Principles of Cloning

Second Edition

Jose Cibelli, John Gurdon, Ian Wilmut,
Rudolf Jaenisch, Robert Lanza,
Michael D. West and Keith H. S. Campbell

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Edited by

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About the Cover:
The first mammal ever cloned from adult somatic cells was a Dorset sheep named Dolly. In this cover twelve cloned sheep can be seen. Four of them are Dolly's clones, the rest are also clones, all produced by Dr. Keith Campbell's team at University of Nottingham, UK. In this specific project Dr. Campbell was testing the hypothesis that cloned sheep can be healthy and have a normal lifespan. Kathryn Campbell, and the Animal Care Staff, at the Bio Support Unit of University of Nottingham, UK, took this picture in February of 2013. Dolly's clones were more than six years old.

Notices
Knowledge and best practice in this field are constantly changing. As new research and experience broaden our understanding, changes in research methods, professional practices, or medical treatment may become necessary.

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In remembrance of Keith Henry Stockman Campbell (23 May 1954–5 October 2012)

Keith Henry Stockman Campbell
Raymond Page, Worcester, MA, USA.

Keith's legacy survives as the work described in many chapters of this book was made possible by his contributions to the field of developmental biology. However, to many, he was much more than a colleague. He was a close and dear friend always with a smile on his face and the life of the party – of which there were many. Keith believed the best ideas occurred after hours and was able to convince many of us to join him in the pursuit. His natural curiosity led him to additional adventures outside the lab including sport fishing, shooting, whitewater rafting, scuba diving and fixing up old Volkswagens. He could also be a bit of an artist in the kitchen. While achieving high stature in science, Keith maintained a blue collar way about him, lacking pretention and always willing to share ideas. While serious about his work, he never took himself too seriously. It was easy to be his friend and colleague. Though he travelled much and could not always be with them, Keith's daughters Lauren and Claire were always a topic of conversation. He was a proud father sharing their accomplishments and wanting the best for them. Keith's life was perhaps the most enriched by his wife Kathryn Campbell, whose unwavering devotion and companionship was palpable when in their company. Keith's legacy not only survives with the work represented in this Edition, but will continue to thrive with work that will be done by generations of scientists in years to come. New treatment tools for today's untreatable diseases as well as a deeper understanding of basic biological processes will be possible only because of Keith's scientific ingenuity, curiosity and search for truth was shared so generously with the rest of us.

This volume is dedicated to the lasting memory of Keith.

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Chapter 11

Enhancing SCNT with Chromatin Remodeling Agents

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Chapter Outline

INTRODUCTION

Since it was first reported in 1997 that a sheep had been cloned (Wilmut *et al*., 1997), more than 10 additional mammalian species have been cloned successfully using somatic cell nuclear transfer (SCNT). The success of SCNT gives promise to applications such as species preservation, livestock propagation, and cell therapy for medical treatment by nuclear transfer embryonic stem cells (NT-ESCs) (Wakayama *et al*., 2001, 2005a). While cloning efficiencies can range from 0% to 20% when measured as offspring born healthy as a proportion of the total SCNT embryos transferred, efficiency rates of only 1–2% are typical for mice. These inefficiencies have limited such practical applications of SCNT. Moreover, many abnormalities in mice cloned from somatic cells have been reported, including abnormal gene expression in embryos (Boiani *et al*., 2002; Bortvin *et al*., 2003; Kishigami *et al*., 2006a), abnormal placentas (Wakayama and Yanagimachi, 1999; Tanaka *et al*., 2001), obesity (Tamashiro *et al*., 2000, 2002), and early death (Ogonuki *et al*., 2002). These have been major issues for SCNT technologies to overcome.

Many trials attempting to overcome the inefficiencies of SCNT have been reported, and these can mainly be divided into four types as follows.

Type 1: improvement by optimizing SCNT protocols in each condition for oocyte activation (Kishikawa *et al*., 1999; Terashita *et al*., 2012), timing of oocyte activation (Wakayama and Yanagimachi, 2001a), timing of enucleation or injection of nucleus (Wakayama *et al*., 2003), and culture medium SCNT embryos (Boiani *et al*., 2005; Campbell *et al*., 2007), as well as a serial cloning (Ono *et al*., 2001).
Type 2: improvement by modified donor cell preparation, including optimized cell cycle, chemical treatment of donor cells (Enright *et al*., 2003), or optimizing donor-cell types (Inoue *et al*., 2003; Wakayama and Yanagimachi, 2001b; Wakayama *et al*., 2005b).
Type 3: improvement by chemical treatment of reconstructed oocytes.
Type 4: improvement by gene manipulation (Inoue *et al*., 2010; Matoba *et al*., 2011) or optimizing the genotype of donor cells (Inoue *et al*., 2003; Wakayama *et al*., 2005b).

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Although more or less significant improvement of SCNT has been reported in each type, the recent dramatic success achieved by treatment of reconstructed oocytes with histone deacetylase inhibitor (HDACi) has confirmed that SCNT can be improved at a practical level by using chemical agents.

ESTABLISHMENT OF A CONCEPT FOR DIRECT TREATMENT OF SCNT EMBRYOS WITH CHEMICAL AGENTS

The inefficiency of SCNT has been supposed to be attributed to epigenetic errors such as DNA methylation (Cibelli, 2007). Consistently, accumulating data have shown abnormal epigenetic modifications (Dean *et al*., 2001; Kang *et al*., 2001; Santos *et al*., 2003; Ohgane *et al*., 2004; Yamagata *et al*., 2007; Inoue *et al*., 2010), gene expression during pre- or post-implantation development (Humphreys *et al*., 2002; Suemizu *et al*., 2003; Inoue *et al*., 2006) and even after birth (Kohda *et al*., 2005), and chromosome segregation possibly by a particular epigenetic status of the centromere in SCNT (Mizutani *et al*., 2012). Therefore, it would be natural to aim at improvement of SCNT by correcting epigenetic modifications with chromatin remodeling agents.

The first success in direct treatment of SCNT embryos with chemical agents occurred accidentally in 2001, when 1% dimethyl sulfoxide (DMSO) in the activation medium was found to improve significantly the frequency of development to the blastocyst stage *in vitro* (Wakayama and Yanagimachi, 2001a), demonstrating that nuclear reprogramming can be enhanced artificially using chemical treatment, and establishing a concept of direct treatment of SCNT mouse embryos with chemical agents. Although the detailed mechanism underlying improvement by DMSO is unclear yet, DMSO treatment affects the DNA methylation status at multiple loci (Iwatani *et al*., 2006), and also possibly improves SCNT through changing epigenetic modifications.

The first success in direct treatment of SCNT embryos themselves with chemical agents occurred accidentally in 2001, when 1% dimethyl sulfoxide (DMSO) in activation medium was found to improve significantly the frequency of development to the blastocyst stage *in vitro*.

DISCOVERY OF THE OPTIMAL TREATMENT OF SCNT EMBRYOS WITH TRICHOSTATIN A

Trichostatin A (TSA), a histone deacetylase inhibitor (HDACi), is representative of a chromatin remodeling agent that enhances the pool of acetylated histones (Yoshida *et al*., 1990) and DNA demethylation (Hattori *et al*., 2004). Enright *et al.* found that 0.08-μM TSA treatment of donor cells increased blastocyst development compared to controls (35.1% vs 25.1%; Enright *et al*., 2003). However, to date, chemical treatment of donor cells has shown minimal improvement in SCNT.

On the other hand, regardless of the report on DMSO as early as 2001, direct treatment of SCNT embryos with TSA was not instantly successful. In 2006, two groups independently discovered the optimum concentration, timing, and period of TSA treatment for cloned mouse embryos (Figure 11.1) (Kishigami *et al*., 2006b; Rybouchkin *et al*., 2006). Eventually this method led to a greater than five-fold increase (e.g., from 0.3% to 6.5%) in the success rate of mouse cloning (Figure 11.2; see also Table 11.1, below) and a doubling in the rate of establishing ntES cell lines (Kishigami *et al*., 2006b). Based on these studies, the best protocol for TSA treatment in mice is: (1) reconstructed oocytes should be continuously exposed to TSA from the time point of oocyte activation for at least 10 h, but before the two-cell stage (Figure 11.3); (2) TSA concentrations of 5–50 nM as are recommended, as TSA becomes effective from 5 nM but shows toxicity at 500 nM (Figure 11.4). Notably, the optimal time window for TSA treatment is narrow and specific, such as the first 10 h after oocyte activation. This finding reveals that this time period is crucial for success of reprogramming of SCNT embryos, which determines their developmental fate, at least in mice. Further, this time period corresponds to that before the initiation of zygotic gene activation (ZGA), implying that this enhancement of reprogramming by TSA is related to change of epigenetic state but is not directly coupled with transcriptional activity during this time period. TSA treatment for longer or shorter time periods, or even different timing, significantly reduces the effectiveness of TSA treatment on developmental potential. It should be also noted that TSA treatment enables around 40% of reconstructed oocytes to produce ntES cells using B6D2F1 cumulus cells. Considering the establishment rate of normal ES cells from fertilized embryos, more than half of somatic cells should have the potential to be reprogrammed into ntES cells, supporting the stochastic model for reprogramming rather than the elite model (Yamanaka, 2009), in which most or all cells have the potential to become at least pluripotent. Further, recently it has been demonstrated that the gene expression profile in cloned neonatal mice shows more normalization by TSA treatment; the total gene expression profile of the TSA clones resembles that of the pups born following fertilization by ICSI (Kohda *et al*., 2012). Thus, TSA treatment evidently enhances nuclear reprogramming to increase the success rate of cloning.

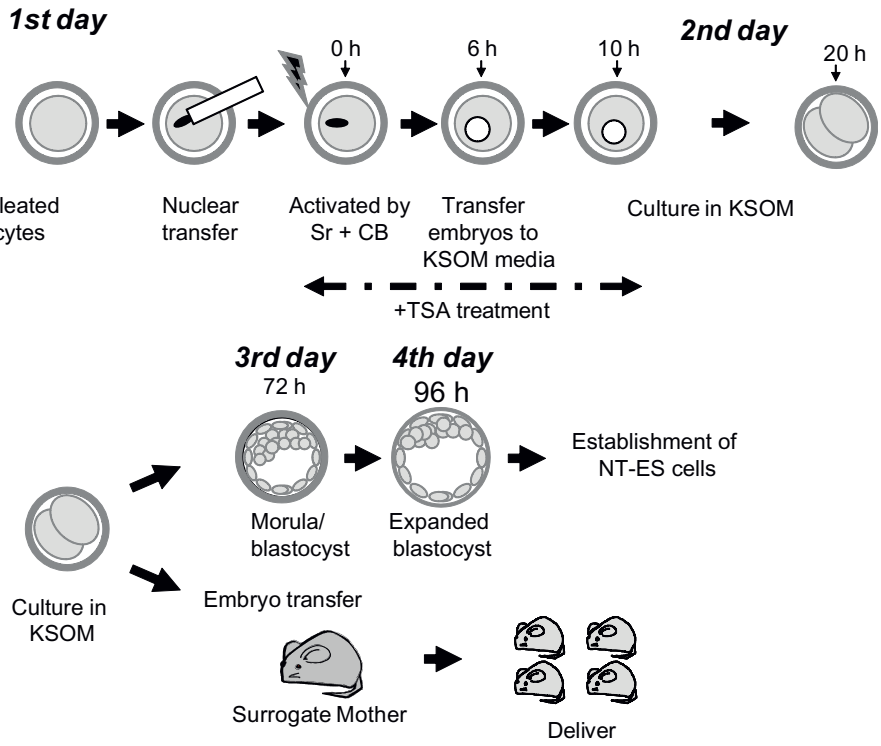

FIGURE 11.1 **An experimental scheme of TSA treatment.** According to the standard procedure of mouse cloning, donor nuclei from somatic cells are injected into enucleated oocytes. These reconstructed oocytes are activated by culture in the Ca^{2+}-free CZB medium including 5-mM Sr^{2+}, as well as TSA for TSA treatment except for the 14-h TSA treatment. After 6 h activation, the activation medium was changed to KSOM medium. For 10 h (or up to 20 h) of TSA treatment, activated oocytes are cultured in KSOM medium including TSA for another 4 h (another 14 h) and transferred into KSOM without TSA. These cloned embryos are cultured in KSOM until subject to embryo transfer into the surrogate mother (2nd day), examination of blastocyst formation (3rd or 4th day) or establishment for NT-ES cells (3rd or 4th day).

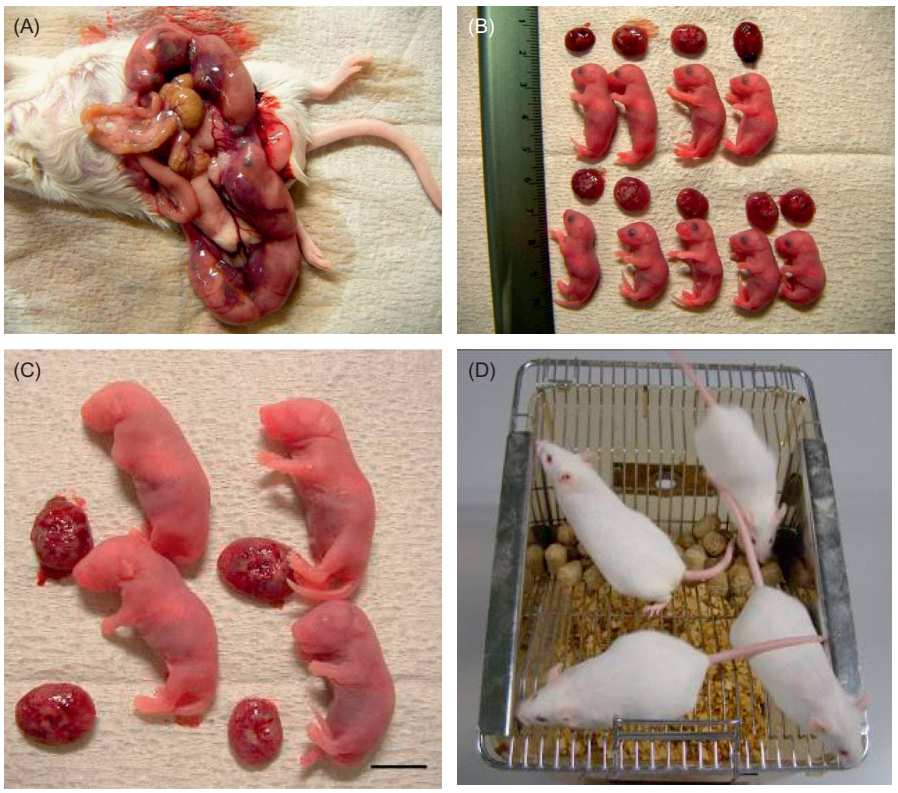

FIGURE 11.2 **Production of "normal" cloned mice after TSA treatment.** TSA treatment often led to multiple conceptions. In one case, five fetuses in one foster were seen at full term (A). Produced cloned mice treated by TSA showed no obvious abnormalities with the exception of a large placenta (B), as also seen in "normal" clones without TSA treatment. (C) TSA treatment also led to the success of cloning ICR mice from adult cumulus cells. After cesarean section, pups showed normal appearance but had large placentas (C). All the pups were weaned normally and on time (D).

TABLE 11.1 Summary of Effect of Histone Deacetylase Inhibitor (HDACi) Treatment on Embryonic Development in Each Species[a]

Species	Donor cell (strain)	HDACi	Conc.	Exposure Time (h)	Development to Blastocyst (vs control)	Development to Term (vs control)	Description	References
Cow	Fibroblast	TSA	50 nM	13	36% vs 30%	N.A.	No improvement	Iager *et al.*, 2008
	Fibroblast	TSA + 5-aza	50 nM + 10 nM	12(TSA) + 72(5-aza)	38% vs 13%	N.A.		Ding *et al.*, 2008
	Fibroblast	TSA	5 nM	20	31% vs 16%	N.A.	Donor-cell line dependent	Akagi *et al.*, 2011
	Fibroblast	TSA + 5-aza	50 nM + 10nM	12(TSA) + 72(5-aza)	N.A.	13% vs 3%		Wang *et al.*, 2011
	Fibroblast	TSA	50 nM	14	42% vs 22%	6.5% vs 7.4% per blast	100% vs 50% postnatal survival	Sawai *et al.*, 2012
Mouse	Cumulus (B6D2F1)	TSA	5 nM	10	75% vs 23 %	6.5% vs 0.3%		Kishigami *et al.*, 2006b
	Fibroblast (ICR)	TSA	5 nM	10	N.A.	4.2% vs 0%		Kishigami *et al.*, 2007
	Cumulus (C57BL/6)	SCR	250 nM	10	N.A.	2.3% vs 0%	Better than TSA	Van Thuan *et al.*, 2009
	Cumulus (C3He)	SCR	250 nM	10	N.A.	0.9% vs 0%	Better than TSA	Van Thuan *et al.*, 2009
	Cumulus (DBA/2)	SCR	250 nM	10	N.A.	6.5% vs 0%	Better than TSA	Van Thuan *et al.*, 2009
	Cumulus (129/Sv)	SCR	250 nM	10	N.A.	9.8% vs 2.4%	Better than TSA	Van Thuan *et al.*, 2009
	Cumulus (B6D2F1)	TSA +5-aza	100 nM + 10 nM	8	69% vs 69% (vs TSA only)	1% vs 13% (vs TSA only) at E10.5		Tsuji *et al.*, 2009
	Cumulus (B6D2F1)	SAHA	1.0 μM	10	70% vs 31%	9.4% vs 2.6%	Better than TSA	Ono *et al.*, 2010
	Cumulus (B6D2F1)	VPA	2 mM	10	57% vs 51%	8% vs 7% (BD129F1)	No effect	Ono *et al.*, 2010
	Cumulus (B6D2F1)	Ox	1.0 μM	10	63% vs 50%	7.5% vs 2.6%	Better than TSA	Ono *et al.*, 2010
	Cumulus (B6D2F1)	CBHA	20 μM	10	70% vs 33%	3.6% vs 0.8%	Better than TSA	Dai *et al.*, 2010
Pig	Fibroblast	TSA	50 nM	24	46% vs 18%	N.A.		Zhang *et al.*, 2007
	Fibroblast	TSA	37.5 nM	24	81% vs 54%	N.A.	Live piglets produced	Li *et al.*, 2008

	Fibroblast (NIH miniature pig)	SCR	500 nM	14–16	21% vs 9 %	1.3% vs0%		Zhao *et al.*, 2009
	Fibroblast (Landrace)	TSA SCR	50 nM 500 nM	10	23% vs 10 % 25% vs 11 %	0.8% vs0.4 % 1.6% vs 0.4%		Zhao *et al.*, 2010
	Fibroblast Bone marrow	TSA	10 ng/ml	10	45% vs 24 % 30% vs 27 %	11% vs 10 % 5% vs 0 %	Dependence on the nuclear cell type	Lee *et al.*, 2010
	Fibroblast	VPA	5 mM	24	41% vs 23%	N.A.	Increasing ICM cell no.	Kim *et al.*, 2011
	Fibroblast	TSA	50 nM	24	30% vs 15%	N.A.	11% vs 0% NT–ES lines	Vassiliev *et al.*, 2011
Rabbit	Fibroblast	TSA	100 nM	6	45% vs 23 %	N.A.		Shi *et al.*, 2008
	Cumulus (Hycole hybrid)	TSA	5 nM	10	79% vs 80 %	5% vs 3%	No adulthood after TSA treatment	Meng *et al.*, 2009
Rat	Fibroblast (SD and Wistar)	TSA	50 nM	10–14	N.A.	0% vs 0%		Sterthaus *et al.*, 2009
Inter-species (oocyte donor)								
Rabbit–Human	Fibroblast	TSA	100 nM	3	5% vs 2 %	N.A.	No significant difference	Shi *et al.*, 2008
Black-footed cat–Domestic cat	Fibroblast	TSA	50 nM	20	3.3% vs 3.3 %	0% vs 0%		Gomez *et al.*, 2011
Long-tailed macaque–pig	Fibroblast	TSA	10 nM	48	23% vs 10 %	N.A.		Qin *et al.*, 2012

TSA, trichostatin A; SCR, Scriptaid; SAHA, suberoylanilide hydroxamic acid; VPA, valproic acid; Ox, Oxamflatin; 5-aza, 5-aza-2′-deoxycytidine; CBHA, m-carboxycinnamic acid bis-hydroxamide.
[a]*The following species were studied: cow, mouse, pig, rabbit, and rat.*

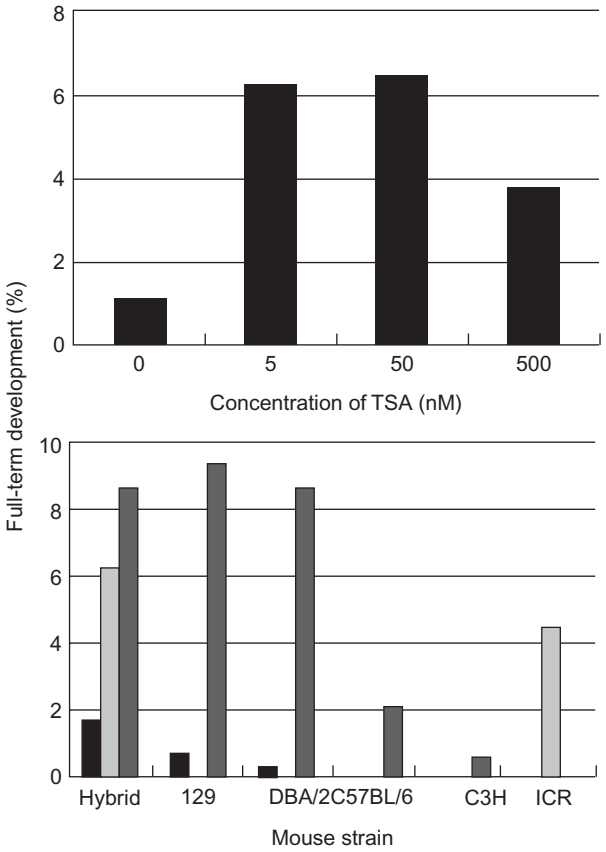

FIGURE 11.3 Effect of TSA treatment on development of cumulus clones, and optimization of exposure time period and concentration of TSA. The development % is shown as the ratios of (expanded) blastocysts after another 72 h, developed from two-cell cloned embryos 24 h after activation. To optimize the exposure time of TSA, 0, 6, 10, 14 and 20 h time periods were used as in Figure 11.1; 14-h TSA treatment was the same as 20-h treatment except for the use of activation medium without TSA.

Reprogramming by TSA is related to a change of epigenetic state but not directly linked to transcriptional activity.

Can TSA treatment of animal cloning be used for any type of cells in mouse? Significant improvement, to a greater or lesser degree, has been observed in cloning from any somatic cells, including cumulus, fibroblast, spleen, and neural stem cells, in mice. However, so far no success has been reported in improving ES-cell cloning by TSA treatment; rather, it appears to sabotage development (Kishigami *et al*., 2006b). The genomes of ES cells should already have an optimal epigenetic state at a reduced DNA methylation state which may be optimal for cloning. Therefore, TSA treatment in ES cloning may be toxic, as is high-dose TSA treatment in somatic cloning. In fact, TSA treatment following somatic cloning also led to a success rate of 6–7%, which is comparable to that of ES cloning

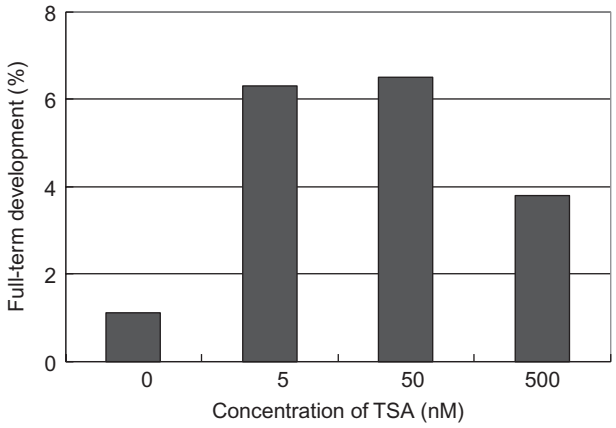

FIGURE 11.4 Optimal concentration of TSA for production of cloned mice. The effect of different concentrations of trichostatin A (TSA) on the efficacy of mouse cloning was examined by SCNT. When 5 or 50 nM of TSA was used, the success rate of cloned mice was significantly increased compare to control.

(~5%) (Wakayama *et al*., 1999). Therefore, it may be possible to interpret that TSA treatment of somatic cloning brings transferred somatic nuclei close to ES-like nuclei.

SCNT USING HDACi TREATMENT

Before the discovery of TSA treatment, most cloned mice were limited to hybrid strains and had never been cloned from outbred or inbred strains (Wakayama and Yanagimachi, 2001b; Inoue *et al*., 2003). Following the establishment of optimal TSA treatment conditions, it was found that TSA treatment was applied for producing cloned mice even from an outbred, supposedly "unclonable," strain (Kishigami *et al*., 2007), demonstrating that apparent "unclonability" of certain mouse strains does not indicate that nuclear reprogramming of those nuclei is impossible. Regardless, most of the important mouse strains have still not been cloned successfully. Subsequently, Scriptaid, another HDACi, was found to increase cloned embryo development not only in hybrid but also in inbred strains, and this allowed us to generate full-term offspring from several inbred mouse strains, such as C57BL/6 and C3H/He (Figure 11.5; Table 11.1) (Van Thuan *et al*., 2009).

Although TSA application resulted in great improvements in SCNT cloning in mice, the effects of TSA treatment on cloning efficiency in other species are still controversial.

Although TSA application resulted in great improvements in SCNT cloning in mice, the effects of TSA treatment on cloning efficiency have been controversial in cow (Iager *et al*., 2008; Wu *et al*., 2008), pig (Li *et al*., 2008;

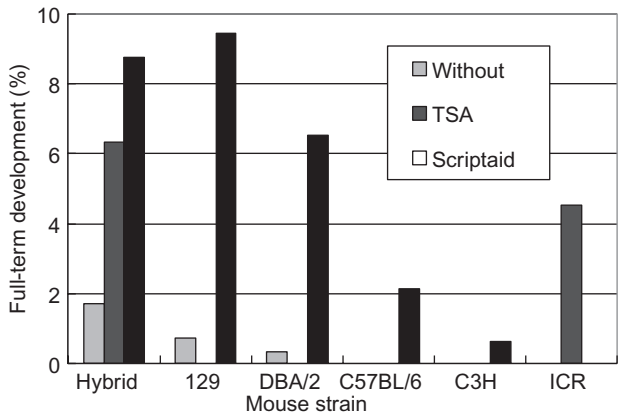

FIGURE 11.5 Production of cloned mice from "unclonable" strains using histone deacetylase inhibitors (HDACis). Without HDACi treatment, cloned mice could be obtained from the hybrid and 129/Sv strains, but with a low success rate. However, when Scriptaid was used, the overall success rate was increased even from inbred strains.

Yamanaka *et al*., 2009), rabbit (Shi *et al*., 2008; Meng *et al*., 2009), and rat (Sterthaus *et al*., 2009) (Table 11.1). Moreover, some groups have reported that TSA treatment had detrimental effects on the *in vitro* and *in vivo* development of the SCNT embryos (Wu *et al*., 2008; Meng *et al*., 2009). In experiments on rabbits, all cloned offspring treated with TSA died within 19 days of birth, whereas the untreated control clones grew to adulthood (Meng *et al*., 2009). On the other hand, it is known that the drug Scriptaid acts as an HDACi but is less toxic than TSA (Su *et al*., 2000). Using this drug, Zhao and colleagues managed to improve the success rate of pig cloning to full term (Zhao *et al*., 2009). To our knowledge, the effects of TSA treatment on full-term development have not been determined in any species other than the mouse. These results suggest that although the use of HDACi drugs can enhance reprogramming in cloned embryos, their toxicity means that the effects depend on the sensitivity of the donor cell type, strain, or species.

THE POSSIBLE MECHANISM UNDERLYING HDACi TREATMENT TO ENHANCE REPROGRAMMING

Although the underlying mechanism of how HDACi treatment improves cloning efficiency remains unknown, it is thought that it can induce hyperacetylation of the core histones, resulting in structural changes in chromatin that permit transcription and enhanced DNA demethylation of the somatic cell-derived genome following SCNT (Kishigami *et al*., 2006b), which is a necessary part of genetic reprogramming (Simonsson and Gurdon, 2004). In fact, several reports have clearly shown that HDACi treatment improved histone acetylation (Wang *et al*., 2007; Yamanaka *et al*., 2009), nascent mRNA production (Van Thuan *et al*., 2009), and gene expression (Tsuji *et al*., 2009) in a manner similar to that in normally fertilized embryos.

However, how histone methylation is modified in TSA-treated cloned embryos is not completely understood. Recently, TSA treatment was found to cause an increase in chromosome decondensation and nuclear volume in SCNT-generated embryos, similar to embryos produced by intracytoplasmic sperm injection (ICSI) (Bui *et al*., 2010). Histone acetylation was increased in parallel with chromosome decondensation. This was associated with a more effective formation of DNA replication complexes in treated embryos. Interestingly, the proportion of SCNT-generated embryos showing an asymmetric expression of nascent RNA between blastomeres was significantly reduced in the TSA-treated group compared with controls at the two-cell stage. These results suggest that the incomplete and inaccurate genome reprogramming of SCNT-generated embryos was improved by TSA treatment.

THE TARGETS OF HDACi TO ENHANCE NUCLEAR REPROGRAMMING

In general, the HDAC enzymes are divided into five categories: class I (HDAC1, 3, and 8), class IIa (HDAC4, 5, 7, and 9), class IIb (HDAC6 and 10), class III (SIRT 1–7), and class IV (HDAC11) (Blackwell *et al*., 2008). As shown in Table 11.1, so far seven classes of HDACi have been examined in our laboratory. TSA, Scriptaid, SAHA, and oxamflatin are inhibitors for class I and IIa/b HDACs (Marks *et al*., 2003; Zhang and Dent, 2005; Mukhopadhyay *et al*., 2006; Blackwell *et al*., 2008; Chuang *et al*., 2009; Codd *et al*., 2009; Kuhn *et al*., 2009). These significantly improved cloning efficiency. On the other hand, APHA is an inhibitor of class I and IIa/b HDACs, but is greater than 10-fold more active against HDAC3 (class I) and HDAC6 (class IIb) than the other HDACs (Mai *et al*., 2003; Blackwell *et al*., 2008). VPA is an inhibitor for class I and IIa HDACs (Chuang *et al*., 2009). However, these two drugs did not improve the mouse cloning success rate (Figure 11.6) (Ono *et al*., 2010).

Sirtinol is an inhibitor of class III HDAC, but its effect on cloning was very limited (Hirata, 2008). Thus, inhibition of class IIb HDACs (HDAC6 and 10, but most likely HDAC10) appears to be very important for improving the success rate in cloning mice (Table 11.2). It is known that the class IIb HDACs preferentially not only target nuclear histone deacetylase but also play important roles in the regulation of heat shock protein (HSP)-mediated vascular endothelial growth factor receptors (Park *et al*., 2008). These other pathways could have negative effects on embryo development or on complete genomic reprogramming following SCNT.

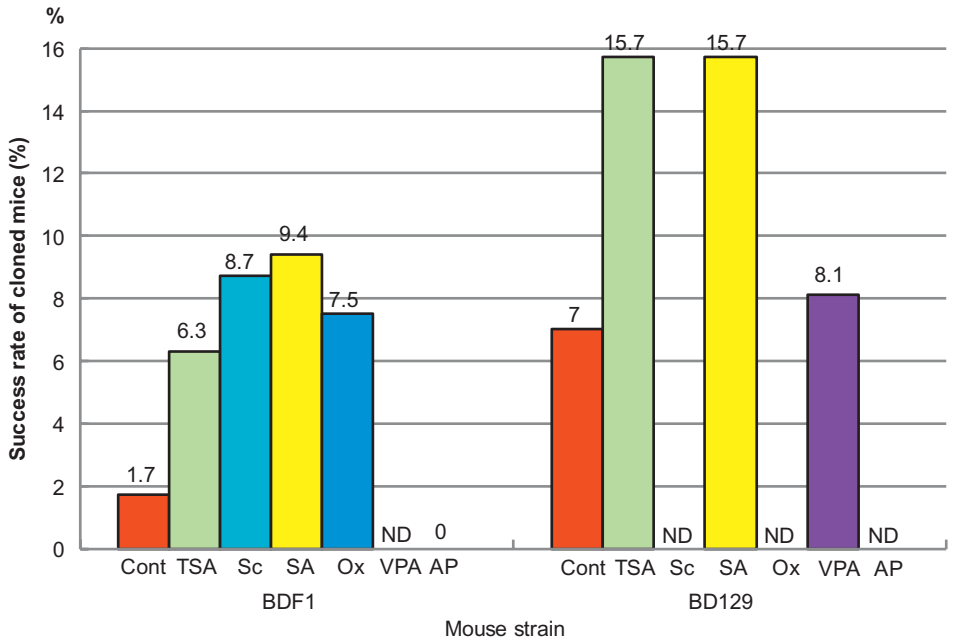

FIGURE 11.6 **Effects of histone deacetylase inhibitor (HDACi) treatment on mouse cloning.** Without HDACi treatment, cloned mice could be obtained but with a low success rate. When trichostatin A (TSA), Scriptaid (Sc), suberoylanilide hydroxamic acid (SA), or oxamflatin (Ox) were used, the success rates were increased with both BDF1 and BD129F1 strains; however, when valproic acid (VPA) or aroyl pyrrolyl hydroxamide (AP) were used, the overall success rate was not increased.

TABLE 11.2 Summary of Improvements in Mouse Cloning Outcome and Characteristics of Each Histone Deacetylase Inhibitor (HDACi)

Class of HDAC	Type of HDAC	Inhibitor						
		TSA	SCR	SAHA	Ox	APHA	VPA	SIRT
Class I	HDAC 1, 2, and 8	●	●	●	●	○	●	×
Class I	HDAC 3	●	●	●	●	●	●	×
Class IIa	HDAC 4, 5, 7, and 9	●	●	●	●	○	●	×
Class IIb	HDAC 6	●	●	●	●	●	×	×
Class IIb	HDAC 10	●	●	●	●	○	×	×
Class III	SIRT 1–7	×	×	×	×	×	×	●
Improvement in cloning?		Yes	Yes	Yes	Yes	No	No	Slight
Reference*		1	2	3	3	2	3	4

TSA, trichostatin A; SCR, Scriptaid; SAHA, suberoylanilide hydroxamic acid; Ox, oxamflatin; VPA, valproic acid; APHA, aroyl pyrrolyl hydroxamide; SIRT, sirtinol; UP, unpublished observation; ●, inhibit normally; ○, 10-fold lower; ×, no effect.
**1, Kishigami et al. (2006b); 2, Van Thuan et al. (2009); 3, Ono et al. (2010); 4, Hirata (2008).*

WHY DO CLONED EMBRYOS REQUIRE HDACi TREATMENT FOR BETTER GENOMIC REPROGRAMMING?

In nature, the oocyte cytoplasm contains reprogramming mechanisms, such as histone acetylation or DNA demethylation, that convert the sperm and oocyte nuclei to a totipotent state (Mayer *et al.*, 2000; Wang *et al.*, 2007; Feil, 2009). However, it is not yet clear whether these reprogramming factors are sufficient to reprogram the somatic cell nucleus, because the potential reprogramming machinery of the oocyte cytoplasm is prepared for the receipt of a gametic nucleus, not a somatic cell nucleus. In general, it is considered that the incomplete reprogramming of somatic

cell nuclei following SCNT arises from poor reprogramming in the oocyte. However, we now think that the oocyte cytoplasm might reprogram the somatic cell nucleus too strongly, or that the somatic cell nucleus is more sensitive to oocyte reprogramming factors than is the gametic cell nucleus. Therefore, by inhibiting a particular HDAC – especially a class IIb HDAC – during reprogramming, the donor nuclei in our studies were possibly reprogrammed more correctly (Kishigami *et al*., 2006b; Van Thuan *et al*., 2009), resulting in a higher success rate for cloning.

FURTHER STUDIES OF AGENTS THAT MAY IMPROVE SCNT

The DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-aza-dC), is one of the major agents affecting epigenetic memory to reduce DNA methylation. Although most attempts to improve SCNT using 5-aza-dC have ended in failure, such as 5-aza-dC treatment of oocytes and donor cells having a negative effect due to toxicity (Jones *et al*., 2001; Vignon *et al*., 2002; Enright *et al*., 2003; Shi *et al*., 2003; Tsuji *et al*., 2009), a combination of 5-aza-dC with TSA successfully increased preimplantation development of cloned bovine embryos and production of cloned calves, but did not do so in mouse (Table 11.1). In addition to histone acetylation and DNA methylation, it was reported that Vitamin C (also known as L-ascorbic acid or L-acerbate), which improves iPS-cell generation (Esteban *et al*., 2010), enhances *in vitro* and *in vivo* development of porcine somatic cell nuclear transfer embryos more than two-fold (Huang *et al*., 2011). Although Vitamin C is well known to be an antioxidant, it is supposed to enhance nuclear reprogramming via several pathways, including histone demethylation and *p53* repression (Shi *et al*., 2010). However, the mechanism in SCNT and its application for other species remains unclear to date. Further study is required to reveal the role of Vitamin C in SCNT.

Vitamin C, which improves iPS cell generation, also enhances *in vitro* and *in vivo* development of porcine somatic cell nuclear transfer embryos.

CONCLUDING REMARKS

The advent of cloning from adult-derived cells in 1997 marked a new departure in the study of key biological problems in NT biology (Wilmut *et al*., 1997), finally developing iPS cells technology in just one decade. Thus, reprogramming technologies have evolved rapidly. However, the reprogramming mechanism underlying SCNT still remains largely unclear after more than 15 years. The success in significant improvement of SCNT by HDACi has provided many clues in understanding the reason for the inefficiency of SCNT. Future study should be focused on identifying target HDACs of HDACis, as well as the target proteins of HDACs and their roles of embryonic development. Literature regarding successful improvement of SCNT development using agents other than HDACi is still limited. Further, this type of approach to find chemical agents to enhance reprogramming in SCNT could lead to understanding of the reprogramming mechanism in SCNT, thus making SCNT a truly practical tool.

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