Preparation of Animals with a High Degree of Chimerism by One-Step Coculture of Embryonic Stem Cells and Preimplantation Embryos

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ABSTRACT

A single-step coculture procedure has been developed that can generate chimeric mice with high efficiency and reproducibility. The procedure involves culture of embryonic stem (ES) cells with 8- to 16-cell embryos in microwells to provide conditions for effective cell-cell-embryo contact. A suspension of ES cells is layered over the microwells, followed by transfer of an embryo without zona pellucida into each microwell. Following overnight culture, the blastocysts are transferred into pseudopregnant recipients. The method has several advantages due to its simplicity and reproducibility: (i) Over 90% of ES-cell contribution in newborns can be obtained frequently and most of the chimeras display germ-line transmission. (ii) The procedure does not require specialized skills or expensive instruments. (iii) All the steps of embryo manipulation can be completed in a relatively short period of time; therefore, a large number of embryos can be manipulated simultaneously. The method was tested with three independent ES cell lines and two different strains of mice with similar results. The technique may be an alternative to microinjection of DNA into zygotes to prepare transgenic lines of small and large mammalian species.

INTRODUCTION

Targeting of genes by homologous recombination in pluripotent embryonic stem (ES) cells (3,10) provides a powerful tool for introducing specific mutations in the genome of animals (1,2,16). In general, a positive-negative selection gene-targeting cassette is prepared in which an exon of the target gene is interrupted by the gene for neomycin resistance (5,9,12). A herpes simplex virus thymidine kinase (HSV TK) gene is fused at either one end or both ends of the genomic sequence as a negative selection marker. The targeting cassette is introduced into ES cells by electroporation, and the cells in which the endogenous gene is disrupted by homologous recombination are selected with G418 and gancyclovir or FIAU (5,12).

The most common method for preparing chimeric animals is by direct microinjection of ES cells into the cavity of the blastocyst (1). Although this method is efficient, it has several limitations. First, it requires specialized training and skills. Second, it needs many expensive and sophisticated instruments. Third, the availability of healthy and well-expanded blastocysts is generally limited.

To fully realize the potential of gene knockout technology, a simple and efficient method is highly desirable. Based on the principle of aggregation of preimplantation embryos (11,19,20), a coculture procedure of ES cells with early embryos (22) and a sandwich procedure (13,14) were recently described to prepare animals with a high degree of chimerism.

In this report we describe a simple one-step coculture method to prepare highly chimeric animals reproducibly. The method does not involve special instruments or specialized skills, can be completed in a relatively short time and a large number of embryos can be manipulated simultaneously.

MATERIALS AND METHODS

Isolation of Embryos

Three- to five-week-old female FVB/N and C57BL/6 strains of mice, purchased from NIH FCRF (Frederick MD, USA), were superovulated with 5 IU of pregnant mare serum gonadotropin (PMSG) (Gestyl; Professional Compounding Centers of America, Houston, TX, USA), followed 48 h later by 5 IU of human chorionic gonadotropin (HCG) (Sigma Chemical, St. Louis, MO, USA), and the females were mated with males from the respective strain. The females were sacrificed 2.5 days postcoitus (p.c.), and 8- to 16-cell embryos were collected by flushing the uterine horns with M16 medium (7) or Brinster's medium (Life Technologies, Gaithersburg, MD, USA). The embryos were stored in the same medium under paraffin oil in a CO₂ incubator at 37°C.

Preparation of ES Cells

Normal ES cells (R1 cells, a gift from Dr. A. Nagy [Mount Sinai Hospital, Toronto, Canada] and RW4 cells from Genome Systems [St. Louis, MO, USA]) or gene-targeted J1 ES cells with a null mutation in the gene for

Table 1. Coculture of FVB/N Embryos with J1 ES Cells

Experiment #	No. of Embryos	No. of Foster Mothers	No. Pregnant	Total No. of Pups	No. of Chimeras
I	31	3	2	7	7(4)
II	22	2	2	4	3(1)
III	44	3	2	6	4(2)

Figures in parentheses represent the number of chimeras that were either dead or killed by the foster mothers.

collagen type II (8), were used for coculture with embryos from the FVB/N and C57BL/6 strains of mice. The cells were cultured over the primary embryonic fibroblast feeders in 60-mm petri dishes following published methods (16) in ES cell medium supplemented with 1000 IU of lymphocyte inhibitory factor (LIF) (Esgro; Life Technologies).

Confluent ES cell cultures were treated with 1 mL of 0.25% trypsin EDTA solution (Life Technologies) for 5–8 min at 37°C in a CO₂ incubator, and the cells were harvested in 6 mL of ES cell medium. The cells were dispersed as single-cell suspensions by repeated pipetting, followed by centrifugation at 1000 rpm (Model TJ-6 Centrifuge Rotor; Beckman Instruments, Fullerton, CA, USA) for 5 min at 4°C. The cells were washed once in 5 mL of medium and resuspended in 5 mL of the same medium in a 50-mL plastic tube. The tube was allowed to stand for 10 min to allow feeder layer cells to settle, after which the top 1 mL of the supernatant was transferred to a 1.5-mL microcentrifuge tube. The cells

were pelleted at 1000 rpm in microcentrifuge tubes for 2 min at room temperature, followed by washing two times in 1 mL of medium A [M16 medium or Brinster's medium with 10 mg/mL final concentration of bovine serum albumin (BSA) (Pentex; Miles, Kankakee, IL, USA)]. The cells were resuspended in the same medium at a concentration of $1.5\text{--}2 \times 10^5$ cells/mL and diluted, as necessary, with the same medium.

Preparation of Coculture Dish

To maximize contact between the ES cells and the embryos, 8–10 microwells, 0.3–0.5-mm outer diameter at the top and 0.1–0.2 mm at the bottom, were constructed in a 0.5-cm circle on a 35-mm petri dish. Figure 1 shows the top view of wells as seen under a stereomicroscope at 60× magnification. The microwells can be easily constructed by pressing the blunt end of a fire-polished glass Pasteur pipet against the bottom of the petri dish (Genome Systems). Seventy microliters of ES cell suspension were layered over the microwells, and the cells were allowed to settle for 10 min at room temperature. The microwell dimensions accommodate 15–20 cells and also prevent adhesion between the embryos during coculture.

Preparation of Zona-Free Morulae and Coculture with ES Cells

About 30–50 healthy 8- to 16-cell morulae were collected in a minimum volume and transferred into a drop of 100 µL acid Tyrode solution (137 mM NaCl; 2.7 mM KCl; 0.5 mM MgCl₂·6H₂O; 5.6 mM glucose; 1.6 mM CaCl₂·2H₂O and 0.4% polyvinylpyrrolidone, pH 2.5) (7) in a 60-mm

Figure 1. Microwells constructed on the surface of a 35-mm petri dish in a circle of about 0.5-cm diameter. The bar is equal to 1 mm.

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petri dish. Up to 100 embryos can be processed easily in the same volume. The embryos were monitored constantly under a stereomicroscope. Once the zona pellucida was dissolved, the solution was diluted with 1 mL of medium A to minimize exposure of the embryos to acidic pH, followed by washing in 5–7 mL of fresh medium. A high concentration of BSA (10 mg/mL) in medium A prevented adhesion of the denuded embryos with each other during subsequent handling before coculture (see Results). One embryo was then transferred to each microwell carrying the ES cells and incubated overnight in a CO₂ incubator at 37°C.

Preparation of Chimeric Animals

After overnight culture, blastocysts were collected from the wells and washed in 5 mL of medium A. Approximately 8–10 embryos were surgically transferred into one uterine horn of 6–8-week-old, 2.5-day p.c. pseudo-pregnant CD1 female mice. The newborn pups were screened for mutation in the type II collagen gene by the polymerase chain reaction (PCR) or for chimerism (based on coat color). Chimeric animals were bred with nor-

Table 2. Coculture of R1 ES Cells with FVB/N and C57BL/6 Embryos

Experiment #	Mouse Strain	No. of Embryos	No. of Foster Mothers	No. Pregnant	Total No. of Pups	No. of Chimeras
I	FVB/N	26	3	1	3	1
II	FVB/N	33	3	2	5	5
III	C57BL/6	29	3	3	12 ^a	6 ^b

^aSix pups were either cannibalized or killed by the mothers. Two pups had very pale skin indicating a high contribution of ES cells.
^bTwo pups were larger in size, displayed high chimerism and died shortly after birth. Four animals that survived to adulthood were >90% chimeric by coat color.

mal animals of the same strain from which the embryos were isolated, and the F1 progeny were analyzed for germ-line transmission of the mutant allele of type II collagen by PCR (8).

RESULTS

Removal of the Zona Pellucida

To allow successful integration of ES cells into the embryo, the zona pellucida was removed by treating the embryos with acid Tyrode solution (7). Since Tyrode solution has an acidic pH of 2.5, a short duration is critical for embryo survival. Approximately 90%–

95% of the embryos developed to healthy appearing blastocysts when the treatment was kept shorter than 40–50 s. Survival decreased to about 20% when exposure was longer than 90 s (data not shown). Therefore, the embryos were monitored constantly under a stereomicroscope during the treatment to prevent prolonged exposure to acidic pH.

Viability of Cultured Embryos

Previously, Wood et al. (22) observed that long-term culture of embryos over the bed of ES cells is detrimental to their survival. To determine whether a high protein concentration would prevent adhesion of zona-free embryos with each other during handling and allow maximum survival of the embryos after coculture, embryo manipulations were performed in Brinster's medium with different concentrations of BSA. Only a minimal amount of adhesion between the embryos occurred when 10 mg/mL BSA were added to the culture medium, and approximately 90%–95% of the embryos developed into blastocysts. Most ES cells appeared to integrate with the embryos after overnight culture, and no sign of cell death inside or outside the microwell was noticed under these conditions. Small aggregates of ES cells attached to the embryos were occasionally seen that also integrated with the embryos following extended cultures.

Contribution of ES Cells to Neonates

Data presented in Table 1 show the results of three independent experiments on coculture of gene-targeted J1 cells with inbred FVB/N embryos

Figure 2. Chimeric animals prepared from coculture of FVB/N embryos (albino) with J1 cells derived from 129Sv (agouti) strain of mice. Three chimeras displayed almost complete ES cell-derived phenotype, whereas the other three were between 70% and 90% chimeric.

Table 3. Coculture of C57BL/6 Embryos with RW4 ES Cells

Experiment #	No. of Embryos	No. of Foster Mothers	No. Pregnant	Total No. of Pups	No. of Chimeras
I	28	3	3	10	8
II	11	1	1	4	4
III	21	3	3	9	6

Table 4. Germ-line Transmission of Chimeric Animals

Experiment #	No. Tested	Sex	No. Transmitted
I	3	M	3
II	2 ^a	M	1
III	2 ^b	M	1

^aThe animal that did not transmit the gene was <50% chimeric.
^bOne chimeric animal that displayed almost 100% contribution of ES cells was sterile.

(albino coat color). A total of 97 embryos were implanted into 8 recipient mothers. Six of the recipients delivered 17 pups, of which 14 pups (83%) were chimeric for the ES cells. Twelve chimeric animals displayed more than 70%–80% contribution of ES cells, as observed by the coat color. From seven chimeras that survived to adulthood, three were almost indistinguishable from the agouti 129 Sv strain of mice from which the J1 cell line was derived, suggesting complete derivation from ES cells (Figure 2). Similarly, the coculture of 59 FVB/N embryos with R1 cells produced 8 pups, of which 6 pups were over 75% chimeric by coat color (Table 2: Experiments I and II).

A high degree of chimerism in the newborns was also observed when R1 cells were cultured with C57BL/6 embryos. Of the 29 embryos that were implanted into three recipient mothers, a total of 12 pups were born (Table 2: Experiment III). Six of these pups were over 90% chimeric. The other six were cannibalized by the mothers before testing. However, the carcasses from two of the cannibalized pups had light pale skin, indicating a high contribution of ES cells.

To further test the efficacy of this method, C57BL/6 embryos were cultured with RW4 ES cells, a cell line derived from the 129SvJ strain of mice with chinchilla background. As shown in Table 3, a total of 23 pups were born

from 60 embryos in three separate experiments. Eighteen pups (80%) displayed more than 90% contribution of RW4 cells, determined by the color of the skin and pink eye pigmentation in contrast to black pigmentation of the host embryos. About 50% of these chimeras had open eyelids and were cannibalized by the mothers. Two of the surviving pups displayed almost complete RW4 ES cell contribution, with chinchilla coat color, whereas a third pup showed only about 50% contribution, with large, white patches on a black background (not shown).

Germ-Line Transmission of ES Cell Markers

Chimeric animals prepared from the coculture of ES cells with a targeted mutation in collagen type II were crossed with normal FVB/N animals, and the progeny were examined by PCR or by Southern blot analysis using ³²P-labeled probes for the collagen type II gene (8). All the fertile chimeras that displayed more than 75% chimerism transmitted the mutant allele to their progeny (Table 4). One chimera that displayed almost 100% contribution of ES cells was sterile and did not produce any progeny. However, the other two high-degree chimeras transmitted ES cell markers to almost 100% of their progeny, suggesting a complete derivation of the germ line in these animals from ES cells.

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DISCUSSION

Gene knockout by means of homologous recombination in ES cells is a powerful system to unravel the function of genes that control development and differentiation in the organism. Techniques that are simple, efficient and reproducible can therefore expedite the

process for functional analysis of specific genes.

The method described here is highly reproducible and is based on the hypothesis that ES cells establish an axis of integration into the embryo (Figure 3). In the method described by Wood et al. (22), embryos are cultured over a bed of ES cells, and the embryos with

adherent ES cells are subsequently collected and transferred into another petri dish. This requires certain manipulations before there is complete integration of ES cells into the embryos. The microwells in the present procedure provide a physical axis for efficient integration of ES cells into the embryos.

This concept is supported by the

Figure 3. Model for the integration of ES cells into a morula. (A) The bottom of the microwell provides an area for tight cell-cell contact and simulates the formation of ES cell aggregates described in previous methods (13,14,22). (B) Transfer of embryos over these cells establishes cell-cell-embryo contact. As the cells of the embryo continue to divide and move around the ES cells (downward arrows), an axis of ES cell integration is established (upward arrow). (C) Further growth of the embryo therefore results in internalization of ES cells into the embryo.

hypotheses proposed by other investigators for the development of preimplantation embryos (6,20,23,24). In preimplantation embryos, the apolar cells on the inner side of morulae are directed to differentiate into the inner cell mass, whereas the outer polar cells are directed to form the trophoctoderm (TE). Therefore, the ES cells in our microwell coculture procedure may localize to the inside of the embryo and contribute predominately to the inner cell mass, whereas the cells from the morula that are on the outside contribute to the formation of TE. It is also possible that ES cells may induce embryonic cells to form TE.

In contrast to previous observations (22), 90%–95% of our embryos survived consistently, and animals with more than 90% chimerism could be generated reproducibly even after overnight culture of embryos with ES cells. This may be attributed to the following: (i) a low concentration of ES cells used for the coculture; (ii) a simple medium that is known to be conducive to the growth of preimplantation embryos (7); and (iii) a small number of ES cells in microwells, which may prevent the overgrowth and abnormal development of the embryo. The breeding of chimeric animals with normal animals revealed that most of the animals with high chimerism were fertile and capable of germ-line transmission.

The microwells described in this procedure can be constructed by simple tools available in any laboratory. The data obtained with two different strains of mice and three independent ES cell lines suggest that the method may be also applicable to other strains of mice and ES cell lines. It may also be an alternative to microinjection of DNA into zygotes (15) to identify novel genes and their regulatory sequences with the gene trap or promoter trap vectors (4,17) or to create transgenic animals for large animal species in which the availability of zygotes is generally limited and the ES cell lines have been established (18,21).

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