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Strain difference in establishment of mouse embryonic stem (ES) cell lines

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ABSTRACT For utilization of the mouse embryonic stem (ES) cells for various purposes, it is desirable that the cell lines are established from various sources such as inbred and mutant mouse strains. So far, however, most ES cell lines used in genetic manipulation have been derived from the 129/Sv strain. We have established ES cell lines from blastocysts of the C57BL/6 strain at high efficiency by use of a fibroblast cell line SL10 as feeder cells, and supplementing the culture medium with 5,000 unit/ml LIF. Using such culture conditions, we have also established a number of ES cell lines from the BALB/c, BXSB/MpJ-*Yaa* and MRL/Mp-*lpr/lpr* mouse strains. Procedure for establishment of ES cell lines had to be modified among the mouse strains, indicating the strain difference. For example, only a few stem cell colonies appeared from the BALB/c blastocysts. Stem cells of the BXSB strain were very sensitive to the trypsinization in subculturing. Most of these cell lines had normal karyotype and produced chimeric mice. Several C57BL/6 cell lines contributed to the germ-line. These results indicate that ES cell lines can be established from various mouse strains, but their characteristics are different among strains.

KEY WORDS: *ES cell line, mouse strains, feeder cells, auto-immune disease model*

Introduction

ES cell lines maintain characteristics of pluripotential stem cells, such as those of the inner cell mass (ICM), in early embryos. They can differentiate into a variety of cell types (Beddington and Robertson, 1989; Suemori *et al.*, 1990) including germ cells (Gossler *et al.*, 1986; Robertson *et al.*, 1986; Hooper *et al.*, 1987). Recently, this unique nature of the ES cells has been utilized in gene targeting experiments to analyze the function of many genes, including c-*abl* (Schwartzberg *et al.*, 1989), *int*-1 (McMahon and Bradley, 1990), IGF-2 (DeChiara *et al.*, 1990) and *Hox-1.5* (Chisaka and Capecchi, 1991).

ES cell lines so far used in such studies include CCE (Robertson *et al.*, 1986), D3 (Gossler *et al.*, 1986), E14 (Hooper *et al.*, 1987), AB1 (McMahon and Bradley, 1990), MBL-5 (Pease *et al.*, 1990), EFC-3 (Nichols *et al.*, 1990), BL/6-3 (Ledermann and Bürki, 1991), J1 (Li *et al.*, 1992), PJ5 (Johnson *et al.*, 1992), F1/1 (Tokunaga and Tsunoda, 1992) and TT2 (Saga *et al.*, 1992). Most of these cell lines are derived from the 129/Sv strain. The only exceptions are F1/1 and TT2 lines from the hybrid between males of the CBA strain and females of the C57BL/6 strain, and BL/6-3 line from the C57BL/6 strain.

129/Sv strain is characterized by a high incidence of spontaneous testicular teratomas or teratocarcinomas, and has served as the source of embryonic carcinoma cell lines (Stevens, 1970). This strain, however, has not been used widely for immunological or oncological studies. Genetic background is very important in various studies using experimental animals. For example, the phenotype of null mutation mice differs sometimes depending on genetic background (Donehower *et al.*, 1992; Ramíres-Solis *et al.*, 1993). Therefore, the establishment of mouse ES cell lines from various inbred and mutant mouse strains is desirable for wider utilization of ES cells. On the other hand, there is a possibility that the 129/Sv strain has unique characteristics, not found in other strains, permitting establishment of stable ES cell lines.

Abbreviations used in this paper: BXSB, BXSB/MpJ-*Yaa;* DMEM, Dulbecco's modified Eagle's medium; dpc, days post coitum; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; ES cells, embryonic stem cells; ESM, ES cell medium; FCS, fetal calf serum; HCG, human chorionic gonadotropin; ICM, inner cell mass; LIF, leukemia inhibitory factor; MRL, MRL/Mp-*lpr/lpr*; PBS, Dulbecco's modified phosphate buffered saline; PMSG, pregnant mare's serum gonadotropin.

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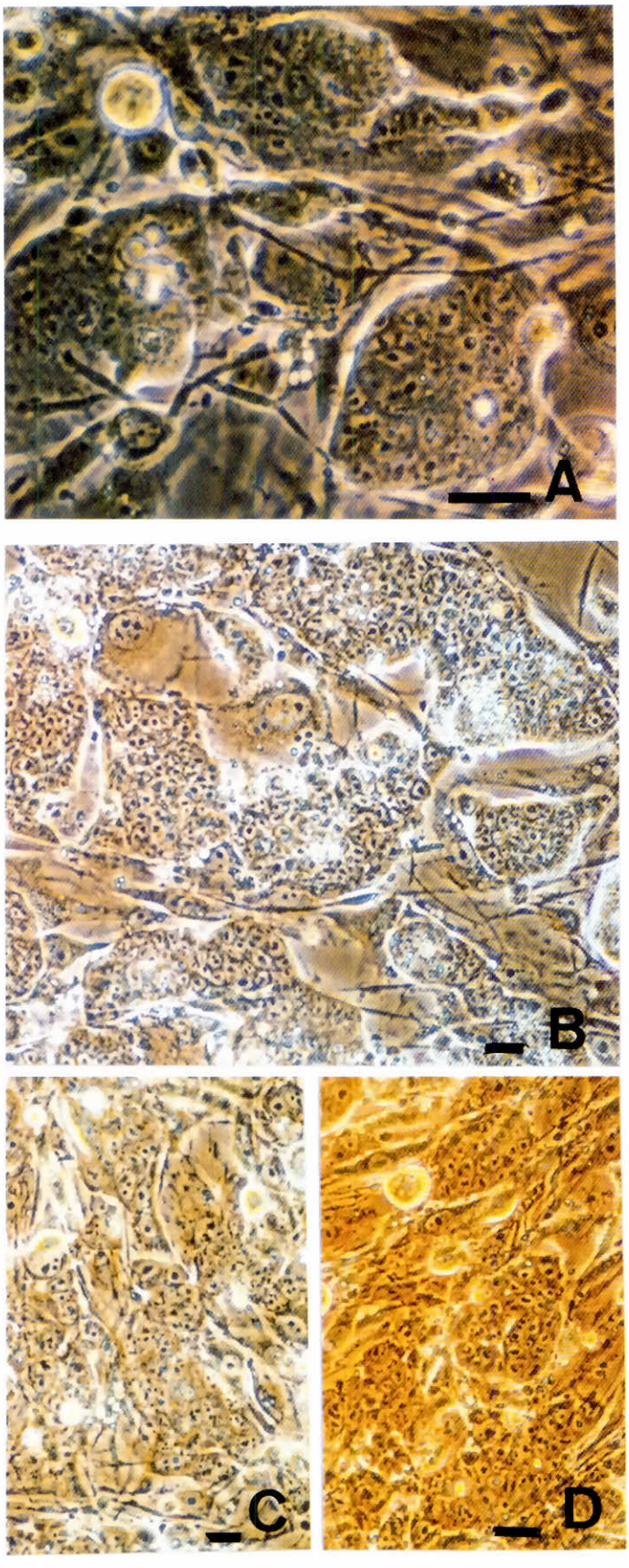


TABLE 1

EFFECTS OF SL10 AND LIF IN ESTABLISHMENT OF ES CELL LINES FROM C57BL/6 STRAIN

Feeder cells	LIF	No. of ICMs used	No. of stem cell colonies appeared	No. of ES cell lines established
STO	(–)	20	0	0
STO	(+)	15	0	0
SL10	(–)	8	2	0
SL10	(+)	15	>12	12

LIF; final concentration was 5,000 unit/ml

In the present study, we tried to establish ES cell lines from several inbred and mutant mouse strains. First, we established many ES cell lines from the C57BL/6 strain. Most of these cell lines had normal karyotype, and some of them produced germ-line chimeras. Then, we used BALB/c, BXSB/MpJ-*Yaa* (Y chromosome-linked autoimmune acceleration) and MRL/Mp-*lpr/lpr* (lymphoproliferation) strains. BXSB and MRL are mutant strains used as models for auto-immune disease. MRL is also known as a unique strain with spontaneous arthritis (Murphy and Roths, 1978). Recently, *lpr* mutation was identified as a deletion of Fas antigen gene mediating apoptosis (Watanabe-Fukunaga *et al.*, 1992). The ES cell lines obtained in this study showed different characteristics during the establishment and maintenance, but most of them had normal karyotype and produced chimeric mice.

Results

Effects of feeder cells

We had previously established ES cell lines from the C57BL/6 strain (Suemori *et al.*, 1990) using a mouse embryonic fibroblast cell line STO as the feeder cells. We tried to obtain better feeder cells for the ES cell line establishment. We transfected STO cells with a vector containing bacterial neo^r gene. Many neo-resistant colonies were expanded and tested for suitableness as feeder cells in culture of an ES cell line CCE. SL10 subline was chosen as the best one to support undifferentiated stem cell colonies of CCE (data not shown). The effects of these feeder cells (STO or SL10) were examined on the basis of efficiency of establishment of C57BL/6 ES cell lines. Our results (Table 1) indicated that use of SL10 and addition of LIF (5,000 unit/ml) were better for ES cells. We therefore used these culture conditions in experiments using the BALB/c, BXSB and MRL strains.

ES cell lines from BALB/c, BXSB/MpJ-Yaa and MRL/Mp-lpr/lpr strains

Table 2 shows results in establishment of ES cell lines from the BALB/c, BXSB and MRL strains. For example, only 18 stem cell colonies appeared from 204 ICMs of BALB/c, indicating a very low efficiency compared to other strains. On the other hand, many stem

Fig.1. Phase contrast photographs *showing* **(A)** *MS12 ES cells from C57BL/6 strain,* **(B)** *ALK2 ES cells from BALB/c,* **(C)** *ES cells from BXSB/MpJ-Yaa, and* **(D)** *LPR4 ES cells from MRL/Mp-lpr/lpr. Scale bars indicate 30 μm.*

TABLE 2

ESTABLISHMENT OF ES CELL LINES FROM THREE MOUSE STRAINS

Mouse strain	No. of ICMs used	No. of stem cell colonies appeared	No. of ES cell lines established
BALB/c	204	18	5
BXSB/MpJ-*Yaa*	40	>>40	1
MRL/Mp-*lpr/lpr*	45	6	0
MRL/Mp-*lpr/lpr**	17	>17	14

ICMs were isolated by immunosurgery of overnight-cultured blastocysts, or 6.5 dpc delayed blastocysts (*).

cell colonies appeared from BXSB ICMs, but they were very difficult to maintain and expand as cell lines. They were very sensitive to trypsin treatment during subculturing. From the MRL ICMs, stem cell colonies appeared but did not expand to the cell lines. Use of the delayed blastocysts, however, caused very efficient establishment of ES cell lines. The morphology of these ES cell lines is shown in Fig. 1.

Karyotype analysis of ES cell lines

We made chromosome preparation, and examined the karyotype of established ES cell lines (Tables 3A-C). All C57BL/6 ES cell lines examined were male (XY), and in most of them, the chromosome number was normal (Table 3A). ES cell lines established from BALB/c (Table 3B) or MRL (Table 3C) strain showed similar characteristics, except that few cell lines had lower normality of the chromosome, and one line was XO. The BXSB ES cell line had abnormal chromosome number (data not shown).

Production of chimeric mice

Using the ES cell lines with normal karyotype, we tested their pluripotency by making chimeric mice with the blastocyst injection method. First, we used the C57BL/6 ES cell lines. We injected MS8 cells into ICR blastocysts, and MS9, MS11 and MS12 cells into BALB/c blastocysts. All of the 4 ES cell lines produced overt chimeras (Table 4A). Some of them showed a relatively high contribution to the coat color (Fig. 2A).

Then, we injected the BALB/c ES cells into C57BL/6 blastocysts. Three of 4 lines tested produced chimeric mice (Table 4B). Most chimeras, however, showed low contribution to the coat color (Fig. 2B). We also injected the MRL ES cells into C57BL/6 blastocysts. All 4 cell lines produced chimeric mice (Fig. 2C). Their coat color contribution was higher than the BALB/c ES cells, but lower than the C57BL/6 ES cells.

Chimeric males were tested for the germ-line chimerism. Three (MS 8, MS11 and MS12) out of the 4 tested C57BL/6 ES cell lines were shown to contribute to the germ-line (Table 5). BALB/c and MRL ES cell chimeras did not produce the ES-cell derived offspring.

Discussion

Our present study showed that the efficiency of the establishment of ES cell lines was different among mouse strains. We could easily establish many ES cell lines from the C57BL/6 strain, but it was difficult in the case of the BALB/c strain. BXSB ICMs produced many stem cell colonies, but it was very difficult to expand into ES cell lines. On the other hand, we could establish MRL ES cell lines from implantation-delayed blastocysts, but not from the usual blastocysts. These results indicate the presence of significant strain differences in the ES cells.

Karyotyping of the established ES cell lines showed that they were male in most cases. One possible reason is that XY cell lines are relatively stable, while XX lines are unstable and have a tendency to become XO. The production of chimeric mice in our study indicated that the efficiency of chimera production and proportion of chimerism in coat color are generally correlated with the ease of ES cell line establishment among various mouse strains.

Three C57BL/6 ES cell lines contributed to the germ-line. The rates of the ES cell contribution in chimeric mice and germ-line transmission depend on the combination of strains of the ES cells and host embryos. For example, 129/Sv ES cell lines produce germ-line chimeras efficiently when combined with C57BL/6 host

TABLE 3A

KARYOTYPE OF ES CELL LINES FROM C57BL/6 STRAIN

Cell line	Karyotype	Modal No. (%)	Chromosome counts				
			38	39	40	41	42
MS8	XY	40 (64%)	3	4	16	2	0
MS9	XY	40 (82%)	3	5	41	1	0
MS11	XY	40 (76%)	0	2	19	3	1
MS12	XY	40 (84%)	3	0	21	1	0

TABLE 3B

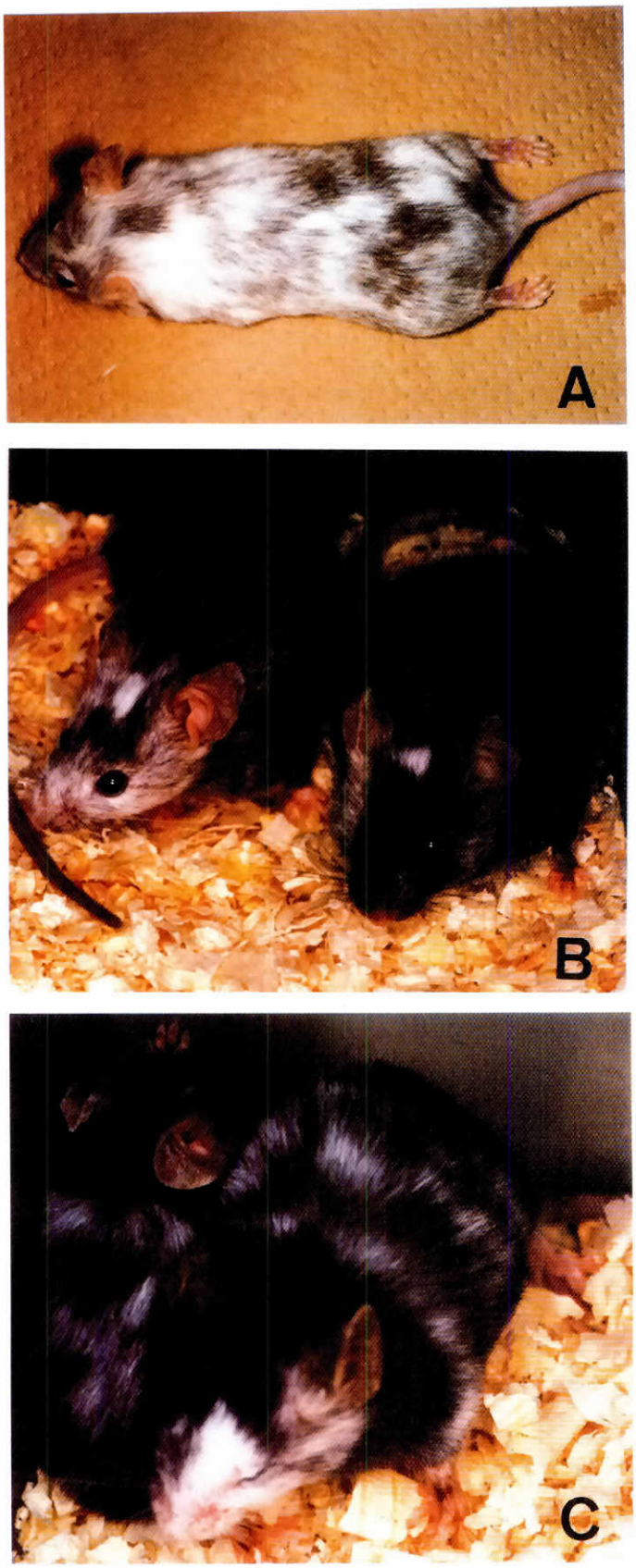
KARYOTYPE OF ES CELL LINES FROM BALB/C STRAIN

Cell line	Karyotype	Modal No. (%)	Chromosome counts				
			38	39	40	41	42
ALK1	XY	40 (67%)	4	5	74	23	5
ALK2	XY	40 (75%)	4	8	98	14	6
ALK3	XY	40 (69%)	2	2	36	9	3
ALK4	XY	40 (81%)	0	5	51	5	2
ALK5	?	40 (45%)	5	18	20	1	0

TABLE 3C

KARYOTYPE OF ES CELL LINES FROM MRL/MP-*LPR/LPR* STRAIN

Cell line	Karyotype	Modal No. (%)	Chromosome counts				
			38	39	40	41	42
LPR1	XY	40 (60%)	5	13	36	6	0
LPR2	XY	40 (79%)	3	5	79	13	0
LPR3	XY	40 (79%)	5	5	76	5	5
LPR4	XY	40 (81%)	5	8	77	3	2
LPR9	XY	40 (87%)	4	0	41	2	0
LPR11	XY	40 (43%)	13	35	69	34	9
LPR12	XO	39 (49%)	12	27	15	1	0



blastocysts, but not with CD-1 or MF-1 blastocysts (Schwartzberg *et al.*, 1989). In the case of the C57BL/6 ES cells, BALB/c blastocysts seem to be better hosts than those of 129/Sv, C3H or ICR (Ledermann and Bürki, 1991; our unpublished data). In addition, we obtained the germ-line chimeras of the C57BL/6 ES cells using ICR or A/J host blastocysts, but efficiency was lower than the BALB/c host.

Recent reports (Lallemand and Brûlet, 1990; Nagy *et al.*, 1990; Tokunaga and Tsunoda, 1992) have suggested that chimera production by the aggregation/injection method using 8-cell stage host embryos gave a higher ES cell contribution in chimeras. We tried to produce chimeric mice with our ES cell lines and the CCE line by using this method, but we did not obtain a higher contribution than the blastocyst injection method, and the birth rate was lower. Thus, the effectiveness of the 8-cell stage aggregation/injection method may be different among the ES cell lines and mouse strains.

The SL10 fibroblast subline, which was derived from the STO cell line, produced more effective feeder cells in the establishment and maintenance of ES cells compared to the parental STO cells. SL10 cells showed a slower growth rate and produced more prominent extracellular matrix (ECM) in culture than STO cells. The effects of such ECM on ES cells are not clear. LIF inhibits differentiation of ES cells (Smith *et al.*, 1988; Williams *et al.*, 1988), and it is present as either a diffusible form (dLIF) or an ECM associated form (mLIF) (Rathjen *et al.*, 1990). These 2 forms of LIF seem to have different functions (Robertson *et al.*, 1993). Over-expression of the dLIF seems to cause no apparent abnormalities in the postimplantation embryos, but that of the mLIF caused abnormal proliferation of tissues and the absence of differentiated mesoderm in embryos (Conquet *et al.*, 1992). Thus, the mLIF is more effective on inhibition of ES cell differentiation. Therefore, ECM of SL10 cells might be related to a larger amount of the mLIF, or they might produce other factors affecting ES cells.

Materials and Methods

Preparation of feeder cells

STO and SL10 fibroblasts were maintained in DMEM (Gibco cat. no. 430-1600) supplemented with 10% FCS. Culture dishes were precoated with 0.1% (w/v) solution of gelatin (Swine skin, type 1, Sigma). The preparation of feeder layers has been described in detail elsewhere by Robertson (1987). Briefly, near-confluent layers of STO or SL10 cells were treated with mitomycin C (10 μg/ml, Wako) for 2-3 h and rinsed with Ca^{2+}-, Mg^{2+}-free PBS three times. These cells were trypsinized with 0.05% trypsin (Difco)-1 mM EDTA in PBS, and suspended in the culture medium. After being centrifuged, they were resuspended at a density of 2.5×10^5 cells/ml. Each well of 24-well plates was seeded with 0.5 ml of the cell suspension. For each well of 12-well plates or 35 mm culture dishes, 1 ml or 2 ml was used for the seeding.

Mice and embryos

C57BL/6 and BALB/c mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). BXSB and MRL mice were purchased from Jackson Laboratory. Mice were maintained on the 12-h light/12-h dark cycle with the dark cycle midpoint at midnight.

Fig 2. Chimeric mice produced with (A) MS12 (C57BL/6, germ-line chimera), (B) ALK4 (BALB/c), (C) LPR9 (MRL/Mp-*lpr/lpr*) ES cell lines. *C57BL/6 mice have black coat color and pigmented eyes. BALB/c and MRL/Mp-lpr/lpr mice have white coat color and unpigmented eyes.*

TABLE 4A

PRODUCTION OF CHIMERIC MICE WITH C57BL/6 ES CELL LINES

Cell line	No. of embryos transferred	No. of mice born (% of transferred)	No. of chimeras (% of mice born)	No. of male (% of overt chimeras)
MS8	22	15 (68%)	5 (33%)	4 (80%)
MS9	9	7 (78%)	3 (43%)	2 (67%)
MS11	24	17 (71%)	5 (29%)	3 (60%)
MS12	22	17 (78%)	2 (12%)	2 (100%)

MS8 cells were injected into ICR blastocysts. Other ES cells were injected into BALB/c blastocysts.

TABLE 4B

PRODUCTION OF CHIMERIC MICE WITH BALB/C ES CELL LINES

Cell line	No. of embryos transferred	No. of mice born (% of transferred)	No. of chimeras (% of mice born)	No. of male (% of overt chimeras)
ALK1	32	7 (22%)	1 (14%)	1 (100%)
ALK2	68	22 (32%)	5 (23%)	1 (20%)
ALK3	14	7 (50%)	0 (0%)	0
ALK4	37	7 (19%)	3 (43%)	0 (0%)

ES cells were injected into C57BL/6 blastocysts.

TABLE 4C

PRODUCTION OF CHIMERIC MICE WITH MRL/MP-*LPR/LPR* ES CELL LINES

Cell line	No. of embryos transferred	No. of mice born (% of transferred)	No. of chimeras (% of mice born)	No. of male (% of overt chimeras)
LPR2	104	23+4* (26%)	9 (39%)	2 (22%)
LPR3	86	20 (23%)	1 (5%)	1 (100%)
LPR4	150	33+5* (25%)	6 (18%)	4 (67%)
LPR9	42	15 (36%)	6 (40%)	3 (50%)

ES cells were injected into C57BL/6 blastocysts. *Mice born but died early before reliable inspection of coat color.

To obtain blastocysts, 4-8 females were induced to superovulate by an injection of 5-10 I.U. PMSG (Teikoku-zoki Co., Japan), followed 46-48 h later by an injection of 5-10 I.U. HCG (Teikoku-zoki Co., Japan), and mated. We used BXSB strain blastocysts from natural mating, because of the low response to superovulation. To obtain delayed blastocysts, pregnant females at 2.5 dpc were anesthetized, ovariectomized, and subcutaneously injected with 0.1 ml of 10 ng/ml Depo-Provera (Sigma) in olive oil. Late morulae and blastocysts were flushed out from uterine horns of 3.5 dpc pregnant females. Delayed blastocysts were isolated from 6.5 dpc pregnant females.

Embryos thus obtained were cultured overnight in ES cell medium (ESM) supplemented with 20% FCS and antibiotics. Delayed blastocysts were cultured for 2-3 h before use. ESM was DMEM supplemented with 1x non-essential amino acids (Gibco), nucleosides (30 nM adenosine, 30 nM guanosine, 30 nM cytidine, 30 nM uridine and 10 nM thymidine, Sigma). Hatched blastocysts, or those denuded with acid tyrode (pH 3) solution, were treated with rabbit anti-mouse antiserum for 1 h, rinsed with M16 medium three times, and treated with guinea pig complement for 30 min to lyse the trophectoderm (Hogan *et al.*, 1986). Such isolated ICMs were rinsed with M16 medium two times and seeded onto feeder cells in ESM supplemented with 20% FCS, 100 μM ß-mercaptoethanol and 5,000 unit/ml LIF (mouse recombinant, Amrad)

ES cell line establishment

ICMs grew vigorously in size for 2-4 days in culture. They consisted of the central mass of stem cells and peripheral primitive endoderm-like cells. They were picked up and transferred into droplets of 0.25% trypsin-0.5 mM EDTA solution for 3-5 min with a mouth-controlled micropipet. Then, they were transferred onto new feeder cells in 24-well culture plates, and dispersed with a narrow micropipet (inner diameter, 25-50 μm) into small aggregates of around 10 cells.

Stem cell colonies appeared by 4-7 days in most cases. When the colonies grew to 200-300 μm, they were dissociated again in a similar method used for the ICM disaggregation. These cells were transferred onto new feeder cells in 24-well plates, or in larger wells thereafter. We judged an ES cell line as established when it was expanded to the size of culturing in 35 mm dishes. Afterwards, such cell lines were maintained by subculturing them every 2-3 days. The culture medium was changed every day (most cell lines) or every 12 h (BALB/c lines).

Karyotype analysis of ES cell lines

ES cells were fed with the fresh medium at 3 h before use, treated with 0.05 mg/ml colcemid for 1 h, and harvested after dissociation. Hypotonic 0.56% KCl solution was added to the pellet of ES cells, and resuspension was left for 13 min at 37°C to allow hypotonization. Then, they were added to the ice-cold fixative solution of absolute methanol-glacial acetic acid (3:1), and spread onto glass-slides. The G-binding was carried out as follows. Glass-slides with the chromosome preparation were kept for 1 week at room temperature, treated with 0.05% trypsin solution in PBS (pH 7.2) for 10 sec, then with 70% and 80% ethanol, and stained with 5% Giemsa solution (Merck) in Sörensen's phosphate buffered solution (pH 6.8) for 10 min.

Production of chimeric mice

ES cells were trypsinized and dissociated into single cells with pipetting in the culture medium. Such ES cell suspension was added to a gelatin-coated dish for 20 min to remove more adhesive feeder cells. Unattached ES cells were transferred to a bacterial petri dish, and incubated for 40 min to allow recovery from the trypsinization. Then, they were transferred into droplets of the injection medium (ESM supplemented with 20% FCS and 20 mM HEPES buffer, pH 8.0) and cooled to 4°C for 15 min. Expanded host blastocysts were injected with 10-20 ES cells at room temperature. ES cells could be kept for 2 h at 4°C and 30 min at room temperature. After a recovery period of 2-3 h in culture, re-expanded blastocysts were transferred into the uterine horns of pseudopregnant 2.5 dpc ICR foster mothers. Chimeric offspring was identified by coat color or eye pigmentation.

TABLE 5

BREEDING OF FERTILE MALE CHIMERAS

Cell line	Male chimera	Coat color chimerism	ES cell-derived progeny/progeny sired
MS8	8-1	40%	1/70
	8-2	20%	3/58
MS11	11-1		0/97
	11-2	70%	3/113
	11-3	30%	12/78
MS12	12-1	20%	3/64
	12-2	40%	3/3
ALK1	ALK1.1	<5%	0/94
ALK2	ALK2.1	<5%	0/69
LPR4	LPR4/1	5%	0/67
LPR9	LPR9/1	30%	0/46
	LPR9/2	10%	0/27

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