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Alteration of DNA Methylation in Oct-4 Gene in Mouse Preimplantation Embryos by the Interference RNA

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ABSTRACT

During early embryo development, Oct-4 is an important transcription factor for the early differentiation. The present study was first examined methylation status in distal enhancer and promoter region of Oct-4 during mouse pre-implantation embryo development. In oocyte and sperm, high methylation was observed in both distal and proximal of promoter in Oct-4. Following fertilization, relatively high methylation level remained until 8-cell stage embryos, but decreased at the morula and blastocyst stage. Specific gene knock down of Oct-4 by siRNA injection into zygote induced higher methylation rates of both distal and proximal region ofpromoter of Oct-4. These results suggest a functional link between the DNA methylation status of distal and promoter region in the Oct-4 gene and the gene sequence-specific transcriptional silencing by exogenous siRNA injection during mouse pre-implantation embryos.

(Key words : DNA methylation, Oct-4; RNA interference, Mouse)

INTRODUCTION

Oct-4 (also known as Oct-3), is a POU domain-containing transcription factor that binds to an octamer sequence, ATGCAAAT. During mouse pre-implantation development, Oct-4 mRNA and protein are present in unfertilized oocytes, and the protein is localized to the pronuclei following fertilization (Scholer *et al.*, 1989; Rosner *et al.*, 1990). Similar to the other maternal m-RNAs, Oct-4 mRNA levels drop dramatically after fertilization (Yeom *et al.*, 1991).

DNA Methylation of genomic CpG dinucleotide is known to be a major epigenetic modification of the genome, and play a key role in embryogenesis by silencing specific gene during development and differentiation. Dynamic alteration of DNA methylation was observed during mouse pre-implantation development. *In vitro* studies demonstrate that DNA methylation severely impairs the efficiency of reporter gene expression by the Oct-4 regulatory elements. DNA methylation in the 1.3kb upstream region of the mouse Oct-4 gene has previously been reported by following RA treatment of mouse OTF9-63 EC cells (Ben-Shushan *et al.*, 1993). A recent study reported that reactivation of the mouse stem cell specific gene Oct-4 depends on demethylation of CpG's in the proximal Oct-4 promoter (Simonsson and Gurdon, 2005). Moreover, CpG dinucleotides in the promoter region of Oct-4 gene, as well as the enhancer region of Oct-4, are predominantly demethylated in the undifferentiated cells when the genes are active (Paromita Deb-Rinker *et al.*, 2005). With differentiation progress, the CpG dinucleotides become substantially methylated (Promita Deb-Rinker *et al.*, 2005).

Small RNA-mediated transcriptional gene silencing was first observed in plants through the use of inverted repeat transgenes or transgenic viruses to generate siRNA homologous to a target promoter (Mette *et al.*, 2000; Jones *et al.*, 2001; Pal-Bhadra *et al.*, 2002). Promoter directed siRNAs also silence specific gene transcription in the yeast *Schizosaccharomyces pombe*, and transcriptional silencing in *Drosophila* has been linked to an Argonaut protein (Pal-Bhadra *et al.*, 2002; Schramke *et al.*, 2003; Pal-Bhadra *et al.*, 2004). Transcriptional gene silencing by siRNAs probably reflects genome defense mechanisms that target chromatin modifications to endogenous silent loci such as transposes and sequences (Mette *et al.*, 2000; Wassebegger *et al.*, 1994; Chan *et al.*, 2004; Zilberman *et al.*, 2003; Volpe *et al.*, 2002). RNA interference transcriptional gene si-

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lencing including DNA methylation in plants (Zilberman *et al.*, 2003; Morel *et al.*, 2000; Mette *et al.*, 2000), and heterochromatin formation in fungi (Volpe *et al.*, 2002; Hall *et al.*, 2002). Recent observation showed that siRNAs can silence target gene at the chromatin level in mammalian cells (Hiroaki *et al.*, 2005). siRNAs targeted to promoters can induce gene transcriptional silencing via DNA methylation in human cells (Morris *et al.*, 2004; Kawasaki *et al.*, 2004). Another recent report also proved unable to detect sequence specific siRNA directed DNA methylation in mouse oocytes targeted with a ~500bp hairpin shRNAs (Svoboda *et al.*, 2004). However, it is not clear whether siRNA influence DNA methylation in mouse pre-implantation embryos.

The map position of Oct-4 on mouse chromosome 17 is between Q and T regions in the Major Histocompatibility Complex (MHC), and it is physically located within 35kb of a class I gene (Yeom *et al.*, 1991). The Oct-4 gene has a GC-rich and TATA-less minimal promoter (Okazawa *et al.*, 1991), and several transactivators and repressors have been reported as the regulators of Oct-4 expression (Pikarsky *et al.*, 1994; Schoorlemmer *et al.*, 1994). However, specific regulatory mechanisms regarding the developmental stage- and cell type-specific expression of the Oct-4 gene have not been conclusively revealed to date. In order to determine whether DNA methylation is involved in the regulation of Oct-4 gene expression, we first examined methylation status in distal enhancer and promoter region of Oct-4 during mouse pre-implantation embryo development. Secondly we determined methylation rates in the Oct-4 gene knock down blastocyst.

MATERIALS AND METHODS

Collection of Mouse Embryo and Sperm

$B_6D_2F_1$ female mice (6 weeks old) were induced to undergo super-ovulation by the intraperitoneal injection of 5 IU pregnant mares' serum gonadotropin (PMSG, sigma, St. Louis, MO, USA), followed by an injection of 5 IU human chorionic gonadotropin (hCG, Sigma) 48 hr later. Unfertilized metaphase II eggs (MII) or one-cell (1C) embryos were collected from ampulla of superovulated female without mating or via mating in a day (20 hr) after the hCG injection. Cumulus cells were removed with 0.1 mg/ml hyarulonidase (Sigma) by pipetting in KSOM medium. In the *in vivo* case, two-cell (2C), four-cell (4C), eight-cell (8C) stage embryos were obtained from oviducts of superovulated females killed 40, 50, or 70 hr after hCG injection, respectively. Morula (MO), and blastocyst (BL) stage embryos were obtained from the uterus of 82 hr or 96 hr after hCG injection. In the *in vitro* case, collected one-cell (1C) embryos were cultured in KSOM medium supplemented with 0.4% (w/v) bovine serum albumin (BSA; Sigma) to produce different developmental stages embryos. Sperm were collected from the $B_6D_2F_1$ of 8-week-old males. Collected embryos and oocytes were washed several times in PBS to remove any adhering maternal cells, snap frozen in liquid nitrogen and stored at −80℃ until used.

RNAi Injection

Female mice (6~8 weeks old) were induced to undergo superovulation by the intraperitonal injection of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG 5 IU., 48 hr apart). Fertilized mouse zygotes were collected from the oviducts 24 hr after the hCG injection. After designing the siRNA that would silence the mouse Oct-4 gene (GenBank number M34381), the chemically synthesized 21-nt sense (5'-GAA CAU GUG UAA GCU GCG GTT-3') and antisense (5'-CCG CAG CUU ACA CAU GUU CTT-3') RNAs were obtained commercially (Ambion, Inc., Houston, TX). The siRNA was produced by annealing of the sense and antisense RNAs for 1 hr at 37℃, after which it was diluted with distilled water to a final concentration of 20 mM and stored at −20℃. Approximate 10 pl siRNA was injected into the cytoplasm of the experimental group zygotes by using a microinjector system (Eppendorf). The control group zygotes were injected with the same volume of culture medium. All zygotes were then cultured in M16 medium supplemented with 0.4% BSA at 37℃.

Genomic DNA Preparation

Five embryos were transferred into 50 μl of lysis buffer (10 mM Tris-HCl (pH 8.0), 150 mM EDTA, 1 % SDS) containing 0.2 μl Proteinase (20 mg/ml) and incubated at 55℃ for 20 min. Then 30 units of RNase were added, and the mixture was incubated for 60 min at 37℃. The identical volume of genomic DNA sample and 3% low-melt agarose were mixed following a bead formation in cold mineral oil. The beads were washed twice in TE (pH 9.0) for 15 min. After being incubated with 500 μl of PMSF solution for 45 min at room temperature, the beads were washed twice in TE (pH 9.0) and restriction enzyme buffer for 15 min at RT. The isolated 3% Agarose bead was digested with *EcoR* I restriction enzyme (Takara, Korea) at 37℃ for 16 hr. The beads were washed twice in 0.33 M NaOH for 15 min. *EcoR* I digested genomic DNAs were denatured in mineral oil for 15 min at 80℃.

DNA Methylation Analysis followed by Bi-sulfite Sequencing

Genomic DNA with methylation CpG was processed using the EZ DNA Methylation-Gold kit™ (Zymo Re-

search, USA). The whole sample was treated with the CT conversion reagent, and purified with the affinity column (Zymo Research, USA). DNA was eluted in 10 μl of elution buffer, and used for two successive rounds of PCR with nested primer (outer and inner) pairs, which are specific to the top strand of mutagenized DNA (Table 1). All PCR reactions were preformed using immolase™ DNA polymerase Mix (Bioline). The condition of the first PCR was as follows: The thermocycling program was 43 cycles of 94℃ for 30 sec, 55℃ for 30 sec, and 72℃ for 1 min, preceded and followed by 10 min of incubation at 94℃ and 72 ℃, respectively. The first PCR product were eluted in 100 μl of water, and used for the second round of PCR reactions.

The entire PCR products were run on a 2% agarose gel to verify to verify the amplification of specific bands, which were then excised from the gel for purification with the affinity column. The electrophoresed PCR fragments were cloned into TOPO TA cloning kits (Invitrogen), and cloned were sequenced for each sample.

Real Time RT-PCR

Messenger RNA was extracted by the one step RT-PCR kit (TOYOBO) according to the manufacturer's instruction. In all experiments histone H2a mRNA was used as an internal standard. First, standard cDNA synthesis was achieved by reverse transcription of the RNA followed by a PCR using thermostable rTth DNA polymerases. After the reverse transcription, a PCR can occur immediately rendering the mid-reaction addition of reagents unnecessary. The threshold cycle (Ct) value represents the cycle number at which sample fluorescence rises statistically above background. The reactions were conducted according to the protocol of the DyNAmo SYBR green qPCR kit, which contains modified Tbr DNA polymerase, SYBR Green, optimized PCR buffer, 5 mM MgCl, and a dNTP mix that includes dUTP (Finnzyme Oy, Espoo, Finland). The PCR

Table 1. Primer sequences for bi-sulfite PCR

Primer set	.	Primer sequences	Size, bp	Annealing temperature (℃)
DE5	1st	5'-GTGACATATTTTAGCAGAAGGTCAGGTG-3'	535	55
		5'-CAAAGGGAGCAGGCACATTCCGCAA-3'		
	2nd	5'-AGAGTGTCTGTGATTTGAGGGACAG-3'	402	55
		5'-GAGTGAGTTCCAGGACAGCCAGGGC-3'		
DE4	1st	5'-GAGTGAGTTCCAGGACAGCCAGGGC-3'	316	55
		5'-CAGCAAGTGTGGTTACTTGCTGAG -3'		
	2nd	5'-CAAAGGGAGCAGGCACATTCCGACA -3'	223	55
		5'-CTCAGCAAGTGTGGTTACTTG -3'		
DE3	1st	5'-GACAAGGAAAGGGAGATGCAGTTAG -3'	447	55
		5'-CCTGGAGGACTCTTGTGTTTAAG-3'		
	2nd	5'-GTTAGCTAAGGAATCTATGCCAGCG-3'	399	55
		5'-GTCTCCTGCTGAGGCTGGCTCAAGG-3'		
Promoter	1st	5'-TGGGCTGAAATACTGGGTTCACCC-3'	533	55
		5'-CATGGCTGGACACCTGGCTTCAG-3'		
	2nd	5'-TAGGACTCTAGACGGGTGGGTAAGCA-3'	490	55
		5'-GGGTGCCCACCTTCCCCATGGCTGG-3'		

protocol involved a denaturation program (95℃ for 10 min) followed by an amplification and quantification program repeated 45 times (94℃ for 10 sec, 60℃ for 30 sec, 72℃ for 30 sec with a single fluorescence measurement), a melting curve program (65~95℃, with a heating rate of 0.2℃/sec and continuous fluorescence measurement) and finally a cooling step to 4℃. Fluorescence density data was acquired after the extension step during PCR reactions that contained SYBR Green. Thereafter, the PCR products were analyzed by generating a melting curve. Since the melting curve of a product is sequence-specific, it can be used to distinguish between nonspecific and specific PCR products. To use the mathematical model, it is necessary to determine the crossing points (CP) for each transcript. CP is defined as the point at which the fluorescence rises appreciably above the background fluorescence. The relative quantification of gene expression was analyzed by the 2-ddCt method (Livak and Schmittgen, 2001). The sizes of the RT-PCR products were confirmed by gel electrophoresis on a standard 2% agarose gel stained with ethidium bromide and visualized by exposure to ultraviolet light.

RESULTS

DNA Methylation Status of the 5'-Flanking Region of the Oct-4 Gene

The Oct-4 gene has two distinct enhancers, a distal enhancer (DE) and proximal enhancer (PE). There is no CpG island at the 5'-region of the Oct-4 gene, although the promoter region is relatively rich in CpG dinucleotide sequences (Hattori *et al.*, 2004). We designed PCR primers that amplify that upstream sequences of Oct-4 (Fig. 1), and analyzed the DNA methylation status of the Oct-4 regulatory region, focusing on these four sites in mouse pre-implantation embryos by target specific siRNA.

Methylation Patterns of Mouse Early Embryos

To trace DNA methylation patterns during early embryogenesis, DNA sample were isolated from mouse pre-implantation embryos, and sperm, liver as the controls. We designed PCR primers that amplify the upstream sequences of Oct-4 (Table 1), and examined the methylation status in mouse pre-implantation embryos. The resultant PCR products were individually cloned and sequenced. As shown in Fig. 2, pre-implantation embryos were heavily methylated in MII (79.1%) and oocyte genomes (52.2%), which were similar to methylation status of sperm (75.9%) and liver cells (56.4 %). This high methylation level was maintained throughout 1-cell to 4-cell stages. Complete DNA demethylation in the Oct-4 distal enhancer was detected in 8-cell stage embryos. These results indicate that while Oct-4 transcription and protein synthesis occurred, active demethylation did occur in the Oct-4 in distal enhancer from 8-cell to blastocyst stage embryos. As shown in Fig. 3, pre-implantation embryos were heavily methylated from MII (100%) to 4-cell (96.9%), which was similar to methylation states of sperm (100%) and liver cells (70%). This high methylation level was maintained throughout 1-cell to 4-cell stages. Reduction of DNA methylation in the Oct-4 proximal promoter was detected in 8-cell stage embryos and further sustaining reduction in blastocysts. These results indicate that the active-demethylation did not occur in the Oct-4 proximal promoter and the passive demethylation appears to be begun at the 8-cell stage.

Specific Knockdown of Oct-4 mRNA by siRNA Injection

Real-time RT-PCR revealed that Oct-4 mRNA was expressed in the mouse zygotes and 2-cell stage embryos but this expression decreased at the 4-cell embryo stage. However, at the 8-cell, morula, and blastocyst stage, Oct-4 gene expression was again significantly increased (data not shown). Injection of Oct-4 siRNA into the zygotes also significantly decreased the relative RNA expression of Oct-4, but not of Histone 2A (H_2A) expression in blastocyst stage embryo (Fig. 4).

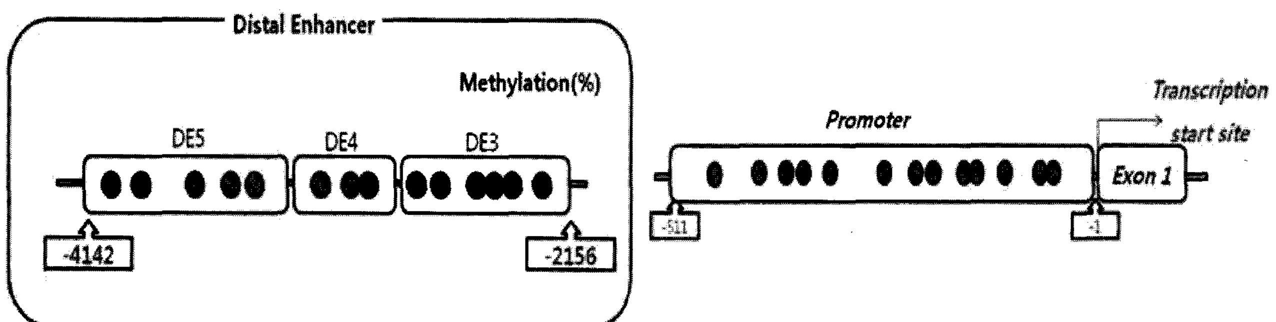

Fig. 1. DNA methylation status of the Oct-4 gene in the mouse. CpG site is shown as an open circle. The CpG-rich region is located at the Oct-4 promoter.

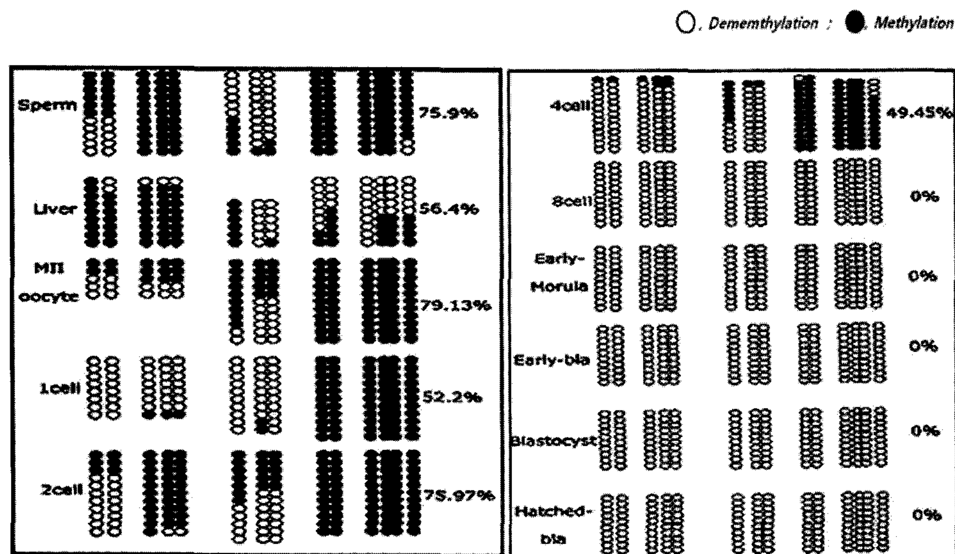

Fig. 2. Methylation analysis of Oct-4 Distal Enhancer. Methylation status of 14 CpGs sites in Oct-4 distal enhancer was determined by bi-sulfite sequencing. White, black circles indicate demethylation and methylation.

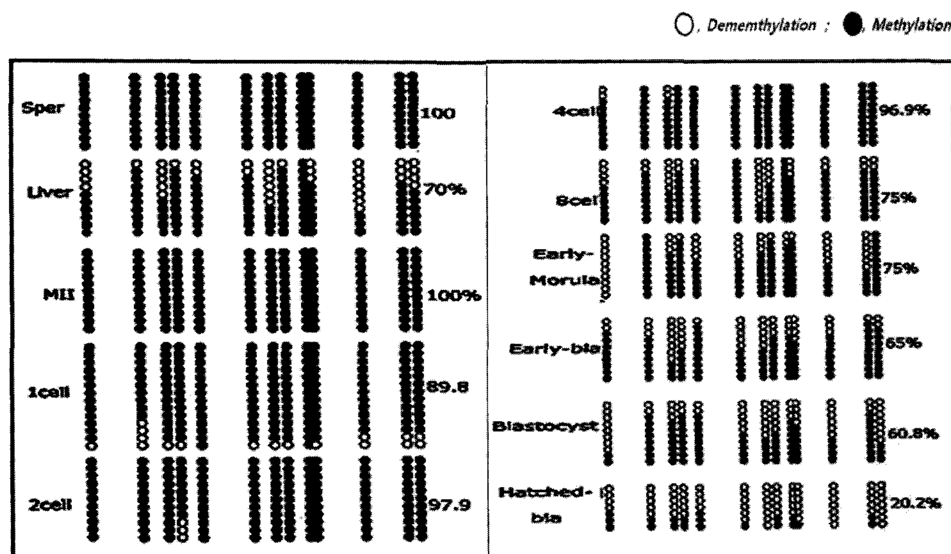

Fig. 3. Methylation analysis of Oct-4 Proximal Promoter. Methylation status of 13 CpGs sites in Oct-4 proximal promoter was determined by bi-sulfite sequencing. White, black circles indicate demethylation and methylation.

Thus, the injection of Oct-4 siRNA into mouse zygotes specifically knocked down the Oct-4 expression during their subsequent *in vitro* development.

Alteration of Methylation Patterns by siRNA Injection

To trace DNA methylation patterns during early embryogenesis, DNA sample were isolated from blastocyst after the injection of Oct-4 siRNA into mouse zygotes, sperm and liver cells DNA were served as controls. The resulting PCR products were individually cloned and sequenced. As shown in Fig. 5, injection of Oct-4 siRNA into mouse zygotes induced the methylation in blastocyst (24.6%). While the DNA samples of injection of buffer into mouse zygotes was completely demethylated in blastocyst (0%). These results were similar to methylation states of *in vivo* blastocyst (0%). As shown in Fig. 6, the Oct-4 gene was hyper-methylated in blastocyst derived from Oct-4 siRNA injected mouse zygotes (93.8%). While injection of buffer into mouse zygotes was methylated in blastocyst (60.4 %).

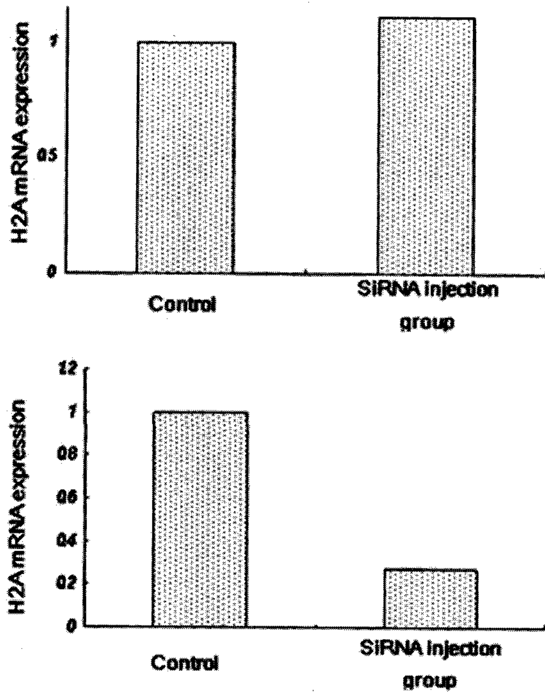

Fig 4. Oct-4 mRNA expression in the blastocyst embryos. Real-time RT-PCR showed the Oct-4 mRNA expression in buffer and Oct-4 siRNA-injected embryos. The experiment was repeated three times.

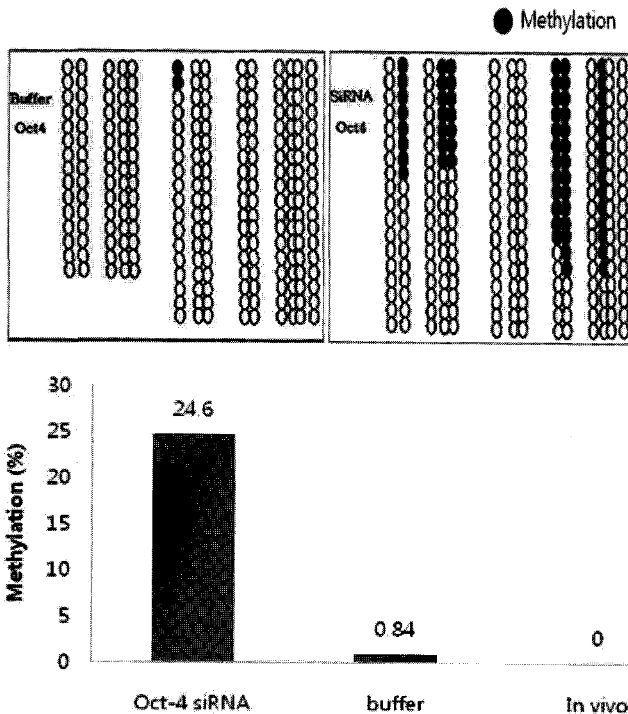

Fig 5. Methylation analysis of Oct-4 Distal Enhancer in blastocyst by siRNA. The upstream regions of the Oct-4 distal enhancer were determined in blastocyst after the injection of buffer and Oct-4 siRNA into mouse zygotes by bi-sulfite sequencing. White, black circles indicate demethylation and methylation.

This results indicate that buffer injected mouse zygotes was similar to methylation states of *in vivo* produced blastocyst (60.8%). These findings show that siRNA-induced transcriptional silencing in pre-implantation embryos is associated with DNA methylation.

DISCUSSION

In the present study, we demonstrated the methylation status in distal enhancer and promoter region of Oct-4 during early developmental mouse embryos. In oocyte and sperm, high methylation was observed in both distal and proximal of promoter in Oct-4. During mouse pre-implantation embryos development, the expression of zygotic Oct-4 gene starts before the 4-cell stage (Nichols *et al.*, 1998; Boiani *et al.*, 2002), consistent with our observation that the methylation was remained until 4-cell stage embryos after fertilization. but decreased during 8-cell, morula and blastocyst stages. specific gene knock down of Oct-4 by siRNA injection into zygote induced methylation of both distal enhan-

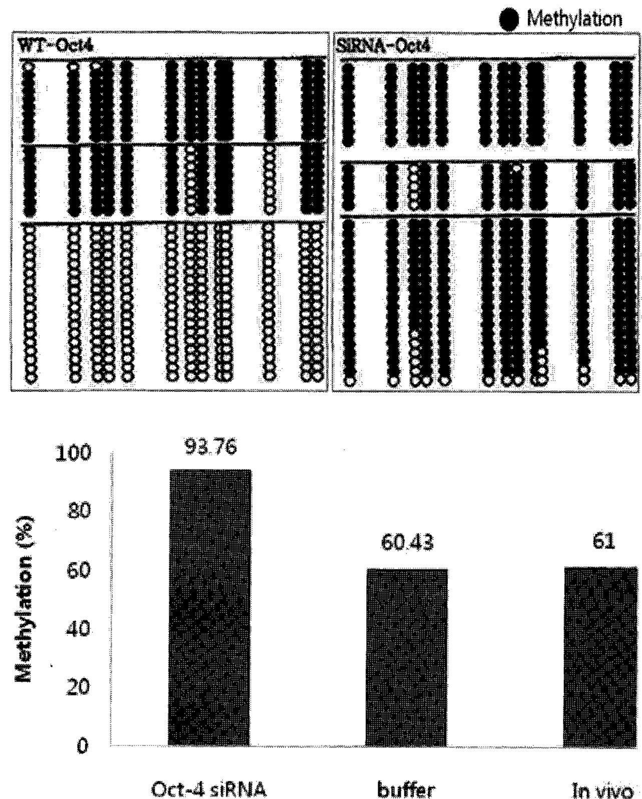

Fig 6. Methylation analysis of Oct-4 Proximal Promoter in blastocyst by siRNA. The upstream regions of the Oct-4 proximal promoter were determined in blastocyst after the injection of buffer and Oct-4 siRNA into mouse zygotes by bi-sulfite sequencing. White, black circles indicate demethylation and methylation.

cer and proximal promoter of Oct-4.

Oct-4 gene expression is dependent on the regulation of three upstream cis-regulatory regions. In the developing embryo, the activity of this enhancer is limited to the primitive ectoderm (Yeom *et al.*, 1991). In undifferentiated cells of the pre-implantation embryos, ES cells and F9 EC cells, the activity of the proximal enhancer is very low (Minucci *et al.*, 1996; Yeom *et al.*, 1996). Distal enhancer is active in undifferentiated cells of the pre-implantation embryo and, later in development, as well as responsible for the lineage-specific expression of Oct-4 in primordial germ cell (Yeom *et al.*, 1996). Recently, reactivation of the mouse stem cell specific gene Oct-4 depends on demethylation of CpGs in the proximal Oct-4 promoter (Simonsson and Gurdon, 2005).

Our study showed that reduction of DNA methylation in the Oct-4 proximal promoter was detected in 8-cell stage embryos and further reduction in blastocyst. Also, complete DNA demethylation in the Oct-4 distal enhancer was detected initially in 8-cell stage embryos. Therefore, this result indicates that the expression of Oct-4 in the developing embryo is dependent on the activities of both the proximal promoter and distal enhancer. The activity of enhancer is temporally regulated by the distal enhancer is active in the ICM, while the proximal enhancer driver expression in the primitive ectoderm. The switch of enhancer activity from distal to proximal occurs around implantation (Yeom *et al.*, 1996).

Epigenetics is the study of meiotically and mitotically heritable changes in gene expression which are not coded for in the DNA (Jablonka *et al.*, 2001; Egger *et al.*, 2004). Three distinct mechanisms appear to be intricately related and implicated in initiating and/or sustaining epigenetic modification; DNA methylation, RNA-associated silencing, and histone modification (Egger *et al.*, 2004). It has recently become clear in human cells that siRNA plays a far more profound and complex role in regulation the expression of genes. This regulatory effect through RNA-associated silencing can be at transcriptional level, and is operable through an RNA interference based mechanism (RNAi) that is specifically mediated by small-interfering RNAs (siRNAs). Specifically, the recent observations suggest that siRNAs can silence target genes at the level of the chromatin in mammalian cells (Kawasaki H *et al.*, 2005). Another recent report also proved unable to detect sequence specific siRNA directed DNA methylation in mouse oocytes targeted with a ~500 bp hairpin shRNAs (Stoboda *et al.*, 2004).

However, our result demonstrated that injection of Oct-4 siRNA into mouse zygote, distal enhancer was methylated in blastocyst (24.6%). While in contrast injection of buffer into mouse zygotes it was completely demethylated in blastocyst stage (0.84%). These results were in good agreement with the methylation status of distal enhancer in *in vivo* derived blastocyst (0%). This study also suggest that Oct-4 proximal promoter was heavily methylated in blastocyst (93.76%), compared with the buffer injected group (60.43%). Taken together, these data show that siRNA-induced gene transcriptional silencing in pre-implantation embryos was associated with DNA methylation.

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