

**MODELS FOR MOUSE CHIMERA PRODUCTION:
AGGREGATION OF ES CELLS WITH CLEAVAGE STAGE
EMBRYOS**

**MODELE DE PRODUCERE A HIMERELOR DE ȘOARECE:
AGREGAREA CELULELOR ES CU EMBRIONI ÎN STADIUL
DE CLIVAJ.**

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In a mutant ES cells ↔ wild-type embryo chimera, ES cells behave more like epiblast cells. They can contribute to the primitive ectoderm layers, which give rise to all the embryonic tissues and some extraembryonic tissues (Beddington and Robertson, 1989), but not to trophoctoderm or primitive endoderm. Using transgenic ES cell lines, aggregated with cleavage stage host embryo, ES cells can integrate randomly in the embryo proper. If they will be take part in the formation of ICM (inner cell mass), it will be possible to obtain germline chimera animals. To generate ES cells ↔ cleavage stage host embryo chimeras, we used (CD-1) mice as donors of host embryos as well as recipients of manipulated embryos. For chimera production, we used fluorescent-labeled ES cell line (CD1/EGFP), because in this case we can follow the fate of ES cells during the embryonic development. We produced the chimers using "aggregation chimera technique". 8 cells stage zona pellucida free, mouse embryos were aggregated in an aggregation plates, with a clump of ES cells (10 – 15 cells. The chimera embryos were cultivated for 24 hours in the incubator (at 37 °C, 5% CO₂ in air). The chimera blastocysts resulted after cultivation, were transferred to the uterus of the 2.5-dpc pseudo pregnant females.

Key words: Es cells, diploid, pseudo pregnant, aggregation

Introduction

Aggregating ES cells with diploid eight-cell stage embryos is a simple and inexpensive means of introducing genetic alterations into mice. Circumventing the transfer of non-chimeric embryos after the aggregation is completed makes the technology more efficient and reduces animal space requirements. A green fluorescent ES cell line now provides a convenient tool to do so, since the incorporation of ES cells into preimplantation stage embryos can be monitored prior to their transfer. ES cell contributions to the embryo can vary, with ES cell contributions into morula and blastocyst stage embryos after overnight incubation

of eight-cell stage embryo– ES cell aggregates depicted. While ES cells will preferentially contribute to the ICM, where they can either represent the entire ICM or just a portion of it they will also occasionally be present in the trophoblast , though it is unclear whether this is a real contribution to the trophoblast lineage or these cells are trapped in this region of the embryo never to proliferate. Studying postimplantation stage chimeric embryos of this type will possibly answer this question.

Materials and Methods

Equipment and reagents

- 8-cell stage embryos after overnight culture
- KSOM and M2 medium in 1 ml syringe with 26G needles
- Embryo-tested mineral oil (Sigma)
- Aggregation needle (BLS Ltd., Hungary)
- 70% Ethanol
- 35 mm tissue culture plates for chimera production (Greiner)
- 30, 60, 100 mm tissue culture plates for fibroblast and embryonic stem cells (Greiner)
- Dissecting microscope
- Acid Tyrode's solution

ES cell line derivation and cultivation

For routine ES cell cultivation, the method of Robertson et al., [1] was used without any modification.

Chimera production

For chimera production we used two components: embryos from CD1 mouse strain, as host embryos, and EGFP expressing ES cells (CD1/EGFP) [2]. We used CD1 mice as donors of host embryos as well as recipients of manipulated embryos and as foster mother.

Micro drops of KSOM medium [3] were placed into a 35-mm tissue culture dish and covered immediately with embryo tested mineral oil. The aggregation needle was washed with 70% ethanol and deep depressions were created by pressing it into the bottom of the plastic dish. 1.5 days old embryos were collected from the oviduct of superovulated females [4]. Superovulation was induced by PMSG (pregnant mare serum gonadotrophin) and HCG (human chorionic gonadotrophin) injections. On the first day at 13 o'clock the females were injected with 7.5 IU PMSG (pregnant mare serum gonadotrophin) and 48 hours later with 7.5 IU HCG (human chorionic gonadotrophin) (i.p.). After that they were put together with the males. Next day in the morning, the mating was controlled by checking the plugs. The day of the plug was considered the first day of pregnancy (Day 0.5). On the second day of pregnancy 2-cell stage embryos were washed from the oviduct using M2 medium, then the embryos were incubated in a thermostate, in microdrops of KSOM in a 35-mm tissue culture dish, at 37°C

and 5% CO₂ until next day, when they reached the eight-cell stage, the first component of chimera production. The zona pellucida of the eight-cell stage embryos was removed by acidic Tyrode's solution [4]. The zona free embryos were placed individually into one depressions of the aggregation plate [4]. On the day of experiment, the medium was removed from the ES cell plates prepared for aggregation. First, the ES cells were washed with Ca/Mg-free PBS, and then a minimal amount of trypsin (SIGMA) was added just to cover the cells. The plate was placed into the incubator for five minutes. After that, 2 ml of culture medium was added to stop the activity of trypsin. 100-200 clumps of loosely connected cells were picked and transferred into a large drop of KSOM medium. Clumps of 10-15 cells were picking up from medium and transferred aside the zona free embryo. After 24 hours most aggregates were at the late morula/early blastocyst stage and were transferred into the uterus of pseudo pregnant females [1, 3].

Results and Discussions

All the experiments started with diploid host embryos produced by super ovulated CD1 females. We obtained 25-30 embryos from one female. When we used not super ovulated females, the number of embryos was lower, around 10. In the first experiment we injected 7 females, 3 females were plug positive and we flushed from the oviducts ~ 100 embryos at 2 cells stage. After overnight culture, the embryos developed to 8-cells stage, we got ~ 80 embryos. From the total amount of 64 chimera morula; we transferred 30 in to the uterine horns of pseudo pregnant females. Five newborn chimera mice have been born and two of them survived the adulthood. In the second experiment, we inject 5 CD1 females, two of them were plug positive and we flushed from the oviducts ~ 85 embryos at 2 cells stage. After overnight culture, the embryos developed to 8-cells stage, we got ~ 65 embryos. From the total amount of 30 chimera morula; we transferred 24 in to the uterine horns of pseudo pregnant females. The pregnant females were subjects for caesarian at 13.5 day of pregnancy. We could get 9 implantations.

Table 1

Data regarding embryo manipulation:

Injected female genotype	CD1	CD1
Injected female number	7	5
Date of plug	05. 24. 2006	05. 26. 2006
Number of plug+ female	3	3
Number of recipients female	2	
Number of gained embryos	110	100
Number of cultured 2 cell stages embryos	100	85
Number of good quality 2.5 day old embryos	80	65
Number of produced chimeras	64	30
Type of chimeras	CD1xCD1/EGFP	CD1xCD1/EGFP

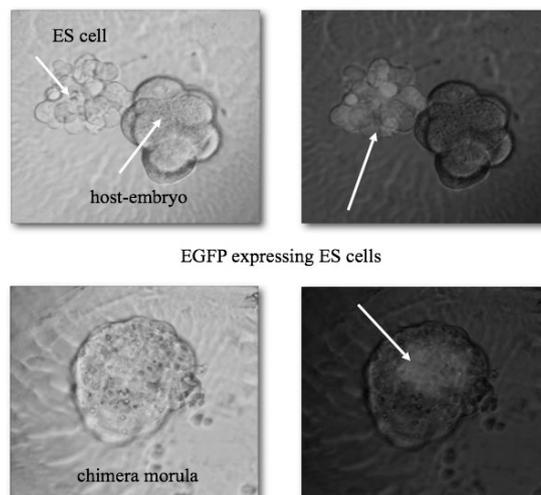
Table 2

Data regarding embryo transfer

Number of transferred chimeras	30	24
Sign of recipient females	TR1, TR2	TR1, TR2
Date of cesarian section	06. 11	06. 06
Newborn	5	Cesarian at 13.5 days
Living newborn	2	-
Resorption (deciduum)	TR1: R(2), L(3) TR2: R(2), L(1)	TR1: R(3), L(1) TR2: R(1), L(4)

Figure 1.

Production of ES cell chimeras (CD1/EGFP ES CD1 host↔embryo)



Conclusions

Trough this experiments we tried to develop optimal methods, which necessary for succesful embryos flashing at 2 cell stage:

1. The time between sacrificing embryo donors and placing the embryos in culture dishes should be kept to a minimum value, ideally no more than 20-30 minutes.
2. It is important to know that 2-cell stage embryos are more sensitive than morulae.
3. From the obtained results we can conclude that the KSOM medium is the appropriate medium for 2 cell stage CD1 embryo cultivation.
4. At chimera production, it is very important to place the ES cell clumps into the depressions, nearly aside the host embryos. In this case, we can be sure that they can form aggregate and the chimera morulas could not attached each other

Acknowledgements:

This work was supported in part by Hungarian-Romanian Intergovernmental Project, No. C18001/09.01.2006.

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La himerele de tip celule ES ↔ embrioni, celulele ES funcționează mai mult ca celule epiblastice. Ele pot contribui la formarea ectodermului primitiv, care dă naștere la toate țesuturile embrionare și la unele țesuturi extraembrionare (Beddington și Robertson, 1989), dar nu la trofoderm sau la endodermul primitiv. Folosind linii de celule ES transgenice agregate cu embrioni în stadiul de clivaj, celulele ES se pot integra la întâmplare în embrionul propriu. Dacă ele vor lua parte la formarea masei interne de celule, va fi posibilă obținerea de animale himeră germinale. Pentru a genera embrioni himeră de tip celule ES ↔ embrion gazdă în stadiul de clivaj, am folosit șoareci din linia CDI ca și donatori de embrioni și ca recipienți ai embrionilor manipulați. Pentru producerea de himere am folosit o linie de celule fluorescentă (CD1/EGFP), pentru ca în acest caz putem urmări soarta celulelor ES pe parcursul dezvoltării embrionare. Noi am produs himere folosind „tehnica de agregare”. Embrioni de 8 celule fără zonă pelucidă au fost agregați, în plăcuțe de agregare, cu 10 – 15 celule ES. Embrionii himeră au fost cultivați pentru 24 de ore în incubator (la 37 °C, 5% CO₂ în aer). Blastociștii himeră rezultați după cultivare au fost transferați în coarnele uterine ale femelelor pseudo gestante.

Cuvinte cheie: celule ES, diploid, pseudo gestante, agregare.