Production of a Germ-line Chimera by Coculture of Zona-free Embryos with Frozen-thawed Embryonic Stem Cells

Hiroshi SUZUKI1,2), Nobuo KAMADA1), Otoya UEDA1), Kouichi JISHAGE1), Hiroki KURIHARA2), Yukiko KURIHARA2), Tatsuhiko KODAMA2), Yoshio YAZAKI2), Sadahiro AZUMA3) and Yutaka TOYODA3)

1)CSK Research Park, INC., 1-135, Komakado, Gotemba, Shizuoka 412, 2)Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113, 3)Department of Reproductive and Developmental Biology, Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo 108, Japan

Abstract. To improve chimera production efficiency, we examined whether frozen-thawed embryonic stem (ES) cells would be useful in a coculture method. A3-1 ES cells were electroporated with the targeting vector pE 10.29NEO-TK. Exon 2 of the endothelin-1 gene in the ES cells was disrupted by homologous recombination. The homologous recombinant was cocultured at a density of 5.5 x 10^5 cells/ml with zona-free 8-cell to morula stage embryos in a coculture medium for 3-3.5 h. Cocultured embryos were transferred into Whitten's medium and cultured overnight. Morula and blastocyst stage embryos were transferred into the recipients on day 2.5 of pseudopregnancy. The development rate to blastocyst after coculture and subsequent overnight cultivation was 62%. As a result of embryo transfer, four male chimeras were obtained. Two of these were identified as germ-line chimeras by a progeny test. These results indicate that frozen-thawed ES cells can be used in coculture to produce chimeras and contribute a germ-line.

Key word: Mouse, Embryonic stem cells, Chimera, Coculture.


Mice which have been gene disrupted by means of gene targeting are useful as tools for analysis of gene function in vivo and as animal models for studying human disease. Although gene disrupted mice are produced via chimeric mice, skillful manipulation techniques and tremendous effort are required for both the culture and injection of embryonic stem (ES) cells used in chimera production. The process of chimera production is composed of the preparation of feeder cells, the culture of ES cells, the recovery of embryos, the injection of ES cells into host embryos and the transfer of manipulated embryos. The majority of man-hours in this process is spent on the culture and injection of ES cells. Recently, a simple method for chimera production, which solved the latter problem, was reported by Wood et al. [1]. To improve the efficiency of this method, we examined whether frozen-thawed ES cells would be useful for the production of chimeras by coculture.
Materials and Methods

Embryos for this experiment were obtained by in vitro or in vivo fertilization. The methods for in vitro fertilization were the same as described by Toyoda et al. [2]. Mature female C57BL/6J mice (CLEA, Japan) were superovulated by injection with 5 i.u. of PMSG (Serotropin, Teikokuzoki, Japan) and 5 i.u. of hCG (Puberogen, Sankyo, Japan) 48 h apart. Their oocytes were obtained 15–16 h after hCG injection. Spermatozoa were collected from the cauda epididymis of C57BL/6J males and suspended in TYH medium. After preincubation for 1–1.5 h, in vitro fertilization was performed by adding a small volume of the preincubated sperm suspension to the medium containing eggs. About 72 h after insemination, embryos that developed to the morula stage were used in coculture after removal of the zona pellucida, as described below. In the case of in vivo fertilization, on day 2.5 of pregnancy, 8-cell to morula stage embryos were flushed from the oviducts of superovulated C57BL/6J females (CLEA, Japan) that had mated with the same strain of males. The zona pellucida of all embryos was removed by treatment with acidified Tyrode’s solution [3]

A3-1 ES cells (129/Sv origin) [4] were electroporated with the linearized targeting vector pE10.29NEO-TK [5]. The pE10.29NEO-TK was constructed from a 7.5 kb of an endothelin-1 (ET-1) genomic fragment encompassing exons 2 to 5. Exon 2 was disrupted by insertion of the neomycin resistance gene, and the genomic fragment was flanked at the 3' end by the herpes simplex virus thymidine kinase gene [6]. One of the homologous recombinant clones, A6, mutated in one of the 2 alleles of the ET-1 locus, was used in this experiment. A6 was expanded by culture on inactivated feeder layers derived from mouse fetal fibroblasts in Dulbecco’s modified Eagle’s medium (DMEM 430-2100EB, Gibco, USA) containing 20% fetal calf serum (FCS, JRH Biosciences, USA), 0.1 mM 2-mercaptoethanol (Sigma, USA) nucleosides, non-essential amino acids [7] and 10^3 units leukemia inhibitory factor (LIF, Amrad, Australia) [8, 9] (SCM + LIF). To obtain a single-cell suspension, ES cells and feeder layers were trypsinized by trypsin-EDTA solution [7]. Trypsin-EDTA was inactivated by an equal volume of the SCM + LIF. The cell suspensions were frozen at a density of 3-5 × 10^6/ml in SCM supplemented with 10% dimethyl sulphoxide (DESO, Sigma, USA) in 1.2 ml cryogenic vials (25703VIAL1, Corning, USA) [7].

To thaw out the ES cells, the vial was retrieved from liquid nitrogen and placed directly in a 37 C water bath. The contents of the vial were transferred to a 15 ml centrifuge tube (25317 CTF15, Corning, USA). Nine ml of SCM+LIF was added to the tube during agitation, and the tube was centrifuged at 1000 rpm for 5 min. The supernatant was aspirated, and then the cells were resuspended in SCM + LIF. After the cell suspension was cultured for 1.5 h on a gelatin coated dish (25010 GEL, Corning, USA), the medium was discarded, and then adhesive cells were trypsinized as described above. The survival rate of the adhesive cells was almost 100%. The cell suspension was centrifuged, and the cells were resuspended in SCM+LIF or DMEM containing 5% FCS and 23 mM lactate (coculture medium) [1]. To remove the feeder cells, the cell suspension was plated onto a tissue culture dish (25020 DISH 100N, Corning, USA) and incubated at 37 C under 5% CO2 in air for 30 min. The ES cells were recovered from the supernatant and resuspended at a density of 5.5–20.0 × 10^5 cell/ml in coculture medium. In some of the experiments, the feeder cells were not removed from the cell suspensions after thawing. The feeder cells accounted for 10% of all the cell suspensions.

Eight zona-free embryos were transferred into 15 μl of the ES cell suspension covered with paraffin oil (Art 7162, Merck, Germany) and then cocultured at 37 C under 5% CO2 in air. Three to 3.5 h after cocultivation, the embryos were transferred to Whitten’s medium [10] supplemented with 100 μM EDTA [11–13] and cultured for 15–16 h. The blastocyst and morula stage embryos were transferred into the uteri of ICR (CLEA, Japan) recipients on day 2.5 of pseudopregnancy. Fetuses were delivered by Cesarian section or natural parturition. Chimeras were identified by coat color and eye pigmentation. To confirm a germ-line transmission of the ES cells, mature chimeras were mated with previously obtained heterozygotes of ET-1 gene disrupted female mice [5]. The progeny were obtained by Cesarian section. Germ-line transmission was identified in the progeny by eye pigmentation and appearance of a specific phenotype, i.e., a craniofacial anomaly, indicating a homozygote of ET-1 gene disruption [5].
Results and Discussion

The results of 6 experiments that produced chimeras by coculture are shown in Table 1. When zona-free embryos at the 8-cell to morula stage were cocultured with frozen-thawed ES cells at a density of 5.5 × 10⁵/ml and 16.4–20.0 × 10⁵/ml, 62% and 27% of the embryos developed to the blastocyst stage after overnight culture, respectively. However, development rates to the blastocysts stage were also significantly different among experiments with the same ES cell density. Although 82–95% of embryos that reached the morula stage 72 h after in vitro insemination developed into blastocysts (Exp.II and III), only 11–25% of the 8-cell to morula stage embryos that were flushed from the oviducts on day 2.5 of pregnancy reached blastocysts (Exp. I and VI) after coculture and subsequent overnight cultivation. Embryonic development after transfer tended to be reduced after high density ES cell coculture. Moreover, no chimeric mice were obtained at a density of 16.4-20.0 × 10⁵/ml. In this study, the most efficient combination of embryo stage and ES cell density yielded a rate of 31% (11/35) of transferred embryos developing to term, and 27% (3/11) were chimeric mice (Exp. I). With regard to A3-1, as 8-cell embryos tended to incorporate too many ES cells, the morula stage has been recommended as the best for the coculture method [14]. Wood et al. [1] reported that 38% (15/40) of transferred blastocysts that were cocultured at the compacted 8-cell stage developed to term, and 40% (6/15) were chimeric. Furthermore, these reports pointed out that too high a contribution from ES cells was not conducive to normal pre-[14] and/or post-[1] implantation development. In the present experiments, however, even though the cocultured morula exhibited a high developmental rate to the blastocyst stage, embryonic development after transfer was severely reduced (Table 1). It was not elucidated whether the completely compacted morula was unable to take in enough ES cells, or whether too many ES cells incorporated into the morula and disturbed embryonic development. The most suitable combination of embryonic stage and ES cell concentration might differ depending on the mouse strain providing the embryos as well as the ES cell lines or their subclones.

When the cocultured embryos that were cultured overnight were transferred to the recipients, chimeric mice with a high degree of chimerism, including that of the germ-line, were obtained. As shown in Table 2, the approximate degree of chimerism ranged from 40% to 100% ES cell contribution, based on the ratio of white and agouti versus black coat colors. These results indicate that the frozen-thawed ES cells can in fact contribute to most tissues, including the germ line, in chimeric mice. Three out of 4 chimeras were tested for germ-line transmission, and 2 of these passed on the ES-cell derived material to 25% or 100% of their progeny.

Table 1. Production of chimeric mice by zona-free embryos with frozen-thawed ES cells (ETKO A6) in coculture

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>ES cells (x 10⁵ cells/ml)</th>
<th>Stage of embryos</th>
<th>No. embryos cocultured</th>
<th>No. (%) embryos developed to:</th>
<th>No. embryos transferred</th>
<th>No. (%) implantation sites</th>
<th>No. (%) newborn</th>
<th>No. (%) chimeras</th>
</tr>
</thead>
<tbody>
<tr>
<td>I*</td>
<td>5.5</td>
<td>8-M*</td>
<td>53</td>
<td>38 (72)</td>
<td>13 (25)</td>
<td>35</td>
<td>18 (51)</td>
<td>11 (31)</td>
</tr>
<tr>
<td>II</td>
<td>5.5</td>
<td>M**</td>
<td>38</td>
<td>1 (3)</td>
<td>31 (82)</td>
<td>35</td>
<td>14 (40)</td>
<td>4 (11)</td>
</tr>
<tr>
<td>III*</td>
<td>5.5</td>
<td>M**</td>
<td>38</td>
<td>1 (3)</td>
<td>36 (95)</td>
<td>13</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Subtotal</td>
<td></td>
<td></td>
<td>129</td>
<td>40 (31)</td>
<td>80 (62)</td>
<td>83</td>
<td>32 (39)</td>
<td>15 (18)</td>
</tr>
<tr>
<td>IV</td>
<td>16.4</td>
<td>8-M*</td>
<td>13</td>
<td>6 (46)</td>
<td>4 (31)</td>
<td>10</td>
<td>7 (70)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>VI</td>
<td>20.0</td>
<td>8-M*</td>
<td>47</td>
<td>14 (30)</td>
<td>18 (38)</td>
<td>47</td>
<td>10 (21)</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Subtotal</td>
<td></td>
<td></td>
<td>97</td>
<td>25 (26)</td>
<td>26 (27)</td>
<td>66</td>
<td>22 (33)</td>
<td>6 (9)</td>
</tr>
</tbody>
</table>

*: Fertilization in vitro.
**: Fertilization in vivo.

The homologous recombinant A6 was cocultured with zona-free embryos in DMEM containing 5% FCS and 23 mM lactate for 3–3.5 h.
In experiment I, 3 out of 11 newborns were male chimeric mice, and 2 of these were recognized as germ-line chimeras. This fact demonstrated that the frozen-thawed ES cell suspension was effective for coculture even without separation from the feeder cells. The majority of time and toil in the process of chimera production is spent on the culture and injection of ES cells. Wood et al. [1] solved the latter problem. The present study solves the former problem because the routine culturing of ES cells and feeder cells is no longer essential for chimera production. Furthermore, the use of frozen-thawed ES cells eliminates the preparation of feeder cells for ES culture. The preparation of feeder cells and the culture of ES cells take 5–7 days prior to the injection of ES cells. Chimera production should be significantly improved by this simplification of conditions.

Acknowledgement

The authors wish to express our gratitude to Dr. K. Boru for his kindness in reading and revising the manuscript.

References

