

Improvement of mouse cloning using nuclear transfer-derived embryonic stem cells and/or histone deacetylase inhibitor

SAYAKA WAKAYAMA and TERUHIKO WAKAYAMA*

Riken Center for Developmental Biology, Kobe, Japan

ABSTRACT Nuclear transfer-derived ES (ntES) cell lines can be established from somatic cell nuclei with a relatively high success rate. Although ntES cells have been shown to be equivalent to ES cells, there are ethical objections concerning human cells, such as the use of fresh oocyte donation from young healthy woman. In contrast, the use of induced pluripotent stem (iPS) cells for cloning poses few ethical problems and is a relatively easy technique compared with nuclear transfer. Therefore, although there are several reports proposing the use of ntES cells as a model of regenerative medicine, the use of these cells in preliminary medical research is waning. However, in theory, 5 to 10 donor cells can establish one ntES cell line and, once established, these cells will propagate indefinitely. These cells can be used to generate cloned animals from ntES cell lines using a second round of NT. Even in infertile and "unclonable" strains of mice, we can generate offspring from somatic cells by combining cloning with ntES technology. Moreover, cloned offspring can be generated potentially even from the nuclei of dead bodies or freeze-dried cells via ntES cells, such as from an extinct frozen animal. Currently, only the ntES technology is available for this purpose, because all other techniques, including iPS cell derivation, require significant numbers of living donor cells. This review describes how to improve the efficiency of cloning, the establishment of clone-derived embryonic stem cells and further applications.

KEY WORDS: *clone, nuclear transfer, reprogramming, ntES cell*

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). While cloning efficiencies can range from 0% to 20%, efficiency rates of only 1%–2% are typical for mice. 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; Inoue *et al.*, 2010), abnormal placentas (Tanaka *et al.*, 2001; Wakayama and Yanagimachi, 1999), obesity (Tamashiro *et al.*, 2002; Tamashiro *et al.*, 2000), early death (Ogonuki *et al.*, 2002) and sex reversal (Inoue *et al.*, 2009). Such abnormalities notwithstanding, success in generating cloned offspring has still opened new avenues of investigation.

Recently, we found that the efficiency of mouse cloning could be enhanced up to fivefold through the addition of the histone deacetylation inhibitor trichostatin A (TSA) into the oocyte activation medium (Kishigami *et al.*, 2006b), even though high concen-

trations of TSA are toxic to embryonic development (Svensson *et al.*, 1998). This result suggests that nuclear reprogramming might be enhanced by chemical treatment and that this method will provide a new approach for practical improvements in mouse cloning techniques and new insights into the genomic reprogramming of somatic cell nuclei. However, the success rate of producing cloned offspring is still too low to apply for practical purposes such as for agriculture or industry. Moreover, this modified method can be used only for 'clonable' strain donor cells from particular strains or cell types. In the mouse, most strains cannot be used to generate cloned offspring, even with the use of TSA. In addition, live donor cells are required for SCNT. It was thought that even extinct species could be resurrected by SCNT from frozen cells, but it is almost impossible to apply current SCNT technology for this purpose.

Abbreviations used in this paper: ES, embryonic stem cell; HDACi, histone deacetylase inhibitor; ntES, nuclear transfer-derived ES cell; SCNT, somatic cell nuclear transfer; TSA, trichostatin A.

*Address correspondence to: Teruhiko Wakayama. Riken Center for Developmental Biology, 2-2-3 Minatojima-minamimachi, Kobe 650-0047, Japan. Fax: 81-78-306-0101. e-mail: teru@cdb.riken.jp

On the other hand, it is known that embryonic stem (ES) cell lines can be established from cloned embryos. The first success in generating ES-like cell lines by SCNT was initially performed in the bovine (Cibelli *et al.*, 1998) and then subsequently in the mouse (Kawase *et al.*, 2000; Munsie *et al.*, 2000). We have shown previously that these ES-like cell lines are capable of differentiating into all three germ layers *in vitro*, or even into spermatozoa and oocytes in chimeric mice (Wakayama *et al.*, 2001). This was the first demonstration that such ES-like cells have the same potential as ES cells from fertilized blastocysts. To distinguish NT-derived ES cell lines from those lines derived from fertilized embryos, the former are referred to as ntES cell lines (Wakayama *et al.*, 2001). Interestingly, such ntES cell lines can be established with success rates 10 times higher than from 'conventional' reproductive cloning (Fig. 1) (Wakayama *et al.*, 2006; Wakayama *et al.*, 2005b; Wakayama *et al.*, 2005c; Wakayama *et al.*, 1998; Wakayama *et al.*, 2001). Although almost all of the cloned mice showed some abnormalities and ntES cells were established using the same NT procedure, ntES cells are transcriptionally and functionally indistinguishable from the ES cells derived from fertilized embryos (Brambrink *et al.*, 2006; Wakayama *et al.*, 2006).

Unfortunately, human ntES cells pose ethical problems because fresh oocytes must be donated by healthy women and the resulting cloned embryos are deprived of their potential to develop into a complete human being. Therefore, although there are several reports proposing the use of human ntES cells as a model of regenerative medicine (Rideout *et al.*, 2002; Tabar *et al.*, 2008), the use of these cells in preliminary medical research is waning. However, for basic biology, the ntES cell techniques can be applied to characterize very rare and specialized cell types in the body, such as olfactory neurons. In theory, 5 to 10 donor cells can establish one ntES cell line and, once established, these cells will propagate indefinitely.

In this review we will describe another use of ntES cells, which is to generate cloned animals from ntES cell lines using a second round of NT (Thuan *et al.*, 2010; Wakayama, 2007; Wakayama *et al.*, 2001). Even in infertile and 'unclonable' strains of mice, we

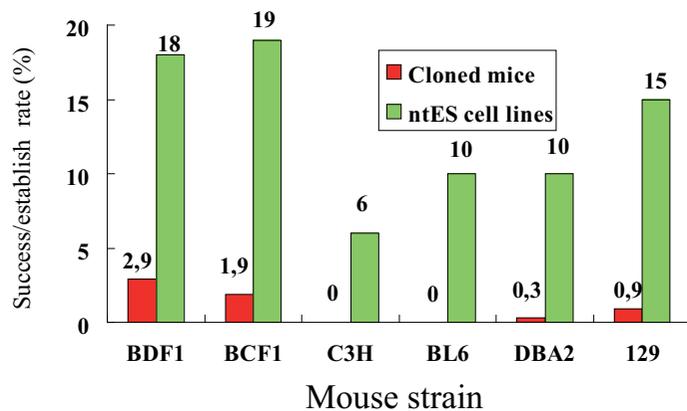


Fig. 1. Comparison of the success rate of cloned mice and establishment rate of nuclear transfer-derived embryonic stem (ntES) cell lines. The rate of ntES cell establishment is nearly 10 times higher than the rate of producing cloned mice. Interestingly, ntES cell lines can be established even from 'unclonable' mouse strains, such as C3H or C57BL/6 (BL6).

can generate offspring from somatic cells by combining cloning with ntES technology (Mizutani *et al.*, 2008; Wakayama *et al.*, 2005a; Wakayama *et al.*, 2005b). Moreover, cloned offspring can be generated potentially even from the nuclei of dead bodies or cells (Ono *et al.*, 2008; Wakayama *et al.*, 2008), such as from frozen extinct animals. Currently, only the ntES technology is available for this purpose, because all other techniques, including induced pluripotent stem (iPS) cell derivation, require significant numbers of living donor cells.

New attempts to improve the efficiency of mouse cloning

Before we start to describe ntES cell technology, we will introduce our recent progress in increasing the success rate of mouse cloning using chemical treatments such as the use of histone deacetylase inhibitors (HDACi). We previously reviewed many different approaches to improve the efficiency of mouse cloning (Wakayama, 2007), but most of those methods had no effect on the overall success rate. However, nearly 10 years ago we accidentally discovered that 1% DMSO could significantly improve the frequency of development to the blastocyst stage *in vitro* (Wakayama and Yanagimachi, 2001a). Although it is not clear why DMSO enhanced the developmental potential of cloned embryos, it was the first demonstration that nuclear reprogramming can be enhanced artificially using chemical treatment.

Epigenetic alterations by chemical treatment

As mentioned above, a recent molecular analysis of cloned embryos revealed abnormal epigenetic modifications such as aberrant DNA methylation and histone modification (Dean *et al.*, 2001; Kang *et al.*, 2001; Ohgane *et al.*, 2001; Santos *et al.*, 2003). Therefore, the prevention of epigenetic errors is expected to improve the success rate of animal cloning. Recently, Iwatani *et al.* discovered that DMSO treatment affects DNA methylation status at multiple loci (Iwatani *et al.*, 2006). DMSO probably enhances the epigenetic reprogramming of cloned embryos during *in vitro* culture. Enright *et al.* have tried to alter the epigenetic status of donor nuclei used for bovine cloning by using two chemicals: 5-azacytidine, an inhibitor of DNA methylation and TSA, an HDACi (Enright *et al.*, 2003). Although the *in vitro* developmental potential was increased significantly with TSA, the cells did not demonstrate full-term development. Because those epigenetically affecting drugs are very toxic (Gurvich *et al.*, 2005; Kishigami *et al.*, 2006c), each drug must be tested pharmacologically for its appropriate exposure, timing, concentration and duration.

Discovery of the optimal HDACi treatment for mouse cloning

In this situation, Kishigami *et al.* discovered by trial and error the optimum concentration, timing and period of TSA treatment for cloning mouse embryos. Eventually this method led to a greater than fivefold increase in the success rate of mouse cloning and a doubling in the rate of establishing ntES cell lines using cumulus cell, tail fibroblast, neural stem cell and spleen cell (Kishigami *et al.*, 2006b). These results were independently and concurrently corroborated by Tsunoda's laboratory (Rybouchkin *et al.*, 2006), showing that the effects of TSA are reproducible. On the other hand, most cloned mice have only been generated from

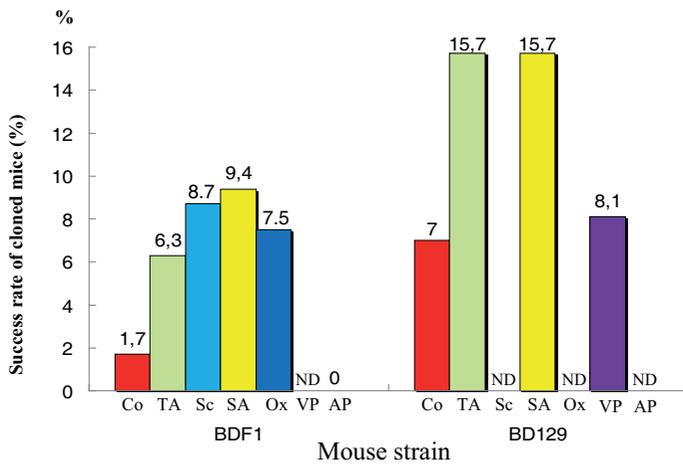


Fig. 2. Effects of histone deacetylase inhibitor (HDACi) treatment on mouse cloning. Without HDACi treatment, cloned mice could be obtained but with a low success rate. When trichostatin A (TSA), scriptaid (Sc), suberoylanilide hydroxamic acid (SA) or oxamflatin (Ox) were used, the success rates were 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.

hybrid strains and have never been cloned from outbred or inbred strains (Inoue *et al.*, 2003; Wakayama and Yanagimachi, 2001b). Although we found that TSA could be used to produce cloned mice even from an outbred supposedly ‘unclonable’ strain (Kishigami *et al.*, 2007), most of the important mouse strains have never been cloned successfully.

Although TSA treatment resulted in great improvements in SCNT cloning in mice, its effects on cloning efficiency are controversial for the bovine (Iager *et al.*, 2008; Wu *et al.*, 2008), pig (Li *et al.*, 2008; Yamanaka *et al.*, 2009), rabbit (Meng *et al.*, 2009; Shi *et al.*, 2008) and rat (Sterthaus *et al.*, 2009). Moreover, some groups have reported that TSA treatment had detrimental effects on the *in vitro* and *in vivo* development of SCNT embryos (Meng *et al.*, 2009; Wu *et al.*, 2008). To our knowledge, the effects of TSA treatment on full-term development have not been determined in any species other than the mouse. In experiments on rabbits, all cloned offspring treated with TSA died within 19 days after 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 *et al.* improved the success rate of pig cloning to full term (Zhao *et al.*, 2009). At the same time, Van Thuan *et al.* found that scriptaid treatment could increase cloned embryo development rates not only in hybrid but also in inbred strains of mice and this allowed us to generate full-term offspring from several inbred strains, such as C57BL/6 and C3H/He (Van Thuan *et al.*, 2009). These results suggest that although the use of HDACi drugs can enhance reprogramming in cloned embryos, because of their toxicity, the effects depend on the sensitivity of the donor strain or species. More recently, we found that two other specific HDACi agents, suberoylanilide hydroxamic acid (SAHA) and oxamflatin, could also improve the full-term development of cloned mice and establishment of ntES cell lines significantly without leading to obvious abnormalities (Ono *et al.*, 2010). Another group found that *m*-carboxycinnamic acidbishydroxamide

(CBHA) also improve the full-term mouse cloning (Dai *et al.*, 2010). However, although valproic acid (VPA) was reported to increase the reprogramming efficiency of mouse fibroblasts by more than 100-fold to establish induced pluripotent stem cells (Huangfu *et al.*, 2008), it had little (Costa-Borges *et al.*, 2010) or no effect (Ono *et al.*, 2010) on the success rate of mouse cloning. Another inhibitors, aroyl pyrrolyl hydroxamide (APHA), also could not improve cloning efficiency (Van Thuan *et al.*, 2009). Fig. 2 summarized the effect of HDACis on mouse cloning between BDF1 and BD129F1 cumulus cells (Kishigami *et al.*, 2006b; Ono *et al.*, 2010; Van Thuan *et al.*, 2009).

Which HDAC has a negative effect on nuclear reprogramming?

In general, the HDAC enzymes are divided into five categories: class I (HDAC 1, 3 and 8), class IIa (HDAC 4, 5, 7 and 9), class IIb (HDAC 6 and 10), class III (SIRT1 to 7) and class IV (HDAC 11) (Blackwell *et al.*, 2008). As shown in Table 1, so far seven classes of HDACi have been examined in our laboratory. TSA, scriptaid, SAHA and oxamflatin are inhibitors for classes I and IIa/b HDACs (Blackwell *et al.*, 2008; Chuang *et al.*, 2009; Codd *et al.*, 2009; Kuhn *et al.*, 2009; Marks *et al.*, 2003; Mukhopadhyay *et al.*, 2006; Zhang and Dent, 2005). These significantly improved the cloning efficiency. On the other hand, APHA is an inhibitor of classes 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 (Blackwell *et al.*, 2008; Mai *et al.*, 2003). VPA is an inhibitor for classes I and IIa HDACs (Chuang *et al.*, 2009). However, these two drugs did not improve the mouse cloning success rate (Ono *et al.*, 2010). Sirtinol is an inhibitor of class III HDAC but its effect on cloning was very limited (Hirata *et al.*, 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 1). It is known that the HDAC IIb preferentially target not only nuclear histone deacetylase but also plays important roles in the regulation of heat shock protein (HSP)-mediated vascular

TABLE 1

IMPROVEMENTS IN CLONING OUTCOME AND CHARACTERISTICS OF EACH HISTONE DEACETYLASE INHIBITOR (HDACi) USED

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 No.		1	2	3	3	2	3	4

Key: TSA, trichostatin A; SCR, scriptaid; SAHA, suberoylanilide hydroxamic acid; Ox, oxamflatin; VPA, valproic acid; APHA or aroyl pyrrolyl hydroxamide; SIRT, sirtinol; UP, unpublished observation; ⊙, inhibit normally; ○, 10-fold lower; ×, no effect.

Reference no 1: Kishigami *et al.*, 2006b; 2: Van Thuan *et al.*, 2009; 3: Ono *et al.*, 2010; 4: Hirata *et al.*, 2008.

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.

How does HDACi treatment 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 after SCNT (Kishigami *et al.*, 2006b), which is a necessary part of genetic reprogramming (Simonsson and Gurdon, 2004). In fact, several reports clearly showed 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. Bui *et al.* found that TSA treatment caused an increase in chromosome decondensation and nuclear volume in SCNT-generated embryos, similar to embryos produced by intracytoplasmic sperm injection (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 reduced in the TSA-treated group significantly compared with controls at the 2-cell stage. Those results suggest that the incomplete and inaccurate genome reprogramming of SCNT-generated embryos was improved by TSA treatment.

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 (Feil, 2009; Mayer *et al.*, 2000; Wang *et al.*, 2007). 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 gametic cell nuclei. Therefore, by inhibiting a particular HDAC—especially class IIb—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.

Combination of ntES cell approaches for mouse cloning

We proposed previously that ntES techniques could be applied to the biological sciences as a novel investigative tool. Potential investigations could be made using only this technique and not with conventional iPS technology. For example, Li *et al.* and Eggan *et al.* generated ntES cells from the nucleus of a single olfactory sensory neuron and then demonstrated that the odorant receptor gene was reset by SCNT, not accompanied by genomic alterations (Eggan *et al.*, 2004; Li *et al.*, 2004). Similarly, monoclonal mice have been generated from ntES cells derived from lymphocyte nuclei indirectly using tetraploid complementation (Hochedlinger and Jaenisch, 2002). Thus, ntES cell techniques can be applied to the characterization of very rare cells in the body. However, here, we propose another application of ntES cells: they can be used for reproduction of offspring from infertile or dead animals by combination with NT or by injection of ntES cells into the blastocyst to form a chimera (Figs. 3,4) (Wakayama *et al.*, 2005b).

Producing offspring from individual mice

The current success rate of generating cloned mice from adult somatic cell nuclei is very low, whereas the generation of cloned mice from ES cell nuclei is relatively high (Wakayama *et al.*, 2005c; Wakayama *et al.*, 1998). We have examined whether the success rate of cloning from somatic cells could be improved through the use of ntES cells established from the somatic cell

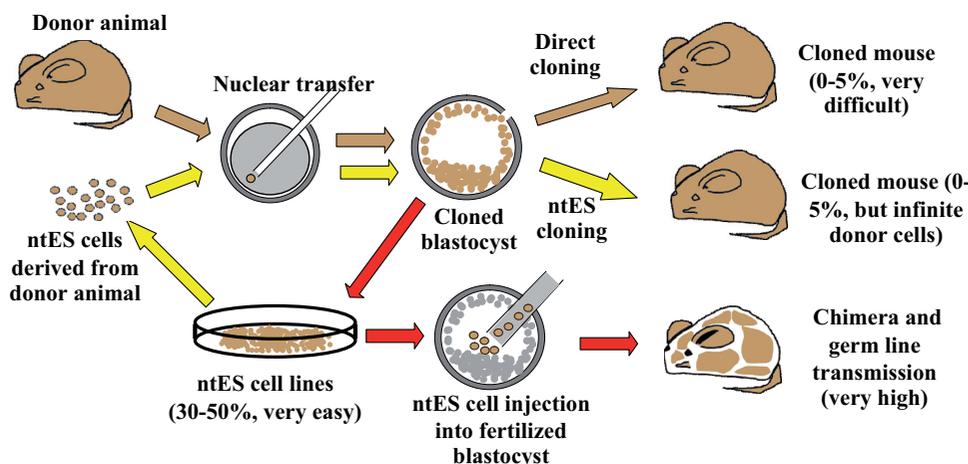


Fig. 3. Three different approaches to generate offspring from infertile, 'unclonable' or frozen dead mice. The blue arrow showed general nuclear transfer method (direct cloning). The success rate of mouse clone is 0-5%. The red arrow showed the establishment of ntES cell lines from cloned blastocysts and making chimera (for germ line transmission). Establish rate of ntES cell lines is very high (20-50%) and most of cell lines can differentiate to germ cell of chimera. The yellow arrow showed serial nuclear transfer method (ntES cloning). The success rate of mouse clone is 0-5%, same as direct cloning. However, donor cell became infinite. Therefore, you can repeat this experiment infinitely.

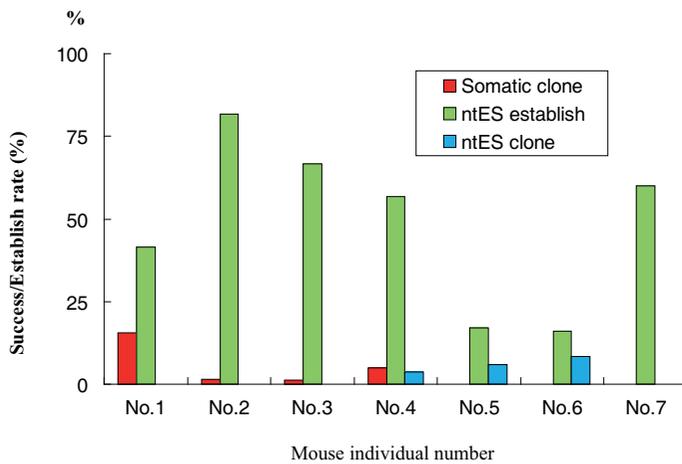


Fig. 4. Success rates of cloning mice and rate of establishing ntES cell lines in different individuals. In this experiment, seven mice were used as donors and cloned mice were obtained from four of them (Nos 1 to 4). The ntES cell lines were established from all donor mice at the same time with a very high success rate. Using a second round of nuclear transfer with ntES cells as the donors, cloned mice were obtained from two of the remaining three donor mice (Nos 5 and 6). Only one donor mouse (No. 7) failed to produce a cloned mouse by either somatic cell nuclear transfer or ntES cell cloning.

nuclei of the same individual (Wakayama *et al.*, 2005b). Although we obtained many cloned mice, the overall success rate of cloning in this way was no better than when using somatic cell nuclei. Importantly, even when using TSA, the current success rate of somatic cell nuclear transfer is still low and in this study, we could obtain cloned mice from four individuals out of seven donor mice. The other three mice have never been cloned. However, we could establish several ntES cell lines from all individuals and healthy cloned mice were obtained from two of the remaining three individuals (Fig. 4) (Wakayama *et al.*, 2005b). Therefore, although using ntES cells as nuclear donors does not ensure a better success rate for mouse cloning than that with somatic cells, we recommend the establishment of ntES cell lines at the same time to preserve and clone valuable individuals. These can be used as an unlimited source of donor nuclei for NT and can therefore complement conventional somatic cell NT cloning approaches (Wakayama *et al.*, 2005b). Unfortunately, if difficulty is experienced in producing cloned mice from a donor cell, it is also experienced when attempting to produce cloned mice by a second round of NT from ntES cell nuclei (Wakayama *et al.*, 2005a).

Producing offspring from infertile mice

The genetically modified mouse is a powerful tool for research in the fields of medicine and biology but they are often unhealthy and infertile. Overcoming this infertile phenotype is a challenge worth undertaking, as the ability to maintain such types of mutant mice as genetic resources would afford numerous advantages for research in human infertility and in the biology of reproduction. For this reason, we have tried to demonstrate that the ntES cell technology can be applied to generate offspring from such infertile mice. For example, senescent mice are often infertile and the cloning success rate decreases with age, making it almost impossible to produce cloned progeny directly from such mice. How-

ever, we succeeded in establishing ntES cell lines from aged mice (nearly 3 years old) regardless of sex or strain. The cloned mice were obtained from these ntES cells by a second round of NT (Mizutani *et al.*, 2008). We also achieved success using a mutant, hermaphroditic, sterile mouse found in our ICR mouse breeding colony (Wakayama *et al.*, 2005a). Although the mutant mouse died accidentally soon after tail-tip biopsy and we failed to produce cloned mice from those cells, we could establish several ntES cell lines. Using those ntES cell lines, we generated chimeric mice by injecting them into normal embryos and finally one diploid chimeric male transmitted most of its mutant mouse genes to the next generation via the ntES cells. Thus, this technique is potentially applicable for the propagation of a variety of animals or important mutant genes, regardless of age or fertile potential.

The possibility of resurrecting an extinct animal

Cloning animals by SCNT provides an opportunity to preserve endangered mammalian species as when viable cells can be collected from frozen bodies, it is possible to generate cloned animals (Hoshino *et al.*, 2009). However, the 'resurrection' of extinct species from permafrost (such as the woolly mammoth) is thought to be impractical, because no live cells will be available. On the other hand, it is known that 'dead' sperm from freeze-drying treatments (Wakayama and Yanagimachi, 1998) or from a whole frozen body (Ogonuki *et al.*, 2006) still possess the complete haploid genome, and when such sperm are injected into oocytes, the resulting embryos can develop to full-term healthy offspring. Recently, successful SCNT using freeze-dried cells was reported. Loi *et al.* generated cloned bovine blastocysts from freeze-dried somatic cells preserved for 3 years at room temperature (Loi *et al.*, 2008). This report was the first to demonstrate that even freeze-dried somatic cells have developmental potential after NT, but the authors could not determine whether the blasto-

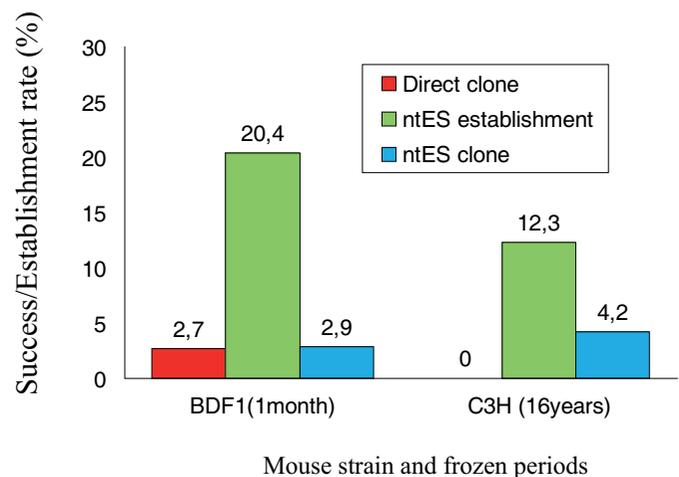


Fig. 5. Production of offspring from frozen dead mice. The outcomes for BDF1 mice frozen for 1 month and C3H mice frozen for 16 years were compared between direct SCNT cloning, the rate of establishment of ntES cells from cloned blastocysts, and ntES cell cloning. For the long-term frozen mice, direct cloning failed, probably because these were an inbred 'unclonable' strain rather than because of the duration of storage. However, several ntES cell lines were established from both sets of tissues and many healthy cloned mice were obtained by successive rounds of NT using these cell lines.

cysts were normal. On the other hand, we established ntES cell lines from freeze-dried mouse cells (Ono *et al.*, 2008). Importantly, after chimera construction experiments, these ntES cell lines could contribute into all organs including germ cells, which suggests that the genomic integrity of somatic cells can be maintained after freeze-drying and that it is possible to produce offspring from such cells using SCNT.

We have also attempted to produce cloned mice from bodies kept frozen at -20°C for up to 16 years without any cryoprotection. These conditions are similar to those of a frozen body recovered from permafrost. Although we could not produce cloned offspring from the somatic cells directly, several ntES cell lines were established from the cell nuclei of most organ cells, irrespective of the duration of preservation. Surprisingly, frozen brain was the best tissue for generating cloned embryos, even though fresh brain tissue has proven to be one of the most difficult sources of nuclei for the successful production of cloned mice (Mizutani *et al.*, 2006; Yamazaki *et al.*, 2001). Finally, healthy cloned mice were produced from these ntES cells by a second round of NT (Fig. 5) (Wakayama *et al.*, 2008). Thus, these techniques could be used to resurrect animals or to maintain genome stocks from tissues that have been frozen for prolonged periods or even when no live cells are available.

Perspectives

The mouse is a most popular experimental animal and the advent of mouse cloning from adult-derived cells in 1998 marked a new departure in the study of key problems in cloning biology. Unfortunately, the success rate of SCNT cloning is still very low and the mechanisms involved in reprogramming the epigenome are not yet clear. Therefore, the SCNT method has been thought of as a 'black box approach' and inadequate to determine the detail of how genomic reprogramming occurs (Thuan *et al.*, 2010). However, only the NT approach can reveal dynamic and global modifications in the epigenome without using genetic modification and can give important hints to the reprogramming mechanism. Moreover, the NT approach remains the only valid way to study biology, especially in generating offspring from a single cell or even from dead cell nuclei. The abnormalities seen in cloned animals pose a major obstacle for application but this could be resolved when the mechanisms of genomic reprogramming are better understood. We believe that the mechanisms of reprogramming will be clarified when cloning efficiency is improved through technical advances.

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