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Simple Methods for Production of Chimeric Mouse by Coculture with TT2 Embryonic Stem Cells

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ABSTRACT

Gene targeting are very useful tools for the research on the gene function *in vivo*, mass production of foreign materials and biomedical approach of therapeutic process. But this process is very complicated and necessary highly skilled technique, because it is very different from ES cell origin, genetic background of embryo, and experimental conditions. We investigated the productivity ability of chimeric mouse after aggregation with TT2 ES cells. Increse of ES cell density caused gradual decrease in embryo development *in vitro* and in the production of chimeric mice *in vivo*. One million ES cell density for the aggregation was very efficient to produce high percentage chimeric mice in their coat color. These results suggested that appropriate cell density plays a key role in the development and production of chimeric mice by a 8-cell aggregation method.

(Key words: Gene targeting, Embryonic stem cells, Chimeric mice, Aggregation method)

Ⅰ. INTRODUCTION

Gene targeting in the mouse species is the most efficient method for eliminating gene function and for establishing null phenotype(Matzuk et al., 1992). The production of chimeric mice is one of the most important steps in the gene targeting. The common method used for producing a chimeric mouse is blastocyst injection(Bradlly, 1987), which introduces ES cells into the blastocoel cavity of 3.5 day embryo using a micromanipulator. This procedure allows direct monitoring while ES cell manipulation procedure, but the number of injection that can be performed is quite limited. An alternative method to introduce ES cells to the host embryo is the aggregation method(Wool et al., 1993; Nagy and Rossant, 1993). This method only requires contact between ES cells and host embryo, which allows further development of the embryo up to the blastocyst, and then transfer them to uterus of the pseudopregnant foster mother. The method is rather simple, only requires no special or complicated equipment, and allows handling of the greater number of embryos. Two types of ES cells, 129J line and TT2, currently used for gene targeting.

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E14, D3, and AB cells derived from 129J types of mouse is the most broadly used, and TT2 ES cells (Yagi et al, 1993) from an F1 embryo between C57 BL/6 and CBA mice has high germline differentiating potency. Two different procedures for aggregation have been published; coculture of embryos on the lawn of ES cells and nestling of the embryo and ES cells(Nagy et al, 1993; Wool et al, 1993; Khillan and Bao, 1997). The reports for effects of ES cells density in the aggregation methods is absent in the previously published reporter.

To improve the efficiency of chimeric production, we examined the effects of ES cell density for development and production of chimeric mice in the condition of embryo-ES coculture, and obtained the results that high ES cell contributed chimeras in the 1×10^6 cell/ml .

Ⅱ. MATERIALS AND METHODS

1. Mice and Embryos

ICR mice were purchased from Charles Rever Inc., Japan, and housed in an environmentally controlled room with a 12-h dark-light cycle. The ICR female mice were superovulated by intraperitonial injection of 5 IU pregnant mare's serum gonadotropin(Teikokuzoki, Japan), followed by the injection of 5 IU human chorionic gonadotropin (Teikokuzoki, Japan) 48 hours later. They were mated with male mice of the same strain. The 8-cell embryos were recovered at 72 hr. post-hCG injection and cultured in M16 media (sigma, USA) in a humidified atmosphere of 5% CO_2 in air at 37 ℃(Hogan et al, 1986).

2. Culture of ES Cells

TT2 ES cells purchased from GIBCO BRL(Japan) and cultured with ES medium [Dulbecco's modified Eagle's medium (high glucose, GIBCO BRL, Japan) supplemented with 20% fetal calf serum (GIBCO BRL, Japan), 10^4 unit/ml leukemia inhibitory factor (AMRAD, ESGRO), 10^{-4}M β -mercaptoethanol, nonessential amino acid (Flow Lab.), and sodium pyrvate (GIBCO BRL, Japan)]. TT2 ES cells(8×10^5 cells) were routinely seeded onto primary embryonic fibroblast feeder prepared from 13.5 day fetus of Balb/C mice in 25-cm^2 flask(Falcon), ES medium was changed every day, and the cells were passaged within 2 or 3 days.

3. Aggregation of Eight-cell Embryos with ES Cells

ES cells were washed with PBS-EDTA, treated with 0.25% trypsin-EDTA (GIBCO, Japan), and then resuspended to a density 0.5×10^6, 1×10^6 and 2×10^6 cells per ml in M16 medium containing 5% FBS (aggregation medium). Small drops(15 μl) of the aggregation medium containing ES cells were placed in a 3-cm plastic dish(Falcon 1008) and covered with liquid paraffin oil. The zona pellucida of 8-cell stage embryos removed with Tyrode's solution(Sigam, St. Louis, USA) and transferred into drop containing ES cells and cultured in 5% CO_2 incubator at 37℃ for 3 hr. Embryos with attached ES cells were transferred to fresh droplets and cultured overnight to morula and blastocysts. The blastocysts were transferred to the uterine horns of pseudopregnant ICR recipient female mice on day 2.5 post coitum.

Ⅲ. RESULTS

1. *In Vitro* Development of 8-cell Stage Embryo Co-cultured with TT2 ES Cells

To examine the *in vitro* development of 8-cell stage embryo cocultured with TT2 ES cells, the 8-cell stage embryos removed zona pellucida with Tyrode's solution and cultured for 3 hr. with ES cells at the density of 0.5×10^6, 1×10^6 and 2×10^6 per ml in the aggregation medium. *In vitro* blas-

Table 1. Effects of ES cell density for development of mouse embryos

No. of ES cells	No. of embryos aggregated[a]	No. of embryos recovered[b]	No. of embryos developed to[c]		
			Abnomal(%)	Morular(%)	Blastocysts(%)
0.5×10^6	283	252(89.0)	20(7.9)	55(21.8)	177(70.2)
1×10^6	297	275(92.6)	31(11.3)	66(24.0)	178(64.7)
2×10^6	236	234(99.1)	38(16.2)	80(34.1)	116(49.5)

[a] indicated the number of embryos for aggregated with ES cells
[b] indicated the % of recovered embryos compared with a
[c] indicated the % of development embryos compared with b

tocyst development of 8-cell stage embryos was significantly higher in the 0.5×10^6 and 1×10^6 ES cells per ml than that of 2×10^6 ES cells per ml (Table 1). In the 0.5×10^6 and 1×10^6 cells per ml culture condition, clumps of 2-6 ES cells were attached to an 8-cell stage embryo. These embryos developed into blastocysts within 20~24 hr. culture in M16 droplets. When 8-cell stage embryos cultured in the 2×10^6 ES cells per ml, clumps of more than 7 ES cells were attached to an 8-cell stage embryo. Developmental ratio of these embryos was decreased within 20~24 hr. culture in M16 droplets.

2. Production of Chimeric Mice using Eight -cell Embryo Cocultured with TT2 ES Cells

We examined the productivity of chimeric mice of 8-cell stage embryos cocultured with TT2 ES cells. When blastocysts were transferred to the uterine horns of pseudopregnant ICR recipient female mice on day 2.5 post coitum, the offspring were significantly higher in 0.5×10^6 and 1×10^6 cells per ml than that of in 2×10^6 cells per ml. The efficiency to generating chimeric mice by coculture with 1×10^6 cells per ml was higher compared with 0.5×10^6 and 2×10^6 cells per ml. When the offspring were classified according to their coat color, 100% chimeric mice were obtained only by co-culture with 1×10^6 cells per ml(Table 2).

Ⅳ. DISCUSSION

In the current studies, we describe the development of 8-cell stage embryo cocultured with TT2 ES cells and obtained 100% chimeric mice by coculture of 8-cell stage embryo and TT2 ES cells. When blastocyst were transferred to the uterine horns of pseudopregnant ICR recipient female mice,

Table 2. Effects of ES cell density for production of chimeric mice

No. of ES cells	No. of embryos transfered	No. of recipient	No. of offspring(%)[a]	No. of chimeras (%)[b]			
				<50%	50~70%	70~90%	90~100%
0.5×10^6	177	16	82(46.3)	7	1	0	0
1×10^6	176	16	84(47.7)	9	6	9	5
2×10^6	116	10	17(14.6)	5	0	0	0

[a] indicates the % of offspring compared with embryos trasferred
[b] indicates the % of coat color of the chimeras

we obtained the offspring in the each cell density (0.5×10^6, 1×10^6 and 2×10^6 cells per ml), and 100% chimeric mice in their coat color were obtained only in the 1×10^6 cells per ml.

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of mouse blastocysts and have totipotentic activity. After microinjection and aggregation into a host embryos, they can develop into any cell lineage, including germ cells(Bradley, 1987). In this studies, 8-cell embryos cocultured with 2×10^6 ES cells per ml showed low development into blastocysts and decreased production of offspring. The reason that why coculture with 2×10^6 cells per ml can not development into blastocysts are unknown.

The efficiency of generating chimeric mice by injection of wild-type R1 ES cells was higher compared to the aggregation method, which was 7% in the previously published reporet(Nagy et al., 1993). In this study, we obtained high efficiency production of chimeric mice by 8-cell embryo with aggregated with 1×10^6 ES cells per ml TT2 ES cells (17%, >70% of coat color of the chimeric mice). This efficiency was comparable to a previous study (Kondoh et al. 1999). In that study, a single embryo of the eight cell-morula stage and a cluster containing 20~25 ES cells were nestled in a hole made by a darning needle and cultured for overnight. We cultured 8-cell embryos with M16 droplets(15 μl) containing 1×10^6 per ml of TT2 ES cells for 3 hr. This methods is simple and reduce the culture time.

Finally, the practical benefit of aggregation method should be emphasized as it may save time and money for generating mutant mouse strain from ES cells and this technique allows non-embryologists to produce gene targeted mice.

V. REFERENCES

1. Bradley, A. 1987. Production and analysis of chimeric mice. In: Robertson EJ, editor, Teratocacinomas and Embryonic stem cells, IRL Press, Oxford, pp.113-152.
2. Hogan, B., Constantini, F. and Lacy, E. 1986. Manipulating the mouse embryo, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
3. Kondoh, G., Yamamoto, Y., Yoshida, K., Suzuki, Y., Osuka, S., Nakano, Y., Morita, T. and Takeda, J. 1990. Easy assessment of ES cell clone poteancy for chimeric development and germ-line competency by an optimized aggregation method. J. Biochem. Biophys. Methods 39:137-142.
4. Khillan, J. S. and Bao, Y. 1997. Preparation of animal with a high degree of chimerism by one -step coculture of embryonic stem cell and preimplantation embryos. BioTechniques 22: 544-549.
5. Nagy, A. and Rossant, T. 1993. Production of completely ES cell-derived fetuses. In: Joyner AL, editor, Gene targeting, IRL Press, Oxford, pp. 147-180.
6. Matzuk, M. M., Finegold, M. J., Su, J-G. J., Hsueh, A. J. W. and Bradley, A. 1992. α-Inhibin is a tumor-suppressor gene with gonadal specificity in mice. Nature. 360:313-319.
7. Wool, S. A., Pascoe, W. S., Schmidt, C., Kemler, R., Evans, M. J. and Allen, N. D. 1993. Simple and efficient production of embryonic stem cell-embryo chimerase by coculture. Proc. Natl. Acad. Sci. USA. 90:4582-4585.
8. Yagi, T., Tokunaga, T., Furuta, Y., Nada, S., Yoshida, M., Tsukada, T., Saga, Y., Takeda, N., Ikawa, Y., and Aizawa, S. 1993. A novel ES cell line, TT2, with high germline-differentiating potency. Anal. Biochem. 214:70-76.

요 약

TT2 Embryonic Stem Cell을 이용한 Chimeric Mouse 생산에 있어서 간단한 공배양방법

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본 연구는 TT2 embryonic stem(ES) cell을 이용하여 chimeric mouse를 생산하는데 있어서 더욱 간편한 공배양방법 개발하기 위하여 수행되었다. 유전자 적중 생쥐의 개발은 유전자의 기능을 연구하는데 매우 중요한 수단으로 이용되고 있다. 이러한 생쥐의 개발에 있어서 chimeric mouse를 생산하는 과정은 ES cell의 종류의 차이는 있지만 주로 배반포기의 수정란에 ES cell을 주입하고 있다. 이 기술은 고가의 미세조작장치 뿐만 아니라 고도의 기술을 요하고 있다. 그러므로 본 연구에서는 TT2 ES cell를 8세포기 수정란과 공배양할 때의 필요로 하는 적절한 ES cell의 수를 검증함으로써 chimeric mouse의 생산 효율을 높일 수 있었다. 각각 0.5×10^6, 1×10^6과 2×10^6/ml의 ES cell을 8세포기의 수정란과 공배양하였을 때 0.5×10^6/ml과 1×10^6/ml에서 높은 배반포기로의 발달율을 나타내었다. 또한 가임신된 생쥐에 이들 배반포기를 이식한 결과 1×10^6/ml에서 높은 chimeric mouse생산 효율을 나타내었다. 이러한 결과는 적절한 수의 ES cell과 수정란을 공배양함으로써 매우 간단하게 효율 좋은 chimeric mouse을 얻을 수 있음을 제시하고 있다.

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