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—Original—

Mice Cloned by Nuclear Transfer from Somatic and ntES Cells Derived from the Same Individuals

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Abstract. The current success rate of cloned mice from adult somatic cell nuclei is very low, whereas it is relatively high for cloned mice from ES cell nuclei. In this experiment, we examined whether the success rate of cloning from somatic cells could be improved via nuclear transfer embryonic stem cells (ntES cells) established from somatic cell nuclei. We obtained 11 cloned mice and 68 ntES cell lines from the somatic cell nuclei of 7 mice, and cloned 41 mice were cloned from the ntES cell nuclei. Unexpectedly, the overall success rate of cloning from ntES cell nuclei in this series was no better than when using somatic cell nuclei. Interestingly, full-term cloned mice were produced only via ntES cells from two individuals, but not by direct nuclear transfer from the somatic cells, and vice versa. Ultimately, we were able to obtain clone mice from 6 out of 7 individuals using either somatic cells or ntES cells. Thus, although ntES cells as donor nuclei do not absolutely assure a better success rate for mouse cloning than somatic cells, to preserve and clone valuable individuals, we recommend that ntES cell lines be established. These can then be used as an unlimited source of donor nuclei for nuclear transfer, and thus complement conventional somatic cell nuclear transfer cloning approaches.
Key words: Clone, ntES cell, Nuclear transfer, Reprogramming

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Cloning mammals by nuclear transfer began more than 20 years ago [1], yet the biology underlying this process remains obscure. We previously developed a microinjection-based nuclear transfer method to generate cloned mice from adult cumulus cells [2, 3] and from tail-tip cells [4]. Ogura *et al*. have reported success in cloning mice from Sertoli cells [5], natural killer T cells [6], and primordial germ cells [7]. However, as with other mammals, the overall efficiency of mouse cloning (the percentage of activated oocytes giving rise to live offspring) in all reports is less than 2%. Cloned embryos generally grow to the blastocyst stage, but then fetal developments tend to arrest, with a failure to establish normal placental tissues, presumably because of impaired genomic reprogramming. We previously showed that the donor strain [8] and treatment of dimethyl sulfoxide (DMSO) are important determinants of cloning efficiency [8, 9]. However, the technical variations that have been isolated so far apparently do not exert a marked influence on the efficiency of cloning. These include the methods of oocyte activation [10], timing of oocyte activation [9], inhibition of cytokinesis [9], and timing of

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enucleation or injection of the nucleus [11].

By contrast, the successful rate of cloning was higher when embryonic stem (ES) cells from the fertilized blastocyst were used as a source of donor nuclei than when somatic cells were used directly [12, 13]. As with somatic cells, success depended on the background genotype of the ES cells [8, 14]; thus 5%–10% of cloned blastocysts produced using the nuclei of F1 generation ES cells developed to full-term mice [12, 13]. ES cells are pluripotent (capable of differentiating into all tissue types except the placenta), and therefore the nuclei might not require as rigorous or complete genomic reprogramming for full-term development is required for as fully differentiated somatic cells.

ES-like cell lines have now been established from somatic cells via nuclear transfer in cows [15], mice [16–18], and humans [19]. These cell lines are considered to possess the same capacities for sufficient differentiation and self-renewal as those of conventional ES cell lines derived from normally fertilized embryos. To distinguish the two, the former are referred to as nuclear transfer ES (ntES) cell lines [20]. We previously showed that ntES cell lines are capable of differentiating into all three germ layers *in vitro*, or even into spermatozoa and oocytes, in chimeric mice produced by injection of ntES cells into the blastocoel of normal embryos [18]. This was the first time that ntES cells were demonstrated to have the same developmental potential as conventional ES cells (from fertilized embryo). Interestingly, ntES cell lines can be established successfully in certain inbred mouse strains [21], such as C57BL/6 and C3H/He, but neither have ever been cloned successfully [8].

If ntES cells have the same potential as conventional ES cells, then in theory, the success rate of cloning mice from their nuclei should also be better than with somatic cells. We demonstrated previously that cloned mice can be generated from ntES cell nuclei, and that the success rate is similar to that for somatic cell cloning [18, 22]. However, because these studies used different donor mouse strains, further study is required to establish this point conclusively. In this study, we examined the production of cloned mice from somatic and ntES cell nuclei from the same mice to determine whether overall cloning efficiency can be improved using ntES cells.

Materials and Methods

Animals

F1 and F2 hybrid generation mice were used as nuclear donors to enhance the genetic diversity between individuals. Three male and two female (2 to 3 mo old) from the BD129 F1 [(C57BL/6 × DBA/2) × 129/Sv] and a male and a female from transgenic (Tg) mice with a BDF2 (BDF1 × BDF1) background, carrying and expressing the gene for green fluorescent protein (GFP) [23], were used for both somatic cell cloning and the establishment of ntES cells. Enucleated BDF1 (C57BL/6 × DBA/2) strain oocytes were used as recipients for nuclear transfer. ICR females were used as surrogate mothers to carry the cloned embryos. All animals (obtained from SLC, Shizuoka, Japan, or bred in Riken) were maintained in accordance with the Animal Experiment Handbook at the Riken Center for Developmental Biology.

Media

Oocytes and embryos were cultured in KSOM medium (Specialty Media, Phillipsburg, NJ, USA) containing amino acids, glucose, and 1 mg/ml bovine serum albumin (BSA) at 37.5 C under 5% CO_2 in water-saturated air. Oocyte manipulations were carried out in Hepes-buffered CZB medium [24], pH7.4 (Hepes-CZB), at room temperature (25–29 C). Ca^{2+}-free CZB containing 5 mM $SrCl_2$ and 5 μg/ml cytochalasin B (an inhibitor of actin polymerization) was used for oocyte activation [25].

Isolation of cumulus cells and oocytes

Mature BDF1 and BDF2 female mice were induced to superovulate by consecutive injections of equine chorionic gonadotrophin (eCG) (5 IU) and human chorionic gonadotrophin (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 27 C. Approximately 2 μl of BDF2 mouse cumulus cell suspension was transferred to 20 μl Hepes-CZB containing 12% (w/v) polyvinylpyrrolidone (PVP; *M*r 360,000, Wako, Japan) and kept at room temperature for up to 3 h before injection. The molecular weight and concentration of the PVP in these experiments is

apparently important; we have not so far been able to produce cloned mice when PVP with a molecular weight 360,000 is substituted with PVP at a lower molecular weight (unpublished observations).

Isolation of tail-tip fibroblasts

Tail tips of five BDF2 mice and one BD129F1 male mouse were isolated, freed of skin, cut into small pieces, divided between two 35 mm dishes, and incubated in 5 mL DMEM (Sigma- Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (Sigma). When incubated for 10 to 14 days at 37.5 C under 5% CO_2 in air, many fibroblasts were seen spreading over the surface of the dish. To detach the cells from the dish, they were treated for 10 min with Ca^{2+}-Mg^{2+}-free phosphate-buffered saline (PBS) containing 0.25% trypsin and 0.75 mM ethylenediaminetetraacetic acid (EDTA). The suspension was pipetted for a few minutes to release the cells from the dish surface and centrifuged (1500 rpm 10 min) to sediment the cells. The cells were then washed three times by the same centrifugation step using DMEM. Before injection, an aliquot of the cell suspension (2 μl) was mixed with Hepes-CZB (about 20 μl) containing 12% (w/v) PVP and left at room temperature for up to 3 h.

Production of cloned offspring using adult somatic cells and ntES cells

Enucleated BDF1 oocytes were injected individually with an adult tail tip, cumulus cell, or ntES cell nuclei [2, 4, 18], activated using Sr^{2+} [25], cultured for 72 h, and allowed to develop to morulae or blastocysts. When some cloned embryos had developed to the 2-cell stage or morulae/blastocyst stage, they were transferred to the oviducts or uteri of pseudopregnant foster mothers (ICR), which had been mated with vasectomized ICR males one or three days previously. All recipient females were euthanized at 19.5 days post-copulation (dpc), and their uteri were examined for the presence of fetuses and implantation sites.

Establishment of ntES cell lines

In this experiment, different pieces of tail or cumulus cells from the same female mice were used as nuclear donors. Cloned embryos were produced as described above, and when they reached the blastocyst stage, they were used to establish ntES cell lines as described previously [18, 21, 22]. All the established ntES cell lines were tested for alkaline phosphatase activity (a primordial germ cell marker) and the ability to form embryoid bodies (evidence of pluripotency).

Statistical analyses

Outcomes were evaluated using Chi-square tests, and $P<0.01$ was assumed to be statistically significant.

Results

Initially, we tried to produce cloned mice directly from the somatic cells of seven BD129F1 and BDF2 individuals (except for one mouse, M7, for which the cells were contaminated with bacteria). The success rates for production of full-term pups per transferred embryo varied between 0% and 15.6%,

Table 1. Effect of cell sources for nucleus donors on the establishment of ntES cell lines and production of cloned animals
Category 1: cloned mice were produced from both somatic and ntES cells

Origin of donor nucleus			No. of enucleated oocytes	No. of normally activated oocytes	No. (%) of oocytes developing to blastocyst*	No. (%) of dead fetuses	No. of successes (% oocytes activated) [% blastocysts formed]	
Animal no. genotype)	Cell source	Tissue/Cell line no.					Live offspring	ntES cell establishment
M1(Male	Somatic cell	Fibroblast	233	132	81 (61.4)[a]	6	4 (3.0) [4.9]	—
BD129F1)	Somatic cell	Fibroblast	101	76	44 (58)[a]	—	—	25 (32.9) [56.8]
	ntES cell	M1–1	332	224	80 (35.8)[b]	2	3 (1.3) [3.8]	—
	ntES cell	M1–2	132	65	13 (20)[b]	4	0	—
	ntES cell	M1–3	151	91	15 (17)[b]	4	0	—
	ntES cell	M1–4	148	90	24 (27)[b]	3	0	—

* Data were analyzed using the Chi-square test. $P<0.01$ between a and b.

Table 2. Effect of cell sources for nucleus donors on the establishment of ntES cell lines and production of cloned animals Category 2: cloned mice were only produced from somatic cells

Origin of donor nucleus			No. of enucleated oocytes	No. of normally activated oocytes	No. (%) of oocytes developing to blastocyst*	No. of dead fetuses	No. of successes (% oocytes activated) [% blastocysts formed]	
Animal no. (genotype)	Cell type (tissue)	Tissue/Cell line no.					Live offspring	ntES cell establishment
M2 (Male BD129F1)	Somatic cell	Fibroblast	198	141	80 (56.7)[a]	0	1 (0.7) [1.3]	
	Somatic cell	Fibroblast	19	13	9 (69)	—	—	6 (46.2) [66.7]
	ntES cell	M2–1	140	121	9 (7.4)[b]	0	0	—
	ntES cell	M2–2	108	61	22 (36)[c]	0	0	—
F3 (Female BD129F1)	Somatic cell	Cumulus	171	150	61 (40.7)	0	1 (0.7) [1.6]	
	Somatic cell	Cumulus	81	18	11 (61)[d]	—	—	9 (50.0) [81.8]
	ntES cell	F3–1	101	55	13 (24)[e]	0	0	—
	ntES cell	F3–2	102	75	18 (24)[e]	0	0	—
M4 (Male BDF2)	Somatic cell	Fibroblast	66	56	33 (59)[f]	0	5 (8.9) [15.6]	—
	Somatic cell	Fibroblast	64	41	29 (71)[f]	—	—	12 (29.3) [41.4]
	ntES cell	M4–1	132	94	28 (30)[g]	0	0	—
	ntES cell	M4–2	124	83	16 (19)[g]	0	0	—

* Data were analyzed using the Chi-square test. $P<0.01$ between a and b, b and c, d and e, f and g.

with a mean rate of 3.0%, which is the similar to the success rates in previous reports [2–11]. We established 68 ntES cell lines from all seven individual mice, with a high success rate (16% to 82%, mean 39%). All established cell lines were positive for alkaline phosphatase staining and formed embryoid bodies, suggesting that these ntES cell lines would be pluripotent. In our previous reports [18, 21], the rates of establishing ntES cell lines were around 20%, irrespective of mouse strain, sex, and cell type, but the BD129F1 and BDF2 strains were not used. To produce cloned mice from ntES cells in the present study, we carried out over 4000 nuclear transfers using 17 ntES cell lines derived from the seven donor mice, and obtained 41 cloned mice. The mean success rate (4.8%) was slightly higher than that from the somatic cells (3.0%).

When the results were analyzed for individual donor mice, they were categorized into four types. In category 1, cloned mice were obtained from both somatic and ntES cell nuclei (donor M1, Table 1). In category 2, clones were obtained only from somatic cell nuclei, but not from ntES cell nuclei (donors M2, F3, and M4; Table 2). In category 3, clones were obtained from ntES cell nuclei but not from somatic cell nuclei (donor F5; Table 3). In category 4, clones were not obtained from either cell type (donor F6; Table 4). The results for donor M7 were incomplete (the somatic cells were contaminated with bacteria) and provisionally placed in category 3 (Table 3). In this case, although the failure of cloned mice was due to a technical accident, we were still able to establish ntES cell lines. Though the rate of establishment of ntES cell lines was relatively low in category 3 (16.7% vs. 41–82%), the success rate for producing clones from ntES cells was the highest (7.4% vs. 0–3.8%). While no clones were obtained from category 4 (donor F6), ntES cell lines were established at a rate similar to the other donors.

The success rates for producing cloned mice from ntES cell nuclei differed between ntES cell lines, even when cell lines were derived from the same donor. Thus, for donor M1, only one (line M1–1) of 4 examined ntES cell lines contributed to produce clones, and its success rate (3.8%) was similar to somatic cell cloning (4.9%). For donor F5, we were unable to produce cloned mice from somatic cells or from the F5–1 ntES cell line, but one cloned mouse was obtained from the F5–2 line (1.9%). However, nine cloned offspring were obtained (8%), when ntES cell nuclei were used from the F5–3 line.

Table 3. Effect of cell sources for nucleus donors on the establishment of ntES cell lines and production of cloned animals Category 3: cloned mice were only produced from ntES cells

Origin of donor nucleus			No. of enucleated oocytes	No. of normally activated oocytes	No. (%) of oocytes developing to blastocyst*	No. of dead fetuses	No. of successes (% oocytes activated) [% blastocysts formed]	
Animal no. (genotype)	Cell type (tissue)	Tissue/Cell line no.					Live offspring	ntES cell establishment
F5 (Female BDF2)	Somatic cell	Cumulus	102	73	28 (38)	0	0	—
	Somatic cell	Cumulus	124	110	53 (48.2)[a]	—	—	9 (8.2) [17.0]
	ntES cell	F5–1	154	91	33 (36)	1	0	—
	ntES cell	F5–2	334	232	54 (23.3)[b]	4	1 (0.4) [1.9]	—
	ntES cell	F5–3	450	330	113 (34.2)	8	9 (2.7) [8.0]	—
M7 (Male BD129F1)	Somatic cell	Fibroblast	ND	—	—	—	—	—
	Somatic cell	Fibroblast	129	102	25 (24.5)	—	—	4 (3.9) [16.0]
	ntES cell	M7–1	309	237	90 (38.0)	6	7 (3.0) [7.5]	—
	ntES cell	M7–2	1081	653	227 (34.8)	8	21 (3.2) [9.3]	—

*Data were analyzed using the Chi-square test. $P<0.01$ between a and b.
For donor M7, somatic cell cloning experiments could not be conducted because of contamination.

Table 4. Effect of cell sources for nucleus donors on the establishment of ntES cell lines and production of cloned animals Category 4: Cloned mice were not produced from somatic cells or ntES cells

Origin of donor nucleus			No. of enucleated oocytes	No. of normally activated oocytes	No. (%) of oocytes developing to blastocyst*	No. of dead fetuses	No. of successes (% oocytes activated) [% blastocysts formed]	
Animal no. (genotype)	Cell type (tissue)	Tissue/Cell line no.					Live offspring	ntES cell establishment
F6 (Female BD129F1)	Somatic cell	Cumulus	187	140	86 (61.4)[a]	0	0	—
	Somatic cell	Cumulus	11	8	5 (63)	—	—	3 (37.5) [60.0]
	ntES cell	F6–1	147	113	36 (31.9)[b]	0	0	—
	ntES cell	F6–2	108	69	21 (30)[b]	0	0	—

*Data were analyzed using the Chi-square test. $P<0.01$ between a and b.

Discussion

The main objective of this study was to evaluate whether the success rate of producing cloned mice from ntES cell lines is higher than when using somatic cell nuclei from the same donor. We predicted that it would be higher since previous studies using different mouse strains showed better success rates using ES cell nuclei from normally fertilized embryos than from somatic cells; moreover, ntES and ES cells have very similar characteristics [18, 21].

Previously, we demonstrated that cloned mice can be generated from the nuclei of ntES cells [18]. We have also reported that mice can be cloned from the ICR strain using ntES cell nuclei [22], although it is very difficult to generate clones from adult somatic cell nuclei in this strain [26, 27]. However, the success rate in that study of ntES cloning was very low, and cloned pups were only obtained from one ntES cell line [22]. Thus, if it is difficult to produce cloned mice from somatic cell nuclei in a particular strain, it would also be difficult to produce clones via a second nuclear transfer from ntES cell nuclei [18, 22]. However, in those previous experiments, the somatic and ntES cell cloning studies used different individual mice, so a direct comparison of success rates was not possible. Therefore, in this study, somatic cells from the same donor mice were used for somatic clone production, establishment of ntES cell lines, and the following clone production from established ntES cells.

As a result of this study, the mean success rates were unexpectedly similar between ntES cell and somatic cell nuclei, and showed high variability

between donors in both cloning approaches. Only five of the 17 examined ntES cell lines contributed to cloned mice successfully. Thus, conversion from somatic cells to ntES cells does not increase the overall success rate of cloning. Moreover, the potential of donor nuclei for creating full-term development differed among ntES cell lines, even when the cell lines were derived from the same individual at the same time. For example, M1–1 ntES cell line showed a success rate similar to somatic cell cloning, whereas three other cell lines (M1–2 to M1–4) failed to form clone pups. The ntES cell lines from donor F5 showed more variation, and no cloned mice were born from nuclei of either somatic or F5–1 ntES cell nuclei. For this donor mouse, only one cloned mouse (1.9%) was born from the F5–2 ntES cell line. On the other hand, the F5–3 ntES cell line showed a high success rate (8%). Thus, ntES cell lines shows different potential, not only according to their genetic background, but also between each line, even those derived from the same individuals. This suggests that these differences were caused by epigenetic differences between donor cell nuclei, even among those from the same individual. We also noted that when the ntES cell establishment rate was lower, the success rate of cloned mice from these ntES cells was actually higher (see category 3).

Previously, we demonstrated that most (up to 80%) ntES cells showed normal karyotypes and germ line transmission, which confirmed their pluripotency [21]. The characteristics of these ntES cells were nearly identical to those of ES cells derived from normally fertilized embryos. It is possible that, most of the somatic nuclei that were transferred to enucleated oocytes might be incompletely reprogrammed, whereas they still showed normal developmental potential to the blastocyst stage [28, 29], and ntES cells have been established from incompletely reprogrammed blastocysts. Therefore, ntES cells may still retain some somatic-style epigenetic status, even if we have never detected any obvious difference between ntES and ES cells to date. The abnormal epigenetic status of the nucleus by incomplete reprogramming may not be visible even when ntES cells are transmitted via chimeric mice [18, 22]. However, the abnormal condition of the nucleus after first nuclear transfer may affect the development of clones via a second nuclear transfer. In other words, most of the ntES cell lines that were studied here may have different potential than ordinary ES cells, probably because of the imperfect nuclear epigenetic status of their nucleus. Further investigation is clearly needed to elucidate this.

In our previous work, the overall rate of ntES cell line establishment ranged from 9% to 18% [18, 21]. In the current study, it ranged from 16% to 82%, with a mean of 39%. This improvement could be explained by better nuclear transfer and ntES cell derivation techniques, such as a newly modified medium [30]. However, different mouse genotypes (either different genotypes or different supplying companies) and—possibly—increased skill of the researchers [31] may have affected the present results. Blelloch *et al*. [32] reported that 44%–57% of cloned blastocysts derived from embryonic carcinoma (EC) and ES cell nuclei could be used to establish ntES cell lines successfully, and suggested that the epigenetic state of the EC/ES cell genomes might be more efficiently reprogrammed than somatic cell genomes [32]. Our results here showed similar success rates from somatic cell nuclei as shown in that report. However, caution is required when comparing results between laboratories, as individual skill levels and laboratory environments will vary and affect the success rate.

In conclusion, the production of cloned mice from ntES cell nuclei in this series was no better than from somatic cells. However, we succeeded in cloning mice from three out of seven individuals via ntES cells. Thus, establishing ntES cell lines gives a better overall chance to clone mice from individual donors. Further, ntES cells will divide indefinitely and are easily cryopreserved. Therefore, for cloning valuable individual mice, we recommend that laboratories establish ntES cells simultaneously with the use of somatic cells as nuclear donors.

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