

Establishment of New Embryonic Stem (ES) Cell Lines from C57BL/6 Strain Mice by Whole Blastocyst Culture Method

Makoto Osonoi*, Sang-Yong Kim, Jutarō Takahashi and Yasuhisa Yasuda

Laboratory of Animal Breeding and Reproduction, Faculty of Agriculture, Iwate University, Morioka, Iwate 020, Japan

Abstract: Two embryonic stem (ES) cell lines (OKB6-I and -II) derived from C57BL/6 strain mice were established by the whole blastocyst culture method, and the net isolation ratio was 3.9% (2/51). They had pluripotency and normal karyotype, and were positive for alkaline phosphatase activity. It was indicated that OKB6-I was a female cell line, and OKB6-II was a male, judged by the size and morphology of their chromosomes. By coculture with ICR embryos, a chimeric mouse and a hermaphrodite were generated from OKB6-II, but no chimera was generated from OKB6-I. OKB6-II \leftrightarrow ICR chimera generated 3 albino type viable offspring, when mated with ICR strain mouse. OKB6-II \leftrightarrow ICR hermaphrodite was sterile, and had a contralateral ovary and testis-like structure.

Key words: Embryonic stem (ES) cell lines, C57BL/6 strain mouse, Whole blastocyst culture method, Chimera, Hermaphrodite.

ES cell lines were firstly established from early mouse embryos by Evans and Kaufman [4], and Martin [12] in 1981. ES cell lines are similar to inner cell mass (ICM) cells; they maintain undifferentiated morphology, and exhibit pluripotency and normal karyotype. Because of these characteristics, ES cells have been used for approaches as gene targeting [9].

To date, ES cell lines have been established from various strains of mice [1, 2, 4, 6, 10, 11, 14, 16, 22], and attempts have been made to isolate from other species [3, 5, 7, 8, 13, 15, 19, 20, 21]. For animal species other than mouse, however, there are few reports that ES cell lines which are able to produce chimeras are established. Even in the mouse, efficiency

of establishment of ES cell lines differed from strain to strain, for example, ES cell lines which were derived from 129 strain mice were numerous [1, 2, 4, 6, 14, 16, 22], but strains such as C57BL/6 [2, 10, 11] or BALB/c [10] were limited. Azuma and Toyoda [1] attempted to establish ES cell lines derived from 129 and C57BL/6 strain mice, and obtained ES cell lines from 129 strain mice, but no lines from C57BL/6 strain mice. 129 strain is characterized by a high incidence of spontaneous testicular teratomas or teratocarcinomas. This characteristic may be favorable for establishment of ES cell lines. Kawase *et al.* [10] reported establishment of ES cell lines derived from BALB/c strain mice, but germ line chimeras were not obtained. There are no other reports of the establishment of ES cell lines derived from this strain, and it seems that BALB/c strain is one of the most difficult strains to establish ES cell lines, but the establishment of ES cell lines from many strains of mice would be useful for various practical applications. We therefore attempted to establish new ES cell lines derived from C57BL/6 strain mice by the whole blastocyst culture method. The cell lines obtained were confirmed *in vitro*, and it was attempted to produce chimeras by coculture method [18, 23, 24].

Materials and Methods

Embryos

C57BL/6 strain mice females were superovulated with 5–7.5 IU of pregnant mare serum gonadotropin followed 46–48 hr later by 5–7.5 IU of human chorionic gonadotropin and were mated with males of the same strain. Embryos were recovered by flushing of uteri with modified Brinster's BMOC-III medium (PLG) on 3.0 days post coitum (dpc). These embryos which were morulae or compacted morulae were cultured in microdrops of PLG

Received: April 15, 1996

Accepted: August 15, 1996

*To whom correspondence should be addressed.

supplemented with 0.5% bovine serum albumin (BSA), for about 48 hr, at 37°C, 5% CO₂ in air.

Preparation of feeder layers

Primary embryonic fibroblasts derived from BALB/c fetuses at 14–16 days in gestation were isolated by the method described by Robertson [17] for use as feeder cells. These fibroblasts were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 5–10% fetal bovine serum (FBS), and they were used as feeder layers within 5 passages. The feeder layers were prepared by standard procedure including treatment with mitomycin C. These feeder layers were used within 7 days.

Establishment and culture of ES cell lines

All procedures for establishment of ES cell lines were based on the method described by Robertson [17].

The embryos which were developed into blastocysts by culture for about 48 hr, were placed on feeder layers in 24 well plate containing 1 ml of ESM (high glucose DMEM supplemented with 20% FBS, 2-mercaptoethanol stock, non-essential amino acids solution and nucleosides stock; see [17] in detail). They were cultured for 5 days until outgrowth of ICMs were suitable to disaggregation. On day 5, ICM clumps which showed good outgrowth were picked up, trypsinized and disaggregated. After disaggregation, they were transferred to 24 well feeder plates. On day 4, ES-like colonies were selected by morphology. They were cultured for further 3 days to observe on their morphology and growth. On day 7 after ICM disaggregation, ES-like colonies were disaggregated by same procedure as that for ICM clumps. After that, wells which contained some ES-like colonies were passaged gradually to larger feeder wells every 3–4 days, and the medium was changed every day. Finally, they were maintained 60 mm feeder dishes. They were passaged every 3–4 days, and the medium was changed every day. They were frozen and thawed, if necessary. After several passages, serum concentration of ESM was decreased into 10%.

Confirmation of ES cell lines in vitro

(i) *Formation of embryoid bodies:* ES-like cell lines were passaged at high density in gelatin-treated 60 mm tissue culture dishes, and cultured for 2–3 days to remove feeder cells. The cell lines were trypsinized for a short time. The dishes were gently rocked until clumps of cells were detached. Sufficient ESM was added to neutralize trypsin. Clumps of cells were transferred to 100 mm non-treated dishes. They were then cultured

for about 10 days, and the medium was changed, if necessary.

(ii) *Alkaline phosphatase activity:* The alkaline phosphatase (Al-p) activity test were carried out by azo dyeing method which used naphthol AS-MX phosphate as the substrate and fast blue RR salt as azo dye. The feeder cells were removed by the same procedure as in the embryoid bodies formation test. The dishes were then fixed with 10% formalin calcium (4°C, 15–20 min). After treatment, the dishes were washed 5–6 times with distilled water, and a reactive solution (0.05 M Tris buffer solution supplemented with substrate and azo dye) was added. The dishes were left at room temperature for 20–30 min and then, they were washed thoroughly with water. After air drying, they were observed by light microscope.

(iii) *Karyotype analysis:* After the feeder cells were removed by the same procedure as embryoid body formation test, ES-like cells were treated by ESM supplemented with 0.05 µg/ml demecolcine for about 1 hr. ES-like cells were then collected by trypsinization. Approximately 1 ml of hypotonic solution (0.56% KCl) were added and cells were resuspended. A suitable amount of hypotonic solution were added, and treated 10–15 min at room temperature. After hypotonic treatment, they were centrifuged (500 rpm, 5 min), and the supernatant was removed. A suitable quantity of ice-cold fixative (absolute methanol 3: acetic acid 1) were dripped onto then while flicking the tube. After pipetting, they were treated for 5 min at room temperature. In the same way, the fixative was changed 3 times, and finally, 1 ml of suspension were prepared. Slides which had been cleaned by soaking in 75% ethanol were prepared, and a drop of suspension was released onto slides. After air drying, the slides were stained by immersing for 13–15 min in 3% Giemsa staining solution. The slides were washed, air dried, and used for analysis.

Production of chimeras

ICR embryos from 8-cell to compact morula stage were recovered by flushing the oviducts and the uteri with PLG. After removing the zona pellucida with protease, the embryos were cultured in PLG supplemented with 0.5% BSA. Feeder cells were removed from ES-like cell line cultures by the same procedure as in the embryoid body formation test or the procedure described by Wood *et al.* [24]. ES-like cells were collected and suspended in coculture medium (mixture of ESM and PLG supplemented with 0.5% BSA at the same quantity as used in this study) to a concentration between 0.5×10^6 cells/ml and 1.0×10^6 cells/ml. The drops of ES-

like cell suspension were placed onto tissue culture dish, and covered with liquid paraffin. About 10 zona-free embryos were dispensed in each drop of ES-like cell suspension. They were cocultured for 3–4 hr at 37°C, 5% CO₂ in air. After coculture, embryos were washed and cultured in PLG supplemented with 0.5% BSA for 1–2 days. Embryos which developed to the blastocyst stage were transferred into uteri of ICR recipients on 2.5 days of pseudopregnancy. Chimerism of offspring were judged by coat color, and germ line transmission was examined by mating with ICR strain mice.

Results

Data of establishment of cell lines were shown in Table 1. Fifty one blastocysts were attached to feeder layers at a high ratio, and indicated expansion of trophoblasts and exposure of ICMs. On day 5, thirty-seven (72.5%) good outgrowths of ICM clumps were picked up and disaggregated. After disaggregation of ICM clumps, some colonies which had different morphology

were obtained. ES-like colonies were identified by their morphology on day 4, and further cultured for 3 days. Seven (13.7%) ES-like colonies which maintained good morphology until day 7 (Fig. 1) were disaggregated again. Identification of other differentiated colonies was not carried out. After that, two cell lines (OKB6-I and-II) were obtained, and the net ratio of isolation was 3.9%.

Two cell lines obtained were confirmed *in vitro*. In an embryoid body formation test, both cell lines showed a boundary of different cell types and formed simple embryoid bodies until day 3 (Fig. 2A). When culture was continued, they gave rise to cavities, and formed cystic embryoid bodies (Fig. 2B). After that, they increased diameter by enlarging cavities. In an Al-p activity test, both cell lines were stained blue (Fig. 3), indicating positive Al-p activity. And, in karyotype analysis, both cell lines had normal number of chromosomes (Fig. 4). Furthermore, judged by the morphology and size of chromosomes, it was suggested that OKB6-I was a female cell line and that OKB6-II was a male (Fig. 5).

By coculture with ICR zona-free embryos, one chi-

Table 1. Establishment of embryonic stem (ES) cell lines from blastocysts of C57BL/6 strain mice

No. of blastocysts cultured	Appearance of ICM clumps (%)	No. of ES-like colonies (%)	ES cell lines (%)
51	37 (72.5)	7 (13.7)	2 (3.9)

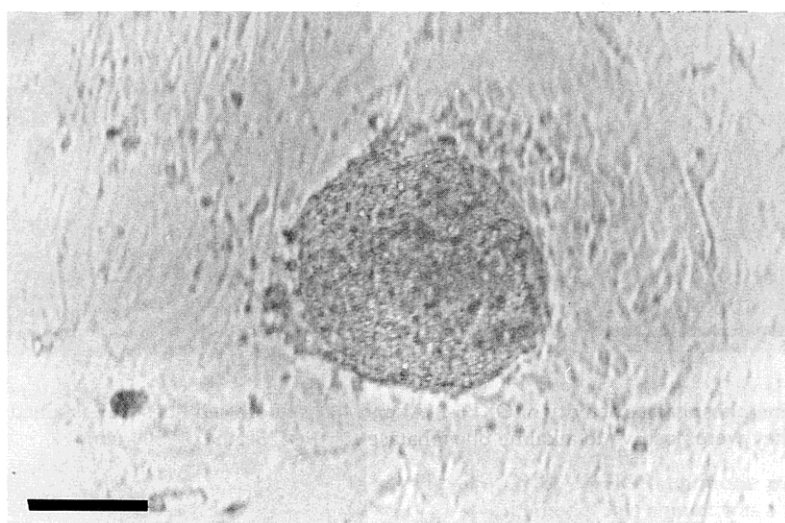


Fig. 1. The morphology of ES-like colony on day 7 after disaggregation of ICM clumps. Scale bar: 100 μ m.

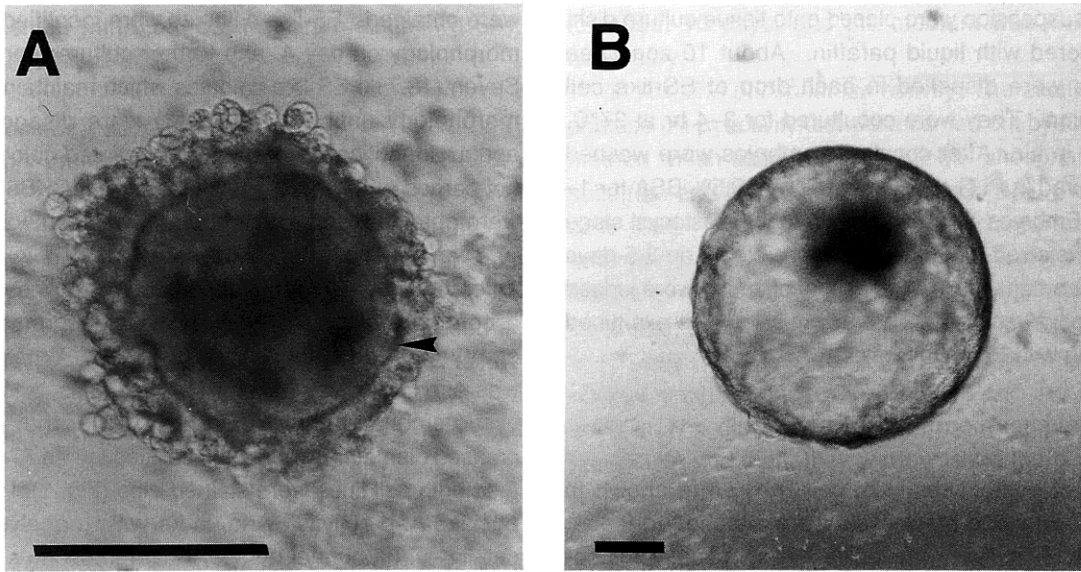


Fig. 2. Development of embryoid body derived from OKB6-I. Embryoid bodies derived from OKB6-II were similar to those derived from OKB6-I. (A) Embryoid body on day 3, which had a boundary of different cell types (an arrow). (B) Embryoid body on day 10, which showed a large cavity. Scale bar: 100 μm .

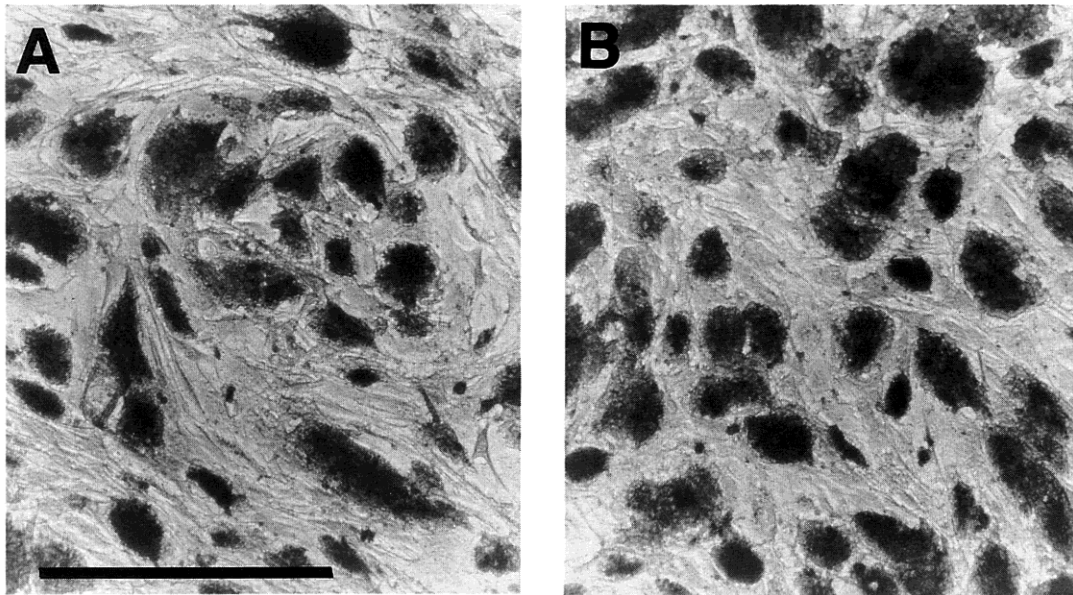


Fig. 3. Alkaline phosphatase activity of OKB6-I (A) and -II (B). Both cell lines were stained blue, indicating that they were positive to alkaline phosphatase activity. Scale bar: 500 μm .

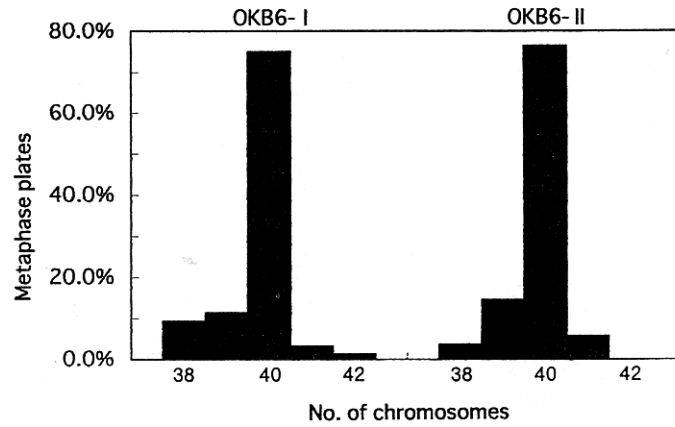


Fig. 4. Histograms which showed chromosome numbers of OKB6-I and -II. In both cell lines, more than 75% of cells had the normal number of chromosomes.

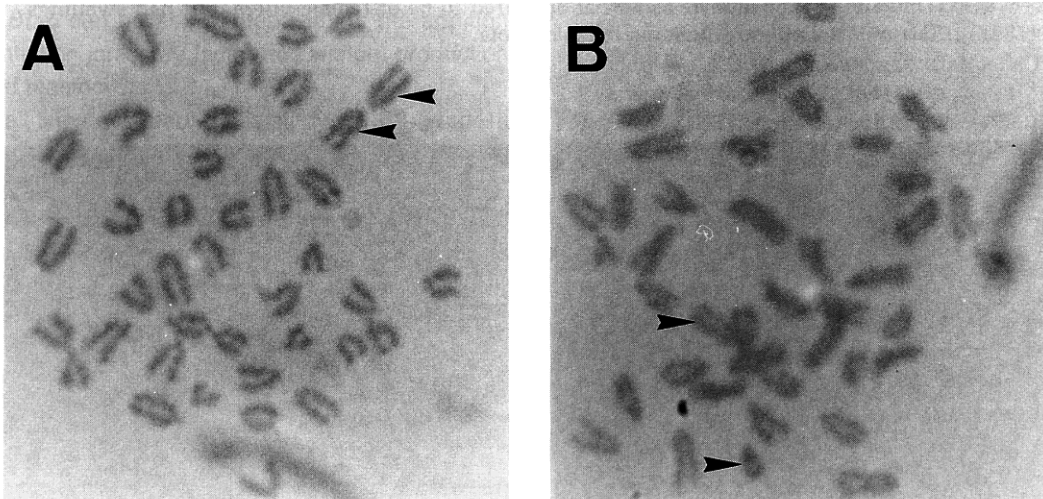


Fig. 5. Karyotype of OKB6-I (A) and -II (B). It was found that OKB6-I was a female cell line, and OKB6-II was a male, judged by size and morphology of chromosomes. Arrows: sex chromosomes.

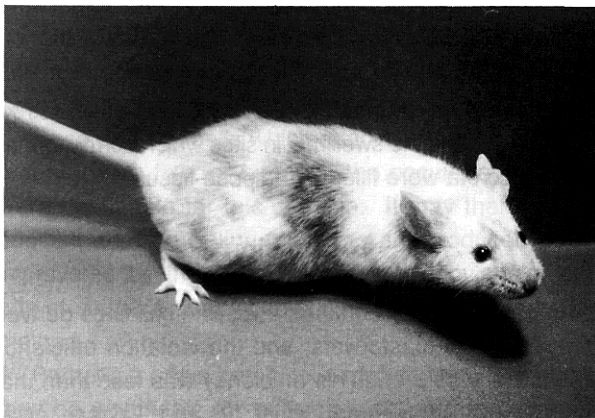


Fig. 6. OKB6-II ⇌ ICR chimera produced by coculture method. This animal was a female, and generated 3 albino type offspring by mated with ICR male. Germ line transmission of OKB6-II in this chimera would be a little, if any.

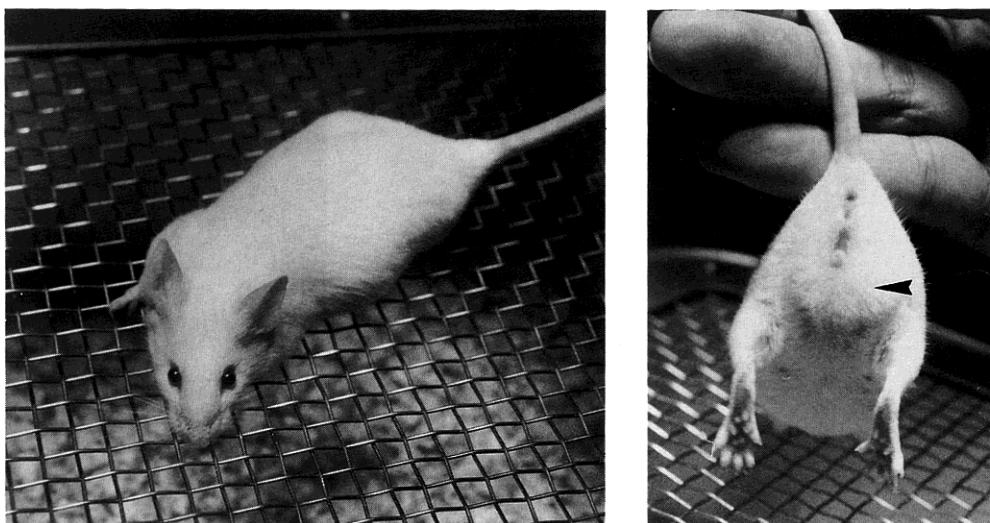


Fig. 7. Appearance of hermaphrodite produced by coculture between OKB6-II and ICR embryos. This albino type animal had what appeared to be of female vulva, but had swellings (arrow) in sites corresponding to male scrota.

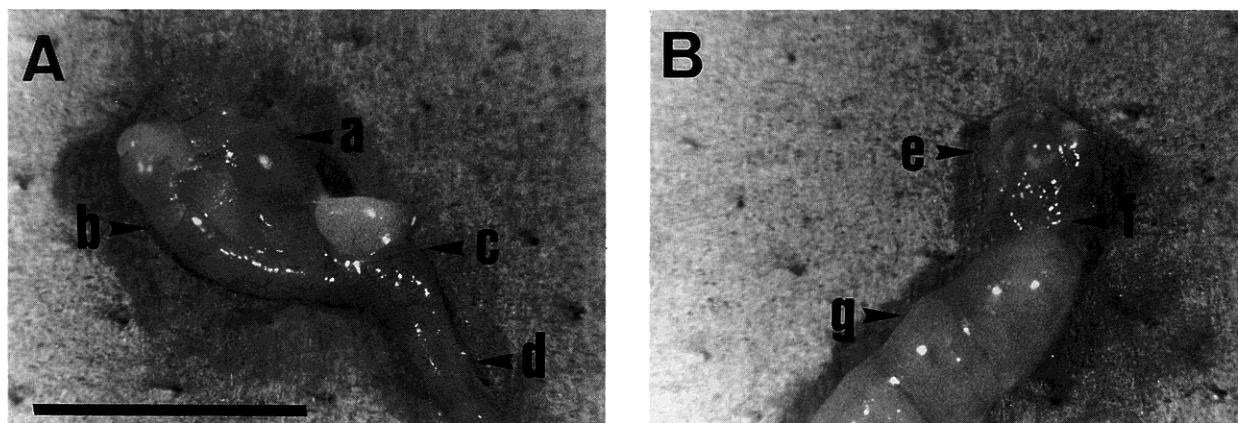


Fig. 8. Internal genital organs of hermaphrodite. The left organs (A) were abnormal; they were composed of testis-like (a), epididymis-like (b), oviduct-like (c) and uterus-like (d) structure. The right organs (B) were female type, showing the ovary (e), oviduct (f), and uterus (g). Scale bar: 10 mm.

mera obtained from OKB6-II (Fig. 6), but no chimera was obtained from OKB6-I. The chimera derived from OKB6-II was female, and had pigmented eyes. In a mating test, this chimera generated 3 albino type offspring. A mouse which showed abnormal sexual differentiation was also obtained from OKB6-II. It had an albino coat and the appearance of female vulva, but might induce hermaphroditism, since it had swellings in sites which corresponded to male scrota (Fig. 7). This animal failed to mate with either a female or a male.

This animal had a contralateral ovary and testis-like structure (Fig. 8), and the swellings in sites which corresponded to male scrota were filled by adipose tissue.

Discussion

In this study, we obtained 2 ES-like cell lines derived from C57BL/6 blastocysts, and the isolation efficiency was 3.9% (Table 1). This efficiency was less than that reported by Doetschman *et al.* [2] and Kawase *et al.*

[10], and similar to that reported by Ledermann and Bürki [11] when they used primary embryonic fibroblasts as feeder cells. But Azuma and Toyoda [1] attempted to establish ES cell lines from 129 and C57BL/6 strain, and reported that ES cell lines derived from 129 strain were able to be established, but not those from C57BL/6 strain. These differences in the isolation ratio may be caused, at least partially, by strain combinations between feeder cells and embryos for the establishment of ES cell lines. Embryonic fibroblasts derived from BALB/c may be suitable for establishment of ES cell lines derived from C57BL/6, but there were no reports of studies on the effects of strain combinations between feeder cells and embryos as materials for the establishment of ES cell lines. But the effects of strain combination may be small, since the establishment of ES cell lines is influenced by various conditions. In addition, our results confirmed that ES cell lines derived from C57BL/6 can be established without the addition of differentiation inhibitory factor and without production of delayed blastocysts.

Two cell lines obtained in this study, OKB6-I and -II, had normal karyotype and pluripotency *in vitro*. It was suggested that OKB6-I was a female cell line, and that OKB6-II was a male, judged by morphology and size of chromosomes. The nature of both cell lines *in vitro* was similar to that of existing ES cell lines reported.

The OKB6-II \leftrightarrow ICR chimera was female, and generated 3 albino type offspring in a mating test. Since the offspring were few, germ line transmission of OKB6-II in this chimera was not determined, but there would be a little, if any.

The hermaphrodite obtained by coculture between OKB6-II and ICR embryos failed to mate with either a male or a female, indicating that this animal was sterility. This animal had a contralateral ovary and testis-like structure (Fig. 7), and the swellings in sites which corresponded to male scrota were filled by adipose tissue. This indicated that the hermaphrodite had an XY component which probably derived from OKB6-II cell line, and it suggested that germ line of this animal might incompletely induced differentiation into testis and scrota, because OKB6-II cells which attached to zona-free ICR embryo were few, or because the coculture condition were unsuitable for this cell line. It may therefore be possible that germ line chimeras are produced by an improvement in the coculture conditions or by other methods of chimera production such as blastocyst injection method.

Perhaps a strain combination for chimera production may be unsuitable for our cell lines, or the method of

chimera production may be unsuitable for them, because only a few chimeras were obtained. It is therefore necessary to apply other methods of chimera production, and to determine the differentiation ability and the germ line transmission of both cell lines.

References

- 1) Azuma, S. and Toyoda, Y. (1991): Production of a germ-line chimeric mouse derived from newly established embryonic stem cells. *Jpn. J. Anim. Reprod.*, 37, 37–43.
- 2) Doetschman, T.C., Eistetter, H., Katz, M., Schmidt, W. and Kemler, R. (1985): The *in vitro* development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. exp. Morph.*, 87, 27–45.
- 3) Doetschman, T., Williams, P. and Maeda, N. (1988): Establishment of hamster blastocyst-derived embryonic stem (ES) cells. *Dev. Biol.*, 127, 224–227.
- 4) Evans, M.J. and Kaufman, M.H. (1981): Establishment in culture of pluripotential cells from mouse embryos. *Nature*, 292, 154–156.
- 5) Graves, K.H. and Moreadith, R.W. (1993): Derivation and characterization of putative pluripotential embryonic stem cells from preimplantation rabbit embryos. *Mol. Reprod. Dev.*, 36, 424–433.
- 6) Handyside, A.H., O'neil, G.T., Jones, M. and Hooper, M.L. (1989): Use of BRL-conditioned medium in combination with feeder layers to isolate a diploid embryonic stem cell line. *Roux's Arch. Dev. Biol.*, 198, 48–55.
- 7) Iannaccone, P.M., Taborn, G.U., Garton, R.L., Caplice, M.D. and Breni, D.R. (1994): Pluripotent embryonic stem cells from the rat are capable of producing chimeras. *Dev. Biol.*, 163, 288–292.
- 8) Inagaki, H., Ryoo, Z.Y., and Sugawara, S. (1993): An establishment of embryonic stem cells from bovine embryos fertilized *in vitro*. *J. Mamm. Ova Res.*, 10, 50–55.
- 9) Joyner, A.L. (1991): Gene targeting and gene trap screens using embryonic stem cells: new approaches to mammalian development. *Bioessays*, 13, 649–656.
- 10) Kawase, E., Suemori, H., Takahashi, N., Okazaki, K., Hashimoto, K. and Nakatsuji, N. (1994): Strain difference in establishment of mouse embryonic stem (ES) cell lines. *Int. J. Dev. Biol.*, 38, 385–390.
- 11) Ledermann, B. and Bürki, K. (1991): Establishment of germ-line competent C57BL/6 embryonic stem cell line. *Exp. Cell Res.*, 197, 254–258.
- 12) Martin, G.R. (1981): Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA*, 78, 7634–7638.
- 13) Masaki, T., Ryoo, Z.Y., Sugawara, S. and Masaki, J. (1991): Isolation of the colony derived from bovine embryo *in vitro* fertilization. *J. Mamm. Ova Res.*, 8,

- 27–33.
- 14) Nichols, J., Evans, E.P. and Smith, A.G. (1990): Establishment of germ-line-competent embryonic stem (ES) cells using differentiation inhibiting activity. *Development*, 110, 1341–1348.
 - 15) Notarianni, E., Laurie, S., Moor, R.M. and Evans, M.J. (1990): Maintenance and differentiation in culture of pluripotential embryonic cell lines from pig blastocysts. *J. Reprod. Fertil., Suppl.*, 41, 51–56.
 - 16) Pease, S., Braghetta, P., Gearing, D., Grail, D. and Williams, R.L. (1990): Isolation of embryonic stem (ES) cells in media supplemented with recombinant leukemia inhibitory factor (LIF). *Dev. Biol.*, 141, 344–352.
 - 17) Robertson, E.J. (1987): Embryo-derived stem cell lines. In: *Teratocarcinomas and Embryonic Stem Cells; A Practical Approach* (Robertson, E.J. ed.), pp. 71–112, IRL press, Oxford.
 - 18) Sugino, T., Azuma, S., Sato, E. and Toyoda, Y. (1993): Production of chimeric mice by coculture of embryonic stem cells and zona-free embryos. *J. Mamm. Ova Res.*, 10, 216–221.
 - 19) Sukoyan, M.A., Golubitsa, A.N., Zhelezova, A.I., Shilov, A.G., Vatolin, S.Y., Maximovsky, L.P., Andreeva, L.E., McWhir, J., Pack, S.D., Bayborodin, S.I., *et al.* (1992): Isolation and cultivation of blastocyst-derived stem cell lines from American mink (*Mustela vison*). *Mol. Reprod. Dev.*, 33, 418–431.
 - 20) Takahashi, Y., Takahashi, A., Matsumoto, K., Nakagata, N., Anzai, M. and Miyata, K. (1994): Establishment of pluripotential cell lines from inner cell mass of rat blastocysts. *Anim. Sci. Technol.*, 65, 826–833.
 - 21) Thomson, J.A., Kalishman, J., Golos, T.G., Durning, M., Harris, C.P., Becker, R.A. and Hearn, J.P. (1995): Isolation of a primate embryonic stem cell line. *Proc. Natl. Acad. Sci. USA*, 92, 7844–7848.
 - 22) Tokunaga, T. and Tsunoda, Y. (1989): Establishment of the mouse embryonic stem cell lines from whole blastocysts and isolated inner cell masses. *Jpn. J. Anim. Reprod.*, 35, 173–178.
 - 23) Wood, S.A., Allen, N.D., Rossant, J., Auerbach, A. and Nagy, A. (1993): Noninjection methods for the production of embryonic stem cell-embryo chimaeras. *Nature*, 365, 87–89.
 - 24) 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.