In vitro development of mouse somatic nuclear transfer embryos: effects of donor cell passages and electrofusion

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Summary

In this study, C57BL/6 adult male mouse ear fibroblast cells and Kunming mouse M2 oocytes were used as donors and recipients, respectively, to investigate the effect of passage number on donor cells and electrofusion times on the *in vitro* development of nuclear transfer (NT) embryos. The results demonstrated firstly that when the ear fibroblast cells from either 2–4, 5–7 or 8–10 passages were used as donors, respectively, to produce NT embryos, the number of passages undergone by the donor cells had no significant effect on the *in vitro* development of NT embryos. The developmental rates for morula/blastocyst were 15.2, 13.3 and 14.0%, respectively, which were not significantly difference ($p >$ 0.05). Secondly, when the NT embryos were electrofused, there was no significant difference between the fusion ratio for the first electrofusion and the second electrofusion ($p > 0.05$). The developmental rates of the 2-cell and 4-cell stages that had undergone only one electrofusion, however, were significantly higher than those that had had two electrofusions (65.7% compared with 18.4% and 36.4% compared with 6.1%; $p < 0.01$), furthermore the NT embryos with two electrofusions could not develop beyond the 4-cell stage. This study suggests that this protocol might be an alternative method for mouse somatic cloning, even though electrofusion can exert negative effects on the development of NT embryos.

Keywords: Ear fibroblast cells, Electrofusion, Mouse, Nuclear transfer

Introduction

Since the first cloning of 'Dolly' (Wilmut *et al.*, 1997), great progress has been made in mammalian somatic cloning, to date, cattle (Kato *et al.*, 1998), goat (Baguisi *et al.*, 1999), mouse (Wakayama *et al.*, 1998), pig (Polejaeva *et al.*, 2000), rabbit (Chesne *et al.*, 2002), cat (Shin *et al.*, 2002), horse (Galli *et al.*, 2003), mule (Woods *et al.*, 2003), rat (Zhou *et al.*, 2003), dog (Lee *et al.*, 2005; Parker *et al.*, 2006) and wolf (Kim *et al.*, 2007) have all been cloned successfully. Mice are one of the most important model animals in developmental biology.

Extensive studies have been performed on mouse NT that have used different types of somatic cells as donors, which include cumulus cells (Wakayama *et al.*, 1998), embryonic stem cells (Wakayama *et al.*, 1999a), tail-tip cells (Wakayama *et al.*, 1999b), immature Sertoli cells (Ogura *et al.*, 2000a), fetal fibroblast cells (Ono *et al.*, 2001), mature T and B cells (Hochedlinger & Jaenisch, 2002) and olfactory sensory neurons (Eggan *et al.*, 2004; Li *et al.*, 2004), and so on.

Mice are usually cloned by direct injection of donor nuclei into enucleated oocytes, a technique that differs from the cloning of other mammals, such as cattle, sheep etc., in which nuclear transfer (NT) is performed by cell fusion. Microinjection might be a better method for NT than is cell fusion, because the donor nucleus is intermingled readily with the recipient ooplasm such that the timing of nuclear transfer and oocyte activation can be controlled precisely (Ogura *et al.*, 2000b). The technique of mouse oocytes microinjection is more difficult than electrofusion, however, especially when large cells are used as donor cells, e.g. mature Sertoli cells and tail-tip fibroblast cells, which are larger and

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more rigid than cumulus cells (Wakayama *et al.*, 1998; Wakayama & Yanagimachi, 1999b). Ogura *et al.* (2000b) cloned mice successfully by electrofusion using tail-tip fibroblasts as donors and demonstrated that NT by electrofusion is a practical approach for mouse somatic cloning.

Previous studies in our laboratory have shown that a 3% sucrose treatment is a well tolerated way to facilitate the enucleation of mouse M2 oocytes, furthermore the enucleated oocytes are competent to support the full-term development of the embryos derived from *in vitro* fertilization, pronuclear transplantation and embryonic nuclear transfer (Wang *et al.*, 2001a, b; Kong *et al.*, 2005; Zhang *et al.*, 2007), respectively. In the present study, with the help of 3% sucrose treatment to facilitate the enucleation of mouse M2 oocytes, we employed adult male mouse ear fibroblast cells as donors to examine the effects of the number of donor cell passages and electrofusion times on the *in vitro* development of NT embryos and to try and establish a new possible method for mouse cloning.

Materials and methods

Preparation of adult male mouse ear fibroblast cells

The adult male C57BL/6 mouse (Institute of Genetics, Chinese Academy of Sciences, Beijing, China) was killed by cervical dislocation and the ear tissues were cut into small pieces. The pieces of tissue were placed into a cell culture bottle and digested with 0.25% trypsin (Sigma) at 4 °C for 12–18 h, next 2 ml DMEM/F12 (Gibco) containing 20% fetal cattle serum (FCS) was added gently. The ear tissues were cultured in 5% CO_2, 95% air at 37 °C for about a week until fibroblast cells developed on the bottom and became 70–80% confluent. After the fibroblast cells had been passaged 2–10 times, they were starved with 0.5% FCS for 2–3 days. Most of the starved cells were at the G_0 stage (Wilmut *et al.*, 1997) and were then digested with 0.25% trypsin for use as donor cells.

Collection of M2 oocytes

Female Kunming mice (Institute of Genetics, Chinese Academy of Sciences, Beijing, China), 8–12 weeks old, were superovulated with 7.5–10 IU equine chorionic gonadotropin (eCG, Tianjin Experimental Animal Center, Tianjin, China) followed by 7.5–10 IU hCG (Institute of Zoology, Chinese Academy of Sciences, Beijing, China) 48 h later. Matured oocytes were collected from the ampullae of oviducts 13–15 h after hCG injection and placed in 200 μl CZB medium containing 300 IU/ml hyaluronidase (Sigma). After complete removal of cumulus cells from the oocytes, they were washed three times in CZB containing 10 mM HEPES and then transferred for manipulation.

Nuclear transfer

The collected M2 oocytes were pretreated in CZB containing 3% sucrose, 5 μg/ml cytochalasin B (CB, Sigma) and 10 mM HEPES for 10 min. The transparent area was used as the position for enucleation (Wang *et al.*, 2001b). The zona pellucida was slit with a glass needle along one-fifth to one-quarter of its circumference (Tsunoda *et al.*, 1986) close to the position of the transparent or spindle. The M2 chromosomes of all recipients were removed with an enucleating pipette (inner diameter about 12 μm) with a non-bevel tip. A fibroblast donor cell arrested at the G_0 stage was inserted into the perivitelline space of the enucleated oocyte.

Electrofusion

The donor–recipient pairs were incubated for 15–30 min in CZB in an atmosphere of 5% CO_2 and 95% air at 37 °C to recover normal osmotic pressure. The fusion of donor–recipient pairs was induced by a DC pulse of 1.6 kv/cm for 10 μs using an ECM2001 (BTX) in 300 mM mannitol containing 0.1 mM MgSO4, 0.1 mg/ml polyvinyl alcohol and 3 mg/ml bovine serum albumin (Ogura *et al.*, 2000b). At 30 min after electrofusion, the donor–recipient pairs were checked for their fusion and the unfused pairs were treated using the same conditions for the second electrofusion.

Activation

The fused pairs were cultured for 1–2 h and then were activated by treatment with 10 mM SrCl2 in Ca^{2+}-free CZB for 6 h. Because the donor fibroblast cells were starved at the G_0 stage, 5 μg/ml CB was added into the activation medium to protect the extrusion of the second polar bodies and to keep the normal 2C DNA complement of the NT embryos (Wilmut *et al.*, 1997; Wakayama *et al.*, 1999a).

In vitro culture

Activated nuclear transfer embryos (pseudo-pronuclear formation) were cultured in CZB medium in 5% CO_2, 95% air at 37 °C.

Data statistic

Data were analysed by chi-squared test.

Table 1 Effects of donor cell passages on the electrofusion and *in vitro* development of mouse NT embryos.

Donor cell passages	No. of experiments	No. of oocytes (%)	No. of fused (%)	Developmental stage			
				2-cell (%)	4-cell (%)	8-cell (%)	M/B (%)
Passages 2–4	8	216	132 (61.1)[a]	91 (68.9)[a]	46 (34.8)[a]	29 (22.0)[a]	20 (15.2)[a]
Passages 5–7	6	155	64 (65.3)[a]	98 (63.2)[a]	30 (30.6)[a]	18 (18.4)[a]	13 (13.3)[a]
Passages 8–10	10	283	164 (58.0)[a]	106 (64.6)[a]	52 (31.7)[a]	32 (19.5)[a]	23 (14.0)[a]

Fusion rate: no. of fused oocytes/no. of oocytes; development rate of embryos at different stages: no. of embryos at different stages/no. of fused oocytes.
M: morula; B: blastocyst.
[a]Values in the same column are not significantly different ($p > 0.05$).

Results

Effects of different numbers of donor cell passages on electrofusion and *in vitro* development of NT embryos

C57BL/6 mouse ear fibroblasts that had undergone 2–10 passages served as donors for NT. These donors were divided into three groups of 2–4, 5–7 and 8–10 passages, respectively. The results showed that the rates of electrofusion and *in vitro* development of the NT embryos were not significantly different ($p > 0.05$) when the fibroblast cells that had undergone different numbers of passages were used as donors. The rates of electrofusion and *in vitro* development of 2-cell, 4-cell, 8-cell and morula/blastocyst were 61.1, 63.2 and 58.0%; 68.9, 65.3 and 64.6%; 34.8, 30.6 and 31.7%; 22.0, 18.4 and 19.5%; and 15.2, 13.3 and 14.0%, respectively (Table 1).

Effects of electrofusion times on the electrofusion and *in vitro* development of NT embryos

After the first electrofusion, the NT embryos were cultured in CZB medium in an atmosphere of 5% CO_2 and 95% air at 37 °C for 30 min. Next, they were checked for fusion. The unfused NT embryos underwent a second electrofusion under the same conditions and fusion numbers were recorded separately. The results showed that the fusion rates between the first time and the second time electrofusion were not significantly different (53.5% compared with 59.8%; $p > 0.05$), but that the developmental competence of NT embryos that had undergone one electrofusion was significantly higher than the embryos that had been electrofused twice. This difference was significant at a high level (65.7% compared with 18.4% and 36.4% compared with 6.1%; $p < 0.01$). Furthermore, the NT embryos that had undergone two electrofusions could not develop beyond the 4-cell stage (Table 2).

Discussion

In 1998, Wakayama *et al.* were the first to succeed in mouse somatic cloning using cumulus cells as donors. Since that time, mice have been cloned successfully from many kinds of somatic cells (Wakayama *et al.*, 1999a,b; Ogura *et al.*, 2000a; Ono *et al.*, 2001; Hochedlinger & Jaenisch, 2002; Eggan *et al.*, 2004; Li *et al.*, 2004). In Dolly's cloning, Wilmut *et al.* (1997) demonstrated that it was crucial for the success of the somatic cloning that the donor cells were arrested at G_0 stage of the cell cycle. Subsequent reports, however, showed that G_0 stage of the donor cells was not necessary for somatic cloning. Wakayama *et al.* (1999a) obtained cloned mice from ES cells at the G_1 and

Table 2 Effects of electrofusion number on the fusion and *in vitro* development of mouse NT embryos.

No. of electrofusions	No. of experiments	No. of oocytes (%)	No. of fused cells (%)	Developmental stage			
				2-cell (%)	4-cell (%)	8-cell (%)	M/B (%)
One electrofusion	7	185	99 (53.5)[a]	65 (65.7)[b]	36 (36.4)[b]	24 (24.2)	16 (16.2)
Two electrofusions	7	82	49 (59.8)[a]	9 (18.4)[b]	3 (6.1)[b]	0 (0)	0 (0)

Fusion rate: no. of fused oocytes/no. of oocytes; development rate of embryos at different stages: no. of embryos at different stages/no. of fused oocytes.
M: morula; B: blastocyst.
[a]Values in the same column are not significantly different ($p > 0.05$).
[b]Values in the same column are highly significantly different ($p < 0.01$).

G_2/M stages of the cell cycle, respectively. Because the chromosomes from the donor cells were duplicated at the G_2/M stage the donor cells contained 4C DNA, therefore when the NT embryos were activated, the $SrCl_2$ activation medium did not contain CB, so that the second polar bodies of the embryos were extruded and the DNA recovered was 2C in the embryos. In our present experiments, we primary cultured adult male mouse fibroblasts and passage cultured up to 10 passages. When the cells became 70–80% confluent, we treated the cells with 0.5% serum starvation to synchronize the cell cycle at the G_0 stage, such that the fibroblasts could serve as donors. CB was added to the $SrCl_2$ activation medium to block extrusion of the second polar body and to keep 2C DNA in the NT embryos (Wilmut *et al.*, 1997; Wakayama *et al.*, 1999a).

Both freshly prepared cells and passage cultured cells could be used as donor nuclei for animal somatic cloning. Passage cultured cells have some advantages in NT experiments, because modifications could be made easily in the cell culture procedure, such as addition of a transgene, cell cycle synchronization and so on. Wakayama *et al.* (1999a) cloned mice from ES cells after 22–33 passages, in order to demonstrate that ES cells with a high passage number could also act as donor nuclei for mouse cloning. In cattle cloning, when cattle ear skin fibroblasts that had undergone 5, 10 or 15 passages were used as donors, the *in vitro* developmental rates of blastocysts from NT embryos that had undergone 10 or 15 passage donors was significantly higher than that of cells that had undergone only five passage. This finding was possibly because there were some epidermal cells mixed in with the fibroblasts that had undergone only five passages and the number of these epidermal cells diminished as the number of passages increased (Kubota *et al.*, 2000). In our present experiments, we used mouse fibroblast cells that had undergone 2–10 passages as donors in order to compare the effect of number of passages on *in vitro* development. Our results suggested that the developmental potential of mouse NT embryos did not change with different donor cell passage number. Our results contrasted with those reported by Kubota *et al.*, the reason for which was unknown.

Mice are usually cloned by direct injection of donor nuclei into enucleated oocytes. The disadvantage of microinjection is that it is more difficult to manipulate than is electrofusion, especially when large cells are used as donor cells, e.g. mature Sertoli cells and tail-tip fibroblast cells, which are larger and more rigid than cumulus cells and resulted in much lower efficiency of mouse NT (Wakayama *et al.*, 1998, 1999b). Ogura *et al.* (2000b) cloned mice successfully by electrofusion using tail-tip cells and demonstrated that NT by electrofusion is practical for mouse somatic cloning. In electrofusion manipulation, the parameters of the electrical pulse are crucial for the survival of NT embryos and these parameters can exert negative effects on the subsequent development of NT embryos. In the present study, we used the same electrical pulse parameters to compare the effects of electrofusion number on the *in vitro* development of NT embryos. Our results showed that electrofusion had negative effects on the NT embryos, as, after two electrofusions, the NT embryos could not develop beyond the 4-cell stage. In the mouse NT experiment, therefore, the second electrofusion was possibly not necessary. Our results suggested that the combination of a 3% sucrose treatment for M2 oocytes to facilitate the enucleation and an electrofusion to induce NT might be an applicable protocol for mouse somatic cloning, even although the efficiency was relatively low.

Acknowledgement

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