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# Birth of Mice after Transplantation of Early Cell-Cycle-Stage Embryonic Nuclei into Enucleated Oocytes[1]

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## ABSTRACT

The present study was conducted to investigate the influence of cell cycle stage of the donor nucleus on chromatin structure and development of mouse embryonic nuclei transplanted into enucleated oocytes. Donor cell-cycle stage was controlled in order to examine, in addition, the developmental potential of nuclei from 2-, 4-, and 8-cell-stage embryos. The cell cycle stage of donor nuclei was classified as early, middle, or late. After nuclear transfer, electrofusion, and activation, early-stage transplants formed a single pronucleus-like structure, but middle-stage transplants formed very irregular types of structures and late-stage transplants extruded a polar body. A high proportion of development to the blastocyst stage (77.8%) and an increased cell number (62.1 cells) were obtained from the early 2-cell-stage transplants as opposed to the middle- (0%) and late-stage (20.8%, 37.0 cells) transplants ($p < 0.001$). With transplantation of early-stage nuclei, high proportions of development to the blastocyst stage and of offspring were obtained from nuclear transplant embryos with a nucleus from a 2-, 4-, or 8-cell-stage embryo. The results confirm that the donor cell-cycle stage critically affects the chromatin structure and development of nuclear transplant embryos. The results also demonstrate that the nuclei from 2-, 4-, and 8-cell-stage mouse embryos in the early stage of each cell cycle can be reprogrammed when transplanted into enucleated mature oocytes.

## INTRODUCTION

A nucleus transplanted into an enucleated mature oocyte undergoes various morphological modifications. These changes are characterized by a premature chromosome condensation (PCC) followed by pronucleus-like formation and swelling of the nucleus upon activation of the oocyte [1, 2]. PCC and nuclear swelling have been considered morphological signs of the reprogramming of transferred nuclei (reviewed in [3, 4]).

In somatic cell hybrids, the morphology of the PCC depends on the cell cycle stage of the donor nucleus. PCC in the G1 and G2 phases results in elongated chromosomes with single- and double-stranded chromatids, respectively. However, PCC in the S phase leads to heterogeneous chromatin elements with small dispersed fragments [5, 6]. Furthermore, PCC in different cell-cycle stages influences spindle structure, chromosome constitution, and in vitro development of nuclear transplant rabbit embryos [7, 8]. It has been reported that spindle and chromosome constitution are normal and that development to the blastocyst stage is improved when blastomere nuclei in G1 phase are transplanted to mature, enucleated oocytes. However, S-phase nuclear-transplant embryos develop poorly compared to G1 phase transplants. Thus, it has been suggested that synchronization of the donor nucleus in the G1 phase is an important factor for successful development of nuclear transplant embryos [7, 8].

The influence of donor cell-cycle stage on developmental capacity of mouse blastomere nuclei transplanted into enucleated oocytes has also been reported [9]. In the mouse, nuclear transplant embryos extrude an extra polar body regardless of the cell cycle stage of the donor nuclei, but full-term development is achieved only by nuclear transplant embryos that received late 2-cell-stage (presumably G2 phase) donor nuclei [9].

The present study was conducted to examine the influence of donor cell-cycle stage on the chromatin structure of the donor nucleus and the development of nuclear transplant mouse embryos. Donor cell-cycle stage was controlled in order to investigate, in addition, the developmental potential of nuclear transplant embryos with nuclei from 2-, 4-, and 8-cell-stage embryos.

## MATERIALS AND METHODS

### *Collection of Oocytes and Embryos*

F1 hybrid (C57BL/6J × CBA) and ICR strain female mice were superovulated with injections of 5 IU of eCG (Serotropin; Teikoku Zoki, Tokyo, Japan), followed 48 h later by 5 IU of hCG (Gonatropin; Teikoku Zoki). Oocytes at metaphase (M phase) II were collected from the ampullae of the oviducts of F1 females 14–15 h after hCG injection with 37°C M2 medium [10]. Cumulus cells were removed by treatment with 300 IU/ml hyaluronidase (Sigma, St. Louis, MO).

To collect donor embryos, the oviducts of ICR females mated with F1 strain males were flushed using 37°C M2 medium. Late 1-cell-stage embryos were collected 29–30 h after hCG injection. Two-cell-stage embryos were collected 44–45 and 48–49 h after hCG injection, respectively. Late 4-cell-stage embryos were collected 59–60 h after hCG injection. The zona pellucidae of late 4-cell-stage embryos were removed after a short pretreatment with 0.5% pronase (Ac-

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tinase; Kaken, Tokyo, Japan) in 37°C BSA-free M2 medium. Blastomeres of 4-cell embryos were isolated by pipetting zona-free embryos in $Ca^{2+}$-free M16 medium [11].

*Enucleation of Oocytes*

All manipulations were performed via an inverted microscope (Diaphot; Nikon, Tokyo, Japan) with Nomarski optics and Narishige micromanipulators at room temperature (25–28°C) for 1–1.5 h. Oocytes were enucleated by removal of the M phase chromosomes arranged in the spindle of the second meiotic division. This system had provided a high enucleation rate (89%) in our previous study (Cheong et al., unpublished results). Oocytes were placed in a drop of M2 medium in a petri dish covered with paraffin oil. The zona pellucidae over the spindle area were slit with a fine glass needle along 10–20% of their circumference. After the zona were slit, the oocytes were placed in a drop of M2 medium containing 5 μg/ml cytochalasin B (CB; Sigma) and 0.1 μg/ml colcemid (Gibco, Grand Island, NY). The oocytes were secured by a holding pipette opposite the slit in the zona. An enucleation pipette (20–25 μm in diameter) with a beveled sharpened tip was inserted into the perivitelline space through the slit, and the spindle area of M phase chromosomes was aspirated into the enucleation pipette with a small amount of cytoplasm.

*Nuclear Transplantation*

Nuclear transplantation was carried out as described previously [12, 13]. A karyoplast from 2- and 4-cell-stage embryos or a blastomere from 8-cell donor embryos was introduced into the perivitelline space of the enucleated oocytes. The manipulated eggs were then cultured in M16 medium containing 100 μM EDTA in an atmosphere of 5% $CO_2$ in air at 37°C for 10–30 min before fusion treatment.

*Electrofusion and Activation*

Membrane fusion was performed by electrofusion as described previously [14], 18–20 h after hCG injection of the oocyte donors. Manipulated eggs were placed in a 0.5-mm round wire, stainless steel electrode chamber filled with 37°C 0.3 M mannitol containing 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$, and 0.05 mg/ml of BSA. The eggs were aligned by exposure to an alternating current (a.c.) pulse of 0.6 MHz, 6 V, for 6 sec; then two direct current (d.c.) pulses of 1.25 kV/cm for 70 μsec (each pulse 1 sec apart) were applied to the chamber via an Electro Cell Fusion instrument (LF 100; Life Tec, Tokyo, Japan). After fusion treatment, eggs were placed in culture (see below) and checked every 10 min. Manipulated embryos were fused usually within 15 min and were undergoing PCC within 30 min. At around the time of onset of PCC (25–30 min after fusion treatment), fused embryos were treated again with 5–6 d.c. pulses of 1.5 kV/cm for 70 μsec (each pulse 1 sec apart) to obtain sufficient activation of oocytes. In a separate experiment, with two d.c. pulses of 1.25 kV/cm for 70 μsec, 90% (27/30) of nuclear transplant mouse embryos were fused and 11% (3/27) of fused embryos were activated, showing a pronucleus-like formation. However, when an additional five to six d.c. pulses of 1.5 kV/cm for 70 μsec were given after fusion treatment of two d.c. pulses of 1.25 kV/cm for 70 μsec, 97% (30/31) of fused embryos were activated, showing a pronucleus-like formation (unpublished data). The average time from oocyte collection until electrofusion with donor cells was 4–5 h.

*In Vitro Culture, Monitoring, and Embryo Transfer*

After fusion and activation treatments, the nuclear transplant embryos were cultured in drops of M16 medium containing 100 μM EDTA and covered with paraffin oil, in an atmosphere of 5% $CO_2$ in air at 37°C. Embryos were monitored every hour for 4 h to assess activation, and their development in vitro was assessed every 8 h. Activated embryos were those showing pronucleus-like formation 3–4 h after fusion treatment [9]. Nuclear transplant embryos that had developed to the blastocyst stage by 96 h after culture were transferred to the uterine horn of Day 3 (2.5 days post-coitum) pseudopregnant recipients (ICR strain). Control blastocysts derived from ICR females mated with ICR males were transferred into the same recipients. The recipients were retained until parturition.

*Preparations*

Whole-mount preparations were made through use of the method of Bedford [15]. Embryos were mounted on a slide, fixed with 10% neutral formalin for 2 h, and stained with 0.25% aceto-lacmoid. Air-dried preparations were made according to Tarkowski [16]. Blastocysts were incubated in M16 medium containing 0.4 μg/ml colchicine for 3–4 h to arrest cleavage division at M phase. They were then treated with a hypotonic solution of 1% sodium citrate for 15 min, fixed with a mixture of methanol and acetic acid (3:1), and stained with 4% Giemsa.

*Experiment 1*

The first experiment investigated the effect of donor cell-cycle stage on the type of pronucleus-like formation and in vitro development of nuclear transplant embryos. Late 1-cell-stage embryos were collected, and most of these were cultured in M16 medium containing 0.1 μg/ml colcemid (Sigma) at 37°C for 1–2 h. Some embryos already in mitosis were stored at 4°C in M2 medium for the same period. Colcemid or cold treatment of embryos for a short time had no adverse effect on development to the blastocyst stage in vitro. Embryos were removed from colcemid or cold and were placed in culture (see above). Cleavage to the 2-cell stage was assessed every 15 min, and newly cleaved embryos at 0.5–1.5 h after release from colcemid or cold were assigned to an experimental treatment. Embryos were im-

TABLE 1. Influence of donor cell-cycle stage on fusion and oocyte activation of manipulated eggs with nuclei from 2-cell-stage embryos.

| Donor cell-cycle stage | Time[a] of activation | No. (%) of oocytes fused/ manipulated[b] | No. (%)[c] of oocytes activated | No. (%) of activated embryos with [d] | | | |
|---|---|---|---|---|---|---|---|
| | | | | 1PN | 1PN + 1PB | 1PN + 2PB ≤ | Others |
| Early | 1.5 hpc | 37/80(46.3)[e] | 36(97.3) | 35(97.2)[e] | 0[e] | 0[e] | 1( 2.8)[e] |
| Middle | 5-6 hpc | 70/82(85.4)[f] | 68(97.1) | 6( 8.8)[f] | 17(24.3)[f] | 16(22.9)[f] | 31(45.6)[f] |
| Late | 47-48 hph | 78/86(90.7)[f] | 77(98.7) | 4( 5.2)[f] | 52(67.5)[g] | 15(19.5)[f] | 6( 7.8)[e] |

[a]hpc: hours post-cleavage; hph: hours post-hCG injection.
[b]Total of five replicates.
[c]Based on the number of fused embryos.
[d]PN: pronucleus; PB: polar body.
[e-g]Values with different superscripts in the same column are significantly different ($p < 0.01$).

mediately used (early stage) or cultured in M16 medium containing 100 μM EDTA at 37°C for 3–4 h before nuclear transfer (middle stage). Nuclear transfer, fusion, and activation were completed within 1.5 and 5–6 h after cleavage for early- and middle-stage donors, respectively. These times are likely to be associated with the G1 and S phases of the second cell cycle, respectively [17, 18]. Two-cell-stage embryos collected 44–45 h after hCG injection were stored at 4°C before nuclear transfer and fusion (47–48 h after hCG injection; late stage). This timing is likely to be associated with the G2 phase of the second cell cycle [18]. Nuclear transplant embryos were monitored every hour for 4 h for assessment of activation and type of pronucleus-like formation. Activated embryos were cultured in vitro for 4 days for assessment of their developmental potential in vitro. Five replicates were performed.

*Experiment 2*

The chromatin structure of the donor nucleus after fusion treatment was investigated. Nuclei from 2-cell-stage embryos in different stages of the cell cycle were transferred and fused to enucleated oocytes. About 1.5 h after fusion treatment, embryos were prepared via the whole-mount technique and examined under a microscope equipped with Nomarski optics.

*Experiment 3*

The potential of donor nuclei in the early cell-cycle stage to direct full-term development of nuclear transplant embryos was determined. The early stage of the second cell cycle was determined as described for experiment 1. Early stages of the third and fourth cell cycles were determined by time after cleavage. Two-cell-stage embryos collected 48–49 h after hCG injection and blastomeres of late 4-cell-stage embryos received low-temperature or colcemid treatment, and cleavage was assessed after release from these treatments as described for experiment 1. Cleaving blastomeres of late 4-cell-stage embryos were isolated by pipetting in $Ca^{2+}$-free M16 medium. Nuclear transfer, fusion, and activation were completed within 1.5 h after cleavage for early 4- and 8-cell donor nuclei to adjust cell stage to the G1 phases of the third [17, 19] and fourth cell cycles [19], respectively. Nuclear transplant embryos were cultured in vitro for 4 days and blastocysts were transferred to recipient females. Six replicates were performed.

*Statistical Analysis*

Data were analyzed by chi-square test or Student's *t*-test.

## RESULTS

*Experiment 1*

Fusion rate was affected by the donor cell-cycle stage. A significantly lower ($p < 0.01$) fusion rate was obtained in the early stage (46.3%) than in the middle (85.4%) and late stages (90.7%). Most of the fused eggs were activated regardless of donor cell-cycle stage. However, types of activation varied and were significantly ($p < 0.01$) affected by the stage of the donor cell cycle (Table 1). Most of the nuclear transplant embryos with nuclei in the early cell-cycle stage (97.2%) showed a single pronucleus-like (1PN) structure. However, transplantation of middle-stage donor nuclei yielded a variety of complex and irregular structures. Transplantation of late-stage donor nuclei resulted primar-

TABLE 2. In vitro development of nuclear transplant embryos with nuclei from 2-cell-stage embryos in different cell-cycle stages.

| Donor cell-cycle stage | No. of embryos cultured | No. (%) of embryos developed to | | | | | Cell number in blastocysts (mean ± SEM) |
|---|---|---|---|---|---|---|---|
| | | 2-cell | 4-cell | 8-cell | Morulae | Blastocyst | |
| Early | 36 | 35(97.2)[a] | 33(91.7)[d] | 31(86.1)[d] | 30(83.3)[d] | 28(77.8)[d] | 62.1 ± 2.2[d] |
| Middle | 68 | 26(38.2)[b] | 5( 7.4)[e] | 0[e] | 0[e] | 0[e] | — |
| Late | 77 | 60(77.9)[c] | 34(44.2)[f] | 25(32.5)[f] | 19(24.7)[f] | 16(20.8)[f] | 37.0 ± 1.6[f] |

[a-c]Values with different superscripts are significantly different ($p < 0.05$).
[d-f]Values with different superscripts in the same column are significantly different ($p < 0.001$).

TABLE 3. Chromatin structure of nuclear transplant embryos in whole-mounts 1.5 h after fusion treatment.

| Donor cell-cycle stage | No. of embryos analyzed | No. (%) of embryos with different no. of chromatin clumps | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | >3 clumps |
| Early | 20 | 20(100.0)[a] | 0[a] | 0[a] | 0[a] |
| Middle | 27 | 2( 7.4)[b] | 6(22.2)[a] | 9(33.3)[b] | 10(37.0)[b] |
| Late | 28 | 1( 3.6)[b] | 18(64.3)[b] | 8(28.6)[b] | 1( 3.6)[a] |

[ab]Values with different superscripts in the same column are significantly different ($p < 0.05$).

ily (67.5%) in the formation of 1PN structure as well as a polar body (1PN + 1PB).

The developmental capacity of nuclear transplant embryos to the blastocyst stage was significantly ($p < 0.01$) affected by the donor cell-cycle stage (Table 2). A very high proportion (97.2%) of embryos with donor nuclei in the early stage of the cell cycle cleaved, and 77.8% of cultured embryos with these nuclei developed to the blastocyst stage. With donor nuclei in the middle and late stages of the cell cycle, however, the proportions of development to the 2-cell stage (38.2% and 77.9%, respectively, $p < 0.05$) and to the blastocyst stage (0% and 20.8%, respectively, $p < 0.001$) were significantly reduced as compared to those for the early-stage nuclei.

Mean cell number in blastocysts after 96 h of culture was significantly lower ($p < 0.001$) for nuclei in the late cell-cycle stage (37.0 cells) than for nuclei in the early cell-cycle stage (62.1 cells). All embryos with early cell-cycle-stage nuclei that were analyzed (21/21) had a diploid chromosome constitution. However, 23% (3/13) of embryos with nuclei in the late cell-cycle stage had a tetraploid chromosome constitution.

*Experiment 2*

Chromatin structure of the donor nucleus was determined in whole mounts 1.5 h after fusion treatment (Table 3). All embryos with nuclei in the early stage of the cell cycle had one clump of chromatin in their cytoplasm (Fig. 1A). However, most of the embryos with nuclei in the middle stage of the cell cycle (70.3%) had three or more clumps of chromatin irregularly dispersed in their cytoplasm (Fig. 1B). Most of the embryos with nuclei in the late stage of the cell cycle (64.3%) showed normal mitosis and had two clumps of chromatin (Fig. 1C).

*Experiment 3*

The developmental potential of 2-, 4-, and 8-cell donor nuclei in the early stage of the cell cycle was examined (Table 4). The rates of fusion, activation, and cleavage were not affected by donor cell stage. Development to the blastocyst stage was similarly high with 2- and 4-cell-stage donor nuclei (78.2% and 71.4%, respectively). However, the developmental potential of 8-cell nuclear donors was sig-

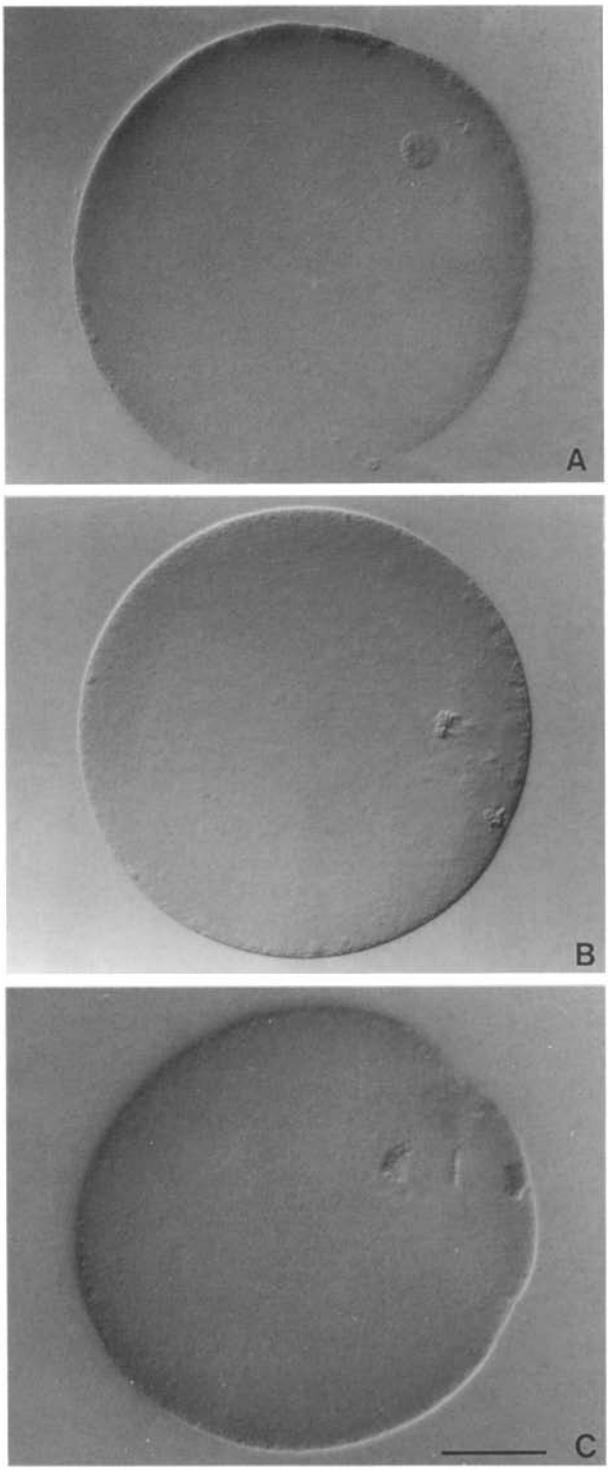


FIG. 1. Chromatin structures of nuclear transplant embryos 1.5 h after fusion treatment. A) Early-stage transplants: one clump of chromatin is seen in the cytoplasm. B) Middle-stage transplants: several clumps of chromatin are dispersed in the cytoplasm; some chromatin clumps are out of focus. C) Late-stage transplants: a normal telophase plate is shown in the cytoplasm. The bar represents 40 μm.

TABLE 4. Development of nuclear transplant embryos with nuclei from 2-, 4-, and 8-cell-stage embryos in the early stage of the cell cycle.

| Stage of donor nuclei | No. (%) of oocytes fused/ manipulated[a] | No. (%) of oocytes activated[b] | No. (%) of embryos developed to | | No. (%) pregnant/ no. of recipient | No. (%) of young/ no. of embryos transferred |
|---|---|---|---|---|---|---|
| | | | 2-cell | Blastocyst | | |
| 2-cell | 48/102(47.1) | 46(95.8) | 45( 97.8) | 36(78.2)[c] | 5/8(62.5) | 10/34(29.4) |
| 4-cell | 43/ 97(44.3) | 42(97.7) | 42(100.0) | 30(71.4)[c] | 4/6(66.7) | 6/27(22.2) |
| 8-cell | 41/ 94(43.6) | 39(95.1) | 36( 92.3) | 18(46.2)[d] | 3/6(50.0) | 3/17(17.6) |

[a]Total of six replicates.
[b]Embryos were activated within 1.5 h post-cleavage for each stage of the donor nuclei; percentages were based on the number of fused embryos.
[cd]Values with different superscripts are significantly different ($p < 0.01$).

nificantly reduced (46.2%, $p < 0.01$). After transfer of blastocysts derived from nuclear transplant embryos to pseudopregnant recipients, 10 (29.4%), 6 (22.2%), and 3 (17.6%) live young were obtained from embryos with 2-, 4-, and 8-cell-stage donor nuclei, respectively. Of the 86 transferred blastocysts derived from ICR mice, 39 (45.3%) developed to term.

## DISCUSSION

The results of the present study confirm that the cell cycle stage of the donor nucleus affects the chromatin structure of the donor nucleus and the developmental ability of nuclear transplant embryos. The nucleus in the early stage of the cell cycle demonstrated great developmental potential to the blastocyst stage. Furthermore, successful development to the blastocyst stage and to term was achieved from transplantation of nuclei from 2-, 4-, and 8-cell-stage embryos in the early stage of each cell cycle, strongly confirming complete reprogramming. To our knowledge this is the first time this has been achieved.

As no direct observations of cell cycle stage were made in the donor nuclei in this study, interpretation of these results depends upon earlier experiments in other laboratories. However, it seems very likely that the stages described as early, middle, and late in our experiments corresponded to G1, S, and G2, respectively. Cell cycle stage of mouse embryos has been established for the first [20, 21], second [17, 18], third [17, 19], and fourth [19] cell cycles.

In the present study, in which the time after cleavage was controlled, high proportions of development to the blastocyst stage as well as a large increase in cell number of blastocysts were obtained in nuclear transplant embryos with nuclei in the early stage of the cell cycle, although the fusion rate was low. The greater developmental potential of nuclear transplant embryos with early-stage donor nuclei may be explained by the pronucleus-like formation type, whole-mount, and chromosome preparations. The early-stage transplantations showed 1PN structure after activation and had a diploid chromosome constitution. Most of the late-stage transplantations showed 1PN + 1PB structure after activation, and some embryos developed to the blastocyst stage with diploid chromosomes. However, our previous findings (Cheong et al., unpublished results) confirmed that the chromosome number of late-stage transplants with 1PN + 1PB varied at the 1-cell stage, and only a small proportion (37.5%) of these nuclear transplant embryos had a diploid chromosome constitution. Chromosome abnormalities in the middle-stage transplants are also consistent with the data on pronucleus-like formation and the whole-mount preparation. A large proportion of chromosome abnormalities were also observed in late S phase transplants in the rabbit [8].

The results of the current study are in contrast with those from a previous mouse nuclear-transplant study [9]. In that investigation most of the nuclear transplant embryos emitted a polar body after activation regardless of the donor cell-cycle stage, and no development to the blastocyst stage or to term was obtained from the early-stage transplants. This discrepancy is presumably due to differences either in the stage of recipient oocytes used or in activation treatment and time. In the present study, M phase II oocytes were used for recipient cells, and electric field-induced fusion and activation were completed within 1.5 h after cleavage for early-stage transplantation. In the study by Kono et al. [9], however, telophase I oocytes were employed for recipient cells, and activation treatment was performed with ethanol at 1.5 h after virus-induced fusion. On the other hand, the fact that pronucleus-like formation type varies with different species cannot be disregarded. The extrusion of a polar body was not observed after activation in rabbits [2, 7, 8, 22], sheep [23, 24], or cattle [25, 26].

In the mouse, 2-cell-stage nuclei transferred to enucleated zygotes [27] and oocytes [9] can develop to term, but the developmental potential of nuclei from 4- or 8-cell-stage embryos is seriously limited. No offspring have been obtained after transfer of these nuclei to enucleated zygotes [13, 27, 28] or oocytes [29, 30]. It has been suggested that the ability of nuclear transplant embryos to develop to the blastocyst stage is affected by the transcriptional activity of the embryonic genome [31], which occurs at the middle 2-cell stage in the mouse [32, 33]. In the present study, however, not only high proportions of development to the blastocyst stage but also normal live young were successfully obtained from the nuclear transplant embryos with nuclei from 2-, 4-, and 8-cell-stage embryos. These results suggest that initiation of transcription has little effect on the devel-

opmental potential of donor nuclei transplanted into enucleated mature oocytes, although reduced development in vitro was observed with the use of an 8-cell nucleus donor. This suggestion is supported by findings in rabbits [2], sheep [23, 24], and cattle [25]. In these species, pre- and post-transcriptional nuclei directed development of nuclear transplant embryos to the morula or blastocyst stage in similar proportions. Reduction in the developmental potential of 8-cell donor nuclei may have been due either to intensive pipetting to separate blastomeres immediately after cleavage or to the advanced transcriptional age of the donor nuclei rather than to transcriptional activation itself.

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