Development of mouse enucleated oocytes receiving a nucleus from different stages of the second cell cycle

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Summary. The influence of the stage of the cell cycle of donor nuclei on the development of mouse oocytes enucleated at telophase I was examined. After nuclear transplantation and activation, a high proportion of the oocytes remodelled a nucleus, emitted a polar body and formed a pronuclear-like nucleus. Most of the reconstituted embryos that received an interphase nucleus 30–32 h or 34–36 h after treatment with human chorionic gonadotrophin (hCG) arrested at the 2-cell stage. The reconstituted embryos were able to develop to blastocysts when nuclei from late 2-cell embryos (44–46 and 48–50 h after hCG) were transferred to the oocytes. The resulting blastocysts were transferred to recipients and ten live young were obtained from the embryos that formed a pronuclear-like nucleus after extrusion of a polar body. Thus, the developmental ability of the reconstituted embryos was critically influenced by the stage of the cell cycle of the donor nuclei.

Keywords: nuclear transfer; mouse; oocytes; cell cycle

Introduction

Nuclear transplantation studies have shown that nuclear cytoplasmic interaction is a key factor for the successful development of reconstituted mammalian embryos to term. The primary factor that influences their developmental ability is the differentiation of the donor nuclei, which occurs in embryonic development. The developmental ability of reconstituted zygotes derived from the transfer of nuclei from 2-cell and more advanced embryos was restricted after their transfer to recipient female mice (McGrath & Solter, 1984; Tsunoda *et al.*, 1986) and rats (Kono *et al.*, 1989). However, single blastomeres of 4-cell embryos and two blastomeres of 8-cell embryos developed to term when fused with enucleated 2-cell embryos (Tsunoda *et al.*, 1987; Kono *et al.*, 1988, 1991a). The transferred nuclei are remodelled by cytoplasmic factors (e.g. maturation-promoting factor) in secondary oocytes (Szollosi *et al.*, 1986, 1988). Remodelling of donor nuclei in reconstituted oocytes gives the resulting embryos greater developmental ability (Willadsen, 1986; Prather *et al.*, 1987; Smith & Wilmut, 1989; Tsunoda *et al.*, 1989). In amphibians, most blastula nuclei were able to develop to tadpoles when transferred to enucleated oocytes, but the developmental ability was rapidly reduced when later-stage donor nuclei were used (reviewed by Gilbert, 1984; DiBerardino, 1987).

The compatibility of cell-cycle phase between donor nuclei and recipient cytoplasm has been suggested as a second factor that influences the developmental ability of reconstituted embryos. Nuclear transplantation studies in *Rana pipiens* have shown that donor nuclei at the G₁ phase of the cell cycle have a reduced capacity to support development beyond the blastula stage (Von Beroldingen, 1981). In mammals, the effect of the stage of the cell cycle of the donor nuclei upon developmental potential was significant when nuclear transplantation was carried out between zygotes and 2-cell mouse embryos (Howlett *et al.*, 1987; Smith *et al.*, 1988, 1990). These results

from nuclear transfer studies demonstrate that the effect of the stage of the cell cycle of donor nuclei used for nuclear transfer must be clarified to advance our ability to clone embryos. We have developed an efficient procedure for nuclear transplantation into enucleated mouse oocytes (Kono *et al.*, 1991b), in which the chromosomes of the oocyte are removed at telophase of the first meiotic division and most of the reconstituted oocytes emit a polar body after activation. In the present study, we examined the developmental ability *in vitro* and *in vivo* of the reconstituted mouse embryos produced using this procedure, when blastomeres from specific stages of the second cell cycle were used as donor nuclei. These studies demonstrate that live young can be produced when nuclei from late 2-cell blastomeres are transferred to enucleated mouse oocytes.

Materials and Methods

Collection of oocytes and embryos

Female agouti F₁ hybrid mice (C57BL/6J × CBA) were used as oocyte donors and female albino mice of strain CD-1 were used to produce 2-cell embryos. They were superovulated by injections of 5 iu pregnant mares' serum gonadotrophin (Peamex, Sankyo Ltd, Tokyo) and 5 iu human chorionic gonadotrophin (hCG, Puberogen, Sankyo Ltd) given 48 h apart. Oocytes at telophase of the first meiotic division and freshly ovulated oocytes at metaphase II were released from the ovarian follicles at 9–11 h after hCG and from the oviducts at 12 h after hCG, respectively. Cumulus cells were removed by treatment with 300 iu hyaluronidase/ml in M2 medium (Quinn *et al.*, 1982) and washed several times. After injection with hCG, CD-1 females were paired with males of the same strain, and 2-cell embryos were collected by flushing the oviducts with M2 medium at 30–32, 34–36, 44–46 and 48–50 h after hCG injection.

Removal of chromosomes from recipient oocytes

Oocytes were collected from ovarian follicles 10 h after hCG injection. The zonae pellucidae of the oocytes were slit with a glass needle along 10–20% of their circumference and placed in a small drop of M2 medium containing cytochalasin B (5 µg/ml) in a micromanipulation chamber. The telophase I chromosomes were removed using an enucleation pipette with unsharpened bevelled tip (Kono *et al.*, 1991b). After aspiration of the first polar body, telophase chromosomes with spindles were aspirated into the enucleation pipette: these were readily identified using differential interference microscopy. By this method, all of the chromosomes from recipient oocytes were removed.

Nuclear transfer

After enucleation, the oocytes were cultured in M16 medium (Whittingham, 1971) for 4–6 h and were then subjected to nuclear transplantation. This gives a high activation rate in the reconstituted eggs (Kwon *et al.*, 1991). Nuclear transplantation was carried out as described by McGrath & Solter (1983) and Tsunoda *et al.* (1986). A karyoplast from a donor 2-cell embryo was introduced with inactivated Sendai virus (HVJ) (haemagglutinating activity 2700 unit/ml) into the perivitelline space of the enucleated and preincubated oocytes. The manipulated oocytes were cultured with M16 medium in an atmosphere of 5% CO₂, 5% O₂ and 90% N₂ at 37°C. Most oocytes fused with the nuclear karyoplast within 15 min. About 60 min after fusion with a karyoplast, the oocytes were activated with 7% ethanol for 7 min at room temperature (Cuthbertson, 1983).

Culture and embryo transfer

After nuclear transplantation and activation, the reconstituted embryos were cultured in individual drops of M16 medium containing 100 µmol EDTA/l for 4 days, covered with paraffin oil, in an atmosphere of 5% CO₂, 5% O₂ and 90% N₂ at 37°C. The development of the embryos was observed twice a day. Reconstituted embryos that developed to the blastocyst stage were transferred to the right uterine horn of pseudopregnant recipients 2.5 days *post coitum* (CD-1 strain). Control blastocysts derived from CD-1 females mated with F₁ males were transferred to the left uterine horn of the same recipients. The offspring were expected to be albino after transfer of the reconstituted embryos.

Chromosome analysis

After culture with colcemid (0.1 µg/ml), the embryos were exposed to 0.4 ml 1% sodium citrate for 1.5 min at room temperature (Yoshizawa *et al.*, 1990). Initial mild fixation was performed by adding 0.01–0.02 ml of acetic acid and alcohol (1:3) into the hypotonic solution and leaving for several minutes. The embryo was placed on a glass slide and 3–5 drops of fixative were dropped on to it. After gentle blowing, the slide was dried and stained with 1% Giemsa.

Results

Pronuclear formation in activated oocytes

There was no effect of stage of the cell cycle of donor karyoplast on the success rate of fusion and activation (Table 1). After oocyte activation, four pathways were observed for the completion of meiosis: (i) one polar body and one pronucleus (1PB + 1PN), (ii) two pronuclei (2PN), (iii) immediate cleavage (IC) and (iv) one pronucleus without polar body (1PN). After activation, most of the reconstituted oocytes emitted a polar body within 2 h and formed one pronucleus, especially when nuclei from 2-cell embryos were transferred at the early and middle period between the first and second mitotic divisions of the embryo.

Table 1. Incidence of different types of activated mouse oocyte after reconstitution with nuclei of 2-cell embryos at different stages of the mitotic period between first and second cleavage

Stage of donor nuclei	Time since injection with human chorionic gonadotrophin (h)	No. of oocytes fused/ no. manipulated	No. of oocytes activated	No. of activated eggs with			
				1PB + 1PN	2PN	Immediate cleavage	1PN
Early	30–32	156/160 (98)	153 (98)	149 (97)	4 (3)	0	0
Middle	34–36	181/183 (99)	180 (97)	168 (93)	6 (3)	4 (2)	2 (1)
Late 1	40–42	303/305 (99)	294 (97)	251 (85)	32 (11)	7 (2)	4 (1)
Late 2	48–50	187/189 (99)	176 (94)	138 (78)	27 (15)	9 (5)	2 (1)

Numbers in parentheses are percentages.

PB, polar body; PN, pronucleus.

Development of the reconstituted embryos

The ability of the reconstituted embryos (Table 2) to develop to the blastocyst stage was affected significantly by the mitotic stage of donor nuclei at transfer. The developmental capacity of the reconstituted embryos that received donor nuclei at the early and middle stage of the mitotic period between first and second cleavage was restricted. Of the reconstituted embryos, 48% (early) and 33% (middle) developed to the 2-cell stage, but most arrested at the 2- or 4-cell stage. In contrast, the embryos that received a nucleus from late 2-cell embryos had a greater capacity to develop into blastocysts.

Table 2. *In vitro* development of mouse oocytes reconstituted with nuclei of 2-cell embryos at different stages of the mitotic period between first and second cleavage

Stage of donor nuclei*	No. of eggs cultured	No. of eggs developed to				
		2-cell	4-cell	8-cell	Morula	Blastocyst
Early	153	73 (48)	9 (6)	1 (1)	0	0
Middle	180	60 (33)	13 (7)	4 (2)	2 (1)	2 (1)
Late 1	294	253 (86)	190 (65)	144 (49)	123 (42)	76 (26)
Late 2	176	155 (88)	100 (57)	77 (44)	63 (36)	44 (25)

*See Table 1 for time since injection with human chorionic gonadotrophin for these stages. Numbers in parentheses are percentages.

Blastocysts were obtained from all the types of reconstituted embryos that received a nucleus from late 2-cell embryos; 102 (1PB + 1PN), 16 (2PN), 1 (IC) and 1 (1PN). There was no significant difference between the developmental rates of 1PN + 1PB embryos (26%, 102/389) and 2PN

embryos (27%, 16/59). The blastocysts derived from 1PB + 1PN embryos had a normal diploid set of chromosomes.

To examine the ability to develop to term, 66 blastocysts derived from the 1PB + 1PN embryos and 16 blastocysts derived from 2PN embryos were transferred to pseudopregnant recipients (Table 3). Of these, ten (15%) albino live young, four males, three females and three unsexed owing to cannibalism, were successfully obtained from 1PB + 1PN embryos, but none from the 2PN embryos. All of the live offspring produced litters of normal size after mating.

Table 3. Development of blastocysts derived from reconstituted mouse embryos after transfer to recipients

Type of reconstituted oocytes	No. of blastocysts transferred	No. pregnant/ no. of recipients (%)	No. of young (%)
1PN + 1PB	66	6/20 (30)	10 (15)
2PN	16	0/4	0

PB, polar body; PN, pronucleus.

Discussion

The present study demonstrated that the stage of the cell cycle of the donor nuclei significantly affects the developmental ability of reconstituted mouse oocytes. According to previous reports (Bolton *et al.*, 1984; Howlett & Bolton, 1985), DNA synthesis is initiated 1 h after the first cleavage division and continues for the next 5 h of the second cell cycle. The embryos in this study were collected at 30–32 h (early), 34–36 h (middle), 40–42 h (late 1), and 48–50 h (late 2) after hCG injection. These times are associated with the G₁, S, middle G₂ and late G₂ phases, respectively. When nuclei from late 2-cell stage embryos were transferred to enucleated oocytes, the reconstituted embryos not only developed to blastocysts, but also produced live young after transfer to recipients. By contrast, the oocytes that received early- and middle-stage nuclei from 2-cell embryos exhibited a more limited ability to develop beyond the 2-cell stage. Similar results have been obtained in amphibians: the influence of stage of cell cycle on the success of nuclear transplantation using synchronized cells was investigated in *Rana pipiens* (Von Beroldingen, 1981). The results showed that G₁ cells have a more limited capacity to support development beyond the blastula stage than nuclei of mid-S, late-S and G₂ cells. It has also been reported that enlargement of transferred nuclei is related to the developmental ability of the reconstituted embryos, and that nuclear enlargement of G₁ and early-S nuclei was less than that of G₂ and late-S nuclei (DiBerardino & Hoffner, 1970; Von Beroldingen, 1981).

One decisive event that occurred after activation was whether the activated oocyte emitted a second polar body and developed a pronucleus. The present results showed that the developmental progress of the reconstituted embryos to blastocyst was improved after the emission of a second polar body and the development of a single pronucleus, in a similar way to that observed after sperm penetration or 1PN + 1PB parthenogenone formation. After activation, we observed four types of pronuclear formation in the reconstituted embryos, as previously observed in parthenogenetic mouse embryos (Kaufman *et al.*, 1973; Webb *et al.*, 1986), in which most of the reconstituted embryos emitted a polar body and formed one pronucleus. This polar body must be emitted as a result of the mitotic division of the transferred nucleus after activation.

Under the present system, when nuclei at the G₂ phase were transferred, diploid nuclei were observed in 1PN + 1PB embryos as a result of mitosis of the donor nucleus and emission of one set of chromosomes into the polar body. These results indicate that successfully remodelled nuclei in

the reconstituted embryos are able to complete to the G₂ to M stage of the cell cycle. Through these post-fusion and activation events, the remodelled nuclei of the reconstituted embryos attain the capacity to develop to the blastocyst stage and to term after transfer to recipient females. The blastocysts derived from 1PB + 1PN were normal diploids. The 2PN embryos that were produced by inhibition of the emission of a polar body, using cytochalasin B treatment, were tetraploids (T. Kono, unpublished data). Moreover, all ten normal live young in this study were obtained from the reconstituted embryos that formed one pronucleus with a polar body, but none was obtained from the embryos with two pronuclei. This also shows that nuclei from late 2-cell mouse embryos, in which transcription of the embryonic genome is initiated (Flach *et al.*, 1982; Bolton *et al.*, 1984), are able to support development to term after transfer to enucleated oocytes.

In contrast, early 2-cell nuclei supported development to blastocysts when transferred to enucleated zygotes, but only a few developed to blastocysts when late 2-cell nuclei were transferred (Howlett, *et al.*, 1987), in which heat-shock proteins of 68–70 kDa that are characteristic of the first embryonic gene activity were detected in both cases. These data and the results in this study indicate that nucleic changes during the second cell-cycle may be reprogrammed by oocyte cytoplasm, but that the ability of zygote cytoplasm is severely limited.

In previous reports, on the other hand, the emission of a polar body from reconstituted mouse oocytes was not observed after activation in sheep (Willadsen, 1986; Smith & Wilmut, 1989), cattle (Prather *et al.*, 1987; Bondioli *et al.*, 1990), rabbits (Stice & Robl, 1988) and mice (Tsunoda *et al.*, 1989). In such nuclear transfer systems, the reconstituted oocytes may have received a G₁ nucleus and developed to term. The ability of the diploid blastocysts derived from the reconstituted embryos to develop to term was low (15%, 10/66). The limited developmental ability of blastocysts derived from oocytes receiving donor nuclei has also been reported in cattle (Bondioli *et al.*, 1990).

It has been reported that mouse thymocyte nuclei are remodelled for 30–60 min after fusion with the ooplasm as a result of exposure to cytoplasmic maturation-promoting factor. During this time, breakdown of the nuclear envelope, premature chromosome condensation and organization of the spindle apparatus occur successively, but the emission of a polar body from extra nuclei occurred in only one case (Czolowaska *et al.*, 1984, 1986). It has been suggested that the extra genomes are reprogrammed by direct exposure to some cytoplasmic factor, perhaps maturation-promoting factor, during this period. Maturation-promoting factor acts in a non-species-specific manner and plays a key role in promoting the M phase within the cytoplasm during meiosis and mitosis (Miake-Lye *et al.*, 1983; Lohka & Maller, 1985; Miake-Lye & Kirschner, 1985). The activity of maturation-promoting factor in mouse oocytes is cyclic during meiosis, exhibiting a high activity in metaphase I oocytes, declining during telophase I and increasing again at metaphase II (Hashimoto & Kishimoto, 1988). Moreover, the nuclear envelope of hybrid and nuclear-transferred oocytes is reconstituted after activation; but, except for a single case in mice (Czolowaska *et al.*, 1986), the emission of a polar body has not been previously reported.

In conclusion, the developmental ability of enucleated oocytes receiving donor nuclei is influenced by the embryonic stage and the stage of the cell cycle of the donor nuclei. However, it has still to be clarified whether controlling the stage of the cell cycle of donor nuclei would enhance the ability of reconstituted oocytes to develop to term.

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