchanged in the treated embryos at the blastocyst stage (Supplementary Figure 3B).

Figure 3 The effects of *Smyd3* knockdown on pre- and post-implantation development. (A) Pairs of representative photos showing the development of preimplantation embryos injected with either siSmyd3-1 or siControl. Embryos were photographed at 36 h after IVF and at 24 h intervals thereafter. (B) The percentage of development at E1.5 ($\geq$ two-cell), E2.5 ($\geq$ four-cell), E3.5 ($\geq$ morula), E4.5 ($\geq$ blastocyst), and E5.5 ($\geq$ hatching) in *Smyd3*-knockdown and control embryos. Data are expressed as means $\pm$ S.E.M. ($n=3$). Twenty to 25 embryos were used in each experiment (63 and 69 embryos in total in siControl and siSmyd3-1 respectively). (C) The numbers of ICM and TE cells were assessed by counting OCT4-positive cells and CDX2-positive cells respectively. Total embryonic cell numbers were obtained by combining the numbers of ICM and TE cells. Data are expressed as means $\pm$ S.E.M. ($n=16$). (D) Photographs of the representative results of outgrowth experiments for control and *Smyd3*-knockdown embryos (arrowhead, ICM-derived colony; arrow, trophoblast cells). (E) The successful percentages of attachment and ICM-derived colony formation in *Smyd3*-knockdown and control embryos after 4 days in culture ($*P<0.05$). Data are expressed as means $\pm$ S.E.M. ($n=6$). Thirteen to 83 embryos were used in each experiment (238 and 243 embryos in total in siControl and siSmyd3-1 respectively).

Knockdown of *Smyd3* does not induce apoptosis during peri-implantation development

Because it has been reported that *Smyd3* knockdown induces apoptosis in human cancer cells (Chen *et al.* 2007), we investigated the effects of *Smyd3* knockdown on apoptosis in mouse peri-implantation embryos. However, there was no difference in the number of TUNEL-positive cells between *Smyd3*-knockdown and control embryos (Fig. 6).

Discussion

SMYD3 methylates both H3K4 and H4K5 (Hamamoto *et al.* 2004, Van Aller *et al.* 2012), recruits RNA polymerase II through an RNA helicase to form a

transcription complex, and elicits its oncogenic effects by activating the transcription of downstream target genes (Hamamoto *et al.* 2004, 2006, Liu *et al.* 2007, 2013). SMYD3 is also involved in apoptosis and the inhibition of cell growth, migration, and invasion (Xu *et al.* 2006, Zou *et al.* 2009). In the present paper, we observed *Smyd3* mRNA expression patterns and protein localization during mouse preimplantation development and showed that *Smyd3* knockdown led to a defect in the ability of *Smyd3* mRNA to attach to a matrix and outgrowth *in vitro* and to a reduction in the number of viable offspring, which suggests that SMYD3 plays important roles during peri-implantation development.

Previous reports have shown that *Oct4* and *Sox2* form an *Oct4/Sox2* complex and bind directly to their own

Table 1 Effect of *Smyd3* knockdown on mouse embryo development.

siRNA injected	No. of trials (number of recipients)	No. of embryos transferred	No. of pregnant mice	No. of live offspring (mean $\pm$ S.E.M.)	Percentage of live offspring
siControl	4	60 ^a	4	31 (7.8 $\pm$ 0.25)	51.7*
siSmyd3-1	4	60 ^a	4	12 (3.0 $\pm$ 0.41)	20.0 [†]

* $^{\dagger}P<0.05$.

^aFifteen embryos were transferred to each recipient at the trial.

Figure 4 *SMYD3* regulates the expression of *Oct4* and *Cdx2* at MGA. (A) qRT-PCR analysis of *Smyd3* mRNA in *Smyd3*-knockdown and control embryos at the four- and eight-cell stages ($*P < 0.05$). Expression levels were normalized to *Gapdh* as an internal control. Data are expressed as means $\pm$ S.E.M. ($n = 3$). (B) Immunostaining of H3K4me3 in *Smyd3*-knockdown and control embryos at the four- and eight-cell stages (red, SMYD3; green, H3K4me3; blue, chromatin). (C and D) qRT-PCR analysis of *Oct4* mRNA and *Cdx2* mRNA in *Smyd3*-knockdown and control embryos at the four- and eight-cell stages ($*P < 0.05$). Data are expressed as means $\pm$ S.E.M. ($n = 3$).

promoter regions in embryonic stem (ES) cells (Chew *et al.* 2005, Okumura-Nakanishi *et al.* 2005). *Oct4*, *Nanog*, and *Sox2* are reported to be regulated via the *Oct4/Sox2* complex and to form a self-reinforcing regulatory loop in ES cells (Mitsui *et al.* 2003, Chew *et al.* 2005, Okumura-Nakanishi *et al.* 2005, Rodda *et al.* 2005). In mouse preimplantation embryos, *Oct4*, *Nanog*, and *Sox2* are required for the maintenance of ICM pluripotency (Nichols *et al.* 1998, Avilion *et al.* 2003, Mitsui *et al.* 2003). Additionally, *Nanog* is known to negatively interact with *Gata6*, and both genes are known to be key regulators in the establishment of EPI and PE fates respectively (Morris *et al.* 2010,

Frankenberg *et al.* 2011, Kang *et al.* 2013, Schrode *et al.* 2014). *Nanog*-deficient mouse embryos are arrested during post-implantation development because of the widespread expression of *Gata6* in the EPI (Mitsui *et al.* 2003, Frankenberg *et al.* 2011). By contrast, *Gata6*-deficient mouse embryos are arrested during post-implantation development because of the widespread expression of *Nanog* in the PE (Morris *et al.* 2010, Schrode *et al.* 2014). In the present study, we showed that *Smyd3* knockdown led to the suppression of the embryonic transcription of *Oct4* during the four-cell stage. In addition, we also showed that in blastocysts, *Smyd3* knockdown abrogates the transcription of other

Figure 5 The effects of *Smyd3* knockdown on the expression of lineage-specific genes in blastocysts. (A) qRT-PCR analysis of the early lineage markers *Oct4*, *Nanog*, *Sox2*, *Cdx2*, *Eomes*, *Gata6*, and *H2afz* in *Smyd3*-knockdown and control blastocyst embryos ($*P < 0.05$). The expression levels were normalized using *Gapdh* as an internal control. Data are expressed as means $\pm$ S.E.M. ($n = 3$). (B) Immunostaining of OCT4, CDX2, NANOG, SOX2, GATA6, and EOMES in *Smyd3*-knockdown and control blastocyst embryos (red, OCT4; green, CDX2; red, NANOG; red, SOX2; red, GATA6; green, EOMES; blue, chromatin).

Figure 6 The effects of *Smyd3* knockdown on the induction of apoptosis. TUNEL-positive cells (green nuclei) were detected. Photographs depict representative results of TUNEL assay for control and *Smyd3*-knockdown embryos before (E3.5–E5.5) and after (E6.5–E7.5) outgrowth. Nuclei are stained with Hoechst dye (blue).

ICM and EPI markers, such as *Nanog* and *Sox2*, and of PE markers, such as *Gata6*. Therefore, it is possible that the lack of pluripotency genes, such as *Oct4*, *Nanog*, and *Sox2*, and of PE-specific genes, such as *Gata6*, could account for the defects observed in *Smyd3*-knockdown embryos, including poor outgrowth *in vitro* and a reduction in the number of viable offspring. Furthermore, *Cdx2* and *Eomes* have been reported to be essential for the specification and differentiation of TE (Russ *et al.* 2000, Strumpf *et al.* 2005). *Cdx2*-knockout embryos fail to hatch from the zona pellucida or to implant *in vivo*, and they also fail to attach to matrix substrates *in vitro*, even when the zona pellucida has been removed (Strumpf *et al.* 2005). In the present study, we showed that *Smyd3* knockdown led to the suppression of the embryonic transcription of *Cdx2* during the eight-cell stages. Additionally, we also showed that in blastocysts, *Smyd3* knockdown abrogates the transcription of TE markers, such as *Eomes*. Therefore, it is possible that the suppression of TE-specific genes, such as *Cdx2* and *Eomes*, could account for a defect in the ability of *Smyd3*-knockdown embryos to attach to a matrix substrate *in vitro*. Furthermore, it has been reported that *Smyd3* knockdown induces apoptosis in cancer (Chen *et al.* 2007). In the present study, however, we showed that the number of apoptotic cells was not increased in *Smyd3*-knockdown embryos. Together, these observations also suggest that SMYD3 plays an important role in peri-implantation embryonic development via the activation of lineage-specific gene expression. However, we demonstrated that in *Smyd3*-knockdown embryos, global H3K4me3 levels appeared

to be unchanged and that developmental arrest did not occur up to the blastocyst stage, even though *Smyd3* knockdown suppressed the nuclear localization of SMYD3 protein at ZGA and MGA. *Smyd3* trimethylates H3K4, and some chromatin remodeling complexes are recruited to H3K4me3 (He *et al.* 2005, Sims *et al.* 2005). These chromatin remodeling factors are involved in an open chromatin state, which correlates with a globally permissive transcriptional state. Within this 'loose' chromatin state, transcription factors can bind to the promoter regions of genes and activate transcription. The decreased expression of lineage-specific genes, such as *Oct4*, *Nanog*, and *Cdx2*, that we observed in the present study echoes results that have been obtained in *Klf5*-null embryos (Lin *et al.* 2010) and in *Hmgpi*-knockdown embryos (Yamada *et al.* 2010), which suggests that SMYD3 functions via these transcription factors to determine lineage specificity. Previous studies have shown that MLL2, one of the H3K4 methyltransferases, affects the global H3K4me3 levels at ZGA (Andreu-Vieyra *et al.* 2010), and the SETD1A/SETD1B methyltransferase complex that modifies H3K4 affects the global H3K4me3 levels at MGA (Bi *et al.* 2011). Additionally, previous studies have demonstrated that a knockdown of SMYD3 expression leads to a selective decrease in H3K4 methylation levels on oncogene promoter regions in cancer cells (Cock-Rada *et al.* 2012, Medjkane *et al.* 2012, Liu *et al.* 2013). From these observations, it is possible that the H3K4me3 level depends on the type of methyltransferase and that SMYD3 modifies H3K4 within the promoter regions of the lineage-specific genes. In addition, a recent report

demonstrated that although global levels of H3K4me3 do not change upon the loss of *Smyd3* in the human breast carcinoma cell line, MCF7, the global levels of H4K5me, a novel chromatin target of *Smyd3*, do change (Van Aller *et al.* 2012). This result indicates that SMYD3 is required for H4K5 methylation in cancer cells. It is also possible that SMYD3 is involved in the regulation of genes in mouse preimplantation embryos through the methylation of H4K5.

In summary, the present results demonstrated that *Smyd3* knockdown does not have a critical effect on early embryonic development or on global H3K4me3 levels, but it does play an important role in early embryonic lineage commitment and peri-implantation development by regulating the expression of *Oct4* and *Cdx2* at MGA. Accordingly, we demonstrated the importance of *Smyd3* as a key regulator of lineage-specific genes in mouse preimplantation embryos.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-15-0019>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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