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The histone deacetylase inhibitor scriptaid enhances nascent mRNA production and rescues full-term development in cloned inbred mice

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Abstract

Since the birth of Cumulina, the first mouse clone produced by somatic cell nuclear transfer (SCNT), the success rate of cloning in mice has been extremely low compared with other species and most of the inbred mouse strains have never been cloned. Recently, our laboratory has found that treatment of SCNT mouse embryos with trichostatin A, a histone deacetylase inhibitor (HDACi), improved the full-term development of B6D2F1 mouse clones significantly. However, this was not effective for the inbred strains. Here, we show for the first time that by treating SCNT embryos with another HDACi, scriptaid, all the important inbred mouse strains can be cloned, such as C57BL/6, C3H/He, DBA/2, and 129/Sv. Moreover, the success of somatic nuclear reprogramming and cloning efficiency via nuclear transfer technique is clearly linked to the competent *de novo* synthesis of nascent mRNA in cloned mouse embryos.

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Introduction

Inbred mouse models serve as a biological test ground and genetic background is an important criterion when selecting a strain for a particular experiment. Moreover, inbred mouse strains such as C57BL/6 are used for transgenesis research to identify functional genes or as genetic models for disease susceptibility. The combination of somatic cell cloning techniques with transgenic technologies using inbred mice will open new fields in biomedical applications. Although cloning mammals by somatic cell nuclear transfer (SCNT) into oocyte cytoplasts have been performed successfully for a decade (Wilmut *et al*. 1997), the success rate is extremely low, especially in mice (Wakayama *et al*. 1998) with a high incidence of developmental anomalies such as placental overgrowth, obesity, umbilical hernias, and neonatal death (Wakayama *et al*. 1999, Eggan *et al*. 2001, Cibelli *et al*. 2002, Tamashiro *et al*. 2002). To date, most of the important inbred mouse strains, such as C57BL/6 and C3H/He, have never been cloned, except for the DBA/2 and 129/Sv strains (Wakayama & Yanagimachi 2001*a*, 2001*b*, Inoue *et al*. 2003), but this was achieved with extremely low efficiency. Recently, our laboratory (Kishigami *et al*. 2006*a*, 2006*b*) and Rybouchkin *et al*. (2006) have found that treatment of SCNT mouse embryos with trichostatin A (TSA), a histone deacetylase inhibitor (HDACi), improved the full-term development of B6D2F1 mouse clones significantly. However, application of this method into SCNT cloned embryos from C57BL/6 and C3H inbred mouse strain could not produce full-term development.

Here we show that the treatment of SCNT embryos with scriptaid, an HDACi with low toxicity, which enhances transcriptional activity and protein expression (Su *et al*. 2000), significantly enhanced the cloning efficiency of inbred mouse strains. Interestingly, cloned two-cell embryos, derived from scriptaid-treated SCNT oocytes, showed significantly higher nascent mRNA expression than controls, with levels close to those of conventionally fertilized embryos. These cloned mice grew to adulthood and showed normal reproductive ability.

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Results

Relationship of histone acetylation with full-term development of cloned embryos

To test whether treatment of SCNT oocytes with HDACis improved the full-term development of cloned mice, we treated SCNT oocytes derived from cumulus cells and oocytes of B6D2F1 mice cultured in KSOM medium with various types of HDACi. These were 50–2000 nM scriptaid, 100 and 200 nM APHA compound 8 (APHA), and 100 nM TSA (Kishigami *et al*. 2006*a*, 2006*b*). Reagents were added for 6 h during oocyte activation and then for 4 h further in culture. A group of control oocytes received 0.5% (v/v) DMSO. The levels of histone H3 acetylation at lysine 9 (aH3-K9) detected in all nuclei of cloned embryos during the first mitotic interphase were higher than control in every treatment group (Fig. 1A and B; $n=40$ embryos per group). However, in the APHA group, the proportion of clones developing to the blastocyst stage was significantly lower and no full-term development was obtained (Fig. 1C and Table 1). In the scriptaid groups, there were high rates of development to the blastocyst stage and these treatments allowed full-term development (3.4, 4.2, 7.6, 6.8, and 4.1%) in repeated experiments with all concentrations (50, 100, 250, 500, and 2000 nM respectively) similar to TSA treatment and was significantly higher than that in the control group (0.5%; $P<0.05$). The HDACi scriptaid improved the full-term development of cloned mice in a dose-dependent manner with the maximum effect at 250 nM (Table 1). Thus, increased histone acetylation in the pronuclei of cloned one-cell embryos appears to be essential for somatic cell chromosome reprogramming, but is not sufficient for the full reprogramming of somatic cells to allow full-term development. Therefore, these results indicate that there may have been other toxic effects of some HDACi, precluding full-term development of cloned embryos. Based on the result of scriptaid concentration on the full-term development, we decided to use 250 nM scriptaid for all subsequent experiments.

Next, we applied scriptaid to SCNT oocytes produced using cumulus cells of a wide range of donor mouse strains. These included hybrid BD129F1 (BDF1 ×129/Sv), and inbred C57BL/6, C3H/He, DBA/2, and 129/Sv mouse strains. SCNT oocytes without treatment were used as controls (containing 0.5% (v/v) DMSO). We obtained full-term development using all donor cell sources (Table 2). Surprisingly, we obtained for the first time full-term development from C57BL/6 (nine cloned mice/394 two-cell embryos, 2.3%) and C3H/He clones (five cloned mice/577 two-cell embryos transferred, 0.9%). By contrast, no full-term development resulted from C57BL/6 or C3H/He cloned embryos in either the control or TSA groups. We also found that with B6D2F1 and 129/Sv mice, scriptaid treatment resulted in placentae that were significantly smaller than non-treated nuclear transfer (NT) controls. But controls and scriptaid NT placentae were still heavier than ICSI and naturally mated controls for the same strain (Table 3). We have never obtained C57BL/6, C3H/He, and DBA/2 cloned pups from non-treated NT controls; therefore, the placental weights in those mouse strains with scriptaid treatment cannot be compared. However, the placenta for inbred strain C57BL/6, C3H/He, and DBA/2, but not 129, was heavier than for B6D2F1 in scriptaid-treated groups. The body weight of cloned pups from the B6D2F1 strain treated with scriptaid were significantly lighter than non-treated NT controls and similar to ICSI and naturally mated controls. But body weights of all of the cloned pups from inbred strains were significantly heavier than naturally mated controls (Table 3).

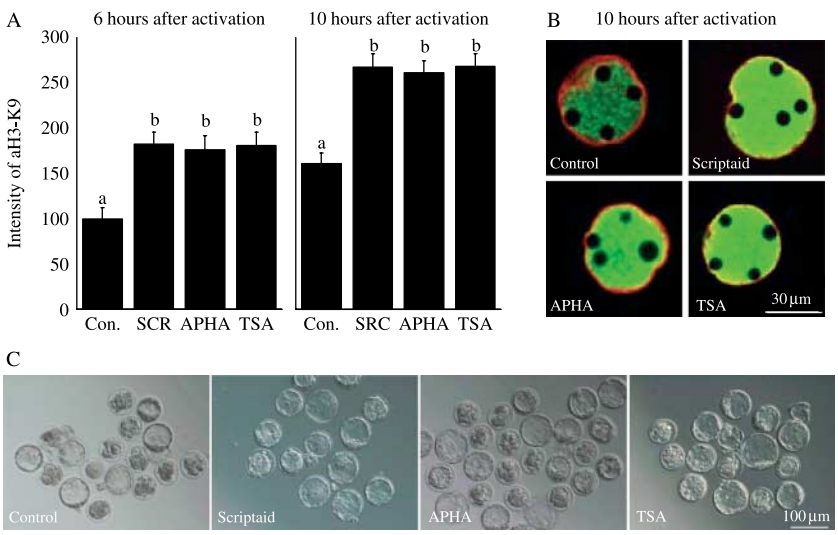

Figure 1 The intensity of histone H3 lysine 9 acetylation (aH3-K9) during the first mitotic interphase of SCNT embryos and the development to the blastocyst stage after being treated with scriptaid (SCR), APHA compound 8 (APHA), and trichostatin A (TSA). The mean value of aH3-K9 intensity of the control embryos (without treatment) at 6-h postactivation was set at 100%, and the fluorescence intensity observed in each sample of the treated groups was expressed relative to this value. (A) The intensity of aH3-K9 at 6 and 10 h after activation (control is without treatment, Con). Bars show the S.E.M. (B) The level of aH3-K9 in the pronuclei of one-cell cloned embryos 10 h after activation. Nuclear membrane is shown in red and aH3-K9 in green. (C) Cloned blastocysts 96 h after activation.

Table 1 Effects of the histone deacetylase inhibitors scriptaid, APHA compound 8, and trichostatin A on the development of cloned B6D2F1 embryos.

		No. of embryos developed to (%)			
Concentration	**No. of SCNT oocytes**	Two cell	Blastocyst	Full term	**No. of placenta only** (%)
Scriptaid (histone deacetylase inhibitor with lower toxicity than trichostatin A, used to enhance protein expression)					
50 nM	130	117 (90)	81 (69)*	4 (3.4)	1 (0.9)
100 nM	130	119 (92)	91 (76)*	5 (4.2)	2 (1.7)
250 nM	160	144 (90)	116 (81)*	11 (7.6)	5 (3.5)
500 nM	150	132 (88)	97 (73)*	9 (6.8)	2 (1.5)
2000 nM	140	123 (89)	79 (72)*	5 (4.1)	3 (2.4)
APHA compound 8 (histone deacetylase inhibitor inducing histone hyperacetylation, growth inhibition, and terminal cell differentiation)					
100 nM	140	128 (91)	49 (38)†	0 (0)	0 (0)
200 nM	280	247 (88)	90 (36)†	0 (0)	0 (0)
Trichostatin A (histone deacetylase; the resulting histone hyperacetylation leads to chromatin relaxation and modulation of gene expression)					
100 nM	140	129 (92)	112 (80)*	6 (4.7)	2 (1.6)
Control (without treatment)					
Control	270	214 (79)	74 (35)†	1 (0.5)	0 (0)

This experiment was repeated five times. SCNT oocytes were treated with reagents for 6 h during the duration of activation and subsequently cultured in KSOM with the same reagents for 4 h further. Numbers of two-cell stage embryos were used to calculate the rates of blastocyst and full-term development. Each group of embryos was cultured to the blastocyst stage and then transferred into pseudopregnant female mice. *,†Values with different superscripts within a column are significantly different ($P<0.05$).

Normal development, reproductive ability, and genotype in cloned inbred mice produced by scriptaid treatment

The postnatal development of all hybrid and inbred cloned mice produced by scriptaid treatment was normal (Fig. 2A and B). All of the cloned inbred C57BL/6, C3H/He, DBA/2, and 129/Sv female mice became pregnant at normal rates of pups when mated with normal male mice (12 ± 1.3, $n=4$; 10 ± 2.2, $n=3$; 10 ± 1.4, $n=6$; 11 ± 1.5, $n=8$ respectively), compared with normal female and male mice of the same strain (11 ± 1.9, $n=5$; 10 ± 1.6, $n=5$; 11 ± 1.3, $n=5$; 12 ± 1.7, $n=5$).

PCR amplification and analyses of microsatellite markers in genomic DNA from the tail of the original donor cell mouse and representative cloned mice from C3H/He (four cloned mice), 129/Sv (five), C57BL/6 (three), and DBA/2 (four) donor strains confirmed that the cloned mice were genetically identical with the donors (Fig. 2C–F).

Scriptaid treatment enhances newly synthesized mRNA levels in cloned embryos

To understand how scriptaid treatment can improve the full-term development of inbred cloned embryos, we examined nascent mRNA expression in two-cell cloned embryos with or without scriptaid treatment compared with fertilized embryos produced by ICSI using B6D2F1

Table 2 Effects of scriptaid on the full-term development of cloned embryos following somatic cell nuclear transfer (SCNT) using different mouse genotypes.

Donor cell genotypes	**Type of HDACi**	**No. of SCNT oocytes**	**No. of two-cell embryos** (%)	**No. of full-term development** (%)	**No. of placenta only** (%)
B6D2F1	TSA	140	129 (92)	6 (4.7)	2 (1.6)
	SCR_{250}	1127	981 (87)	59 (6.0)	15 (1.5)
	Control	270	214 (79)	1 (0.5)	0 (0.0)
BD129F1	TSA	457	298 (65)	22 (7.4)	0 (0.0)
	SCR_{250}	356	244 (69)	7 (2.9)	0 (0.0)
	Control	110	63 (57)	2 (3.2)	0 (0.0)
C57BL/6	TSA	132	123 (93)	0 (0.0)	0 (0.0)
	SCR_{250}	432	394 (91)	9 (2.3)	6 (5.8)
	Control	300	231 (77)	0 (0.0)	0 (0.0)
C3H/He	TSA	127	107 (84)	0 (0.0)	0 (0.0)
	SCR_{250}	681	577 (85)	5 (0.9)	0 (0.0)
	Control	312	220 (71)	0 (0.0)	0 (0.0)
DBA/2	SCR_{250}	157	139 (89)	9 (6.5)	0 (0.0)
	Control	186	142 (76)	0 (0.0)	0 (0.0)
129/Sv	SCR_{250}	214	187 (87)	19 (9.8)	2 (1.9)
	Control	147	124 (84)	4 (2.4)	0 (0.0)

This experiment was repeated ten times. SCNT oocytes were activated with scriptaid at 250 nM (SCR_{250}) for 6 h during activation and subsequently cultured in KSOM supplemented with SCR_{250} for 4 h further. Numbers of two-cell stage embryos were used to calculate the rates of full-term development.

Table 3 Body and placental weights of newborn cloned mice derived from somatic cell nuclear transfer embryos using different donor cell genotypes and treated with 250 nM scriptaid for 10 h.

	Cloned and ICSI pups			Natural mating pups		
		Average weight (g)			Average weight (g)	
Donor cell genotypes	No. of pups	Body (mean±S.E.M.)	Placenta (mean±S.E.M.)	No. of pups	Body (mean±S.E.M.)	Placenta (mean±S.E.M.)
Cloned treated SCR250						
B6D2F1	37	1.40±0.17*	0.177±0.02*	18	1.37±0.11	0.109±0.009
C57BL/6	8	1.38±0.19*	0.214±0.09†	10	1.18±0.04	0.082±0.004
C3H/He	5	1.46±0.14*	0.276±0.07‡	12	1.24±0.09	0.083±0.008
DBA/2	9	1.42±0.17*	0.261±0.07‡	5	1.02±0.14	0.128±0.027
129/sv	19	1.57±0.14*	0.168±0.02*	22	1.19±0.09	0.107±0.008
Cloned without SRC						
B6D2F1	4	1.82±0.15†	0.347±0.12§			
129/sv	4	1.37±0.21*	0.201±0.09†			
Control (ICSI fertilized)						
B6D2F1	40	1.41±0.08*	0.127±0.02‖			

*,†,‡,§,‖Values with different superscripts within a column are significantly different ($P<0.05$).

mice. In ICSI embryos, 74% showed high nascent mRNA expression (Fig. 3A and D; $n=100$). By contrast, only 2% of untreated cloned embryos showed high levels of nascent mRNA (Fig. 3B and D; $n=100$). Interestingly, almost 40% of untreated cloned two-cell embryos were completely negative for nascent mRNA in one of the cells and half of the remaining embryos showed very low levels (Fig. 3B; $n=100$). Surprisingly, when we treated SCNT oocytes with scriptaid for 10 h during and after activation, 29% of the cloned two-cell embryos showed high levels of nascent RNA with a similar pattern to ICSI-fertilized embryos (Fig. 3C and D; $n=100$).

Clearly, the full-term development of cloned embryos using scriptaid treatment is strain dependent (Fig. 3E). To test the different expression levels of new transcriptional activity between hybrid and inbred mouse strains, we compared the nascent mRNA expression at the two-cell stages of B6D2F1, C57BL/6, C3H/He, DBA/2, and 129/Sv strain cloned embryos that had been treated with scriptaid. Only 9 and 4% embryos of the C57BL/6 and C3H/He strain clones exhibited high nascent mRNA at class I (Fig. 3E and F). By contrast, 21 and 23% embryos of the DBA/2 and 129/Sv strain cloned two-cell embryos exhibited high nascent mRNA at this level (Fig. 3E and F; $P<0.05$). Although we did not track nascent mRNA expression levels from the two-cell embryo to full term, statistical analysis of the correlation coefficient between two variables of the full-term development and the level of nascent mRNA expression at the two-cell stage in fertilized embryos and in cloned embryos with different mouse strains shows that the two variables are closely related ($r=0.966$). Thus, an

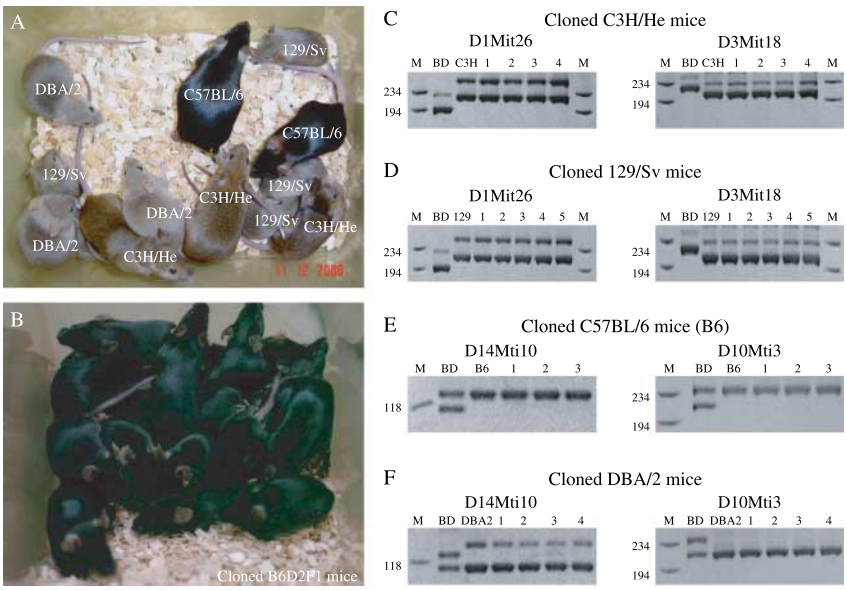

Figure 2 Cloned mice and genomic analysis. (A) Healthy cloned inbred C57BL/6, C3H/He, DBA/2, and 129/Sv strain mice produced with scriptaid treatment. (B) Healthy cloned B6D2F1 mice produced by scriptaid treatment. (C–F) PCR analysis of microsatellite markers in genomic DNA from the tail of a mouse providing oocytes (BD strain), donor cells (C3H/He, 129/Sv, C57BL/6, and DBA/2 strains), and four C3H/He clones (C, lanes 1–4), five 129/Sv clones (D, lanes 1–5), three C57BL/6 clones (E, lanes 1–3), and four DBA/2 clones (F, lanes 1–4).

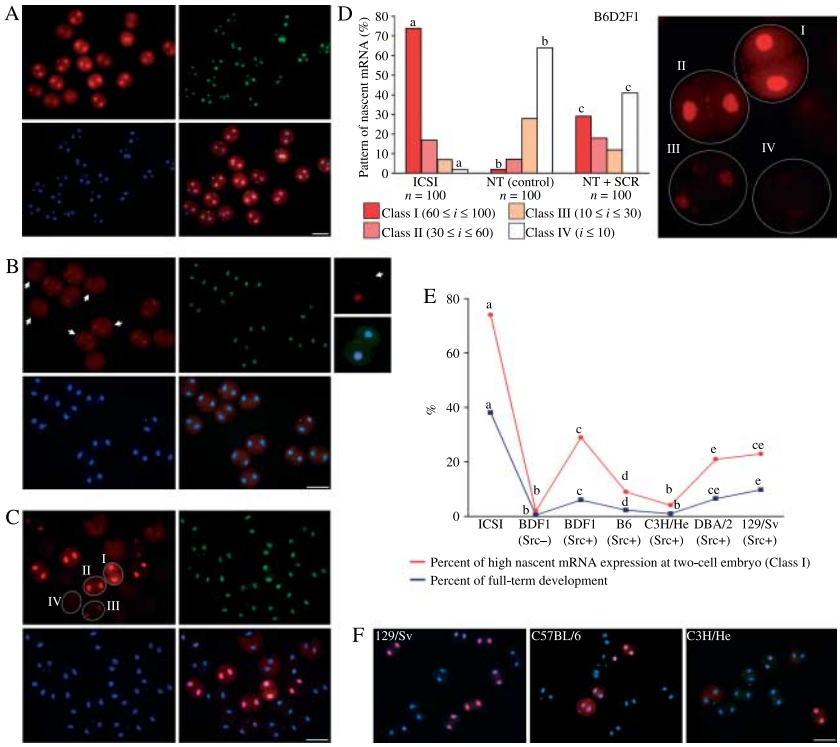

Figure 3 Nascent mRNA expression at the two-cell stage embryo of ICSI-fertilized and SCNT clones with or without scriptaid treatment. Embryos were subjected to electrical permeabilization in the presence of BrUTP, cultured for 1 h and collected for the detection of BrU-labeled transcripts by immunofluorescence confocal microscopy. (A) Immunofluorescent microscopy images of 18 fertilized two-cell embryos. (B) Twelve cloned B6D2F1 embryos without scriptaid treatment. Arrows indicate the cloned embryos with asymmetric expression of nascent mRNA. (C) Eighteen cloned two-cell embryos with scriptaid treatment on the same slide. (D) Classification of nascent mRNA based on the intensity of nuclear fluorescence in the two-cell embryos of fertilized (ICSI), cloned without (NT control) and with scriptaid treatment (NT+SCR). Maximum nuclear fluorescence of fertilized embryos at each repeat experiment was set as 100%. Class I showed intensity of nuclear fluorescence (i) with $60\% < i \leq 100\%$; class II, $30\% < i \leq 60\%$; class III, $10\% < i \leq 30\%$; class IV, $i < 10\%$). The data were obtained from four repeated experiments in which 100 embryos were analyzed for each group. (E) Frequencies of high nascent mRNA expression at the two-cell stage and full-term development of cloned embryos from hybrid B6D2F1 and from different inbred mouse strains. (F) Immunofluorescent microscopy images of cloned two-cell embryos on the same slide produced from 129/Sv, C57BL/6, and C3H/He strain donor cells. Nascent mRNA is shown in red, aH3-K9 in green, and DNA in blue. Different letters in graph with the same color indicate significantly different values ($P < 0.05$). The correlation coefficient between two variables of the full-term development and the level of nascent mRNA expression at the two-cell stage in cloned embryos with different mouse strains are closely related ($r = 0.966$). Bar = 100 μm.

increase in nascent mRNA expression at the two-cell stage of cloned embryos will result in a corresponding increase in the rate of offspring (Fig. 3E and F). Taken together, our results clearly suggested that treatment of SCNT oocytes with 250 nM scriptaid enhances nascent mRNA transcriptional activity in cloned two-cell embryos and that this increases the rate of development to term. As with full-term development, the nascent mRNA expression levels in cloned two-cell embryos depended on the genotype of the donor cell.

Scriptaid has lower toxicity for embryo development

The new cloning method using TSA significantly increased the cloning success rate, but it is very inconvenient for the embryologist: for example if we collect oocytes at 0900 h, finish enucleation and NT at 1300 h, and activate the SCNT oocytes at 1400 h, we must change the culture medium at midnight (2400–0100 h), due to the toxicity of TSA (Kishigami *et al.* 2006*a*, 2006*b*, Rybouchkin *et al.* 2006). As shown in Table 4, we found that treatment of ICSI-fertilized embryos with 250 nM scriptaid, for up to 48 h, did not inhibit *in vitro* or *in vivo* development. However, treatment with 100 nM TSA for 24 h resulted in a significant decrease in the development to the blastocyst stage and to full term (Table 4). Thus, scriptaid was not toxic for development when embryos were treated within 48 h. Based on this finding, we used scriptaid for 24 h on B6D2F1 and C57BL/6 cloned embryos. This new approach also worked well, because full-term offspring were obtained without reduced success rate in both strains (8.7%, $n = 264$ blastocysts transferred and 2.7%, $n = 221$ blastocysts versus 8.9%, $n = 191$ blastocysts and 2.6%, $n = 232$ blastocysts in groups treated for 10 h respectively).

Table 4 Normal development of fertilized embryos treated with 250 nM scriptaid for 10, 24, and 48 h.

Type of HDACi	Time of treatment (h)	No. of embryos treated	No. of embryos developed to (%)		Neonatal death	Average term weight (g)	
			Blastocyst	Full term		Body (mean±S.E.M.)	Placenta (mean±S.E.M.)
TSA	10	79	43 (54)*	21 (27)*	0	1.39±0.14	0.122±0.03
	24	80	26 (32)†	11 (14)†	2	1.27±0.21	0.107±0.04
	48	78	0 (0)‡	0 (00)‡	0	–	–
Scriptaid	10	80	80 (100)§	34 (43)§	0	1.42±0.09	0.124±0.03
	24	80	79 (99)§	37 (46)§	0	1.41±0.11	0.126±0.02
	48	80	78 (98)§	33 (44)§	0	1.42±0.12	0.124±0.02
Control (ICSI)		80	80 (100)§	38 (48)§	0	1.41±0.09	0.127±0.03

All the fertilized embryos were treated with 100 nM TSA or 250 nM scriptaid for 10, 24, and 48 h after sperm injection. The control group was untreated. *,†,‡,§Values with different superscripts within a column are significantly different ($P<0.05$).

Discussion

A variety of approaches have been used with the aim of improving the efficiency of SCNT cloning: changing the timing and conditions of activation and fusion (Park *et al*. 2001, Wakayama & Yanagimachi 2001*a*, 2001*b*, Martinez Diaz *et al*. 2003), treating donor cells or SCNT embryos with dimethyl sulfoxide (Wakayama & Yanagimachi 2001*a*, 2001*b*); TSA, or 5-aza-2′-deoxycytidine (Enright *et al*. 2003, Kishigami *et al*. 2006*a*, 2006*b*, Rybouchkin *et al*. 2006); altering the timing of removal of the oocyte chromosomes (Wakayama *et al*. 2003); serial NT (Wakayama *et al*. 2005); removal of donor cell cytoplasm (Van Thuan *et al*. 2006*a*, 2006*b*); and clone–clone aggregation (Boiani *et al*. 2003). Recent work (Kishigami *et al*. 2006*a*, 2006*b*, 2007) has shown that treatment of oocytes with TSA after NT can enhance the generation of cloned mice from hybrid B6D2F1 and outbred ICR mice. However, application of these methods to inbred mouse strains such as C57BL/6 and C3H/He has never been successful. Here, we report that treatment of SCNT oocytes with scriptaid for 10 or 24 h can produce cloned mice from all inbred strains examined and that these are viable and fertile. The placentae of cloned B6D2F1 and 129/Sv mice were significantly lighter than those of untreated control clones, although they were still heavier than the placentas of ICSI-fertilized and naturally mated fetuses. In addition, our results clearly show that the full-term developmental ability following SCNT in inbred mouse strains is donor cell strain dependent.

What is the mechanism involved in the reprogramming of somatic nuclei by scriptaid? We believe that during preimplantation development, the synthesis of new proteins to supplement those that had accumulated in the oocyte cytoplasm during oogenesis is essential for normal development. This process depends on transcription to produce new mRNA species. To test this, we examined the expression of nascent mRNA of cloned embryos at the two-cell stage. Interestingly, we found that only 2% of conventionally cloned two-cell embryos showed a high level of nascent mRNA, whereas scriptaid treatment resulted in a significant increase in transcriptional activity ($P<0.01$) and nascent mRNA expression pattern closely resembled those of ICSI-fertilized embryos, although at a lower rate. Kim *et al*. (2002) reported that at the late one-cell stage (12-h postactivation), however, nascent mRNA expressed with nearly the same intensity as that of parthenogenetic embryos. Here, we also found that at 12–16 h after activation, the transcription in cloned embryos was weakly detected and the same in both with or without scriptaid treatment (data not shown). Thus, the increase in histone acetylation in one-cell cloned embryos did not affect the transcriptional activity at the one-cell stage. At the two-cell stage cloned embryos, however, transcriptional activities were increased by scriptaid treatment during the first cell cycle. Even when conventional cloned embryos developed to the two-, four-, eight-cell stages, cleavage, compaction, and blastulation were delayed compared with ICSI-fertilized embryos and scriptaid-treated cloned embryos, and most of these cloned embryos degenerated by 9 days after implantation (Wakayama & Yanagimachi 1999). In the present study, our data show that the levels of nascent mRNA expression at the two-cell stage were correlated with the rate of full-term development in cloned embryos. One recent study on global gene expression in bovine SCNT cloned embryos at the blastocyst stage has shown that they are similar to *in vitro* fertilized embryos (Smith *et al*. 2005). However, that study examined total RNA expression in blastocysts, not the newly synthesized mRNA necessary for the production of proteins during the early embryonic genome activation to maintain the postimplantation development of embryo. Therefore, although cloned embryos can develop to the blastocyst stage at a high rate, the survival to birth is very poor in all species studied to date.

Importantly, the genotype of the donor cell had significant effects on new transcriptional activity at the two-cell stage of these SCNT cloned embryos. Significantly, more embryos showed positive levels of nascent mRNA when the donor cells were from the B6D2F1, DBA/2, and 129/Sv strains than embryos from C57BL/6 and C3H/He mice. Moreover, the levels of nascent mRNA

detected at this stage closely paralleled the rate of full-term development, regardless of the donor mouse strain. Although scriptaid could support full-term development of inbred cloned embryos, TSA could not. In the ICSI-fertilized embryo experiment, 100 nM TSA for 10 h resulted in 27% *in vivo* development to term; which was significantly less than for the control (and scriptaid) indicating its toxicity. Development was further reduced with TSA treatment for 24 h and was zero after 48 h (Table 4). Su *et al*. (2000) also reported that scriptaid is not lethal to cells even at high concentrations, whereas TSA is cytotoxic even at its optimal concentration. Moreover, TSA at its minimal toxic concentration was less efficient than scriptaid at inhibiting endogenous histone deacetylation (Su *et al*. 2000). When higher concentration or longer exposure time to TSA such as 500 nM and 20 h was used, full-term development of cloned embryos was obtained but dramatically reduced (Kishigami *et al*. 2006*a*, 2006*b*). In the present study, we could obtain offspring from B6D2F1 cloned embryos even if the concentration of scriptaid was increased to 2000 nM. Those results clearly indicate that scriptaid is much less toxic for embryos than TSA. It seems likely that embryos from inbred mice are more sensitive to TSA than those from hybrid mice, so only scriptaid could support their development. Interestingly, when BD129F1 hybrid mice (a triple-cross between C57BL/6, DBA/2, and 129/Sv) were used as donors, TSA treatment showed a higher success rate than scriptaid. This may suggest that the cloning success rate is affected not only by the toxicity of HDACi, but also by the reprogrammable potential of donor mouse strains, as indicated by the differences in the rates of nascent mRNA expression.

In conclusion, this report shows for the first time that treatment of SCNT oocytes with scriptaid during the first embryonic cell cycle can rescue the development of clones produced from important inbred mouse strains such as C57BL/6 and C3H/He, and can significantly increase the numbers of cloned offspring in the 129/Sv, DBA/2, and B6D2F1 strains. Moreover, treatment of cloned embryos with scriptaid for 24 h produced the same rate of full-term development compared with treatment for 10 h. This finding is very convenient as it overcomes logistical constraints to the cloning protocol. We believe that this scriptaid method will greatly advance the generation of transgenic inbred mice from cell lines or the rescue of strains of infertile mice via SCNT cloning.

Materials and Methods

Animals

B6D2F1 females were used as oocyte donors and B6D2F1, BD129F1, C57BL/6, 129/Sv, C3H/He, and DBA/2 were used as somatic cell donors. B6D2F1 male and female mice were used as a source of spermatozoa and oocytes for the production of embryos via ICSI. Females from the ICR strain were used as recipients for embryo transplantation. All mice were maintained in accordance with the Animal Experimental Hand Book at the Center for Developmental Biology, RIKEN-Kobe, Japan.

Production of cloned offspring using somatic cells from different mouse strains

After collection, the cumulus cells were dispersed with 0.1% hyaluronidase in drops of Hepes-buffered CZB (Hepes-CZB; Chatot *et al*. 1989) medium. The oocytes were transferred to new drops of Hepes-CZB and were denuded of almost all cumulus cells by gentle pipetting. Collected oocytes showing homogeneous ooplasm were selected and cultured in new drops of KSOM medium containing nonessential and essential amino acids (KSOMaa, Specialty Media Inc., Phillipsburg, NJ, USA) at 37 °C in an atmosphere of 5% CO_2 in air until use. After enucleation and NT, the reconstructed SCNT oocytes were activated by 10 mM $SrCl_2$ in Ca^{2+}-free CZB medium in the presence of 5 μg/ml cytochalasin B supplemented with 5–100 nM TSA (TSA group) or 50, 100, 250, 500, or 2000 nM scriptaid; 100 or 200 nM APHA compound 8 (APHA group) for 6 h, then subsequently cultured in KSOM with TSA or scriptaid or APHA at the same concentrations as above for the next 4 h. After three washes in KSOM, cloned embryos were cultured in the same medium for development. To examine the toxicity of HDACi, SCNT, and ICSI, one-cell embryos were continuously exposed to 100 nM TSA or 250 nM scriptaid either for 24 or 48 h from the time of activation or fertilization respectively, and the preimplantation development rates of cloned and fertilized embryo were examined from the two-cell to the expanded blastocyst stages. To produce offspring, two-cell embryos at 24 h or morulae and blastocysts at 72 h after SCNT or ICSI were transferred to pseudopregnant ICR surrogate mothers.

Preparation of TSA, scriptaid, and APHA

TSA, scriptaid, and APHA (Sigma) were dissolved in DMSO and prepared as 200× stock solutions and stored at −20 °C. These were added to the activation or culture media at a 1:200 dilution according to each experiment's protocol.

PCR analysis of genomic DNA

The microsatellite markers D1Mit26, D3Mit18, D14Mti10, and D10Mti3 were used to screen the genomic DNA of cloned C3H/He, 129/Sv, C57BL/6, and DBA/2 strain mice respectively. All DNA markers were amplified by primer pair sequences obtained from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). DNA samples of the cloned mice, donor cell mice, or donor oocyte mice (B6D2F1) were extracted from tail biopsy samples. PCR was carried out for 30 cycles and products were separated on 3% agarose gel before visualization.

Detection of nascent mRNA in embryos

Each group of embryos (<20) was washed twice in electropermeabilization medium (EP medium, 0.25 M D-glucose, 100 μM $CaCl_2{\cdot}2H_20$, 100 μM $MgSO_4$, 0.1%

polyvinylpyrrolidone) and then transferred into a drop of EP medium containing 10 mM BrUTP (Sigma) for 2 min. For EP, treated embryos were transferred into 10–20 μl EP medium containing 10 mM BrUTP between two parallel stainless-steel electrodes in a chamber and two electric pulses of 250 V/cm for 80 μs were applied from an Electro Cell Fusion (Bex LF101L, Tokyo, Japan) with a 2-min interval between pulses. Two minutes after permeabilization, embryos were cultured in CZB medium for 1 h at 37 °C in an atmosphere of 5% CO_2. The embryos were fixed in 4% paraformaldehyde in PBS containing 0.1% polyvinyl alcohol (PBS–PVA). The fixed embryos were washed twice in PBS–PVA and stored overnight at 4 °C in PBS supplemented with 3% BSA (PBS–BSA) and 0.1% Triton X-100 (Nacalai Tesque Inc., Kyoto, Japan). New transcripts incorporating BrUTP were visualized by indirect immunofluorescence (below).

Immunofluorescence procedures

These were as described by Van Thuan *et al.* (2006*a*, 2006*b*). Primary antibodies used here were mouse monoclonal anti-bromodeoxyuridine (Roche Diagnostics, Mannheim, Germany) and rabbit polyclonal anti-acetyl-histone H3-K9 (Upstate Cell Signaling Solutions, Charlottesville, VA, USA). The secondary antibodies were Alexa-Fluor-568-labeled goat anti-mouse IgG and Alexa-Fluro-488-labeled chicken anti-rabbit IgG antibodies. The DNA was stained for 30 min with 2 μg/ml 4,6-diamidino-2-phenylindole (Molecular Probes Inc., Eugene, OR, USA). To diminish errors in measuring nuclear volumes, the embryos in each repeat experiment were mounted on glass slides in the same volume (9 μl) of VectaShield antibleaching solution. Before quantification of fluorescence, the expression levels of nuclear histone acetylation and nascent mRNA in all entire embryos on the same slide were observed and recorded under an epifluorescence microscope.

Quantification of fluorescence intensity by laser-scanning confocal microscopy

Fluorescence was detected using a Fluoview FV1000 confocal scanning laser microscope (Olympus), and signals from the nuclei were quantified using Fluoview FV 1.4a software as follows. The mean of five regions of nucleus pixel value was subtracted from the mean of five regions of cytoplasmic pixel value and multiplied by the nuclear volume ($v=4\pi r^3/3$) to yield the relative values to compare histone acetylation and BrUTP incorporation. To quantify histone acetylation, the mean value of the control embryo (without treatment) at 6-h postactivation was set arbitrarily as 100%, and the fluorescence intensity observed in each sample of treated groups were expressed relative to this value. For quantitation of BrUTP, the maximum value for the fertilized embryos in each repeat experiment was set as 100% and the fluorescence intensities observed in other embryos were expressed relative to this value. We categorized nascent mRNA expression into four classes based on the fluorescent intensity (i) of each two-cell embryo (class I, $60\% < i \leq 100\%$; class II, $30\% < i \leq 60\%$; class III, $10\% < i \leq 30\%$; class IV, $i < 10\%$).

Statistical analyses

Student's *t*-test was used to calculate the significance of any differences between experimental groups in the immunofluorescence studies. Each experiment was repeated at least four to ten times per treatment. The data were subjected to arcsine transformation for each replication. The transformed values were analyzed using one-way ANOVA, and $P<0.05$ was considered to be statistically significant. The correlation coefficients between two variable rates of full-term development and nascent mRNA expression were statistically analyzed by *r* value.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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