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—Review—

How to Improve the Success Rate of Mouse Cloning Technology

Nguyen Van THUAN[1)], Satoshi KISHIGAMI[2)] and Teruhiko WAKAYAMA[3)]

[1)]Department of Animal Bioscience and Biotechnology, Konkuk University, Seoul 143-701, South Korea, [2)]Department of Genetic Engineering, KINKI University, Wakayama 649-6493 and [3)]Center for Developmental Biology, RIKEN Kobe, Kobe 650-0047, Japan

Abstract. It has now been 13 years since the first cloned mammal Dolly the sheep was generated from somatic cells using nuclear transfer (SCNT). Since then, this technique has been considered an important tool not only for animal reproduction but also for regenerative medicine. However, the success rate is still very low and the mechanisms involved in genomic reprogramming are not yet clear. Moreover, the NT technique requires donated fresh oocyte, which raises ethical problems for production of human cloned embryo. For this reason, the use of induced pluripotent stem cells for genomic reprogramming and for regenerative medicine is currently a hot topic in this field. However, we believe that the NT approach remains the only valid way for the study of reproduction and basic biology. For example, only the NT approach can reveal dynamic and global modifications in the epigenome without using genetic modification, and it can generate offspring from a single cell or even a frozen dead body. Thanks to much hard work by many groups, cloning success rates are increasing slightly year by year, and NT cloning is now becoming a more applicable method. This review describes how to improve the efficiency of cloning, the establishment of clone-derived embryonic stem cells and further applications.

Key words: Clone, ntES cell, Nuclear Transfer, Reprogramming

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Since it was first reported in 1997 that a sheep had been successfully cloned [1], the mouse [2], bovine [3], goat [4], pig [5, 6], gaur [7], mouflon [8], domestic cat [9], rabbit [10], horse [11], mule [12], rat [13], African wildcat [14], dog [15], ferret [16], wolf [17] red deer [18], sand cat [19], ibex [20] and camel [21] are among the mammalian species that have been generated successfully as cloned animals using somatic cell nuclear transfer (SCNT). While cloning efficiencies can range from 0 to 20%, efficiency rates of just 1–2% are typical in mice. Moreover, many abnormalities in mice cloned from somatic cells have been reported, including abnormal gene expression in embryos [22–24] abnormal placentas [25, 26], obesity [27, 28], and early death [29]. Such abnormalities notwithstanding, success in generating cloned offspring has still opened new avenues of investigation.

If cloning success rates depend on the donor cell type, we should be able to determine what is most important for reprogramming. For this reason, many different donor cell types have been examined in efforts to understand genomic reprogramming and how to improve the success rate of cloning by SCNT. The most popular cell types as nuclear donors are cumulus cells [2], tail tip cells (probably fibroblasts) [25, 30], Sertoli cells [31] and embryonic stem (ES) cells [32]. As completely differentiated cells, natural killer T cells [33] and granulocyte [34] have been used. As partially undifferentiated cells, fetal neuronal cells [35], hematopoietic stem cells [36], neuronal stem cells [37, 38] and keratinocyte stem cells [39] have also been used. Immature primordial germ cells were also examined [40]. Unfortunately, all cell types show very low success rates for full-term cloning.

If we could produce higher success rates using the same donor cell type, this would suggest that genomic reprogramming could be enhanced artificially. Therefore, a variety of different methods have been trialed, such as oocyte activation [41], cell fusion *vs*. nuclear injection [30], enucleation timing [42], and inhibition of cytokinesis [43] (for review see [44]). However, again, most of these trials have failed to improve the success rate of cloning mice. Recently, we found that the efficiency of mouse cloning could be enhanced by up to five-fold through the addition of the histone deacetylation inhibitor trichostatin A (TSA) into the oocyte activation medium [45], even though high concentrations of TSA are toxic to embryonic development [46]. This result suggests that nuclear reprogramming might be enhanced by chemical treatment, and 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.

On the other hand, we have shown that nuclear transfer-derived ES cell lines possess the same potential as do ES cells produced from fertilized blastocysts [47]. To distinguish nuclear transfer-derived ES cell lines from those lines derived from fertilized embryos, the former are referred to as ntES cell lines [48]. Although ntES cells have been shown to be equivalent to ES cells, there are ethical objections concerning human cells because fresh oocytes must be donated by healthy women and the resulting cloned embryos would be deprived of their potential to develop into a complete human being. To avoid these ethical objections, several approaches have been attempted and some problems have been solved. For example, aged oocytes from failed *in vitro* fertilization (IVF) attempts were used instead of freshly donated oocytes [49].

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Correspondence: T Wakayama (e-mail: teru@cdb.riken.jp)

The use of induced pluripotent stem (iPS) cells for cloning has no ethical problems and is a relatively easy technique, whereas ntES cells pose serious ethical problems and cloning from them is extremely difficult. Therefore, although there are several reports proposing the use of ntES cells as a model of regenerative medicine [50, 51], 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. Another example is to generate cloned animal from ntES cell lines using a second round of NT [48]. Even in infertile and 'unclonable' strains of mice, we can generate offspring from somatic cells by combining cloning with ntES technology [52–54]. Moreover, cloned offspring can be generated potentially even from the nuclei of dead bodies or cells [55, 56], 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. Previously we have reviewed and described details of the features of cloning and ntES cell technology for both basic and applied investigations up to 2007 [44]. Here, we will describe recent updates.

Abnormalities Found in Cloned Mice

The current SCNT method causes numerous abnormalities in cloned mice that are probably related to the efficiency of successful cloning. We review these clone-specific abnormalities briefly below before considering how we can improve the efficiency of cloning.

Abnormalities in embryonic and extraembryonic lineages

Most cloned embryos die during the pre- and peri-implantation periods of gestation [57] and almost all clones showed abnormal placentas when full-term offspring were examined (Fig. 1A). When imprinted gene expression was examined in full-term placentas, some of the genes showed aberrant expression [58]. Therefore, it was thought that the abnormal establishment of extraembryonic lineages within the developing embryo caused the inefficiency of cloning. However, loss of and reduced pluripotency in the inner cell mass (ICM) lineage of cloned blastocysts has also been reported [22]. We have previously examined the trophectoderm (TE) and ICM lineages using Cdx2 and Oct4, key molecules for the specification of TE and ICM fate, respectively [24]. The frequency of Oct4 expressing blastocysts was as low as 50%, as previously reported [22]; however, regardless of Oct4 expression, the majority of the cloned blastocysts (>90%) expressed Cdx2 normally. This result was supported by a recent report in which trophoblast stem (ntTS) cells from cloned mouse blastocysts were established with an efficiency as high as that from fertilized blastocysts [59]. A comprehensive analysis of the transcriptional and epigenetic traits demonstrated that these ntTS cells were indistinguishable from control TS cells. These results suggest that even though SCNT cloned embryos have a reduced potential to produce the ICM lineage, the TE lineage can be established and maintained relatively normal.

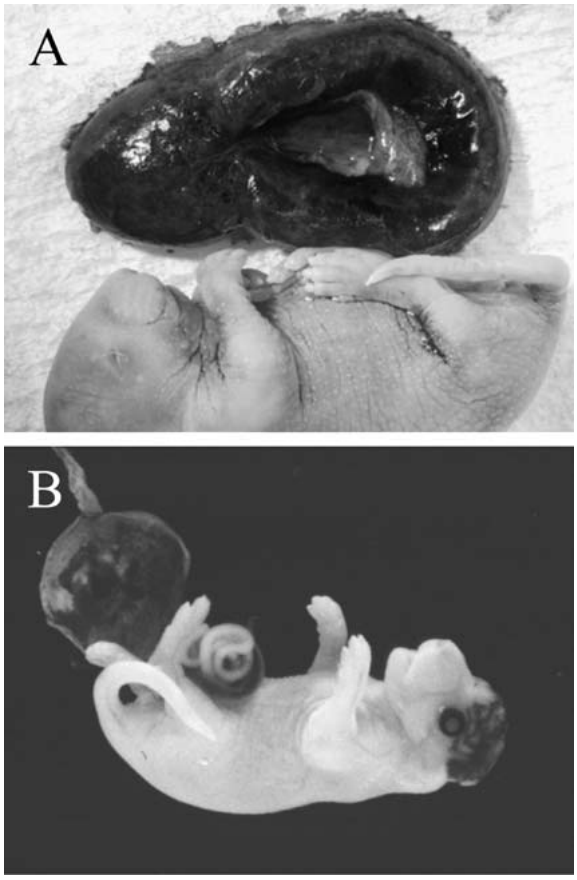

Fig. 1. Abnormalities in cloned mice. (A) All cloned mice were born with an extremely large placenta and this was the heaviest in our records. (B) A few cloned mice showed several other abnormalities. This pup was born alive but showed intestinal herniation and lacked a skull.

Ogura's group also obtained similar results using a different approach [60]. Embryos aggregated with diploid (2n) cloned and tetraploid (4n) fertilized embryos developed hyperplastic placentas. By contrast, placentas of the reciprocal combination—2n fertilized and 4n cloned embryos—were less hyperplastic. This result suggests that embryonic rather than extraembryonic tissues had more impact on the onset of placental hyperplasia, and that the abnormal placentation in clones occurs in a non cell-autonomous manner.

Epigenetic abnormalities in cloned offspring and their placentas

Recent molecular analyses of cloned embryos have revealed abnormal epigenetic modifications such as DNA methylation and histone modifications [61–63]. Moreover, several abnormalities were found not only in early embryos, but also in full-term offspring (Fig. 1B. Therefore, many cloned animals died soon after birth from respiratory failure or from other abnormalities [32, 64] and many cloned mice die prematurely because of liver necrosis, tumors, and pneumonia [29]. These abnormalities presumably reflect a dysregulation of gene function in cells, which may be

accounted for by aberrant genomic reprogramming—the failure to completely reset the differentiated state of the host cell [58, 65–67]. These findings indicate that a cloned mouse is not a perfect copy of the original mouse in terms of placental development, body weight, or the methylation status of its genomic DNA [63].

However, even though most cloned mice are abnormal and show placentomegaly or skewed X-chromosome inactivation in different tissues or individuals [68], some of them are able to develop into adulthood, which indicates that perfect reprogramming or maintenance of the methylation state is not essential for the overall success of mammalian cloning. Interestingly, those epigenetic abnormalities were corrected with increased age and became similar to those seen in normal mice [69]. Hence, some clones survive as long as non-clones. For example, the first cloned mouse, Cumulina, died at the age of 2 years and 7 months, which is slightly longer than the lifespan of an average mouse (News in Brief; Nature 2000, 405:268). Importantly, those cloned mice-specific abnormalities are not heritable and are absent from the progeny of both clones mated with clones and clones mated with wild type mice [27, 70]. This implies that epigenetic abnormalities, such as imprinting and/or reprogramming phenomena caused by the SCNT procedure, can be corrected during gametogenesis. Therefore, even if cloned animals from endangered species die from their abnormalities, we still have the possibility to rescue them by collecting the gonads because the gametes are probably epigenetically normal [71].

Genetic abnormalities in cloned mice and their offspring

As mentioned above, it was thought that most abnormalities in cloned animals arise epigenetically. However, Kawasumi *et al*. [72] reported that nuclear fragmentation was observed in 30% of 2-cell cloned mouse embryos, which suggests that genetic abnormalities might also cause cloning inefficiency. Recently two important papers were published. Balbach et al. found a short tailed mouse when they did cumulus cell NT cloning [73]. Although they failed to identify the genetic abnormality, this phenotype was inherited to the naturally mated offspring. Inoue et al. found more clear evidence of genetic abnormality caused by NT [74]. They reported the accidental birth of a female mouse following SCNT using a male donor. This 'male-derived female' clone grew into a normal adult and produced offspring by natural mating with a littermate. Chromosomal analysis revealed that the female clone had a 39, X karyotype, indicating that the Y chromosome had been deleted in the donor cell or at some early step during the SCNT procedure. For these reasons, we began to examine the genetic constitution of cloned embryos using a newly developed live cell imaging system [75], and found that more than half of the cloned embryos showed aberrant chromosome segregation during preimplantation development (unpublished). If this result is common to all cloned embryos, it may suggest that the inefficient cloning success rate is not only caused by epigenetically incomplete reprogramming but also arises from genetic damage caused by the SCNT procedure or occurring in the somatic cell donor.

Where are the Genomic Reprogramming Factors?

Zygotes are inappropriate recipients

Historically, the first report of cloned mammals was with mice, which were generated by the coordinated microinjection of ICM nuclei into zygotes and the immediate removal of the preexisting pronuclei [76]. However, this replacement procedure has been controversial as all subsequent attempts failed to reproduce the result [77]. Therefore, enucleated metaphase II oocytes have been the preferred recipients in almost all cloning procedures performed during the past 20 years. We have demonstrated that the use of zygotes causes serious damage to the transferred donor nuclei, and we have concluded that there is no firm reason for accepting the claim of mouse cloning success using zygotes [78].

Enucleation removes factors beneficial for developmental potential from the zygote

Modlinski *et al*. [79] demonstrated clearly that critical genomic reprogramming factors are present inside the zygotic pronuclei rather than in the cytoplasm and that these reprogramming factors are removed upon enucleation. The authors developed an alternative method for enucleation that allows for the removal of the pronuclear envelope with the chromatin attached and leaves the reprogramming factors in the zygotic cytoplasm. Egli *et al*. [80] and Piliszek *et al*. [81] confirmed this finding with different methods, but thus far no clones have been born from adult somatic cells using this method, suggesting that reprogramming factors are present in zygotes but that their efficiency is lower than the factors in oocytes.

Ogushi *et al*. [82] reported that there is a vital factor inside the zygote nuclei that is essential for any embryo development, regardless of the integrity of the reprogramming. This suggests that if the nuclei are removed from a zygote, the essential factors are also removed and therefore the subsequent cloned embryo fails to develop even if complete reprogramming occurs. Modlinski's group confirmed this using selective enucleation and concluded that nuclear material is essential for the cleavage division [83]. Moreover, we found that the cytoplasmic extract from enucleated oocytes promoted somatic cell reprogramming, and the success rate of producing cloned offspring increased [84]. This suggests that at least some genomic reprogramming factors are located in the cytoplasm and that a vital factor essential for full-term development is present in the nucleus.

Can we enhance the oocyte's reprogramming capacity?

One possible explanation of the low success rate of cloning is that the reprogramming factor level of each oocyte is insufficient or not properly adapted for the receipt of a somatic cell nucleus, because it is naturally prepared only for the receipt of a haploid male gamete. If this is simply caused by an insufficiency of the reprogramming factor(s), cloning success rates might be improved by adding supplementary oocyte cytoplasm before the nuclear transfer procedure. For this reason, we constructed oocytes with volumes two to nine times greater than normal by the electrofusion or mechanical fusion of enucleated oocytes, and injected somatic cell nuclei (Fig. 2). However, none of these giant oocytes could

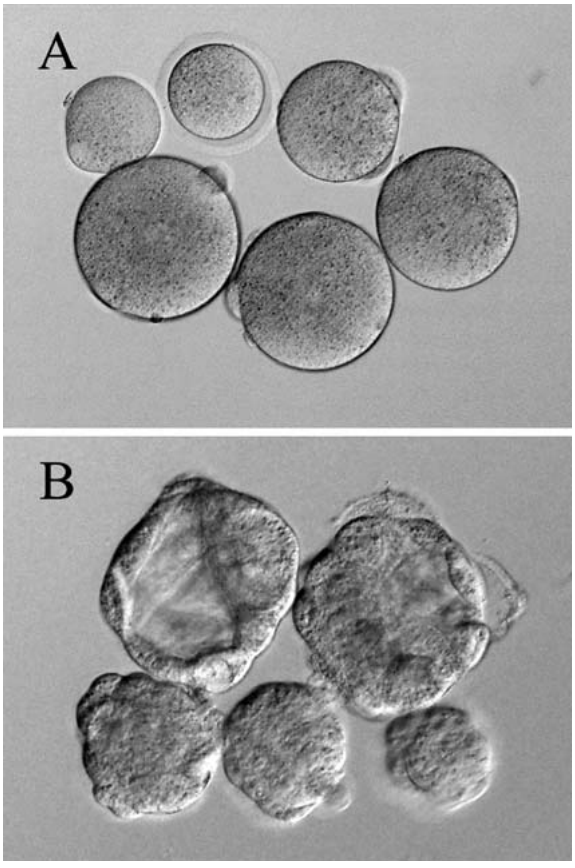

Fig. 2. Large oocytes and embryos generated by electrofusion. (A) One to five times normally sized oocyte. (B) Fertilized giant embryos at four days of culture *in vitro*.

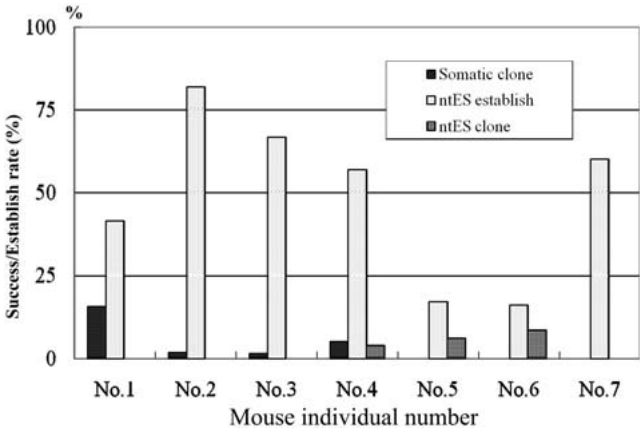

Fig. 3. Success rate of cloning mice and rate of establishing nuclear transfer embryonic stem (ntES) cell lines in different individuals. In this experiment, seven mice were used as donors and the cloned mice were obtained from four of them (No. 1 to 4). The ntES cell lines were established from all 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 out of the remaining three donor mice (No. 5 and 6). Only one donor mouse (No. 7) failed to produce a cloned mouse by either somatic cell nuclear transfer (SCNT) or ntES cell cloning.

support the development of mice cloned by SCNT. These results suggest that oocytes with extra cytoplasm do not have enhanced reprogramming potential [85].

ntES Cells: intrinsic problems and solution

The first success in generating ES-like cell lines by SCNT was initially performed in the bovine [86] and then subsequently in the mouse [87, 88]. We have previously shown that these ES-like cell lines are capable of differentiating into all three germ layers in vitro or into spermatozoa and oocytes in chimeric mice [48]. This was the first demonstration that ntES-like cells have the same potential as ES cells from fertilized blastocysts. Such ntES cell lines can be established with success rates 10 times higher than from 'conventional' reproductive cloning (Fig. 3, Somatic clone *vs*. ntES establsih) [47, 48, 54, 89]. Interestingly, although almost all the cloned mice showed some abnormalities and ntES cells were established using same nuclear transfer procedure, ntES cells are transcriptionally and functionally indistinguishable from ES cells derived from fertilized embryos [47, 90]. Recently, Chang *et al*. reported that abnormal methylation profiles of certain imprinted genes could be observed in both ntES and ES cell lines after long-term culture *in vitro* [91]. We also discovered that the methylation status at the upstream differentially methylated region of U2af1-rs1 was changed significantly in ntES cells, but not in ES cells [92]. These results suggest that NT affects the epigenetic status of a few gene regions in common and that a change in the methylation status of U2af1-rs1 allows us to distinguish ntES cells from ES cells.

Solution of the ethical problem for human ntES cell derivation

The use of human ntES cells has raised ethical objections because fresh oocytes must be donated by healthy women and the cloned embryos would be deprived of their potential to develop into a complete human being. Several different approaches have been proposed to overcome these problems. For example, human IVF is now routinely performed in infertility clinics with high successful fertilization rates (60–70% in humans, greater than 70% in mice) [93–95], but some oocytes still fail to fertilize for unknown reasons. However, if such aged fertilization failure (AFF) oocytes could be used as recipient oocytes to generate ntES cell lines, then this would reduce or even eliminate any ethical concerns over oocyte donation and embryo destruction. In mouse experiments, the efficiency of producing cloned mice decreased with increased oocyte age [96] and the fetuses never developed to full term when AFF oocytes were used [49]. However, surprisingly, ntES cell lines could be established from AFF oocytes with the same efficiency as fresh oocytes even when stored for 24 h after IVF (Table 1), and those ntES cell lines showed the differentiation potential to be normal, the same as ES cells [49]. Therefore, the use of AFF oocytes for NT can reduce or eliminate ethical concerns regarding oocyte donation and embryo destruction.

Egli *et al*. also developed a similar method but they used enucleated polyspermic mouse zygotes, which also cannot develop

Table 1. Success rate of cloned mice and ntES cell lines from IVF failed (AFF) oocytes

	Preserved period after IVF (Temperature)	Percent of survived oocyte after NT	Percent of blastocyst	Percent of established ntES cell lines	Percent of cloned offspring
Fresh control	—	81	56	16	2
AFF oocyte	6 h (37 C)	61	46	20	0
	24 h (25 C)	27	15	29	0

because they have a triploid phenotype [80]. Alternatively, ES cell lines have been established nondestructively by removing single blastomeres or polar bodies from fertilized embryos [97, 98]. However, this can only be applied to subsequent generations, not to direct therapy for the donor.

New Attempts to Improve the Efficiency of Mouse Cloning

We previously reviewed many different approaches to improve the efficiency of mouse cloning [44] but most of those methods had no effect on the overall success rate. However, nearly ten years ago, we accidentally discovered that 1% DMSO could significantly improve the frequency of development to the blastocyst stage *in vitro* [43]. 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.

Fig. 4. Production of cloned mice using the histone deacetylase inhibitor (HDACi) trichostatin A (TSA). This picture shows 13 cloned mice generated in just one experiment from a single donor mouse.

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 [61–63, 99]. 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 [100]. Probably, DMSO enhances the epigenetic reprogramming of cloned embryo during *in vitro* culture. Enright *et al*. have tried to alter the epigenetic status of donor nuclei used for cow cloning by using two chemicals: 5-azacytidine, an inhibitor of DNA methylation, and trichostatin A (TSA), a histone deacetylase inhibitor (HDACi) [101]. Although the *in vitro* developmental potential with TSA was increased significantly, the cells did not demonstrate full-term development. Because those epigenetically affecting drugs are very toxic [102, 103], each drug must be tested pharmacologically for its appropriate exposure, timing, concentration, and duration.

Discovery of optimal treatment of HDACi for mouse cloning

In this situation, by trial and error, one of us (SK) discovered the optimum concentration, timing and period of TSA treatment for cloned mouse embryos. Eventually this method led to a greater than five-fold increase in the success rate of mouse cloning (Fig. 4) and a doubling in the rate of establishing ntES cell lines [45]. These results were independently and concurrently obtained by Tsunoda's laboratory [104], showing that the effects of TSA are reproducible. On the other hand, previously, most cloned mice have only been generated from hybrid strains and have never been cloned from outbred or inbred strains [105, 106]. Although we found that TSA could be used to produce cloned mice even from an outbred supposedly 'unclonable' strain [107], most of the important mouse strains have never been cloned successfully.

Although TSA application resulted in great improvements in SCNT cloning in mice, the effects of TSA treatment on cloning efficiency are controversial in the bovine [108, 109], pig [110, 111], rabbit [112, 113] and rat [114]. Moreover, some groups have reported that TSA treatment had detrimental effects on the *in vitro* and *in vivo* development of the SCNT embryos [109, 112]. 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 [112]. On the other hand, it is known that the drug scriptaid acts as an HDACi but is less toxic than TSA [115]. Using this drug, Zhao *et al*. could improve the success rate of pig cloning to full term [116]. At the same time, one of us (NVT) found that scriptaid treatment could 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 (Fig. 5) [117]. 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.

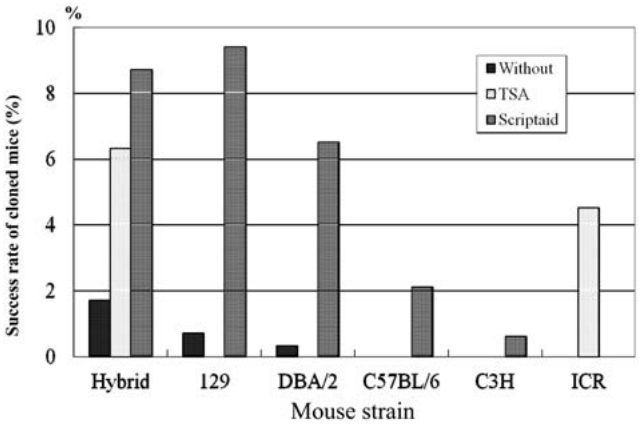

Fig. 5. Effects of TSA and scriptaid treatment on inbred mouse cloning. Without HDACi treatment, cloned mice could be obtained from hybrid and 129/Sv strains, but with a low success rate. Only one cloned mouse was obtained from the DBA/2 strain, but this animal never reproduced. When TSA was used, the success rates for hybrid and outbred strains were increased but we have never succeeded in producing full-term cloned mice from inbred strains. However, when scriptaid was used, the overall success rate was increased even from inbred strains.

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 [45], which is a necessary part of genetic reprogramming [118]. In fact, several reports clearly showed that HDACi treatment improved histone acetylation [111, 119], nascent mRNA production [117] and gene expression [120] in a manner similar to that in normally fertilized embryos. However, more study is required to understand the mechanisms. On the other hand, another HDACi, APHA (3-(1-methyl-4-phenylacetyl-1*H*-2-pyrrolyl)-*N*-hydroxy-2-propenamide) had no effect [117] and sirtinol had very little effect [121] for mouse cloning. In general, HDACs 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). It is known that TSA and scriptaid can inhibit the class I and IIa/b HDAC forms [122], whereas APHA can inhibit only class I [123] and sirtinol can inhibit only class III [124]. This suggests that inhibition of the class II HDAC is very important for the success of mouse cloning.

Effects of serial cloning

We and others have demonstrated that re-cloned animals can be generated from the somatic cells of cloned animals [125–129]. However, in most reports, the overall trend of the cloning efficiency is downward: in the mouse, six successive generations is the upper limit, whereas in bovines, the second generation is the upper limit. It is unclear why subsequent cloning is not possible. At the cellular level, there was no evidence of telomere shortening in the peripheral blood lymphocytes of cloned mice [126]. One possibility is the extremely low success rate of cloning animals. If the success rate is only few percent, then cloning is very unlikely to succeed in the production of the next generation, purely by chance. However, now we have raised the average production rate of cloned animal using HDACi. Therefore, we tried serial cloning experiments again using TSA treatment, and we have obtained 15 successive generations of cloned mice without decreasing the success rate (ongoing and unpublished results). We now believe that animal cloning can be repeated indefinitely.

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 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 choice is reset by SCNT and is not accompanied by genomic alterations [130, 131]. Similarly, monoclonal mice have been generated from ntES cells derived from lymphocyte nuclei indirectly using tetraploid complementation [132]. Thus, ntES cell techniques can be applied to the characterization of very rare cells in the body. Once ntES cells from these rare cell nuclei are established, the cells can proliferate indefinitely. However, here we propose another application of ntES cells, in which they can be used for reproduction from infertile or dead animals.

Producing offspring from individual mice

The genetically modified mouse is a powerful tool for research in the fields of medicine and biology but they are often infertile after genetic modification. 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 crucial to research in human infertility and to the biology of reproduction. Unfortunately, the success rate of somatic cell cloning is very low. Even in cases in which the cloning of a sterile mouse is successful, because of their infertile phenotype it will still be necessary to clone all subsequent generations. On the other hand, the rate of establishing ntES cells is nearly 10 times higher than the success rate of producing cloned mice (Fig. 2) [48, 89]. Converting from somatic cells to ntES cells does not improve the overall success rate of cloning by a second round of nuclear transfer [48, 53, 89]. However, we recommend the establishment of ntES cell lines at the same time to preserve the donor genome, because these lines can then be used as an unlimited source of donor nuclei for NT [54].

For example, because of the low success rate in producing cloned mice, only a few individual donor mice generated clones. However, we were ultimately able to obtain cloned mice from six out of seven individual donors by using either direct somatic cells or serial nuclear transfer of ntES cells (Figs. 2, 6) [54]. On the other hand, 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. However, we

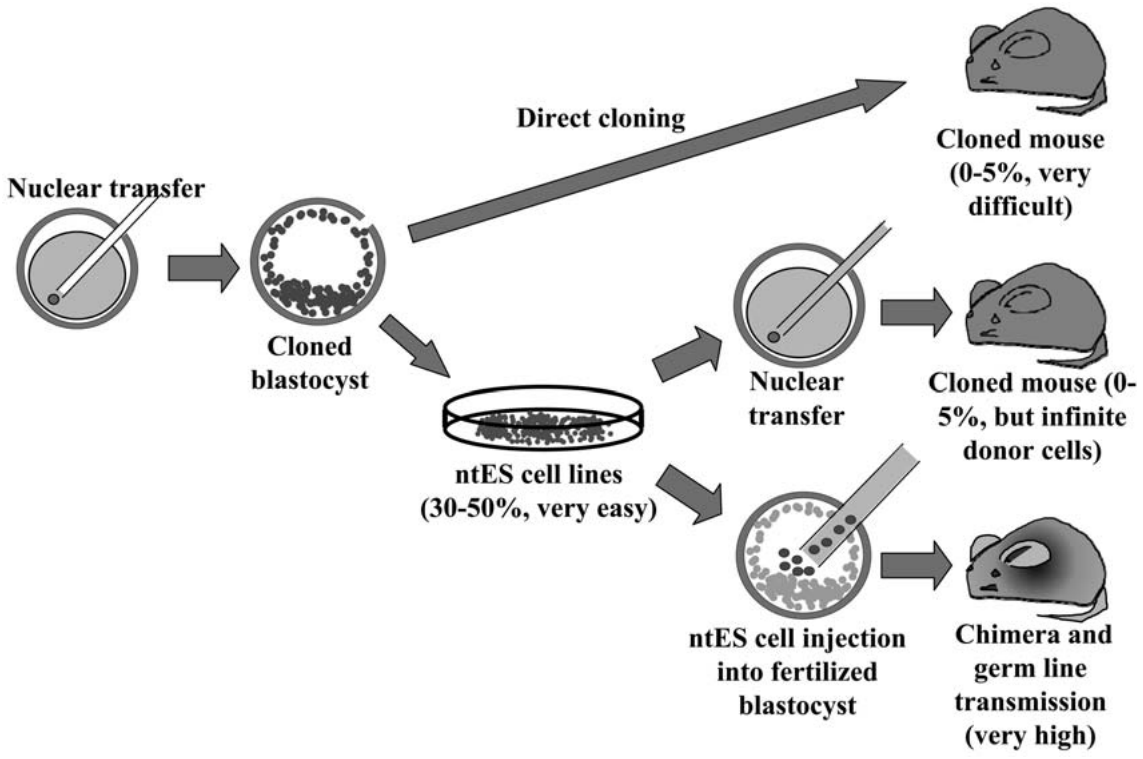

Fig. 6. Three different approaches to generate offspring from infertile, 'unclonable' or dead frozen mice.

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 (Fig. 7) [53]. We also achieved success using a mutant, hermaphroditic, sterile mouse found in our ICR mouse breeding colony [52]. 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 (Fig. 6). Thus, this technique is potentially applicable for the propagation of a variety of animals or important mutant genes, regardless of age or fertile potential.

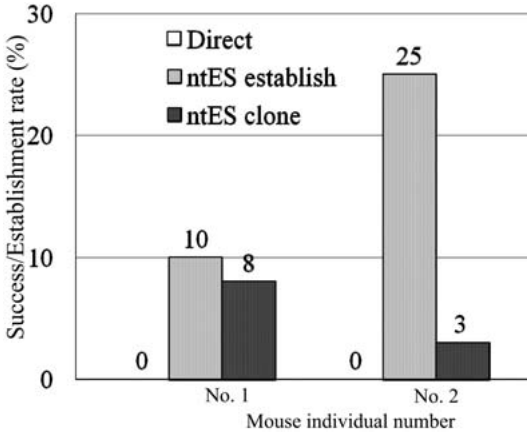

Fig. 7. Production of offspring from aged infertile mice. The outcomes for two aged mice (both 2 years and 9 months) were compared between direct SCNT cloning, the ntES cell establishment rate from those cloned blastocysts and ntES cell cloning. In both animals, direct cloning failed, probably because of the difficulty in genomic reprogramming of aged somatic cells. However, several ntES cell lines were established from both animals, and many healthy cloned mice were obtained by successive rounds of NT using the ntES cell lines.

The possibility of resurrecting an extinct animal

Cloning animals by SCNT provides an opportunity to preserve endangered mammalian species. When live cells can be collected from frozen bodies, it is possible to generate cloned animals [133]. However, the 'resurrection' of extinct species from permafrost (such as the woolly mammoth) is thought to be impractical, because no live cells could be available. On the other hand, it is known that 'dead' sperm from freeze-drying treatments [134] or from a whole frozen body [135] 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 three years at room temperature [136]. This report was the first to demonstrate that even freeze-dried somatic

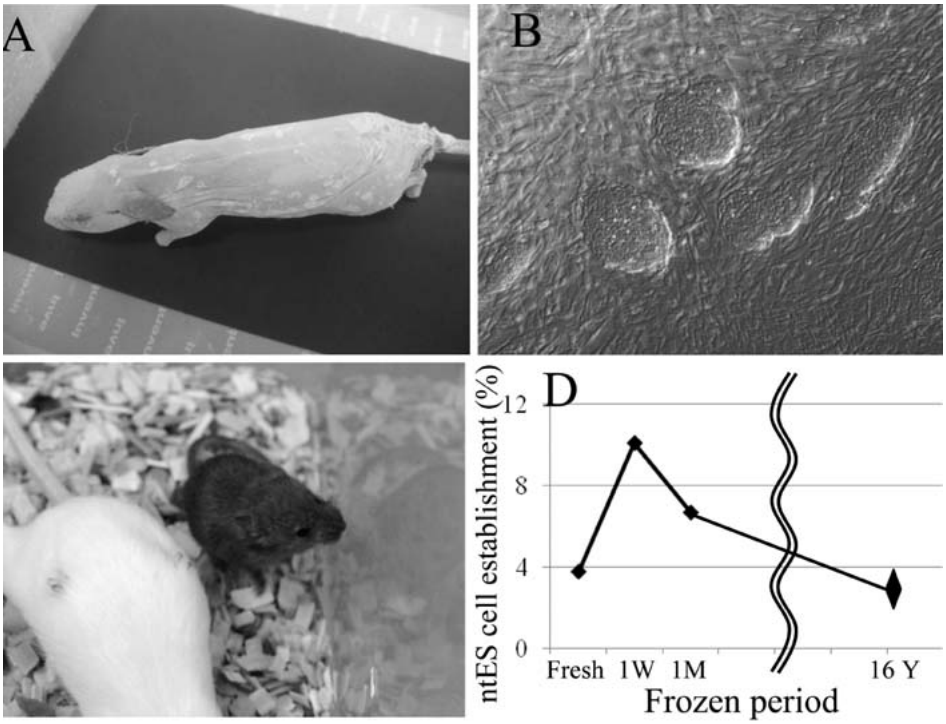

Fig. 8. Cloned mice produced from frozen dead bodies. (A) As a model of extinct animals frozen in permafrost, we used frozen dead mouse bodies as nuclear donors. These had been kept at –20 C for 16 years without any cryoprotectant. Although we could not produce cloned mice directly from this material by SCNT, ntES cell lines could be established (B). (C) Healthy cloned mice were obtained after a second round of NT using these ntES cell lines. (D) Although the rate of establishment of ntES cell lines decreased with increased storage period, these tissues frozen for 16 years retained the genetic potential to establish ntES cell lines.

Table 2. Difference between ntES cell and iPS cells

	Difficulty	Genetic modification	Ethics problem	Minimum no. donor cells	Donor cell condition
ntES cell	Difficult	No	Yes	<10 cell	Any cell, even dead
iPS cell	Easy	Yes?	No	A lot	Live cell only

cells have developmental potential after NT, but the authors could not determine whether the blastocysts were normal. On the other hand, we established ntES cell lines from freeze-dried mouse cells [55]. 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.

In addition, we have attempted to produce cloned mice from bodies kept frozen at –20 C for up to 16 years without any cryoprotection (Fig. 8). These conditions are similar to those of a frozen body recovered from permafrost. Although we could not produce cloned offspring from the somatic cell 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 [35, 38]. Finally, healthy cloned mice were produced from these ntES cells by a second round of NT (Fig. 8) [56]. 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 biological 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. 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. Therefore, the use of iPS cells for genomic reprogramming and for regenerative medicine is currently a 'hot topic' in this field, but we still believe that the NT approach, far from being outmoded, remains the only valid way to study biology, especially in generating offspring from a single cell or even dead cell nuclei (Table 2). Using this system, we have demonstrated that nuclear DNA is much more stable than we thought, and it looks as though we might be closer to realizing a big dream: the resurrection of extinct species by cloning. On the other hand, the abnormalities seen in cloned animals pose a major obstacle for application but this may 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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