

Gradual DNA Demethylation of the *Oct4* Promoter in Cloned Mouse Embryos

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ABSTRACT During differentiation, somatic cell nuclei acquire unique patterns of epigenetic modifications, such as DNA methylation, which affect the transcriptional activity of specific genes. Upon transfer into oocytes, however, the somatic nucleus undergoes reprogramming of these epigenetic modifications to achieve pluripotency. *Oct4* is one of the critical pluripotency regulators, and is expressed in the germ line, including the pluripotent early embryonic cells. Previous studies showed that the upstream regulatory sequences of the *Oct4* gene are distinctly methylated in somatic cells, and the DNA methylation of the regulatory sequences suppresses the transcriptional activity. Thus, successful reprogramming of the somatic cell nucleus to gain pluripotency must be accompanied by the demethylation of the *Oct4* regulatory sequences. Here, we investigated the methylation pattern of the *Oct4* promoter during early development of cloned mouse embryos. We found that the *Oct4* promoter was only gradually demethylated during the early cleavage stages and that the ineffective demethylation of the promoter was associated with developmental retardation. We also found that the upstream sequences of the other pluripotency regulators, namely *Nanog*, *Sox2*, and *Foxd3*, were considerably undermethylated in cumulus cells. These results suggest that the *Oct4* gene, as compared to the other pluripotency regulators, needs to undergo extensive demethylation during nuclear reprogramming, and that the failure of such demethylation is associated with inefficient development of cloned somatic cell embryos. *Mol. Reprod. Dev.* 73: 180–188, 2006.

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INTRODUCTION

Successful cloning of various mammalian species demonstrates that nuclei of differentiated somatic cells are genetically equivalent to zygotic nuclei (Wilmut et al., 1997; Kato et al., 1998; Wakayama et al., 1998; Baguisi et al., 1999; Polejaeva et al., 2000; Chesne et al.,

2002; Shin et al., 2002; Zhou et al., 2003). The potential of differentiated adult cells that can be reprogrammed into pluripotent embryonic cells promises to advance regenerative medicine. Particularly, the blastocysts that are cloned from patients' somatic cell nuclei may be used to derive embryonic stem (ES) cells, which can be induced to differentiate into various types of functional cells to replace damaged or lost tissues (Gurdon et al., 2003; Mombaerts, 2003; Hwang et al., 2005). Currently, however, the efficiency of full-term development from reconstructed oocytes is between 0 and 5%, and that of successful ES cell derivation is less than 10% (Hochedlinger and Jaenisch, 2002; Yanagimachi, 2002; Mombaerts, 2003; Hwang et al., 2005). Thus, the complete reprogramming of adult somatic cell nuclei appears to be an extremely rare event.

Several transcription factors play critical roles in the establishment and maintenance of pluripotency during normal embryogenesis. The *Oct4* (*Pou5f1*) gene encodes POU-domain DNA-binding protein and is expressed in pluripotent embryonic cells and germ cells (Pesce and Scholer, 2001). The expression of *Oct4* is essential for the establishment of pluripotency because *Oct4*-deficient embryos and ES cells spontaneously differentiate into trophectoderm cells (Nichols et al., 1998; Niwa et al., 2000). The other transcription factors, such as *Nanog*, *Sox2*, and *Foxd3*, are also essential for pluripotency (Hanna et al., 2002; Avilion et al., 2003; Chambers et al., 2003; Mitsui et al., 2003). For the successful nuclear reprogramming of the somatic cell nucleus, the proper reactivation of these pluripotency regulators is critical. However, the expression pattern of *Oct4* is abnormal in many cloned embryos that are produced from adult somatic cells (Boiani et al., 2002; Bortvin et al., 2003). This implicates that the inefficient development of

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cloned somatic cell embryos may be partly due to the aberrant expression of *Oct4*. Currently, however, the cause of such insufficient *Oct4* reactivation in cloned embryos is unknown.

Methylation of DNA is a major epigenetic modification of the genome, and is involved in transcriptional suppression of specific genes (Jaenisch and Bird, 2003; Shiota, 2004; Caiafa and Zampieri, 2005). In vitro studies demonstrate that DNA methylation severely impairs the efficiency of reporter gene expression that is driven by the *Oct4* regulatory elements. The endogenous *Oct4* promoter is essentially unmethylated in ES and embryonal carcinoma (EC) cells, which robustly express *Oct4*. In contrast, the promoter is distinctly methylated in somatic cells, in which *Oct4* is not expressed (Gidekel and Bergman, 2002; Hattori et al., 2004; Marikawa et al., 2005). Thus, to reactivate *Oct4* properly in cloned embryos, somatic cell nuclei may need to undergo extensive demethylation of the *Oct4* promoter during nuclear reprogramming.

To understand the mode of DNA demethylation during nuclear reprogramming, we analyzed the change in the methylation pattern of the *Oct4* promoter in cloned somatic cell embryos. The results indicate that the *Oct4* promoter is gradually demethylated during pre-implantation development with the biased efficiency on the CpG positions near the transcription start site. Also, the inefficient demethylation of the *Oct4* promoter is associated with developmental retardation at early cleavage stages. Furthermore, we show that the upstream sequences of other pluripotency regulator genes, *Nanog*, *Sox2*, and *Foxd3*, are significantly less methylated than the *Oct4* promoter in somatic cells, which implicates that the demethylation of the *Oct4* gene is one of the critical events during nuclear reprogramming.

MATERIALS AND METHODS

Collection of Gametes, Embryos, and Cultured Cells

B6D2F1 (C57BL/6 × DBA/2) mice (National Cancer Institute, Frederick, MD) were used to obtain gametes and embryos. Mature motile sperm was collected as follows: dense sperm syrup was squeezed from the caudae epididymis and was transferred into mKSOM^{AA} medium (Alarcón and Marikawa, 2003). After 20 min of incubation at 37°C, the upper layer of the medium, containing motile sperm, was recovered. Cumulus-oocyte complex was isolated from the oviducts of super-ovulated females, and cumulus cells were separated from oocytes using 0.1% hyaluronidase (Nagy et al., 2003). Normal and cloned embryos were produced as previously described (Alarcón and Marikawa, 2003; Yamazaki et al., 2003). Each developmental stage of cleavage stage embryos was collected to contain at least 100 cells. For example, a minimum of 25 embryos for the 4-cell stage, and a minimum of 14 embryos for the 7- to 8-cell stage, were collected as one sample. Also, about 100 cumulus cells were manually collected as one sample.

For the blastocyst stage embryos, 10 were put together as one sample, which may contain more than 300 cells. The data shown in Figure 2A,B were obtained using two independent collections of samples, which yielded consistent results. Animals were maintained according to the guidelines of the Laboratory Animal Service at the University of Hawaii and those prepared by the Guide for the Care and Use of Laboratory Animals of the National Research Council (Committee to Revise the Guide, Institute of Laboratory Animal Resources Council, Commission on Life Sciences, National Research Council, 1996). The protocol of animal handling and treatment was reviewed and approved by the Animal Care and Use Committee at the University of Hawaii. NIH3T3 (embryonic fibroblast) and Hepa1-6 (hepatoma) cell lines were obtained from American Type Cell Culture (Manassas, VA), and were maintained according to the vendor's instruction.

Bisulfite DNA Methylation Analysis

The extraction of genomic DNA and bisulfite mutagenesis sequencing analysis were conducted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) and EZ DNA Methylation Kit (Zymo Research, Orange, CA), respectively. For sperm DNA collection, the Nuclei Lysis Solution was supplemented with dithiothreitol (EMB Biosciences, San Diego, CA) at a final concentration of 60 mM. Although DNA extraction and bisulfite analyses were essentially performed as previously described (Marikawa et al., 2005), the procedures were modified to analyze considerably small amounts of samples in this study. Specifically, after digesting each sample in the ProteinaseK/SDS solution, genomic DNA was precipitated together with 25 µg of linear acrylamide (Ambion, Inc., Austin, TX) in isopropanol. Linear acrylamide serves as a carrier and co-precipitates with DNA. The whole co-precipitant was treated with the CT conversion reagent, and purified with the affinity column (Zymo Research). DNA was eluted in 14 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). The condition of the first PCR was as follows: the initial denaturation at 94°C/3 min, 5 cycles of [94°C/2 min, 55°C/1 min, and 72°C/1 min], followed by 20 cycles of [94°C/1 min, 55°C/30 sec, and 72°C/30 sec], and the final extension at 72°C/3 min. The first PCR product was purified using the affinity column to remove the outer primers. The PCR products were eluted in 14 µl of water, and used for the second round of PCR reactions: the initial denaturation at 94°C/5 min, 25 cycles of [94°C/1 min, 55°C/30 sec, and 72°C/30 sec], and the final extension at 72°C/3 min.

All PCR reactions were performed using JumpStart REDAccuTaq LA DNA Polymerase Mix (Sigma, St. Louis, MO). The entire PCR products were run on an agarose gel to verify the amplification of specific bands, which were then excised from the gel for purification with the affinity column. The purified PCR products

TABLE 1. Primer Sequences and the Location of Genomic Sequences That Are Amplified by These Primers

	Forward primer	Reverse primer	Region amplified ^a
<i>Oct4</i>			
Outer	5'-agg tgt aat ggt tgt ttt gtt ttg gtt ttg-3'	5'-taa ccc atc acc ccc acc taa taa aaa taa-3'	-515 to +33
Inner	5'-tat ggg ttg aaa tat tgg gtt tat tta tat-3'	5'-tct aaa acc aaa tat cca acc ata a-3'	-483 to -3
<i>Nanog</i>			
Outer	5'-aat tta tta agg tag tty gag ttt taa g-3'	5'-aat caa aaa aaa ata aac crc aac ctt c-3'	-745 to -151
Inner	5'-tag gat ata ggt ttt ttt ttt aga ttt g-3'	5'-aaa aca aaa cac caa cca aat caa aat a-3'	-717 to -187
<i>Sox2</i>			
Outer	5'-tta gtt gga tag tyg ttt tga att att t-3'	5'-aat act ttt ccc ttt tta caa aca ctc t-3'	-812 to -367
Inner	5'-ggg ttt tgt ttt att ttg gtt tta gtt t-3'	5'-tct ctt ctc tac ctt aac aac tcc taa t-3'	-782 to -392
<i>Foxd3</i>			
Outer	5'-gta aag agg gga ggg tag ggt agt aag a-3'	5'-act act act crc rac ccc raa aat ttc t-3'	-836 to -290
Inner	5'-gyg gag agg gta ggg tgg taa gaa gtt t-3'	5'-crc acc ccc tca ctt cac caa cct tcc t-3'	-796 to -325

^aThe positions are relative to the translation start site (+1).

were ligated into the pGEM-T Easy plasmid (Promega) for sequencing.

Analysis of *Nanog*, *Sox2*, and *Foxd3* Upstream Sequences

The upstream sequences of *Nanog*, *Sox2*, and *Foxd3* were encoded in the mouse BAC clones RP23-180N22 (*Nanog*), RP23-423J10 (*Sox2*), and RP23-35D18 (*Foxd3*) from the NCBI database (www.ncbi.nlm.nih.gov/entrez). The translation start sites were based on the complete cDNA sequence data AB093574 (*Nanog*), U31967 (*Sox2*), and AF067421 (*Foxd3*) from the NCBI database. The TFSEARCH database (www.cbrc.jp/research/db/TFSEARCH) was used to analyze the DNA sequences for consensus binding sites of the known transcription factors with the threshold score above 90.0.

RESULTS

Methylation Status of the *Oct4* Promoter During Normal Development

In this study, the methylation pattern of the *Oct4* promoter was analyzed using the bisulfite mutagenesis sequencing method. The bisulfite method provides more information than the assays using methylation-sensitive restriction enzymes. It reveals the methylation status of all CpG sites within the genomic regions that are amplified by specific PCR primers. It also describes the methylation patterns of individual alleles, such that each sequence obtained by the bisulfite method represents a single strand of DNA that was present in the original samples (Clark et al., 1994).

We first examined the methylation status of 16 CpG sites in the *Oct4* promoter (Hattori et al., 2004; Marikawa et al., 2005) in normal development at early stages, namely from gametes to blastocysts. The promoter was only mildly methylated in sperm (i.e., 16.5% of the total CpG sites examined were methylated), and essentially unmethylated in oocytes (3.4%), 8-cell stage embryos (4.0%) and blastocysts (1.6%) (Fig. 1). Thus, the methylation level of the *Oct4* promoter was significantly low during normal pre-implantation development.

Methylation Pattern of the *Oct4* Promoter in Cloned Embryos

To produce cloned somatic cell embryos, we used cumulus cells as nuclear donors in this study. While the cumulus cell is considered to be one of the most effective somatic cells for successful cloning (Wakayama and Yanagimachi, 2001; Tian et al., 2003), a significant proportion of the cloned cumulus cell embryos exhibited severe developmental delay or arrest at the pre-implantation stage. The majority (95.2%; n = 585) of reconstructed oocytes became 2-cell stage embryos in 24 hr after activation, but only 51.4% (n = 488) of the 2-cell stage embryos gave rise to 4-cell stage embryos in

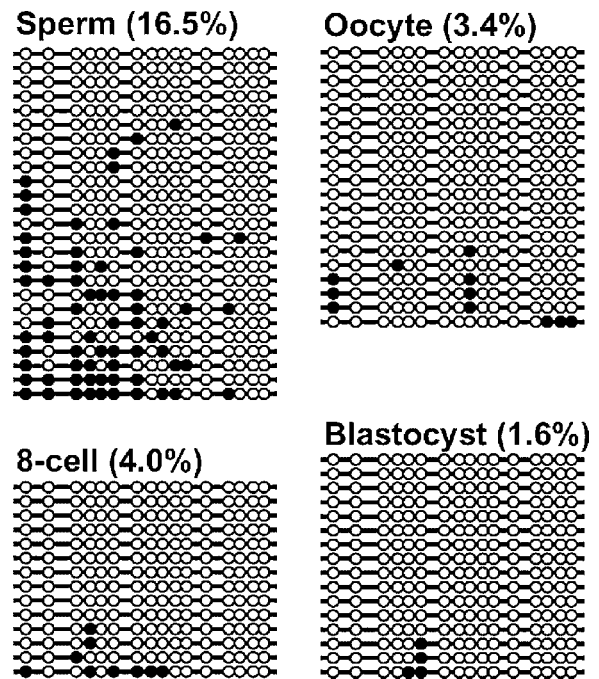


Fig. 1. The DNA methylation pattern of the *Oct4* promoter in gametes and normal embryos, as determined by bisulfite analysis. An open circle represents an unmethylated CpG, and a solid circle represents a methylated CpG sequence. Each horizontal line represents an individual allele of the *Oct4* promoter, which contains 16 CpG sequences. The percentage indicates the methylation level, calculated as the number of methylated CpG per total number of CpG sequences.

44 hr. Among those 4-cell stage embryos, only 22.9% (n = 201) divided further to reach the 7- to 8-cell stage in 56 hr after activation. The rest of the 4-cell stage embryos either divided partially (consisting of five to six cells) or did not divide (consisting of four cells). Many of the 7- to 8-cell stage embryos (71.4%; n = 14), however, gave rise to the blastocyst with an expanded blastocoel cavity in 4 days after activation. Thus, the pre-implantation development of cloned cumulus cell embryos is highly variable and inefficient, which is consistent with previous reports (Wakayama et al., 1998; Chung et al., 2002).

Importantly, the developmental impairment of cloned somatic cell embryos at pre-implantation stages is unlikely to be caused by the experimental manipulations, such as enucleation and nuclear injection. The cloned embryos that are produced from primordial germ cells develop efficiently to the morula and blastocyst stages even though the same experimental manipulations are applied to oocytes (Kato et al., 1999; Boiani et al., 2002; Lee et al., 2002; Yamazaki et al., 2003; Miki et al., 2005). Therefore, it is likely that the distinct property of the somatic cell nucleus, namely the epigenetic status, contributes to the inefficiency of pre-implantation development of cloned embryos.

We then examined the methylation status of the *Oct4* promoter during development of the cloned somatic cell embryos (Fig. 2A). The *Oct4* promoter was highly methylated in the donor cumulus cells (79.3%). In cloned cumulus cell embryos at the 4-cell stage (44 hr after activation), the *Oct4* promoter was less but still distinctly methylated (40.9%). At later stages, the methylation level became significantly lower, namely 23.2% at the 7- to 8-cell stages (56 hr) and 5.9% at the blastocyst stage (4 days). These results indicate that the *Oct4* promoter is gradually demethylated during pre-implantation development and that the methylation level is significantly higher in cloned embryos than in normal embryos at the early cleavage stages.

Inefficient Demethylation of the *Oct4* Promoter in Delayed Cloned Embryos

To determine the relationship between the level of demethylation and developmental efficiency, we examined the *Oct4* promoter in the cloned embryos that exhibited significant delay in development. As described above, some of the cloned embryos that had been at the 4-cell stage at 44 hr after activation gave rise to the 7- to 8-cell stage in 56 hr. However, the remainder of the 4-cell stage cloned embryos gave rise only to 4- to 6-cell stages in 56 hr. We found that the methylation level of the *Oct4* promoter was higher in those delayed embryos (33.8%) than in the 7- to 8-cell stage embryos (23.2%) (Fig. 2A,B). Furthermore, we compared the cloned embryos that displayed a more substantial extent of developmental delay. When the 2-cell stage cloned embryos at 24 hr after activation were cultured for an additional 32 hr, they developed to varying degrees, and the number of blastomeres ranged from three to eight cells. The embryos were categorized into four groups,

namely the 3-cell, 4-cell, 5- to 6-cell, and 7- to 8-cell stages, and were analyzed for the methylation pattern of the *Oct4* promoter. The methylation level paralleled the extent of developmental delay: highest in the 3-cell embryos and lowest in the 7- to 8-cell embryos (Fig. 2A,C). Thus, the developmental delay of cloned embryos was associated with the high level of *Oct4* promoter methylation.

Biased Demethylation Along CpG Positions

To gain an insight into the demethylation mechanisms of the *Oct4* promoter in cloned embryos, we measured the methylation frequency of individual 16 CpG positions (Fig. 3A), and examined which CpG positions are more efficiently demethylated during the early cleavage stages. The methylation frequency was determined based on the bisulfite analysis data shown in Figure 2A and was compared between the donor cumulus cells and the 4-cell stage cloned embryos (Fig. 3B). While the methylation frequency decreased in all CpG positions by the 4-cell stage, the degree of demethylation was more pronounced at the CpG positions that are located near the transcription start site. Specifically, the methylation frequency at the CpG positions between 2 and 11 dropped from 80–90% in the cumulus to 40–60% in the 4-cell stage embryo. In contrast, the decrease in the methylation frequency was more substantial at positions 13 and 14, which flank the transcription start site, dropping from 80–90% to 20–30%. This result implicates that the demethylation of the *Oct4* promoter in cloned embryos is biased along the CpG positions and is more efficient near the transcription start site.

Methylation Status of Other Pluripotency Regulator Genes in Somatic Cells

While the *Oct4* gene is the focus of the present study, the establishment of pluripotency requires the expression of several other genes, namely *Nanog*, *Sox2*, and *Foxd3* (Hanna et al., 2002; Avilion et al., 2003; Chambers et al., 2003; Mitsui et al., 2003). Thus, the effective development of cloned embryos necessitates the proper reactivation of these pluripotency regulators in the somatic cell nucleus, and the methylation status of their regulatory sequences may affect the efficiency of gene reactivation during nuclear reprogramming. Thus, we investigated the methylation status of the upstream sequences of these pluripotency regulator genes in somatic cells.

The *Nanog* promoter was recently identified and characterized in ES cells and F9 embryonal carcinoma cells. It is located immediately upstream of the transcriptional start site and contains several consensus sequences for binding of known transcription factors, namely *Sp1*, *AP-1*, *Oct1*, *Oct4*, and *Sox2* (Kuroda et al., 2005; Rodda et al., 2005; Wu and Yao, 2005). The promoters of the *Sox2* and *Foxd3* genes have not yet been functionally characterized. To locate the regions that are potentially important for transcriptional regulation,

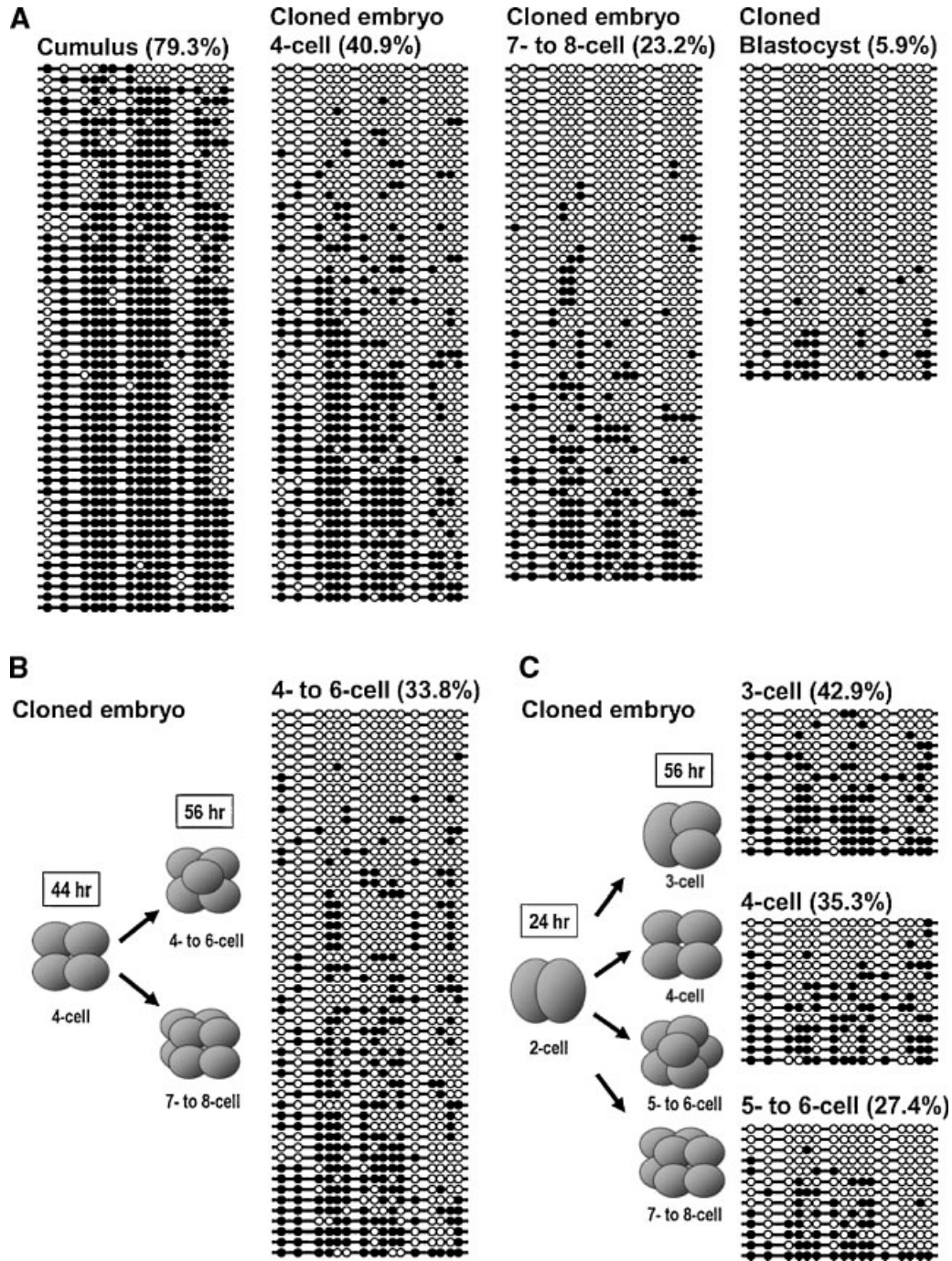


Fig. 2. The DNA methylation pattern of the *Oct4* promoter in cloned cumulus cell embryos. **A:** The methylation pattern in donor cumulus cells and in the cloned embryos that displayed normal timing in development: the 4-cell stage at 44 hr after activation, 7- to 8-cell stage at 56 hr, and blastocyst stage in 4 days. **B:** The methylation pattern in the delayed cloned embryos, which gave rise to the 4-cell stage in 44 hr

after activation, but reached only the 4- to 6-cell stage in 56 hr. Their methylation level is higher compared to the 7- to 8-cell stage cloned embryos shown in (A). **C:** The methylation pattern in the delayed cloned embryos, which were at the 2-cell stage in 24 hr after activation, but did not reach the 7- to 8-cell stage at 56 hr. The more severe the developmental retardation, the higher the methylation level.

we analyzed the upstream sequences of these genes using the TFSEARCH software for the DNA motifs recognized by the known transcription factors (see “Materials and Methods”). The *Sp1* (GGGGCGGG; centered at -643) and *E2F* (TTTCGCGC; centered at -588) target sites are found in the *Sox2* upstream

sequence, and the *CREB* (TGACGTTA, centered at -557) and *E2F* (TTTCGCGG; centered at -421) are present in the *Foxd3* upstream sequence. These target sites contain CpG sequences, whose methylation is implicated to interfere with the binding of transcription factors (Iguchi-Ariga and Schaffner, 1989; Huang and

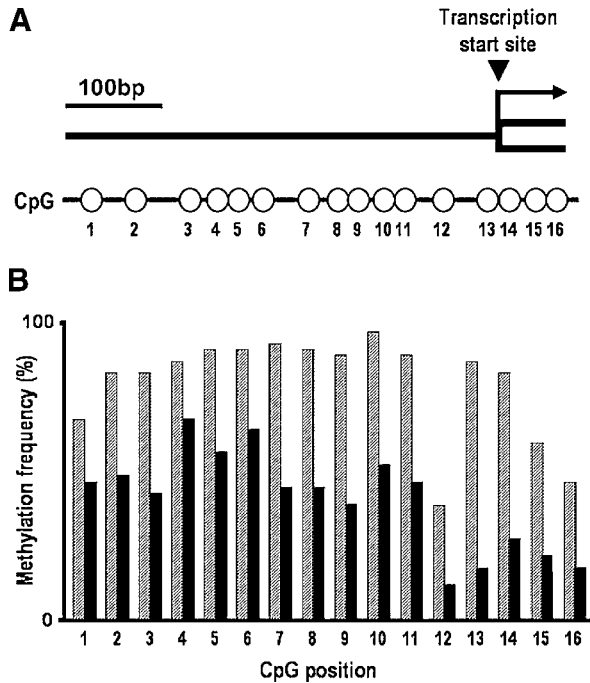


Fig. 3. Changes in the methylation pattern of the *Oct4* promoter in cloned embryos along the CpG positions. **A:** The position of 16 CpG sequences with respect to the structure of the *Oct4* gene. The transcription start site is located between the CpG positions 13 and 14. **B:** Comparison of methylation frequencies along the CpG positions between donor cumulus (gray bar) and cloned 4-cell stage embryos (black bar). The measurement of methylation frequency is based on the data shown in Figure 2A.

Prystowsky, 1996; Mummaneni et al., 1998; Martinowich et al., 2003). Thus, we designed PCR primers that amplify the upstream sequences of *Nanog*, *Sox2*, and *Foxd3* that contain the potential binding sites of these transcriptional regulators (Table 1), and examined the methylation status in somatic cells.

The upstream regions of *Nanog*, *Sox2*, and *Foxd3* were heavily methylated in somatic cell lines, NIH3T3 and Hepa1-6, as 77.0%–96.8% of the CpG sites were methylated (Fig. 4). This is comparable to the methylation status of the *Oct4* promoter in these cell lines: 52.2% in NIH3T3 and 90.2% in Hepa1-6 (Marikawa et al., 2005). In cumulus cells, however, the upstream regions of *Nanog*, *Sox2*, and *Foxd3* were markedly less methylated (19.0%), while the *Sox2* and *Foxd3* sequences were essentially unmethylated. This is in significant contrast to the methylation level (79.3%) of the *Oct4* promoter in cumulus cells (Fig. 2A).

DISCUSSION

In the present study, we showed that the promoter of the critical pluripotency regulator *Oct4* was gradually demethylated in the cloned cumulus embryos during early cleavage stages, and that a significant portion (40.9%; Fig. 2A) of the CpG sites were still methylated at the 4-cell stage. During normal development, the expression of zygotic *Oct4* gene starts before the 4-cell stage (Nichols et al., 1998; Boiani et al., 2002; Sebastiano et al., 2005), consistent with our observation that the

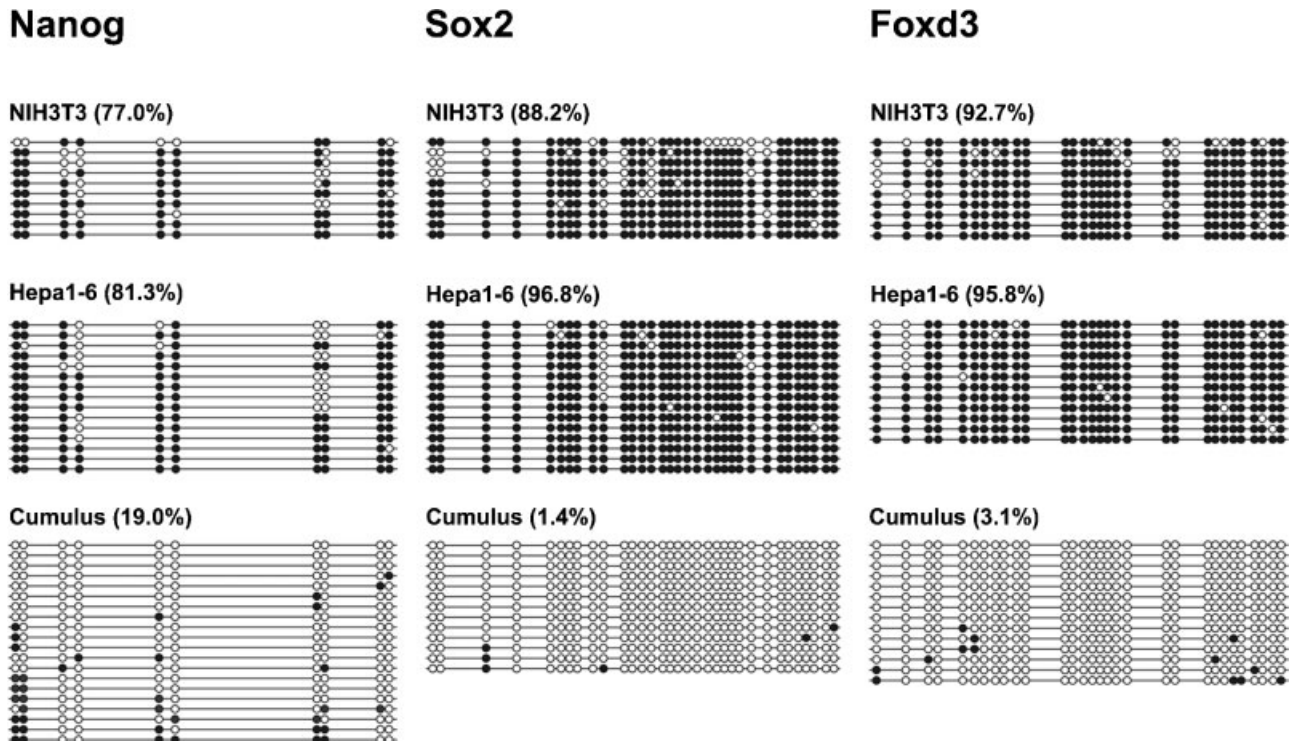


Fig. 4. DNA methylation pattern of pluripotency regulators, *Nanog*, *Sox2*, and *Foxd3*, in NIH3T3, Hepa1-6, and cumulus cells. The locations of the analyzed DNA sequences are indicated in Table 1.

Oct4 promoter is considerably under-methylated in gametes and early embryos. While the importance of *Oct4* expression at the cleavage stages has not been determined, the exact level of *Oct4* expression is critical for ES cells to maintain pluripotency (Niwa et al., 2000). The ectopic promoter methylation at the early cleavage stages may lead to the insufficient reactivation of the *Oct4* gene in cloned embryos, because abnormal *Oct4* expression is observed in many cloned cumulus embryos (Boiani et al., 2002; Bortvin et al., 2003).

Aberrant patterns of DNA methylation have been found in many cloned embryos of various species (Bourc'his et al., 2001; Dean et al., 2001, 2003; Kang et al., 2001, 2002; Mann et al., 2003; Santos et al., 2003; Senda et al., 2004; Nolen et al., 2005). It is not clear, however, whether the aberrant DNA methylation is the cause or effect of abnormal development of cloned embryos. Possibly, somatic cell nuclei that have failed to undergo efficient DNA demethylation are unable to support early development due to the insufficient reactivation of critical embryonic genes. However, the ineffective demethylation of the *Oct4* gene alone is unlikely to be the cause of developmental delay, because *Oct4*-deficient embryos do not exhibit significant delay at the early cleavage stages (Nichols et al., 1998). Alternatively, demethylation of DNA may be a result of delayed development, or more specifically, impaired cell divisions. Passive DNA demethylation occurs as the failure of the maintenance methylation after DNA replication (Razin and Riggs, 1980). If DNA demethylation in cloned embryos depends on a passive mechanism, the inefficient DNA demethylation is simply the result of developmental delay, which may be caused by other unknown factors. Currently, it is unclear whether cloned mammalian embryos utilize the passive mechanism of DNA demethylation, although our result on the *Oct4* promoter is in agreement with this possibility. A recent study, however, showed that the frog *Xenopus* oocyte contains an active mechanism of DNA demethylation, because the *Oct4* promoter was demethylated in the absence of DNA replication after the transplantation of mammalian somatic nuclei (Simonsson and Gurdon, 2004). The present study shows that even if an active demethylation mechanism exists in the mouse oocyte, it is highly ineffective to demethylate the *Oct4* promoter in the somatic cell nucleus.

While the demethylated status of the *Oct4* promoter correlated with the developmental progress of cloned embryos, the methylation level of individual promoter alleles were considerably heterogeneous in a group of embryos. For example, within the 4-cell stage cloned embryos (Fig. 2A), some alleles were totally unmethylated, while some other alleles were heavily methylated, with up to 13 CpG sites methylated among the 16 sites. Because each genomic DNA sample was obtained from a collection of cloned embryos (see "Materials and Methods"), it is not clear whether the heterogeneity of the *Oct4* promoter alleles exists among embryos or within an individual embryo. Importantly, however, the

methylation level of the *Oct4* promoter is considerably heterogeneous in somatic cell populations in the adult body (Fig. 2A; Marikawa et al., 2005). Therefore, some cloned embryos are derived from the somatic cell nuclei that contain a heavily methylated allele of the *Oct4* promoter, while others are from the nuclei with less methylated alleles. It is possible that the heterogeneity observed among cloned embryos partly originated from the varied methylation status of the *Oct4* promoter in donor somatic cell nuclei.

Our study also showed that demethylation is more prominent at the CpG positions that are located near the transcription start site in the cloned embryos at the 4-cell stage (Fig. 3). There are two possibilities for how the sequences near the transcription start site of the *Oct4* gene are more efficiently demethylated. One is that the transcription initiation complex (Bushnell et al., 2004), which physically interacts with the transcription start site, plays a critical role in DNA demethylation, possibly by interfering with the maintenance methylation after DNA replication. Several transcription factors, such as *NF- κ B* and *Sp1*, are implicated in the demethylation of specific promoter sequences in such a manner (Brandeis et al., 1994; Macleod et al., 1994; Matsuo et al., 1998; Hsieh, 1999). Interestingly, the methylation frequency of the CpG position 12 of the *Oct4* promoter is noticeably lower than the other CpG positions in somatic cells, including cumulus cells (Figs. 2A and 3B), liver, spleen, NIH3T3, and Hepa1-6 cells (Marikawa et al., 2005). The CpG position 12 is situated within the consensus binding site (GGGGCGGGG) for the ubiquitous transcription factor *Sp1*, suggesting that the binding of *Sp1* is involved in the low methylation level of position 12 in somatic cells. An alternative possibility for the mechanism of the biased demethylation is that certain sequence elements located near the transcription start site facilitate DNA demethylation independently from transcription. Such demethylation elements have been found in the proximal enhancer of the *Oct4* genes, which operate in P19 EC cells (Gidekel and Bergman, 2002). However, how those demethylation elements function or whether they are involved in the *Oct4* demethylation in cloned embryos is currently unknown.

We demonstrated that the upstream sequences of *Nanog*, *Sox2*, and *Foxd3* were markedly less methylated in cumulus cells (Fig. 4). While the effect of methylation on the expression of these genes has not been characterized in mouse embryos or ES cells, the correlation between the promoter methylation and down-regulation of *Nanog* expression is observed in human NT2 cells, which differentiate into neurons and astrocytes in response to retinoic acid (Deb-Rinker et al., 2005). Thus, the methylation of the upstream sequences may exert inhibitory effects on the expression of *Nanog*, *Sox2*, and *Foxd3*, as in the case for *Oct4* (Gidekel and Bergman, 2002; Hattori et al., 2004; Marikawa et al., 2005). Because the upstream sequences of *Nanog*, *Sox2*, and *Foxd3* are already under-methylated in the donor cumulus cells, the reactivation of these genes during nuclear reprogramming may be more efficient compared

to *Oct4*, whose promoter is heavily methylated in the donor cells (Fig. 2A; Marikawa et al., 2005).

The present study revealed that the *Oct4* promoter in cloned cumulus cell embryos is gradually demethylated during early cleavage stages with a bias near the transcription start site. Whether this pattern of DNA demethylation is unique to the *Oct4* promoter or occurs also to other genes is unknown. While we found that *Nanog*, *Sox2*, and *Foxd3* were not heavily methylated in the cumulus cell, there are likely other genes that are methylated in somatic cells and need to be demethylated for efficient development of cloned embryos. The identification of such genes is critical to elucidate the mechanisms of epigenetic remodifications in cloned embryos and to understand why only a few percentage of reconstructed oocytes are able to develop efficiently.

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