Inheritable Histone H4 Acetylation of Somatic Chromatins in Cloned Embryos

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A viable cloned animal indicates that epigenetic status of the differentiated cell nucleus is reprogrammed to an embryonic totipotent state. However, molecular events regarding epigenetic reprogramming of the somatic chromatin are poorly understood. Here we provide new insight that somatic chromatins are refractory to reprogramming of histone acetylation during early development. A low level of acetylated histone H4-lysine 5 (AcH4K5) of the somatic chromatin was sustained at the pronuclear stage. Unlike in vitro fertilized (IVF) embryos, the AcH4K5 level remarkably reduced at the 8-cell stage in cloned bovine embryos. The AcH4K5 status of somatic chromatins transmitted to cloned and even recombined embryos. Differences of AcH4K5 signal intensity were more distinguishable in the metaphase chromosomes between IVF and cloned embryos. Two imprinted genes, Ndn and Xist, were aberrantly expressed in cloned embryos as compared with IVF embryos, which is partly associated with the AcH4K5 signal intensity. Our findings suggest that abnormal epigenetic reprogramming in cloned embryos may be because of a memory mechanism, the epigenetic status itself of somatic chromatins.

To obtain developmental competence of cloned embryos to term, the differentiated cell nucleus should be subject to epigenetic reprogramming processes including chromatin remodeling and DNA methylation during preimplantation development. In the somatic cell nuclear transfer, many clinical anomalies such as high abortion rates, increased body weight, and early death after birth have been reported (1–4). These developmental failures could be because of aberrant reprogramming in the reciprocal interactions between donor nuclei and enucleated oocyte cytoplasm (5). In this context, it has been demonstrated that reprogramming of DNA methylation (6–9), expression of imprinted and nonimprinted genes (10–13), X-chromosome inactivation (14, 15), and telomerase activity (16) are incomplete in cloned embryos during early development as compared with normal embryos. Especially, abnormal epigenetic reprogramming may lead to faulty or differential gene expression, thereby resulting in developmental defects of cloned embryos.

An intrinsic difference in the early embryonic development between fertilization and somatic cell cloning may arise from distinct chromatin architectures between spermatozoa and somatic cells. In fact, activation and remodeling in a reconstructed oocyte with a somatic cell nucleus might obviously differ from the processes of normal fertilization. In mammary, maternal and paternal genomes should reprogram to reach a totipotent state for normal development. During fertilization, the sperm genome remodels in the oocyte cytoplasm after replacement of its pro- tamines with oocyte histones. In the somatic cell nuclear transfer, however, remodeling of somatic chromatins may be entirely discriminated because of a paucity of sperm factors and incomplete microenvironment within the enucleated oocyte cytoplasm. Differences of chromatin remodeling can be also because of diverse histone variants between germ and somatic cells (17, 18). For instance, a variant subtype of histone linker H1, H1foo, is relatively enriched in the chromatin of mouse oocytes (19) and rapidly replaced with somatic H1 linker histone after fertilization or somatic cell nuclear transfer (20, 21). However, how the maternal and paternal genomes reprogram to set chromatin structure is still poorly understood.

The histones play essential roles in chromatin structure and transcriptional regulation through modifications of the histone amino termini, including acetylation, methylation, phosphorylation, and ubiquitination (22). Among these histone modifications, acetylation and deacetylation, which are catalyzed by specific enzymes, histone acetyltransferases (HATs)2 and deacetyltransferases (HDACs), respectively, are closely related to transcription activity (23). Hyperacetylation of histones could facilitate the access of some transcription factors to nucleosomes (24, 25). In the interphase, the histone acetylation is involved in gene expression (26), cellular function (27), and DNA replication (28, 29). During mitosis, histone acetylation may function as an epigenetic mark, a histone code, by which information about genomic function is transmitted from one generation of cells to the next (30). The histone H4 is acetylated in order at lysine 16, lysine 8 or 12, and lysine 5 (31), implying that acetylation at lysine 5 corresponds to the hyperacetylation state (25, 32).

In early embryonic development, activation of the embryonic genome is a critical event for onset of transcription. It has been known that activation of the embryonic genome occurs during the 2-cell stage in the mouse (33), but transcription actually initiates at mid to late 5 phase of the 1-cell embryo (34, 35). In the bovine, initial genomic activation occurs from 8- to 16-cell embryos (36, 37). Interestingly, transcriptional and translational activities of some genes are observed as early as the 2-cell stage in the bovine (38–40). Histone hyperacetylation results in increased expression of transgenes in early stages of mouse embryos (41), indicating that histone acetylation may be involved in embryonic gene expression. Several genes that are required for development.
opment are abnormally expressed in cloned bovine embryos (42), although it is unclear whether abnormal gene expression is directly correlated to the epigenetic reprogramming in cloned embryos.

In this study, the acetylation status of histone H4 at lysine 5 (AcH4K5) was examined to monitor epigenetic reprogramming of somatic chromatin in cloned embryos. As results, we found the anomaly of cloned embryos in the dynamic modulation of histone H4 acetylation. Histone acetylation status of somatic cell chromatin that were seldom reprogrammed in the enucleated oocyte cytoplast and its levels were arbitrarily fluctuated in early cloned embryos. Along with histone acetylation, two imprinted genes were aberrantly expressed in cloned bovine embryos as compared with normal embryos. Here we suggest that epigenetic marks such as DNA methylation and histone acetylation abnormally reprogram in cloned embryos throughout early development, thereby resulting in dysregulation of gene expression. These observations may hint at the reasons of the developmental defects that are familiar with cloned animals in the somatic cell nuclear transfer.

EXPERIMENTAL PROCEDURES

In Vitro Maturation and in Vitro Fertilization (IVF)—Bovine ovaries were collected from a local slaughterhouse and transported to the laboratory in 0.9% saline at 25–30 °C. Cumulus-oocytes complexes were obtained through a disposable 10–ml syringe with 18-gauge needle from follicles of 2–6 mm in diameter. Approximately 50 oocytes were incubated in 500 μl of the in vitro maturation medium in a 4-well multidish (Nunc, Roskilde, Denmark) under paraffin oil for 20 h at 38.5 °C in an atmosphere of 5% CO2. The medium used for oocyte maturation was TCM-199 (Invitrogen, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen), 10 μg/ml FSH-P (Folltropin-V, Vetepharma, London, UK), 0.6 mM cysteine, 0.2 mM sodium pyruvate, and 1 μg/ml estradiol-17β. After in vitro maturation, 10 oocytes were fertilized with frozen-thawed sperm at a concentration of 2 × 10⁶ cells/ml in 50 μl of fertilization medium (43). When sperm was added to the fertilization drops, 2 μg/ml heparin, 20 μM penicillamine, 10 μM hypotaurine, and 1 μM epinephrine (PHE) were also added.

Somatic Cell Nuclear Transfer and in Vitro Culture—Bovine ear skin fibroblasts were used as donor cells for nuclear transfer. Donor cells were plated into a six-well plate and cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 3 mg/ml bovine serum albumin (fatty acid free). After 24 h, cells were extracted following the manufacturer’s instructions using the Dynabeads mRNA Direct Kit (Dynal). After thawing, the samples were lysed in 50 μl of lysis/binding buffer (Dynal) at room temperature for 10 min. Then, 10 μl of Dynabeads oligo(dT)₂₅ were added to each sample. The beads were hybridized for 5 min and separated from the binding buffer using the Dynal magnetic separator. Poly(A) mRNAs and beads were washed in buffers A and B (DYNAL), and were separated by adding 11 μl of diethyl pyrocarbonate-treated water. Poly(A) mRNAs were reverse transcribed in a total volume of 20 μl containing using 2.5 μM random hexamer primer, 1× reverse transcription buffer, 20 IU of RNase inhibitor, 50 IU Moloney murine leukemia virus reverse transcriptase enzyme (Promega, Madrid, Spain), 5 mM MgCl₂, and 1 μM of each dNTP. After denaturation of the secondary RNA structure at 70 °C for 5 min, the cDNA was complete by the following conditions at room temperature for 10 min, at 42 °C for 60 min to allow the reverse transcription of RNA, and at 93 °C for 1 min to denature the enzyme.

Real-time PCR—The quantification of all gene transcripts was carried out in three replicate by quantitative real-time reverse transcriptase PCR on the MJ Opticon™2 system (MJ Research Inc., Reno, NV) using SYBR Green, a double-stranded DNA-specific fluorescent dye. The primer sequences for each gene are as follows: B-actin (GeneBank™ accession number AY141970), 5′-GGTATCGCTTTCGTTGTAAT-TATGT-3′ (sense), 5′-GCTGTCATTCATAAACAAAAAACA-3′.
Effects of TSA on histone H4 acetylation of bESF. A, immunostaining of bESF for HDAC2 and AcH4K5. Cells were stained with DAPI for chromatin (blue) and immunostained with anti-HDAC2 or anti-AcH4K5 (red). In TSA-treated cells, intensity of the HDAC2 signals was markedly weak, whereas AcH4K5 intensity was strong as compared with normal cells. Scale bar represents 50 μm. B, quantification of HDAC2 or AcH4K5/DNA intensity in normal and TSA-treated cells. TSA-treated cells showed reduced HDAC2 activity and enhanced AcH4K5 intensity. C, Western blot analysis of HAT1, Tip60, and HDAC2 in TSA-treated cells using specific antibodies, respectively. D, localization of AcH4K5 during the cell cycle in normal and TSA-treated cells. I, interphase; P, prophase; PM, prometaphase; M, metaphase; EA, early anaphase; LA, late anaphase; ET, early telophase; LT, late telophase. Scale bar represents 10 μm. TSA-treated cells showed reduced HDAC2 activity and enhanced AcH4K5 states. The quantitative value is expressed as the mean ± S.E. Sample sizes (n) are indicated within the corresponding column and supplemental Table 1. a and b superscripts denote significant differences (p < 0.05).

Within this region of the amplification curve, each difference of one cycle is equivalent to a doubling of the amplified product of the PCR. The sample ΔC(TSAn-ΔC(TSan)) value was calculated by the difference between the β-actin C(TSan) value and the sample C(TSan) value. To evaluate expression levels of the imprinted genes between each developmental stage in IVF, NT, and TSA-NT embryos, the ΔC(TSan) value of IVF morula was used as the control ΔC(TSan) (ΔC(TSan)) value. For comparison of gene expression levels on each developmental stage among IVF, NT, and TSA-NT embryos, the ΔC(TSan) value of the IVF embryo was employed as the control ΔC(TSan) (ΔC(TSan)) value. -Fold differences of the gene expressions were determined by using the formula, 2^-ΔC(TSan).
The membrane was blocked with PBS containing 5% nonfat milk powder at room temperature for 1 h and washed 5 times with TPBS (PBS, 0.1% Tween 20) at 4 °C for 30 min. The samples were incubated in PBS containing 5% nonfat milk powder, with the antibodies for HAT1, Tip60, and HDAC2 at 4 °C for 2 h, respectively. After washing with TPBS, the sample was exposed to the secondary antibody (anti-rabbit horseradish peroxidase conjugated-antibody) diluted at 1:5,000 in PBS containing 5% nonfat milk powder. After incubation at 4 °C for 2 h, the membrane was washed with TPBS and then developed using the ECL system (Pierce) as recommended by the manufacturer.

**Statistical Analysis**—All data were analyzed by analysis of variance using the SAS package. Quantification of images was determined using an image analyzer system by the ratios of AcH4K5 or HDAC2 to DAPI DNA signals. Individual signal intensity was pooled for each group and analyzed by t test of the multiway analysis of variance. In IVF, NT, and TSA-NT embryos, the level of histone H4 acetylation was expressed as the mean value of total signal intensities pooled from single blastomeres and then a difference was tested by Duncan analysis of multiway analysis of variance. Significant differences of relative expression for *Ndn* and *Xist* genes were evaluated by using the Duncan test. A value of $p < 0.05$ was considered as significant.

**RESULTS**

Profiles of Histone H4 Acetylation in Somatic Cells—Although aberrant reprogramming of DNA methylation is responsible for low efficiency of animal cloning (6, 7), molecular mechanisms involving reset of somatic-type histone modifications during early embryogenesis are still unclear. For this, we first examined the AcH4K5 of donor somatic cells.
After treatment with TSA intensity of AcH4K5 signals in bESF was significantly increased (Fig. 1, A and B), which was approximately twice compared with the signals of normal cells (p < 0.05). Western blot analysis represented that the HDAC2 expression level was markedly reduced at 24 h and further decreased at 60 h in TSA-treated cells, whereas histone acetyltransferases, HAT1 and Tip60, were constitutively expressed (Fig. 1C). To chase histone acetylation changes during cell cycle, AcH4K5 signal intensity was measured at each cell cycle stage. In normal cells, intensity of AcH4K5 signals profoundly reduced from the prometaphase to the early telophase and then increased after the late telophase (Fig. 1D). In TSA-treated cells, intensity of AcH4K5 signals gradually increased by the early anaphase, then reduced at the late anaphase and the early telophase, and enhanced at the late telophase (Fig. 1D).

Weak Histone H4 Acetylation of NT Embryos at the Pronuclear Stage—Remodeling of parental genomes or somatic cell chromatins can be monitored by investigating changes of epigenetic marks such as DNA methylation and histone modifications. In this study, chromatin remodeling occurring during pronuclear formation was compared in terms of histone H4 acetylation between germ cells and somatic cells. Both genomes of sperm and metaphase II-arrested oocytes matured in vitro did not exhibit any signal of AcH4K5 in the bovine (data not shown). Only a few oocytes (3 of 15) showed AcH4K5 signals in the sperm chromatin 7 h after insemination, although the sperm penetration was observed in almost all of the oocytes (14 of 15). The sperm chromatin was initially acetylated in most zygotes (36 of 44) 8 h after insemination, whereas maternal chromatin remained inert on histone H4 acetylation (Fig. 2A). The AcH4K5 signals were detected both in male and female pronuclei 10 h after insemination and thereafter further increased as the zygote developed. Thus, appearance of AcH4K5 is temporally asynchronous between sperm and oocyte genomes. This asynchronous histone H4 acetylation indicates distinct mechanisms in chromatin remodeling between the sperm and oocyte genomes.

In the somatic cell NT, AcH4K5 signals were not observed immediately after electrofusion, and then detected in most NT oocytes (6 of 8) 3 h after electrofusion or 1 h after activation (Fig. 2B), whereas most of the TSA-NT (nuclear-transferred with TSA-treated cell) oocytes (9 of 11) displayed AcH4K5 signals even 30 min after electrofusion. During pronuclear development, NT embryos showed a lower AcH4K5 level than IVF and TSA-NT embryos (Fig. 3, A and B, p < 0.05). This difference might be because of a low acetylation state of the donor cell itself because no difference was detected in HDAC2 activity at the pronuclear stage between IVF and NT eggs (supplemental Fig. S1). As shown in Fig. 3D, a proportion (20%, 6 of 30) of NT embryos allocated to a presumptive normal range of the AcH4K5/DNA signal ratio (0.65–0.85), which belongs to the mean value of IVF embryos (73%, 16 of 22) and TSA-NT (51%, 18 of 35) embryos (p < 0.05). Most NT oocytes (73%, 22 of 30) were assigned to a lower AcH4K5/DNA signal ratio (<0.65), whereas approximately half of the TSA-NT oocytes (43%, 15 of 35) were assigned to a higher AcH4K5/DNA signal ratio (>0.85). Thus, NT oocytes were abnormally remodeled in terms of histone acetylation at the pronuclear stage as compared with IVF zygotes. In addition, a difference was detected in the AcH4K5/DNA signal ratio between male and female pronuclei of IVF zygotes (Fig. 3D).
Aberrant Histone H4 Acetylation of Somatic Chromatin in Early NT Embryos—To evaluate the profiles of histone H4 acetylation status during preimplantation development, AcH4K5 signals were measured at various developmental stages in IVF, NT, and TSA-NT embryos. Quantification of AcH4K5/DNA signal intensity and sample sizes (n) at each developmental stage are denoted in supplemental Table 1. C, proportion of 8-cell embryos with a presumptive normal range of AcH4K5/DNA signal ratio in IVF, NT, and TSA-NT embryos. a, b, and c superscripts denote significant differences between IVF, NT, and TSA-NT embryos (p < 0.05). Scale bar represents 50 μm.

FIGURE 4. Intensity of AcH4K5 signals in IVF, NT, and TSA-NT embryos during preimplantation development. A, microscopic images of AcH4K5 signals at various developmental stages. B, profiles of AcH4K5 signals in IVF, NT, and TSA-NT embryos. Quantification of AcH4K5/DNA signal intensity and sample sizes (n) at each developmental stage are denoted in supplemental Table 1. C, proportion of 8-cell embryos with a presumptive normal range of AcH4K5/DNA signal ratio in IVF, NT, and TSA-NT embryos. a, b, and c superscripts denote significant differences between IVF, NT, and TSA-NT embryos (p < 0.05). Scale bar represents 50 μm.

Aberrant Reprogramming of Somatic Chromatins in Clones

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Relationship of Histone H4 Acetylation and Gene Expression during Preimplantation Development—To test whether histone H4 acetylation is correlated to embryonic gene expression, expression levels of Ndn and Xist genes, which are expressed in 8–16 cell bovine embryos (44, 45), were comparatively evaluated at various developmental stages between IVF, NT, and TSA-NT embryos using quantitative real-time PCR. In IVF embryos, expression of the Ndn gene was highly enhanced at the 8-cell stage as compared with other developmental stages (Fig. 5A; p < 0.05). In NT and TSA-NT embryos, expression of the Ndn gene gradually increased by the 8-cell stage and then decreased to the blastocyst stage. This expression pattern resembled that of IVF embryos, although there was no difference in the expression levels among developmental stages (Fig. 5A). The results suggest that transcription machinery for the imprinted genes may not work well in cloned embryos. Partly, the expression levels of the Ndn gene varied at the respective developmental stages among IVF, NT, and TSA-NT embryos (Fig. 5B). At the 8-cell stage, IVF embryos showed a higher Ndn expression level, about 5–8-fold, than NT and TSA-NT embryos (p < 0.05). Ndn expression in IVF embryos were higher at the 2-cell stage than NT embryos, but lower at the morular stage (p < 0.05). Expression profiles of the Xist gene were similar to those of the Ndn gene during preimplantation development (Fig. 5C). Likewise with the Ndn gene, the Xist gene was highly expressed at the 8-cell stage of IVF embryos during preimplantation development (p < 0.05). Unexpectedly, expression levels of the gene significantly increased at the 4-cell stage of NT embryos (Fig. 5D). Thus, expression patterns of two imprinted genes were approximately consistent with profiles of the AchH4K5 signal levels shown in Fig. 4B, indicating that histone H4 acetylation may affect expression of the imprinted genes in early embryos.

Memory of Somatic Histone H4 Acetylation Status in Early NT Embryos—Metaphase chromosomes in bovine IVF zygotes strongly stained with anti-AchH4K5 antibody at the first mitosis just before the first cell division, whereas AchH4K5 signal intensity of NT oocytes was vague at the first M phase during chromatin remodeling (Fig. 6A). Unlike NT oocytes, AchH4K5 intensity in TSA-NT oocytes was strong at the first M phase (Fig. 6, A and B). Weak AchH4K5 intensity reappeared in the metaphase chromosomes of early cleavage stage NT embryos (Fig. 6C). In the mitotic stages, early cleavage stage NT embryos had a lower AchH4K5 intensity than IVF embryos, whereas TSA-NT embryos exhibited intense AchH4K5 intensity (Fig. 6D), as in the first M phase (Fig. 6A). Therefore, we suggest new insight that the acetylation status of donor chromatin remembers in the process of epigenetic reprogramming before the first cell division and during preimplantation development.

We next wondered whether histone H4 acetylation status of early embryos passes on the next generation. To address this question, single blastomeres of the embryos (16–32 cell stage) were individually transferred into enucleated oocytes and then AchH4K5 intensity of the resulting recloned embryos was measured at the 8-cell stage. As shown in Fig. 6E, AchH4K5 intensity was also weak in the recloned embryos derived from single blastomeres of the NT embryos, whereas strong in those derived from IVF and TSA-NT embryos. Thus, differences were displayed in the AchH4K5 intensity among recloned embryos derived from IVF, NT, and TSA-NT blastomeres, respectively (p < 0.05). These...
results indicate that the weak acetylation status of embryonic chromatins derived from somatic cells transmits to the next.

**DISCUSSION**

Normal birth of a cloned animal implies that a somatic cell chromatin introduced into a recipient oocyte might properly implement remodeling as well as epigenetic reprogramming during early embryogenesis. Many studies have demonstrated that developmental defects of cloned embryos are because of abnormal reprogramming of epigenetic marks, especially DNA methylation, during early development (5, 7, 46). So far, however, little information is known as to reprogramming of histone acetylation in the cloned embryos. To our knowledge, this study is the first report that histone H4 acetylation reprogramming of somatic chromatins is aberrant in the cloned bovine embryos, thereby leading to abnormal expression of some embryonic genes. Here we suggest that correct reprogramming of histone H4 acetylation in the preimplantation stage may be a prerequisite process for mammalian development.

**Weak Histone Acetylation Status of Somatic Chromatins Reappears in Reconstructed 1-Cell Oocytes**—In the bovine, hyperacetylation of histone H4 initially occurred in male pronucleus shortly after fertilization and then in female pronucleus (Fig. 2A), like in the mouse (47). Histone acetylation of the male pronucleus appears to arise from the replacement of protamines with oocytic histones during sperm decondensation. The differential histone H4 acetylation level between male and female pronuclei (Figs. 2A and 3D) may be responsible for distinctive configurations of the respective chromatin. The first mitotic chromosomes strongly stained with AcH4K5 antibody in the bovine zygote, although no AcH4K5 signal was detected in the MII-arrested oocyte (Fig. 6A). The results indicate that histone deacetylase activity might be deficient during the M phase of the zygote.

TSA inhibits HDAC activity by interacting with its catalytic site, which leads to increased amounts of hyperacetylated histones (48). The level of AcH4K5 signals in TSA-treated cells was significantly enhanced and its level persisted even in mitosis (Fig. 1, A and D). In contrast, AcH4K5 signals were dramatically reduced in normal cells during mitosis (Fig. 1D). A paucity of anti-AcH4K5 in mitotic cells is consistent with the result that AcH4K5 signals are not observed on mitotic stages of the cell cycle in human neuroblastoma cells (49). Our results demonstrate that histone H4 at lysine 5 is hyperacetylated in the interphase and hypoacetylated throughout the mitotic stages in normal cells. AcH4K5 signals in the reconstructed oocytes with somatic cells did not appear immediately after electrofusion and then appeared vaguely after recon-
... but only in female blastocysts (45, 52). The 8–16 cell embryos (45). It is expressed in both male and female morula, expression of the imprinted gene, is normally expressed in newborn mouse brain and in a variety of human tissues (44). During bovine preimplantation development, maternal mRNAs or proteins, which are synthesized and accumulated during oogenesis, were gradually replaced with de novo embryonic transcripts after embryonic genes are activated. To determine whether histone H4 acetylation correlates to embryonic genome activation, expression of two imprinted genes was examined after each developmental stage in IVF, NT, and TSA-NT embryos using the quantitative real-time PCR method. Acetylation States during Preimplantation Development — The histone acetylation status further was clearly remembered in the pronuclear stage, representing a presumptive normal range of the AcH4K5/DNA signal ratio, decreased to 11% at the 8-cell stage. Therefore, the events of epigenetic reprogramming appear to be aberrant in the somatic cell nuclear transfer.

Abnormal Expression of Embryonic Genes Relates to Histone H4 Acetylation States during Preimplantation Development — In normal embryonic development, maternal mRNAs or proteins, which are synthesized and accumulated during oogenesis, are gradually replaced with de novo embryonic transcripts after embryonic genes are activated. To determine whether histone H4 acetylation correlates to embryonic genome activation, expression of two imprinted genes was examined after each developmental stage in IVF, NT, and TSA-NT embryos using the quantitative real-time PCR method. Xist (X-inactivation specific transcript gene), a paternally imprinted gene, is initially transcribed in 8–16 cell embryos (45). It is expressed in both male and female morula, but only in female blastocysts (45, 52). The Ndn gene, a maternally imprinted gene, is normally expressed in newborn mouse brain and in a variety of human tissues (44). During bovine preimplantation development, its transcripts are detected in 8–16 cell embryos (52). Thus, expression of the Xist or Ndn genes may correlate with embryonic activation in the bovine embryo. It is known that expression of imprinted genes is influenced by histone modifications (53, 54). Abnormal expression of imprinted genes may be associated with the high neonatal mortality in cloned animals (55). In this study, expression of two imprinted genes, Xist and Ndn, was abnormal especially at the 8-cell stage in cloned embryos as compared with IVF embryos (Fig. 5). The results suggest that the embryonic genome may be abnormally activate in cloned embryos, and that somatic chromatin is reset in the oocyte cytoplast itself. This behavior of the embryonic genome may be responsible for abnormal histone H4 acetylation during preimplantation development. Thus, the aberrance of genomic activation in cloned embryos may be because of abnormal reprogramming of histone modifications, eventually leading to developmental failures of cloned embryos.

Somatic Histone H4 Acetylation Status Memorizes in Preimplantation Embryos — Speculation that epigenetic marks of the chromatin transmit information about genomic function from one cell generation to the next has been suggested (22, 30). Although weak in NT oocyte, the AcH4K5 signal in the TSA-NT oocyte was strong at the pronuclear stage (Fig. 3, A and B). This result indicates that donor chromatin is resistant to histone acetylation reprogramming before the first cell division. The histone acetylation status further was clearly remembered in the metaphase chromosomes of NT and TSA-NT embryos during preimplantation development (Fig. 6, A and C). From these observations, we suggest new insight that the machinery for memorizing epigenetic marks, such as histone H4 acetylation, probably exists in the oocyte or embryonic cytoplasm during preimplantation development.

To determine whether epigenetic status of the embryonic chromatin is inheritable in embryogenesis, single blastomeres of IVF, NT, and TSA-NT embryos were individually transferred into enucleated oocytes and then the acetylation status of the resulting recloned embryos were investigated at the 8-cell stage. Intriguingly, AcH4K5 intensities were still low in the recloned embryos as compared with those from IVF or TSA-NT embryos (Fig. 6E). During early embryonic development, therefore, histone modifications may be regulated by a mechanism for encoding and transmitting information about genomic function from one cell generation to the next, namely a histone code (56), although how the epigenetic states maintain and stably inherit throughout preimplantation development is unclear.

From our findings, it assumes that the epigenetic state of the somatic chromatin is reset in the oocyte cytoplasm itself. This behavior of the somatic chromatin may give rise to an aberrant reprogramming during early development, thereby leading to abnormal expression of embryonic genes in cloned embryos. In addition, we suggest that epigenetic reprogramming of cloned embryos may be dependent on the status of donor cell chromatin. Understanding the epigenetic reprogramming of differentiated cell nuclei during early development will contribute to elucidating the mechanisms related to various cellular functions such as differentiation, apoptosis, and aging.

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