Factors Affecting Survival of Reconstructed Mouse Embryos after Nuclear Transfer

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Reconstruction of mouse embryos was performed by injection of donor genetic material from differentiated cells of various types (cumulus cells, cardiomyocytes, and epithelial cells) into recipient cells (mature oocytes and zygotes). A medium for microsurgery was selected, which enhanced survival of both embryonic and somatic cells during the reconstructive manipulations. Special preparation of somatic cells to transplantation was carried out, which employed factors synchronizing the cells in a certain phase of the cell cycle in order to enhance their capacity to maintain the development of reconstructed embryos. The processes of nucleus reprogramming in specialized cells under the action of cytoplasmic factors of oocytes and zygotes were examined. During *in vitro* culturing of reconstructed embryos, the most successful development was observed in embryos implanted with donor material from cumulus cells. Mouse embryos reconstructed with a certain genome and subsequent production and use of stem cells are considered as the model system for developing the basic principles of replacement therapy.

Key Words: *nuclear transfer; reconstructed embryos; cumulus cells; culture medium*

Isolation of embryonic stem cells (ESC) from the inner cell mass of mammal blastocysts [7,12,15,19], including human blastocysts [19], stimulated the development of methods and technologies of embryo reconstruction with implanted genetic apparatus from differentiated cells, since this technique directly leads to replacement therapy based on the use of stem cells with genome identical to patient's genome [5,8,9,11,18].

Ample experimental data are now accumulated on the developmental processes in reconstructed embryos of various species [1,14,16,17,20]. The basic problem confronted all these works is massive death of these embryos at various stages of their development. This can be explained by, first, necrosis during reconstruction resulting from mechanical damage to cells during nuclear transfer, and second, apoptosis, *i.e.* triggering of the intrinsic genetic program sorting out the embryos with developmental abnormalities.

The major factors determining the development of reconstructed embryos are the processes of remodeling of implanted nuclei and reprogramming of their genetic material. Inadequate or incomplete reprogramming is responsible for developmental arrest and death of the majority of reconstructed embryos [6,8,13,14].

This paper describes the data on reconstruction of early mouse embryos and their subsequent development. The following differentiated cells were used as the donors of genetic materials: cardiomyocytes, cumulus cells, and epithelial cells. The study focused on the optimization of experimental technique and culture media for improving the efficiency of embryo reconstruction.

MATERIALS AND METHODS

Mouse strains BALB/c (albino) and (CBA×C57B1/6) F_1 (agouti) were used. The cells of renal epithelium

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and cardiomyocytes were obtained by enzymatic disaggregation of tissues from neonatal mice according to the following technique. In decapitated 1-3-day-old pups, the peritoneum was opened and the kidneys and heart were aseptically isolated and washed in DMEM culture medium. The isolated organs were minced to small fragments (0.5×0.5 mm), which were treated with 0.25% trypsin for 15-20 min at 37°C. The cells were centrifuged for 2 min at 1500 rpm and cultured in DMEM with 10% FCS.

The cells were synchronized in G_0 phase of the cell cycle by culturing the cells in serum-free medium for 1-10 days. Synchronization of the cells in G_1 phase was achieved by subculturing of the cell monolayer at a density of 100,000/cm^2 in a medium containing FCS.

We also examined nonsynchronized cardiomyocytes taken from mixed culture on day 1-10 after isolation. The optimum time of culturing for cardiomyocytes was 9 days. Fractionation of the mixed primary culture was based on differences in adhesion time to the culturing surface.

Cumulus cells from mice aging 2.0-2.5 months were isolated simultaneously with oocytes 12.5 h after superovulation, which was carried out routinely. Zygotes were isolated 11-12 h after natural ovulation. For transferring the nuclei of somatic cells into enucleated oocytes and zygotes we used a method combining microsurgery with electrostimulated fusion of cells [2,3]. The state of reconstructed embryos was assessed by the results of *in vitro* culturing.

RESULTS

At the first stage of the experiments we obtained reconstructed mouse embryos with donor nuclei from somatic cells (cardiomyocytes, cumulus cells, and epithelial cells). A total of 370 oocytes and 143 zygotes were operated, and 334 reconstructed embryos were obtained, which contained nuclei of cumulus (n=98), epithelial (n=136) and cardiac (n=100) cells. The count of embryos survived microsurgery was 70%.

Figs. 1-3 show somatic cells, whose nuclei were used for embryonic reconstruction. Injection of the donor cell (karyoplast) under the embryonic shell (zona pellucida) of enucleated oocyte (cytoplast) is shown in Fig. 4, and Fig. 5 shows a donor cell under the zona pellucida. The genetic material of donor cells was transferred directly to enucleated cells after presentation of electric pulse to the contact region between the karyoplast and cytoplast. Figs. 6 and 7 show electrostimulated fusion resulting in the formation of reconstructed embryos (Fig. 8).

A search was performed for the optimal experimental conditions ensuring highly efficient reconstruction of mouse embryos. This search was aimed at preventing cell death by necrosis or apoptosis. We mainly focused on the most adequate composition of the medium for microsurgery, which would improve survival of both embryonic and somatic donor cells. The decisive factor was osmolarity of the medium (total ion concentration).

Osmolarity of the medium chosen in microsurgical experiments on oocytes was about 140 mosmol/liter, *i.e.* almost 2-fold lower than osmolarity of routine microsurgery media (290-320 mosmol/liter). For zygotes, osmolarity of the microsurgery medium was 190-220 mosmol/liter. These media virtually completely excluding cases of necrotic cell death and considerably improved the efficiency of reconstruction process, including electrofusion of the nucleus-donating somatic cells with enucleated oocytes and zygotes.

Particular attention was given to functional state of the donor cells and to factors synchronizing these cells in a certain phase of the cell cycle for increasing the probability of their reprogramming and potency to maintain the development of reconstructed embryos. The experiments on synchronization were carried out using renal epithelial cells. The cells were cultured in a serum-free medium for 1-10 days. This procedure synchronized the cells in G_0 phase of the cell cycle. The best reconstruction results were obtained for epithelial cells synchronized in G_0 phase for 2-3 days.

We studied remodeling and reprogramming processes in somatic cell nuclei under the effect of cytoplasm of enucleated mature oocytes and zygotes. Structural and functional alterations in the donated nuclei were observed in most operated embryos a few hours after reconstruction: specifically, marked enlargement of the nuclei, appearance of different number of nucleoli, and some other processes, which were characteristic of normal zygotes during the formation of pronuclei (Fig. 9).

It was previously considered that in contrast to cytoplasm of mature oocytes, zygotic cytoplasm induces insignificant changes in the nuclei of differentiated cells, which are insufficient for reprogramming. However, structural alterations in donated nuclei and results of subsequent development of reconstructed embryos suggest that not only oocytic, but also zygotic cytoplasm can stimulate remodeling and reprogramming of the transferred nuclei of differentiated cells. These data refute the current views that only the cytoplasm of unfertilized ovules (metaphase II oocytes) contains specific factors triggering reprogramming of transferred somatic nuclei. Fertilized oocytes (zygotes) also contain factors promoting the corresponding structural and functional rearrangements of the transplanted nuclei.

At the next stage of this study we elaborated *in vitro* methods of culturing reconstructed embryos to

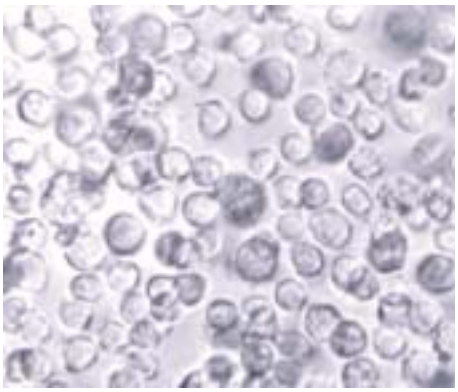

Fig. 1. Cumulus cells, ×450.

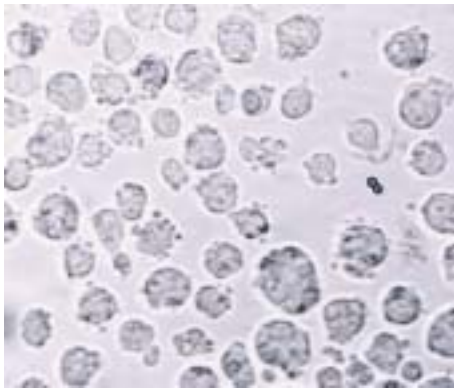

Fig. 2. Epithelial cells, ×450.

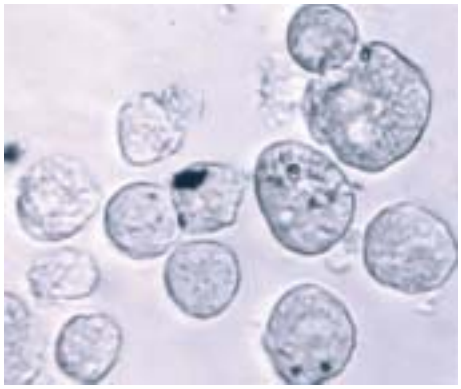

Fig. 3. Cardiomyocytes, ×1500.

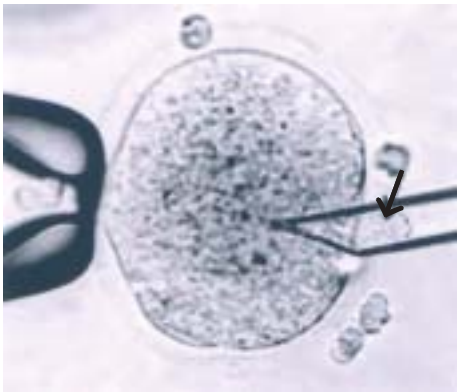

Fig. 4. Injection of epithelial cell (arrow) into enucleated oocyte, ×500.

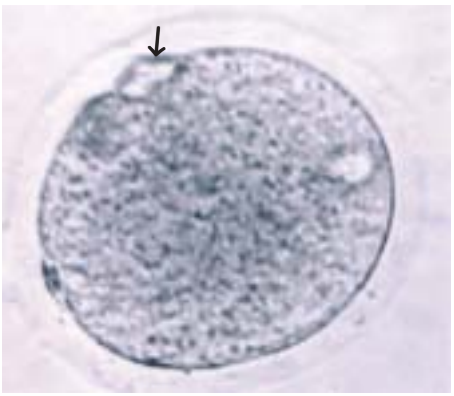

Fig. 5. Epithelial cell (arrow) under zona pellucida, ×600.

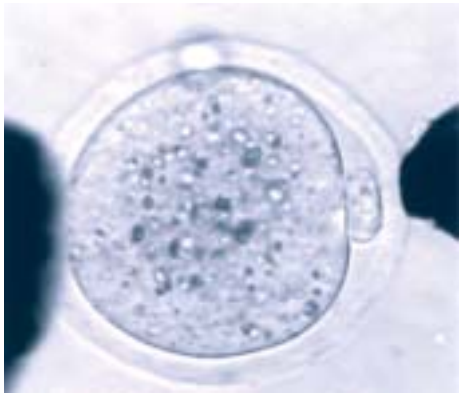

Fig. 6. Electrostimulation, ×500.

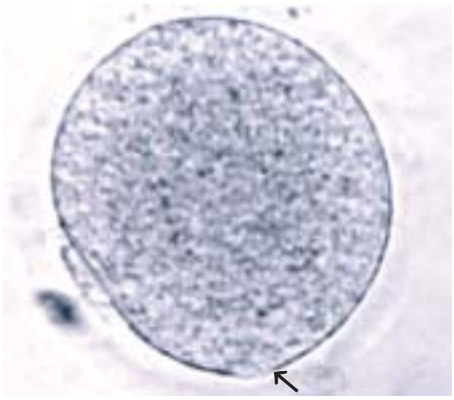

Fig. 7. Fusion of epithelial cell with enucleated oocyte, ×600.

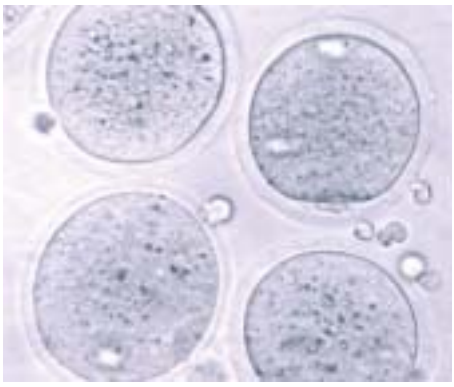

Fig. 8. Reconstructed embryos, ×300.

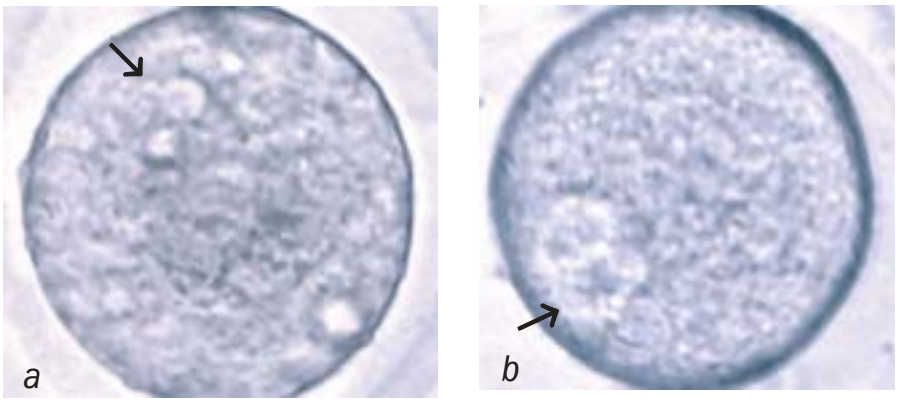

Fig. 9. Remodeled nuclei (arrows) of differentiated cells, ×650.

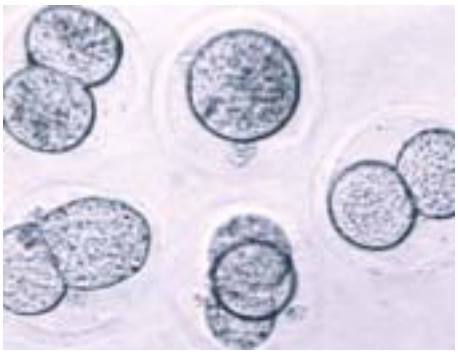

Fig. 10. *In vitro* culturing (day 2) of embryos reconstructed with nuclei of epithelial cells, ×275.

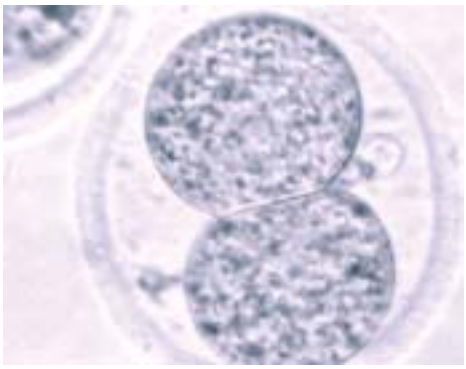

Fig. 11. Reconstructed embryo at two-cell stage, ×600.

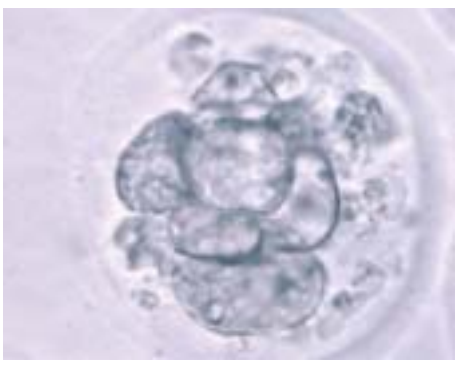

Fig. 12. Abnormal development of reconstructed embryo, ×600.

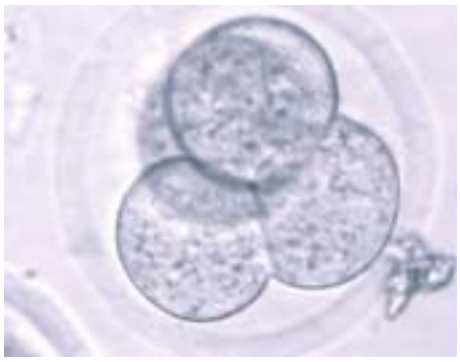

Fig. 13. Reconstructed embryo at four-cell stage, ×600.

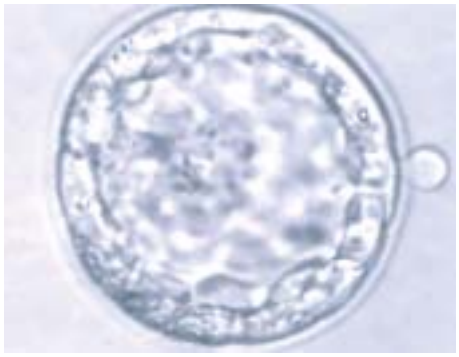

Fig. 14. Embryo reconstructed with cumulus cell nucleus at the blastocyst stage, ×600.

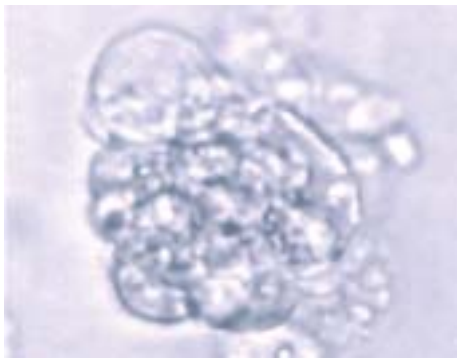

Fig. 15. Inner cell mass isolated from reconstructed embryo at the blastocyst stage, ×700.

the blastocyst stage. The most successful development demonstrated embryos implanted with cumulus cell nuclei. These embryos developed to morula and blastocyst stages (12%), while the development of embryos donated with other nuclei was arrested at the earlier stages. Figs. 10-14 show reconstructed embryos on days 2-5 of their *in vitro* development. Fig. 15 demonstrates the cells of the inner cell mass isolated from the blastocyst.

It was previously established that the development of reconstructed embryos would be surely successful and would lead to the best results only for implantation of embryonic nuclei, including embryonic stem cell nuclei. We showed that the nuclei of differentiated cells characterized with close functional relation to the embryonic cells *in vivo*, also have certain advantages for reconstruction.

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REFERENCES

1. I. S. Lagutina and V. V. Galat, *Ontogenez,* **32**, No. 3, 180-195 (2001).
2. L. M. Chailakhyan, B. N. Veprintsev, T. A. Sviridova, and V. A. Nikitin, *Biofizika*, **32**, No. 5, 874-887 (1987).
3. L. M. Chailakhyan and T. A. Sviridova-Chailakhyan, *Nauka Ross.*, No. 2, 10-15 (2001).
4. T. A. Chailakhyan, E. F. Vikhlyantseva, T. N. Chebotareva, and L. M. Chailakhyan, *Dokl. Ross. Akad. Nauk*, **338**, No. 4, 1-3 (2003).
5. A. Colman and A. Kind, *Trends Biotechnol.*, **18**, 192-196 (2000).
6. W. Dean, F. Santos, M. Stojkovic, *et al.*, *Proc. Natl. Acad. Sci. USA*, **98**, No. 24, 13 734-13 738 (2001).
7. M. J. Evans and M. H. Kaufman, *Nature*, **292**, 154-156 (1981).
8. K. Hochedlinger and R. Jaenisch, *N. Engl. J. Med.*, **349**, No. 3, 275-286 (2003).
9. W. S. Hwang, Y. J. Ryu, J. H. Park, *et al.*, *Science,* **303**, No. 5664, 1669-1674 (2004).
10. A. Jonneau and J. P. Renard, *Curr. Opin. Gen. Dev.*, **13**, No. 5, 486-491 (2003).
11. M. Körbling and Z. Estrov, *N. Engl. J. Med.*, **349**, No. 6, 570-582 (2003).
12. G. Martin, *Proc. Natl. Acad. Sci.*, **78**, 7634-7638 (1981).
13. N. Ogonuki, K. Inoue, Y. Yamamoto, *et al.*, *Nat. Genet.,* **30**, 253-254 (2002).
14. W. M. Rideout, K. Eggan, and R. Jaenisch, *Science,* **293**, No. 5532, 1093-1098 (2001).
15. J. Rossant, *Stem Cells*, **19**, No. 6, 477-482 (2001).
16. A. Rybouchkin, B. Heindryckx, J. Van der Elst, and M. Dhont, *Reproduction*, **124**, 197-207 (2002).
17. D. Solter, *Nat. Rev. Genet.*, **1**, 199-207 (2000).
18. A. Surani, *Curr. Opin. Genet. Dev.*, **13**, No. 5, 445-447 (2003).
19. J. A. Thomson, J. Itskovitz-Eldor, S. S. Shapiro, *et al.*, *Science*, **282**, 1145-1147 (1998).
20. T. Wakayama and R. Yanagimachi, *Mol. Reprod. Dev.*, **58**, 376-383 (2001).