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Differential Development of Rabbit Embryos Derived From Parthenogenesis and Nuclear Transfer

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ABSTRACT Parthenogenetic development (PA) is often used as a model to investigate activation protocols for nuclear transfer (NT) embryos. The objective of this study was to compare the development, as well as the dynamics of the nuclear materials and microtubules of PA and NT embryos following similar activation treatment. Our results demonstrate that, during parthenogenesis, activation through either electrical pulses or chemical stimulation alone resulted in low cleavage rates and compromised development. A combination of two sets of electrical pulses and a 2-h-exposure to chemical activation medium (5 μg/ml cycloheximide (CHX) and 2 mM 6-dimethylaminopurine (6-DMAP) in KSOM+0.1% BSA) could effectively activate rabbit oocytes, and resulted in a 99% (n = 73) cleavage rate with greater than 60% (n = 73) developing to blastocysts at day 4. However, the same activation protocol following NT resulted in only 65–72% of oocytes cleaved (depending on donor cell type), with less than 20% developing to the blastocyst stage. The differences observed between NT and PA embryos subjected to the same activation protocol were also evident in terms of the time required for their development to the blastocyst stage, as well as the cell numbers present in blastocysts at day 6. Furthermore, laser confocal microscopy revealed that pronuclear formation in the NT embryos was delayed by comparison to that in the parthenotes. In conclusion, our study suggests that an effective protocol for parthenogenesis cannot promise a comparable outcome for NT embryos. *Mol. Reprod. Dev. 68: 58–64, 2004.* © 2004 Wiley-Liss, Inc.

Key Words: embryo development; cloning; oocyte activation; rabbit

INTRODUCTION

Nuclear transfer (NT), or cloning, involves transferring a donor nucleus into an enucleated oocyte. NT using donor cells from adult animals provides a practical method for reproduction of animals that possess an obvious superior phenotype (McClintock, 1998; Wolf et al., 1998). By using transfected somatic or ES cells as nuclear donors, NT provides a valid cell-mediated approach for producing transgenic animals (Schnieke et al., 1997; Cibelli et al., 1998). When combined with gene targeting, NT offers an even more powerful method for genetic modification (McCreath et al., 2000; Denning et al., 2001). In addition to practices such as genetic modifications, xenotransplantation (Dai et al., 2002; Lai et al., 2002), disease models, and breeding programs (McClintock, 1998; Trounson, 2001), the technology of animal cloning can also be applied in basic research such as nucleus–cytoplasm interaction, cell differentiation, and gene reprogramming (Kono, 1997; Rideout et al., 2001).

Rabbit oocytes have proved to be an ideal model for many types of studies due to their large size, elasticity, and ease of handling, as compared to those fragile, small mouse oocytes. The cytoplasm of rabbit oocytes is more transparent than those from most domestic animals such as pig, cattle, and sheep. The gestation period of the rabbit is 1 month, much shorter than that of other domestic species. The rabbit, therefore, also provides an excellent model for basic research elucidating NT mechanism (Collas and Robl, 1990; Yang et al., 1992; Mitalipov et al., 1999; Yin et al., 2000; Dinnyes et al., 2001; Chesne et al., 2002).

The successful application of the techniques of NT reported in several mammalian species, as well as exciting progress on ES cell research and genetic modifications, promises a bright future for animal cloning (Schnieke et al., 1997; Wilmut et al., 1997; Cibelli et al., 1998; Kato et al., 1998; Wakayama et al., 1998, 1999; Kubota et al., 2000; McCreath et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000; Denning et al., 2001; Chesne et al., 2002; Dai et al., 2002; Lai et al., 2002; Shin et al., 2002). However, there are still several questions related to NT. In most studies, the total efficiencies for

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NT were extremely low (Wilmut et al., 1997; Wakayama et al., 1998; Wakayama and Yanagimachi, 1999; De Sousa et al., 2001). Many abnormal phenomena such as large size fetuses and placentas, as well as aberrant patterns of X chromosome inactivation were found in cloned animal (Wakayama et al., 1998; Young et al., 1998; Rideout et al., 2001; Xue et al., 2002).

Activation is a critical factor for further development in parthenogenesis and NT (Ozil, 1990). Previous studies on NT with embryonic blastomeres as donor cells has shown that insufficient activation of the reconstructed oocytes was an important factor contributing to the low efficiency in rabbit cloning (Collas and Robl, 1990). The purpose of this study was to select an ideal activation protocol for parthenogenesis, and then to investigate the development of cloned rabbit embryos by using similar activation protocols.

MATERIALS AND METHODS

Oocyte Recovery

All experiments were carried out in accordance with the guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Connecticut. Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Matured Dutch-belted or New Zealand White female rabbits were superovulated with two 3-mg and four 4-mg subcutaneous injections of follicle stimulating hormone (FSH, Folltropin-V, Vetrepharm Canada, Inc., London, Ont., Canada) given 12 hr apart, followed by i.v. injections of 200 IU of human chorionic gonadotropin (hCG) 12 hr after the final dose of FSH. At 16 hr after administration of hCG, the animals were laparotomized, and the oviducts were flushed with Dulbecco's modified PBS (DPBS; Gibco, Grand Island, NY) containing 3 mg/ml BSA. The cumulus cells were removed by exposure to hyaluronidase (300 IU/ml in DPBS) and followed by pipetting.

Culture of Somatic Cells

Somatic cells were cultured following the protocol described in our previous studies (Liu et al., 1999, 2000, 2001). For the culture of cumulus cells, cumulus-oocyte complexes (COC) with multiple layers of cumulus cells were collected from one individual and seeded in DMEM/F12 plus 10% (v/v) FBS in 25 ml cell culture flasks. After the cumulus cells attached to the bottom of the flasks, the oocytes were washed out with culture medium. For the culture of fibroblast cells, tissue biopsies were collected from the ear of an adult female rabbit. The fur was removed with a sterile scissor and the surface was cleaned with 70% (v/v) alcohol for 30 sec. The tissue was cut into small pieces and incubated in 0.25% (w/v) trypsin at 4°C for 4–6 hr. Then most of trypsin was removed and the tissue was incubated at 39°C for a further 30 min. The trypsinized pieces were seeded in 25-ml cell culture flasks with DMEM/F12 plus 10% (v/v) FBS. After 2 to 3 days, a fibroblast cell layer would be attached to the flask bottom. The culture medium was changed every 3 days.

Enucleation

Oocytes freed of cumulus cells were enucleated according to our laboratory's routine protocol (Kubota et al., 1998; Liu et al., 2002); all micromanipulation tools were prepared in our laboratory. Briefly, oocytes were placed into a 30-μl droplet of DPBS plus 20% (v/v) FBS and secured with a holding pipette, a small cut was made in the zona pellucida above Pb1 with an enucleation needle. Thirty percent of the cytoplasm surrounding Pb1 was expelled by pressing the oocyte with the enucleation needle. The expelled cytoplasm, but not the enucleated oocyte itself, was stained with 1 μg/ml Hoechst 33342, to confirm that the nuclear materials of the oocyte were removed.

Nuclear Transfer

Cumulus or fibroblast cells were cultured at full confluency for 3–5 days and detached by 0.25% (w/v) trypsin and 1 mM EDTA. Single donor cells were injected into the subzonal space of enucleated oocytes by a flat-tipped transfer pipette (internal diameter, 20–25 μm).

Electrofusion and Activation

Oocytes in parthenogenesis experiments or oocyte–somatic cell pairs in NT experiments were transferred into Zimmerman fusion medium (reviewed in (Wolfe, 1992)) in a 2-mm fusion chamber. Various activation treatments were compared for parthenogenesis. The detailed protocols will be described in the following section of Experimental Designs. Similar activation protocols were applied in NT experiments.

In Vitro Culture

Some embryos, derived from either parthenogenesis or NT, were cultured in KSOM supplemented with 0.1% (w/v) BSA for the first 2 days, then replaced with KSOM plus 1% (w/v) BSA for the remaining culture in a humidified atmosphere of 5% O_2: 5% CO_2: 90% N_2 at 39°C. Embryos at day 6 were stained with Hoechst 33342 and the cell numbers of blastocysts was recorded. Some blastocysts were fixed for double staining.

Embryo Transfer

Following activation, some embryos derived from NT were transferred into the oviducts of recipient does, whose ovulation was synchronized with that of the oocyte donors by injection of 1.2-μg GnRH analogue (Cystorelin).

Immunohistochemistry

Oocytes or embryos were fixed in 2% formaldehyde at 39°C for at least 30 min. They were then washed in washing buffer (PBS containing 3 mM NaN_3, 0.01% Triton X-100, 0.2% nonfat dry milk, 2% normal goat serum, 0.1 M glycine, and 2% BSA) three times, 15 min each, and left in washing buffer overnight at 4°C for blocking and permeabilization. Oocytes or embryos were

then double stained to visualize microtubules and DNA. Briefly, samples were incubated in mouse anti-α-tubulin (1:200) for 4 hr at 37°C or overnight at 4°C. After three washes in washing buffer, the oocytes or embryos were incubated in FITC-conjugated goat anti-mouse IgG (1:200) for 4 hr at room temperature. Finally, the samples were washed, stained for DNA with 7.5 μM propidium iodide, mounted on glass slides, and examined with a laser scanning confocal microscope (Leica TCS SP2, Mannheim, Germany).

Experimental Design

Experiment 1: Comparing activation protocols during parthenogenesis. Oocytes at the metaphase II stage were randomly divided into five groups and subjected to different activation treatments as follow: (1) two electrical pulses (EPs; 3.2 KV/cm for 20 μsec, with 0.1 μsec interval), followed 1 hr later, by another EPs; (2) incubation in combined activation medium (CAM; 5 μg/mal cycloheximide (CHX) and 2 mM 6-dimethylaminopurine (6-DMAP) in KSOM + 0.1% BSA) for 2 hr; (3) EPs, followed by CAM for 1 hr, then a second set of EPs and further CAM for 1 hr; (4) a first set of EPs, then 1 hr later, a second set of EPs, and placed into CAM for 1 hr; and (5) without electrical pulse or CAM as control. Following the activation treatments, the oocytes were washed and incubated in culture medium. Some oocytes from different activation treatments were fixed for subsequent double staining.

Experiment 2: Comparison of nuclear transfer using different donor cells. Two kinds of somatic cells, cumulus cells and ear fibroblasts, were used as donor cells in NT. The activation protocol determined to be most effective for parthenogenesis was utilized in the NT experiment.

Experiment 3: Transfer of embryos derived from nuclear transfer. Some embryos produced in experiment 2 were transferred to recipient animals. To increase the chance of maintaining a pregnancy, both fused as well as some nonfused embryos were transferred into a recipient doe.

Statistical Analysis

Each experiment was replicated at least three times. All data were analyzed by the chi-square test.

RESULTS

Development of PA Embryos

Parthenogenetic development (PA) of rabbit MII oocytes subjected to different activation protocols is summarized in Table 1. The activation treatment consisting of two sets of electrical pulses, resulted in 82 % of oocytes cleaved, and 32 and 42% developed to blastocyst stage at day 4 and 6, respectively. The oocytes activated by chemicals alone had significantly lower rates of cleavage and blastocyst development, as well as having a fewer number of cells in the blastocyst, than those activated by electrical pulses. It is apparent that the PA of oocytes would be improved by a method of activation which combines both electric pulses and chemicals. There does not seem to be any considerable difference between the two combination activation treatments; however, the embryo quality, as evidenced by the cell numbers, was the greatest in those blastocysts derived from oocytes activated by two sets of electrical pulses followed by exposure to the combined chemical activation medium. The embryos which developed from oocytes receiving a 2 hr exposure to chemical activation medium had larger cell numbers than those which had only a 1 hr exposure to chemical activation. Very few (5 %) oocytes cleaved and no blastocyst formed in the control group without activation.

Development of NT Embryos

In Experiment 2, we examined the development of oocytes that were enucleated and then had either a cumulus cell or a fibroblast cell transferred into it and subjected to activation using the protocol found most effective for parthenogenetic activation, i.e., two sets of electrical pulses plus two 1-hr exposures to chemical activation medium. There were no significant differences found between these two types of donor cells,

TABLE 1. Development of Rabbit Oocytes With Different Activation Protocols

Treatment[a]	Number of oocytes	Number cleaved (%)	Number of blasts (%)[b] (day 4)	Number of blasts (%)[c] (day 6)	Cell number/ blasts (day 6)
EPs/EPs	65	53 (82)[f]	21 (32)[e]	27 (42)[e]	159 ± 11^{d}
CAM2h	74	21 (28)[e]	5 (7)[d]	8 (11)[d]	115 ± 41^{f}
EPs/CAM1h/Eps/CAM1h	73	72 (99)[f]	48 (66)[f]	47 (64)[f]	162 ± 5^{d}
EPs/EPs/CAM1h	68	65 (96)[f]	37 (54)[f]	41 (60)[f]	137 ± 14^{e}
Control	61	3 (5)[d]	0 (0)[d]	0 (0)[d]	—

[a]EPs/EPs: two electrical pulses (EPs; 3.2 KV/cm for 20 μsec, with 0.1 μsec interval), followed 1 hr later, by another EPs; CAM2h: incubation in combined activation medium (CAM; 5 μg/ml cycloheximide (CHX) and 2 mM 6-dimethylaminopurine (6-DMAP) in KSOM + 0.1% BSA) for 2 hr; EPs/CAM1h/EPs/CAM1h: EPs, followed by CAM for 1 hr, then a second set of EPs and further CAM for 1 hr; EPs/EPs/CAM1h: a first set of EPs, then 1 hr later, a second set of EPs, and placed into CAM for 1 hr; control: without electrical pulse or CAM.
[b]Embryos with an obvious cavity were considered as blastocysts (blasts).
[c]Only embryos with cell number above 60 were considered blastocysts (blasts).
[d,e,f]Data within the same columns with different superscripts are significantly different ($P < 0.05$).

TABLE 2. Development of Rabbit Embryos Derived From Nuclear Transfer (NT) and Parthenogenesis (PA) Following the Same Activation Protocol

Embryo type	Number NT	Number (%) fused	Number (%) cleaved	Number (%) blasts[d] (day 4)	Number (%) blasts[e] (day 6)	Cell number/ blast (day 6)
NT-control[f]	45	—	12 (27)[a]	0 (0)[a]	0 (0)[a]	—
NT-cumulus cells	79	37 (47)	24 (65)[b]	2 (5)[a,b]	3 (8)[a,b]	89 ± 3^{a}
NT-fibroblasts	183	99 (54)	71 (72)[b]	10 (10)[b]	18 (18)[b]	110 ± 17^{b}
PA	—	73	72 (99)[c]	48 (66)[c]	47 (64)[c]	162 ± 5^{c}

The activation protocol used here is electrical pulses (EPs; 3.2 KV/cm for 20 μsec, with 0.1 μsec interval), followed by combined activation medium (CAM; 5 μg/ml cycloheximide (CHX) and 2 mM 6-dimethylaminopurine (6-DMAP) in KSOM + 0.1% BSA) for 1 hr, then a second set of EPs and further CAM for 1 hr.
[a,b,c]Data within the same columns with different superscripts are significantly different ($P < 0.05$).
[d]Embryos with an obvious cavity were considered as blastocysts (blasts).
[e]Only embryos with cell number above 60 were considered blastocysts (blasts).
[f]NT-control: oocytes were enucleated without injection of any type of donor cells.

regarding their rates of fusion, cleavage, or blastocyst development at days 4 and 6 (Table 2). The average cell number in blastocysts derived from fibroblast cells, however, was higher than those from cumulus cells (110 vs. 89). Without injection of any type of donor cell, 27% of the enucleated oocytes cleaved, but none developed into blastocysts (Table 2).

The developmental outcomes of embryos derived from NT, using either cumulus cells or fibroblast cells as donor cells, were unmatched with those from parthenogenesis with the same activation protocol (Table 2). Approximately 68% of oocytes form NT cleaved, whereas more than 95% of oocytes cleaved parthenogenetically when activated by a combination of electrical pulses and chemicals. Utilizing a similar activation protocol, about 60% of oocytes developed into blastocysts through parthenogenesis at day 4, more than five times as many as those from NT (5–10%) at the same time (day 4). If cultured for an additional 2 days, more NT embryos developed to the blastocyst stage, however, the day 6 blastocyst rates (8–18%) of NT derived embryos remained less than one third of that achieved through parthenogenesis. Furthermore, the cell numbers in blastocysts derived from NT were much lower than those from parthenogenesis (Table 2).

Dynamics of Nuclei and Microtubules in PA or NT Embryos

Typical changes observed in microtubule organization and nuclear morphology during parthenogenesis following different activation protocols are presented in Figure 1.

Electrical pulses alone. One hour after the first set of electrical pulses, the oocytes typically remained in MII stage, displaying a clear metaphase spindle with the chromosomes aligning on the metaphase plate (Fig. 1a). The oocytes progressed into telophase 1 hr after the second set of electrical pulses, evidenced by the chromosomes separating and moving to the two poles of the spindle (Fig. 1b). Many oocytes formed one or two pronuclei within 2 to 4 hr after activation by two sets of electrical pulses (Fig. 1c,d).

Chemical activation alone. When the oocytes were exposed to chemical activation medium without any electrical stimulus, they displayed a microtubule network consisting of many cytoasters and an orderly distribution of chromosomes (Fig. 1e,f). Two to four hours after the oocytes were removed from chemical activation medium, the microtubule network as well as the cytoasters disappeared and, instead, the spindle reformed with chromosomes aligned on the metaphase plate (Fig. 1g,h). Most of the oocytes treated with chemical activation alone did not form pronuclei.

Two sets of electrical pulses plus two 1-hr exposures to chemical activation medium. If oocytes were subjected to one set of electrical pulses prior to being exposed to chemical activation medium for 1 hr, they displayed a cortically distributed microtubule network and a condensed pronucleus (Fig. 1i). The pronucleus increased in size after the second set of electrical pulses, and another 1-hr exposure to chemical activation medium was applied to the oocytes (Fig. 1j). Two and four hours after cessation of the combined activation, the oocytes formed one or two swollen pronuclei (Fig. 1k,l).

Two sets of electrical pulses plus a 1-hr exposure to chemical activation medium. Similar pronucleus formation was observed in the group exposed to only 1 hr exposure to chemical activation combined with two sets of electrical pulses (Fig. 1m–p).

Typical changes in microtubule organization and nuclear morphology after NT following the combined activation protocol found most effective for parthenogenesis are presented in Figure 2. Following electrofusion and a 1-hr exposure to chemical activation medium, the constructed oocyte displayed a strongly stained microtubule network distributed throughout the cortex as well as a lighter stained microtubule network inside the cytoplasm. The donor nuclei were present at the periphery of the oocytes, with a microtubule aster nearby (Fig. 2a,b). Following the second set of electrical pulses and an additional 1-h exposure to chemical activation medium, the aster which appeared close to the donor nucleus along with the inside microtubule

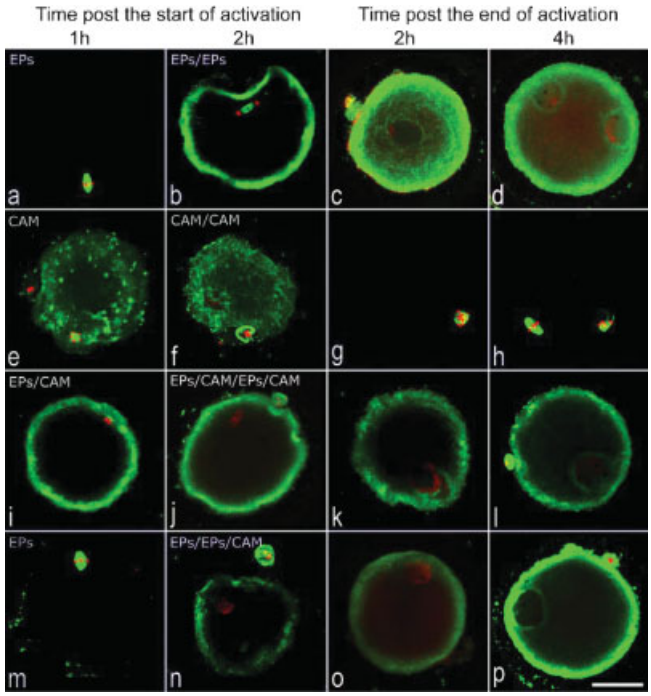

Fig. 1. Dynamics of nuclei and microtubules during parthenogenesis with different activation protocols. EPs, a set of two electrical pulses (3.2 KV/cm for 20 μsec, with 0.1 μsec interval); CAM, incubation in combined activation medium (5 μg/ml cycloheximide and 2 mM 6-dimethylaminopurine in KSOM + 0.1%BSA) for 1 hr. Green = α-tubulin, red inside the cytoplasm = DNA, red on the cortex = actin. See Results for detailed description. Bar = 50 μm.

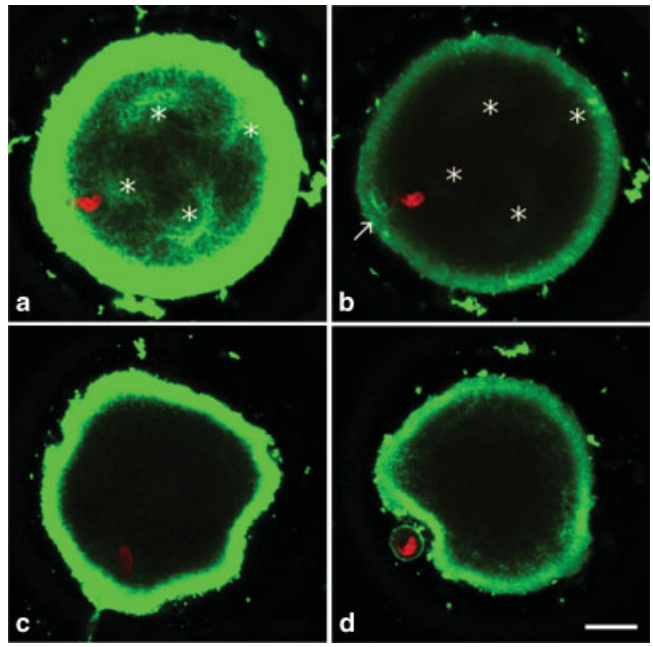

Fig. 2. Dynamic of nuclei and microtubules during nuclear transfer. **a**,**b**: NT oocyte at 2 hr post fusion, note that microtubule asters (*) formed in the cytoplasm of the reconstructed oocytes and an aster (arrow) close to the fused somatic nucleus; the green signals in panel a were enhanced by software to show the cytoplasmic microtubule asters (*). **c**: NT oocytes at 4 hr post fusion, no aster formed close to the somatic nucleus. **d**: Unfused oocyte, note that the somatic cell is in the perivitelline space of the enucleated oocyte. Bar = 25 μm.

network disappeared, while the cortical microtubule network remained (Fig. 2c). The cortical microtubule network of enucleated oocytes also formed after activation even though the donor nucleus was not fused with the ooplasm (Fig. 2d).

Embryo Transfer

Enucleated oocytes reconstructed with cumulus cells, following electrofusion and a combination activation treatment, were transferred into the oviducts of synchronized recipient does. Transfer of 196 fused reconstructed oocytes, together with 218 unfused oocytes, into 4 recipients results in no full term development (Table 3).

DISCUSSION

Activation of an oocyte is a step critical for its further development, including parthenogenesis and NT. Many kinds of activation stimulation, such as electrical pulses (Ozil, 1990), inositol 1,4,5-triphosphate (Mitalipov et al., 1999), 6-DMAP (Yin et al., 2000; Chesne et al., 2002), and cooling (Chang, 1954), have been utilized to induce in rabbit oocyte activation. The aim of artificial activation is to mimic the processes occurring in natural fertilization, i.e., the intracytoplasmic calcium surges, with the accompanying decrease in activity of maturation/M-phase promoting factor (MPF), mitogen-activated protein kinase (MAPK), and cytostatic factor (CSF), and the onset of maternal RNA transcription (Machaty and Prather, 1998; Liu and Yang, 1999). Electrical pulses could induce obvious ionic calcium influx, while CHX, a protein synthesis inhibitor, and 6-DMAP, a kinase inhibitor, were assumed to promote pronuclear formation (Liu et al., 1998a; Liu and Yang, 1999). Neither two sets of electrical pulses nor chemical activation alone were sufficient for activating rabbit oocytes. The advantage of combined activation on parthenogenesis was also shown in bovine oocytes (Liu et al., 1998b); while activation with electrical pulses alone worked well in pigs (Zhu et al., 2002). If oocytes

TABLE 3. Development of Cloned Embryos After Transfer Into Recipients

Number NT	Number of oocytes before fusion	Number (%) fused	Number of embryos transferred			Number of recipient	Number developed to term
			Fused	Unfused	Total		
435	429	197 (46)	196	218	414	4	0

were exposed to chemical activation medium only, without any electrical pulses, the nuclei did not change a great deal, while the pattern of microtubules changed dramatically. Once an oocyte was removed from the chemical activation medium, the MII spindle reassembled and the chromosomes were arranged on the spindle plates. Electrical stimulation alone could induce pronuclear formation, however, further development was compromised when compared to that achieved by combining electrical and chemical stimulation. In addition, combined chemical treatment (CHX + 6-DMAP) (this study and Chesne et al., 2002) with electrical pulses significant improved blastocytst development over CHX or 6-DMAP plus electrical pulses alone (Mitalipov et al., 1999; Dinnyes et al., 2001). Perhaps electrical pulses plus the combined chemical treatment which stops protein synthesis as well as phosphorylation will induce more complete activation leading nonreversable reduction of MPF (Liu et al., 1998a,b; Liu and Yang, 1999).

Parthenogenesis has often been used as a model to investigate activation protocols for NT (Yin et al., 2000; Dinnyes et al., 2001). However, even using the same activation protocols, the developmental consequence for NT embryos is not comparable to PA. The cleavage rate of NT oocytes was significantly lower than that of parthenotes activated by combination of electrical pulses and chemicals. Using the same protocol, about 60% of oocytes developed into blastocysts via parthenogenesis, while only 5–10% of oocytes from NT developed to the blastocyst stage at day 4. With two additional days culture, more NT embryos developed into blastocysts, however, the total remained less than one third of the number of blastocysts from parthenogenesis. Whereas most blastocysts formed at day 4 during parthenogenesis, approximately half of the blastocysts from NT did not form until day 6. The quality of the parthenogenesis-derived blastocysts was superior to that of blastocysts originating through NT as evidenced by their much greater cell numbers.

Many factors may explain the developmental differences observed between parthenogenetic and nuclear transferred embryos. First, oocytes which underwent parthenogenesis were preserved intact, neither the zona pellucida, which is especially important for implantation in the rabbit, nor the cytoplasm, a pool for numerous regulatory factors were disrupted. In contrast, the embryos undergoing a NT endure invasive micromanipulation at least twice: during enucleation when a slit is made in the zona pellucida through which about one third of the cytoplasm is expressed, and again when a donor cell is injected into the subzonal space in order to transfer a somatic nuclei. Second, it takes some time to complete the micromanipulations requisite for NT. Since rabbit oocytes are known to be sensitive to mechanical manipulations as well as temperature fluctuations, prolonged exposure to room temperature during micromanipulation would be detrimental to the further development of cloned rabbit embryos. Third, the nuclear materials in embryos undergoing parthenogenesis were at metaphase prior to activation, while most donor nuclei were at interphase when introduced into the cytoplasm of enucleated oocytes which, prior to activation would be at metaphase. The incompatibility of cell cycles could compromise reprogramming, since a donor interphase nuclei is placed into a metaphase ooplasm. Moreover, we have observed that it takes longer for pronuclear formation in NT embryos than in parthenogeneisis (our unpublished data).

The question remains whether or not centrosome inheritance is a critical factor for the success of somatic NT in various species of mammals. The mechanism of centrosome inheritance in mammals has long been an enigma of developmental biology (Karr, 2001). Paternal centrosomal inheritance was evidenced in domestic species such as sheep (Le Guen and Crozet, 1989; Crozet et al., 2000), cattle (Navara et al., 1994), and pig (Kim et al., 1996). In contrast, centrosomes were considered maternally inherited in rodents (Schatten et al., 1991). It has been assumed that the centrosome in rabbits was paternally inherited, however, recent studies suggest that the centrosome in rabbit zygotes probably originated from both parents (Terada et al., 2002). Our study has shown that, 1 hr after electrofusion, an obvious microtubule aster was formed close to the donor cell however, within another 1 hr, it had disappeared. It is unclear whether this aster is due to somatic centrosomes which accompanied the donor nuclei, or is due to some unknown factor(s) in the ooplasm which interacted with the donor nuclei. It is unclear whether the lower cleavage rates achieved in nuclear transfer, as compared to parthenogenesis, is related to the functions of centrosomes. Additionally, we are unable to ascertain whether centrosome disruptions cause many NT derived embryos to become fragmented and/or divide abnormally.

There are many variables and factors which affect embryo transfer. The zona pellucida of preimplantation rabbit embryos is known to be very important for implantation. It is likely, therefore that micromanipulation which necessarily disrupts the zona pellucida during enucleation and again when transferring donor cells, would compromise the developmental potential and implantation rate. Since embryos derived through nuclear transfer grow much slower than those from parthenogenesis, or those grown in vivo, the incompatibility of the transferred embryos' cycle to that of the recipient animal's could further contribute to the failure of embryo transfer. Transferring reconstructed embryos into recipients retarded by nearly one day, as well as launching a specific activation protocol, led to the first success of somatic nuclear transfer in rabbit (Chesne et al., 2002). However, the differential development from parthenogenesis and NT was also obvious in that study.

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