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Current Status of Chromosomal Abnormalities in Mouse Embryonic Stem Cell Lines Used in Japan

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We performed chromosomal analysis on 540 mouse embryonic stem (ES) cell lines obtained during 2001 to 2004 from 20 institutions in Japan. Overall, 66.5% of the ES cell lines showed normal chromosomal numbers, but 15.9%, 9.1%, and 2.8% showed modal chromosomal numbers of 41, 42, and 39, respectively. When we karyotyped 88 ES cell lines selected arbitrarily from the 540 lines, 53 (60.2%) showed normal diploid karyotypes; the sex chromosome constitution of 52 lines was XY, with the remaining 1 being XX. Among 35 ES cell lines showing abnormal karyotypes, trisomy of chromosome 8 (41, XY, +8) was dominant (51.4%), 14.3% had trisomy 8 with loss of one sex chromosome (40, XO, +8), and 11.4% had trisomy 8 together with trisomy 11 (42, XY, +8, +11). Karyotypic abnormalities including trisomy 8 and trisomy 11 occurred in 88.6% and 17.1% of ES cell lines, respectively. The XO sex chromosome constitution was observed in 25.7% of all abnormal ES cell lines. Of the 88 selected ES cell lines, 60 lines were established from strain 129 animals, 17 from F_1 progeny of C57BL/6J × CBA (called TT2 in this study), and 11 from C57BL/6J mice. Normal diploid karyotypes were observed in 58.3% of lines derived from 129, 58.8% of those from TT2, and 72.7% of C57BL/6J. The relatively high incidence of abnormalities in chromosomal number and karyotype in ES cell lines used in Japan suggests the importance of chromosomal analysis of ES cells for successful establishment of new animal models through germline transmission.

Abbreviations: ES, embryonic stem cell; PCR, polymerase chain reaction

Mouse embryonic stem (ES) cells are totipotent cells that are derived from the inner cell mass of a preimplantation mouse embryo.[1,11] Abnormalities of chromosomal number and karyotype gained during passage in culture are reported to influence the totipotent state and ability to contribute to the germ line.[16] However ES cell lines with approximately 40% normal karyotype and cystic embryoid body formation within 7 d after suspension culture were capable of germ line transmission in chimeric mice.[15] Mouse ES cell lines recently established from various mouse strains are now widely used for introducing targeted mutations and other genetic alterations into mouse germ lines. In this study, we examined the current status of the cytogenetic quality (chromosomal number and karyotype) of ES cell lines used in Japan as a means of quality assurance of ES cell lines for successful establishment of new animal models through germline transmission.

Materials and Methods

ES cell lines. We subjected 540 mouse ES cell lines obtained from 20 academic or industrial institutions during 2001 to 2004 in Japan to contracted chromosomal analyses as trust business.

Cell culture. ES cells were cultured in ES cell culture medium consisting of Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 20% fetal calf serum (Invitrogen), 2 mM L-glutamine (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 1.0 mM sodium pyruvate (Invitrogen), 55 μM 2-mercaptoethanol (Invitrogen), and 10^3 U/ml mouse leukemia inhibitory factor (Chemicon, Temecula, CA) on mitomycin C (Sigma, St. Louis, MO)-treated feeder layers derived from mouse fetal fibroblasts at 37 °C with 5% CO_2 in air. The culture medium was changed every day.

Chromosomal number analysis. ES cells at 2 to 3 d after passage were arrested in metaphase by addition of colcemid (Sigma; final concentration, 0.02 μg/ml) to the culture medium for 25 min. The cells were treated with a 0.25% trypsin–ethylenediaminetetraacetic acid solution (Invitrogen) for 3 min at room temperature. After vigorous pipetting, the single-cell suspension was centrifuged at 250 × *g* (1200 rpm) for 5 min, and the pellet was treated with 0.075 M KCl solution (Invitrogen) at room temperature. After 20 min, the hypotonic solution was mixed with a solution of methanol and acetic acid (3:1) with gentle pipetting. Then, the suspension was washed 3 times by centrifugation at 180 × *g* (1000 rpm) for 5 min prior to spreading the cells on slides. The chromosome slides were prepared by a routine air-drying method, kept overnight at room temperature, and stained with 5% Giemsa solution for 5 min. The chromosomal numbers of 50 well-spread metaphases were counted for all ES cell lines, except those for which 49, 46, or 34 metaphases were examined. The highest frequency of chromosomal number in an ES cell line was defined as its 'modal chromosomal number.' In this report, when differences between frequencies of 2 or 3 different chromosomal numbers were <10%, the lines were described as being bi-modal or tri-modal.

Karyotype analysis. A total of 88 ES cell lines selected arbitrarily from the 540 ES cell lines underwent karyotype analysis by using a modified method of the Q-banding technique;[13] these 88 lines comprised 60 derived from the 129 mouse strain, 17 from TT2, and 11 from C57BL/6J. The remaining 452 ES cell lines underwent analyses for chromosomal number only. The chromosome slides were rinsed with distilled water, stained with 0.01 μg/ml Hoechst 33258 for 5 min, and then stained with 5.0 μg/ml of quinacrine mustard for 20 min. Stained slides were

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mounted in a mount solution for observation by a Leica fluorescent microscopy (Leica Microsystems, Wetzlar, Germany). Karyotype analysis was performed on 3 to 10 metaphases in each ES cell line, and metaphases with the modal chromosomal number were analyzed preferentially.

Microbiological tests. All lines were tested for mouse hepatitis virus (MHV) and *Mycoplasma* spp. MHV status was determined by polymerase chain reaction (PCR) assay. The NP gene of the virus was reverse-transcribed into cDNA by using oligo (dT) primer (Pharmacia, USA) and was amplified by PCR as described previously.[4,7] Lines were assayed for *Mycoplasma* spp by a culture method with PPLO broth, for which the culture medium and growth condtions have been described previously.[3]

Results

Of 540 ES cell lines examined for chromosomal number, 359 (66.5%) showed a normal diploid chromosomal number of 40, but 86 (15.9%) had 41 chromosomes and 15 (2.8%) had 39 chromosomes (Table 1). Average frequencies of the cells with 39, 40 and 41 modal chromosomal numbers were >73.0%, and the highest frequency seen was for the 40 modal number (79.5%). ES cell lines with modal numbers of 42, 43, and 44 accounted for 49 (9.1%), 11 (2.1%), and 2 (0.4%) lines, respectively, and the average frequencies of cells with these modal chromosomal numbers were approximately 67%. In addition, 14 (2.6%) ES cell lines showed bi- or tri-modal chromosomal numbers, and the average frequencies of cells with these modal numbers were 22.0% to 38.5%. For the 4 (0.8%) ES cell lines whose modal number was in the tetraploid range (70, 80, and 81), the frequencies of cells with these modal chromosomal numbers were <55%.

The karyotypes of 88 selected ES cell lines are summarized in Table 2. Of the 88 cell lines, 52 (59.1%) showed a normal diploid chromosome component with XY, whereas only 1 (1.1%) had XX sex chromosomes with a normal karyotype. The remaining 35 (39.8%) ES cell lines showed abnormal karyotypes. Of these 35, 18 (51.4%) showed 41 chromosomes with XY including trisomy of chromosome 8 (trisomy 8; 41, XY, +8; Figure 1a). Trisomy 8 also was found with absence of 1 of the sex chromosomes (40, XO, +8) in 5 (14.3%) ES cell lines (Figure 1b). Double trisomy (trisomy 8 associated with trisomy 6, 11, or 14) was seen in 8 (22.9%) lines (Figures 1c and 1d). Trisomy 11 and 14 were seen in 1 (1.1%) ES cell line each. Another (1.1%) ES cell line showed 40 chromosomes with XO including a small metacentric chromosome (Table 2); the origin of this abnormal chromosome could not be identified, but it may be an isochromosome of Y. In addition, 1 ES cell line had 39 chromosomes with XO associated with no other abnormalities. Absence of 1 sex chromosome (XO) with or without other abnormalities (number and karyotype) was found in 9 (25.7%) ES cell lines. Pseudodiploidy, having the normal chromosomal number[40] but an abnormal karyotype, occurred in 7 (20.0%) ES cell lines.

We compared the karyotype patterns according to the mouse strain from which the lines derived (Table 3). Normal chromosome constitutions occurred in 58.3% (35 of 60) of the lines from the 129 strain, 58.8% (10 of 17) of those from TT2, and 72.7% (8 of 11) of those from C57BL/6J. Abnormal karyotypes including trisomy 8 were seen in 38.3% (23 of 60) of the lines from the 129 strain, 41.2% (7 of 17) of those from TT2, and 9.1% (1 of 11) of those from C57BL/6J. Abnormal sex chromosome constitutions (XO) occurred in 11.7% (7 of 60) of the lines from the 129 strain and 18.2% (2 of 11) of those from C57BL/6J but in none of those from TT2.

Table 1. Number (%) of mouse embryonic stem (ES) cell lines with different modal numbers of chromosomes and number (%) of cells with the various modal numbers among the 540 ES lines examined

Modal number	No. (%) of ES lines	No. of cells observed	No. (%) of cells with modal number
39	15 (2.8)	734	537 (73.1)
40	359 (66.5)	17949	14275 (79.5)
41	86 (15.9)	4296	3170 (73.9)
42	49 (9.1)	2450	1631 (66.6)
43	11(2.1)	530	359 (67.7)
44	2(0.4)	100	67 (67.0)
79	2(0.4)	100	54 (54.0)
80	1 (0.2)	50	26 (52.0)
81	1(0.2)	50	26 (52.0)
38/40	1 (0.2)	50	16 (32.0)/14 (28.0)
39/40	1 (0.2)	50	11 (22.0)/14 (28.0)
40/41	2 (0.4)	100	29 (29.0)/28 (28.0)
40/42	2 (0.4)	100	31 (31.0)/34 (34.0)
41/42	4 (0.7)	200	77 (38.5)/75 (37.5)
42/43	2 (0.4)	100	28 (28.0)/26 (26.0)
40/41/42	2 (0.4)	100	24 (24.0)/23 (23.0)/23 (23.0)
Total	540 (100.0)	26959	

Discussion

Our results indicate that abnormal modal chromosomal numbers occurred in 181 (33.5%) of the 540 ES cell lines evaluated, and 35 (39.8%) of the 88 ES cell lines analyzed showed abnormal karyotypes. The relationship between chromosomal abnormalities and passage conditions in ES cell lines is unclear, because we lack information about those conditions. Therefore, we cannot exclude the possibility that some of the ES cell lines with the same abnormal karyotypes are clonal derivatives.

Liu and coworkers reported a surprisingly high rate (75.9%, 22 of 29) of chromosome abnormalities in ES cell lines, primarily trisomy 8 (77.3%, 17 of 22).[8] It was reported that all 4 ES cell lines transfected with pENL and pSTneo plasmids showed trisomy 8, although the parental ES line had the normal 40 chromosomes.[14] In the present study, 31 (88.6%) of the 35 ES cell lines showing abnormal karyotypes included trisomy 8. This result confirms the previous finding that trisomy 8 is the leading contributor to abnormal karyotype patterns of ES cell lines.[8,14] Liu and coworkers mentioned that the ES cells carrying tisomy 8 had a higher growth rate than did normal cells, and this factor very likely contributed to the expansion of abnormal cell populations.[8] They also suggested that chromosome 8 and the syntenic human chromosomes 8 and 16 carry oncogenes such as Jun b and d, Lyl-1 and Ras 15-2, -6, and trisomy 8 or 16 in humans has been associated with acute lymphoblastic leukemia.[8]

The presence of an isochromosome 8, contributing to trisomy 8 in 4 of 17 ES cell lines showing abnormal karyotypes[8] is noteworthy. In our study, 2 ES cell lines with a modal number of 42, 1 with a modal number of 41, and 1 with a modal number of 43 showed abnormal karyotypes including isochromosomes of 8, resulting in trisomy or tetrasomy of chromosome 8. Because the chromosomal abnormalities in these 4 ES cell lines are very complicated and because the karyotype analyses have not yet been completed, these 4 ES cell lines were excluded from the present results of karyotype analyses of 88 ES cell lines. Detailed results of karyotype analyses of these ES cell lines including isochromosome 8 will be reported separately.

In the present study, 6 of 35 ES cell lines showing abnormal karyotypes included trisomy 11. Trisomy 11 previously was reported to occur in 3 of 22 ES cell lines showing abnormal karyo-

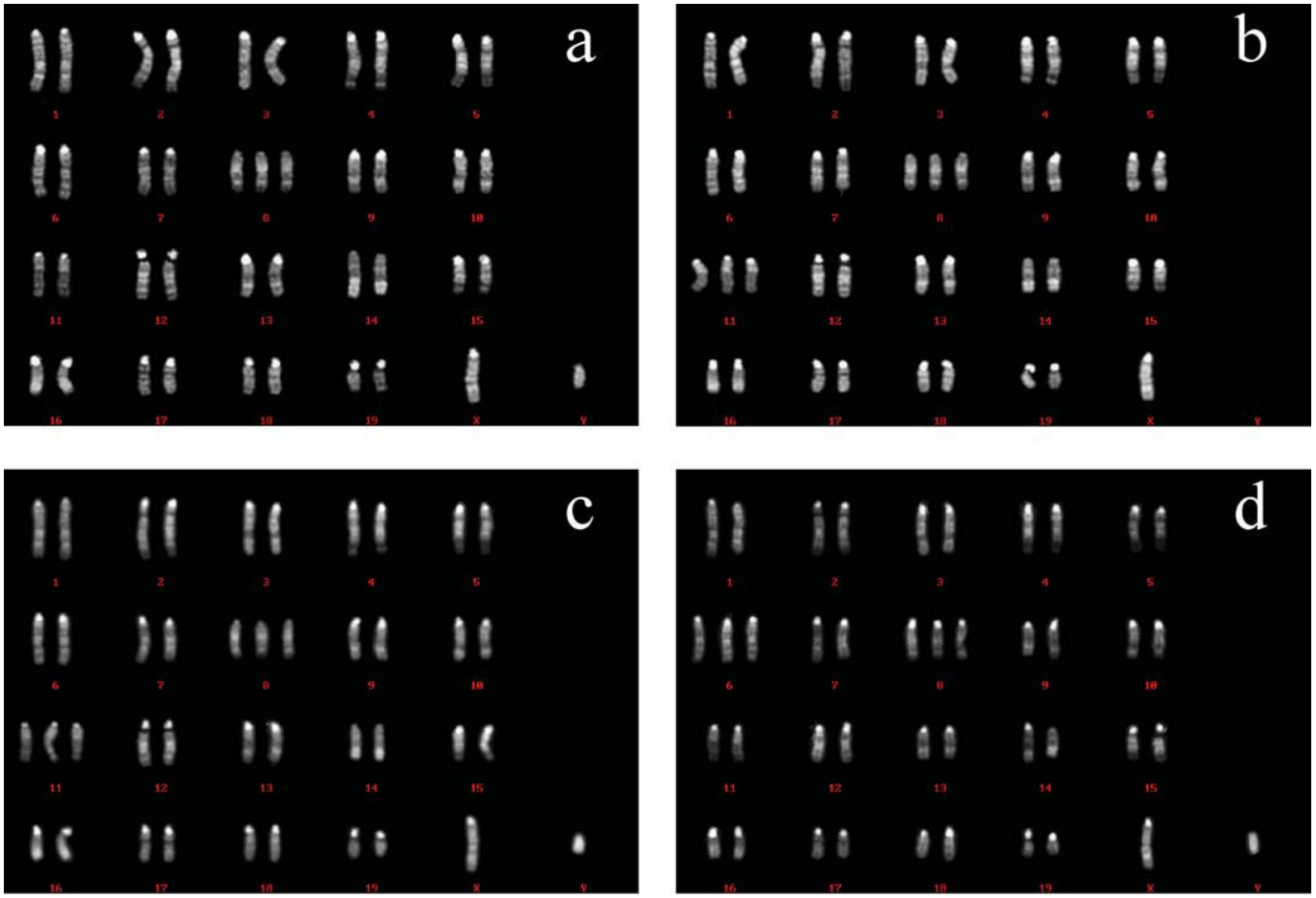

Figure 1. Q-banding karyotype of mouse embryonic stem cell lines showing (a) 41 chromosomes with XY including trisomy of chromosome 8, (b) 40 chromosomes with XO including trisomy of chromosome 8, (c) 42 chromosomes with XY including double trisomy of chromosomes 8 and 11, and (d) 42 chromosomes with XY including double trisomy of chromosomes 6 and 8.

types.[8] These findings suggest that trisomy 11 may be related to acceleration of growth and thus (when present with trisomy 8) further cell growth advantage in ES cells.

It is quite interesting that of 53 ES cell lines with normal chromosomes, only 1 had XX sex chromosomes, and all others were XY. Of 35 ES lines with abnormal karyotypes, 26 lines were XY and 9 lines were XO. In particular, 2 of the 9 XO lines included a minor XY population, and 1 ES line included a minor XX population, but the remaining 6 ES cell lines had no such minor populations (data not shown). Excluding the 6 ES cell lines lacking information on the original sex chromosome constitution, only 2 of 82 ES cell lines had XX sex chromosomes. Because the ratio of XX and XY sex chromosomes in fertilized eggs should be 1:1 theoretically, this predominance of XY in ES cells may correlate with unbalanced segregation during the establishment of ES cell lines in culture. One possible explanation of this unbalanced ratio of XY and XX is the inactivation of 1 of the 2 X chromosomes.

In mammals, dosage compensation ensures equal X chromosome expression between males (XY) and females (XX) by transcriptionally silencing 1 of the X chromosomes in females.[10] In the prevailing view, the XX zygote inherits 2 active X chromosomes, and X inactivation does not occur until after implantation. Because mouse ES cells are established as a cell line from the inner cell mass of mouse blastocysts before implantation, the presence of 2 active X chromosomes in female embryos may correlate with causation of unbalanced segregation of XX ES cells. Recent studies indicate that a single X chromosome is transcription-poor throughout pre-implantation female development, and X chromosome inactivation seems to be a biphasic process, with 'leaky' silencing occurring in the preimplantation period and followed by more complete silencing in postimplantation cells.[5] Therefore the essential nature of X chromosome inactivation may explain why XX ES cells are karyotypically unstable.[5] At present, the possibility that artificial events additionally contribute to the extremely high ratio of XY ES cell lines observed can not be completely excluded.

In the present study, loss of 1 of 2 sex chromosomes occurred in 9 ES cell lines: 2 had a loss of Y chromosome and 1 had a loss of X chromosome. No available information was obtained in the remaining 6 as mentioned previously. None of the embryonal carcinoma cell lines was reported to have a normal karyotype, and loss of the Y chromosome was seen in all cell lines in one study[1] and in 1 ES cell line in another.[8] In addition, loss of 1 of 2 X chromosomes[14] and deletion of the distal portion of 1 of the 2 X chromosomes have been recognized.[6,11,14] Total or partial loss of sex chromosome may be an alternative or additional way to promote the growth of ES cells in culture.

It was reported that prolonged passage in culture reduced the potential for germline transmission of the ES cell population as

Table 2. Summary of karyotype analyses of 88 mouse embryonic stem (ES) cell lines

Modal number	Karyotype	No. (%) of ES cell lines
39	39, XO	1 (1.1)
40	40, XY	52 (59.1)
40	40, XX	1 (1.1)
40	40, XO, +8	5 (5.7)
40	40, XO, +14	1 (1.1)
40	40, XO, +ssm	1 (1.1)
41	41, XY, +8	18 (20.5)
41	41, XY, +11	1 (1.1)
41	41, XO, +8, +11	1 (1.1)
42	42, XY, +6, +8	2 (2.3)
42	42, XY, +8, +11	4 (4.5)
42	42, XY, +8, +14	1 (1.1)
Total		88 (100.0)

ssm, small-sized metacentric chromosome.

a whole.[12] Euploid ES cell lines cultured in vitro for more than 20 passages were reported to become rapidly aneuploid and the ability of these lines to contribute to the germ line was lost when the proportion of euploid cells dropped below 50%.[9] It was suggested that approximately 40% of normal karyotypes are capable of germline transmission in chimeric mice.[15] In addition, most ES cell lines with a normal karyotype gave rise to a high percentage of chimeras and contributed to germline transmission whereas no ES cell lines with trisomy 8 produced chimeras that transmitted the mutation to the germ line.[8] We also have preliminary data suggesting that there was no germline transmission in ES cell lines in which the population with a normal diploid chromosomal number dropped below 75% (data not shown). The present results together with the described information on germline transmission suggest that not only the number of chromosomes but also the karyotype should be monitored during quality assurance testing of ES cells.

Three mouse strains—129, TT2, and C57BL/6J—were used for establishment of ES cell lines examined in this study. ES cell lines with abnormal karyotypes came from all 3 strains. The incidence of ES cell lines with abnormal karyotypes including trisomy 8 was relatively low (9.1%) for C57BL/6J compared with 129 (38.3%) and TT2 (41.2%). Abnormal sex chromosomes (XO) were seen in lines from 129 and C57BL/6J but not TT2. Because only a few of the ES lines we evaluated were derived from TT2 and C57/BL/6J, further study will be needed to clarify the correlation between abnormal karyotypes and strain difference.

All of the samples we used in this study were free from mouse hepatitis virus and *Mycoplasma* spp. We can eliminate *Mycoplasma* contamination as a potential causative factor of the chromosome abnormalities observed in the ES cells examined here, although *Mycoplasma* infection is known to induce chromosomal changes in cell lines.[2]

The high rate of chromosomal abnormalities in the ES cells used in Japan and studied here suggests that quality assurance monitoring will contribute to the establishment rate of new animal models through germline transmission using gene targeting methodologies.

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Table 3. Comparison of karyotypes of embryonic stem cell (ES) lines derived from different mouse strains

Modal number	Karyotype	No. (%) of ES lines in different strains		
		129	TT2	C57BL/6J
40	40, XY	35 (58.3)	10 (58.8)	8 (72.7)[a]
40	40, XO, +8	5 (8.3)	0 (0.0)	0 (0.0)
41	41, XY, +8	13 (21.7)	4 (23.5)	1 (9.1)
42	42, XY, +8, +11	2 (3.3)	2 (11.8)	0 (0.0)
42	42, XY, +6, +8	1 (1.7)	1 (5.9)	0 (0.0)
42	42, XY, +8, +14	1 (1.7)	0 (0.0)	0 (0.0)
Variable	Variable	3 (5.0)[b]	0 (0.0)	2 (18.2)[c]
Total		60 (100.0)	17 (100.0)	11 (100.0)

[a]Including an ES line with 40, XX.
[b]40, XO, +ssm (see Table 2); 41, XY, +11; 41, XO, +8, +11.
[c]39, XO; 40, XO, +14.

References

1. **Evans MJ, Kaufman MH.** 1981. Establishment in culture of pluripotential cells from mouse embryos. Nature **292:**154–156.
2. **Fogh J, Fogh H.** 1973. Chromosome changes in cell culture induced by mycoplasma infection. Ann N Y Acad Sci **255:**311–329.
3. **Goto K, Itoh T, Ebukuro S, Kagiyama N, Harasawa R.** 1993. Characterization of *Mycoplasma* strains isolated from house musuk shrews (*Suncus murius*) and their infectivity in mice and rats. Exp Anim **42:**363–369.
4. **Goto K, Nozu R, Kunita S, Terada E, Itoh T.** 1995. Genotyping of mouse hepatitis virus strains by restriction endonuclease analysis of amplified nucleocapsid protein genes. Exp Anim **44:**159–161.
5. **Huynh KD, Lee JT.** 2003. Inheritance of a pre-inactivagted paternal X chromosome in early mouse embryos. Nature **426:**857–863.
6. **Kaufman MH, Robertson EJ, Handyside AH, Evans MJ.** 1983. Establishment of pluripotential cell lines from haploid mouse embryos. J Embryol Exp Morphol **73:**249–261.
7. **Kunita S, Terada E, Goto K, Kagiyama N.** 1992. Sequence analysis and molecular detection of mouse hepatitis virus using the Polymerase chain reaction. Lab Anim Sci **42:**593–598.
8. **Liu X, Wu H, Loring J, Hormuzdi S, Disteche CM, Bornstein P, Jaenisch R.** 1997. Trisomy 8 in ES cells is a common potential problem in gene targeting and interferes with germ line transmission. Dev Dyn **209:**85–91.
9. **Longo L, Bygrave A, Grosveld FG, Pandolfi PP.** 1997. The chromosome make-up of mouse embryonic stem cells is predictive of somatic and germ cell chimaerism. Transgenic Res **6:**321–328.
10. **Lyon MF.** 1961. Gene action in the X-chromosome of the mouse (*Mus musculus* L.). Nature **190:**372–373.
11. **Martin GR.** 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A **78:**7634–7638.
12. **Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC.** 1993. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc Natl Acad Sci U S A **90:**8424–8428.
13. **Nesbitt MN, Francke U.** 1973. A system of nomenclature for band patterns of mouse chromosomes. Chromosoma **41**:145-158.
14. **Park J-I, Yoshida I, Tada T, Takagi N, Takahashi Y, Kanagawa H.** 1998. Trisomy 8 does not affect differentiative potential in a murine parthenogenetic embryonic stem cell line. Jpn J Vet Res **46:**29–35.
15. **Suzuki H, Kamada N, Ueda O, Jishage K, Kurihara Y, Kurihara H, Terauchi Y, Azuma S, Kadowaki T, Kodama T, Yazaki Y, Toyoda Y.** 1997. Germ-line contribution of embrionic stem cells in chimeric mice: influence of karyotype and in vitro differentiation ability. Exp Anim **46:**17–23.
16. **Ueda O, Jishage K, Kamada N, Uchida S, Suzuki H.** 1995. Production of mice entirely derived from embryonic stem (ES) cells with many passages by coculture of ES cells with cytochalasin B induced tetraploid embryos. Exp Anim **44:**205–210.