Oct-4 Regulates DNA Methyltransferase 1 (Dnmt1) Transcription by Direct Regulatory Element Binding

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Abstract: The transcription factor Oct4 plays a pivotal role for the development of mouse preimplantation embryo, and DNA methyltransferase 1 (Dnmt1) maintains the changes of DNA methylation during mammalian early embryonic development. However, little is known of the role of Oct4 in DNA methylation in mouse. In the present study, Kunming white mice were used as an animal model to elucidate the correlation between DNA methylation and Oct4 during mammalian embryonic development. The expression of Dnmt1 and Oct4 were initially studied by real-time PCR, exhibiting different patterns during mouse preimplantation stage. Moreover, by using promoter assay and Chip analysis, we found that the transcriptional activity of Dnmt1 in mouse NIH/3T3 cells and CCE cells were both regulated by Oct4 through direct binding to -554 to -294 fragment of upstream regulation element of Dnmt1. Then, the downregulation of Dnmt1 expression level and enzyme activity by mouse Oct4 were further confirmed by transfecting Oct4 siRNA into mouse CCE cells. Our results indicate that the function of Oct4 is involved in DNA methylation by regulating Dnmt1 transcription, especially during the early stages of mouse preimplantation embryo development.

Keywords: Dnmt1; Oct4; transcription regulation; Kunming white mouse

1. Introduction

It is well known that the transcription factors Oct4, Sox2, Klf4, and c-Myc (OSKM factors, also called Yamanaka factors) are essential for mammalian embryo development, especially in the preimplantation embryonic stage (Li et al., 2005)[1]. A previous study has found that the abilities of self-renewal and multipotency of mammalian embryonic stem cells (ESC) and adult stem cells cannot perform their functions to maintain the normal development processes without the activities of these factors (Kashyap et al., 2009)[2]. Recently, a novel type of stem cells, induced pluripotent stem (iPS) cells, was successfully generated through retroviral introduction of the abovementioned critically important genes. The highest efficiencies of induced pluripotency were achieved when combinations of all four factors were realized (Takahashi et al., 2006)[3]. Although little information is available on the molecular biology events during these processes, we still have reason to believe that these four factors have important functions in the embryonic development, maintaining pluripotency and differentiation potential (Li et al., 2005)[1]. However, many studies have been reported that adult mouse neural stem cells and human fetal neural stem cells could be directly reprogrammed to iPS cells by ectopic expression of Oct-4 alone, suggesting that Oct-4, but not the other three factors, is capable of reprogramming mammalian somatic cells to pluripotency (Kim et al., 2009a, b; )[4, 5]. Oct-4 has been demonstrated to be a key transcription factor controlling pre-implantation development in the mouse embryo (Li et al., 2005)[1]. It is noteworthy that Oct-4 kinetics was identified as a predictive measure of developmental cell lineage patterning in the early mouse embryo using fluorescence decay after photoactivation (Plachta et al., 2011)[6]. Meanwhile, the specification of pluripotent cell identity requires the embryonic genome to express Oct-4 during mouse development.
In the mouse blastocysts, Oct4 is required for the expression of multiple epiblast and primitive endoderm genes as well as for the operation of multiple metabolic pathways essential for the continued growth of the preimplantation embryo (Frum et al., 2013)[8]. These findings suggest that Oct-4 has crucial roles in the early stages of development and differentiation as evidenced elsewhere (Jerabek et al., 2014)[9].

DNA methylation is an important epigenetic modification event during mouse embryonic development as well as in the processes of somatic cell reprogramming and gene silencing (Shi et al., 2009; Saitou et al., 2012; Meissner et al., 2013)[10-12]. Dnmt1 is the major DNA methyltransferase responsible for methylating hemi-methylated cytosines in CpG sequences, and it also acts as a maintenance methyltransferase that maintains genome-wide methylation patterns during genomic DNA replication (Chen and Riggs, 2011; Jurkowska et al., 2011)[13, 14]. Many studies have shown that Dnmt1 plays a crucial role in the normal mammalian development, as well as in cell proliferation and survival (Tsumura et al., 2006)[15]. Mutation of Dnmt1 results in extensive demethylation of the genome DNA, embryonic lethality, loss of imprinting, and alterations in X chromosome inactivation during mouse embryonic development, while the absence of Dnmt1 leads to the death of ES cells (Klose and Bird, 2006)[16]. The knockdown of Dnmt1 in germline cells leads to their immediate apoptosis (Takashima et al., 2009)[17]. During mouse oogenesis and preimplantation development, both transcripts and protein encoded by Dnmt1 were expressed and responsible for the maintenance of methylation at the preimplantation stages other than the eight-cell embryo (Hutnick et al., 2010)[18].

The expression of Oct4 and DNA methylation have been demonstrated to be key factors in mammalian preimplantation embryo development and cell reprogramming (Gu et al., 2011)[19]. However, of the results on the correlation between mouse Oct4 and Dnmt1 in these processes are still contradictory. Here, we used Kunming mice as a model to examine the expression of Dnmt1 and Oct4 during the preimplantation stage. Luciferase promoter assay and Chromatin immunoprecipitation (ChIP) was performed to investigate the binding of Oct4 to the cis-regulation element of Dnmt1. Finally, RNAi of Oct4 in mouse CCE cells was carried out to examine whether the expression level and enzyme activity of Dnmt1 was regulated by Oct4 in vitro. Our results are consistent with those of other reports; thus, we have the reason to believe that Oct4 is involved in DNA methylation by regulating the transcription of Dnmt1 as well as in human mesenchymal stem cells (Tsai et al., 2012)[20]

2. Results

2.1 Mouse Dnmt1 and Oct4 expression in preimplantation embryos from the zygote stage

Quantitative PCR results showed that the expression level of mouse Dnmt1 in the embryos was increased from the zygote to 4-cell stage and dramatically decreased from the 8-cell stage onwards (Fig. 1A), whereas the level of Oct4 increased steadily during the mouse preimplantation embryo stage (Fig. 1B).

![Fig. 1. Expression pattern of mouse Dnmt1 (A) and Oct4 (B) in preimplantation embryos detected by real-time PCR. Data are expressed as the mean ± SE of three replicates, and the values sharing the same letters are not significantly different at P < 0.05.](image)
2.2 Oct4 regulates mouse Dnmt1 gene transcription in vitro

Based on the prediction by the three online programs, a potential Oct4 binding site (Octamer motif, TTTTGCAT/ATGCAAAA) was found on the -475 to -468 bp region relative to the Transcription start site (TSS). To confirm the potential Oct4 binding site, four luciferase reporters were constructed and transfected in two different cell lines. The analysis results showed that the relative luciferase activities were higher in the groups that were co-transfected with Dnmt1-P1-1, -2, -3 and Oct4 than in the separate control (Fig. 2A, B and E). Deletion of the upstream region from -1228 to -554 had no effect on the activation mediated by Oct4 (Fig. 2B and E), indicating that the three constructions (Dnmt1-P1-3) did not affect the Oct4 function in the transcriptional activity of Dnmt1. In contrast, a decline in the relative luciferase activity in the shortest Dnmt1 promoter (-P4) was detected, indicating that the Oct4 positive regulatory element in the region between -554 bp and -294 bp relative to TSS could be lacking. These findings are consistent with the results predicted by the web tools. As expected, mutation of this element to CCCCATGC remarkably decreased the promotor activity of Dnmt1 and transcription activation efficiency of Oct4 (Fig. 2C and F). This result suggests that TTTTGCAT could be the possible binding site for Oct4 in the mouse Dnmt1 promoter region. Furthermore, the co-transfection of Dnmt1-P3 and Oct4 increased the luciferase activity in a dose-dependent manner (Fig. 2D and G).
Fig. 2. Promoter analysis of mouse Dnmt1 in NIH3T3 and CCE cells by luciferase assay. (A) Schematic structures of mouse Dnmt1 gene constructs used in this study. (B and C, E and F) Oct4 enhanced the promoter activity of mouse Dnmt1; Oct4 -pcDNA3.1 (100 ng) plasmid was co-transfected with a series of sequential deletion constructs (Dnmt1 P1-4) and mutant of the mouse Dnmt1 promoter (500 ng/well) into NIH3T3 and CCE cells, respectively; (D and G) 10, 50, 100, 200, and 500 ng of Oct4-pcDNA3.1 expression plasmid was co-transfected with mouse Dnmt1-P3 promoter (500 ng/well) into NIH3T3 and CCE cells, respectively. The total amount of the transfected plasmid, including the pRL-TK control vector (100 ng/well), was adjusted to 1.0 µg with pcDNA3.1 empty vectors. Firefly and Renilla luciferase activities were measured 48 h after the transfection. The relative luciferase activity was calculated by dividing the activity of firefly luciferase by the activity of Renilla luciferase. The data are presented as the mean ± SD for triplicate transfections.

2.3 Oct4 direct binding to the regulatory elements of mouse Dnmt1

Next, we examined whether Oct4 binds to the Dnmt1 promoter in vitro. Chip assay was employed to investigate the binding affinity. The chromatin (from 4×10⁵ NIH/3T3 cells) was immunoprecipitated with the anti-RNA Pol II antibody (as a positive control), anti-IgG antibody (as a negative control), and the specific antibody against mouse Oct4. As shown in Fig. 3, the anti-RNA Pol II and anti-Oct4 antibodies precipitated the proteins bound in vivo to the specific amplified sequence of the mouse Dnmt1 promoter in the region between -554 and -294. Conversely, the non-specific IgG antibody failed to precipitate in vivo the proteins bound to this sequence, suggesting that mouse Oct4 has the potential to bind to the fragment of Dnmt.

Fig. 3. Chromatin immunoprecipitation assay conducted to confirm the binding of Oct-4 to the promoter of mouse Dnmt1 in vivo. Anti-RNA Pol II and anti-Oct4 antibodies precipitated proteins bound in vivo to the specific amplified sequence of the mouse Dnmt1 promoter (between -554 to -294 bp region), whereas non-specific IgG (negative control antibody) failed to do. 213-bp length of PCR products were resolved on 1.5% agarose gel and stained with ethidium bromide to visualize the bands.

2.4 Dnmt1 expression was downregulated by Oct4 RNAi in CCE cells

Further, we investigated whether the knockdown of Oct4 in CCE cells can decrease the mRNA level and enzyme activity of Dnmt1. To determine the transfection efficiency, 20 nM Red Fluorescent Oligo was transfected into CCE cells. As depicted in Fig. 4A, B and C, we revealed that the efficiency reached >85%, indicating that these conditions can be used to perform the subsequent experiments.

It is noteworthy that the morphology of CCE cells was obviously changed 48 h after the transfection with the three siRNA of Oct4 (R1, R2, and R3) (Fig. 4 D, E, and F), indicating that the self-renewal and undifferentiated state of CCE cells were not maintained. Real-time PCR and Western blot were used to measure the mRNA and protein levels of Oct4 in transfected siRNA CCE cells. The Oct4 mRNA and protein level in R1, R2, and R3 transfected-groups dramatically decreased, while that in the control remained unchanged (Fig. 5A and B). The results showed that both R2 and R3 reduced Oct4 mRNA by 95% of the blank control at 48 h post-transfection. In addition, the protein levels of Oct4 were also obviously reduced in R2 and R3 transfected-groups (Fig. 5 A and B). A Dnmt1 assay was also performed to detect the enzyme activity of Dnmt1 in the transfected siRNA CCE cells. The value of the final OD (subtract the 655 nm ODs from 450 nm ODs) of R2 and R3 transfected-groups...
measured by microplate reader were 1.5 to 2 times lower than those of the negative control. The change rates and the amounts of Dnmt1 in the three siRNA CCE were approximately 60–80% of those of the negative control (Fig. 5C and D). These results indicated that the decline of Dnmt1 activity is dependent on the function of Oct4 in CCE cells.

Fig. 4. Effect of mouse Oct4 knockdown in CCE cells. (A) and (B), CCE cells were transfected without or with Negative control alone at 48h, respectively. More than 85% of cells were successfully transfected with Alexa Fluor® Red Fluorescent Oligo (C). (D), (E), and (F), the self-renewal and undifferentiated state of CCE cells were not maintained when transfected with the three siRNA of Oct4 (R1, R2, and R3) when tested at 48 h after the transfection. The arrows indicate the normal CCE mouse ES cells. The images were photographed by Leica DMI3000B with 200x magnification.

Fig. 5. Dnmt1 assay showed decrease of Dnmt1 amount in CCE cells transfected with mouse Oct4 siRNA. (A) and (B), the mRNA and protein level of Oct4 were downregulated by the three mouse Oct4 siRNA. (C) and (D), The final OD (subtract the 655 nm ODs from 450 nm ODs), and the amount of Dnmt1 was reduced in mouse Oct4 siRNA R1, R2, and R3 transfected-group when compared with the negative control. Results are presented as the mean ± SD. The final ODs from triplicate transfected samples were measured by microplate reader. * and **, statistically significant difference obtained with the Student’s t-test for the comparison with the negative control at P < 0.05 and 0.01.
3. Discussion

In China, Kunming white mice are widely used as model animals due to their specific characteristics and certain advantages over other mouse strains. The dynamic expression and cellular localization of Dnmt1 and Oct4 in preimplantation embryos of other mouse strains have been reported in previous studies (Li et al., 2005; Hirasawa et al., 2008)[1, 21], but little is known about the mRNA expression pattern of Dnmt1 and Oct4 in preimplantation embryos of Kunming white mouse, a distinctive specific Chinese animal model. In this study, we first examined the expression of Dnmt1 and Oct4 during preimplantation embryo stages. The results showed that the mRNA level of Dnmt1 before the 4-cell stage was relatively higher than that from the 8-cell stage onwards, further indicating that Dnmt1 is a maintenance methyltransferase in the per-implantation period (Jurkowska et al., 2011; Smith et al., 2012)[14, 22], whereas the genomic remethylation in the inner cell mass (ICM) and hypomethylated state in the trophectoderm of the blastocyst are achieved passively by decreasing the expression of Dnmt1 or caused by de novo methyltransferases (Dnmt3a and Dnmt3b) (Nakanishi et al., 2012)[23].

In a previous study, Oct4 was observed to be located in the ICM cells of blastocysts (Ding et al., 2012)[24]. It is worth noting that in Kunming white mouse the mRNA expression level of Oct4 increased with embryonic development and peaked during the blastocyst stage as has also been reported in other mouse strains (Li et al., 2005)[1]. Inspired by these previous and present data, we speculate that some correlation may exist between Oct4 and the transcription of Dnmt1. Fortunately, by using bioinformatic analysis, an Octamer motif was predicted in the region between -475 bp and -468 bp relative to TSS. Therefore, promoter analysis was performed to investigate whether or not Dnmt1 transcription is regulated by Oct4. Sequential deletion promoters of mouse Dnmt1 were isolated and cloned into luciferase reporter vectors, including luciferase plasmid bearing a mutation in the -475/-468bp Oct4 binding motif. Using transfection assays, we found that no significant difference was present in the luciferase activities of mouse Dnmt1 among the Dnmt1 P-1 to P-3 groups when co-transfected with mouse Oct-4, but significant differences were observed between Dnmt1 P-1 to P-3 groups and the respective control treatments. On the other hand, the luciferase activity of Dnmt1 P-4 was obviously lower than those of Dnmt1 P-1 to -3 groups, indicating that there is a cis-regulatory element of Oct4 in the mouse Dnmt1 promoter region (the region from -554 to -294 bp) as well as detected in mutant reporter. These data indicate that mouse Dnmt1 gene expression might be regulated by Oct4 through direct binding to the promoter region of Dnmt1.

In the processing of MSC proliferation, Dnmt1 was upregulated by Oct4 through direct binding to its promoter, leading to the decreased expression of p16 and p21 and the genes associated with development and lineage differentiation (Tsai et al., 2012)[20]. Breast cancer–associated gene1 (BRCA1) can bind to the DNMT1 promoter through a potential OCT1 site in both mouse and human cells (Shukla et al., 2010)[25]. During the processes of cell transformation and tumorigenesis, mouse Dnmt1 transcription is regulated by both E2F-Rb-HDAC-dependent and -independent pathways (Kimura et al., 2003)[26]. We employed Chip assay and sensitive two-color EMSA assay (Fig. S1) to confirm that the DNA fragment between -554 to -294 bp of Dnmt1 was bound by Oct4 directly in vitro. Furthermore, in our study, silencing of Oct4 expression significantly reduced the expression and enzyme activity of Dnmt1 in CCE cells, while the overexpression of Oct4 in NCI-H157 cells obviously increased the amount of Dnmt1 in NCI-H157 cells (a line of human non-small cell lung cancer cells with a high mRNA level of endogenous Dnmt1) (Fig. S2). As can be seen in Fig. S3, the fluorescence intensity of Dnmt1 in Oct4-overexpressed group was higher than that of the control; the bright field image shows the promoted proliferation of NCI-H157 in Oct4-overexpressed group (Fig. S3). These abovementioned previously reported findings, together with our data, reveal that Dnmt1 gene expression in vivo is regulated by Oct4. On the other hand, the DNA methylation status of mouse Oct4 gene upstream region has been considered essential for its gene expression; i.e., the Oct-4 enhancer/promoter region was hypomethylated in ES cells, and the expression of Oct-4 mRNA in the Dnmt1knplacenta was detected but not in the wild-type placenta (Hattori et al., 2009)[27]. In addition, aberrant Oct4 gene expression was identified in another examination (Gu et al., 2011)[19]. These results indicate that the maintenance of DNA methylation status by Dnmt1 in mice might be...
accompanied with a change in the expression level of Oct4 mRNA. Based on these results, we have reason to believe that DNA methylation catalyzed by Dnmt1 is modulated through Dnmt1 expression, regulated by Oct4, whereas the special and temporal profiles of Oct4 are influenced through the DNA methylation status of its promoter and DNA methylation-mediated gene silencing.

4. Materials and methods

4.1. Animals and Collection of embryos

Briefly, all animals were maintained in a photoperiod of 14-h light and 10-h dark at 20–25 °C for at least two weeks before use. Zygotes, 2-cell, 4-cell, 8-cell embryos, morulae, and blastocysts were collected for this study as described previously (Wu et al., 2012a, b)[28, 29]. All animal studies were approved by the Institutional Animal Care and Use Committee at Fuyang Teachers College (Fuyang, Anhui Province, China). This study was conducted in strict accordance with the recommendations in the Regulations on the Management of Laboratory Animals in China promulgated in 1988. Kunming white mice that were used as a model in the present examination were purchased from the Experiment Animal Center of Anhui Medical University (Certification of quality # 34000200000077, 34000200000078).

4.2 RNA extraction and real-time PCR

Fifty embryos were used for each time point, and three replicates were performed for each stage. Total RNA extraction, DNAs synthesis from all the samples, and real-time PCR were conducted according to the manufacturer’s instructions as described previously (Wu et al., 2012a)[28]. Gapdh was used as an internal control. The threshold cycle (Ct) was defined as the fractional cycle number by the method of global minimum. The ratio change in the Oct4 and Dnmt1 relative to Gapdh control gene was determined by the 2-△△Ct method (Wu et al., 2012)[28]. Data were expressed as the mean ± SE for the three replicates. A Kruskal-Wallis test, which were conducted with GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA), was used to determine if there was a significant difference among the means (P < 0.05). All primers used for the study are listed in Table 1.

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Purpose</th>
</tr>
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<tr>
<td>Dnmt1-F</td>
<td>CCTAGTCCGTGGCTACGAGGAGAA</td>
<td>For real-time PCR</td>
</tr>
<tr>
<td>Dnmt1-R</td>
<td>TCTCTCTCTCTGAGCAGCCGACTCA</td>
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<tr>
<td>Gapdh-F</td>
<td>ATTCAACGGCACAGTCAGAGG</td>
<td></td>
</tr>
<tr>
<td>Gapdh-R</td>
<td>GGTCCTCAGTGGCCCAAGA</td>
<td></td>
</tr>
<tr>
<td>Dnmt1-P1F</td>
<td>CGACGCGTTATACTACTTCTATTGG</td>
<td>For promoter assay</td>
</tr>
<tr>
<td>Dnmt1-P2F</td>
<td>CGACGCGTTAGACACTACAGAACC</td>
<td></td>
</tr>
<tr>
<td>Dnmt1-P3F</td>
<td>CGACGCGTGCTGACCTCAAATCAGA</td>
<td></td>
</tr>
<tr>
<td>Dnmt1-P4F</td>
<td>CGACGCGTAAAGGCTGTAGGACC</td>
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<tr>
<td>Dnmt1-Pwt</td>
<td>CGACGCGTACAGAGTCTCTGTGCTTTGGCAT</td>
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<td>Dnmt1-Pmu</td>
<td>CGACGCGTACAGAGTCTCTGTGACcccctgc</td>
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<td>Dnmt1-PR</td>
<td>CCCAAGCTTGGCCGAACCCGGAAGCAGCA</td>
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<tr>
<td>Oct4-F</td>
<td>CGGATTGCCACCACCGCTGGACACCTGG</td>
<td>For pcDNA3.1 expression plasmid</td>
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<tr>
<td>Oct4-R</td>
<td>CGGCTCAGTCAGTTGGGATGCGAT</td>
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<tr>
<td>Dnmt1-CF</td>
<td>GCCACCACCGCCTACCTTT</td>
<td>For Chip</td>
</tr>
<tr>
<td>Dnmt1-CR</td>
<td>GCCACCACCGCCTACCTTT</td>
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Underlined bases show the restriction sites
4.3 Plasmid constructs

The 1228-bp, 931-bp, 554-bp, and 294-bp fragments of 5'-flanking regions of mouse Dnmt1 gene were generated by PCR and subcloned into the pGL3-Basic Vector (Promega Corp., Madison, WI, USA) within the Mlu I and Hind III sites. The software Promoter 2.0 Prediction Server (http://www.cbs.dtu.dk/services/Promoter/) was used to predict the transcription start sites of mouse Dnmt1. Luciferase plasmid bearing a mutation in the -475/-468bp Oct4 binding motif was constructed by PCR-mediated mutagenesis using primers containing the mutations. The software TFSEARCH ver.1.3. (http://www.cbrc.jp/research/db/TFSEARCH.html) and PROMO searching tools (http://alggen.lsi.upc.es/) were employed to analyze all possible binding sites on the sense and antisense chain of the mouse Dnmt1 promoter. Mouse Oct4 were amplified and cloned into the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA, USA) using gene-specific open reading frame (ORF) primers as described in our previous study (Wu et al., 2012a)[28] All inserted sequences were further confirmed by sequencing in Life Technologies Corporation (Shanghai, China).

4.4 Cell culture and luciferase assay

Using Lipofectamine 2000 (Invitrogen), mouse fibroblast cell line NIH/3T3 cells were transfected with the following plasmids: (1) 500 ng of normal or truncated constructs or mutant of the mouse Dnmt1 promoter which were cloned into the pGL3-Basic luciferase reporter vector; (2) 10, 50, 100, 200, and 500 ng of Oct4-pcDNA3.1 expression plasmid; and (3) pRL-TK (Promega), at 100 ng/well. Renilla luciferase from pRL-TK was employed as an internal control for transfection efficiency. Firefly luciferase and Renilla luciferase readings were obtained using the Dual-Luciferase Reporter Assay System (Promega) and GloMax® 20/20 Luminometer (Promega). Cell culture, transient transfection, and luciferase assays were performed as reported previously (Wu et al., 2012b)[28]. For CCE mES cells, a mESC line derived from the 129/Sv mouse strain, a gift from Prof. Sijin Liu (Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences), was cultured and transfected with the same plasmids as in NTH/3T3 cells by using Effectene Transfection Reagent (Qiagen) as described previously (Chang et al., 2012; Ko et al., 2009)[30, 31].

4.5 Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed with the Imprint® Chromatin Immunoprecipitation Kit (Cat. No. CHP1, Sigma) according to the manufacturer’s instructions. Briefly, a total of 4×10⁶ NIH/3T3 cells were cross-linked in 1% formaldehyde at room temperature for 10 min. The isolated nuclei were lysed and followed by solubilization with shearing buffer containing a protease inhibitor cocktail (Sigma). The chromatin was then sonicated and immunoprecipitated. The Antibodies used for ChIP studies were anti-RNA Pol II antibody anti-IgG antibody provided in the Kit, and anti-Oct4 antibody (Abcam, ab19857). After reverse cross-linking and DNA purification, DNA from input (1:10 diluted) or immunoprecipitated samples was assayed by PCR, and the products were separated by 1.8% agarose gel electrophoresis. The primers used for ChIP analysis PCR reaction are presented in Table 1.

4.6. RNA interference (RNAi), and transfection

Lipofectamine™ RNAiMAX (Invitrogen Life Technologies) was used to transfect the Stealth RNAi siRNA against Oct4 into CCE cells. The Stealth™ RNAi Negative Control Duplexes (Invitrogen Life Technologies) were used as a negative control. BLOCK-i™ Alexa Fluor® Red Fluorescent Oligo (Invitrogen) was utilized to facilitate the assessment and optimize the delivery of double-stranded RNA oligonucleotides into the CCE cells. These siRNA sequences were submitted to a BLAST search to ensure that only the mouse Oct4 gene was targeted. The sequences of the three synthesized oligonucleotides were: R1 sense 5’CCAAUGCCUGAAGUGGAGAGGU-3’ and anti-sense 5’ACCUCUCCCAAUCACCGGCAUUUGG-3’; R2 sense, 5’CCCCAGAAGAAAGCGAAGCUACAU-3’ and anti-sense, 5’AUGCUAGGUUCGUUCUCUCUGCGG-3’; R3 sense, 5’CCAUUCAGCGUGGCUAGGAAGGA-3’ and anti-sense,
5’UCCUUCUCUUAGCCCAAGCUGAUUGG-3’. RNAi transfection was conducted according to the manufacturer’s instructions. Gene knockdown assays were performed after the complexes were added to the cells and incubated for 48 h at 37 °C in a CO2 incubator.

4.7 Gene knockdown and Dnmt1 assay

Real-time PCR and Western blotting were conducted to investigate the mRNA and protein expression levels of mouse Oct4 and confirm whether Dnmt1 was downregulated. Total RNA extraction, cDNAs synthesis, and real-time PCR were performed as described above. Total proteins were extracted from the RNAi-transfected CCE cells following the procedure detailed in the manual of the “NE-PER Nuclear and Cytoplasmic Extraction Reagents” (#78833, Thermo Fisher Scientific, Waltham, MA, USA). The protein concentration was measured by a bicinchoninic acid assay via a NanoDrop 2000 spectrophotometer. Western blotting analysis was performed as described previously (Wu et al., 2016)[32]. The antibody against mouse Dnmt1 was purchased from Abcam (ab13537). For Dnmt1 assay, the RNAi-transfected CCE cells was initially lysed with NE-PER Nuclear and Cytoplasmic Extraction Reagent, and the EpiQuik DNMT1 assay kit (Epigentek, P-3011; Epigentek, Farmingdale, NY, USA) was used to detect the amount of Dnmt1 according to the manufacturer’s instructions described previously (Wu et al., 2016)[33].

5. Conclusions

 Taken together, our results demonstrate that Oct4 plays an important role in the transcription of Dnmt1 by direct binding to the specific site on the Dnmt1 promoter, and the total amount of Dnmt1 in CCE cells is reduced by Oct4 as evidenced by the knockdown assay. These findings might reveal the correlation between Oct4 and Dnmt1 during the stages of mouse preimplantation embryo development and provide new insights into the mechanism of the early stages of mammalian embryonic development.

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Authors’ Contributions: F.R Wu and W.Y Li designed and coordinated the study. Y. Z cloned the Oct4 cDNA, Dnmt1-P1-4-pGL3-Basic Vector, and performed promoter assay. Q.Q Wu and B.Ding performed Chip and EMSA assay. D.K Li prepared cell lines and RNAi knockdown assay. F.R Wu and R.Wang performed WB and Dnmt1 assay. Y. Liu performed all microscopy. F.R Wu and W.Y Li wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of interest: The authors declare that they have no competing interests.

Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Oct4</td>
<td>octamer-binding transcription factor 4</td>
</tr>
<tr>
<td>Sox2</td>
<td>(sex determining region Y)-box 2</td>
</tr>
<tr>
<td>Klf4</td>
<td>Kruppel-like factor 4</td>
</tr>
<tr>
<td>Dnmt1</td>
<td>DNA (cytosine-5)-methyltransferase 1</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
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<td>base pair</td>
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<td>Embryonic stem cell</td>
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<td>Chromatin Immunoprecipitation</td>
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<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
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References


21. Hirasawa R, Chiba H, Kaneda M, Tajima S, Li E, Jaenisch R, Sasaki H. Maternal and zygotic Dnmt1 are necessary and sufficient for the maintenance of DNA methylation imprints during...