Successful Mouse Cloning of an Outbred Strain by Trichostatin A Treatment after Somatic Nuclear Transfer

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Abstract. Although the somatic cloning technique has been used for numerous applications and basic research of reprogramming in various species, extremely low success rates have plagued this technique for a decade. Further in mice, the “clonable” strains have been limited to mainly hybrid F1 strains such as B6D2F1. Recently, we established a new efficient cloning technique using trichostatin A (TSA) which leads to a 2–5 fold increase in success rates for mouse cloning of B6D2F1 cumulus cells. To further test the validity of this TSA cloning technique, we tried to clone the adult ICR mouse, an outbred strain, which has never been directly cloned before. Only when TSA was used did we obtain both male and female cloned mice from cumulus and fibroblast cells of adult ICR mice with 4–5% success rates, which is comparable to 5–7% of B6D2F1. Thus, the TSA treatment is the first cloning technique to allow us to successfully clone outbred mice, demonstrating that this technique not only improves the success rates of cloning from hybrid strains, but also enables mouse cloning from normally “unclonable” strains.

Key words: Mouse, Nuclear transfer, Outbred, Trichostatin A

The success of somatic cell cloning in the mouse brings promise to applications such as species preservation, livestock propagation, and cell therapy for medical treatment using nuclear transfer embryonic stem cells (NT-ES cells) [1–4]. However, mouse cloning by somatic cell nuclear transfer (SCNT) has been inefficient, with success rates of 2% or less since the first cloned mouse, “Cumulina”, was born in 1997 [5]. Further, the success rates of mouse cloning heavily depends on their genetic backgrounds [6, 7], and because of that, current “clonable” mouse strains have been limited to hybrid F1 strains, such as B6D2F1 and B6C3F1. Actually, to date neither inbred except 129 [6, 7] nor outbred strains have been used for mouse cloning [6, 7]. In particular, there are few reports describing outbred mouse cloning. For example, the ICR strain is a popular outbred strain as a practical laboratory mouse and is used in various research fields including toxicology and pharmacology studies, but mouse cloning from the ICR strain is reported to be possible only using fetal cells [8, 9] or NT-ES cells [2] with success rates of 3% or less. In other words, no one is able to clone ICR directly from adult mice. Currently, it remains
unclear why cloning of F1 strains exclusively can occur. There are two possibilities. One possibility is that the efficiencies of reprogramming of the nuclei from such “unclonable” strains may simply be much lower than those from F1 hybrid. The second is that there may be some strain-specific irreversible genetic or epigenetic factors, and that the “unclonable” strains are truly “unclonable”.

Although we have tried several new methods, so far none exert a marked influence on the efficiency of cloning, including the methods of oocyte activation [10, 11], inhibition of cytokinesis [11], recloning via NT-ES cells [3], and timing of enucleation or injection of the nucleus [12]. Recently, we and others have published a new cloning method embryos are treated with trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, following nuclear transfer [13, 14], which leads to 2–5 times higher success rates for both reproductive and therapeutic cloning [13], suggesting that TSA enhances reprogramming of transferred somatic nuclei in oocytes. However, this newly established technique, called the TSA cloning method, has not been thoroughly evaluated in terms of mouse strain and donor cell types since the first report of TSA treatment described cloning of only cumulus cells from the B6D2F1 mouse. In this study, we applied the TSA cloning method to an outbred strain, ICR, and obtained a substantial number of cloned ICR mice, demonstrating that the TSA cloning method makes it possible to clone either sex from both cumulus and fibroblast cells of the outbred strain. These findings also provide a method to practically clone a popular outbred strain as well as knowledge regarding the mechanism for nuclear reprogramming from different genetic backgrounds.

Materials and Methods

Animals

ICR strain mice (aged 2–3 mon) were used as somatic cell donors. B6D2 F1 females (C57BL/6 × DBA/2 hybrids; 8–10 weeks of age) were used as oocyte donors. Surrogate females were ICR females mated with vasectomized males of the same strain. All animals were obtained from SLC (Shizuoka, Japan) and were maintained in accordance with the Animal Experiment Handbook at the Riken Center for Developmental Biology.

Isolation of donor cells and oocytes

For the preparation of cumulus cells and oocytes, female mice were induced to superovulate by consecutive injections of equine chorionic gonadotropin (eCG; 5 IU) and human chorionic gonadotropin (hCG; 5 IU), which were given 48 h apart. Thirteen to 15 h after hCG injection, cumulus-oocyte complexes were collected from the oviducts, and cumulus cells were dispersed by a 10-min treatment with 0.1% (w/v) bovine testicular hyaluronidase (300 USP units/mg) in Hepes-CZB at 37 C. For the preparation of tail-tip fibroblasts, the tail tips of ICR males were isolated, freed of skin, cut into small pieces, divided among 35-mm dishes, and incubated in 5 ml of DMEM (Sigma, St Louis, MD, USA) supplemented with 10% fetal calf serum (Sigma). These dishes were incubated for 10 to 14 days at 37 C under 5% CO2 in water-saturated air until fibroblasts attached and were fully grown. These cultured fibroblasts were detached from the dish by trypsin treatment, suspended in the same culture media, and washed three times with phosphate-buffered saline (PBS, pH 7.2).

Nuclear transfer followed by TSA treatment and production of cloned offspring

Nuclear transfer from adult cumulus or tail tip cells was performed as described previously [5, 15]. Briefly, collected B6D2F1 oocytes were enucleated and injected individually with an adult cumulus or tail tip cell from ICR mice via a Piezo-driven micromanipulator (PrimeTech Corporation, Ibaraki, Japan). TSA treatment of cloned embryos also followed the previous protocol [13, 16]. Briefly, after nuclear transfer, the reconstructed oocytes were activated by 10 mM SrCl2 in Ca2+-free CZB medium in the presence of 5 µg/ml cytochalasin B and 5 or 50 nM TSA for 6 h., and then cultured in KSOM medium containing the same concentration of TSA for 4 h more (total: 10 h-TSA treatment). After TSA treatment, KSOM without TSA was used to enable development to 2-cell stage embryos before they were transferred to pseudopregnant ICR surrogate mothers. For control cloning the same protocol was used without TSA. All the recipient females were euthanized at 19.5 days post coitum (dpc), and the cloned pups were raised by lactating ICR foster mothers.

Statistical analysis

The data of the offspring production rates were
Results

Successful mouse cloning from outbred strain after TSA treatment

First, we tried cloning female ICR mice from cumulus cells using the standard method [5], but failed to clone any mice (Table 1). In contrast, 5 nM TSA treatment for 10 h after nuclear transfer resulted in 8 pups (4.5%) out of 194 reconstructed oocytes (Fig. 1a and Table 1). The mice cloned from the B6D2F1 cumulus cells after TSA treatment were delivered with a 5.4% success rate (Table 1), which is similar to our previous results [13]. Although the placentas and body masses of the cloned ICR offspring are relatively larger than those of the cloned B6D2F1, with mean body weights of 2.08 g vs. 1.61 g and mean placenta weights of 0.37 g vs. 0.32 g, respectively (Fig. 1a and Table 1), all of the cloned female ICR mice were weaned and lived for more than 6 months (Fig. 1b). Thus, ICR cloning from cumulus cells showed a comparable success rate to that of B6D2F1 cloning.

Second, we tried to clone an ICR male using tail tip fibroblast cells, but again failed to clone any mice without TSA treatment. On the other hand, 5 nM TSA treatment after nuclear transfer allowed us to clone 5 healthy males (4.2%) out of 120 reconstructed oocytes with a mean body weight of 1.91 g and a mean placenta weight of 0.34 g (Fig. 1c and Table 1). Again, all the cloned ICR male mice were weaned and lived for more than 6 months (Fig. 1c). This data indicates that TSA treatment after NT using either cumulus cells or fibroblast cells enhances the success rates of somatic cloning. Further, we also examined the effect of higher TSA concentration, 50 nM, but obtained only one cloned male. This result is consistent with the previous observation that higher concentrations of TSA reduced the success rates of mouse cloning from cumulus cells.

Discussion

This study investigated whether the TSA cloning
method could be helpful for mouse somatic cloning from an outbred strain other than a hybrid F1 strain using adult fibroblast cells (male) as well as cumulus cells (female). Our results clearly indicate that the TSA cloning method makes it possible to clone ICR mice without any overt abnormalities and with success rates of 4–5% that are comparable to the 5–7% success rates of B6D2F1 [13]. Our results also demonstrate that the TSA cloning method can be used for both male and female mouse cloning using cumulus and fibroblast cells. To our knowledge, this is the first report of cloning mice directly from an adult outbred strain. Thus, the TSA cloning method increases the success rates for mouse cloning in currently used strains, that is, the hybrid F1 strains, and now, the previously “unclonable” outbred strain.

Although the mechanism underlying how the TSA cloning method improves cloning remains unknown, it is believed that TSA treatment enhances DNA demethylation of a somatic cell-derived genome after NT [13, 17], a necessary part of genetic reprogramming [18]. Actually, accumulating data suggests that TSA selectively inhibits DNA methylation depending on the cell type and genomic region [17, 19, 20]. Further, even in the same locus, demethylation or the effect by TSA is different depending on the developmental stage and cell type [20]. Regardless, we succeeded in cloning mice using different somatic cells from both sexes via the TSA cloning method, suggesting the common mechanism by which somatic nuclei are efficiently reprogrammed by TSA functions in a cell type- and sex-independent manner.

It has not been clear why the success rate of mouse somatic cell cloning varies, for example, from 0% to over 3% of the cloning success rates from cumulus cells, depending on genetic background [6, 7]. However, the similar success rates between ICR and B6D2F1 using the TSA cloning method suggests that 1) the dependence of success rates of mouse cloning on genetic background is simply due to different efficiencies of reprogramming during one-cell stage development, rather than some strain-specific irreversible genetic or epigenetic factors and that 2) regulation of HDAC activities after SCNT is key for efficient reprogramming among strains. Taken together, it is reasonable to believe that the transferred nuclei from different strains have different susceptibilities to reprogramming factors including HDACs, which results in different epigenetic statuses for factors such as DNA methylation, producing different success rates for mouse cloning among strains. Research that supports this idea report that different DNA methylation statuses for gene expression are found among different strains [21] and also that epigenetic statuses, including DNA methylation, of transferred pronuclei are established differently through nucleocytoplasmic interaction of different strains [22, 23]. More importantly, TSA treatment of cloned embryos can even prevent such strain-specific reprogramming errors. If this is the case in any strain, it should be possible to clone even “unclonable” inbred strains, such as C57BL/6 and C3H/He [6], which have never been cloned before. Future study will focus on cloning more “unclonable” strains as well as the mechanism underlying “unclonability”. Thus, the TSA cloning method provides valuable insight into reprogramming of different strains.

TSA treatment of normal fertilized embryos is also known to cause detrimental effects and teratogenicity on both pre- [24] and post-implantation development [25, 26]. Regardless, the success of the TSA cloning method indicates that TSA treatment is helpful for mouse cloning. How does this apparent discrepancy occur? Our recent observations indicate that the helpful or harmful impact of TSA on the developmental rates of treated embryos heavily depends on their nuclear derivations [27]. For example, in terms of preimplantation development, TSA treatment is harmful to fertilized embryos generated by intracytoplasmic sperm injection (ICSI) but is not harmful to fertilized embryos generated by round spermatid injection (ROSI) or parthenogenetic embryos, and yet significantly helpful for somatic cloned embryos [13, 27]. Therefore, TSA treatment of embryos itself is not always detrimental.

Our success of ICR cloning using the TSA cloning method also confirms the helpful effect of TSA treatment in mouse cloning. Further, TSA treatment of cloned embryos provides insight into reprogramming of different strains and cell-types and, at the same time, a stabilized cloning method independent of those characteristics.
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References

1997; 3: 45–53.


