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ISOLATION AND CHARACTERISTICS OF PRIMORDIAL GERM CELLS IN CHICKEN

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S u m m a r y

Optimized conditions for isolation of primordial germ cells in chicken and their description are given. Trypsinization (with 0.05 % trypsin) was found to be the most effective method of disaggregation in chick embryos for isolation of their embryonic cells. The maximum number of primordial germs cells can be isolated from 6 days-old embryos, followed by separation of the resulting cell suspension by adhesion after 90 minutes of their cultivation with the use of chicken embryonic fibroblasts as feeder layer. During incubation of primordial germ cells on feeder layer cells remained viable for a longer time, and ranged from 5 to 7 days, depending on the type of cells used as feeder layer (finite cell line STO, recently separated and incubated for several passages in chicken embryo fibroblasts).

Keywords: chicken, embryos, primordial germ cells, conditions for isolation.

A significant current advance in creation of transgenic bird has been obtained through the use of various methods of gene transfer (1-7), but production of transgenic chickens is still concerned to certain problems. Peculiarities of bird reproduction and development (8) reduce the efficiency of microinjection commonly performed on animal cells, which necessitates applying some alternative methods of directed gene transfer. One of such methodological approaches is actively developed in recent years – using pluripotent stem cells (PSC) of different types (including primordial germ cells - predecessors of differentiated germ cells) as target cells for the transfer of recombinant DNA (9). Genetic modification of primordial germ cells (PGCs) with following transplantation into chicken embryos in vivo is considered as a promising method for target genetic modification of bird gonads and creation of transgenic bird.

In this connection, the subject of this research was isolation and characterization of primordial germ cells of chickens as target cells for genetic engineering.

Technique. Experiments were performed on chicken embryos the cross Ptichnoe at developmental stages from the 4^{th} to 8^{th} day of incubation. Disaggregation of the embryos was performed using two methods: mechanical dissociation of cells and enzymatic treatment of tissues. Dissociation of the cells was performed by pipetting in DMEM medium ("Pan-Eco", Russia) for 5 minutes (first variant) or in trypsin solutions of different concentrations (0,05; 0,10; 0,15; 0,25%) preheated to 37°C for 7-10 min (second variant).

The resulting suspensions of cells were visually examined with an optical microscope ("Nikon", Japan), magnification S400, for the following parameters: the number of cells in suspension, the number of unit cells, the number of cell conglomerates, the number of live and dead cells. PGCs were identified by PAS-staining for glycogen (10).

Adhesive capacity of chicken PGCs to other types of embryonic cells was evaluated by short-term culturing (from 15 to 120 min) in Petri dishes with DMEM medium. After the incubation, the culture medium containing the cells not adhered to the substrate cells was gently transfused from the Petri dishes into centrifuge tubes, and then centrifuged (200 g, 5 min). The precipitate was resuspended in DMEM (240 cells/ul), 50 ul suspension was applied on a glass slide to prepare a smear, which was dried and stained for glycogen (PAS-reaction) to reveal PGCs. Upon the obtained results, the percentage of stained cells (PGCs) relative to the total number of examined cells was calculated.

The culture of derived PGCs was maintained using DMEM medium with high concentrations of glucose (4,5 g/l) and fetal bovine serum (15%) supplemented with glutamine (2 mM), 2-mercaptoethanol (10^{-6} mM) and gentamicin (50 ug/ml). The suspension of cells isolated from the 6-day-old chicken embryos was plated in Petri dishes (d=100 mm) and cultured for 18-24 hours, after which the supernatant containing predominantly red blood cells was poured off and the cells adhered on the surface of Petri dishes were removed with trypsin solution (0,25%) and re-plated. After 90 minutes of culturing necessary for adhesion of embryonic fibroblasts, PGCs-containing supernatant was transferred to a new Petri dish with a feeder layer or without it. Three variants of feeder layer were prepared: a continuous culture of mouse embryonic fibroblasts the line STO, freshly isolated chicken embryonic fibroblasts, and chicken embryonic fibroblasts cultured for several passages.

Viability of the cells was assessed by a conventional trypan blue (0,5%) staining (11).

Statistical processing of data was performed using a standard computer program Microsoft Excel.

Results. The effects of three factors (the method of disaggregation of the embryos, the method of separation of PGCs from other types of cells, and the age of embryos used as a source of PGCs) were studied in respect to their influence on the efficiency of isolation of PGCs from chicken embryos.

In the case of mechanical disaggregation of the embryos, the resulting suspension consisted of both separate cells and cell conglomerates amounted to, respectively, 69 and 31%. So, mechanical treatment resulted in incomplete disaggregation of tissues while the minimum reduce in cell viability (less than 1% of dead cells).

The method of enzymatic treatment provided the suspension containing mostly separate cells with singular cell aggregates. These few groups of cells (0,1%) were revealed only by staining with 0,05 and 0,10% trypsin. However, the yield of viable cells was lower than in the variant of mechanical treatment. Using 0,05% trypsin, it was observed the maximum amount of viable cells in the suspension - 95%, which is slightly lower (4%) than in the case of mechanical treatment. Considering this fact, as well as the possibility of obtaining the suspension containing nearly 100% of unit cells, isolation of PGCs for further studies was conducted using 0,05% trypsin solution.

Cytological examinations of the specimens derived from chicken embryos on the 4^{th}-8^{th} day of incubation revealed age-

dependant changes in the number of PGCs in the gonads. In 4-day-old embryos, the number of PGCs per embryo was only 302±18 pcs, but on the 6th day it increased to 4080±70 cells, and then sharply reduced to 1217±76 (3,2-fold; 7th day) and 940±37 (4,3-fold; 8th day), probably, due to the start of differentiation of PGCs in gonads at this period of embryogenesis with subsequent partial loss of their morphological features (eg., some cells were not stained by PAS-reaction for glycogen).

The population of PGCs derived from chicken embryos, as a rule, is heterogeneous due to the presence of other cell types, which requires additional purification of PGCs culture, eg., through separation of cells by adhesion.

The comparison of adhesive capacity of PGCs and other types of embryonic cells at short-term (15-120 min) culturing has shown that in primary cell population of chicken embryos, the proportion of PGCs did not exceed 5,0±0,1% (Fig. 1). Separation by adhesion increased the proportion of PGCs in the cell population. Separation of cells after 90 min of culturing provided the highly homogenous cell population with proportion of PGCs equal to 80,7±3,4%. When the time of culturing was less than 90 min, the incubation in a growth medium (with subsequent separation) resulted in the cell population containing only 26,5-67,9% PGCs.

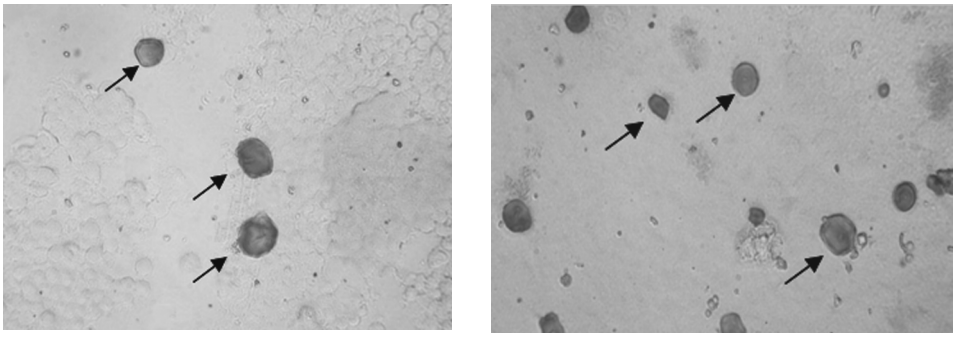

Fig. 1. Suspension of embryonic cells of chickens (cross Ptichnoye) derived before and after (respectively, left and right) **separation of cells by adhesion** (primordial embryonic cells are pointed by arrows). PAS-staining for glycogen, magnification S400.

Optimization of conditions for isolation of PGCs from chick embryos allowed to obtain the cell population consisting mainly of PGCs. These spherical cells with diameter ranging from 14 to 26 um were morphologically distinct from other types of embryonic cells.

The evaluation of maintaining the culture of PGCs in DMEM with the supplements has shown that in Petri dishes without a feeder layer the cells retained viability for 1-2 days while being a suspension almost not attached to the substrate. Staining of PGCs with 0,5% trypan blue revealed no reduce in viability during 8 h of culturing. Increasing the time of incubation caused the decline in amount of viable cells up to 0,1% after 48 hours of culturing (Fig. 2).

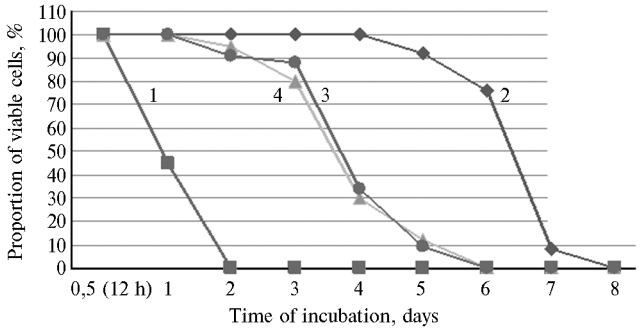

Fig. 2. Time of incubation of primordial embryonic cells of chickens (cross Ptichnoye) depending on a feeder layer: 1 – no feeder layer, 2 – freshly isolated embryonic fibroblasts of chicken, 3 – continuous culture of embryonic fibroblasts of mouse the line STO, 4 – cultured embryonic fibroblasts of chicken.

PGCs cultured on a feeder layer retained viability for a longer time. The time of culturing PGCs ranged from 5 to 7 days depending on the type of cells in a feeder layer - a continuous cell culture of mouse the line STO, freshly isolated embryonic chicken fibroblasts, and embryonic chicken fibroblasts cultured for several passages.

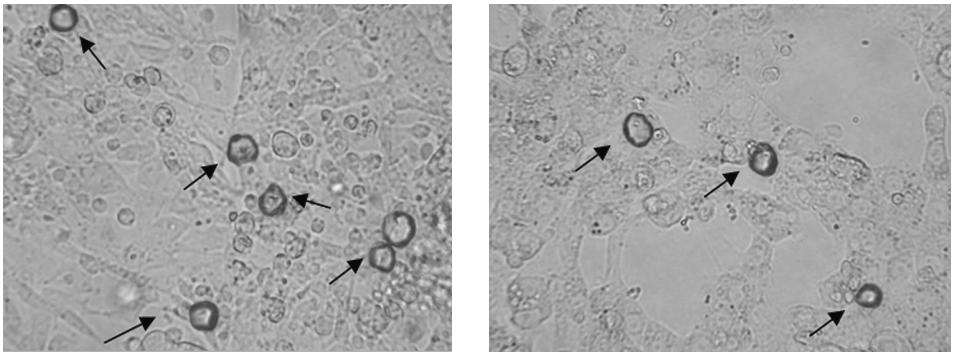

Fig. 3. Primordial germ cells of chickens (cross Ptichnoye) cultured on different feeder layers – embryonic fibroblasts of chicken (left) **and continuous culture of embryonic fibroblasts of mouse the line STO** (right) (primordial embryonic cells are pointed by arrows). Magnification S400.

The maximum time of culturing PGCs (7 days) was established using the feeder layer of freshly isolated chicken embryonic fibroblasts. In this variant, the primary isolated suspension of embryonic cells was plated in Petri dishes (d = 100 mm) and cultured for several days without separation of cell types, which resulted in a feeder layer formed by fibroblasts and PGCs were adhered on it (Figure 3). When fibroblasts formed a monolayer, PGCs were transferred on a new feeder layer – the cultured chicken embryonic fibroblasts treated with mitomycin C. Then, the re-plated PGCs attached to the feeder cells, but after 18-24 h of incubation they

separated from the feeder layer and then, being a suspension of cells, retained viability for 1-2 days.

In the variants of culturing PGCs on feeder layers of cultured chicken embryonic fibroblasts and embryonic fibroblasts of mouse the line STO, PGCs maintained viability for more than 5 days. It wasn't observed any significant difference in duration of culturing PGCs on these feeder layers. Besides, in all cases PGCs showed a tendency almost similar to the abovementioned: after the transfer on a feeder layer, the cells firstly attached to it, then in 36-48 h separated and then were cultured as suspension for 2-3 days.

Thus, this research has allowed to develop the optimized conditions for isolation of primordial germ cells (PGCs) from chicken embryos. The effective method of disaggregation of embryonic cells including PGCs has been established – enzymatic treatment of the embryo with 0,05% trypsin solution. At the same time, the proportion of PGCs in resulting cell culture depends on the age of the embryo and the method of separating PGCs from other cell types. The maximum amount of PGCs can be derived from the 6-day-old embryos, with following separation of the cell suspension by adhesion after 90 minutes of incubation with a feeder layer of cultured chicken embryonic fibroblasts.

REFERENCES

1. Ivarie R., Avian Transgenesis: Progress Towards the Promise, *Trends Biotechnol*., 2003, vol. 21, pp. 14-19.
2. McGrew M., Sherman A., Ellard F., and Lilico S., Efficient Production of Germline Transgenic Chickens using Lentiviral Vectors, *EMBO Reports*, 2004, vol. 5, pp. 728-733.
3. Sang H., Prospects for Transgenesis in the Chick, *Mechanisms of Development*, 2004, vol. 121, pp. 1179-1186.
4. Volkova N.A., Tomgorova E.K., Bagirov V.A., Beloglazov D.V., Zinovyeva N.A., Volkova L.A., and Ernst L.K., Effective Transformation of Chicken Cells in vitro and in vivo by Gene Construction on the Basis of Retroviral Vectors, *S.-kh. biol.,* 2009, vol. 6, pp. 44-48.
5. Ernst L.K., Volkova N.A., and Zinovyeva N.A., Some Aspects in the Use of Transgenic Technologies for Farm Animals Breeding, *S.-kh. biol.,* 2009, vol. 2, pp. 4-9.
6. Scott B.B., Velho T.A., Sim S., and Lois C., Applications of Avian Transgenesis, *ILAR J.,* 2010, vol. 51, no. 4, pp. 353-61.
7. Byun S.J., Kim S.W., Kim K.W., et al., Oviduct-Specific Enhanced Green Fluorescent Protein Expression in Transgenic Chickens, *Biosci. Biotechnol. Biochem*., 2011, vol. 75, no. 4, pp. 646-649.
8. Petitte J. and Mozdziak P., Production of Transgenic Poultry, in *Transgenic Technology: a Laboratory Handbook*, Pinkert C.A., Ed., New York, 2002, pp. 279-306.
9. Petitte J., Liu G., and Yang Z., Avian Pluripotent Stem Cells, *Mechanisms of Development*, 2004, vol. 121, pp. 1159-1168.
10. Romeis B., *Mikroskopicheskaya tekhnika* (Microscopy Technique), Moscow, 1953.
11. *Zhivotnaya kletka v kul'ture. Metody i primenenie v biotekhnologii* (Animal Cells in Culture. Methods and Application in Biotechnology), Dyakonov L.P., Ed., Moscow, 2009.