NEW POSSIBILITIES OF MAMMAL CLONING


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Key words: mammal cloning, embryonic stem cells, chimeras, lab mice

Abstract. Knowing the role of human genes in cell and tissue, economy can't be achieved only if genes of interests are transferred into the genome of animals realizing the humanization of experimental animal models. Because until this moment this can't be achieved only at the beginning of life of the individual, there was the need of creating embryos from cells which genome contained previously transferred genes of interest. In conclusion, in creating and developing cloning methods we have to consider specific methods used in embryology, molecular biology and transgenesis.

Pluripotent embryonic stem cells or even adult stem cells can be used not only for realizing transgenesis for genes of interests, but also for their further” transformation” into embryos.

INTRODUCTION

Finding new ways of mammal cloning would help us very much understanding the role of each human gene in the cell and tissue structure and function, on one side, and would make a huge step in animal science efficiency. Many methods have been already tried in order to solve this issue.

Preimplanational embryo blastomeres totipotency has suggested the idea that their repeated dissociation from morulas and their separate „in vitro” cultivation could become a way of mammal cloning. Experiments performed by Willadsen et al (4) demonstrated that this is possible until the stage of 4-8 cells. Because these are losing stepwise their totipotency, this method of cloning stays valable until the embryo reaches 4-8 blastomeres.

Embryonic bisection in the stage of blastocyst and their transfer, after a previous cultivation of few hours in the thermostat, into the uterine corns of foster mothers, reproductively synchronous with the embryonic age, has constituted a remarkable progress in domestic animal cloning progress, applicable even in bull farms in order to obtain identical twins (1).

Embryonic nuclear transfer or transfer of primitive somatic cells (fibroblasts) or specialized cells (mammary cells) and their cultivation in the vitellus of enucleated oocytes has constituted the third generation of methods in which farm animal cloning has been tried. This method was successfully applied both in sheep cloning (sheep Dolly and Polly), as well as in bull cloning and other mammal species (2, 3, 1).

In our work we will describe a method used by our team to obtain lab mice from embryonic stem cells. Because until this moment, embryonic stem cells can be achieved from mice, humans, sanguine monkey and bovines (cells similar to embryonic stem cells), it is reasonable to say that this method can be used in animal cloning.
MATERIAL AND METHODS

Our research has been realized in embryologic lab of Agricultural Biotechnological Center from Godollo-Hungary in collaboration with the animal genetic lab of the Faculty of Animal Science and Biotechnologies Timisoara.

In order to achieve mice from pluripotent embryonic stem cells we used a variant of the working technology specific for obtaining chimeras. For this we used 8 cell embryos, which were carrying in their genome the genetic marker of coloring in white the hair. After removing the zona pellucida, we aggregated them with 8- 10 embryonic stem cells, which were marked for the brown color of the hair.

The working steps were the following: 1) Obtaining embryos of 8 blastomeres 2) Obtaining embryonic stem cells 3) „In vitro” embryonic aggregation with embryonic stem cells and obtaining chimeras 4) Transfer of chimerical embryos into the uterine corns of pseudogestant foster mothers 5) Examination of the results.

In fig. 1 we show the main steps of obtaining chimerical mice from embryonic stem cells.

Fig. 1 Figure showing the aggregation between a host embryo and a colony of embryonic stem cells

WORKING TECHNOLOGY

1) Obtaining 8 cell embryos

As embryo donors we used mice females with white fur (CD1) between 5-6 weeks of age. In order to obtain a large number of embryos, these were superovulated. This was realized by intraperitoneal injection of 7 UI PMSG (pregnant mare serum). After 46- 48 hours females were i.p injected with 7 UI HCG (human corionic gonadothropin). Immediately after HCG administration, females were placed in separate cages with a male, for pairing. In the following days females were inspected for the presence of the vaginal plug. Those who presented vaginal plug (a sure sign that the coupling took place) have been chosen for embryo donors. Embryos were harvested by ooduct perfusion with PBS after these were excised from the pelvic cavity of sacrificed females at 1,5 days post coitum.
2) Obtaining embryonic stem cells

Embryonic stem cells (R1E line) have been obtained from a specialized firma and were kept in liquid nitrogen. Four days before their aggregation with the embryo, stem cells were defreezed and cultivated for 48 hours in the thermostat, on fibroblasts feeder layer. 48 hours before the embryonic aggregation they were trypsinysed for 3 minutes at room temperature, after the enzyme has been inactivated with 5 ml ES media (fig 2).

Colonies of ES cells on fibroblast layer     Colonies of ES cell after triyspinisation

Fig. 2 Stem cell colonies R1E before and after trypsinisation

3) Realizing the aggregate between an embryo and embryonic stem cells

CD1 embryos have been harvested from donor females 1,5 days after pairing, at the age of 8 blastomeres. Afterwards we removed the zona pellucida, by their immersion into Tyrode acid media at pH= 2.5. In order to achieve chimeras we had to approach a diploid embryo denudated by its transluclide zone and 8-10 pluripotent stem cells. For this we prepared plastic sterile Petri dishes on which bottom we performed with the help of a metal shaft, by pressure, many depressions with a very small diameter. In each of this small depressions we placed an embryo together with 8-10 pluripotent embryonic stem cells with the purpose of forcing them to adhere intimately one of another. The bottom of the Petri dish has been previously covered by KSOM culture media. At the end, Petri dishes were placed in thermostat at 37°C at 5% CO2 saturated by water steam. After 24 hours of cultivation, most of the aggregates (embryos plus stem cells) have formed one single embryo in the stage of late morula or early blastocyst. We show their aspect in fig. 3

Fig. 3 CD1 embryo aggregation with stem cells leading to the formation of chimerical embryo type CD1xR1; ES at 2, 5 days and 3, 5 days of in vitro development (original)
Embryos, which have aggregated with stem cells and have formed morulas and chimerical blastocysts, have been transferred into the uterine corns of pseudogestant females in order to continue their development until the time of delivering.

4) Aggregated chimerical embryo transfer into the uterine corns of foster mothers (embryos x stem cells)

In order to successfully realize the transfer of embryos aggregated in the uterine corns of foster mothers it was necessary, first, that these were brought in the stage of pseudogestation.

Preparing pseudopregnant females

As embryo recipients we used CD 1 females. 8-12 weeks old females were prepared for pairing, over night, with vasectomyzed males. The next day they were checked for the presence and absence of vaginal plug. The females, which had vaginal plug were placed separately in individual cages. They were used as embryo recipients two days later (2.5 d pc)

Aggregated embryo transfer procedure

We first anesthetized the mouse female by intraperitoneal injection (0.6-0.7 ml avertin 0.2%) after which it was put in abdominal position on a 9 cm Petri dish in order to be easily moved under the microscope.

We aspirated then the embryos in the pipette in the following way: a part with media followed by a gas bubble, then another part media, a gas bubble, media with embryos and finally a gas bubble with a small amount of media. Until used, the pipette was fixed by pressing it into a peace of plastiline placed on the base of the microscope and taking care not to touch the surrounding environment.

In the postero median part, at the level of the lumbar vertebrae, we performed an incision of 1 cm into the skin of the female with the help of a scissor for external use. Through this incision, with the help of a forceps with sharp tip, the muscle was perforated. With the help of a forceps with blunt tip we evidenced the genital tract (ovary, ooduct and uterus), without touching the blood vessels.

Using a Serafine forceps we took out the fat, which surrounded the ovaries, the genital tract was descending at the middle of the back letting the ovary, the ooduct and the incipient part of the uterus to stay out of the body.

![Image of mouse with genital tract and uterine corns](image)

**Evidencing the genital tract** (original)  **Perforation of the uterine corn** (original)

Fig. 4 Moments of the transfer process of chimerical embryos into the uterine corns of the foster mother.
We perforated the uterine corn with the help of a hypodermic needle (26 thick), near to the utero-tubal junction, creating a hole of few millimeters through which we introduced the tip of the pipette containing the embryos. Once the pipette was in the lumen of the cornus, the embryos were released by pressure inside it, and the genital tractus was reintroduced in the pelvic cavity. The other uterine corn was approached in the same manner.

The wound was closed with the help of suture clamps. At the end of the procedure the female was placed on the heating plate until it recovered from anesthesia, and then it was placed in a clean cage. The average number of transferred embryos/receptor female was 14-16 (7-8 embryos in each uterine corn).

RESULTS AND DISCUSSIONS

We obtained 84 chimerical embryos from embryos harvested from CD1 mice, which presented the genetic marker of white hair color, aggregated with pluripotent embryonic stem cells R1/E line, carrying the genetic marker of brown hair. After their transfer into the uterine corns of pseudogestant foster mothers, at delivery time we obtained 15 mice babies, which presented white fur spotted with brown hair. We show this aspect in fig. 2

We believe that the white hair results from embryonic cells, which presented the white color marker, and the brown comes from cells descending from stem cells possessing the brown hair marker. The way that the white and brown colors are distributed along the entire body, head, feet, leads us to the conclusion that in embryonic and later fetus and pups formation, participate both the embryonic cells (white hair marker) and also the pluripotent stem cells (brown hair marker), which were added to it.

From our research we conclude that the mixture of 8 days old embryonic blastomeres with embryonic stem cells leads to the formation of chimeras. Chimerical tissues contain both cells belonging to white mice (embryos containing the genetic marker white) and also cells belonging to stem cells containing the genetic marker brown. If this phenomenon takes place in a similar manner also in the constitution of gonads, then the chimerical animal of both sexes must produce sperm and functional oocytes specific to both couple of parents. This means that from pairing chimerical animals between them, we have to obtain mice belonging to both lines of mice that participated in their formation.
Testing the chimerical characteristics of gonads

In order to convince ourselves that such situation takes place also in the constitution of ovaries and testis, we paired chimerical females (white and brown hair) with chimerical mails or white mails, brothers of these females, and we obtained both mice with white fur and also mice with brown fur. This objective prove shows that chimerical characteristics of these both lines is found not only in cells composing hair and skin, but also in cells forming gonads.

Isolated pluripotent embryonic stem cells aggregated with 8 days embryonic blastomeres, participate also in the formation of gonads, leading at puberty to the formation of genetically identical gamets with those from the pure line from where they have been isolated. This fact leads us to the conclusion that pluripotent embryonic stem cell usage in forming chimerical embryos (which can be isolated from every individual and can be "in vitro" cultivated in millions of copies), builds up a new generation of methods in cloning mammals.

In another work we will demonstrate that in the case of tetraplod embryos aggregated with stem cells we can produce mice from stem cells in one generation. We proved in multiple experiments that we performed, that tetraploid embryonic cells form only the placenta and the fetal layers, and that descendents of stem cells participate in forming the organism of the future fetus or newborn.

CONCLUSIONS

From our experiments we conclude the following:

1) If pluripotent stem cells are aggregated with 8 days embryonic blastomeres and are cultivated in the thermostat and then transferred into the uterus of a foster mother, their descendents are differentiating and disseminating in all tissues contributing to the formation of the future individual.

2) Offspring of embryonic stem cells participate along embryonic stem cells, with whom they were mixed, also in the constitution of testis and ovaries, giving birth to chimerical gonads.

3) Germ cells from testis and ovaries, offspring of the two couples of parents give birth both to sperm and functional oocytes, genetically similar to the lines where they came from.

4) Pairing chimerical animals between them or chimeras with other animals belonging to pure lines gives birth to mice genetically similar with pure lines, which have participated to the constitution of chimerical animals.

5) From embryonic stem cells we obtain animals, which carry in their genome all genes that can be found in the individual where they have been harvested. This way, this method that uses aggregation of embryonic stem cells with 8 days old blastomeres, becomes a feasible method of mammal cloning.

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