

Inefficient reprogramming of the hematopoietic stem cell genome following nuclear transfer

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Summary

In general, cloning undifferentiated preimplantation embryos (blastomeres) or embryonic stem cells is more efficient than cloning differentiated somatic cells. Therefore, there has been an assumption that tissue-specific stem cells might serve as efficient donors for nuclear transfer because of the undifferentiated state of their genome. Here, we show that this is not the case with adult hematopoietic stem cells (HSCs). Although we have demonstrated for the first time that mouse HSCs can be cloned to generate offspring, the birth rates (0-0.7%) were lowest among the clones tested (cumulus, immature Sertoli and fibroblast cells). Only 6% of reconstructed embryos reached the morula or blastocyst stage *in vitro* (versus 46% for cumulus clones; $P < 5 \times 10^{-10}$). Transcription and gene expression analyses of HSC clone embryos revealed that

they initiated zygotic gene activation (ZGA) at the appropriate timing, but failed to activate five out of six important embryonic genes examined, including *Hdac1* (encoding histone deacetylase 1), a key regulator of subsequent ZGA. These results suggest that the HSC genome has less plasticity than we imagined, at least in terms of reprogrammability in the ooplasm after nuclear transfer.

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Key words: Embryos, Hematopoietic stem cells, Mice, Nuclear transfer, Zygotic gene activation

Introduction

The epigenetic status of the genome determines the function and the fate of cells without modifying the genetic information. In principle, the epigenetic memory is inherited by the daughter cells of the same cell lineage *in vivo*, but may be gradually modified with cell differentiation. Recent nuclear transfer studies clearly indicate that epigenetic modifications accumulated during cell differentiation can be fully reprogrammed into the pluripotent state of early preimplantation embryos, although the efficiency of producing healthy young is still low (<5%) using somatic cells (reviewed by Tamada and Kikyo, 2004) and fetal germ cells (Miki et al., 2005). One exception to this clone-associated difficulty is nuclear transfer using embryonic stem (ES) cells. The birth rate following nuclear transfer from ES cells is as high as 20% in the mouse, although it varies greatly according to the cell line used and the number of passages (Wakayama et al., 1999; Eggan et al., 2001). Following nuclear transfer, bovine ES-like cells also gave rise to offspring at a very high rate (three calves per seven transferred) (Saito et al., 2003). By contrast, producing clones from T-cell or B-cell lymphocytes by nuclear transfer was very inefficient. Clonal mice with lymphocyte-specific markers were born only when the cloning procedure was combined with tetraploid embryo complementation, in which tetraploid cells complement the early development of clones, especially that of the extra-embryonic tissues (Hochedlinger and Jaenisch, 2002). Very recently, we produced

cloned mice directly from the nucleus of natural killer T (NKT)-cell lymphocytes by single-step nuclear transfer, but not from peripheral T cells (Inoue et al., 2005). Thus, one might speculate that differentiated somatic cells possess extensive chromatin modifications, which may be less efficiently reprogrammed when introduced into oocyte cytoplasm compared with undifferentiated cells. The most typical cells before extensive differentiation in adults are tissue-specific stem cells. However, as far as we know, there has been no study of the cloning of adult tissue-stem cells.

Hematopoietic stem cells (HSCs) are the longest-studied and best-understood mammalian adult stem cells. Recently established methodology for the purification of HSCs enables the direct and precise examination of their biological and biochemical properties, especially those related to self-renewal and differentiation (reviewed by Ema and Nakauchi, 2003; Akashi, 2005). At present, it is known that even a single HSC can fully reconstitute the hematopoietic compartment of lethally irradiated adult mice, indicating that the purification of HSCs does not perturb their biological normality and stem cell features (Osawa et al., 1996). Furthermore, although still controversial, recent studies have reported that the genomic plasticity of HSCs enables them to switch between hematopoietic and non-hematopoietic lineages (Krause et al., 2001; Wagers et al., 2002). Therefore, it is expected that these purified HSCs could be used as nuclear donors because they have a relatively undifferentiated genome, which is considered

better for somatic cell cloning than that of differentiated cells. In this study, we examined the reprogramming efficiency of the HSC genome after nuclear transfer cloning. It is known that the complexity of potentially contributory technical factors associated with cloning experiments might obscure the reprogramming efficiency of the donor genome. Therefore, we carefully assessed embryos reconstructed with HSCs for their developmental ability *in vitro* and *in vivo*, and for gene expression patterns, and compared these with those of embryos reconstructed with other somatic cells (cumulus cells, immature Sertoli cells and fibroblast cells) of the defined genetic backgrounds under strictly controlled experimental conditions as described previously (Inoue et al., 2002; Inoue et al., 2003).

Results and Discussion

Development of HSC clone embryos *in vivo* and *in vitro*

We isolated HSCs from B6D2F1 (C57BL/6Cr×DBA/2) females and (B6×129)F1 (C57BL/6Cr×129/Sv-*ter*) males so that we could compare their cloning efficiency with that of B6D2F1 cumulus cells, which are the standard donors for mouse cloning (Wakayama et al., 1998; Inoue et al., 2002), and with that of (B6×129)F1 immature Sertoli cells, which are the most efficient donors so far examined (Inoue et al., 2003), respectively. We also used tail-tip fibroblasts because they can be donors common to both sexes (Ogura et al., 2000b). Isolated HSCs, which have a smooth, round surface and a relatively small diameter, are easily nuclear transferred by injection. We experienced no technical problems in reconstructing oocytes. The rates of successful nuclear transfer, either by injection (HSCs, cumulus cells or immature Sertoli cells) or electrofusion (tail-tip fibroblasts), were approximately 70–80%, irrespective of the cell type used. The majority of embryos thus reconstructed were cultured for 48 hours and transferred into recipient females. In all experimental groups, about 90% of cloned embryos developed to the 2-cell stage within the first 24 hours (Table 1). This reflected their predominant G0–G1 population. However, during the next 24 hours, many HSC-derived embryos in either genotype failed to develop to the 4-cell stage, whereas the majority of other clones reached the 4-cell stage (Table 1). Some embryos from HSCs and cumulus cells were cultured until 96 hours. Development of HSC clone embryos into blastocysts was very poor (5.9%; 13/220) compared with that of cumulus clones (45.8%, 88/192; $P < 5 \times 10^{-10}$) (Table 1). When 4-cell embryos were transferred into recipient females, two normal-looking offspring were born from HSCs from (B6×129)F1 males (Fig. 1), but not from



Fig. 1. Two pups cloned from (B6×129)F1 HSCs. The pups were normal in appearance and showed active movement shortly after caesarian section, but were cannibalized by the foster mothers within 24 hours. They had enlarged placentas, as observed for other somatically cloned mouse pups (Inoue et al., 2002).

B6D2F1 females (Table 1). Cloned offspring were born in all other groups, (B6×129)F1 Sertoli cells being the most numerous, which is consistent with our previous report (Inoue et al., 2003). Thus, HSC clones showed the lowest developmental potential both *in vitro* and *in vivo* among the clones tested (cumulus, immature Sertoli and fibroblast cells) in two defined genetic backgrounds.

Transcription activity and gene expression in HSC clone embryos

As shown in the preceding section, the earliest developmental arrest of HSC embryos was found at the transition from 2-cell to 4-cell stage. In general, the donor genome should be reprogrammed by nuclear transfer to activate embryo-specific genes at the appropriate time, as occurs in normal fertilized embryos. This switch from maternal to embryonic control of the genome is commonly referred to as zygotic (or embryonic) gene activation (ZGA or EGA). In mice, early minor ZGA has been observed in the late 1-cell stage but the major ZGA occurs at the 2-cell stage (Telford et al., 1990). Therefore, it is very likely that reconstructed mouse embryos would arrest their development if they failed to initiate ZGA in a proper manner. To elucidate this possibility, we first examined transcriptional activity in HSC clone embryos for the initial ZGA (12 hours after oocyte activation) using a 5-bromouridine 5'-triphosphate (BrUTP) incorporation assay (Kim et al., 2002). As shown in Fig. 2, the levels of anti-BrUTP fluorescence, which reflect the transcriptional activity in HSC clone embryos, were not significantly different from those of control parthenogenetic or

Table 1. Development of embryos cloned from HSCs and other somatic cells

Strain	Donor cell	Cultured	2 cells (%)	4 cells (%)	Morulae and blastocysts (%)	Embryos transferred (ET)*	Implanted (% per ET)	Offspring (% per ET)
BDF1 (female)	HSC	444	384 (86.5)	151 (34.0) [†]	13/220 (5.9) [§]	109	24 (22.0) [¶]	0 (0.0)
	Cumulus	1141	1029 (90.2)	746 (65.4) [†]	88/192 (45.8) [§]	545	219 (40.2) [¶]	8 (1.5)
	Fibroblasts	358	348 (97.2)	279 (77.9) [†]	–	279	151 (54.1) [¶]	2 (0.7)
B6×129 (male)	HSC	637	563 (88.4)	302 (47.4) [‡]	–	302	90 (29.8) ^{**}	2 (0.7) ^{††}
	Sertoli	175	155 (88.6)	104 (59.4) [‡]	–	81	36 (44.4) ^{**}	6 (7.4) ^{††}
	Fibroblasts	192	172 (89.6)	126 (65.6) [‡]	65/94 (69.1)	155	81 (52.3) ^{**}	3 (1.9)

*Embryos were transferred at the 4-cell stage, except for 65 out of 155 embryos from B6×129 fibroblast clones, which were transferred at the morula or blastocyst stage.

[†] $P < 1 \times 10^{-10}$; [‡] $P < 0.005$; [§] $P < 5 \times 10^{-10}$; [¶] $P < 0.0005$; ^{**} $P < 0.05$; ^{††} $P < 0.01$ (HSC vs other donor cells).

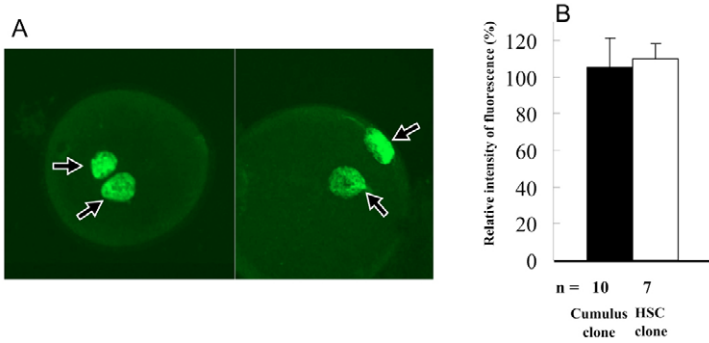


Fig. 2. Transcriptional activity of 2-cell HSC clone embryos assessed by a BrUTP incorporation assay. (A) Incorporation of BrUTP was detected by a specific antibody in the nuclei (arrows) of cumulus clone embryos (left), HSC clone embryos (right) and parthenogenetic embryos (not shown) at 12 hours post-activation. (B) The fluorescent intensity in the nuclei, which depicts transcriptional activity, was not different between cumulus and HSC clone embryos ($P>0.05$), and both were at the control level (parthenogenetic embryos=100%).

cumulus clone embryos. This indicates that HSC clone embryos initiated ZGA normally in terms of its timing and the initial transcriptional level; in other words, they successfully activated the so-called ‘zygotic clock’.

The result from the BrUTP incorporation assay did not preclude the possibility that the developmental arrest of HSC clones might have been caused by failure to activate genes of the major ZGA at the 2-cell stage. We selected six zygotic genes – *Dppa2*, *Dppa3*, *Dppa4*, *ERV-L*, *Hdac1* and *eIF-1A* – according to previous studies on global or specific gene expression analysis (De Sousa et al., 1998; Hamatani et al., 2004; Evsikov et al., 2004). We examined individual embryos because cloned embryos generally show diverse gene expression patterns, even under strictly controlled experimental condition. The transcription levels of these genes in each embryo were examined by real-time quantitative reverse

transcriptase (RT)-PCR using specific primers (Table S1, supplementary material). The gene expression patterns of HSC cell embryos, cumulus cell embryos, in vitro fertilization (IVF) embryos and metaphase II (MII) oocytes are summarized in Fig. 3. Of the genes examined, *Dppa2*, *Dppa3*, *ERV-L* and *eIF-1A* showed significantly lower expression levels in HSC and cumulus clone embryos than in IVF embryos (Fig. 3). By contrast, *Hdac1* was downregulated in HSC clone embryos and normally expressed in cumulus clone embryos (Fig. 3).

We then analyzed the expression levels of other major histone deacetylase genes – *Hdac2* and *Hdac3* – in HSC clone embryos. As shown in Fig. 3, both *Hdac2* and *Hdac3* were detected in MII oocytes, but their expression levels decreased by the 2-cell stage in IVF and cumulus clone embryos. The expression profiles of three histone deacetylase genes in this study are consistent with those reported previously by Schultz

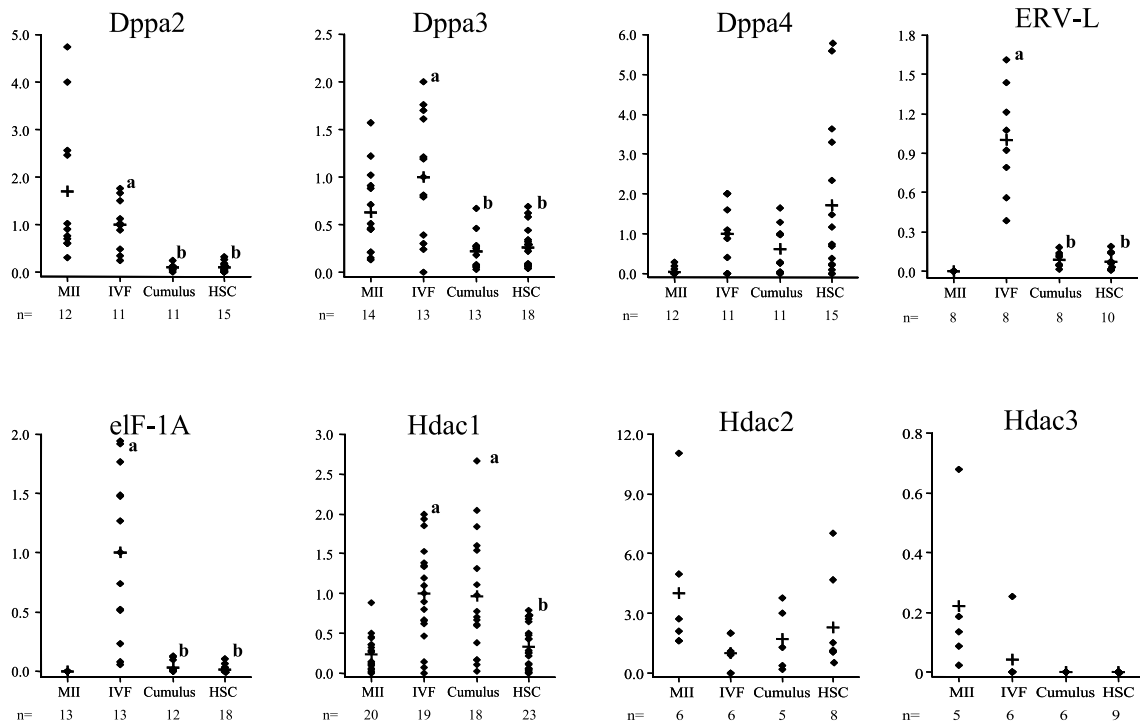


Fig. 3. Quantification by real-time RT-PCR of mRNA expression of various genes in single oocytes and embryos. Genotype (B6D2F1)-matched unfertilized MII oocytes, 2-cell IVF embryos, 2-cell cumulus clone embryos and 2-cell HSC clone embryos were analyzed. *Dppa2*, *Dppa3*, *Dppa4*, *eIF-1A*, *ERV-L* and *Hdac1* are considered zygotically activated embryos according to previous studies. Except for *Hdac3*, values are expressed relative to those in the IVF group (value=1). For *Hdac3*, the values are expressed relative to *Hprt* values because of lack of *Hdac3* expression in the IVF group. Values with different letters (a and b) differ significantly ($P<0.05$; Scheffe’s F test).

et al. (Schultz et al., 1999). They found that *Hdac1* was expressed at low levels in MII oocytes and 1-cell embryos, but at high levels in 2-cell and 4-cell embryos. By contrast, the patterns of *Hdac2* and *Hdac3* demonstrated high levels of transcripts in MII oocytes, followed by degradation until the 4-cell stage. Thus, in mouse embryos, *Hdac1* is the major zygotically activated histone deacetylase gene. Indeed, only *Hdac1* is sensitive to α -amanitin among the histone deacetylase genes and has the most number of interactions with other genes in a gene network of mouse 2-cell embryos (Zeng and Schultz, 2005). Consequently, this gene is probably the key regulator of the correct pattern of gene expression required for further development, because global histone hyperacetylation by histone deacetylase inhibitor (trichostatin A) leads to a complete developmental block at the 2-cell stage (Ito et al., 2000; Ma et al., 2001).

We then examined whether the levels of histone acetylation were affected in HSC clone embryos by immunostaining using antibodies specific to histone H3 acetylated on lysine 9 (H3K9), histone H4 acetylated on lysine 8 (H4K8) and histone H3 acetylated on lysine 14 (H3K14). According to data by Stein et al. (Stein et al., 1997), H3K9 and H4K8 are sensitive to histone deacetylase inhibitors whereas H3K14 is non-sensitive. As expected, HSC clone embryos were more strongly stained with the antibodies for H3K9 and H4K8, but not with the antibody for H3K14, as compared with cumulus clone embryos and intracytoplasmic sperm injection (ICSI) embryos (Fig. 4). This finding clearly indicates that the low *Hdac1* activity in HSC clone embryos leads to a phenotypic modification at the chromatin level, and probably leads to low developmental potency of the embryos. Interestingly, Kishigami et al. have recently reported that development of

clones was significantly improved when reconstructed oocytes were exposed to trichostatin A during oocyte activation (Kishigami et al., 2006). As the zygotic transcription has not yet started by this stage, this treatment might have enhanced reprogramming of the genome by increasing the accessibility of 'reprogramming factors' to DNA with hyperacetylated histones.

The expression of *Dppa2*, *Dppa3*, *ERV-L* and *eIF-1A* was significantly repressed in both HSC and cumulus clone embryos as compared with that of IVF embryos (Fig. 3). The *eIF-1A* gene is also a key regulator of ZGA because eIF-1A (formerly known as eIF-4C) is the major translation initiation factor in mouse 2-cell embryos and is thought to function in mRNA and initiator tRNA recruitment (Rhoads, 1993; De Sousa et al., 1998). *Dppa2* and *Dppa3* are genes with sequence similarities to the *Oct4* gene, which plays an essential role in the control of developmental pluripotency. *Dppa3*, also known as *PGC7* (Sato et al., 2002) and *Stella* (Saitou et al., 2002), is essentially a maternal-effect gene but is further activated during preimplantation development (Payer et al., 2003). *ERV-L* is an endogenous retrovirus gene that shows a high expression coincidentally with ZGA, although its roles in embryonic development are unclear (Evsikov et al., 2004). It is very probable that these zygotically activated genes had a cumulative effect and were responsible, at least in part, for the lower development potential of cloned embryos compared with normally fertilized IVF embryos (more than 90% blastocyst rates). We postulate that the low *Hdac1* activity found in HSC clone embryos might have further compromised their gene expression pattern, leading to the extremely low cloning efficiency.

Unlike normal fertilization, successful nuclear transfer cloning requires silencing of genes that had been actively transcribed in the donor cells. In a recent study of cloning cultured myoblast cells, the reconstructed embryos continued to express muscle-specific genes and showed a greater preference for cell culture media than embryo culture media, indicating inheritance of somatic cell phenotypes (Gao et al., 2003). We examined HSC clone embryos for the transcription of *Endomucin*, *CD45* and *c-kit*, which are known to represent active genes of HSCs (Akashi et al., 2003). RT-PCR revealed that these genes were not expressed in HSC clone embryos at the 2-cell or blastocyst stage (Fig. 5). The absence of CD45 protein was also confirmed by immunofluorescence of HSC-derived blastocysts (data not shown). Normal gene suppression upon nuclear transfer was also confirmed for cumulus cell clones (Fig. 5). Thus, we can assume that the active state of donor-specific genes was successfully converted to the repressive state following nuclear transfer under our experimental conditions.

Low plasticity of HSC genome

This study is the first to demonstrate the birth of cloned pups following nuclear transfer using HSCs. We expected that cloning efficiency would be better with HSCs than with other differentiated somatic cells because it has been suggested that the HSC genome possesses sufficient plasticity to differentiate into non-hematopoietic as well as hematopoietic lineages (Krause et al., 2001). However, in vitro development of HSC clone embryos was very

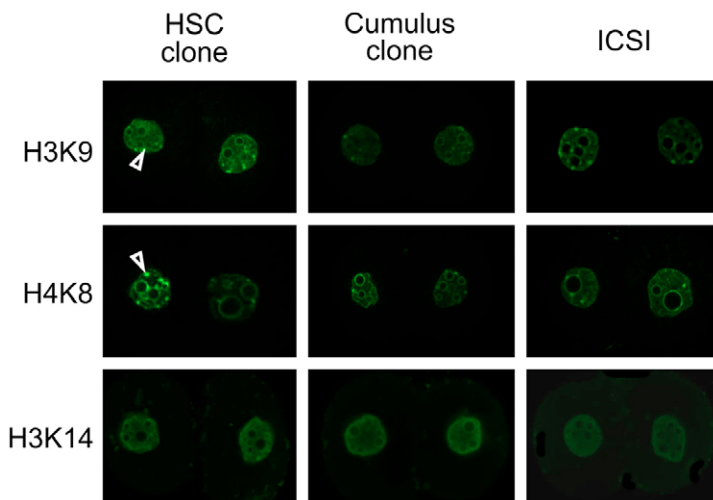


Fig. 4. Fluorescent images of 2-cell embryos stained with antibodies that recognized acetylated histones. Histone H3 acetylated on lysine 9 (H3K9) and H4K8, but not H3K14, are known to be sensitive to histone deacetylases. HSC clone embryos were more intensely stained for H3K9 and H3K14 than cumulus clone embryos or intracytoplasmic sperm injection (ICSI) embryos. Dot-like areas were frequently noted in HSC clone embryos (arrowheads). In HSC clone embryos, the two blastomeres were often stained differentially for H4K8. There was no difference in the staining pattern for H3K14 among the three types of embryos. 6-12 embryos were observed in each group.

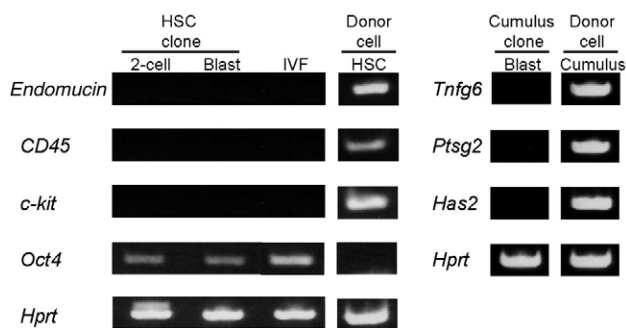


Fig. 5. Detection of donor-cell-specific gene expression in cloned embryos by RT-PCR. Transcription of donor-cell-specific genes was repressed in HSC clone and cumulus clone embryos. Each lane represents a single embryo, but results were confirmed by at least four replicates.

poor and the birth rates per transfer were no better than those of clones from other cell types in both genotypes (B6D2F1 and B6 \times 129). We have also found that the efficiency of ES cell establishment from HSC clone embryos was also poor (2 lines from 13 blastocysts, 15%) as compared with that from cumulus clone embryos (13/34, 38%) (unpublished). Consistent with these findings, HSC clone embryos failed to activate five out of six zygotically activated genes. As we used markers of CD34^{low}c-Kit⁺Sca-1⁺Lin⁻ (CD34⁺KSL) for donor cell isolation, the cell suspension was considered to be highly enriched for HSCs and their immediate progeny, which have multi-differentiation potency. We confirmed that the HSCs we isolated had the capacity for long-term hematopoietic lineage reconstitution in lethally irradiated mice after single-cell transplantation. When 3, 5 and 10 isolated cells were transplanted, 100% (4/4), 100% (3/3) and 67% (2/3), respectively, of recipient mice showed long-term reconstitution (Table S2, supplementary material). This result is comparable with that of a previous report (Osawa et al., 1996). Thus, the genome of HSCs has a lower genomic plasticity in terms of the ability to be reprogrammed in the MII cytoplasm.

The extremely low cloning outcome using HSCs as donor cells is in contrast with the efficient birth of clone pups and establishment of ES cells following nuclear transfer with NKT cells, a lymphocyte population in the same hematopoietic lineage (Inoue et al., 2005). Therefore, the presumptive notion that there is a reciprocal correlation between nuclear transfer cloning efficiency and cell differentiation status might not always be true, at least for cells within the hematopoietic lineage.

Why was *Hdac1* repressed in HSC clone embryos?

As mentioned previously in this paper, low *Hdac1* activity might have been one of the causes of the very poor development of HSC clone embryos. Generally, hyperacetylation of histones results in a transcriptionally active state by increasing the accessibility of transcriptional factors to DNA (Grunstein, 1997). This state can be readily achieved by low histone deacetylase activity. It is hypothesized that HSCs express multiple genes at a low level, probably as a result of a wide-open chromatin structure that enables random access of multiple transcriptional factors (Akashi et al., 2005; Zipori, 2004). HSCs decrease transcriptional accessibility as they

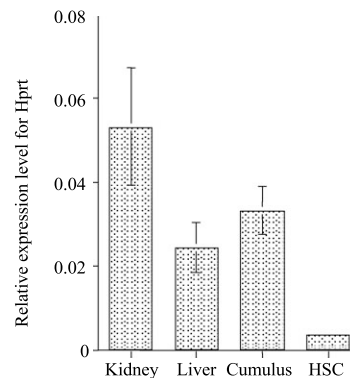


Fig. 6. Real-time RT-PCR quantification of *Hdac1* expression in donor HSCs, cumulus cells and other somatic cells freshly isolated from B6D2F1 female mice. Values are expressed relative to the *Hprt* expression level, which was assigned a value of 1.

differentiate in a stepwise manner to restrict the developmental potentials (Akashi et al., 2003). In an additional series of experiments, we undertook a quantitative analysis for *Hdac1* expression in donor HSCs that were freshly prepared from bone marrow tissue. Interestingly, the expression of *Hdac1* mRNA in HSCs was lower than that of other somatic cells (Fig. 6). Indeed, a recent study presented evidence that histone deacetylase inhibitors can preserve stem cell characteristics in mouse and human HSCs in culture (Young et al., 2004). Thus, the epigenetic state of the HSC genome, which might reflect its stem-like nature, is perhaps resistant to reprogramming and could be inherited through nuclear transfer, although further analytical studies are needed to confirm this hypothesis.

Materials and Methods

Animals

All animals, including oocyte donors, cell donors and embryo transfer recipients were maintained under specific-pathogen-free conditions at the Bioresource Center (RIKEN, Japan). They were provided with water and commercial laboratory mouse chow ad libitum and were housed under controlled lighting conditions (daily light period: 0700 hour to 2100 hour). All animals were maintained in accordance with the guidelines of RIKEN.

Nuclear donor cells

HSCs, cumulus cells, immature Sertoli cells and fibroblast cells were prepared as described previously (Osawa et al., 1996; Wakayama et al., 1998; Ogura et al., 2000a; Ogura et al., 2000b) with slight modifications. HSCs were isolated from the bone marrow of 8- to 10-week-old B6D2F1 and (B6 \times 129)F1 mice. Bone marrow cells were stained with a lineage marker (Lin) cocktail consisting of biotinylated anti-Gr-1, -Mac-1, -B220, -CD4, -CD8 and -Ter119 antibodies. Lin⁺ cells were depleted using Dynabeads M-280 streptavidin (SA) (DynaL Biotech). The remaining cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD34, phycoerythrin (PE)-conjugated anti-Sca-1, allophycocyanin (APC)-conjugated anti-c-Kit and biotinylated anti-Lin antibodies, followed by staining with SA-APC-Cy7. CD34^{low}c-Kit⁺Sca-1⁺Lin⁻ (CD34⁺KSL) cells, which are highly enriched for HSCs, were sorted into 200 μ l of serum-free medium S-Clone (Sanko Junyaku) containing 1% fetal bovine serum, mouse stem cell factor (SCF) (10 ng/ml; KIRIN), and human thrombopoietin (TPO; 100 ng/ml; KIRIN) using a fluorescence-activated cell sorter (FACS) Vantage SE (BD Biosciences). All antibodies were purchased from BD Biosciences Pharmingen and eBioscience. The sorting gates for CD34⁺KSL cells on FACS profiles are shown in Fig. S1, supplementary material. CD34⁺KSL cells represented 0.0154% \pm 0.0057% and 0.0106% \pm 0.0016% of bone marrow mononuclear cells in B6D2F1 ($n=8$) and (B6 \times 129)F1 ($n=6$) mice, respectively. The sorted CD34⁺KSL cells were subjected to a competitive repopulation assay (Osawa et al., 1996). Cumulus cells were collected from cumulus-oocyte complexes of B6D2F1 mice that had been superovulated, as described below. Immature Sertoli cells were isolated from newborn testes of (B6 \times 129)F1 males at 2-5 days after birth by treatment with 0.1 mg/ml collagenase (Sigma Aldrich) and 0.2 mg/ml trypsin (Sigma Aldrich). Adult fibroblast cells were collected from tail-tip tissue of B6D2F1 females and

(B6×129)F1 males at 2–4 months of age. Tail-tip fibroblasts adhered to the bottom of the dish and became confluent within two weeks of culture.

Nuclear transfer

Nuclear transfer was carried out as described previously (Wakayama et al., 1998; Ogura et al., 2000a; Ogura et al., 2000b). B6D2F1 females at 7–10 weeks of age were superovulated with an injection of 7.5 IU pregnant mare serum gonadotrophin (PMSG) and 7.5 IU human chorionic gonadotrophin (hCG) at intervals of 48 hours. Mice were sacrificed and the oviducts were removed 16 hours after the hCG injection. Cumulus-oocyte complexes collected from the oviducts were freed of cumulus cells by 0.1% bovine testicular hyaluronidase in KSOM medium (Lawitts and Biggers, 1993). Oocytes were washed three times with fresh KSOM medium and were incubated at 37.5°C in an atmosphere of 5.5% CO₂ in air. MII oocytes were enucleated with a small amount of cytoplasm in Hepes-buffered KSOM medium containing 7.5 mg/ml cytochalasin B at 37°C using a piezo-driven micromanipulator (PrimeTech). After repeated washes with fresh medium, the enucleated oocytes were incubated in KSOM for 0.5–2 hours. Nuclei from hematopoietic cells, cumulus cells and immature Sertoli cells were transferred into enucleated oocytes by direct injection using a piezo-driven micromanipulator. Nuclei from tail-tip fibroblasts were transferred by electrofusion (2300 V/cm, 9 μs) in 300 mM mannitol medium containing 50 mM MgCl₂ and 0.1 mg/ml polyvinyl alcohol. Oocyte-donor cell pairs were aligned by AC current (200 V/cm, 2 MHz, 10–20 seconds) before the electrofusion pulse. Oocytes reconstructed by injection or electrofusion were cultured in KSOM for 1–2 hours, activated with Ca²⁺-free KSOM containing 3 mM SrCl₂ and 5 mg/ml cytochalasin B for 1 hour, and then cultured in the presence of 5 mg/ml cytochalasin B for 5 hours. After washing, the embryos were further cultured in KSOM at 37.5°C under 5.5% CO₂ in air until gene expression analysis or embryo transfer. Reconstructed embryos that reached the 4-cell stage by 48 hours in culture were transferred into the oviducts of day-one pseudopregnant ICR females mated with vasectomized males. On day 20, the recipient females were examined for the presence of fetuses, and live pups were nursed by lactating ICR females.

Measurement of in vitro transcriptional activity

In vitro transcriptional activity was measured as described previously (Kim et al., 2002). Briefly, reconstructed embryos at 12 hours post-activation were permeabilized in phosphate buffer (PB) containing 0.05% Triton X-100 and labeled with BrUTP for 15 minutes. After washing with PB three times, the embryos were fixed with 3.7% paraformaldehyde and stained with anti-BrUTP monoclonal antibody (Boehringer-Mannheim). Subsequently, they were incubated in PBS containing 0.5 mg/ml anti-mouse IgG antibody conjugated with Texas Red (Jackson ImmunoResearch) for 45 minutes. Fluorescent label was quantified with a confocal laser-scanning microscope (Carl Zeiss).

Gene expression analysis of 2-cell and blastocyst embryos

The zona pellucidae of reconstructed 2-cell stage (24–26 hours after activation) or blastocyst (96–98 hours after activation) embryos were removed by treatment with acidic Tyrode's solution. Each embryo was transferred into a 0.2 ml PCR tube, and cDNA was extracted by Cell-to-cDNA II (Ambion). For primer sequences and PCR conditions, see supplementary material. For quantitative analysis using 2-cell embryos, the PCR products amplified with primers were purified with a Freeze 'N Squeeze Spin Column (BioRad) and tenfold serially diluted products were used as the external standards for the real-time PCR. The ABI Prism 7900HT was used to determine the levels of cDNA with a QuantiTect SYBER Green PCR Kit (QIAGEN). The level of the *Hprt* gene in each embryo was used for the endogenous reference. Temperature conditions for amplification were as follows: an initial activation step of 94°C for 15 minutes, followed by 50 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds, and a final step at 72°C for 2 minutes. For the gene expression analysis of blastocysts, purified cDNAs were amplified with Ex Taq DNA Polymerase Hot-Start Version (Takara) using the primer sets listed in Table S1, supplementary material. The amplified products were separated by 2% agarose gel electrophoresis.

Expression analysis of donor-cell-specific genes in cloned embryos

Tissue and cell samples (liver, kidney, cumulus cells and HSCs) for gene expression analysis were collected from B6D2F1 adult females. Immediately before collection of the liver and kidneys, cells in the blood vessels were thoroughly removed by perfusion of animals under deep anesthesia. Cumulus cells and HSCs were prepared as described elsewhere in the methods section. After homogenization, RNAs of these samples were purified with ISOGEN (Wako). cDNAs were synthesized with reverse transcriptase (Takara) and quantitative analysis was carried out as described above with primers shown in Table S1, supplementary material.

Immunostaining for acetylated histones in 2-cell embryos

2-cell embryos were fixed in PBS containing 0.1 mg/ml polyvinyl alcohol and 4% paraformaldehyde for 1 hour and washed in PBS supplemented with 1 mg/ml BSA (Sigma-Aldrich) (PBS-BSA) at room temperature. Fixed embryos were then

immersed overnight with PBS-BSA containing 0.5% Triton X-100 for blocking and permeabilization at 4°C. The embryos were incubated with a rabbit polyclonal antibody against acetyl-histone H3-Lys9 (H3K9), H3-Lys14 (H3K14) or K4-Lys8 (H4K8) (Upstate) for 1 hour. After washing in PBS-BSA, the embryos were stained with FITC-conjugated anti-rabbit IgG antibody (Sigma-Aldrich) for 1 hour. For labeling nuclear DNA, 5 mg/ml DAPI (Nacalai Tesque) was used. Immunostaining was performed at room temperature. Following thorough washing, the embryos were mounted on slide glasses using antifading mounting medium (Vectashield; Vector Laboratories) and observed using a confocal scanning laser microscope (Digital Eclipse C1).

Statistical analysis

Development of embryos in vitro and in vivo was compared between groups using Fisher's exact probability test. The relative transcription levels of embryos or donor cells determined by quantitative real-time RT-PCR were analyzed by one-way ANOVA followed by a post-hoc procedure using Scheffe's F test for multiple comparisons between the groups where appropriate. Other methods of statistical analysis, when appropriate, are indicated in the Results and Discussion.

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Table S1. Primer sequences for gene expression analysis

	Forward primer	Reverse primer	Product size	Ref.
Endomucin	5'-acaactgaaggtcccctaagg-3'	5'-ttggtttcccctgtgcagag-3'	461 bp	
CD-45	5'-gaaatcttatatcgcggtgt-3'	5'-tttagatgcagactcagggt-3'	162 bp	
c-kit	5'-acatccgtgaactccatgtg-3'	5'-cactccagaatcgtcaactc-3'	150 bp	
Oct4	5'-aggcaggagcagcagtggaag-3'	5'-gctggcgaactgttctagctcc-3'	156 bp	Goldin <i>et al.</i> 2003
Tnfr6	5'-gaagacatcattagcacaggaa-3'	5'-aaggctatacagagaaccct-3'	310 bp	
Pts2	5'-gtctgatgatgatccacca-3'	5'-aagctgtactcctgtcttca-3'	322 bp	
Has2	5'-gtggtttacaactcctctag-3'	5'-cacttattgatgagaaccac-3'	162 bp	
Dppa2	5'-taccaagcacctgtctccgc-3'	5'-ccctgcttcattctggcctcc-3'	198 bp	
Dppa3	5'-atcgggaagaattaggagct-3'	5'-tttactttattcctcagccct-3'	222 bp	
Dppa4	5'-aggaagtcagcaccaccgtagt-3'	5'-aagctggaagaggccaatggct-3'	263 bp	
ERV-L	5'-ggaatgaaggtatgggtcaatcc-3'	5'-ccttcacctcagccagcac-3'	71 bp	Hayashi <i>et al.</i> 2003
eIF-1A	5'-aagaagtctgaaggcctatg-3'	5'-cagagaacttggaatgtagc-3'	170 bp	Kageyama <i>et al.</i> 2004
HDAC1	5'-tgctcgtcgtggacttac-3'	5'-gtagggcagctcattaggatcg-3'	67 bp	Hayashi <i>et al.</i> 2003
HDAC2	5'-gtcaattggctggaggactac-3'	5'-agacggtcatcagcgatct-3'	193 bp	
HDAC3	5'-gactacatcgactcctgca-3'	5'-atcaatgtagagcaccggag-3'	312 bp	
Hprt	ABI Mm00446968_m1			

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Table S2. Repopulating activities of CD34⁻KSL cells

No. transplanted CD34 ⁻ KSL cells	No. positive mice	% chimerism in positive mice
3	4/4 (100%)	0.9, 3.8, 9.2, 11.8
5	3/3 (100%)	0.1, 19.7, 22.9
10	2/3 (67%)	0.3, 14.8

The sorted CD34⁻KSL cells from (B6×129)F1(Ly5.2) mice were transplanted into lethally irradiated (B6×129)F1(Ly5.1/5.2) mice together with 2×10⁵ bone marrow competitor cells from (B6×129)F1(Ly5.1/5.2) mice. Twelve weeks after transplantation, peripheral blood cells were stained with antibodies against Ly5.1 and Ly5.2, as well as lineage markers (Gr-1, Mac-1, B220, CD4 and CD8), and analyzed on a FACS for multilineage reconstitution and percentage chimerism of cells (Ly5.2) derived from CD34⁻KSL cells.