EXAMINATION OF THE GERM CELL CHIMERA FORMING POTENTIAL OF MOUSE EMBRYONIC STEM CELLS

EXAMINAREA CAPACITĂŢI CELULELOR EMBRIONARE PLURIPOTENTE PENTRU FORMAREA ORGANISMELOR HIMERE GERMINALE

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The aim of this study was to examine the factors, which influence the chimera forming potential of mouse embryonic stem cells (ES cells). In our work, we examine the chimera producing ability of R1 and R1/E mouse ES cell lines. We found that the passage number affects chimera-forming capability of the ES cells. With the increasing of the passage number, it could be getting less chimera animal, and only the R1/E ES cell line derived cells could contribute to the germ cells. At first, we compared the marker of pluripotency using immunostaining and RT PCR, but we could not find any difference between the R1 and R1/E cell in this way. At chromosome analysis, we found, that the number of aneuploid cells, in R1 ES cell line, dramatically increased after 10 passages. We thought that the reason is that during the cell division Y chromosome could not arrange correctly between the two newly derived progeny cells. To prove our conception, we made X and Y-chromosome FISH analyses. We found, that the aneuploid R1 and R1/E ES cells contain only one X and one Y chromosome, so not the loss of Y chromosome cause the problem at the germ cell formation. At last, we made the karyotype analysis of R1 and R1/E ES cells at different passages. The karyotype analysis demonstrated that in the case of R1 ES cell line, the 41 and 42-chromosome containing cells hold trisomy. With the increasing of the passages number, the number of trisomy containing aneuploid cells increased. The aneuploid ES cells can contribute to the different tissuses of chimera animals, but cannot form viable germ cells.

Key words: Es cells, chimera, FISH, karyotype

Introduction

The methods developed for transgenic mouse production were improved in the last few decades. With the establishment of the first mouse embryonic stem cell lines became possibly to develop new method for introduction external genetic information into the mouse genome. Although mouse ES cells have supported discovery in myriad research fields, their value was established as a tool to enable

targeted mutagenesis. Several years earlier, it was demonstrated that, when they were injected into mouse blastocysts, genetically altered ES cells could generate transgenic offspring. The application of these techniques with homologous recombination technology thus provided scientists with a controlled process to generate an unlimited variety of transgenic mice with engineered, predetermined genomes.

Matherial and Metods

Superovulation of mice

To get 2-cell stage embryos, females were superovulated with intraperitoneal injection of pregnant mare's serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) at specific times (1). After hCG injection, the females were caged individually with the appropriate males. Matting was verified by the presence of a vaginal plug the following morning. On the day of the plugs, a group of CD1 females were mated with vasectomised CD1 males, to produce pseudopregnant females, to act as embryo recipients.

Embryo collection and cultivation

We collected the embryos flushing them from the oviducts of superovulated females using M2 medium. After washing we cultured them in CO_2 incubator for 24 h (5% CO_2, 37^0C) in KSOM medium, respectively until eight cell stage (1).

ES cells and tissue culture

The ES cells lines, used for producing chimeras in this study, were R1 and R1/E ES cell lines (1). Culture was carried out following the standard protocol of Robertson et al. (2).

Chimera production

The zona pellucida of the eight-cell stage embryos was removed by acidic Tyrode's solution. The zona free embryos were placed individually into one depressions of the aggregation plate. Clumps of 10-15 ES cells were picking up from medium and transferred aside the zona free embryo. After 24 hours most aggregates were transferred into the uterus of pseudo pregnant females.

Karyotyping method

The cells were passed to the gelatin-covered plate one day before the karyotyping. The next day, colcemid or vinblastine solution was added to the plate for 2.5 hour to block the cells in metaphases. The supernatant was removed and the pellet was resuspended in 4 ml of warm (37°C) hypotonic solution (0, 56%KCl)(drop by drop) and the suspension was kept on RT, for 10 minutes. Suspension was centrifugated at 1000 rpm for 7 min, on RT, than the supernatant was removed. 0.5 ml of freshly prepared fixative (methanol: acetic acid (3:1), 4°C)(drop by drop) was added. The cells were centrifuged again at 1000 rpm for 7 min, (at RT), the supernatant was carefully removed and after we added 4 ml fixative, swirling the cells (these two steps were repeated for two times). The cellular suspension was stored at 4°C (or -20°C). Before analyzis, the cells were

centrifuged again at 1000 rpm (200g) for 7 min, (at RT), the supernatant was carefully removed and 1 ml fixative was added. From the far distance we droped 3-5 drops of suspension to a cold and wet slide. The slides were dried at the room temperature and stained using Giemsa stain (80 ml MQ, 20 ml PBS, 2,5 ml Giemsa (SIGMA)), for 10 minutes. The slides were washed under flowing tap water for 1 minute, dried and covered. We didn't stain the slides, if we wanted to use the slides for FISH analyzis.

FISH techniques

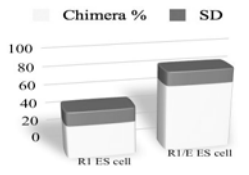
We used „maturated" slides for FISH analyzis. It means, that after splitting, the slides with attached chromosomes were kept on RT from three up to one week. First we washed the slides in 2xSSC/0.5% NP40 for 10 min. After that we put the slides in to the protease solution for 10 min at 37˚C. After protease treatment we washed slides in 1xPBS, for 5 min. to stop the protease reaction. At dehidratation we washed the slides in ethanol (70-90-100 %) for 2 min. each, after dehidration we dried the slides and added the probe to the selected area. Next step was the denaturation. To denaturat the DNA we kept the slides at 72 ˚C, for two minutes. After that we kept the slides at room temperature for 5 min. Hybridization was done by keeping the slides at 37 ˚C during the next 16-24 hours. On the second day we removed the glass coversleep from the slides (in 2xSSC, at 37 ˚C). For removing the probe, we washed the slide for 10 min. at 37 ˚C, using 50 % formamid/2xSSC. After we washed it for 5 min. 1xPBS. We stained the chromosomes in DAPI-VECTA solution (10-15 µl/ 22x22 mm cover slip. Visualization was done using UV light under 100x oil objective magnify.

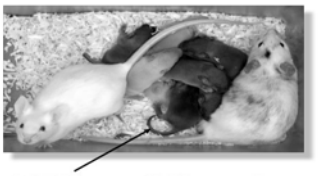
Results and Discussions

In our work, we examined the chimera forming capability of R1 and R1/E mouse ES cell lines. We considered that the passage number affects the chimera-forming capability of the ES cells. With increased the passage number, the rate of viable chimera animals decreased and only the R1/E ES cell line derived cells could contribute to the germ cells *(Figure 1.)*.

Figure 1.
Examination of R1/E and R1 ES cell line chimera forming ability

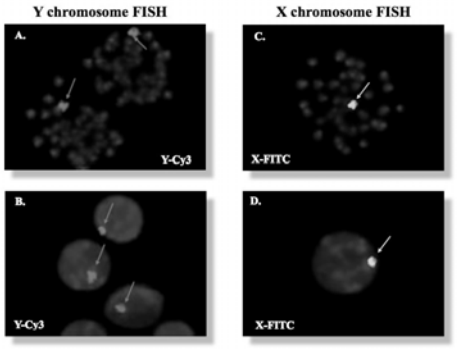
ES cell line	Newborn/ transferred % (±SD)	Chimera/ transferred % (±SD)	Chimera/ newborn % (±SD)	ES% (±SD)	Germ cell chimera
R1	19,8±11,0	6,8±3,3	35,2±13,6	28,3±16,1	-
R1/E	32,2±11,4	21,2±6,7	70,2±13,6	62,5±16,3	+





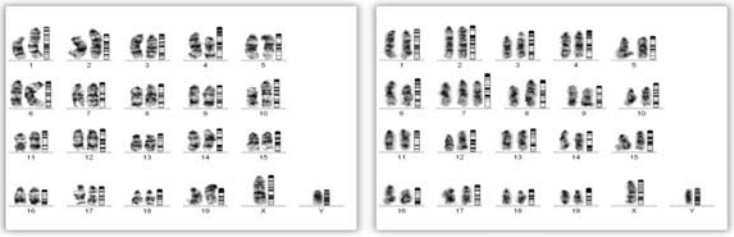
R1/E ES germ cell chimera male and his transgenic progenies

Figure 2.
FISH analysis of R1/E and R1 ES cells



At first, we compared the markers of pluripotency using immunohistocemistry and RT PCR, but we could not find any difference between the R1 and R1/E cell line in this way. The results of chromosome analysis showed that the number of aneuploid cells in R1 ES cell line dramatically increased after six passages. The euploid cells in mouse contain 40 chromosomes. During the passages, the number of cells with 38, 42 and more chromosomes increased. First we thought that during cell division Y chromosomes could not arrange correctly between the two newly derived progeny cells, and after cell division it could became one 39 X0 cell, and one 41 chromosome containing XYY cell and so on. To prove our conception we made X and Y-chromosome FISH analyses *(Figure 2.)*. We found, that the aneuploid R1 and R1/E ES cells contain only one X and one Y chromosome, so not the loss of Y chromosome cause the problem at germ cell formation. At last, we made the karyotype analysis of R1 and R1/E ES cells at different passages. The karyotype analysis demonstrated that in the case of aneuploid R1 ES cells the 41 and 42-chromosome containing cells hold trisomies. With increased passage number the number of trisomic cells increased. The aneuploid ES cells can contribute to the different tissues of chimera animals but canno

Figure 3.
Kariogram of R1 ES cells (p23)



40 chromosomes

41 chromosomes (Chr7 trismoy)

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Scopul acestui studiu a fost examinarea factorilor care influenţează potenţialul de formare al himerelor, de către celulele stem embrionare de şoarece (ES).În experimente, noi am examinat abilitatea liniilor de celule ES R1 şi R1/E pentru producerea himerelor. Noi am descoperit ca numărul pasajului liniei celulare, afectează capacitatea acesteia de formare a himerelor. Odată cu creşterea numărului pasajului, au putut fi obţinute mai puţine animale himeră, şi doar celulele derivate din linia de celule ES R1/E au putut contribui la linia germinală. În primă fază, noi am comparat markerul pentru pluripotenţă folosind imuno colorarea şi RT PCR – ul dar in acest mod nu am găsit nici o diferenţă între liniile R1 şi R1/E.La analiza cromozomilor, am observat că numărul de celule aneuploide în cazul liniei ce celule R1, creşte dramatic după cel de al 10 - lea pasaj. Am crezut ca motivul este faptul că pe parcursul diviziunii celulare, cromozomul Y nu s-a putut directiona corect între cele două celule descendente noi. Pentru a demonstra concepţia noastră, am făcut analiza FISH a cromozomilor X şi Y. Am descoperit că, celulele aneuploide R1 şi R1/E conţin doar un cromozom X şi un cromozom Y şi deci, nu pierderea cromozomului Y u cauzat probleme în formarea liniei germinale.În final, am analizat cariotipul celulelor R1 şi R1/E la diferite pasaje. Analizele cariotipice au demonstrat că, în cazul liniei celulare R1, cromozomii 41 şi 42erau conţinuti de celule ce prezentau trisomie. Odată cu creşterea numărului de pasaje, numărul trisomiilor conţinute de celule, a crescut. Celulele ES aneuploide pot contribui la formarea diferitelor ţesuturi în animalele himeră, dar nu pot forma celule germinale viabile.

Cuvinte cheie: celule ES, himere, FISH, cariotip