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H3K27me3 may be associated with Oct4 and Sox2 in mouse preimplantation embryos

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ABSTRACT. As a core member of polycomb repressive complex 2, the transcription and enzyme activity of enhancer of zeste homolog 2 (Ezh2) is directly involved in the trimethylation of lysine 27 on histone H3. In this study, the fluorescence intensity of H3K27me3 in mouse *in vivo* morulae and blastocysts was compared by indirect immunofluorescence staining. We found that demethylation of H3K27me3 occurred during the blastocyst stage. Real-time polymerase chain reaction was performed to investigate *Ezh2* expression in oocytes and in preimplantation embryos. *Ezh2* expression peaked during the zygote stage and gradually decreased from the 2-cell stage, exhibiting an inverse pattern when compared with *Oct4* and *Sox2* mRNA in mouse preimplantation embryos. To understand the role of development-related genes on the transcription of mouse *Ezh2*, a promoter assay was performed in NIH/3T3 cells. *Ezh2* expression was markedly suppressed by Oct4 and Sox2 alone in a dose-dependent manner, while Ezh2 promoter activity in co-transfection with Nanog, Klf-4, and c-Myc groups showed no significant change as compared with the control.

Our data suggest that the demethylation of H3K27me3 is caused by the degressive expression and activity of *Ezh2* in blastocysts, leading to increased expression of developmentally important transcription factors. We also observed negative effects of Oct4 and Sox2 on the transcription of *Ezh2* and identified Oct4 and Sox2 as novel negative regulators of Ezh2 at the post-translation level in a mouse preimplantation embryo.

Key words: *Ezh2*; Development-related genes; Mouse; Transcriptional regulation; Trimethylation of H3K27

INTRODUCTION

Trimethylation of lysine 27 on histone H3 (H3K27me3), which is catalyzed by polycomb repressive complex 2 (PRC2), is an important histone modification and considered to be an epigenetic marker of maternally derived chromatin mediating gene silencing in mammals (Park et al., 2009). Enhancer of zeste homolog 2 (Ezh2) is a histone methyltransferase and a core component of PRC2 (Sauvageau and Sauvageau, 2010). Several studies have indicated that the activity of Ezh2 may be associated with H3K27me3, but can also play key roles in embryonic development, tumor cell proliferation, and the physiology of several cell types and tissues. During mouse embryogenesis, Ezh2 is essential for establishing the first differentiated cells, the trophectoderm, and the pluripotent epiblast cells (Erhardt et al., 2003); its protein was also found to exhibit tissue specificity and depended on the embryo's stage of development (Zhu et al., 2011). In mouse somatic cloned embryos, inadequate expression of *Ezh2* in inner cell mass (ICM) cells led to defective chromatin structure, particularly inadequate H3K27me3 (Zhang et al., 2009). In mouse and human, T-cell leukemia development was suppressed by Ezh2 and its associated genes (Simon et al., 2012). Moreover, Ezh2 is thought to be a therapeutic target for treating cancer (Qi et al., 2012).

Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells are widely used in the study of developmental biology. The expression profiles of developmental genes, such as *Oct4*, *Sox2*, and *Nanog*, are closely related to the totipotency and pluripotency of ES and iPS cells, respectively (Boyer et al., 2005; Takahashi and Yamanaka, 2006). A recent study found that the epigenetic status of the Nanog promoter was directly regulated by Ezh2, indicating that Ezh2 can affect the equilibrium between self-renewal and differentiation in ES cells. In addition, the loss of *Ezh2* and higher levels of *Nanog* are synchronously observed in iPS cells (Villasante et al., 2011). In murine Oct4(+)Sca-1(+)Lin(-)CD45(-) very small embryonic-like stem cells, similarly to other pluripotent stem cells, the pluripotent states are maintained through an Ezh2-dependent bivalent domain-mediated epigenetic mechanism (Shin et al., 2012). At the onset of transcription factor-induced reprogramming, iPS cells lacking Ezh2 efficiently silenced the somatic transcriptome and differentiated into tissues derived from the 3 germ layers (Fragola et al., 2013).

Although many studies have shown that *Ezh2* and Ezh2-mediated H3K27me3 contribute to mammalian development and the status of ES and iPS cells, little is known regarding the upstream regulator of *Ezh2* and the relationship with development-related genes. First, we examined H3K27me3 by using indirect immunofluorescence staining and by determining the expression pattern of *Ezh2* using real-time polymerase chain reaction (PCR) in mouse preimplantation embryos and compared these results with the known expression patterns of

Oct4, *Sox2*, and *Nanog*. Furthermore, we investigated whether the promoter activity of *Ezh2* was influenced by these development-related genes to determine the pathway of early stage mammalian embryonic development.

MATERIAL AND METHODS

Collection and culture of oocytes and embryos

Kunming white mice were used as model in the present study. The purchasing and rearing methods and the collection and culture of oocytes and embryos were performed according to previously described methods (Wu et al., 2012a,b). MII stage oocytes, zygotes, 2-, 4-, 8-, and 16-cell embryos, morulae, and blastocysts were collected for this study as described previously (Wu et al., 2012a,b).

Immunofluorescence histochemistry for detection of H3K27me3

Morulae and blastocysts generated by *in vivo* fertilization were washed in 1X phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde in 1X PBS, and permeabilized in 0.2% Triton X-100 in PBS for 30 min at room temperature. After blocking in PBS containing 1% bovine serum albumin for 1 h at room temperature, the embryos were incubated overnight at 4°C in a 1:100 dilution of primary antibodies against H3K27me3 (Catalog No. ab6002, Abcam, Cambridge, UK). Subsequent steps were also performed as described previously (Wu et al., 2012a). Samples without primary antibody were included as negative controls. Fluorescence was detected using a Leica confocal laser scanning microscope (TCS-SP5) according to manufacturer instructions.

Real-time PCR

Five zygotes or embryos were used for each time point and 5 samples (replicates) were collected from each stage. Total RNA was extracted and cDNAs were synthesized according to the protocols of the RNeasy Plus Micro Kit and Sensiscript RT Kit (Qiagen, Hilden, Germany). Quantitative PCR was carried out according to manufacturer instructions of FastStart Universal SYBR Green Master (Roche, Basel, Switzerland). The threshold cycle (Ct) was defined as the fractional cycle number by the method of global minimum. *Gapdh* was used as an internal control. The ratio change in the *Ezh2* gene relative to *Gapdh* control gene was determined using the $2^{-\Delta\Delta Ct}$ method. Data are reported as means ± SE for the 3 replicates. The GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA) was used to identify significant differences. All primer sequences used for the present study are listed in Table 1.

Cell culture, transfections, and luciferase assay

Briefly, the promoter (-2106 bp) of the mouse *Ezh2* gene was generated by PCR using the primers listed in Table 1 and subcloned into the pGL3-Basic Vector (Promega Co., Madison, WI, USA). Mouse *Oct4*, *Sox2*, *Nanog*, *Klf-4*, and *c-Myc*-pcDNA3.1 expression vectors (Invitrogen, Carlsbad, CA, USA) were used as described previously (Wu et al., 2012b).

The constructs and orientation of the insert were confirmed by direct sequencing; plasmids used in transfection experiments were purified using a QIAfilter Plasmid Midi Kit (Qiagen). NIH/3T3 cells (mouse embryonic fibroblast cell line) were transfected using Lipofectamine (Invitrogen) with the following plasmids: 1) 500 ng of the mouse *Ezh2* promoter cloned into the pGL3-Basic luciferase reporter vector; 2) 100 ng *Oct4*, *sox2*, *Nanog*, *Klf-4*, and *c-Myc* -pcDNA3.1 expression plasmid (Invitrogen); and 3) pRL-TK (Promega), at 100 ng/well. Cell culture, transient transfections, luciferase assays, and statistical analysis were performed as reported previously (Wu et al., 2012b).

Table 1. List of primer sequences used in real-time PCR and promoter analyses.

Primer	Sequence (3'-5')	Purpose
Ezh2-F	ACCAAGAGTGGAAGCAGCGG	For real-time PCR
Ezh2-R	CACTGGTGACTGAACACTCCCT	
Gapdh-F	ATTCAACGGCACAGTCAAGG	
Gapdh-R	GGTCCTCAGTGTAGCCCAAGA	
Ezh2-PF	CGACGCGTTCGCATCCTTTCATCTATTTTAC	For promoter assay
Ezh2-PR	CCCAAGCTTGCAGATAAAGTTAGTGCGGTTC	
Oct4-F	CGGAATTCGCCACCATGGCTGGACACCTGG	For pcDNA3.1 expression plasmid
Oct4-R	CCGCTCGAGTCAGTTTGAATGCATG	
c-Myc-F	CGGGATCCGCCACCATGAGTGTGGGTCTT	
c-Myc-R	CCGCTCGAGTCATATTTCACCTGG	
Nanog-F	CGGAATTCGCCACCATGCCCCTCAACGTG	
Nanog-R	CCGCTCGAGTTATGCACCAGAGTT	
Sox2-F	CGGGATCCGCCACCATGTATAACATGATGGA	
Sox2-R	CCGCTCGAGTCACATGTGCGACAGGGGC	
Klf-4-F	CGGGATCCGCCACCATGAGGCAGCCACCTGGC	
Klf-4-R	CCGCTCGAGTTAAAAGTGCCTCTTCATGT	

Underlined bases show the restriction sites.

RESULTS

Modification of H3K27me3 in morulaes and blastocysts of *in vivo* mouse embryos

Several *in vivo* morulae and blastocysts were collected to confirm the modification of H3K27me3. As shown in Figure 1, a dramatic change in H3K27me3 was observed between morulae and blastocysts. H3K27me3 was positively detected in morulaes, while very weak signals were observed in blastocysts. H3K27me3 modifications gradually disappeared from the morula stage; demethylation of H3K27me3 occurred at this stage.

Expression patterns of Ezh2 in mouse preimplantation embryos

The expression patterns of *Ezh2* in mouse MII oocytes and preimplantation embryos were detected by real-time PCR. *Ezh2* was expressed more strongly in zygotes than in oocytes. As shown in Figure 2, the levels of *Ezh2* gradually decreased from the 2-cell stage. At the blastocyst stage, weaker but persistent expression of *Ezh2* was also detected. As a core member of PRC2 that catalyzes H3K27me3, the activity of mouse Ezh2 must be the lowest in blastocysts. These results are also consistent with the modification of H3K27me3 as shown in Figure 1.

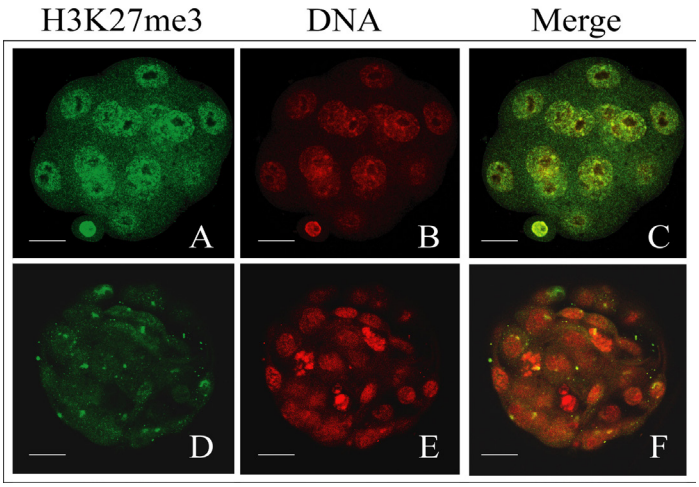

Figure 1. Trimethylation of lysine 27 on histone 3 (H3K27me3) in morulae and blastocysts of *in vivo* mouse embryos. Embryos were immunostained with specific antibodies against H3K27me3. H3K27me3, green; DNA, red. Scale bar = 20 μm.

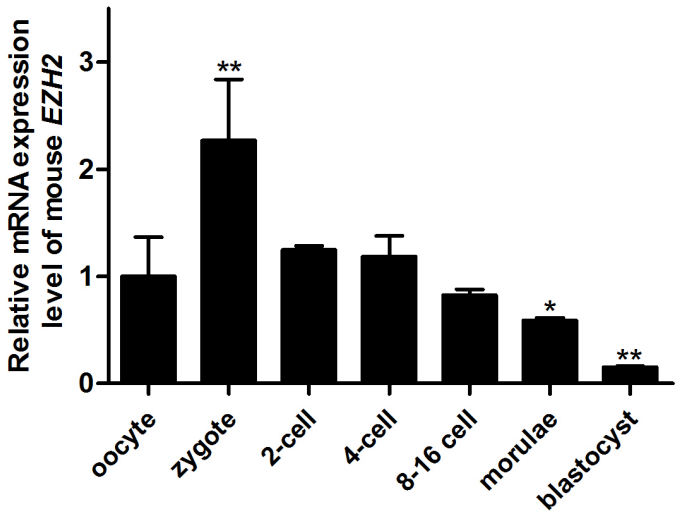

Figure 2. Expression patterns of mouse *Ezh2* in MII stage oocytes and preimplantation embryos by real-time PCR. Data are reported as means ± SE for 3 replicates. *P < 0.05; **P < 0.01 as compared with oocytes.

Promoter analysis of mouse Ezh2

A 2106-bp 5'-flanking of mouse *Ezh2* was cloned into the pGL-3 basic vector, and promoter analysis was performed to investigate the effect of Oct4, Sox2, Nanog, Klf-4, and c-Myc on the promoter activity of mouse *Ezh2*. The results showed that activity of the mouse *Ezh2* promoter was downregulated by Oct4 and Sox2 alone in NIH/3T3 cells, while *Ezh2* promoter activity in cells co-transfected with Nanog, Klf-4, and c-Myc groups did not show any significant change compared with the control (Mock) (Figure 3). To further examine whether

the transcription of *Ezh2* was regulated by Oct4 and Sox2 in a dose-dependent manner, 10-200 ng *Oct4* and *Sox2* expression vectors were co-transfected with the mouse *Ezh2* promoter. As shown in Figure 4, the inhibitory effects on the mouse *Ezh2* promoter were gradually tightened when the amounts of Oct4 and Sox2 were increased; this indicates that mouse *Ezh2* expression was repressed by Oct4 and Sox2 in a dose-dependent manner (Figure 4).

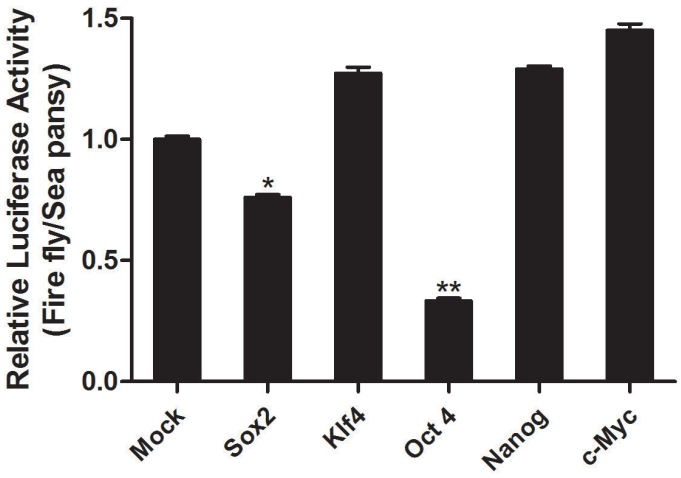

Figure 3. Promoter analysis of mouse *Ezh2* in NIH/3T3 cells by luciferase assay. *Oct4*, *c-Myc*, *Nanog*, *Klf-4*, and *c-Myc*-pcDNA3.1 (100 ng) were co-transfected with mouse *Ezh2* promoter (500 ng/well) into NTH/3T3 cells. The total amount of transfected plasmid, including the pRL-TK control vector (100 ng/well), was adjusted to 1.0 µg with empty vectors. Firefly and *Renilla* luciferase activities were measured 48 h after transfection. Relative luciferase activity was calculated by dividing firefly luciferase activity with *Renilla* luciferase activity. Results are reported as means ± SD for triplicate transfections. *P < 0.05; **P < 0.01 as compared with Mock by one-way analysis of variance.

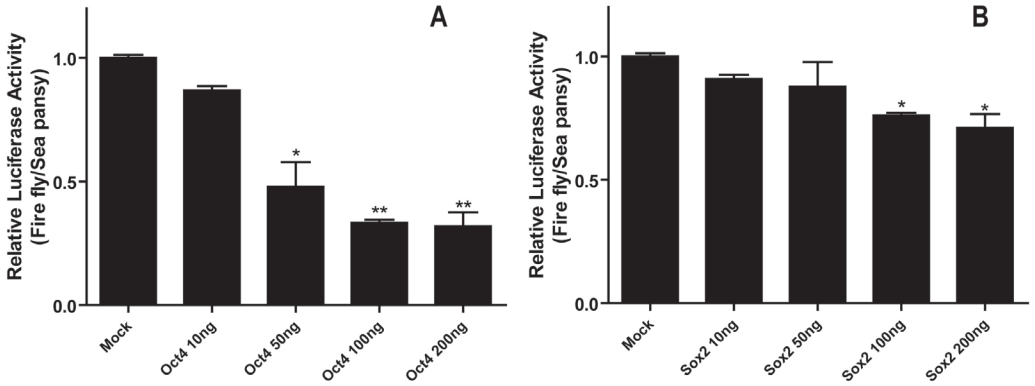

Figure 4. Dose dependence of Oct4 and Sox2 on mouse *Ezh2* promoter activity. First, 10-200 ng Oct4 (**A**) and Sox2 (**B**) were co-transfected with mouse *Ezh2* promoter construct (500 ng/well) into NIH/3T3 cells. The protocol for transient transfections and luciferase assay is described in the figure legend of Figure 3. *P < 0.05, **P < 0.01 as compared with Mock by one-way analysis of variance.

DISCUSSION

In this study, H3K27me3 modification was further confirmed in morulae and blastocysts of *in vivo* mouse embryos by immunofluorescence staining. We found that the intensity of H3K27me3 in morulae was more extensive than that in blastocysts and that demethylation of H3K27me3 occurred during the morula stage. These results are consistent with those of a previous report (Zhang et al., 2009). Several studies have suggested that the promoters of inactive or weakly expressed genes are often enriched with H3K27me3, and these genes must undergo demethylation of H3K27me3 when expressed (Dahl et al., 2010). It is well known that many development-related genes are upregulated in blastocysts compared to morulae (Tanaka and Ko, 2004). Demethylation of H3K27me3 during the blastocyst stage was synchronized with the modification of H3K4me3 (Wu et al., 2012a), indicating that many development-related genes were activated in this stage to maintain the progress of embryonic development and implantation during the latter stage.

The methytransferase activity of Ezh2 is closely associated with H3K27me3 in mammals (Zhu et al., 2011). mRNA expression of *Ezh2* was observed in whole mouse embryos at day E9.5 and in the fetal liver, brain, and skeletal muscles at day E13 or day E17 (Laible et al., 1997). In the present study, the expression pattern of *Ezh2* in mouse oocytes and *in vivo* preimplantation embryos were investigated by real-time PCR. Expression of *Ezh2* was detected in oocytes, further indicating that *Ezh2* is a maternal factor (Erhardt et al., 2003). During the entire preimplantation and embryo development periods, the highest level of *Ezh2* peaked during the zygote stage and gradually decreased from the 2-cell stage onwards, while *Ezh2* was expressed at the lowest level in blastocysts. The gradually decreasing *Ezh2* mRNA expression may correspond to the gradually decreased enzymatic activity of Ezh2. These results are consistent with staining data obtained by immunofluorescence histochemistry. Therefore, Ezh2 may be involved in mouse embryonic development by mediating the modification of H3K27me3.

Oct4, Sox2, Nanog, Klf-4, and c-Myc correlated with the development potential of preimplantation embryos, the maintenance of ES cell pluripotency, and iPS cell totipotency (Li et al., 2005; Takahashi and Yamanaka, 2006; Suzuki et al., 2009). Oct4, Sox2, Nanog, and Klf-4 proteins were observed to be in the ICM cells of blastocysts; the fluorescence intensity increased with embryonic development, peaking during the blastocyst stage (Rodda et al., 2005; Zhang et al., 2009; Keramari et al., 2010). These gene expression profiles may have been caused by demethylation of H3K27 during the blastocyst stage. However, no c-Myc signal was detected in blastocysts (Suzuki et al., 2009). A previous study reported that the Ezh2 protein was observed in ICM cells according to immunofluorescence results (Zhang et al., 2009), indicating that Ezh2 and development-related genes are co-localized in mouse blastocysts. Using promoter analysis, we found that the promoter activity of *Ezh2* was suppressed by Oct-4 and Sox2 alone in NIH/3T3 cells in a dose-dependent manner. These results were consistent with the expression of *Ezh2* determined by real-time PCR. In fact, this was not the first evidence that the transcription of epigenetic modifying enzyme genes is regulated by these master transcription factors. In mesenchymal stem cells, Oct4 and Nanog upregulated *DNA methyltransferase 1* by directly binding to its promoter to maintain self-renewal and its undifferentiated state (Tsai et al., 2012). The H3K9me2 and H3K9me3 demethylase genes, *Jmjd1a* and *Jmjd2c*, are positively regulated by Oct4 in ES cells (Loh et al., 2007). *Ezh2* expression is controlled by Sox4 during the epithelial-mesenchymal transition in normal and cancerous

breast epithelial cells (Tiwari et al., 2013). In T47D cells, FOXP3 could downregulate *Ezh2* protein level to promote mammosphere formation, cell growth, directional migration, and colony formation (Shen et al., 2013). These reports, together with our data, demonstrate that epigenetic modifying enzyme genes are direct transcriptional targets of key development-related genes (Oct4 and Sox2). In contrast, the transcription activity of these development-related genes also depended on the epigenetic modification catalyzed by epigenetic modifying enzymes.

Taken together, our study showed that *Ezh2* mRNA expression was inversely correlated with Oct4 and Sox2, but Oct4 and Sox2 negatively affect *Ezh2*; additionally, Oct4 and Sox2 were negative regulators of *Ezh2* primarily on a post-translation level. These data illustrate the correlation between development-related genes and epigenetic modification in mouse embryonic development and cell reprograming.

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