—Research Note—

Inheritance of Histone H3 Methylation in Reprogramming of Somatic Nuclei Following Nuclear Transfer

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Abstract. Successful cloning requires reprogramming of epigenetic information of the somatic nucleus to an embryonic state. However, the molecular mechanisms regarding epigenetic reprogramming of the somatic chromatin are unclear. Herein, we transferred NIH3T3 cell nuclei into enucleated mouse oocytes and evaluated the histone H3 dimethyl-lysine 4 (H3K4me2) dynamics by immunocytochemistry. A low level of H3K4me2 in the somatic chromatin was maintained in pseudo-pronuclei. Unlike *in vitro* fertilized (IVF) embryos, the methylation level of nuclear transfer (NT) embryos was significantly increased at the 8-cell stage. NT embryos showed lower H3K4me2 intensity than IVF embryos at the 2-cell stage, which is when the mouse embryonic genome is activated. Moreover, the H3K4me2 signal was weak in the recloned embryos derived from single blastomeres of the NT embryos, whereas it was intense in those from IVF embryos. Two imprinted genes, *U2afbp-rs* and *Xist*, were abnormally transcribed in cloned embryos compared with IVF embryos, and this was partly correlated to the H3K4me2 level. Our results suggest that abnormal reprogramming of epigenetic markers such as histone acetylation and methylation may lead to dysregulation of gene expression in cloned embryos.

Key words: Gene expression, Histone H3 methylation, Mouse, Somatic cell nuclear transfer, *U2afbp-rs*, *Xist*
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Reprogramming of differentiated somatic nuclei to a totipotent state by transfer into oocyte cytoplasm is not efficient. Although in some experiments a considerable proportion of reconstructed embryos might reach the blastocyst stage, the potential developmental ability to full-term is very limited. To improve the efficiency of nuclear transfer (NT), it is necessary to understand the mechanisms of the reprogramming process.

The reprogramming events following transfer of somatic nuclei into oocyte cytoplasm occur at the epigenetic level. Histone acetylation and methylation are two important epigenetic modalities [1–4]. It is known that acetylation of histone plays important roles in transcription activity [5] and histone methylation at K9 associates with gene silencing [6, 7]. Recently, histone H3K4 di-methylation (H3K4me2) has been found to be a positive chromatin marker associated with promoters of active genes [8, 9]. Similar studies have found that H3K4me3 is enriched at fully activated promoter regions, while H3K4me2 is correlated with the basal transcription-permissive state [10]. Demethylation of H3K4 catalyzed by lysine-specific demethylase 1 (LSD1) is associated with transcriptional repression [11]. In addition, H3K4 di- and trimethylation of reprogrammed somatic genomes may be independent of gene activity and may represent one of the major events that occur during somatic genome reprogramming towards a transcriptional activation-permissive state [12].

In early embryonic development, activation of the embryonic genome is a crucial event for onset of transcription during preimplantation development [13]. It has been determined that the activation occurs at the 2-cell stage in the mouse [14], but transcrip-

tion actually launches at the late S phase in the 1-cell embryo [15, 16]. Our recent studies found that H3K4 hypermethylation results in increased expression of embryonic genes in the 2- and 4-cell stages of mouse embryos [17]. This indicates that histone methylation may be involved in embryonic gene expression. Tong *et al.* reported that several genes required for embryonic development are aberrantly expressed in cloned mouse embryos [18]. However, it is unclear whether aberrant gene expression in cloned embryos is directly related to epigenetic reprogramming.

Imprinted genes are a suitable model system for the study of epigenetic control of gene expression because their allelic expressions are essentially controlled by this mechanism. *Xist* (X-inactivation specific transcript) gene, a paternally imprinted gene, is initially expressed at the 1- to 2-cell stages [19]. It is expressed in both male and female morula, but only in female blastocysts [19, 20]. The *U2afbp-rs* (U2af-binding protein related sequence) gene, a maternally imprinted gene, is expressed predominantly in the adult mouse brain and also preferentially in other adult mouse tissues [21]. During mouse preimplantation development, its transcripts are detected in 2-cell embryos [22]. Thus, expression of *Xist* and *U2afbp-rs* may correlate with embryonic activation in the mouse embryo. It is known that expression of imprinted genes is influenced by histone modifications [23–25]. Abnormal expression of imprinted genes may be associated with the high neonatal mortality in cloned animals [26, 27].

In this study, the methylation status of H3K4 was examined by immunocytochemistry to monitor epigenetic reprogramming of somatic chromatin in cloned mouse embryos. We also studied the expression of *U2afbp-rs* and *Xist* in cloned and IVF embryos by real-time PCR.

Materials and Methods

Animals

B6D2F1 female mice (3–4 weeks old) and ICR male mice (10–12 weeks of age) were obtained from the Experimental Animal Breeding Center of Nanjing Medicinal University (Nanjing, China). The donor NIH3T3 cells were purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). All experimental protocols were approved by the Animal Ethics Committee of Jiangsu University. All animals were bred under controlled temperature ($25 \pm 2^\circ\text{C}$), lighting (lights on from 0700 to 1900 h) and for at least 1 week prior to use.

Materials

All reagents and chemicals used in this study were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA) unless otherwise mentioned. For handling and culture of mouse oocytes and embryos, mWM [28] and KSOM [29] media were prepared, respectively.

In vitro fertilization and embryo culture

Female mice were treated with 10 IU pregnant mare serum gonadotrophin (PMSG) followed by 10 IU human chorionic gonadotrophin (hCG) 48 h later. Mature oocytes were collected from oviducts 14–16 h after hCG injection, placed in mWM medium and treated with 0.1% hyaluronidase until the cumulus cells dispersed. During IVF, the MII oocytes were inseminated with capacitated sperm from an ICR male mouse. Two hours after insemination, the fertilized eggs were washed with KSOM medium and then cultured in fresh KSOM medium supplemented with 3 mg/ml bovine serum albumin (BSA) at 37°C in an atmosphere of 5% CO_2 .

Nuclear transfer

Enucleation and cell transfer were carried out on the heated stage at 37°C (controlled by a constant temperature device) of an inverted microscope (IX71; Olympus, Tokyo, Japan) using a piezo micro manipulator controller (PMAS-CT 150; Prime Tech, Ibaraki, Japan). Self-fabricated holding pipettes (80–100 μm in diameter), enucleation pipettes (10–14 μm in diameter) and injection pipettes (12–15 μm in diameter) were sterilized by exposure to UV light. A group of 10–15 oocytes were placed into a drop of HEPES-buffered KSOM medium containing 5 $\mu\text{g}/\text{ml}$ cytochalasin B under mineral oil. The oocytes were then held with the metaphase plate at 3 o'clock, as clearly observed using an inverted microscope. A few piezo pulses (speed 1–2, intensity 2–3) were then used to advance the enucleation pipette while applying very slight negative pressure, and the chromosomes were gently aspirated. After removal of the cytochalasin B, the oocytes were cultured in fresh KSOM medium.

The nuclei of NIH3T3 cells were introduced into the enucleated oocytes by electrofusion at 1500 V/cm for 30 μsec using an Electro Cell Manipulator (ECM2001; BTX, San Diego, CA, USA) in 300 mM mannitol that contained 0.1 mM MgSO_4 , 0.1 mg/ml polyvinyl alcohol and 3 mg/ml BSA, as described previously [30]. At 2 h after electrofusion, fused embryos were activated by 6 h exposure

to 10 mM strontium chloride in calcium-free KSOM medium supplemented with 5 $\mu\text{g}/\text{ml}$ cytochalasin B. Activated embryos with clear pseudo-pronuclei were washed and cultured in KSOM medium at 37°C in 5% CO_2 in air. When embryos developed to the 4-cell stage, they were transferred into KSOM medium supplemented with 1 mM glucose.

Recloned embryo production

Donor blastomeres at the morula stage were dissociated from IVF and NT embryos. For dissociation, the morulae were treated with 0.5% pronase at 37°C for 3–5 min to remove the zonae pellucidae. After washing the embryos in three changes of KSOM medium, they were transferred into Ca^{2+} - and Mg^{2+} -free Dulbecco's PBS (Gibco, Paisley, Scotland) droplets containing 0.5% FCS for 15–20 min before repeated dissociation with a fine-bore pipette. Individual blastomeres of the same embryo were placed into 20 μl droplets of KSOM-PVP.

The blastomeres were inserted into the perivitelline spaces of the enucleated oocytes. Fusion of donor-recipient pairs was induced by a DC pulse of 1,500 V/cm for 30 μsec in 300 mM mannitol containing 0.1 mM MgSO_4 , 0.1 mg/ml polyvinyl alcohol and 3 mg/ml BSA. Subsequently, the fused pairs were cultured for 2 h and then activated by treatment with 10 mM strontium chloride in Ca^{2+} -free KSOM containing 5 $\mu\text{g}/\text{ml}$ cytochalasin B for 6 h. The reconstructed embryos were cultured in KSOM medium. After culture for 2 days, cleaved embryos were subjected to immunocytochemical analysis.

Extraction of mRNA and real-time PCR

The quantification of two gene transcripts was carried out by quantitative real-time PCR. The primer sequences for each gene are as follows: 5'-GAAGAGTGGCTGCTGAGGG-3' (sense) and 5'-AGAAGGGTGGGACTTGACG-3' (antisense) for *U2afbp-rs* (GenBank accession number S69507); 5'-AATAATGCGACAG-GCAAAGG-3' (sense) and 5'-TCCCGCTCATTTTCCATTAG-3' (antisense) for *Xist* (GenBank accession number AF104906); 5'-GAGGGGGAAATCGTGCGTGACATCAA-3' (sense) and 5'-GGAACCGCTCGTTGCCAATAGTGA-3' (antisense) for β -actin (GenBank accession number NM_007393). All PCR reactions were performed using a Fluorescent Real-time Cycler System (MJ Opticon® 2; MJ Research, Waltham, MA, USA) and SYBR Green I (Invitrogen, Carlsbad, CA, USA) as a double-stranded DNA specific fluorescent dye.

The total RNA was isolated from 20 embryos at various developmental stages using a TRIzol RNA isolation kit (Invitrogen). Reverse transcription was conducted by adding 0.5 $\mu\text{g}/\mu\text{l}$ oligo-dT primer (Promega, Madison, WI, USA), 10 mM dNTP, 0.1 M DTT, 40 units Moloney murine leukemia virus reverse transcriptase (Promega) and 20 units RNasin. Samples were incubated using the following program: 42°C for 60 min, 52°C for 30 min and 94°C for 2 min. The parameters for PCR reaction began with an initial step of 95°C for 1 min followed by 40 cycles of 94°C for 10 sec, 52°C – 58°C for 20 sec and 72°C for 20 sec. After each PCR run, a melting curve analysis was performed for each sample to verify that a single specific product was generated. Amplicon size was confirmed by ethidium bromide-stained 2% agarose gel electrophoresis. Neg-

ative controls, comprised of the PCR reaction mix without nucleic acid, were also run with each group of samples. The relative numbers of each sample were determined using a standard curve obtained by amplifying a 10-fold dilution series of cDNA that was prepared by PCR.

Immunostaining and quantification of fluorescence intensity

The NIH3T3 cells, oocytes and embryos were immunostained with antibodies against dimethyl-lysine 4 of histone H3 (Upstate Biotechnology, Lake Placid, NY, USA). The cells were fixed with 3.7% paraformaldehyde for 30 min, permeabilized with 0.5% Triton X-100 for 15 min and then incubated with the anti-methyl histone for 1 h at room temperature or overnight at 4 C. The antibodies that bound to the oocytes were probed with FITC-conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA). Counterstaining was conducted with DAPI to visualize the DNA. Fluorescence was detected using a Leica epifluorescence light microscope. Quantification of fluorescence intensity was determined using an image analysis system (SigmaScan-pro V5.01; SPSS, Chicago, IL, USA) by the ratios of H3K4me2 to DNA signals.

The level of H3K4me2 was expressed as the mean value of total signal intensities pooled from single blastomeres, and differences were tested by independent-samples *t*-test. Significant differences in gene expression were analyzed by ANOVA. All data were performed by analysis of variance using SPSS 11.5 software. A value of $P < 0.05$ was considered statistically significant.

Results

Profile of H3K4me2 in somatic cells and oocytes

Although abnormal reprogramming of DNA methylation is related to low efficiency of cloning [31, 32], the molecular mechanisms regarding rearrangement of somatic-type histone modifications in early development are unclear. For this reason, H3K4me2 was first examined in the donor somatic cells. The signal of H3K4me2 was detected in the germinal vesicles (GV) of GV-stage oocytes and the chromosomes of MII-stage oocytes and somatic cells. Especially, weak signals were observed in the somatic chromosomes (Fig. 1).

Differential H3K4me2 of NT embryos at the pronuclear stage

The methylation of H3K4 was also examined in normal and cloned 1-cell embryos. A weak signal of H3K4me2 was detected in female pronuclei 12 h after insemination, but not in the paternal chromatin (Fig. 2b). Thereafter, the H3K4me2 signals further increased as the zygote developed. In the NT embryos, weak H3K4me2 signals were observed immediately after electrofusion and remained until 8 h after electrofusion (Fig. 2a). During pronuclear development, NT embryos exhibited weaker H3K4me2 signals than the IVF embryos (Fig. 2c, $P < 0.05$).

Abnormal histone H3 methylation of somatic chromatin in preimplantation NT embryos

To evaluate the histone H3 methylation patterns during preimplantation development, H3K4me2 signals were tested at various

developmental stages of IVF and NT embryos. The signals were observed at all stages of preimplantation embryos (Fig. 3a), but the quantitative patterns varied between the two kinds of embryo (Fig. 3b). In the IVF embryos, the level of H3K4me2 suddenly increased at the 2-cell stage and then markedly declined by the 8-cell stage (Fig. 3b; $P < 0.05$). Unlike the IVF embryos, the methylation level of the NT embryos was significantly increased from the 2-cell to 8-cell stage and then decreased at the blastocyst stage ($P < 0.05$). In addition, the intensity of H3K4me2 at various stages in the NT embryos was significantly lower than in the IVF embryos with the exception of the 8-cell stage (Fig. 3b).

Inheritance of somatic histone H3 methylation in preimplantation NT embryos

We next investigated whether an aberrant H3 methylation state of early embryos was transmitted to the next generation. Single blastomeres of the embryos were transferred into enucleated oocytes, and then the H3K4me2 intensity was examined at the 2-cell stage in successive cloned embryos. As shown in Fig. 4, the H3K4me2 signal was also weak in the successive cloned embryos from the blastomeres of the NT embryos, whereas the signal was strong in those from the IVF embryos.

Correlation of histone H3 methylation and gene expression during early development

To test whether histone H3 methylation is related to embryonic gene expression, the transcription levels of the *U2afbp-rs* and *Xist* genes transcribed in preimplantation mouse embryos [19, 22] were evaluated at various stages in normal and cloned embryos. Transcription of the *U2afbp-rs* gene in normal embryos was highly enhanced at the 2-cell stage and then declined to a very low level by the 8-cell stage (Fig. 5a; $P < 0.05$). In the NT embryos, transcription of the *U2afbp-rs* gene was transiently increased by the 2-cell stage. In addition, the transcription levels of *U2afbp-rs* varied at the respective developmental stages between IVF and NT embryos (Fig. 5b). The *U2afbp-rs* transcription level in the IVF embryos was approximately 3-fold higher at the 2-cell stage compared with the NT embryos but was lower at the 8-cell and blastocyst stages ($P < 0.05$). The transcription pattern of the *Xist* gene resembled that of the *U2afbp-rs* gene during early development (Fig. 5c). The *Xist* gene was highly transcribed at the 2-cell stage in normal embryos, but in the NT embryos, the transcription levels of *Xist* significantly increased at the 4-cell stage (Fig. 5d; $P < 0.05$).

Discussion

Previous studies have confirmed that developmental defects in cloned embryos are due to aberrant reprogramming of epigenetic markers, such as DNA methylation and histone modification [31, 33–35]. To date, however, little information has been available about reprogramming of histone methylation in cloned embryos. In this study, the profiles of H3K4me2 and its inheritance were investigated during early development of NT mouse embryos. Before fertilization, H3K4me2 was present in the maternal genome and was maintained until after fertilization, whereas it was absent from the paternal chromatin. Our results are consistent with the

Fig. 1. Immunostaining of H3K4me2 in NIH3T3, GV-stage and MII-stage oocytes. Cells were stained with DAPI for chromatin (blue) and immunostained with anti-H3K4me2 (green). Twenty-one and 18 oocytes were examined at the GV- and MII-stage, respectively, and representative examples are shown. The scale bars denote 50 μ m for NIH3T3 and 10 μ m for GV- and MII-stage oocytes.

Fig. 2. Intensity of H3K4me2 signals in IVF and NT embryos during pronuclear formation. (a) Distribution of H3K4me2 signals in IVF and NT zygotes at various times after insemination. (b) Microscopic images of IVF and NT embryos at the pronuclear stage. IVF zygotes 16 h after insemination and NT embryos 12 h after electrofusion were immunostained with anti-H3K4me2, respectively. m, male pronucleus; f, female pronucleus. (c) Comparison of H3K4me2/DNA intensity between IVF and NT zygotes. The numbers of embryos examined were: 23, 34, 21 and 28 for the IVF 8, 12, 16 and 20 h 1-cell embryos, respectively, and 22, 16, 13 and 18 for the NT 0.5, 3, 8 and 12 h 1-cell embryos, respectively. The superscripts a–c and e and f represent significant differences between IVF and NT zygotes, respectively (P<0.05). The scale bar denotes 10 μ m.

Fig. 4. The levels of H3K4me2 in recloned mouse embryos derived from single blastomeres of IVF and NT embryos. Sixteen and 12 blastomeres were examined in the recloned embryos derived from IVF and NT blastomeres, respectively. The scale bar denotes 50 μ m.

Fig. 3. Intensity of H3K4me2 in IVF and NT embryos during preimplantation development. (a) Microscopic images of H3K4me2 in IVF and NT embryos. BL, blastocyst. The numbers of blastomeres examined were 42, 56, 76 and 52 for the IVF 2-cell, 4-cell, 8-cell and blastocyst embryos, respectively, and 12, 25, 43 and 38 for the NT 2-cell, 4-cell, 8-cell and blastocyst embryos, respectively. The superscripts a–c represent significant differences between IVF and NT embryos (P<0.05). The scale bar denotes 50 μ m.

Fig. 5. Transcription patterns of the *U2afbp-rs* and *Xist* genes in IVF and NT embryos during early development. (a) Transcription levels of *U2afbp-rs* at various developmental stages. (b) Comparative expression levels of *U2afbp-rs* at each developmental stage for IVF and NT embryos. (c) Relative transcription patterns of *Xist* at various developmental stages. (d) Comparative expression levels of *Xist* at each developmental stage for IVF and NT embryos. The results represent the mean $\pm$ SEM (n=3).

recent findings of Sarmiento *et al.* [36]. The difference in histone H3 methylation level between female and male pronuclei may be responsible for distinctive formation of the respective chromatin. In addition, zygotes derived from NT showed lower H3K4me2 signals than those derived from IVF during chromatin remodeling. These data suggest that aberrant methylation reprogramming of somatic chromatins occurs as early as the 1-cell stage in NT embryos.

To explore epigenetic reprogramming of somatic chromatins, the patterns of H3K4me2 were determined at various developmental stages during preimplantation development. In IVF embryos, H3K4me2 signals increased at the 2-cell stage and then significantly decreased until the 8-cell stage. However, NT embryos showed lower H3K4me2 intensity than IVF embryos at the 2-cell stage, which is when the mouse embryonic genome was activated. Therefore, NT embryos exhibited low methylation levels at the 2-cell stage, indicating that genome reprogramming was incomplete and inaccurate.

In the analysis of gene expression in normal and NT embryos at various developmental stages, we found that the *U2afbp-rs* and *Xist* genes were aberrantly expressed in NT embryos compared with

normal embryos. The expression profiles of these genes were closely consistent with the patterns of the H3K4me2 levels. Therefore, we suggest that the aberrant gene expression in NT embryos may be due to abnormal histone methylations during early development. Further work is required to determine the molecular mechanisms of how H3K4me affects expression of embryonic genes. In previous studies, H3K4me has been shown to affect transcription of imprinted [20, 24] and non-imprinted genes [8, 11, 12]. Moreover, a recent study reported that H3K4 hypermethylation results in re-expression of aberrantly silenced genes, which is important in the development of colon cancer [11]. Taken together, rectifying reprogramming of histone H3 methylation at the early stage may be a strategy to improve cloning efficiency.

It is possible that epigenetic markers of the chromatin transmit genome information from one generation to the next. To confirm this possibility, single blastomeres of IVF and NT embryos were transferred into enucleated oocytes, and then the H3K4me2 levels were tested at the 2-cell stage. Interestingly, the H3K4me2 intensities were still low in the successive cloned embryos compared with those from IVF embryos. These results suggest that histone modifications may be seldomly corrected during reprogramming of the

embryonic genome. However, how the epigenetic statuses are maintained and stably inherited throughout preimplantation development is still unclear.

Based on these findings, we suggest that the epigenetic state of the somatic chromatin is rearranged in the oocyte cytoplasm. This behavior of the somatic chromatin may give rise to abnormal reprogramming during preimplantation development, thus leading to aberrant transcription of embryonic genes in NT embryos. Our results will be helpful for developing new strategies to improve cloning efficiency.

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