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Methods of Production of Transgenic Mice by Manipulating Fetal Germ Line Cells

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It has been unclear when the segregation of germ line cells to somatic line cells ocurrs in mouse embryos. It is as cells with a high alkaline phosphatase activity within the extraembryonic mesoderm cells near the base of allantois in egg-cylinder stage enbryos (about 7.0 dpc) that we can first identify germ line cells. And then, these cells invade into the endoderm epithelium of the yolk sac adjacent the base of allantois, which translocates to the epithelium of the hind gut by the passive migration during the gut formation. As the result of this morphogenetic movement, they are transferred near the genital ridges (primordial gonads). And they migrate from the gut epithelium into the mesenchyme of the dorsal mesentery , and towards the genital ridge with active migration.They first arrive at the genital ridges along the mesonephroi at about 10.5 dpc. By 12.5 dpc, most of them have become lodged in the primordial(indifferent) gonads. The germ cells from the first detectable origin until the settlement in indifferent gonads are called primordial germ cells (PGCs). About this stage(12.5 dpc), the sex difference of gonads become morphologically recognizable. As soon as the sexual differentiation can be detected, the sex of the individual is called male or female , the gonad is called a (fetal) ovary or a testis. The germ cells in the ovary are initially called oogonia, and in the testis spermatogonia(or more precisely, prospermatogonia). In the 13.5 dpc fetal ovaries, about 4 % of the oogonia stop active mitosis and start meiosis (they are called oocytes), and by 15.5 dpc almost all have entered the first meiotic division. By 18.5 dpc, all oocytes have attained the diplotene to dictyotene stage of the first meiotic division , and arrested the progress of meiosis. In fetal testes, on the other hand, spermatogonia stop active mitosis at about 14 dpc and take a rest till the birth.

To produce transgenic (Tg) mice, generally, fertilized eggs and embryonic stem (ES) cells are used as the vehicles to introduce foreign genes. However, these methods have some demerits to apply them to producing Tg livestock. We had surveyed candidates to serve as vehicles for manipulating genetically in exchange for those cells.

ES cells are used to produce knockout or knockin mice by the technique called "homologous recombination." Such homologous recombination events occur rarely in mammalian cells, and seems to occur via the mechanism of DNA repair. Usually, homologous recombination occur in cells just prior to meiosis. It is only the PGCs and gametogonia that are able to enter meiosis among the germ line cells. If "homologous recombination constructs" can be introduced into these cells, homologous reconbination frequency will be increased. Among these cells, PGCs and oogonia are transitory cells in embryos as compared with spermatogonia which exist not only in embryos but also in adult males as stem cells within the seminiferous tubules of testes. Since PGCs and gametogonia proliferate more actively in embryos, it seemed for us to find out easily the culture conditions of these embryonic cells like ES cells. We had started to develop the technology for obtaining offspring from manipulated PGCs and gametogonia and thier culture conditions in vitro.

1. Method of obtaining offspring from oogonia

Fetal ovaries of 12.5 dpc embryos contain only oogonia as germ cells. We dissociated them into dispersed cells with trypsin and EDTA, and cultured for 2 days with a gyratory shaker to produce reaggregates which were called "reconstituted ovaries." And we transplanted them into the ovary sites of ovariectomized adult females. We have successfully obtained offspring derived from transplanted reconstituted ovaries. This suggests that we are able to manipulate the mammalian germ line by using oogonia as vehicles and targets of various manipulation in vitro.

2. Method of obtaining offspring from spermatogonia

We dissociated fetal testes of 12.5-15.5 dpc (mainly 12.5 dpc) embryos into single cells with the enzymatic treatment described above, and cultured for 2-3 days into Matrigel to reconstruct cord-like structure called "reconstituted seminiferous tubules." We transplanted them into the space among seminiferous tubules of germ cell-deficient W mutant male. They were coupled with normal females for 3 months. Since we could not obtain offspring, host testes were examined histologically. We found out many tubules containing many germ cells of various stages of spermatogenesis via sperm

among germ cell-free host tubules. However, we could not detect the interconnection of transplants-derived tubules with host tubules. Recently, Yanagimachi and his colleague succeeded in obtaining offspring by the electrofusion of round spermatids with ovulated ova and the sperm injection. This suggested that offspring could be obtained derived from fetal spermatogonia by combining our method with Yanagimachi's method, because spermatids and spermatozoa in germ cell-free host could be isolated easily.

Brinster and his colleague recently reported that they could obtain transplanted spermatogonia-derived offspring by injecting the mixture of spermatogonia and Sertoli cell precursors into the lumen of seminiferous tubules of Busulfan-treated adult males and mating them with normal females. Another merit of this method is that the lumen of seminiferous tubules are one of the immunologically privileged places. If this phenomenon is not restricted to mice, it will be an interesting method for the production of Tg livestock, since livestock are not inbred generally. We tried to simplify the Brinster's methods.

3. A simplified method of intraseminiferous tubule transplantation

Disposable glass-made Pasteur pipettes which were attached to a silicon tube connected with a mouthpiece were employed. The diameter of the tips used were a little larger than that of host tubules. By the hand-held mouth-controlled injection pipette, the mixture of dispersed cells of newborn testes (B6 strain as isografts , C3H strain as allogfafts) was injected into host seminiferous tubules of W mutant mice (B6 strain). Host testes were histologically examined 2 months after the transplantation. In both testes, tubules containing many germ cells were found among host germ cell-free tubules.

Now, one of the problems which need to be solved is the culture condition in vitro to select targeted cells and proliferate them.

4. The culture condition of migrating PGCs

Several substances, such as stem cell factor (SCF), leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), tumor necrosis factor a (TNFa) and forskolin, were reported as growth and/or survival factors of mouse PGCs. However, the in vitro growth rates of PGCs by their factors compares lower than in vivo rate. On

the other hand, the condition reported by Matsui et al. and Resnick et al., those are the combination of LIF, SCF and bFGF, results in the transformation of PGCs into ES-like cells which were called embryonic germ (EG) cells. Actually, they proliferateactively to form ES cell-like colonies. By introducing them into blastocysts, germline chimeras can be obtained. However, the EG cells are undifferentiated cells, not germ line cells. We tried to develop the PGC culture condition to proliferate similarly to in vivo and not to transform into EG cells. When migrating PGCs derived from 8.5 dpc embryos were cultured onto the feeder cells expressing membrane-bound SCF and maintained in the medium where forskolin and the BRL (Buffalo rat liver cell) conditioned medium added, they had actively proliferated similarly to in vivo. We must assure them of PGCs, although they continue to express c-kit and Oct-3 proteins. For this purpose, we constructed reconstituted ovaries by mixing cultured PGCs and isolated somatic cells of fetal ovaries and transplanted them into adult ovaries. Histological examination showed that the grafts contained oocytes.It is unclear, however, whether or not the culture condition for migrating PGCs will be suitable to nesting PGCs in gonads, oogonia or spermatogonia of fetuses, newborns, or adults.