Effect of enucleation procedures and maturation conditions on the development of nuclear-transferred rabbit oocytes receiving male fibroblast cells

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Enucleated oocytes matured *in vitro*, from which chromosomes were removed by treatment with ionomycin and demecolcine, were used as recipient oocytes for nuclear transfer of fibroblast cells from a mature male rabbit. The enucleated oocytes with donor nuclei were electrically activated 2 h after fusion. The potential of nuclear-transferred oocytes matured *in vitro* and ovulated oocytes to develop into blastocysts was high (33–55%), except for oocytes cultured for 8.0 (19%) and 8.5 h (25%) *in vitro*. After transfer of nuclear-transferred oocytes to recipients, ten of 62 (16%) and one of eight (13%) recipients that received *in vitro*-matured and ovulated oocytes,

respectively, had 19 (1%) and one (0.6%) implantation sites at the time of laparotomy on days 8–17 after transfer. Four fetuses, including two with beating hearts, were obtained on day 15 of gestation after transfer of nuclear-transferred oocytes matured *in vitro*. The reason for the low efficiency of fetus production was not clear. One possibility is chromosomal abnormalities of nuclear-transferred oocytes, as most (21 of 22) of the oocytes had chromosomes dispersed along the spindle fibre at the first cell cycle. This is the first report of successful production of fetuses after nuclear transfer of rabbit somatic cells.

Introduction

Cloned sheep (Schnieke *et al.*, 1997; Wilmut *et al.*, 1997), mice (Wakayama *et al.*, 1998; Kato *et al.*, 1999), cows (Kato *et al.*, 1998; Wells *et al.*, 1999), goats (Baguisi *et al.*, 1999) and pigs (Batthausen *et al.*, 2000; Onishi *et al.*, 2000; Polejaeva *et al.*, 2000) have been produced after nuclear transfer of somatic cells. The potential of enucleated oocytes receiving somatic cells to develop into young *in vivo* varies among species; it is relatively high in cows, sheep and goats, but low in mice and pigs. To date, no fetuses or young have been obtained after nuclear transfer of rabbit somatic cells. A relatively high percentage (13–30%) of enucleated rabbit oocytes receiving fibroblast or cumulus cells from adult animals develops into blastocysts (Mitalipov *et al.*, 1999; Yin *et al.*, 2000; Dinnyes *et al.*, 2001), but the implantation rate after transfer to recipients was only 2% (Yin *et al.*, 2000) and no fetuses were obtained. Although the reason for the failure of nuclear-transferred rabbit oocytes to develop into fetuses is not clear, one reason might be insufficient remodelling of somatic cell nuclei in rabbit oocytes. Better reprogramming of nuclei should occur in the cytoplasm of younger oocytes compared with aged oocytes, as the developmental potential of activated rabbit oocytes decreases with age (Stice and Robl, 1988; Collas and Robl, 1990). In bovine

nuclear transfer, *in vitro*-matured oocytes have been used as recipient oocytes soon after maturation. Mouse oocytes are also recovered immediately after ovulation. However, it is technically difficult to collect a large number of rabbit oocytes soon after ovulation because the timing of ovulation varies from 10.5 h to 14.0 h after injection of LH (Harper, 1963). In most reports in which cloned animals have been obtained, somatic cell nuclei are fused with or injected into mechanically enucleated oocytes at the second metaphase stage. Baguisi and Overstrom (2000) reported improved efficiency of production of cloned mice by direct injection of cumulus cells into chemically enucleated oocytes.

In the present study, an improved procedure is reported for production of nuclear reconstructed rabbit embryos and successful production of rabbit fetuses after nuclear transfer of fibroblast cells from a mature male into chemically enucleated oocytes matured *in vitro*, which is technically more demanding than obtaining *in vivo*-matured oocytes.

Materials and Methods

In vitro maturation of oocytes

Mature Japanese white female rabbits were treated with six consecutive s.c. injections of 0.4 iu FSH (Antrin; Denka Pharmaceutical Co. Ltd, Tokyo) given 12 h apart (Yin *et al.*, 2000). The rabbits were killed and the ovaries were removed aseptically at 12 h after the final dose of FSH. Fully grown oocytes were collected by rupturing follicles 1–3 mm

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Fig. 1. Nuclear transfer procedures.

in diameter in TCM-199 supplemented with 3 mg BSA ml⁻¹ and Hepes. The cumulus–oocyte complexes (COCs) were washed twice in maturation medium consisting of Earle's balanced salt solution (EBSS-complete; Maurer, 1978) supplemented with 10% heat-treated FBS. Fewer than 50 COCs were cultured in 500 µl of maturation medium in a four-well dish (Nunc, Roskilde) under paraffin oil (Nakarai, Kyoto) in an atmosphere of 5% CO₂ in humidified air at 39°C for 6–14 h. The cumulus oophorus cells surrounding the oocytes were removed in PBS containing 300 iu bovine testes hyaluronidase ml⁻¹ (Sigma Chemical Co., St Louis, MO) and corona cells were removed by repeated gentle pipetting with a small-bore pipette. Oocytes with a polar body were classified as mature. In some experiments,

ovulated oocytes, which were recovered from superovulated females 14–16 h after injection of hCG (Sankyozoki Co. Ltd, Tokyo), were used.

Preparation of donor cells

Fibroblast cells were obtained from ear skin tissue of a mature Dutch-belted male rabbit. The tissue was cut into 1–3 mm fragments and placed in Dulbecco's modified Eagle's medium (Nikken Biomedical Laboratory, Kyoto) with 10% FBS. After 7–14 days of culture, confluent cells were treated with trypsin–EDTA solution and passaged through culture, and used for nuclear transfer within seven passages. The fibroblast cells were induced into a quiescent state by culturing them in 0.5% FBS for 3–14 days (serum starvation; Kato *et al.*, 1998) or by culture in 10% FBS for 14–21 days (contact inhibition; Kato *et al.*, 2000). When these cells were cultured with bromodeoxyuridine (BrdU) medium, the incorporation rate was low (0.5–1.0%), but it was high (54%) in rapidly dividing cells. Analysis of the numbers of chromosomes in donor cells revealed that 41 of 50 cells had 44 normal diploid chromosomes.

Micromanipulation

In vitro-matured and ovulated oocytes were activated with 5 µmol ionomycin l⁻¹ for 3–5 min followed by 0.6 µg demecolcine ml⁻¹ (Sigma Chemical Co.) in maturation medium (Wako, Kyoto) for between 30 min and 2 h to induce extrusion of the second polar body (Fig. 1). A preliminary study revealed that extrusion of the second polar body started 0.5 h after activation under such conditions and that the condensed chromosome mass was allocated to the polar body. When oocytes were cultured for 20 h after treatment, the chromosomes remained in a condensed form and did not change into a pronucleus. The ionomycin- and demecolcine-treated oocytes were incubated in EBSS containing 7.5 µg cytochalasin B ml⁻¹ (Sigma Chemical Co.) and 0.6 µg demecolcine ml⁻¹ for 15 min, and the maternal chromosomes were then eliminated by removing the second polar body with a small volume of the surrounding oocyte cytoplasm. Chromosomes were removed mechanically from one group of oocytes (Yin *et al.*, 2000).

A single donor cell was introduced into the perivitelline space of enucleated oocytes, and membrane fusion of the donor cell and recipient oocyte was induced by two direct current pulses of 1.5 kV cm⁻¹ for 20 µs in Ca²⁺-free Zimmerman cell fusion medium. After electrical stimulation, reconstructed oocytes were washed in Ca²⁺- and Mg²⁺-free EBSS medium containing 3 mg BSA ml⁻¹ and left in the medium for 15 min. Fused oocytes were cultured for 2 h in EBSS-complete containing 10% FCS and 5 µg cytochalasin B ml⁻¹, and were then activated by electrical stimulation in Zimmerman cell fusion medium after treatment with 6-dimethylaminopurine (6-DMAP; Sigma Chemical Co.) for 2 h as described by Yin *et al.* (2000).

Table 1. *In vitro* development of nuclear-transferred rabbit oocytes

Source of oocytes	Time after <i>in vitro</i> culture (h)	Number of oocytes (%)					Mean number of cells in blastocysts (number of blastocysts examined)
		Used	Fused	Cultured	Cleaved	Developed into blastocysts	
<i>In vitro</i> -matured	8.0	398	292 (73) ^a	93	69 (74) ^a	18 (19) ^a	106 ± 89 ^a (8)
	8.0*	72	52 (72)	50	35 (70) ^a	10 (20) ^a	75 ± 24 ^a (10)
	8.5	549	442 (81) ^b	87	71 (82) ^{ab}	22 (25) ^a	83 ± 77 ^a (22)
	9.0	241	172 (71) ^a	46	43 (93) ^b	18 (39) ^{ab}	55 ± 21 ^a (16)
	10.0	128	111 (87) ^b	55	49 (89) ^a	30 (55) ^b	90 ± 43 ^a (24)
	11–14	128	116 (91) ^b	102	80 (78) ^a	34 (33) ^{ab}	95 ± 39 ^a (26)
<i>In vivo</i> -matured	–	123	102 (83) ^b	40	39 (98) ^b	21 (53) ^b	77 ± 43 ^a (11)

*The maternal chromosomes of oocytes were removed mechanically.

^{ab}Values within the same column with different superscripts are significantly different ($P < 0.01$).

Table 2. *In vivo* development of nuclear-transferred rabbit oocytes

Source of oocytes	Number of oocytes transferred	Number of recipients	Number of implantation sites (%)	Number of recipients with implantation sites (%)	Number of fetuses (live)
<i>In vitro</i> -matured	1877	62	10 (16)	19 (1)	4 (2)
<i>In vivo</i> -matured	160	8	1 (13)	1 (0.6)	0

In vitro culture of nuclear-transferred oocytes and embryo transfer

Nuclear-transferred oocytes were cultured in EBSS-complete medium for 6 days in an atmosphere of 5% CO₂ in air at 39°C and the numbers of blastocyst cells were counted. Immediately after activation or 18 h after *in vitro* culture, 5–34 nuclear-transferred oocytes at the one- to four-cell stage were transferred to each oviduct of recipients into which 30 iu hCG had been injected i.v. 12–18 h before. Laparotomies were performed on recipients 8–17 days after transfer to examine the number of swellings in recipient uteri (implantation sites). The recipients with at least one implantation site were considered pregnant. All swellings in pregnant recipients except for one were dissected carefully at the time of laparotomy using forceps to examine the fetuses.

Immunofluorescence localization of chromatin and microtubules

The localization of chromatin and microtubules in nuclear-transferred oocytes was examined according to previously reported procedures (Pinto-Correia *et al.*, 1993), except that propidium iodide (Sigma Chemical Co.) was used instead of Hoechst. In brief, whole mounts of nuclear-transferred oocytes at 2 h after ionomycin and demecolcine treatment, oocytes at 2 h after ionomycin treatment and intact oocytes were treated with anti- α -tubulin monoclonal antibody (Sigma Chemical Co.) and 1 μ g propidium iodide ml⁻¹.

Microsatellite analysis

The genomes of cloned fetuses, the recipient female and nuclear donor cells were typed for microsatellites using five primers (M77195, M33582, X99887, X99889 and X99891). Genomic DNA was extracted from the fetal membranes of four cloned fetuses, the muscle cells of the recipient female and the fibroblast cells used for donor nuclei.

Statistical analysis

Statistical differences were determined using chi-squared analysis for the *in vitro* development and Student's *t* test for the number of blastocyst cells. $P < 0.05$ was considered significant.

Results

The proportion of oocytes with a first polar body after *in vitro* maturation culture for 6, 7, 8, 9 and 10 h was 0, 45, 78, 82 and 89%, respectively. Most of the oocytes used were cultured for 8–10 h, but oocytes cultured for 11–14 h were also used in some experiments for convenience.

In vitro and in vivo development of nuclear-transferred oocytes

The *in vitro* developmental potential of oocytes receiving fibroblast cells is shown (Table 1). As there were no significant differences in the potential of oocytes matured *in vitro* receiving quiescent fibroblast cells induced by serum starvation and contact inhibition to develop into blastocysts

Fig. 2. Cloned fetuses on day 15 of gestation obtained after nuclear transfer of fibroblast cells from a mature male rabbit. (a) Fetus with a beating heart, 0.14 g body weight. (b) Fetus with a beating heart, 0.21 g body weight. (c) Dead fetus, 0.14 g body weight. (d) Dead fetus, 0.09 g body weight.

(27% for serum starvation and 21% for contact inhibition), the data from the two procedures are combined. Fusion rates were slightly, but significantly, different among groups (71–91%). The developmental potential of nuclear-transferred oocytes the chromosomes of which were removed by either chemical or mechanical procedures was not different. The developmental potential increased with the duration of *in vitro* maturation and culture, and reached a maximum at 10 h after culture, which is similar to the results obtained with *in vivo*-ovulated oocytes. There were no significant differences in the mean number of blastocyst cells among groups.

The implantation rates of nuclear-transferred oocytes were not significantly different between *in vitro*- and *in vivo*-matured oocytes (Table 2). A total of 2037 nuclear-transferred oocytes was transferred to 70 recipient rabbits, and data from the serum starvation and contact inhibition experiments were combined as the number of pregnancies was too small to analyse separately. Of 62 recipients that received *in vitro*-matured nuclear-transferred oocytes, ten had implantation sites at the time of laparotomy. One recipient that received oocytes cultured *in vitro* for 8 h after

receiving fibroblast cells (passage 3) that were serum-starved for 4 days had six implantation sites with four fetuses (two live; Fig. 2) on day 15 of gestation. The other eight recipients had 1–3 implantation sites on days 8–17, but none had fetuses. The remaining recipient had one large swelling but the presence of a fetus was not confirmed and the pregnancy was not allowed to proceed to term. On day 31, the recipient was re-examined, but had one absorbed implantation site without a fetus. Of ten recipients that received nuclear-transferred ovulated oocytes, one had a single small implantation site on day 15, but no fetus.

Immunofluorescence localization of chromatin and microtubules

As spindle formation patterns and chromosome allocation were similar in *in vivo*- and *in vitro*-matured oocytes, the data are presented together. A total of nine intact oocytes, 12 ionomycin-treated oocytes and 27 nuclear-transferred oocytes were examined. All intact oocytes recovered 18 h after hCG administration or matured *in vitro* for 8–14 h had metaphase chromosomes with a typical

Fig. 3. Immunofluorescence localization of microtubules (green) and chromosomes (orange). (a) Intact rabbit oocyte recovered at 18 h after hCG injection. (b) Ionomycin-treated rabbit oocyte at 2 h after treatment. (c) Nuclear-transferred rabbit oocyte with spread chromosomes at 2 h after fusion. (d) Nuclear-transferred rabbit oocyte with chromosomes of normal appearance.

barrel-shaped meiotic spindle close to the plasma membrane (Fig. 3a). The oocytes treated with ionomycin and cultured for 2 h extruded the second polar body, but seven of 12 oocytes still had metaphase chromosomes with a spindle (Fig. 3b) and the remaining five had a pronucleus. However, all oocytes treated with ionomycin for 3 min and demecolcine for 0.5–2.0 h had one condensed chromosome mass within the second polar body (Fig. 4). After treatment with ionomycin, the metaphase chromosomes started to move into anaphase, but the microtubules were disorganized by the demecolcine treatment before complete separation occurred. The entire chromosome mass then moved into a second polar body and remained in a condensed form (Fig. 4). Most of the nuclear-transferred oocytes (22/27) formed a spindle, but chromosomes were dispersed along the spindle fibres (21/22; Fig. 3c); however, one oocyte had chromosomes with a normal appearance (Fig. 3d).

Microsatellite analyses

Microsatellite DNA analysis confirmed that all four fetuses were genetically identical to each other and to the somatic cells used for donor nuclei. In addition, the cloned fetuses were not genetically related to the recipient rabbit.

Fig. 4. Rabbit oocytes matured *in vitro* were stained with anti- α -tubulin monoclonal antibody and propidium iodide at 2 h after ionomycin and demecolcine treatment. A condensed chromosome mass is visible within the second polar body.

Discussion

Since the first successful report by Wilmut *et al.* (1997), who obtained a live lamb after nuclear transfer of adult somatic

cells, somatic cell-cloned animals have been produced in sheep, mouse, cows, goats and pigs, but not in rabbits. To our knowledge, this is the first reported success of live fetuses at mid-gestation obtained after nuclear transfer of rabbit somatic cells. In addition, *in vitro*-matured oocytes have never before been used for nuclear transfer. One recipient that received nuclear-transferred *in vitro*-matured oocytes had six implantation sites and four fetuses. Two of the fetuses had beating hearts at the time of dissection on day 15 of gestation and a morphologically normal appearance. The development of the other two fetuses was retarded. All recipients except for one were killed on days 8–17 of gestation because the pregnancy rate was low (11/70, 16%) and the number of implantation sites per pregnant recipient was small (1–4). Therefore, it is not clear whether the live fetuses would have developed to term. All four fetuses were obtained from one of 70 females that received nuclear-transferred *in vitro*-matured oocytes. The other ten recipients with implantation sites had no fetuses. Although the number of oocytes transferred and number of recipients for evaluation of implantation success were different between *in vitro*-matured oocytes (1877 oocytes to 62 recipients, mean = 32) and *in vivo*-matured oocytes (160 oocytes to eight recipients, mean = 20), the proportions of recipients with implantation sites and nuclear-transferred oocytes implanted were not significantly different between the two groups. Thus, it is not clear from the present data whether the lack of *in vivo* development is related to the potential of *in vitro* development of these two types of reconstituted embryos. The potential of the *in vitro*-matured oocytes cultured for 8.0 and 8.5 h to develop into blastocysts was significantly lower than that of oocytes cultured *in vitro* for 10 h, probably due to the low sensitivity of the oocytes to parthenogenetic stimulation soon after maturation. Baguisi and Overstrom (2000) presented a brief report of the high potential of cumulus cells to develop to young after direct injection of a cytoskeleton-modifying agent into enucleated oocytes, but no comprehensive papers on this new methodology have been published. The results of the present study demonstrate that enucleation induced with demecolcine after activation with ionomycin is also possible in rabbit oocytes. The potential of nuclear-transferred *in vitro*-matured oocytes cultured for 8 h, the chromosomes of which were chemically removed, to develop into blastocysts (19%) was the same as that of mechanically enucleated oocytes receiving the same type of donor cells. Maternal chromosomes of oocytes were easily eliminated by simply removing the second polar body and the enucleation efficiency was 100% (18/18). The maturation promoting factor (MPF) activity in these enucleated oocytes was considered to be high because all enucleated oocytes receiving fibroblast cells at the G0–G1 phase had metaphase chromosomes.

The nuclear-transferred oocytes have a high potential to develop into blastocysts, but the reasons for the low efficiency of implantation are not clear. One hypothesis is that there is asynchrony between the developmental stage

of nuclear-transferred embryos and the physiological states of the recipients. As the potential of rabbit embryos to develop into young decreases markedly with the duration of *in vitro* culture (Maurer, 1978), the nuclear-transferred embryos were transferred to recipients immediately or at 18 h after culture. In general, it is considered that the highest rates of survival are obtained after embryo transfer to recipients that are synchronized with donors (Chang, 1950); therefore, the nuclear-transferred embryos were transferred to synchronized recipients into which hCG had been injected 12–18 h previously. However, retarded rabbit embryos, such as those obtained after freezing and thawing, had a higher rate of survival if they were transferred to recipients that were ovulating after the donors ovulated rather than to those that were synchronized or earlier ovulating recipients (Tsunoda *et al.*, 1982). As the window of implantation in rabbits is considered to be narrow (Adams, 1982), the nuclear-transferred embryo–recipient synchrony with respect to survival of fetuses should be examined further.

Another possible reason for the low implantation efficiency is abnormalities of spindle formation and chromosome arrangement in nuclear-transferred oocytes. When nuclear-transferred oocytes were examined 30 min after fusion, donor chromosomes were condensed, but only faint spindle formation was observed at 1 h after fusion. As we expected to increase the probability of remodelling of the donor nuclei and the full formation of transient spindle formation before parthenogenetic activation, the exposure time of donor nuclei to recipient oocyte cytoplasm was prolonged by 2 h. The procedures for removal of chromosomes from recipient oocytes were simple but might have removed the MPF preferentially binding to chromosomes, thereby resulting in a MPF concentration that was too low for the alignment of chromosomes in the metaphase plate. Thus, chromatin fragments were scattered along the microtubules in most nuclear-transferred oocytes. Chromosomal analysis of six blastocysts that developed from nuclear-transferred oocytes revealed that three had 44 normal diploid chromosomes, but that the other three had 39, 42 and 45 chromosomes. Disarrayed chromosomes have also been observed in mouse nuclear-transferred oocytes at 3 h after injection (Wakayama *et al.*, 1998). The potential of nuclear-transferred mouse oocytes to develop into morulae and blastocysts is relatively high (58–67%), but the potential to develop to young after transfer to recipients is low (2.0–2.8%; Wakayama *et al.*, 1998). Such chromosomal abnormalities are also observed in oocytes receiving nuclei from preimplantation embryos, even if oocytes are activated at the time of nuclear transfer (Pinto-Correia *et al.*, 1993). These abnormalities might have an adverse effect on the viability of nuclear-transferred oocytes during or after implantation. A possible method to overcome such chromosomal abnormalities in the first cell cycle and to promote sufficient reprogramming of donor nuclei would be to fuse donor nuclei with oocytes and then remove the oocyte chromosomes several hours after fusion.

The results of the present study demonstrate that chemical enucleation is effective for nuclear transfer of rabbit somatic cells. The demecolcine treatment of oocytes might have residual effects that persist during subsequent embryonic and fetal development, as the developmental potential of mouse two-cell embryos treated with demecolcine, but not with nocodazole, for 12.5–14.5 h is reduced after transfer to the recipient (Kato and Tsunoda, 1992). However, no adverse effects were observed in *in vitro* development of rabbit 16-cell stage embryos (Collas *et al.*, 1992). Further studies are required to examine the effects of concentrations of demecolcine or other reagents with low toxicity, such as nocodazole, on the potential of chemically enucleated nuclear-transferred oocytes to develop to term in other mammals.

Note added in proof: after completion of this article, successful production of cloned rabbits was reported after nuclear transfer of adult somatic cells.

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