Establishment of Germ-line Competent C57BL/6J Embryonic Stem Cell Lines

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Objective To establish C57BL/6J embryonic stem (ES) cell lines with potential germ-line contribution

Methods ES cells were isolated from blastocyst inner cell mass of C57BL/6J mice, and cultured for 15 passages, and then injected into blastococels of ICR mice blastocysts to establish chimeric mice.

Results Three ES cell lines (mC57ES1, mC57ES3, mC57ES7) derived from the inner cell mass of C57BL/6J mice blastocysts were established. They were characteristic of undifferentiated state, including normal XY karyotype, expression of a specific cell surface marker “stage-specific embryonic antigen-1” and alkaline phosphatase in continuous passage. When injected into immunodeficient mice, mC57ES1 cells consistently differentiated into derivatives of all three embryonic germ layers. When mC57ES1 cells were transferred into ICR mice blastocysts, 4 chimeric mice have been obtained. One male of them revealed successful germ-line transmission.

Conclusion We have obtained C57BL/6J ES cell lines with a potential germ-line contribution, which can be used to generate transgenic and gene knock-out mice.

Key words: C57BL/6J mice; embryonic stem (ES) cell; ICR mice blastocysts; germ-line competent

Embryonic stem (ES) cells, derived from the inner cell mass of preimplantation embryos, have normal and stable karyotypes, express high levels of telomerase and phosphatase activity, and express conserved epitope markers. Under certain conditions, these cells are capable of unlimited, undifferentiated proliferation in vitro. In chimeras with intact
embryos, mouse ES cells contribute to a wide range of adult tissues, including germ cells, providing a powerful approach to introducing specific genetic changes into the mouse germ line[6]. Gene targeting has been widely used, particularly in mouse ES cells, to make a variety of mutations in many different loci so that the phenotype consequences of specific genetic modifications can be assessed in the organism. Now, hundreds of knock-out mice models of human genetic diseases have been generated, which will be convenient for us to study gene function in a mammalian organism.

The past decade has seen rapid exploitation of ES cells to propagate mutations created by gene targeting, taking advantage of the possibility of transmitting the altered gene through the germ-line of a chimera. However, as genetically and functionally relevant backcrossing (from 129 mice to, for example, C57BL/6) is only practical under specific circumstances where close linkage of other important and modifying genes to the targeted gene can be determined or avoided, the necessity for ES cells from strains other than 129 is increasingly evident. Efforts to generate stable ES cell lines from mouse strains other than 129 have led to the isolation of C57BL/6 and BALB/c ES cells. Ledermann et al.[7] successfully obtained a C57BL/6 ES cell line, and generated germ-line-transmitting chimeras. There have been reports in China about the establishment of C57BL/6J ES cell lines with different characters[8, 9]. The C57BL/6 mice ES cell lines have wide applications in immunological studies and production of transgenic models. Lemckert et al.[10] successfully generated gene knock-out mice in the C57BL/6 mouse strain using targeted C57BL/6 ES cell lines. We reported here the successful establishment of valuable germ-line competent C57BL/6J ES cell lines which can be used for gene targeting.

Materials & Methods

Embryo recovery and ES cell line isolation

The chemicals used for cell culture, were purchased from GIBCO/BRL, except for those particularly mentioned. C57BL/6J mice were purchased from the Experimental Animal Center, Shanghai Institute of Planned Parenthood Research (SIPPR). Blastocyst stage embryos were flushed from uterine horns of 3.5 days post-coitus (dpc) females. Mouse embryonic fibroblasts (MEF) cells derived from 14 dpc embryos were used as feeder layers, and cells were subcultured no more than 4 passages. Blastocysts initially were cultured in one well of a 4-well tissue culture plate (Nunc) containing mitomycin C treated MEF feeders. The cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM; no pyruvate, high-glucose formulation) supplemented with 15% FBS, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 1 % nonessential amino acid stock, 0.1 mmol/L β-mercaptoethanol, 100 u/mL of penicillin, 100 µg/mL of streptomycin and 1000 u/mL of recombinant mouse leukemia inhibitory factor (mLIF, CHEMICON). Cultures were grown at 37°C under an atmosphere of
5% CO₂ and 95% air.

After being cultured for 7-8 d on the feeder layer, the adequately proliferated ICM-derived clumps were disaggregated into small clumps with 0.05% trypsin-0.53 mmol/L EDTA (GIBCO/BRL) and then replated on feeder layer in fresh medium and observed for colony formation. After 5 d’s culture, undifferentiated colonies were individually selected, trypsinized again, and cultured in the same DMEM-supplemented medium. Early passage cells were frozen and stored in liquid nitrogen.

**Identification of the embryonic stem cells**

Following 48 h culture, ES cells were fixed for detection of alkaline phosphatase activity with 4% paraformaldehyde for 20 min at room temperature and then stained with NBT-BCIP substrate (Zymed) at 37°C for 30 min. Feeder layer cells were used as the control. Fifteenth passage mC57ES1 cell line cultured on a layer of MEF was used for examination of cell surface marker expression. The SSEA-1, SSEA-3 and SSEA-4 antigens were detected by immunocytochemistry with specific monoclonal antibodies. The MC480 (SSEA-1), MC631 (SSEA-3), MC813-70 (SSEA-4) monoclonal antibodies were purchased from DAKO Corporation. Antibodies were detected by using FITC labeled anti-mouse secondary antibody (Sigma). After being incubated for 30 min, the mixture was washed and observed under Olympus fluorescence microscope.

Fifteenth passage ES cells were incubated in growth media with 0.4 µg/mL of colchicine (Sigma) for 1 h they were trypsinized, resuspended in 0.075 mol/L KCl, and incubated for 30 min at 37°C, then fixed in 3:1 methanol/ acetic acid for 3 times, the cells were further karyotyped with a standard G-banding technique and Lucia chromosome figure analysis software was used to analyse their karyotypes.

**Differentiation experiment**

An amount of 10⁶ mC57ES1 cells (passage 14) were plated on gelatin-treated 35 mm tissue culture plate (Falcon) in growth media in the absence of mLIF. In order to avoid ES cells clumps attaching to the wall, the medium was changed every other day and blown gently. After 3 weeks of suspension culture, embryoid bodies and differentiated cells were observed.

An amount of 10⁷ mC57ES1 cells (passage 14) were injected into rear leg muscles of 4-week-old male Balb/c-nu mice (Laboratory Animal Center, Chinese Academy of Sciences). Twenty one d after injection, the teratomas were fixed in Bouin’s mixture, embedded with paraffin, and sectioned (5 µm) using a Leica ultramicrotome. After being stained with HE, the resulting differentiated cells of the teratomas were observed under microscope.

**Production of chimeras**

The mC57ES1 cells (passage 14) were passaged two d before injection. Before injection, ES cells were harvested and plated twice on gelatine-coated (0.1%) 35 mm tissue culture plate to remove fibroblasts and resuspended in M2 (Sigma) containing 10%FBS and 1 000 u/mL
of mLIF. ICR blastocysts were micro-injected with ES cells at 37℃. Each blastocyst received 10-15 ES cells. Injected blastocysts were reimplanted into F1 (ICR × C57BL/6J) pseudopregnant (d 2.5) foster mothers. Totally 8-9 blastocysts were implanted in one uterine horn.

**Results**

**Three pluripotent embryonic stem cell lines derived from C57BL/6J blastocysts were obtained**

Eleven fine blastocysts were recovered from two 3.5 dpc C57BL/6J female mice. Three pluripotent embryonic stem cell lines (mC57ES1, mC57ES3, mC57ES7) derived from C57BL/6J blastocysts were established. These cells had compact colony morphology, smooth and clear borderline, which was similar to previous literatures (Figure 1-3).

The cultured 15th passage of mC57ES1 cells possessed high levels of alkaline phosphatase activity (Figure 4). mC57ES cells expressed SSEA-1 (Figure 5), but did not express SSEA-3, SSEA-4, which was consistent with the characteristics of undifferentiated mouse embryonic stem cells. Each of the three mC57ES cell lines was XY karyotype, and had a rate of normal diploid cells above 80% (Figure 6, 7).

The synergism of feeder cells and mLIF prohibited mC57ES1 cells from differentiation and generation of embryoid bodies. When ES cells was removed from fibroblast feeders or mLIF withdrawed, rhythmic beating myocardial-like cells and EBs were observed 7-10 d later (Figure 8).

Paraffin sections of teratomas revealed that mC57ES1 cells can differentiate into cells of a variety of types, including blood vessels, muscle cells, cartilage, epithelial cells, fat cells, lymphocytes and neural cells derived from the three embryonic germ layers (Figure 9).

**Four chimeric mice were obtained**

Altogether 28 ICR blastocysts were micro-injected with mC57ES1 cells, and were reimplanted into 3 pseudopregnant foster mothers. Eight offsprings were born and alive, among whom 4 were chimeric mice (3 males, 1 female). Each of these chimeric mice has black eyes, but different mixture of color on hairs of the head and back, mainly of black and white or black and pale-grey. The degree of coat color ranged between 50%-95%. These mice were then mated with female ICR mice. According to the coat color of their litters, one male was identified as germ-line chimera (Figure 10).

**Discussion**

In 1981, pluripotent embryonic stem cells derived from the inner cell mass of mouse blastocyst stage embryos were isolated and conditions defined for their propagation and
maintenance in culture⁴. Up to now, isolation of embryonic stem cell lines has been attempted in the hamster (1988)¹¹, mink (1998)¹², pig (1994)¹³, rhesus monkey (1995)¹⁴, rabbit (1996)¹⁵, sheep (1997)¹⁶ and human (1998)¹. Using stringent culture conditions and expression of specific differential genes, ES cells may directionally differentiate into a wide variety of cell types in culture, including myocardial cell¹⁷, haematopoietic stem cells¹⁸, neural cells¹⁹, sperm²⁰ and oocyte²¹. Cell-type-specific differentiation and the isolation of lineage-restricted stem cells could serve as a source of cells for transplantation. Genetic modification of these pluripotent stem cells may allow the generation of universal cells or cells that have been customized to meet individual requirements. Obviously, these embryonic stem cells could increase our understanding of many diseases which are hazardous to human.

Figure 1  An attached and proliferated C57BL/6J blastocyst ICM (× 100)
Figure 2  After being cultured for 5 d, an ICM-derived clump suitable for isolation (× 400)
Figure 3  mC57ES1 colonies growing on MEF (× 100)
Figure 4  AKP positive mC57ES1 colonies growing on MEF (× 100)
Figure 5  mC57ES1 cells express epitope recognized by antibodies against SSEA-1 (× 400)
Figure 6-7  Karyotype of the 15th passage of mC57ES1 line, 40: XY
Figure 8  An embryoid body of mC57ES1 cells cultured in vitro (× 400)
Culture condition plays an important role in maintaining the growth of ES cells. During initial cell line isolation, we used 25% FBS and 5,000 u/mL mLIF to benefit inner cell mass, leading to enough proliferation and undifferentiation. mC57ES cells had a high nucleus/cytoplasm ratio, tightly compacted multicellular colonies morphology with smooth, clear borderline and vague cellular outlines, which resembles ES cells derived from 129 strain, but is but is distinguishable from “MESPU” that is large and slabby and grows slowly. Each of the 3 ES cell lines was strongly dependent on feeder layers, whose $10^4$-$10^5$ cells/cm² could keep their stem characteristics and grow normally. When removed from feeder layers, mC57ES cells still couldn’t maintain undifferentiation stage, even in the presence of 5,000 u/mL mLIF. The colony morphology became flat and loose and differentiated cells appeared in the colony margin.

ES cells need to be karyotyped and evaluated after continuous passage. Normal diploid karyotype rate of good embryonic stem cell lines is usually about 80%[22]. Normal diploid
karyotypes rates of our ES cell lines (mC57ES1, mC57ES3, mC57ES7) derived from the inner cell mass of C57BL/6J mice blastocysts are all above 80%. These cells expressed a series of cell surface markers (alkaline phosphatase+, SSEA-1+, SSEA-3- and SSEA-4-) that characterize undifferentiated mouse ES cells. Paraffin sections of teratomas revealed that mC57ES1 cells can differentiate into a variety of cell types derived from the three embryonic germ layers. mC57ES cells with undifferentiated state and developmental pluripotentiality can be applied to genetic manipulations.

So far, over 1,000 genetic mutant mice models with different mutations have been established, while 129 ES cells lines were the most commonly used in gene targeting research. The targeted ES cells were injected into C57BL/6 or BALB/c blastocysts to produce gene knock-out mice. However, large quantities of available embryos can’t be retrieved from the above two strains mice, which baffled a high probability of the knock-out mice production. It is becoming increasingly obvious that a pure genetic background is highly desirable for the evaluation of effects of gene targeting with insufficient backcrossing producing potentially conflicting results\cite{23, 24}. The genetic control of regulatory and compensatory processes is still insufficiently understood and the genes that control them are relatively undefined. Thus, concerns have been raised about the ambiguity of results derived from backcrossed animals and also the exclusive use of the 129 mouse strains in biomedical research. The availability now of ES cells derived from the C57BL/6 mouse strain presents workers with a valuable alternative\cite{10}. The significant advantage of the production of gene knock-out mice directly in mouse strains other than 129 is the use of ICR strain of blastocysts donor, which results in high yields of healthy embryos and good embryonic development that enable good levels of germline competence.

Due to the low technical level in generating stable ES cell lines, there are currently only a few reports in China about studies on the generation of gene knock-out mice. In our study, we paid special attention to the culture conditions. Before injection, mC57ES1 cells must grow for no more than 40 h in the presence of 1,000 u/mL mLIF and low density of feeder layers (10^4 cells/cm^2). Four chimeric mice with high degree of coat color ranging between 50%-95% were obtained in our study. Mouse ES cells in chimeras sometimes contribute to germ cells, thus providing a vehicle for introducing genetic changes into the germ-line\cite{6}. The degree of coat color chimerism correlates with the degree of germ-line contribution. When above 85% of the coat in a series of any chimera was judged as the germ-line-competent chimera, genetic features of ES cells could be transferred to every tissue and organ\cite{25}. One germ-line competent C57BL/6J male chimeric mouse was obtained in our study, which proved our success in establishing an embryonic stem cell line that could be used to create transgenic and gene knock-out mice.
References


