Production of Mice Entirely Derived from Embryonic Stem (ES) Cell with Many Passages by Coculture of ES Cells with Cytochalasin B Induced Tetraploid Embryos

Otoya UEDA, Kouichi JISHAGE, Nobuo KAMADA,
Satomi UCHIDA, and Hiroshi SUZUKI

CSK Research Park Inc., Fuji Gotemba Research Laboratories, Chugai Pharmaceutical Co., Ltd.,
1–135 Komakado, Gotemba, Shizuoka 412, Japan

Abstract: Mice entirely derived from ES cells were obtained from aggregates of TT2 ES cells and cytochalasin B induced tetraploid embryos. Tetraploid embryos were cocultured with ES cells in a well on the Multiplate-Terasaki. After embryo transfer of the aggregates, the male newborns were recovered normally after Cesarean section and reached adulthood. The male mice exhibited complete pigmentation of the eye and coat, suggesting ES cell contributions alone. Alkaline phosphatase-1 analysis yielded no evidence of tetraploid cells in the kidney or liver. The TT2-derived males were fertile, produced normal offspring, and exclusively transmitted the TT2 genotype to their progeny. This result clearly shows that ES cells are able to support complete fetal development.

Key words: chimera, ES cells, tetraploid

Introduction

Gene disrupted mice, which are produced via chimeric mice, are useful as a tool for the analysis of gene function *in vivo*, and as an animal model for human disease. Chimeric mice are produced from genetically manipulated ES cells and host embryos. ES cells alone can not develop to term since ES cells are capable of colonizing trophoctoderm and primitive endoderm derivatives at a low frequency [2]. Therefore, complementary functional activity by host cells is required in chimeras during the early stages of development. Recent advances which use such cells in methods for producing chimeras have lead to a recently established [22] and improved [14, 19] coculture methods.

Cytochalasin B treatment arrests cell division but permits nuclear division to continue [3]. Furthermore, tetraploid embryos as induced by cytochalasin B can implant at a high frequency, but only a few of these tetraploid embryos develop to term [12]. Postnatal survival of tetraploid embryos has not been reported. Tetraploid embryos can be rescued so as to develop to maturity by aggregating them with normal diploid embryos to produce diploid/tetraploid chimeric mice [5]. In diploid/tetraploid aggregates, the tetraploid cells are selected against during development of the fetal tissues in most cases [5], but persist in extraembryonal membranes [17].

According to these observations, it would appear that the aggregates of ES cells cytochalasin B-induced tet-

(Received 2 February 1995 / Accepted 17 April 1995)

Address corresponding: H. Suzuki, CSK Research Park Inc., Fuji Gotemba Research Laboratories, Chugai Pharmaceutical Co., Ltd., 1–135 Komakado, Gotemba, Shizuoka 412, Japan

raploid embryo are capable of developing to term. Furthermore, it seemed highly probable that the young can potentially be made up of exclusively ES cells components. Nagy, *et al.* has reported that ES cells aggregated with tetraploid embryos, which were produced by electrofusing 2-cell stage blastomeres, were able to develop into viable embryos [7]. These embryos were entirely ES cell-derived and most of the extraembryonic cell lineages tetraploid-derived [7]. However, the totipotency of ES cells was lost with extended culture, i.e., over 14 passages, *in vitro* [8]. We describe in this report that mice, which were solely of ES cell lineage, were obtained from cytochalasin B induced tetraploid embryo and TT2 ES cells aggregates. These TT2 ES cells had undergone 18 passages of extended culture. The result shows that TT2 ES cells have sustained the potential to contribute many tissues including germ cells in resultant chimeric mice even after they had undergone many passages.

Materials and Methods

Strains of mice used for embryos and embryonic stem cells

ICR mice purchased from CLEA Japan were used in this study. The animals were kept in a barrier unit at $24 \pm 2^\circ\text{C}$ with a relative humidity of 50–60% under a lighting regimen of 14L: 10D (lights on 05:00 to 19:00 hr), and whole air exchanged 12–15 times/hr. They were housed in polycarbonate cages (TP-106; $175 \times 245 \times 125$ mm, TP-102; $215 \times 320 \times 140$ mm, TOYORIKO, Japan). They were allowed to access standard laboratory chow (CA-1; CLEA Japan) and tap-water.

TT2 ES cells [23] were kindly supplied by Dr. Aizawa of Lab. of Morphology, Inst. of Physical and Chemical Research, Kumamoto Univ., Japan via Mr. Saitoh of Life Technologies Oriental, Inc., Japan. TT2 were expanded by culture on mitomycin C-treated feeder layers derived from mouse fetal fibroblast in Dulbecco's modified Eagle's medium (DMEM 430–2100EB; Gibco, USA) containing: 20% fetal calf serum (FCS; JRH Bioscience, USA), 0.1 mM 2-mercaptoethanol (Sigma, USA), nucleosides, non-essential amino acids [9] and 10^3 units leukemia inhibitory factor (LIF; Amrad, Australia) [10, 21] (SCM + LIF). We continued counting the additional passage

number after we obtained the TT2 (passage 12). The TT2 were frozen at every passage and stored as described previously [14, 19]. This study was performed using ES cells from passage 18. After thawing the TT2, the cryoprotectant was removed from the cell suspensions by centrifugation. The ES cells were resuspended in SCM + LIF and cultured for 2 days as described above.

Production of tetraploid embryos

Female mice were superovulated with 5 IU of PMSG (Serotropin, Teikoku Hormone Mfg. Co., Japan) and 5 IU of hCG (Puberogen, Sankyo, Japan) injected 48 hr apart, and the oocytes were obtained 15–16 hr after the hCG injection. Sperm was collected from the cauda epididymis of males, suspended in 400 μl of TYH medium [18], covered with paraffin oil (Art 7162; Merck, USA), and equilibrated overnight at 37°C in 5% CO_2 in air. After preincubation for 1.5 hr, oocytes were fertilized *in vitro* with a small volume of sperm suspension (150 cells/ μl) added to the medium. Six hours after insemination, fertilization was confirmed by the presence of a second polar body as well as both male and female pronuclei. Fertilized eggs were treated with 5 $\mu\text{g}/\text{ml}$ of cytochalasin B (Lot 113H4049; Sigma, USA) [11] overnight in Whitten's medium [20] supplemented with 100 μM EDTA [1, 4] after being washed twice. After the treatment with cytochalasin B, the mono-cell embryos were separated and considered as tetraploids. The embryos were washed 4 times and cultured for a further 2 days in Whitten's medium supplemented with 100 μM EDTA. The compacted and compacting 4-cell stage embryos were used as aggregation chimeras. The cytochlasin treated embryos were analyzed for ploidy at the blastocyst stage, the result being that, all of the blastocysts were shown to be tetraploids.

Coculture of ES cells with tetraploid embryos and embryo transfer

To obtain a single cell suspension, ES cells and feeder layers were trypsinized by trypsin-EDTA solution [9]. Trypsin-EDTA was inactivated by an equal volume of SCM + LIF. The cell suspension was centrifuged and then the cells were resuspended at a density of 7,500 cells/20 μl , i.e., 7,500 cells/well, in Whitten's medium

supplemented with 10% FCS on a Multiplate-Terasaki (Sumitomo Bakelite Co., Ltd., Japan) [13, 15]. The feeder cells were not removed from the cell suspensions [14, 19].

The compacted and compacting embryos were decompacted by brief exposure of 0.02% EDTA in phosphate buffered saline (PBS). The zona pellucidae of these embryos were removed by treatment with 0.5% pronase (Actinase E; Kakenseiyaku, Japan) [6]. The zona-free embryos were washed twice in Whitten's medium supplemented with 100 μ M EDTA and then transferred into 20 μ l of the ES cell suspension in a well of the Multiplate-Terasaki. One (indicated as 4N/ES) or two embryos, which were aggregated with each other (indicated as 4N/4N/ES), were cocultured with the ES cells in a well covered with paraffin oil. After 3 hr incubation at 37°C under 5% CO₂ in air, the successful aggregates were transferred into Whitten's medium supplemented with 100 μ M EDTA on the Multiplate-Terasaki and cultured for 24 hr. Control embryos were also treated with EDTA and pronase without cytochalasin B. A single embryo (indicated as 2N/ES) at 72 hr after insemination was cocultured with ES cells in a control experiment.

The morula to blastocyst stage embryos were transferred into the uteri of ICR recipients on day 2.5 of pseudopregnancy. The methods for embryo transfer were the same as described by Suzuki *et al.* [15]. Recipients were routinely subjected to a Cesarean section on day 19.5 of pregnancy. Live fetuses were counted as well as the number of early post implantation resorptions and embryos dying at or after mid-gestation. Resorptions above 4 mm in diameter at term were considered as mid-gestation dead embryos [8]. Fetuses that recovered after the Cesarean section were placed with foster mothers. Chimeras were identified by coat color and eye pigmentation, since tetraploid embryos were albino, while the ES cells were derived from agouti embryos. To confirm a germ-line transmission of the ES cells, mature chimeras were mated with ICR female mice.

Biochemical markers

The polymorphic alkaline phosphatase-1 (*Akp1*) gene on chromosome 1 was used to distinguish between the tetraploid embryos and ES cell-derived components of

the chimera. The ES cell lines were derived from both *Akp1^a/Akp1^a* CBA and C57BL/6, and the tetraploid components were produced from *Akp1^b/Akp1^b* ICR embryos. Isoenzyme analysis was performed in ICLAS Monitoring Center, Central Institute for Experimental Animals, Japan as described below. Tissues surgically removed were homogenized in a five fold volume of distilled water. The homogenates were centrifuged at 15,000 rpm at 4°C for 1 min and 0.6 μ l of this supernatant was analysed by cellulose acetate electrophoresis in Tris-EDTA-Borate buffer (10.9 g of Tris aminometane, 3.1 g of Boric acid, 0.6 g of EDTA 2Na and made up to 1,000 ml with distilled water, pH 8.6) at room temperature for 45 min at 100 V. The membranes were stained with a mixture of reagent A (50 mg of beta-naphthylphosphate, 20 mg of fast blue RR salt and 50 mg of fast violet B), reagent B (10 ml of 0.1 M Tris-HCl) with reagent C (50 μ l of 1 mg/ μ l MgCl₂ and 50 μ l of 1 mg/ μ l MnCl₂) and incubated at 37°C for 20 min. Destain and fixation were performed with 0.5% acetic acid solution for 10 min.

Karyotype analysis

ES cells at 2 days after passage were arrested in metaphase by adding colcemid (0.1 μ g/ml at final concentration) to the culture medium for 1.5 hr at 37°C under 5% CO₂ in air. The cells were treated with trypsin-EDTA for 5 min at room temperature. After vigorous pipetting, single cell suspension was centrifuged at 1000 rpm for 5 min. The pellet was exposed to hypotonic shock by 0.56% KCl solution for 30 min at room temperature. After centrifugation at 500 rpm for 5 min, the hypotonic solution was removed, and the pellet fixed with methanol/acetic acid, 3:1 (vol/vol) via gently pipetting. After 10 min the suspension was centrifuged at 500 rpm for 5 min. Fixation was performed twice prior to spreading the cells on slides. The slides were air-dried overnight, stained in freshly made 5% Giemsa for 30 min and rinsed with distilled water.

Results

After the treatment with cytochalasin B for 16 hr, almost all eggs were in the mono-cell stage. Seventy-three percent of the cytochalasin B treated eggs, as shown in Table 1, developed to compacted or compact-

ing 4-cell stage at 72 hr after insemination. This rate was not significantly different from that of the non-treated control. When pairs of cytochalasin B treated embryos were cocultured with TT2 ES cells, as shown in Table 2, 84 out of 137 pairs were recovered successfully as aggregate embryos. 62% of these aggregates (4N/4N/ES) developed to the blastocyst stage after overnight cultivation. When a single cytochalasin B treated embryo was cocultured with TT2, 61% of the embryos (4N/ES) developed to blastocyst. These results were not significantly different as compared with control (2N/ES) value (64%), respectively.

As a result of embryo transfer of the ES cell-cytochalasin B treated embryo aggregates, five male newborns recovered normally after Cesarean section on day 19.5 of pregnancy and three of them reached adulthood (Table 3). As shown in Fig. 1, 4N/4N/ES aggregates resulted in similar numbers of mid-gestation fetuses as compared with 2N/ES (control) aggregates. In contrast, 4N/ES resulted in markedly reduced numbers of mid-gestation fetuses as compared with both 2N/ES and 4N/4N/ES. Furthermore, in com-

parison to control, only 13% and 11% of 4N/4N/ES and 4N/ES aggregates, respectively, were capable of survival to term. The male mice produced from this experiment showed complete pigmentation of the eye and coat color, suggesting extensive or entire ES cell contributions. The male mice were tested for germline chimerism and confirmed transmission of ES-derived germ cells. All of three TT2-derived males were fertile, siring 66, 15 and 70 offspring respectively, and exclusively transmitted the TT2 genotype to their progeny.

Akp1 assay of the kidney and liver tissues confirmed the TT2 origin of the mouse. Electrophoresis of these tissues derived from the males showed *Akp1^u/Akp1^a*.

Chromosome spreads were prepared from passages 14, 22 and 25. A slight decrease was detected in the frequency of spreads compared to the normal number of chromosomes (Fig 2.)

Discussion

Both the ES cells and cytochalasin B induced tetraploid embryo aggregates developed to term and were viable after birth (Table 3). Progeny test and *Akp1* analysis revealed that the mice were entirely derived from the ES component. *Akp1* analysis, in present study, showed no evidence of tetraploid cells in the kidney and liver.

It has previously been shown that the ES cells aggregated with tetraploid embryos (which were produced by electrofusing 2-cell stage blastomeres) were able to develop into viable fetus where the embryonic cells were entirely derived from ES cells and where most of

Table 1. *In vitro* development of embryos treated with cytochalasin B 72 hr after insemination

Treatment	No. of embryos cultured	No. (%) of embryos developed
Cytochalasin B	492	358 ¹⁾ (73)
None	140	109 ²⁾ (78)

¹⁾ Compacted or compacting 4-cell. ²⁾ Morula.

Table 2. Effect of coculture of embryonic stem cells with cytochalasin B induced tetraploid embryos on subsequent development *in vitro*

Type of aggregate	No. of embryos (pairs) cultured	No. (%) of aggregates recovered	No. (%) of aggregates develop to :		
			Morula	EB-SB	Blastocyst
4N/4N/ES ¹⁾	137	84 (61)	15 (18)	10 (12)	52 (62)
4N/ES ²⁾	62	62 (100)	6 (10)	6 (10)	38 (61)
2N/ES ²⁾	64	64 (100)	12 (19)	2 (3)	41 (64)

¹⁾ Two embryos were aggregated with each other on the ES cells in a well of a Multiplate-Terasaki, and cocultured for 3 hr. ²⁾ One embryo was cocultured with the ES cells in a well for 3 hr. The successful aggregates were cultured in Whitten's medium supplemented with 100 μ M EDTA for 24 hr.

Table 3. Developmental potency of the aggregates of ES cells and tetraploid embryos after embryo transfer

Type of aggregates	No. of aggregates transferred	No. (%) of implantations	No. (%) of newborn	No. (%) of survive	No. (%) of animals reached adulthood
4N/4N/ES	75	36 (48) ^a	4 (5) ^a	4 (5) ^a	2 (3) ^a
4N/ES	48	19 (40) ^a	2 (4) ^a	1 (2) ^a	1 (2) ^a
2N/ES	49	15 (31) ^a	9 (18) ^b	9 (18) ^b	8 (16) ^b

Values with the same superscripts are not significantly different in the same column at $P>0.05$.

Fig. 1. Embryonic death after implantation in TT2-tetraploid embryo aggregates.

the extraembryonic cell lineages were tetraploid-derived, although a very small residual contribution from the tetraploid cells could not be entirely ruled out [7]. Furthermore, Nagy *et al.* used R1 ES cell lines derived from early passages with electrofusion derived tetraploid embryos to form aggregates and obtained mice which were entirely derived from ES cells [8]. However, the R1 ES cells lost this totipotency upon extended culture *in vitro*. No animal survived to adulthood from ES cells obtained from later than 14 passages [8]. Moreover, even when early passage cells have been used, many ES cell-tetraploid aggregates die before developing to term. Only 3.8% of transferred aggregates have survived after Cesarean section [8]. In our present study, five out of 123 (4.1%) transferred blastocysts, which were derived from cytochalasin B induced tetraploid embryos and TT2 ES cells at passage 18, developed to term and three of them reached adulthood

Fig. 2. Distribution of metaphase spreads with different chromosome numbers at passages 14, 22 and 25.

(Table 3). In these TT2 ES cells, metaphase spreads indicated that high percentages of the normal number of chromosomes were maintained in spite of extended culture *in vitro* (Fig. 2). This may be a reason for the maintained totipotency in our TT2 ES cells. There are also other benefits of inducing tetraploidy with cytochalasin B, namely; simple method, reproducibility, and that many embryos can be treated simultaneously.

As reported by Nagy *et al.* [8], this approach cannot be considered as feasible to routinely achieve germ-line transmission from genetically manipulated ES cells. Nevertheless, this system is useful for testing the totipotency of newly established ES cell lines or subclones, and to study the development of genetically modified ES cells such as defects that relate to extraembryonic lineage.

Acknowledgments

The authors wish to express our gratitude to Dr. P. Kowalski-Saunders for his kindness in reading and revising the manuscript and to Dr. H. Katoh for his technical assistance.

References

1. Abramczuk, J., Solter, D., and Koprowski, H. 1977. The beneficial effect of EDTA on development of mouse one-cell embryos in chemically defined medium. *Dev. Biol.* 61: 378–383.
2. Beddington, R. S. P. and Robertson, E. J. 1989. An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. *Development* 105: 733–737.
3. Carter, S. B. 1967. Effects of cytochalasins on mammalian cells. *Nature* 213: 261–264.
4. Hoshi, M. and Toyoda, Y. 1985. Effect of EDTA on the preimplantation development of mouse embryos fertilized *in vitro*. *Jpn. J. Zootech. Sci.* 56: 931–937.
5. Lu, T. Y. and Markert, C. L. 1980. Manufacture of diploid/tetraploid chimeric mice. *Proc. Natl. Acad. Sci. USA*, 77: 6012–6016.
6. Mintz, B. 1962. Experimental study of the developing mammalian egg: Removal of zona pellucida. *Science* 138: 594–595.
7. Nagy, A., Gocza, E., Diaz, E. M., Prideaux, V. R., Ivanyi, E., Markkula, M., and Rossant, J. 1990. Embryonic stem cells alone are able to support fetal development in the mouse. *Development* 110: 815–821.
8. Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J. C. 1993. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci.* 90: 8424–8428.
9. Robertson, E. J. 1987. Embryo-derived stem cell lines. pp. 71–112. *In: Teratocarcinomas and Embryonic Stem Cells* (Robertson, E. J. ed.), IRL, Oxford, UK.
10. Smith, A. G., Heath, J. K., Donaldson, D. D., Wong, G. G., Moreau, J., Stahl, M., and Rogers, D. 1988. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* 336: 688–690.
11. Snow, M. H. L. 1973. Tetraploid mouse embryos produced by cytochalasin B during cleavage. *Nature* 244: 513–515.
12. Snow, M. H. L. 1975. Embryonic development of tetraploid mice during the second half of gestation. *J. Embryol. Exp. Morph.* 34: 707–721.
13. Suzuki, H., Togashi, M., Miyai, T., and Okamoto, M. 1987. Viability of zona-free mouse embryos derived from eggs fertilized *in vitro*. *J. Mamm. Ova Res.* 4: 84–91.
14. Suzuki, H., Kamada, N., Ueda, O., Jishage, K., Kurihara, H., Kurihara, Y., Kodama, T., Yazaki, Y., Azuma, S., and Toyoda, Y. 1994. Production of a germ-line chimera by coculture of zona-free embryos with frozen-thawed embryonic stem cells. *J. Reprod. Dev.* 40: 361–365.
15. Suzuki, H., Togashi, M., Moriguchi, Y., and Adachi, J. 1994. Relationship between age-related decline in fertility and *in vitro* fertilization rate in IVCS mice. *J. Reprod. Dev.* 40: 107–116.
16. Suzuki, H., Togashi, M., Adachi, J., and Toyoda, Y. 1995. Developmental ability of zona-free mouse embryos is influenced by cell association at the 4-cell stage. *Biol. Reprod.* 53: 78–83.
17. Tarkowski, A. K., Witkowska, A., and Opas, J. 1977. Development of cytochalasin B-induced tetraploid and diploid/tetraploid mosaic mouse embryos. *J. Embryol. Exp. Morph.* 41: 47–64.
18. Toyoda, Y. and Takasugi, M. 1982. Early development of mouse embryos fertilized *in vitro*. pp. 57–64. *In: Genetic Approaches to Developmental Neurobiology* (Tsukada, ed.), Univ. Tokyo Press, Japan.
19. Ueda, O., Kamada, N., Jishage, K., Uchida, S., and Suzuki, H. 1995. Factors affecting the efficiency of chimera production by coculture of zona-free embryos with frozen-thawed embryonic stem cells. *J. Reprod. Dev.* (in press).
20. Whitten, W. K. 1971. Nutrient requirements for the culture of preimplantation embryos *in vitro*. *Advan. Biosci.* 6: 129–141.
21. Williams, R. L., Hilton, D. J., Pease, S., Willson, T. A., Stewart, C. L., Gearing, D. P., Wagner, E. F., Metcalf, D., Nicola, N. A., and Gough, N. M. 1988. Myeloid leukemia inhibitory factor (LIF) maintains the developmental potential of embryonic stem cells. *Nature* 336: 684–687.
22. Wood, S. A., Pascoe, W. S., Schmidt, C., Kemler, R., Evans, M. J., and Allen, N. D. 1993. Simple and efficient production of embryonic stem cell-embryo chimeras by coculture. *Proc. Natl. Acad. Sci. USA*, 90: 4582–4585.
23. Yagi, T., Tokunaga, T., Furuta, Y., Nada, S., Yoshida, M., Tsukada, T., Saga, Y., Takeda, N., Ikawa, Y., and Aizawa, S. 1993. A novel ES cell line, TT2, with high germline-differentiating potency. *Analy. Biochem.*, 214: 70–74.