Bioresource Engineering Division

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Goal
To develop genetics-related techniques, especially those essential for maintenance and supply of laboratory mice and stem cell lines at a high quality in RIKEN Bioresource Center.

Activities
I. Development of mouse somatic nuclear transfer techniques
II. Development of microinsemination techniques
III. Development of reliable cryopreservation techniques for mouse embryos or gametes
IV. Development of new stem cell lines

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**Specific aim**

I. Development of mouse somatic nuclear-transfer techniques

1) Cloning mice from hematopoietic stem cells

It is often assumed that tissue-specific stem cells can be used as efficient donors in cloning experiments. However, we have found that embryos reconstructed from hematopoietic stem cells (HSCs) show very poor development *in vivo* and *in vitro* compared with those reconstructed from cumulus cells. Gene expression analysis of two-cell HSC-cloned embryos revealed that five of the six zygotically activated genes examined had failed to activate. Importantly, a comparison with cumulus clones suggested that Hdac1 (histone deacetylase-1), a key gene in subsequent embryonic gene activation, was specifically repressed in HSC clones. These results suggest that the genomes of HSCs are less plastic than we imagined, at least in terms of their reprogrammability in the ooplasm after nuclear transfer.

![Figure 1. Repressed expression of zygotic genes in cumulus cell (CC) and hematopoietic stem cell (HSC) clone embryos.](image-url)
2) Interspecies nuclear-transfer cloning using monkey somatic cells

The quality of recipient oocytes is the critical factor for successful somatic cell cloning. However, it is often difficult to prepare several high-quality fresh oocytes at a time in some species. We examined the feasibility of interspecies nuclear transfer using monkey somatic cells and rabbit oocytes. About 50%–70% of the reconstructed oocytes developed to the 2–4-cell stage, irrespective of the cell type, whereas only 1% developed to blastocysts. When cytoplasts prepared from monkey somatic cells were introduced into reconstructed oocytes, the efficiency of their development into blastocysts was significantly improved (P < 0.005).

II. Development of microinsemination techniques

1) Screening for sterility-related genes in mutant mice

To screen for dominant male-sterility-related genes, male germ cells from sterile males among the progeny (G1) of mutagen (N-ethyl-N-nitrosourea)-treated mice were used for microinsemination. The G2 mice thus obtained after natural matings were then examined. We concluded that sterility was not attributable to genetic causes because all the offspring examined were fertile. We are now investigating the offspring from a second sterile line.

Figure 2. Enucleation of a recipient rabbit oocyte (Left). Arrowhead indicates the oocyte chromosomes. An ES-like cell colony derived from the inner cell mass of a rabbit-monkey interspecies nuclear transfer blastocyst (Right)

Figure 3. An elongated spermatid retrieved from a testis of a sterile G1 male (arrow). Only a few spermatids were found the testicular cell suspension but offspring were successfully obtained following microinsemination.
2) Microinsemination using male germ cells from epididymides and testes stored in freezers

The fertilization capacity of male germ cells retrieved from epididymides and testes stored at -80°C up to one year without cryoprotectant was examined using a microinsemination technique. Microinsemination was performed with epididymal/testicular spermatozoa and round spermatids by direct injection into oocytes. Normal pups were obtained irrespective of the method of cryopreservation or the cell type used. For transportation experiments, frozen testes within a polypropylene cryotube were placed in a polystyrene foam case filled with dry ice and were transported from the U.K. to Japan by air and land. Microinsemination was performed after the testes were thawed and the spermatogenic cells collected. Normal pups were obtained after embryo transfer. These results indicate that male mouse germ cells retain their nuclear integrity even after the epididymides/testes are frozen in freezers. Because this cryopreservation technique is very simple and allows storage at –80°C, it may allow us to transport male mouse germ cells internationally on dry ice, even when the senders are not specialists in cryopreservation.

3) Production of offspring from GS cells or mGS cells

The contribution of embryonic stem (ES)-like cells (mGS cells) derived from germline stem (GS) cells to normal embryonic development was examined by their microinjection into blastocysts. The chimeric mice thus obtained were used for microinsemination experiments to confirm the germline transmission capacity of mGS cells. After transfer of the microinsemination-derived embryos, donor-origin pups were obtained. In another study, we assessed the normality of GS cells that had been cultured under anchor-free conditions for a long period. Following microinsemination with round spermatids of cultured GS cell origin, normal pups were obtained. This indicates that GS cells can be cultured under floating-culture conditions without loss of their germline stem-cell capacity.
4) Microinsemination with primary spermatocytes by single nuclear transfer
We found that cumulus-free in vitro maturation (IVM) in mice can be considerably improved by using an IVM medium consisting of MEM and TYH media (1:1 mixture). A high rate (23.8%) of development from germinal vesicle oocytes to full-term fetuses following in vitro fertilization and embryo transfer to foster mothers was achieved using this medium. When this IVM system was applied to metaphase I (MI) oocytes injected with spermatocytes, offspring were first obtained without cytoplasmic replacement at MII.

III. Development of reliable cryopreservation techniques for mouse embryos/gametes

1) Efficient cryopreservation of mouse embryos of different strains
In our routine protocol, embryos cryopreserved (vitrified) in EFS40 solution are thawed in 0.5 M sucrose–PB1 medium and equilibrated in the same medium for 5 min. This thawing step requires great skill and experience. We have found that embryos can be handled more easily after thawing if they are thawed and equilibrated for 3 min in 0.75 M sucrose–PB1 medium. The rates of embryos retrieved and the rates of embryos with normal morphology were significantly improved to 98%–100% and 95%–100%, respectively, and this was consistent among all the mouse strains tested (C57BL/6, BDF1, BALB/c, and FVB).

2) Development of sperm cryopreservation techniques
The freezing rate for mouse sperm was optimized for their most efficient cryopreservation. When sperm were frozen with 18% raffinose solution as cryoprotectant at rates between –10 °C/min and –450 °C/min, about 20% of motile sperm were retrieved after thawing. No motile sperm were retrieved after freezing at a rate of –0.7 °C/min or –3000 °C/min. The freezing rate was most critical when the temperature decrease was between the refrigeration temperature (4 °C) and –50 °C.

3) Development of oocyte cryopreservation techniques
Mature unfertilized oocytes from BDF1 mice were cryopreserved in 16% DMSO + 16% ethylene glycol + Ficoll + sucrose–PB1 in a cryo-top rather than a cryotube after equilibration in 8% DMSO + 8% ethylene glycol–PB1 for 3 min. Ninety-eight percent of oocytes were morphologically normal after recovery in 0.5 M sucrose. When the surviving oocytes were treated in 2.5 M SrCl2–CZB medium to determine their
developmental capacity, 94% and 69% of oocytes developed parthenogenetically to the two-cell and blastocyst stages, respectively.

IV. Development of new stem cell lines
We have established ES cells and nuclear-transfer embryonic stem (ntES) cells from several mouse strains, as experimental models for human regenerative medicine and the conservation of mouse genetic resources. We also aim to develop methods of gene targeting, and the effective derivation of GS cells and embryonic germ (EG) cells in laboratory animals.

1) Establishment of mouse ES cell lines
Generally, mouse ES cell lines are established from 129 strains. If ES cell lines can also be established from strains other than these 129, they would provide a valuable resource for biomedical research. We have established 37 ES cell lines from six mouse strains including C57BL/6 and F1 hybrids between wild and laboratory strains. Furthermore, 32 ntES cell lines have been established from five donor cell types. Their pluripotency and germline transmission capacity are now being investigated.

2) Establishment of germline stem cells
We are establishing new GS cell lines from mice, rabbits, and monkeys. We have successfully established GS-like cell colonies from newborn gonadal cells from both male and female rabbits. These cells produce stem-cell marker proteins and have alkaline phosphatase activity. Their ability to differentiate into germ cells will be examined with in vivo experiments. Preliminary experiments have revealed that they can be transfected with viral vectors, but further technical improvements are necessary.

3) Characterization of oocytes and stem-like cells isolated from neonatal mouse ovaries
We have found that oocytes collected from neonatal ovaries can develop in vitro for up to 18 days in the presence of stem cell factor and start to degenerate thereafter. They produce a normal-looking zona pellucida and express the markers of growing oocytes. Without treatment with stem cell factor, round colonies consisting of stem-like cells were formed. After they were transplanted into ovaries, some successfully proliferated and preferentially colonized the cortex. More detailed histological examinations are now underway.

4) Derivation of EG cell lines
Embryonic germ cells are multipotent stem cells that are derived from the primordial germ cells of embryos at 8.5–12.5 days post-coitus (dpc). Because of the difficulties involved in establishing EG cell lines from post-12.5-dpc embryos, we have generated EG cell lines from 14.5-dpc female embryos. These EG cell lines exhibit stem-cell marker expression and multipotency. It will be interesting to understand the character of the constituent cells of developing mouse gonads. Further characterization will be undertaken.

Figure 7. Staining for the alkaline phosphatase activity in mouse ntES cells, rabbit GS-like cells, and mouse EG cells (from the left).
Original Papers (*Peer reviewed Journal)


Oral Presentations


