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Brief Report

# Successful Serial Recloning in the Mouse over Multiple Generations

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## SUMMARY

**Previous studies of serial cloning in animals showed a decrease in efficiency over repeated iterations and a failure in all species after a few generations. This limitation led to the suggestion that repeated recloning might be inherently impossible because of the accumulation of lethal genetic or epigenetic abnormalities. However, we have now succeeded in carrying out repeated recloning in the mouse through a somatic cell nuclear transfer method that includes a histone deacetylase inhibitor. The cloning efficiency did not decrease over 25 generations, and, to date, we have obtained more than 500 viable offspring from a single original donor mouse. The reprogramming efficiency also did not increase over repeated rounds of nuclear transfer, and we did not see the accumulation of reprogramming errors or clone-specific abnormalities. Therefore, our results show that repeated iterative recloning is possible and suggest that, with adequately efficient techniques, it may be possible to reclone animals indefinitely.**

Animals have been cloned from a number of species and organs (Thuan et al., 2010; Wakayama et al., 1998; Wilmut et al., 1997) and even from frozen cadavers (Wakayama et al., 2008). In some mammalian species, it is also possible to produce recloned animals with somatic cell nuclei derived from previously cloned animals (Cho et al., 2007; Kubota et al., 2004; Kurome et al., 2008; Wakayama et al., 2000; Yin et al., 2008). In principle, this type of approach could be useful for the large-scale production of superior-quality domesticated animals and for research into genomic reprogramming (Graf, 2011). Previously, we proposed that repeated rounds of genomic reprogramming via serial cloning might lead to an increase in efficiency over successive generations because of the selection of easily reprogrammable cells. Disappointingly, however, it has been found that the success rate in fact decreased with each iteration. In one study, only one cloned mouse was produced in the sixth generation from more than 1,000 nuclear transfer attempts—but it was cannibalized by its foster mother (Wakayama et al., 2000). We have never succeeded in understanding the reason for this failure of recloning over successive generations. Similar results have been reported in cattle, where serial nuclear transfer failed to produce a third generation (Kubota et al., 2004). The recloning of cats (Yin et al., 2008) and pigs (Cho et al., 2007; Kurome et al., 2008) has also been studied, but those attempts reached only the second and third generations, respectively.

One possible explanation for this limit on the number of recloning attempts is an accumulation of genetic or epigenetic abnormalities over successive generations. It is well known that cloned animals frequently show several abnormal phenotypes (Inoue et al., 2002; Ogonuki et al., 2002; Wakayama and Yanagimachi, 1999) caused by genomic reprogramming errors at the time of somatic cell nuclear transfer (Inoue et al., 2010; Yang et al., 2007). Thus, if a donor nucleus from a cloned animal is already epigenetically abnormal, the additional abnormalities introduced during a subsequent round of reprogramming might lead to embryo failure. Another possibility is that cloned animals contain only a few normal or reprogrammable somatic cells and that recloning was successful only when those cells were selected by chance, but the number of such cells drops over successive generations. A more straightforward explanation would simply be that the inherent success rate of cloning was too low for it to be reliable over repeated generations. In the prior studies, this was the case, and we were unable to investigate these possibilities (Wakayama et al., 1998), and the reason, therefore, remained unclear. Recently, we were able to improve the success rate of mouse cloning up to 5-fold by limiting the accumulation of epigenetic abnormalities by using a histone deacetylase inhibitor, trichostatin A (TSA) (Kishigami et al., 2007; Kishigami et al., 2006; Thuan et al., 2010). In the present study, we attempted serial mouse cloning again with the

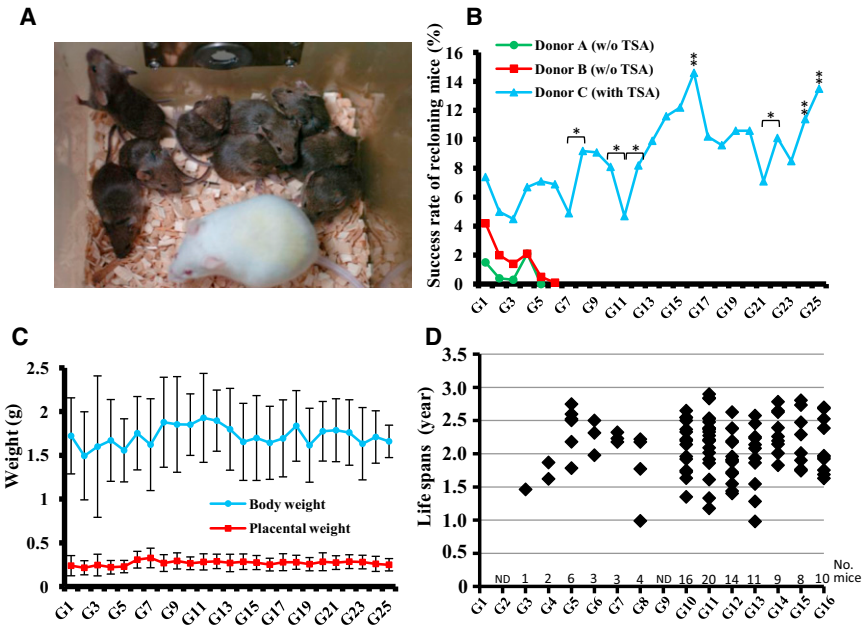


**Figure 1. Production of Recloned Mice from the Somatic Cell Nuclei of Previously Cloned Mice**

(A) A group of nine 20th generation (G20) recloned pups (brown coats) were born in a single experiment.

(B) The success rate of mouse recloning in each generation with and without (w/o) the use of trichostatin A (TSA) during nuclear transfer. The data for donor A and donor B are from a previous study (Wakayama et al., 2000). *, significant differences between generations ($p < 0.05$). **, significant difference between this generation and G1 ($p < 0.05$).

See also Table S1.

(C) Mean body and placental weights of recloned mice through successive generations are shown. There were no significant correlations between these weights and generation numbers ($r = 0.0029$ and 0.0013, respectively). Error bars designate the SD.

See also Figure S2.

(D) Lifespans of recloned mice in successive generations are shown. The lifespan of G1, G2, and G9 clones could not be measured because the mice were used for other experiments. Each dot represents an individual recloned mouse.

addition of TSA and examined the reprogramming capacity and phenotypes of the recloned mice.

We used four BD129F1 female mice (BDF1 × 129/Sv) as nuclear donors, and the first generation of cloned mice (hereafter termed G1) was produced from the cumulus cell nuclei of those donors. These mice were produced in our laboratory with three-way crosses between C56/BL6, DBA/2, and 129/Sv strains. Thus, the progeny of each mouse can be identified by genotyping. The recipient oocytes were collected from adult BDF1 females or, in subsequent generations, from the recloned donor mice themselves for examination of the effect of a heterogeneous oocyte cytoplasm. The donor mouse that showed the highest success rate in producing G1 clones was selected as the original donor and used to initiate the serial mouse cloning experiment. The second generation of cloned mice (G2) was produced from the cumulus cells of a G1 clone when it was 3 months old. This study commenced in December 2005, and we aimed to use our original nuclear transfer procedure without any modifications throughout the entire duration. However, given the time frame of this experiment, some changes were unavoidable, such as in the quality of the media used and the skill of the experimentalists involved, and these changes could potentially affect the success rate or phenotype of the recloned mice. To control for such variation, we produced cloned control (CC) mice from other donors with the same genetic origin (BD129F1) for use as technical or time-matched comparisons. We also generated fertilized normal control (NC) mice of the same genetic background (BD129F1) by intracytoplasmic sperm injection into oocytes to mimic the in vitro manipulation and culture stresses applied to cloned embryos.

The success rates of serial recloning varied between generations; for example, the average success rates of recloning attempts in G3, G7, and G11 were very low (4%–5%), whereas the success rates for the next generations of each of these (G4, G8, and G12) were 1.5- to 2-fold higher. G16 showed the highest success rate, but in the next generation, the success rate decreased by one-third (Figures 1A and 1B). This variation was observed not only between generations but also within experiments. In G10, G18, and G25, the maximum success rate was over 20%, but the minimum rate was only 3%–4%. (Table S1 available online). Therefore, although we saw significantly higher cloning success rates in recent generations (G16, G24, and G25) than in G1, the high variation even within generations makes it difficult to draw any clear conclusions about changes in success rate. Nevertheless, we have been able to conduct repeated recloning over 25 generations, and, to date, 581 recloned mice have been generated from one original donor mouse (Table S1 and Figure 2B).

During the course of this experiment, we tested whether a complete matching of the donor nucleus and recipient oocyte would improve efficiency by injecting donor nuclei into the donor's own oocytes instead of BDF1 oocytes, but this approach did not increase the success rate of cloning (Table S1). Thus, it seems that, at least for mouse cloning, genetic heterogeneity between the donor nucleus and the recipient oocyte cytoplasm does not influence the quality of genomic reprogramming and full-term development. All of the cloned mice were female, and genotyping confirmed that all of the generations of clones were derived from the original single donor mouse (Figure S1).

Cloned mice frequently show placentomegaly (Tanaka et al., 2001; Wakayama and Yanagimachi, 1999; Lin et al., 2011), and some have increased body weight (Tamashiro et al., 2002) or die early as a result of respiratory failure (Ogonuki et al., 2002). Therefore, we measured the body and placental weights of all the cloned mice at the time of caesarian section. The mean body weight in each generation was within the normal range of naturally derived mice (Figure 1C). The mean placental weight in each generation was 0.22–0.32 g, which is 2- to 3-fold heavier than the placentas of normal control mice, consistent with

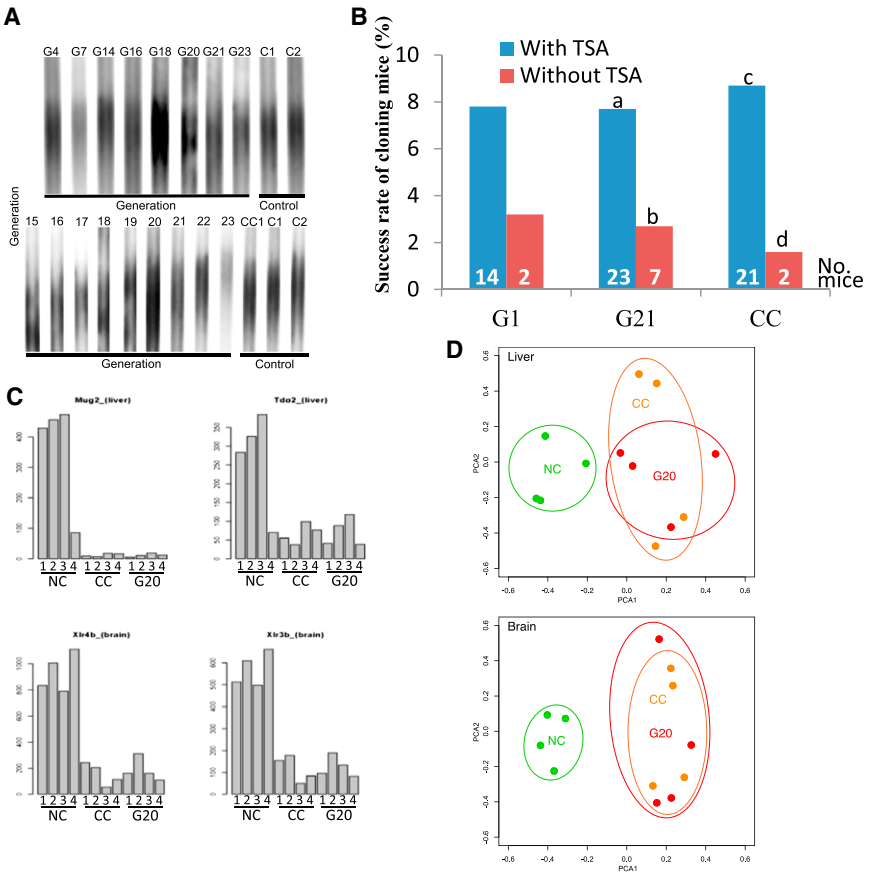


**Figure 2. Telomere Length, TSA Dependence, and Gene Expression Patterns in Control and Recloned Mice**

(A) Telomere lengths among generations of recloned mice sampled at 3 months of age (upper panel) or sampled at the same time. The G15 recloned mouse was the oldest (2 years and 8 months) and the G23 mouse was the youngest (3 months) (lower panel). C1, C2, and CC1 were naturally conceived, age-matched controls and cloned control (CC) mice at 3 months of age, respectively.

(B) The effect of TSA treatment for the production of cloned and recloned mice is shown. G1 and CC cloned mice were generated from naturally conceived mice, and G21 recloned mice were generated from G20 recloned mice. All experiments were performed with or without TSA. (a) versus (b), (c) versus (d); $p < 0.01$.

(C and D) Gene expression profiles in the neonatal liver and brain of normal control (NC), CC, and cloned (G20) mice. (C) shows the level of gene expression; Mug2 and Tdo2 were selected for the liver sample (upper), and Xlr4b and Xlr3b were selected for the brain sample (lower). (D) shows principal component analysis in the liver (upper) and in the brain (lower) in which the horizontal and vertical axes represent principal components (1) and (2), respectively. The dots represent individual NC, CC, and G20 cloned mice.

previous findings for cloned mice. However, neither body nor placental weight increased over successive generations, indicating that abnormalities do not accumulate (Figure 1C). In fact, when placentas from the G20 clones were examined histologically, expansion of the spongiotrophoblast layer—an abnormality specific to cloned mice (Tanaka et al., 2001)—was reduced in comparison with that seen in the CC mice (Figure S2).The lifespan of the cloned animals was also within the normal range. Unlike in initial reports, in this study, the majority of the pups (517/545, 94.9%) commenced respiration spontaneously and grew to adulthood. The average lifespan of mice in the G1 to G16 was about 2 years (ongoing), similar to that of naturally conceived mice (Figure 1D).

Fertility can also be used as an indicator of normal development in mice. To examine the fertility of our cloned mice, we selected four G20 clones randomly at the time of weaning and mated them with normal BDF1 male mice produced via natural mating. All the clones gave birth naturally to normal litter sizes, and pups lacked any abnormalities; the mean age at first birth was about 2 months, similar to that of naturally generated mice (Table S2).

Telomeres are vital for maintaining chromosomal integrity and genomic stability in normal cells in vivo, and they shorten with each cell division. In normal reproduction, the telomeres are repaired by telomerase in the germline, but cloned animals develop from somatic cells directly and, therefore, miss this step. Telomere lengths have been examined in cloned animals of several species (Konishi et al., 2011; Lanza et al., 2000; Miyashita et al., 2011; Shiels et al., 1999; Wakayama et al., 2000), and most reports have concluded that the telomeres of cloned animals are repaired during genomic reprogramming. In this study, we examined telomere lengths in the recloned mice at 3 months of age and compared them with those of age-matched control mice. We also collected samples from earlier generations of recloned mice still living at the same time, which were older at the time of collection. As shown in Figure 2A, these experiments revealed that there was no evident shortening of telomeres in the recloned mice of any generation or at any age.

Previous studies have also identified abnormal gene expression profiles in cloned mice, with a high degree of heterogeneity occurring between individuals (Kohda et al., 2005; Kohda et al., 2012). To examine the effect of serial cloning on these profiles, we analyzed the gene expression profiles of the G20 cloned mice compared to CC mice and NC mice. The brain and liver were collected from four newborn pups. The gene expression profiles of the G20 clones differed from those of NC mice, but these differences were similar to those observed in CC mice (Figures 2C and 2D). Thus, it seems that genes that were not successfully reprogrammed in the first round of nuclear transfer were still not reprogrammed, even after successive rounds (Figure 2C).

Finally, to address the possibility that serial cloning might enhance the inherent reprogramming susceptibility of the donor nuclei, we examined the effect of TSA treatment on the success

rate of cloning after serial cloning. When nuclear transfer was performed with the use of G20 cumulus cells without TSA, the success rate of producing G21 clones was only 3%, similar to that of G1, and was significantly lower than the success rate achieved with nuclear transfer with TSA treatment (8%) (Figure 2B). Thus, the somatic cell nuclei of recloned mice still required TSA for effective reprogramming, as in control experiments, and recloning did not appear to increase the reprogrammability of somatic cell nuclei, even when it was repeated 25 times.

There has been a longstanding question in the field about whether serial cloning over many generations is possible at all and, if so, whether it would lead to either an increase in reprogramming efficiency or the accumulation of abnormalities that prevent successful serial recloning. Our current study answers this question by showing that serial nuclear transfer cloning can be performed over at least 25 generations without evident introduction of genetic or epigenetic changes that have a negative impact on viability. Moreover, the genomic reprogramming efficiency also did not increase through successive generations, suggesting that serial recloning does not select for somatic cells that are more amenable to reprogramming or introduce genomic changes that increase the efficiency of the process. In other words, the barrier to reprogramming of somatic cells was maintained at the same level through this serial cloning experiment.

In this study, we also found that successive recloning over multiple generations produced phenotypically normal fertile mice with normal lifespans. Thus, there seems to be no inherent reason why recloning in mice should fail, and it seems most likely that the previous failures in serial recloning (Wakayama et al., 2000) can be attributed to the low success rate of the cloning techniques being used at that time, leading to an accidental end to the serial recloning expierment. Even with our improved procedure, the cloning success rate varied from 2% to 25% through the 25 generations that we examined. Thus, with further improvement to nuclear-transfer cloning techniques, unlimited animal recloning in many different animal species might in fact be possible.

## ACCESSION NUMBERS

The gene expression data sets reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database at accession number GSE43476.

## SUPPLEMENTAL INFORMATION

Supplemental Information contains Supplemental Experimental Procedures, two figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.01.005.

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