Developmental Capacity of Reconstituted Mouse Embryos: Influences of Nucleus and Cytoplasm Sources

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ABSTRACT. This study was undertaken to examine the developmental capacity of reconstituted mouse embryos, and the influences of nucleus and cytoplasm on the development of these embryos following reciprocal pronuclear transplantation between in vitro 2-cell blocked and nonblocked embryos. Karyoplast containing pronuclei was transferred into the perivitelline space of the enucleated zygote and fused to cytoplasm with electrofusion. Maximum fusion rate was obtained when a field strength of 1.5 kV/cm was used. The fusion rates were high (86.2±3.2 to 90.6±2.0%) regardless of the strains of donor nucleus and recipient cytoplasm. Developmental rates of reconstituted embryos to the blastocyst stage, which were similar to that of the F1 (C57BL/6J × CBA) control were high when F1 embryos were used as the cytoplasm recipients (88.8±1.5 and 91.9±2.0%). When ICR embryos were used as the recipient cytoplasm, developmental rates were significantly reduced (71.5±2.9 and 54.1±3.2%), and affected by the source of nucleus. There were no significant differences in the cell number of embryos that developed to blastocysts and in the developmental rates to live young among the embryos reconstituted with different nuclei and cytoplasm, and the ICR control. The results of this study show that the development of reconstituted embryos is hardly affected by nuclear transplantation and electrofusion procedures. It is indicated that the recipient cytoplasm, rather than the donor nucleus, has the greater influence on the in vitro development of the reconstituted embryos to the blastocyst stage.—KEY WORDS: electrofusion, mouse, nuclear transplantation, pronuclear stage.

Nuclear transplantation in mouse embryos has been widely used to investigate nucleo-cytoplasmic interactions during early embryo development [6, 12, 18] and the possibility of cloning [8, 16]. Although it has been suggested that it is difficult to apply the results from mouse embryos to other mammals directly [14, 15], much of the basic information about mammalian embryonic development can be provided by nuclear transplantation in mouse embryos.

Pronuclear transplantation between mouse zygotes has often been used as a basis of the nuclear transplantation technique [9, 10, 21]. In the mouse, while pronuclear transplantation has no deleterious influence on embryo viability, there is a substantial decrease in the ability of the reconstituted embryos to develop to the blastocyst stage when a nucleus derived from a two-cell or later stage embryo is transferred [11]. Thus, the estimation of the efficiency of the nuclear transplantation technique itself, and investigation of the roles of parental genomes during embryogenesis have been achieved by the pronuclear transplantation between mouse zygotes [19, 20].

Assessment of the roles of nucleus and cytoplasm in early embryonic development has also been examined by pronuclear transplantation [2, 17]. It has been suggested that the preimplantation embryo development in vitro and 2-cell block are controlled by the cytoplasmic source [5, 13]. Others have suggested that the nucleus is of equal importance [17], or is solely responsible [7] for embryo development in vitro.

The present study was conducted to examine the developmental capacity in vitro and in vivo of reconstituted mouse embryos following pronuclear transplantation with electrofusion. In addition, the influences of nucleus and cytoplasm on the in vitro and in vivo development of reconstituted embryos were investigated by reciprocal nuclear transplantation between 2-cell blocked and nonblocked embryos.

MATERIALS AND METHODS

Embryo recovery: ICR and F1 (C57BL/6J × CBA) females were superovulated with intraperitoneal injections of 5 IU of PMSG, followed 48 hr later by 5 IU hCG. They were then mated with males of the same strain. One-cell embryos were collected from the ampulla of the oviducts by flushing with M2 medium [4] approximately 20–22
hr after hCG injection. Cumulus cells were removed by treating the embryos with 300 IU/ml hyaluronidase in M2 medium. The embryos were placed in drops of M16 medium [22] containing 100 μM EDTA under paraffin oil in an atmosphere of 5% CO₂ in air at 37°C until the micromanipulation.

Micromanipulation: Micromanipulation was carried out using an inverted microscope (Diaphot, Nikon, Tokyo, Japan) with Nomarski's contrast and Narishige micromanipulators. Holding pipettes (outer diameter; 80–100 μm, inner diameter; 10–15 μm) and beveled, sharpened enucleation pipettes (outer diameter; 20–25 μm) were prepared as described previously [10].

Embryos were incubated for 15–20 min prior to micromanipulation in M16 medium containing 5 μg/ml of cytochalasin B (CB; Sigma, St. Louis, MO, U.S.A.) and 0.1 μg/ml colcemid (Gibco, Grand Island, NY, U.S.A.). The non-disruptive microsurgery procedure as described originally by McGrath and Solter [10] was employed. Groups of donor and recipient embryos (about 10 per group) were placed apart in a drop of M2 medium containing cytoskeletal inhibitors (5 μg/ml CB and 0.1 μg/ml colcemid). The pronuclei were removed from recipient embryos, and karyoplasts containing pronuclei from donor embryos were inserted into the perivitelline space of enucleated embryos by using an enucleation pipette. In an experiment, a small portion of cytoplasm, approximately equal to the volume of cytoplasm carried along with the nucleus, was transferred to non-enucleated embryos to examine the effects of the cytoplasm transferred with the nucleus. After micromanipulation, manipulated embryos were washed and incubated in drops of M16 medium containing 100 μM EDTA under an atmosphere of 5% CO₂ in air at 37°C for 1 hr before electrofusion treatment.

Electrofusion: Membrane fusion was performed by electrofusion as described previously [3]. Briefly, four to six manipulated embryos were washed in a 0.3 M mannitol solution containing 0.1 mM MgSO₄ and 0.05 mM CaCl₂, before being subjected to the electric field. They were then placed between two parallel, stainless-steel wire electrodes (φ 500 μm) that were glued onto a glass slide 0.5 mm apart, overlaid with the same solution. The embryos were aligned by exposure to an alternating current (a.c) pulse of 600 kHz, 6 V, for 6 sec, after which fusion was initiated by applying three direct current (d.c) pulses of 1.0 to 2.0 kV/cm for 70 μsec, each 1 sec apart. Power was provided by an Electro Cell Fusion instrument (LF-100, Life Tec, Tokyo, Japan).

Embryo culture in vitro: After the electrofusion procedure, manipulated embryos were washed in M16 medium containing 100 μM EDTA, then placed under paraffin oil in drops of the same medium and incubated in a humidified atmosphere of 5% CO₂ in air at 37°C. Most manipulated embryos were fused within 30 min of fusion treatment. After 1 hr, they were assessed for fusion, and fused embryos were further incubated for 4 days to examine their developmental capacity in vitro. Control embryos, which were exposed to the manipulation medium for 1 hr, were also incubated to compare development in vitro.

Cell counts and embryo transfer: Some reconstituted and control embryos that developed into blastocysts at 96 hr after culture were fixed with a mixture of methanol and acetic acid (3:1), stained in 4% Giemsa, and their cell numbers were counted. Some reconstituted and control embryos that developed to the blastocyst stage were transferred to the uteri of Day 3 pseudopregnant ICR strain females to examine their viability in vivo. The recipients were kept until parturition.

Statistical analysis: Data on fusion and in vitro development were analyzed by Duncan's multiple comparison test. Cell number was analyzed by Student's t-test, and data on live young produced was analyzed by Chi-square test.

RESULTS

Electrofusion conditions: Overall statistical analysis showed that fusion rate was significantly (P<0.01) affected by field strength. When the field strengths of 1.0 and 1.5 kV/cm were used, fusion rates (84.8±1.4 and 90.1±3.2%) were equally high (Table 1). The field strength of 2.0 kV/cm resulted in a significant (P<0.01) low rate (72.0±2.5%) due to the increase in the number of degenerated embryos. There was no significant difference in the developmental rates of the three groups. Therefore, the field strength of 1.5 kV/cm which gave the maximum fusion rate was employed for electrofusion in the following experiments.

Developmental capacity in vitro: The proportion of karyoplasts that fused with recipient cytoplasm was high (86.2±3.2 to 90.6±2.0%) regardless of the strains of nucleus donors and cytoplasm recipients.
NUCLEAR TRANSPLANTATION IN MOUSE EMBRYOS

Table 1. Effect of electric field strength on the fusion rate of karyoplasts with recipient cytoplasm

<table>
<thead>
<tr>
<th>Field strength (kV/cm)</th>
<th>No. of embryos manipulated(^a)</th>
<th>% (mean±SEM) of embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fused</td>
<td>Nonfused</td>
</tr>
<tr>
<td>1.0</td>
<td>46</td>
<td>84.8±1.4(^d)</td>
</tr>
<tr>
<td>1.5</td>
<td>48</td>
<td>90.1±3.2(^d)</td>
</tr>
<tr>
<td>2.0</td>
<td>45</td>
<td>72.0±2.5(^d)</td>
</tr>
</tbody>
</table>

\(a\) The karyoplasts and cytoplasm were derived from ICR and F1 (C57BL/6J×CBA) embryos, respectively. Numbers include 5 replicates.
\(b\) The karyoplasts, not cytoplasm, were destroyed in all cases.
\(c\) Based on the number of fused embryos.
\(d\), \(e\), \(f\), \(g\) Values with different superscripts in the same column are significantly different (P<0.01).

Table 2. Development in vitro of reconstituted and control embryos

<table>
<thead>
<tr>
<th>Nucleus donor</th>
<th>Cytoplasm recipient</th>
<th>No. of embryos manipulated(^a)</th>
<th>% (mean±SEM) of embryos fused</th>
<th>% (mean±SEM)(^b) of embryos developed to 2-cell</th>
<th>Blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICR</td>
<td>F1</td>
<td>113</td>
<td>87.3±1.3</td>
<td>100</td>
<td>88.8±1.5(^d)</td>
</tr>
<tr>
<td>F1</td>
<td>F1</td>
<td>103</td>
<td>90.6±2.0</td>
<td>100</td>
<td>91.9±2.0(^d)</td>
</tr>
<tr>
<td>F1</td>
<td>ICR</td>
<td>109</td>
<td>86.2±3.2</td>
<td>97.2±1.4</td>
<td>71.5±2.9(^d)</td>
</tr>
<tr>
<td>ICR</td>
<td>ICR</td>
<td>102</td>
<td>89.4±1.9</td>
<td>98.6±1.4</td>
<td>54.1±3.2(^d)</td>
</tr>
<tr>
<td>Control F1</td>
<td>—</td>
<td>100</td>
<td>—</td>
<td>100</td>
<td>96.1±1.6(^d)</td>
</tr>
<tr>
<td>Control ICR</td>
<td>—</td>
<td>107</td>
<td>—</td>
<td>100</td>
<td>66.9±3.4(^d)</td>
</tr>
</tbody>
</table>

\(a\) Numbers include 8 replicates.
\(b\) Based on the number of fused embryos.
\(c\), \(d\), \(e\), \(f\), \(g\) Values with different superscripts in the same column are significantly different (P<0.01).

Statistical analysis revealed that developmental rate to blastocyst was significantly (P<0.01) affected by the nucleus and cytoplasm with interactions between these two factors. In vitro development of reconstituted embryos was very greatly influenced by the source of cytoplasm (Table 2). When the F1 embryos were used as cytoplasm recipients, high developmental rates (88.8±1.5 and 91.9±2.0%) to the blastocyst stage were obtained regardless of the strains of nucleus donor. These values were not significantly different from that of the F1 control. However, when the ICR embryos were used as cytoplasm recipients, the developmental rates of reconstituted embryos significantly (P<0.01) decreased (71.5±2.9 and 54.1±3.2%), although cleavage rates were very high (97.2±1.4 and 98.6±1.4%). A F1 nucleus in ICR cytoplasm significantly (P<0.01) enhanced the development of the reconstituted embryos compared with an ICR nucleus, but could not completely overcome the detrimental effect of ICR cytoplasm. To examine the effect of F1 cytoplasm carried along with the nucleus, approximately equal volume of F1 cytoplasm was transferred to ICR embryos, but no enhancement in the in vitro development of ICR embryos was observed (33/54, 61.1%) compared to that of ICR embryos fused with ICR cytoplasm (30/52, 57.7%).

The mean cell number of reconstituted embryos that developed to the blastocyst stage was not affected by the micromanipulation procedure nor by the source of nucleus and cytoplasm (Table 3). The mean cell number of embryos reconstituted with ICR nucleus and ICR cytoplasm was significantly (P<0.05) lower than that of the F1 control, but not of the ICR control.

Developmental capacity in vivo: The proportions of recipient mice that became pregnant were similar (average 66.7%) in all groups (Table 4). Of the 136 reconstituted embryos that were transferred to the recipient, 47 (34.6%) gave rise to live young. There was no significant difference in the rates of development to live young between each experimental group and the ICR control group. Although a
Table 3. Cell numbers of reconstituted and control embryos that developed to the blastocyst stage at 96 hr after micromanipulation

<table>
<thead>
<tr>
<th>Nucleus donor</th>
<th>Cytoplasm recipient</th>
<th>No. of embryos analyzed</th>
<th>Cell numbers (mean±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICR</td>
<td>F1</td>
<td>22</td>
<td>75.2±4.1&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>F1</td>
<td>F1</td>
<td>25</td>
<td>77.5±2.6&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>F1</td>
<td>ICR</td>
<td>20</td>
<td>73.9±3.7&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ICR</td>
<td>ICR</td>
<td>16</td>
<td>69.3±4.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control F1</td>
<td></td>
<td>28</td>
<td>83.1±3.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control ICR</td>
<td></td>
<td>20</td>
<td>78.0±3.9&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

a), b) Values with different superscripts are significantly different (P<0.05).

Table 4. Development of reconstituted and control embryos to term after transfer

<table>
<thead>
<tr>
<th>Nucleus donor</th>
<th>Cytoplasm recipient</th>
<th>No. pregnant/No. of recipient used (%)</th>
<th>No. of young/No. of embryos transferred (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICR</td>
<td>F1</td>
<td>4/6(66.7)</td>
<td>12/36(33.3)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>F1</td>
<td>F1</td>
<td>5/7(71.4)</td>
<td>17/44(38.6)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>F1</td>
<td>ICR</td>
<td>3/5(60.0)</td>
<td>11/32(34.4)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ICR</td>
<td>ICR</td>
<td>3/5(60.0)</td>
<td>7/24(29.2)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control F1</td>
<td></td>
<td>6/8(75.0)</td>
<td>30/52(57.7)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control ICR</td>
<td></td>
<td>5/8(62.5)</td>
<td>23/48(47.9)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

a), b) Values with different superscripts are significantly different (P<0.05).

significant (P<0.05) difference in developmental rates to live young was obtained between the reconstituted embryos with ICR nuclei and F1 controls, developmental capacity in vivo was hardly affected by reciprocal pronuclear transplantation between different strains.

**DISCUSSION**

Electrofusion parameters defined in the previous electrofusion experiment of mouse two-cell embryos [3] were applied successfully for the fusion of the karyoplasts with enucleated embryos. However, when the fusion pulse strengths of 2.0 kV/cm was applied, the karyoplast degeneration increased. It has been reported that the larger the cells, the greater the field strength and the longer the exposure times to the field pulse are required to fuse the cells [23]. It seems that karyoplasts are sensitive to the higher intensity electric stimulus because of their very small size compared with the cytoplasm.

The source of the cytoplasm strongly influences the development of reconstituted embryos in vitro. The cytoplasm of F1 embryos, but not ICR embryos, has a great potential to support the in vitro development of reconstituted embryos regardless of the strain of nucleus. These results contrast with those of Kono and Tsuchida [7] who found that the development in vitro of the reconstituted embryos was affected by the source of donor nuclei but not by recipient cytoplasm. This difference may be due to the number of embryos used in the experiments. Only a small number of embryos with fused karyoplast were tested for developmental capacity in vitro in their experiment. It seems that the differences in developmental capacities in vitro between F1 and ICR recipient cytoplasm may be related to the in vitro 2-cell block. The importance of the cytoplasm in early development of mouse embryos in relation to the in vitro 2-cell block has previously been demonstrated [5, 13].

The results of this study, however, suggested that the cytoplasm was not solely responsible for the in vitro development of embryos. Although the influence of the nuclei on the in vitro development of embryos was restricted by the source of cytoplasm, the nucleus did have a certain influence when the blocking ICR embryos were used as cytoplasm recipients. This suggest that the nucleus partially determines the ability of embryos to develop in vitro. However, the influence of the nucleus was extremely restricted in F1 cytoplasm. Other researchers [17] reported that the nucleus was of equal importance as that of the cytoplasm for the successful development of embryos cultured from one-cell stage. These differences may be due to the difference in the donor nuclei. In their experiment, a B6D2 × ICR strain was used as the donor nucleus.

The present results also showed that the reciprocal nuclear transplantation did not affect the cell numbers of reconstituted embryos that developed to the blastocyst stage. It seems that some differences in the cell numbers between reconstituted and control embryos may be due to the delay of first cleavage caused by micromanipulation and electrofusion procedures for about 3 hr (data not shown).

The reconstituted embryos that overcame 2-cell block and developed to the blastocyst stage could also develop into normal live young. The in vivo development of the reconstituted embryos transferred to recipient mice at the blastocyst stage was not affected by the strains of donor nucleus and recipient cytoplasm. This result is in agreement with that of Kono and Tsuchida [7]. In this study, the rates of development of reconstituted embryos to
full term was slightly lower than that of control embryos. This may be due to some reconstituted embryos that either fused with the second polar body as well as with the karyoplast, or had small number of cells.

The results of this study show that embryonic development is hardly affected by the nuclear transplantation with electrofusion. It was also indicated that the source of cytoplasm had the main influence on the developmental ability of reconstituted embryos to the blastocyst stage in vitro, while the effect of the donor nucleus was very small and restricted by the source of recipient cytoplasm. Neither cytoplasm nor nucleus affected the cell number of embryos that developed to the blastocyst stage and their subsequent development in vivo.

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