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The Production of Chimeric Mice by Embryonic Stem Cell Carrying Human Luteinizing Hormone Gene

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사람 LH 호르몬유전자를 도입한 배아주세포에 의한 카이미라 생쥐 생산

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적 요

최근 의약적으로 유용한 단백질을 대량 생산키 위한 실현 가능한 방법이 유전자변환 가축의 이용과 관련되어 발전되어 왔다. 이러한 유전자 변환동물은 이종의 단백질을 유즙속으로 분비시키는 생체반응기로서 이용되고 있다. 이러한 전략적 목적을 위해 현재 유전자 변환동물의 생산을 위한 이용에 있어 여러가지 방법들이 보고되고 있다. 그러나 ES 세포의 사용이 이러한 방법들 사이에서 가장 실질적인 것으로 추정되고 있다. 본 실험에서는 유전자 구축을 위해 사람 황체호르몬(human luteinizing hormone; hLH)의 전사를 유도하기 위해 각각 2.2 및 0.5 kb의 토끼 β-casein promoter 단편을 이용하여 생쥐의 유선에 hLH를 발현시키도록 조절하고 발현이 thymidine kinase(TK) promoter에 의해 좌우되는 neo 유전자를 selectable marker로서 plasmid속에 삽입하였다. 그 결과 생긴 구축 유전자는 각각 pCas 2.2와 pCas 0.5로 명명하였다. 구축된 유전자로 2×10^7의 TT-2 ES 세포를 170V, 550μF로 100μg의 선상 plasmid에 의해 electroporation 시켰다. 감염된 colony들은 250μg / mℓ G418을 함유하는 ESM 배양액에서 선별 7일 이후에 회수하여 성공적으로 감염된 ES세포는 PCR 및 Southern blot에 의해 확인되었고 그들 중 나머지는 trypsin 처리 후 각각 미세조작과 공배양 기술을 사용하여 ICR 생쥐의 8세포기 수정란 속에 도입하였다. 결국 24시간 동안 37℃, 5% CO_2에서 배양된 배반포를 chimera의 생산을 위해 위임신 유기된 foster생쥐 자궁에 이식시켰다. pCas 0.5 및 pCas 2.2로 각각 감염시킨 4×10^5의 ES 세포로부터 G418 선발처리 이후 400 및 275개의 ES 세포 colony가 생존하였으며, 3개의 ES 세포의 colony의 genome속에 임의적으로 plasmid가 삽입된 것을 Southern blot에 의해 확인되었다. 총 13 chimera 생쥐가 3 colony로부터 생산되었으나 germ-line chimera는 현재 조사중이다. chimera 생산빈도는 공배양 기술보다 주입방법에서 현저히 높았다.

INTRODUCTION

Recently, a feasible method for producing large amounts of pharmaceutically useful proteins has been developed by producing transgenic farm animals(Wright *et al.*, 1991). This animal transgenesis has been achieved via microinjection into one of the pronuclei(Wall *et al.*, 1991), viral transfection(Stewart *et al.*, 1985) or the use of embryonic stem(ES) cell. It would be more controllable to use ES cells in various genetic

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manipulations including specific gene targeting for the production of transgenic animals. In this report, transfection of ES cells and subsequent construction of chimeric embryos by incorporating the ES cells were employed to produce chimeric mice containing a human gene which was manipulated to express specifically in mammary gland.

MATERIALS AND METHODS

Plasmid construction

Plasmid was manipulated to give tissue-specific expression in mammary gland. Thus the plasmids contained 2.2 or 0.5 kb fragments of rabbit β-casein promoters, respectively. Human LH gene was fused to the promoter with termination signal. For the selection of transfected ES cells *neo* gene was ligated to the thymidine kinase promoter. The resulting constructs were designated pCas 2.2 and pCas 0.5, respectively(Fig. 1).

1. Culture of ES cells

T-2 ES cells were maintained as described previously(Tokunaga and Tsunoda, 1992). 2.4×10^7 of ES cells(TT-2, F1/1) were electroporated with 100μg of linearized plasmid at 170V and 550μF. Transfected clones of ES cells were isolated in 7 days of incubation in ES medium containing 250μg/ml G418. ES cells were cultured on primary embryonic fibroblast cell feeder layer inactivated with mitomycin C treatment. Dulbecco's Modified Eagle's Medium supplemented with 20%(V/V) fetal calf serum, 10^{-4}M 2-mercaptoethanol and nonessential amino acid solution, was used for maintenance of the ES cells.

2. Southern blot analysis

To demonstrate transfection in TT-2 cells PCR and Southern blot using *Pst* I fragment of hLH as a probe were performed according to the established method.

3. Production of chimeric embryos

Transfected TT-2 cells were suspended in sDMEM and about 15 cells were microinjected into the ICR 8-cell embryos as a clump. Some of the TT-2 cells were aggregated by coculture method(Wood *et al*, 1993) after the removal of

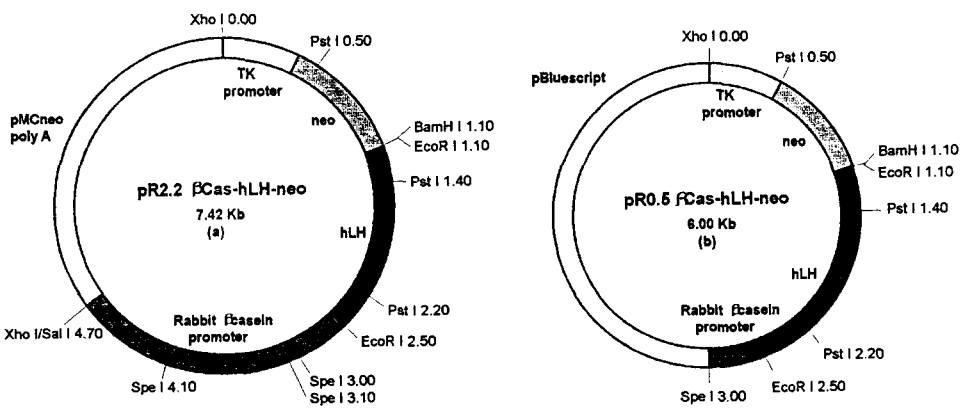

Fig. 1. Construction of plasmids for the expression of hLH gene under control of different length of β-casein promoter of rabbit. 2.2 kb(a) and 0.5 kb(b) β-casein promoter elements were linked to hLH encoding gene, respectively. G418 gene derived by TK-promoter was used as a selectable marker.

the zona pellucida in acid Tyrode's solution.

4. Production of chimeric mice

Manipulated 8-cell embryos were cultured, in M16 medium for 24 h and blastocysts were transferred into the uterus of pseudopregnant foster mother and allow to deliver at term.

RESULTS AND DISCUSSIONS

In this experiment 400 and 275 colonies out of 4×10^5 ES cells transfected with pCas 0.5 and pCas 2.2, respectively were survived on G418 selective medium(Table 1). Transfected colonies were divided into aliquats for both storage and Southern blot. In the process of transfection and selection, it seems that there is no differences between the plasmids used. Consequently, 3 colonies of ES cells whose genomes have random insertion of either plasmid were confirmed by Southern blot(Fig. 2). Transfected colonies as evidenced by the blot were subsequently used for making chimaeric embryos. Two methods were both employed to make chimeric embryos. 8-cell injection method showed higher frequency of live pubs than coculture one does, although these two methods showed similar frequency of chimera production(Table 2). Thus two methods may be used without differences. Total 13 chimeric mice were produced from 3 colonies of ES cells(Fig. 3), but germ-line chimerisms are now under investigation.

ABSTRACT

Recently, a feasible method for producing large amounts of pharmaceutically useful proteins has been develped that involves the use of transgenic farm animals. Thus, such transgenic animals are employed as bioreactors in which the heterologous proteins are secreted into the

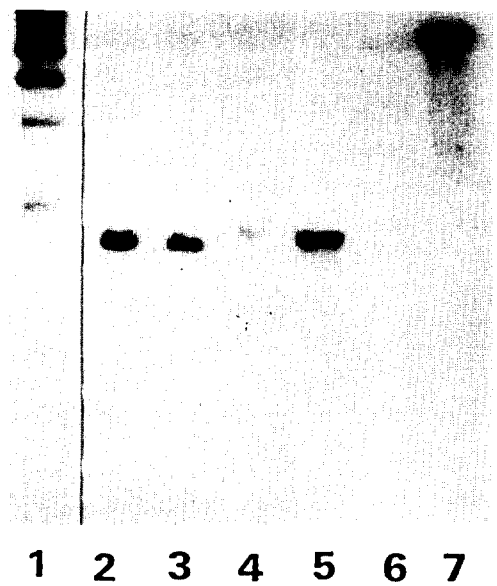

Fig. 2. Genomic Southern blot hybridization of transfected mouse ES colonies. Genomic DNA of ES colones were extracted, restricted with EcoRI and fractionated on 0.8% agarose gel. Pst I fragment of human LH use as a probe was gel-purified and labeled with biotin by the random priming method. Lane 1 : molecular size marker, Lane 2, 3, 4 and 5 : transfected ES colonies, Lane 6 : genomic DNA of untransfected ES cells, Lane 7 : the plasmid DNA restricted with Xho I.

Table 1. Number of ES colonies survived after treatment of G418

Plasmid	No. of ES cells electroporated	No. of ES colonies survived
pRβCas 2.2-hLH-neo	4×10^5	275
pRβCas 0.5-hLH-neo	4×10^5	398

Table 2. Efficiency of production of chimeras between co-culture techniques and 8-cell injection of ES cell

Methods	Name of ES colonies*	No. embryos transferred	No. of recipients	No. of pups born (%)	No. of chimera born (%)
Co-culture	11[a]	87	7	31 (35.6)	6 (19.4)
	14[b]	72	5	18 (25.0)	0 (0.0)
	15[b]	66	4	16 (24.2)	1 (6.3)
8-cell injection	11[a]	60	4	36 (60.0)	3 (8.3)
	14[b]	60	3	32 (53.3)	3 (9.3)
	15[b]	107	7	59 (55.1)	3 (5.1)

* ES cells were introduced human LH β-subunit gene with the regulatory region ($-0.5kb^a$ or $-2.2kb^b$) of rabbit β-casein gene.

Fig. 3. Male chimeric mice obtained by injection of ES cells into 8-cell embryos and co-culture techniques. The extent of contribution of ES cells was less than typical germ-line chimeric mice judged by their coat color (agouti derived from ES cells).

milk. For the purpose of this strategy, several methods currently in use for producing transgenic animals are introduced. But various advantage in using ES cells has been most effectively recommended among these methods. We have approached the problem of expressing human luteinizing hormone(hLH) in the lactating mammary gland of mouse by employing 2.2 and 0.5 kb fragments of rabbit β-casein promoter, respectively, to induce transcription of hLH in ap-

propriate gene constructions. Neo gene whose expression is governed by Thymidine Kinase (TK) promoter was also introduced into the plasmid as a selectable marker. The resulting construct was named pCas 2.2 and pCas 0.5, respectively. 2.4×10^7 of TT-2 ES cells were electroporated with 100㎍ of linearized plasmid at 170V and 550㎌. Transfected colonies were isolated after 7-days of selection in ESM containing 250㎍/㎖ G 418. Successfully transfected ES cells were confirmed by PCR and Southern blot, and the rest of them were trypsinized and then introduced into 8-cell embryo of ICR mouse using micromanipulation and coculture techniques, respectively. Finally, blastocyst embryos, cultured in M16+BSA at 37℃, 5% CO_2 in air for 24h, were transferred into the uteri of pseudopregnant recipients for the production of chimeras. In this experiment, 400 and 275 colonies of ES which were transfected with pCas 0.5 and pCas 2.2, respectively. Consequently, integration of the plasmid randomly into the genome of 3 colonies of ES cells was confirmed by Southern blot. Total 13 chimeric mice were produced from these 3 colonies, but germ-line chimerisms are now under investigation. The frequency of chimera production was very similar between two methods, but that of live pubs was significantly higher in the injection method than coculture technique.

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