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Isolation of a Germline-Transmissible Embryonic Stem (ES) Cell Line from C3H/He Mice

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ABSTRACT—We have isolated three embryonic stem (ES) cell lines from C3H/He mice using mouse STO cells as a feeder layer. One ES cell line (H-1) was male, and two (H-2 and H-3) were female, as determined by polymerase chain reaction, *in situ* hybridization, and karyotype analyses. All were immunocytochemically reactive with a C3H strain-specific antibody. Injection of cells from the female ES H-3 line into C57BL/6 blastocysts yielded four chimeras with slight coat color chimerism. All chimeras were male, and as expected, no germline-transmission was observed. By contrast, when male ES H-1 cells were injected into the perivitelline space of 8-cell C57BL/6 embryos, one male mouse with overt coat color chimerism was recovered, and it produced ES H-1-derived offspring exclusively. This germline-transmissible C3H/He cell line represents a novel addition to those ES lines currently employed for gene manipulation studies of development.

INTRODUCTION

Mouse embryonic stem (ES) cells have been used as vehicles for introducing targeted mutations into mice (Thomas and Capecchi, 1987; Doetschman *et al.*, 1987), and they also provide a model system of cell differentiation *in vitro* (Doetschman *et al.*, 1985). Most of the ES cell lines currently available, including D-3 (Doetschman *et al.*, 1985), EK.CCE (Robertson *et al.*, 1986), E14 (Handyside *et al.*, 1989), and AB1 (McMahon and Bradley, 1990), are derived from different substrains of 129/Sv mice. However, characterization of the 129/Sv strain for laboratory investigations is incomplete and its genetic background differs from one substrain to another. If there were pluripotent ES cell lines from common inbred strains, the biological consequences caused by gene knockouts could be analyzed more readily and precisely in relation to a well defined genetic background. Until recently, there have been relatively few attempts to establish ES cell lines from other strains with exception of C57BL/6 (Ledermann and Bürki, 1991; Kawase *et al.*, 1994), BALB/c (Kawase *et al.*, 1994), (C57BL/6 × CBA) F_1 (Tokunaga and Tsunoda, 1992; Yagi *et al.*, 1993), and MRL/MP-*lpr*/*lpr* strains (Kawase *et al.*, 1994). Here we report a germline-transmissible ES cell line from C3H mice, one of the most well characterized inbred mouse strains commonly used for physiological and pathological investigations.

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MATERIALS AND METHODS

Mouse strains and embryos

Adult mice were maintained under guidelines for hygiene, ventilation, and nutrition in the Animal Care Facility at the National Institute of Animal Health (NIAH) on 12hr light/ 12hr dark cycle. C3H/HeJah mice were obtained from the Institute for Medical Research, University of Tokyo in 1963, propagated by sister-brother mating (Mannen *et al.*, 1994), and used as a source of blastocysts for culture. C57BL/6Ncrj and Crj: CD-1(ICR) mice were purchased from Charles River Co., Japan. For natural matings, C3H/He females were placed in cages with males in the evening and were examined for vaginal plugs the following morning. The day on which a vaginal plug was detected was designated as day 0.5 of pregnancy (E0.5). For the induction of superovulation, females were given five I.U. of pregnant mare serum gonadotropin (Teikoku Zoki, Tokyo, Japan) by intraperitoneal injection, followed by five I.U. of human chorionic gonadotropin (Teikoku Zoki) after a 48 hr interval. On E3.5, C3H/He blastocysts were collected by flushing the uterine cavity with DMEM supplemented with 10% fetal calf serum (FCS). Pseudopregnant ICR females were prepared by mating with vasectomized males as described (Hogan *et al.*, 1986).

Blastocyst culture and establishment of ES cell lines

All reagents were obtained from Sigma, St. Louis, MO, USA, unless otherwise specified. Single C3H/He blastocysts were cultured at 37°C in 35 mm plastic culture dishes containing ES medium in a humidified atmosphere of 95% air and 5% CO_2. ES medium was composed of DMEM (code #05919, Nissui, Tokyo, Japan) supplemented with 10% heat inactivated FCS (Cell Culture Lab., Oratories, OH, USA), glucose (4.5 g/l), 30 mM adenosine, 30 mM cytidine, 30 mM uridine, 30 mM guanosine, 10 mM thymidine, non-essential amino acids (Gibco BRL, Gaithersburg, MD, USA), 2-

mercaptoethanol (10^{-4} M), leukemia inhibitory factor (LIF, 10^3 unit/l, Amrad Co. Melbourne, Australia), penicillin G (31 mg/l), streptomycin sulfate (50 mg/l), and sodium bicarbonate (3.7g/l). Mouse STO fibroblasts (Martin and Evans, 1975) were treated with mitomycin C (Robertson, 1987) and seeded into the dishes on the second day of blastocyst culture. Blastocysts hatched after 2 days of culture and attached to the dish surface. After 5 days, the inner cell mass (ICM) of the blastocyst had proliferated to form a round aggregate at the center of the trophoectodermal cell sheet, which had attached and spread on the dish surface. The ICM was washed with PBS(-) and treated with trypsin-EDTA solution (0.001% trypsin and 1 mM EDTA) in PBS(-) at 37°C for a few minutes. After trypsin was inactivated by adding ES medium, the ICM was dissociated into small clumps by gentle pipetting, and the clumps were seeded into a new 35 mm dish with fresh STO feeder cells. This dissociation was designated as the first passage (P1). Culture medium was replaced every other day. Seven to ten days after dissociation of the ICM, small round colonies that were expected to contain stem cells were observed in some dishes, and these were trypsinized and seeded into new 35 mm dishes. ES cells were propagated as required, harvested, suspended in freezing medium at ca. 1×10^7 cells/ml, and slowly frozen at -80°C. Freezing medium was composed of DMEM containing 10% FCS, 10% DMSO, 1% HEPES at pH 7.4. For teratoma formation, ca. 1×10^7 ES cells were suspended in 1 ml PBS(-) and intraperitoneally injected into normal adult C3H/He females. After two to three months, solid tumor masses were recovered, fixed with Bouin's solution, embedded in paraffin, and examined histologically.

Immunocytochemistry

ES cells cultured on Lab-Tek chamber slides (Nunc Inc., Naperville, IL, USA) were fixed with a mixture of 95% ethanol and acetic acid (99:1, v/v) and were processed for immunocytochemistry as described (Kitani *et al.*, 1991). Anti-C3H specific antibody (Kusakabe *et al.*, 1988) was applied for 30 min. at room temperature. This antibody specifically recognizes one of the variants of the heat shock protein 70 family in this strain (Michikawa *et al.*, 1993). After washing three times with cold PBS(-) for 5 min., slides were incubated for 30 min. at room temperature with a second antibody, FITC-conjugated anti-mouse IgG (No. 6250, TAGO, Burlingame, CA, USA) diluted 1:100. Then, slides were washed with cold PBS(-), and coverslips were mounted using 90% non-fluorescent glycerol in PBS(-). Normal mouse IgG_1 was used as negative control. Photomicrography was performed with an Olympus BH2-RFK fluorescence microscope.

Generation of chimeras

Chimeric mice were produced by injecting 15-25 C3H/He ES cells into each blastocyst cavity (Bradley, 1987) or 10-15 cells into the perivitelline space of 8-cell stage embryos (Tokunaga and Tsunoda, 1992) from superovulated C57BL/6 females. Passage numbers of injected ES cells were between P6 and P12. Injected embryos were cultured overnight in M16 medium, and viable embryos were transferred into the uterine cavity of pseudopregnant ICR females as described (Hogan *et al.*, 1986). Chimeras were identified by the agouti coat color characteristic of C3H/He mice, and they were mated with C57BL/6 mice to assess germline-transmission of the injected C3H/He ES cells.

Analysis of karyotype and sex chromosomes

For karyotype analysis, ES cells were allowed to incorporate 5-bromodeoxyuridine (100 μg/ml) for 8-9 hr. During the last 1 hr, Colcemid (40 ng/ml) also was present. Harvested cells were treated 0.075 M KCl, fixed with methanol: acetic acid (3:1,v/v), and spread onto clean slides. Chromosome slides were stained with freshly prepared acridine orange and examined with a fluorescence microscope.

To determine the sex of the ES cell lines, polymerase chain reaction (PCR) was used to amplify a mouse Y chromosome-specific repetitive sequence (pY353/B) (Bradbury *et al.*, 1990) from ES cell DNAs. For *in situ* hybridization, ES cell preparations were spread on glass slides and were hybridized with a digoxigenin-labeled pY353/B probe. Hybridization was detected by an alkaline phosphatase labeled anti-digoxigenin antibody (Boehringer, 1093274) according to the manufacturer's instructions. Photomicrography was performed with a Nikon Nomarski microscope.

RESULTS

Blastocyst culture and establishment of ES cell lines from C3H/He mice

Table 1 shows the incremental efficiencies of establishment of ES cell lines from C3H/He blastocysts. Two types of ovulation were used. From superovulated C3H/He females, a total of 124 blastocysts were cultured in 11 experiments. Successful growth of 100 inner cell masses (ICMs) (81%), 33 primary ES-like colonies (27%), and 1 permanent cell line (0.8%) was observed. From naturally ovulated C3H/He females, a total of 91 blastocysts were cultured in 5 experiments. In this group successful growth of 77 ICMs (85%), 6 primary ES-like colonies (7%) and 2 permanent cell lines (2%) was obtained. There appeared to be little difference in the efficiencies of establishment of cell lines between these two types of ovulation.

These cell lines established from C3H/He blastocysts (termed H-1, H-2 and H-3) were confirmed as ES cell lines by the following observations. All cell lines formed colonies of tightly packed cells with prominent nucleoli were formed (Fig.1A). This is a typical morphological feature of ES cells in culture (Robertson, 1987). The cells were maintained on an STO feeder layer by passaging them every 2-3 days at a split ratio of 1:10. Each ES cell line was considered to be clonal, because it was derived from a single blastocyst culture (See Materials and Methods). The cell lines were neoplastic when transplanted into C3H/He mice. After two to three months of intraperitoneal injection of ca.1×10^7 cells into normal C3H/He mice, all of the cell lines developed into solid tumor masses. These tumor masses contained undifferentiated as well as differentiated cells. The differentiated cells appeared to derive from all three embryonic germ layers including neural cells and cornified epithelial cells (ectodermal origin), striated muscle cells and chondrocytes (mesodermal origin), ciliated columnar epithelial cells (endodermal origin), and other unidentified tissues (Fig. 2A, B). All of the C3H/He ES cell lines were immunocytochemically reactive with a anti-CSA antibody that recognizes with a C3H-specific antigen (Kusakabe *et al.*, 1988) (Fig.1B). STO feeder cells were not immunoreactive. A finely granular immunofluorescence was

Table 1. Establishment of permanent cell lines from C3H/He strain mice

Ovulation	Cultured blastocysts	ICM growth	Primary colonies	Established cell lines
Hormonal	124	100	33	1
Natural	91	77	6	2

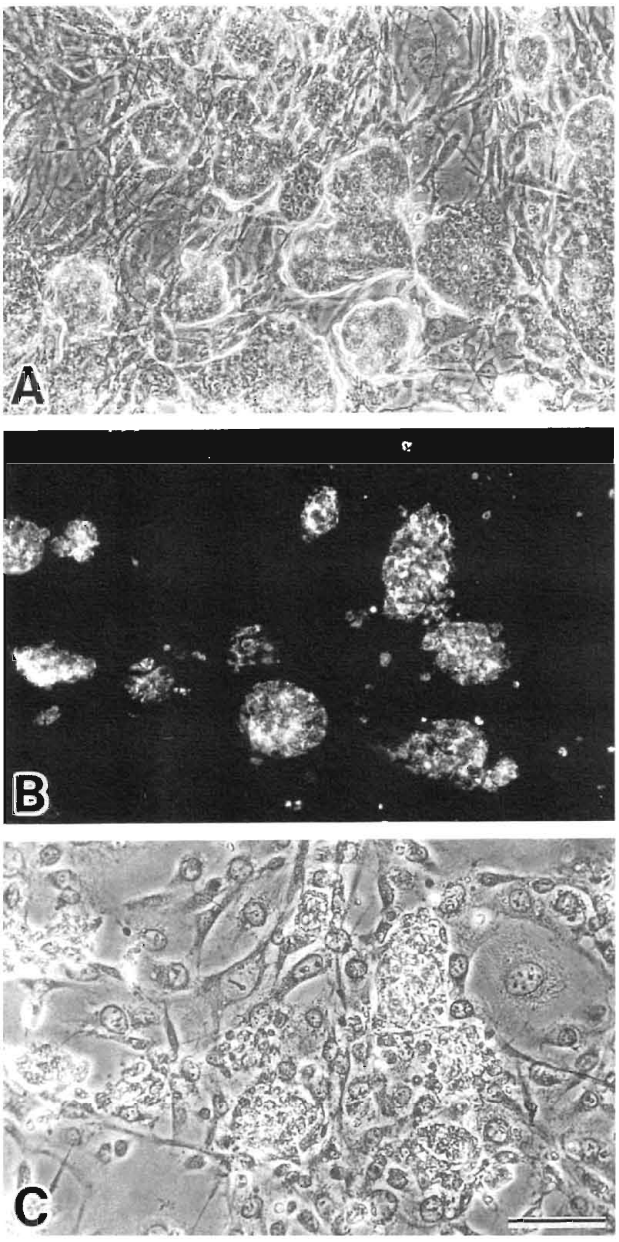

Fig. 1. (A) Phase contrast micrograph of the H-1 ES cells established from C3H/He blastocysts. The ES cell line showed typical morphological characteristics of ES cells in culture, e.g., formation of compact colonies from tightly packed small cells with prominent nucleoli. (B) The H-1 ES cell line was immunocytochemically stained with anti-CSA antibody, while STO feeder cells were not stained. (C) Phase contrast micrograph of (B). Bar=100 μm.

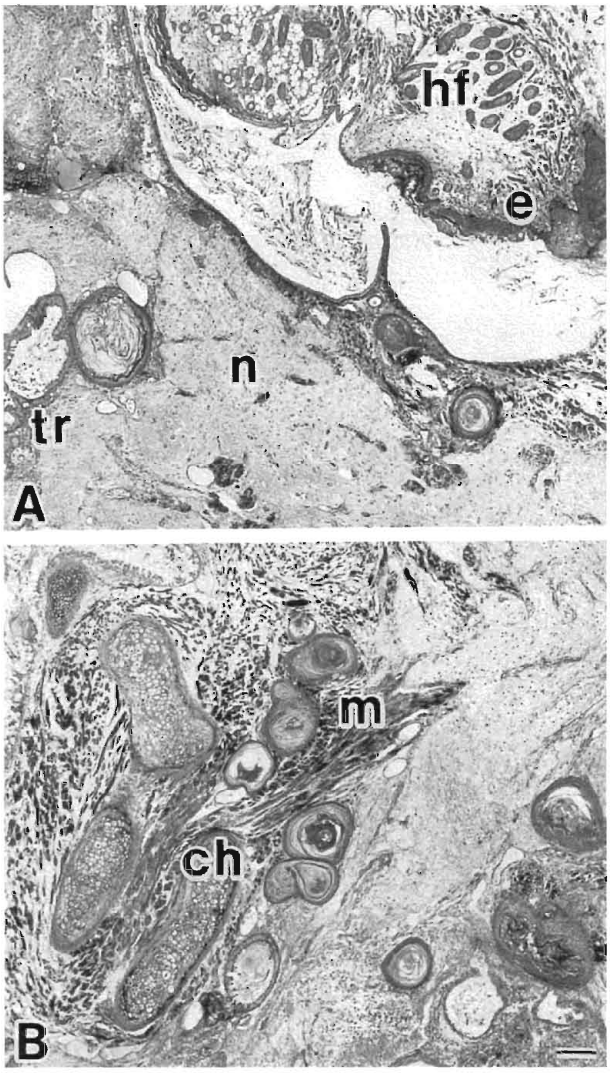

Fig. 2. Histological section of a solid tumor formed by H-1 ES cells after intraperitoneal injection into normal C3H/He mice. Undifferentiated stem cells and various differentiated cells are observed. (A) Neural cells (n), cornified epithelial cells (e), hair follicles (hf) and tracheal epithelial cells (tr). (B) Striated muscle cells (m) and chondrocytes (ch). Bar=100 μm.

detected in the cytoplasm of the ES cells, in accordance with the known localization of this antigen in mitochondria (Michikawa *et al.*, 1993).

Generation of chimeric mice and germline-transmission

The potential of the ES cells to form germline chimeras was tested by two methods. After blastocyst-injection, chimeric mice were obtained only from H-3 cells (Table 2A). Injection of H-3 cells into 89 C57BL/6 blastocysts resulted in 30 offspring, and 4 of them showed only slight chimerism in abdominal coat color. Each male chimera was mated with four or five C57BL/6 females, and 30 to 36 offspring were obtained from each chimeric father. None of the pups showed C3H/He agouti coat color, indicating that no germline-transmission had occurred in these male chimeras. This result is in accordance with the fact that female ES cells do not differentiate into sperm in chimeric males. H-1 and H-2 cells, did not generate chimeric mice after they were injected into 60 and 48 C57BL/6 blastocysts, respectively (Table 2A).

The eight-cell embryo injection method also was tested. It has been reported that male ES cells may convert the sex of female host embryos, resulting in a male distorted sex-ratio in the postnatal chimeras (Bradley *et al.*, 1984). In this way, chimeric males may transmit their genotype to a number of

Table 2A. Generation of chimeric mice by blastocyst-injection

ES Cell Line	Blastocyst injected (C57BL/6)	Progeny born	Chimeric mice	Male chimeras	Germline chimeras
H-1	60	23	0	–	–
H-2	48	4	0	–	–
H-3	89	30	4	4*	0**

*C3H/He agouti coat color was sparsely distributed in the abdomen as thin bands in these chimeras.
**After mating with 4 or 5 C57BL/6 females, a total of 30 to 36 offsprings were obtained from each chimeric father. In each case, all of the pups showed C57BL/6 black coat color, indicating that no germline-transmission was evident with these male chimeras.

Table 2B. Generation of Chimeric mice by 8-cell embryo-injection

ES Cell Line	8-cell embryo injected (C57BL/6)	Progeny born	Chimeric mice	Male chimera	Germline chimera
H-1	41	10	1	1	1*

*Mating of this male chimera with C57BL/6 females produced a total of 55 offsprings from 8 litters, and all the pups showed agouti coat color typical of C3H/He strain.

offspring. Tokunaga and Tsunoda (1993) have reported efficient production of germline chimeras by injecting male ES cells into 8-cell stage embryos. Since our preliminary experiments indicated that only H-1 ES cells were male (see below), we applied the 8-cell embryo injection method to these ES cells . After H-1 ES cells at passage number 10 were injected into the perivitelline space of 8-cell embryos from C57BL/6 (41 embryos in three experiments), 1 male chimera with overt coat color chimerism was obtained among 10 postnatal offspring (Table 2B). This chimera had an agouti coat color that was extensively intermingled throughout whole body (Fig. 3A), suggesting that the distribution of H-1 ES cells was widespread in various internal organs. This male chimera was mated with 8 C57BL/6 females. A total of 55 offspring were born, and all had agouti coat color (Fig. 3B). These results indicate that H-1 ES cells had exclusively contributed to the differentiation of germ cells in this male chimera.

Analysis of karyotype and sex chromosomes

Male ES cells have been used for most gene knockout studies (Thomas and Capecchi, 1987; Doetschman *et al.*, 1987), because male ES cell lines are karyotypically more stable than female lines, and germline transmission of male ES cells can be assessed more easily than that of female ES cells. The sex type of these ES cell lines was determined by PCR using Y chromosome specific primers and *in situ* hybridization. With the PCR method, a male-specific band was amplified from H-1 cell DNA but not H-2 or H-3 cell DNA (data not shown). Figure 4 shows *in situ* hybridization with a digoxigenin-labeled pY353/B probe. Male specific dots were detected in the nuclei of H-1 cells (Fig. 4A). No signal was observed in the nuclei of H-3 (Fig. 4B) or H-2 cells (data not shown).

A normal euploid karyotype is an essential prerequisite for ES cells to differentiate into functional germ cells in chimeric mice. The low efficiencies with which our C3H/He ES cells formed germline chimera may be due to low proportions of normal euploid cells in these cell lines. Accordingly, the karyotype was examined at the 10th passage by an R-banding technique after BrdU incorporation (Table 3). The sex chromosome constitution was XY in H-1, and XX in H-2 and H-3 confirming the results obtained by PCR and *in situ* hybridization. In H-1, 32 out of 42 cells examined had 40 chromosomes, but 14 cells contained a long marker chromosome most probably derived from chromosome 3. The karyotype was apparently normal in 18 H-1 cells, and 9 hyperploid cells were characterized by trisomy 11. Approximately half of the cells in mitosis were tetraploid in H-2. Only 13 of 68 cells in the diploid range had 40 chromosomes, and most of the remaining cells had an extra chromosome 11. In H-3, more than half of the cells examined had 41 chromosomes. Here again, trisomy 11 was observed most frequently. Thus, it may be concluded that the high proportion of abnormal karyotypes in these cell lines was at least partly responsible for the low frequencies of germline chimeras after injection into blastocysts or the perivitelline space of 8-cell embryos. Recloning of these C3H/He ES cells is currently in progress.

DISCUSSION

We have established a germline-transmissible male ES cell line (H-1) from C3H/He blastocysts. H-1 cells exhibited highly efficient germline-transmission when injected into 8-cell embryos despite the relatively low proportion of normal diploid cells in this cell line. These cells had typical ES-like morphology in culture, and they were maintained as

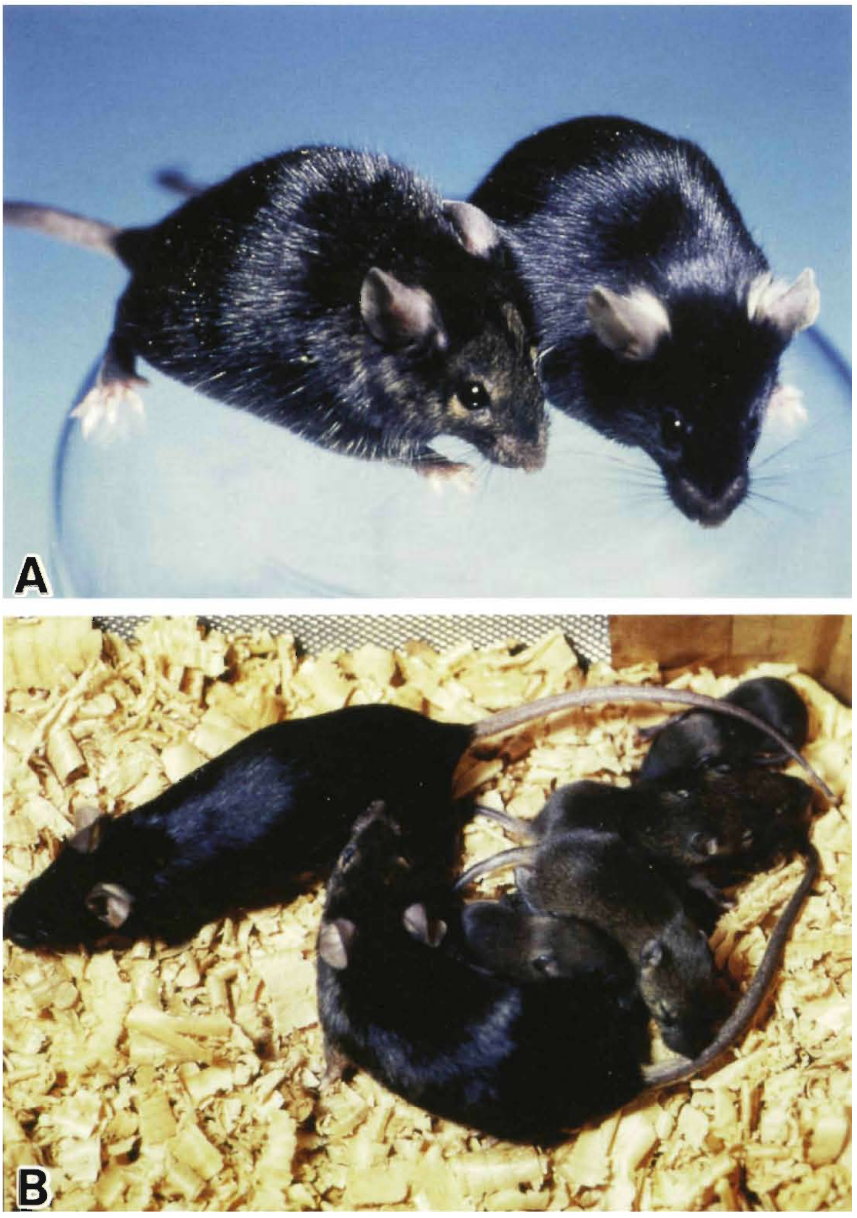

Fig. 3. (A) A male chimera (left) obtained after injection of H-1 cells into the perivitelline space of 8-cell embryo from C57BL/6. This male chimera was mated with C57BL/6 female (right) to examine germline-transmission of the H-1 cells. (B) All of the pups from this mating showed agouti coat color typical of the C3H/He strain, indicating germline-transmission of H-1 cells.

undifferentiated lines on STO feeder cells in medium supplemented with LIF. The cell line exhibited the ability to form teratocarcinomas with differentiated cell types from all three embryonic germ layers, and it had a male (XY) karyotype. In addition, the H-1 ES cell line was immunoreactive with anti-CSA antibody, so it should be possible to trace the developmental fate of the stem cells after injection of these C3H/He ES cells into appropriate anti-CSA-negative host embryos such as those from the C57BL/6 strain.

Two female ES cell lines (H-2 and H-3) also were obtained from the C3H/He strain. These showed characteristics similar to the H-1 cell line in culture, namely teratoma formation and immunoreactivity with anti-CSA antibody. However, we could not demonstrate their abilities to produce germline chimeras. Blastocyst-injections yielded 4 chimeras only with ES H-3 cells. All chimeras were male, and as expected, no germline-transmission was observed in any of these chimeras. Low efficiencies of H-2 and H-3 cell lines for chimera formation may be due in part to a low proportion of normal diploid cells in these cell lines.

Injection of male H-1 cells into 8-cell C57BL/6 embryos resulted in one chimeric male that exhibited exclusive germline-transmission. Conversely, no chimeras were obtained after injection of blastocysts with H-1 cells. This may indicate that the stage of host embryos at the time of injection determines the extent of colonization of C3H/He ES cells in chimeric

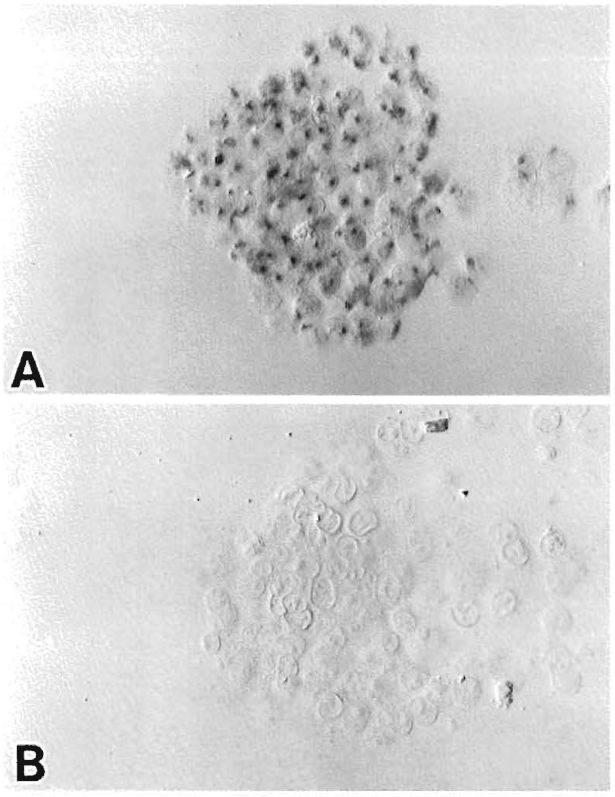

Fig. 4. *In situ* hybridization of cell preparations from ES cells with digoxigenin-labeled PCR product of pY353/B. Specific signals were visualized by alkaline phosphatase labeled anti-digoxigenin antibody system and photographed with Nomarski optics. (A) In the preparation from H-1 ES cells, specific signals were detected in the nuclei. (B) In the preparation of H-3 ES cells, no signal was observed in the nuclei.

animals. Tokunaga and Tsunoda (1992) reported highly efficient production of germline chimeras after injection of male (C57BL/6 × CBA) F_1 ES cells (F1/1) into the perivitelline space of 8-cell host embryos. Yagi *et al.* (1993) obtained similar results with another male (C57BL/6 × CBA) F_1 ES cell line (TT2). The present study demonstrates that the 8-cell embryo injection method is also applicable to ES cells from the C3H/He strain. H-1 cells may be in an earlier developmental stage as compared to ICM cells (Yagi *et al.*, 1993). Consequently, they may not be incorporated into the host embryo at the blastocyst stage. By contrast, when H-1 cells are injected into an 8-cell embryo (Tokunaga and Tsunoda, 1992), donor cells may be effectively incorporated and localized to the center of the ICM in the chimeric blastocyst, resulting in an exclusive germline-transmissible chimera. Strain combinations between ES cells and host embryos may be another factor influencing the degree of chimerism and subsequent germline-transmission (Schwartzberg *et al.*, 1989; Ledermann and Bürki, 1991). Although there was no comparative study of strain combinations for C3H/He ES cells in chimera production, it is clear that C57BL/6 may be used as a host strain for the C3H/He ES cell line by 8-cell embryo injection method.

Availability of a germline-transmissible male H-1 ES cell line from the common inbred C3H/He strain will permit targeted mutation of specific genes on a well characterized genetic background, thus avoiding the confounding effects caused by background genes from 129 strains (Gerlai, 1996). In addition, various congenic as well as mutant substrains with a C3H-background (Lyon, 1989) may further extent the range of experimental possibilities for future studies of developmental genetics.

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REFERENCES

Bradbury MW, Isola LM, Gordon JW (1990) Enzymatic amplification of a Y chromosome repeat in a single blastomere allows identification of the sex of preimplantation mouse embryos. Proc Natl Acad Sci USA 87: 4053–4057

Bradley A (1987) Production and analysis of chimaeric mice. In "Teratocarcinomas and Embryonic Stem Cells - A Practical Approach" Ed by EJ Robertson, IRL Press, Oxford, pp 113–151

Bradley A, Evans M, Kaufman MH, Robertson E (1984) Formation of germ-line chimeras from embryo-derived teratocarcinoma cell lines. Nature 309: 255–256

Doetschman TC, Eistetter H, Katz M, Schmidt W, 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 Morphol 87: 27–45

Doetschman T, Gregg RG, Maeda N, Hooper ML, Melton DW, Thompson S, Smithies O (1987) Targetted correction of a mutant HPRT gene in mouse embryonic stem cells. Nature 330: 576–578

Table 3. Karyotype of ES cell lines from C3H/He strain mice

ES Cell line	Passage number	Karyotype	Chromosome count							% euploid
			39	40	41	42	43	44	80	
H-1	10	XY	1 (1)*	32 (14)*	9 (1)*					42.9
H-2	10	XX	3	13	51		1		65	9.8
H-3	10	XX	1	14 (1)*	18			1		38.2

( )* Cells having a long marker chromosome.

Gerlai, R (1996) Gene-targeting studies of mammalian behavior: is it the mutation or the backgroud genotype? Trends Neurosci 19: 177–181

Handyside AH, O'Neill GT, Jones M, Hooper ML (1989) Use of BRL-conditioned medium in combination with feeder layers to isolate a diploid embryonal stem cell line. Roux's Arch Dev Biol 198: 48–55

Hogan B, Costantini F, Lacy E (1986) Manipulating the Mouse Embryo -A Laboratory Manual. Cold Spring Harbor Laboratory.

Kawase E, Suemori H, Takahashi N, Okazaki K, Hashimoto K, Nakatsuji, N (1994) Strain difference in establishment of mouse embryonic stem (ES) cell lines. Int J Dev Biol 38: 385–390

Kitani H, Shiurba R, Sakakura T, Tomooka Y (1991) Isolation and characterization of mouse neural precursor cells in primary culture. In Vitro Cell Dev Biol 27A: 615–624

Kusakabe M, Yokoyama M, Sakakura T, Nomura T, Hosick HL, Nishizuka, Y (1988) A novel methodology for analysis of cell distribution in chimeric mouse organs using a strain specific antibody. J Cell Biol 107: 257–265

Ledermann B, Bürki, K (1991) Establishment of a germ-line competent C57BL/6 embryonic stem cell line. Exp Cell Res 197: 254–258

Lyon MF (1989) Rules for nomenclature of inbred strains. In "Genetic Variants and Strains of the Laboratory Mouse" Ed by MF Lyon, AG Searle, Oxford University Press, Oxford, pp 632–648

Mannen H, Tsuji S, Fukuta K, Goto N (1994) Identification of sublines of inbred strains of mice and assessment of genetic relationships between substrains or sublines by DNA fingerprinting. Exp Anim 43: 512–526

Martin GR, Evans MJ (1975) Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies *in vitro*. Proc Natl Acad Sci USA 72: 1441–1445

McMahon AP, Bradley A (1990) The *Wnt*-1 (*int*-1) proto-oncogene is required for development of a large region of the mouse brain. Cell 62: 1073–1085

Michikawa Y, Baba T, Arai Y, Sakakura T, Tanaka M, Kusakabe M (1993) Antigenic protein specific for C3H strain mouse is a mitochondrial stress-70 protein. Biochem Biophys Res Commun 196: 223–232

Robertson E, Bradley A, Kuehn M, Evans M (1986) Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. Nature 323: 445–448

Robertson, EJ (1987). Embryo-derived stem cell lines. In "Teratocarcinomas and Embryonic Stem Cells - A Practical Approach" Ed by EJ Robertson, IRL Press, Oxford, pp 71–112

Schwartzberg PL, Goff SP, Robertson EJ (1989) Germ-line transmission of a c-*abl* mutation produced by targeted gene disruption in ES cells. Science 246: 799–803

Thomas KR, Capecchi MR (1987) Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell 51: 503–512

Tokunaga T, Tsunoda Y (1992) Efficacious production of viable germ-line chimeras between embryonic stem (ES) cells and 8-cell stage embryos. Develop Growth Differ 34: 561–566

Yagi T, Tokunaga T, Furuta Y, Nada S, Yoshida M, Tsukada T, Saga Y, Takeda N, Ikawa Y, Aizawa S (1993) A novel ES cell line, TT2, with high germline-differentiating potency. Anal Biochem 214: 70–76

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