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Factors Affecting the Productivity of Germ-line Chimeras from J1 Embryonic Stem Cells

Kim S. U., B. S. Koo, S. Jeong, T. H. Lee, S. L. Yu, Y. I. Nam,[1] J. L. Kim,[2] B. H. Hyun,[1] H. S. Shin,[2] K. K. Lee, B. C. Sang[3] and D. Y. Yu†
Korea Research Institute of Bioscience and Biotechnology

J1 배아주세포를 이용한 효율적인 생식선 이행 카이미라의 생산

김선욱 · 구본실 · 정상균 · 이태훈 · 유성란 · 남윤이[1] · 김정림[2] · 현병화[1] · 신희섭[2] · 이경광 · 상병찬[3] · 유대열†
한국생명공학연구원 동물발생공학연구실

ABSTRACT

This experiment was designed to improve the production efficiency of germ-line chimeric mice from phospholipase C (PLC)-β3 or peroxiredoxin (Prx) Ⅱ -targeted (Δ) ES cells by the investigating the manipulation conditions and characteristics of J1 ES cells. Four targeting clones were isolated to investigate the karyotypical and morphological stability prior to injection. All clones (ΔPLCβ-3 C3, ΔPrx Ⅱ C3, C10 and I5) showed more than 80% euploidism, however, most of ΔPLCβ-3 C3 clones were extensively differentiated compared to the other clones. Nine of 13 ΔPrx Ⅱ chimeras appeared to have at least 80% chimerism, whereas ΔPLCβ-3 C3 chimeras had 20% chimerism at most. Therefore, the morphological stability of ES cells under stable euploidism might mainly affect the production rate of high-coat chimeric mice. To increase the collection rate of injectable blastocysts (IBs), 5 to 10 week -aged C57BL/6J female mice were sacrificed at 3.5 days post-coitum. Ten week-aged mice were the most optimal IB donors by showing the highest collection rate (2.94/mouse) of injectable blastocysts without increase of non-injectable embryos (0.29/mouse). Foster mothers might be another factor because ICR×C57BL/6J F1 foster mother showed more increased productivity in litter size (2.8 vs. 5.6) and chimera (0 vs. 35.3%) than those of ICR foster mothers. In conclusion, the efficient production of germ-line chimeras mainly depends on the maintenance of ES cell morphology during targeting procedure, and the establishment of manipulation conditions might be a key point to maximize it.

(Key words : Embryonic stem cells, Chimerism, Germ-line chimera, Morphology, Karyotype)

† Correspondence and reprint request : Laboratory of Animal Developmental Biotechnology, Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Yusong, Taejon 305-600, Korea, Tel; 042-860-4422, Fax; 042-860-4608, E-mail; dyyu10@mail.kribb.re.kr

[1] 한국생명과학연구원 실험동물실 (Laboratory of Experimental Animal Resources, ICLAS Monitoring Subcenter Korea, Korea Research Institute of Bioscience and Biotechnology)

[2] 포항공과대학교 생명과학과 (Department of Life Science, Pohang University of Science and Technology)

[3] 충남대학교 낙농학과 (Department of Dairy Suence, Chungnam National University)

Ⅰ. INTRODUCTION

Murine embryonic stem (ES) cells were isolated from inner cell masses (ICMs) of blastocysts in mice (Evans and Kaufman, 1981; Martin, 1981). ES cells are pluripotent and can contribute to somatic and germinal development when they are introduced into the preimplantation embryos (Bradley et al., 1984). Thus, ES cells may give a feasible opportunity to produce transgenic animals by cell-mediated gene transfer. ES cells have been widely used to produce the genetic disease models by the alteration of specific genomes of mice. J1 ES cells used in this study were derived from ICMs of male agouti 129/terSv blastocysts and used for targeted mutation of β2-microglobin (Zijlstra et al., 1989), DNA methyltransferase (Li et al., 1992), and Ca^{2+} channel alpha(1A)-subunit (Jun et al., 1999). However, there were few reports on technical investigations for efficient production of knock-out mice from J1 ES cells.

The chromosome complement of ES cells is pivotal in the efficient production of germ-line chimeras. It was known that ES cells almost invariably display a modal distribution of chromosomes and that aneuploid cells tend to increase with increasing culture time (Robertson, 1987; Mitalipov et al., 1994). The increase of anuploid cells may inhibit ES cells from differentiating into germ line. Therefore, the euploidism can be served as one of major factors for efficient gene targeting.

C57BL/6J mice have been frequently used to efficiently derive the germ-line transmitters from ES cells of 129 strain (Schwartzberg et al., 1989). As C57BL/6J mice have its poor reproductive capacity, CD-1 mice are alternatively used as a blastocysts donor, but they showed a limited germ line compatibility with clonal ES lines tested (Papaioannou and Johnson, 1993; Schwarzberg et al., 1989). To efficiently produce the chimeras, the collection rate of C57BL/6J embryos needs to be improved.

The production of high coat-chimeric mice for high incidence of germ line transmission is central to the knock-out experiment. Although J1 ES cells were extensively used in knockout experiment, the conditions influencing on the production of high-coat chimeric mice from J1 ES cells are pooly understood.

In this report, we purposed to establish the conditions of J1 ES culture and manipulation techniques for efficient production of mice with high coat pigmentation by investigating the factors that can influence a gene targeting efficiency.

Ⅱ. MATERIALS AND METHODS

1. Culture of ES Cells

The culture medium for J1 ES cells was Dulbecco's modified Eagle's medium (DMEM; high glucose, GibcoBRL) containing 10% fetal bovine serum (FBS; HyClone, defined), 5% ES-qualified FBS (GibcoBRL), 0.1 mM of β-mercaptoethanol (Sigma), nonessential amino acids (GibcoBRL) and antibiotics (GibcoBRL). Murine leukemia inhibitory factor (LIF; GibcoBRL) was not used during routine passage of J1 ES cells, but was added to 500 U/ml during selection and cloning culture. The G418-resistant feeder layers were prepared from mouse embryonic fibroblast (MEF) carrying *neo* gene by mitotical inactivation using mitomycin C (10 μg/ml). J1 ES cells were seeded onto the MEF feeder layer prepared 24 h before. ES cells were passaged every 48 h by the treatment of 0.25% trypsin/5.3 mM EDTA for 5 min.

2. Preparation of Targeting Clones

Electroporation was performed using Bio-Rad Gene Pulser apparatus under following conditions,

mixture of 15~20 μg linearized targeting DNA and 2×10^7 cells in a volume of 800 μl, 250~270 voltage and 500 μF capacitance. Electroporation medium was 20 mM Hepes (pH 7.05), 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose and 0.1 mM β-mercaptoethanol. Electroporated cells were cultured at a density of $2.5 \sim 3 \times 10^6$ cells per plate. Nontransformant and most of random integrated ES colonies were removed by double-selection with 200 mg/ml of active G418 (GibcoBRL) for 6 days and with 2 μM gancyclovir for 4 days. Morphologically normal ES colonies were picked, transferred onto 96-well culture dish, and subsequently passaged onto 24-well culture dish. Half of the expanded cultures were cryopreserved in LN_2 and the others were used to determine the homologous recombination. Homologous event was detected by Southern hybridization. Targeting clones were karyotyped to investigate the euploidism.

3. Karyotype Analysis

Exponentially growing ES cells were treated with 0.02 μg/ml of colcemid (GibcoBRL) for 1 h. The cells individualized with 0.25% trypsin/5.3mM EDTA were treated with a hypotonic solution of 0.56 % KCl for 10 min at room temperature and the resulting swollen cells were fixed at least four times with freshly prepared ice-cold Carnoy's fixative (3 : 1 volume of absolute methanol to glacial acetic acid) for 10 min, respectively. The fixed cells were dripped onto clean glass-slide and dried for 24 h. After staining with 4% Giemsa dye (GibcoBRL) for 5 min, the spreads were observed microscopically.

4. Production of Chimeras

Blastocysts were prepared from C57BL/6J female mice of 3.5 days post-coitum (d.p.c.) after natural mating with males of the same strain. C57BL/6J female mice of 5 to 10 week-age were sacrificed to collect stable number of injectable embryos. Medium for embryo collection and injection was DMEM containing 10% FBS (HyClone, defined) and 20 mM Hepes (pH 7.2). Fifteen ES cells were introduced into the blastocoel cavity via junction among trophectodermal cells of blastocyst. Eight to twelve chimeric blastocysts were transferred into the uterus of pseudopregnant ICR or ICR(♀) × C57BL/6(♂) F1 hybrid (IBF1) foster mothers. Chimerism was judged by coat pigmentation. All chimeras were back-crossed with C57BL/6J mice for the examination of the germ-line transmission.

Ⅲ. RESULTS AND DISCUSSION

Gene targeting is a feasible method in order to study the functions of genes. We purposed to establish the targeting conditions and to produce chimeras with high chimerism from J1 ES cells.

ES cells have typical morphologies *in vitro*, such as a sharpness of colony boundary, a compacted cell-cell adhesion, a large nucleus, high sensitivity to alkaline phosphatase stain, etc. It is important to maintain the characteristics of ES cells throughout targeting procedure. LIF (1,000 U/ml) alone could not support the proliferation of ES cells without apparent differentiation during selection period. We experienced that most of J1 colonies extensively differentiated during selection because wild type MEF without *neo* was degenerated by G418 selection pressure. MEF containing *neo* could support the proliferation of J1 ES colonies without extensive differentiation during selection period (personal observation).

C57BL/6J mice are widely used and commercially available and differ from 129 mice in coat colour and at other genetic loci which are useful as markers. Their embryos proved to be compatible hosts for 129 ES cells; chimera formation and most importantly germ-line transmission could be efficiently obtained (Papaioannou and Johnson, 1993).

C57BL/6J mice are high ovulator (Hogan et al., 1986), but they are not practically suitable embryo donor in a lot of laboratories. Lemckert et al. (1997) reported the strategy in order to yield of healthy Balb/c blastocysts (Lemckert et al., 1997). They overcame the problem of low embryo collection rate by harvesting Balb/c embryos at the early morula stage and maturing them to blastocysts by *in vitro* culture. In the previous experiment, five to six week-aged C57BL/6J mice did not produce sufficient number of fully-expanded blastocysts by artificial superovulation with gonadotropin (data not shown). Non-injectable embryos (NIE), such as zona-free and fragmented blastocysts, were frequently collected. The main reason of the drawback might result from non-permissive mating and sexual immaturation of C57BL/6J female mice. Therefore, 5 to 10 week-aged C57BL/6J mice were tested by natural mating to collect healthier embryos than those of artificial superovulation (Table 1). Seven to 10 week-aged females, except only 8 week-age, inclined to show relatively fewer collection rates of NIEs than those of 5 to 6 week-aged mice. Especially 10 week-aged mice could show the highest collection rate (2.94/mouse) of IBs per mouse without the increase of NIEs (0.29/mouse). In subsequent experiment, 11 to 13 week-aged female mice could exhibit similar fecundity of reproduction compared with 10 week aged mice (data not shown). Therefore, karyotypically stable clones were injected into the blastocysts of at least 10 week-aged C57BL/6 female mice.

Table 1. Relationships between injectable embryos and the age of mice

Age of mice (weeks)	No. of mice sacrificed	No. of embryos collected (*NEC)	
		IB	NIE
5	92	75 (0.82)	81 (0,88)
6	25	15 (0.60)	9 (0.40)
7	23	32 (1.39)	32 (1.39)
8	34	19 (0.56)	7 (0.21)
9	15	20 (1.34)	13 (0.87)
10	17	50 (2.94)	5 (0.29)

Abbreviations are IB, injectable blastocysts; NIE, non-injectable embryos.
* NEC = no. of embryos collected per mouse.

One PLCβ-3 (ΔPLCβ-3 C3) and 3 Prx Ⅱ targeting clones (ΔPrx Ⅱ C3, C10, and I5) were isolated and used to produce chimeras. Chimeric embryos between ΔPrx Ⅱ C3 clone and C57BL/6J blastocysts could better develop to term in the uterus of pseudopregnant IBF1 than in those of ICR foster mothers (Table 2). There was no difference in pregnancy rate (71.4 vs. 75.0%), but litter size (2.8 vs. 5.6%) and chimera productivity (0 vs 35.3%) were higher in IBF1 than those of ICR. It suggests that a compatibility might exist between chimeric blastocysts and strains of foster mothers.

It was reported that chromosome make-up is critical to transmit ES cells to germ line (Liu et al., 1997) and none of the ES cell clones with more than 50% of chromosomally abnormal metaphases was transmitted to the germ line (Longo et al., 1997) and that A3-1 ES cells which exhibit cystic embryoid body forming ability at 7 day suspension

Table 2. Compatibility between chimeric embryos and strains of foster mothers

Strain of FM	No. of chimeric embryos transferred	No. of pregnant /no. of FM (%)	No. of new born (litter size)	No. of chimeras / no. of new born(%)
ICR	75	5/7 (71.4)	14/5 (2.8)	0/14 (0)
IBFI	36	3/4 (75.0)	17/3 (5.6)	6/17 (35.3)

Abbreviation is FM, foster mother.

Table 3. Production of germ-line chimeras derived from PLCβ-3 or Prx Ⅱ-targeted ES cells

Clones		Eup. (%)	Level of Morph.	No. of BLs injected	No. of chimeras among pups(%)	*Chimerism						No. of GLTs (%)
						Male			Female			
						A	B	C	A	B	C	
PLCβ -3	C3	>80	+	256	15/71 (21.1)	7	–	–	8	–	–	0
Prx Ⅱ	C3	80.7	+++++	155	7/39 (17.9)	–	1	5	–	–	1	5 (71.4)
	C10	82.4	++++	137	3/18 (16.7)	–	1	2	–	–	–	0
	I5	87.5	++++	55	3/12 (25.0)	–	–	2	–	1	–	0

Abbreviations are Eup., euploidism; Morph., Morphology; BL, blastocyst; GLT, germ-line transmitter.
*A= $<$ 20%; B= 40~60%; C= $>$ 80% coat chimerism.

culture and proximately 40% of normal karyotype were capable of germ line transmission in chimeric mice (Suzuki et al., 1997). Therefore, it might be a critical point to investigate the relationships between the characteristics of ES cells and the production of germ-line chimeras. The results concerned with the derivation of germ-line chimeras were summarized in Table 3.

Each 4 clones was graded by euploidism (% of normal karyotypes) and morphology of colonies. The rate of euploidism was judged by counting more than 30 metaphases. The morphological levels of the colonies were divided into 5 sub-groups by checking the frequency of injectable ES cells with finely-shaped membrane and small diameter (data not shown). ΔPrx Ⅱ C3, C10 and I5 showed more than 80% euploidism, however, mophologically differentiated cells were predominant in ΔPLCβ-3 C3 clone (morphological level = 1). Fifteen chimeras (21.1%) obtained from ΔPLCβ-3 C3 clone had less than 20% coat chimerism. Besides, the male chimeras (7/15, 46.7%) were not primarily produced from the ΔPLCβ-3 C3 clone. In contrast, most of chimeras produced from the other three clones with stable morphology (morphological level = 4~5), ΔPrx Ⅱ C3, C10, and I5, had high -coat chimerism (9/13, 69.2%) as well as high incidence of male chimeras (11/13, 84.6%). However, there were no significant differences in the production rates of chimeras (21.1, 17.9, 16.7 and 25.0 %) from the 4 clones. These results suggest that the morphological stability of ES cells under stable euploidism (at least 80%) might mainly affect the rate of high-coat chimeric mice. Five of 10 chimeras with more than 80% chimerism could also generate germ-line transmitters as a report that high chimerism can give rise to a high incidence of germ line contribution of ES cells (Suzuki et al., 1997). From these investigations, the maintenance of J1 ES cells without the loss of the karyotypical and morphological characteristics during cloning periods was the most important factor throughout gene targeting procedures.

In conclusion, it is important to establish the conditions for the systematic maintenance of ES cell clones until blastocyst injection. Above all, the karyotypical and morphological stability seemed to be a major critical point for the production of high -coat chimeras from J1 ES cells. In addition, the elevated collection rate of blastocysts and the suitable choice of foster mothers might be factors to be considered to improve the efficiency for the production of chimeras.

Ⅴ. 요 약

본 실험은 phospholipase C (PLC)-β-3 및 peroxiredoxin (Prx) Ⅱ 유전자가 적중 (Δ)된 J1 마우스 배아주 (embryonic stem) 세포로 부터 생식선이행 카이미라 마우스생산을 위한 제반조건 및 배아주세포의 배양조건을 확립하기 위하여 수행되었다. 80% 이상의 정상핵형을 보이는 유전자 적중된 4개의 클론 (ΔPrx Ⅱ C3, ΔPrx Ⅱ C3, C10 및 I5)으로부터 카이미라를 생산하였을 때 형태적으로 분화정도가 높은 클론 (ΔPLCβ-3 C3)의 이용은 카이미라의 생산율 (21.1%)과는 무관하게 카이미리즘 (< 20%)이 낮았고, 수컷 카이미라의 생산 빈도 (7/15, 46%)도 낮아지는 것으로 나타났다. 그러나 형태적으로 안정된 3개의 클론 (ΔPrx Ⅱ C3, C10 및 I5)은 80% 이상의 높은 카이미리즘을 지닌 마우스 (9/13, 69.2%)를 생산하였고, 수컷 카이미라의 생산율 (11/13, 84.6%)도 증대된 것으로 나타났다. 따라서 80% 이상의 정상핵형을 지닌 배아주세포를 형태적으로 안정하게 유지하는 것이 카이미리즘이 높은 마우스를 생산하는데 결정적 요인으로 작용할 수 있는 것으로 확인되었다. 미세주입용 배반포를 효율적으로 생산하기 위해 5∼10주령 사이의 C57BL/6J 암컷마우스를 교배한 결과, 10주령 마우스가 미세주입가능한 3.5일령 배반포를 가장 많이 생산 (2.94개/마리)하였다. 또한 미세주입된 배반포를 이식하기 위해 ICR 및 ICR×C57BL/6J F1 (IBF1)위임신 대리모를 사용하였을 때, IBF1이 복당 산자수 (2.8 vs. 5.6)가 많았고 카이미라 생산율 (0 vs. 35.3%)도 매우 높았다. 따라서 공여마우스의 주령 및 대리모의 선택이 카이미라 생산효율 향상에 중요한 요인으로 부각되었다. 결과적으로 핵형이 안정된 ES 세포를 동정하는 것은 물론 클로닝 과정중에 형태적으로 분화가 없도록 ES 세포를 배양하는 것이 카이미리즘이 높은 마우스를 생산하고 아울러 생식선 이행 빈도를 증가시키는데 결정적인 역할을 하는 것으로 확인되었다.

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