—Review—

Development of reproductive engineering techniques at the RIKEN BioResource Center

Atsuo OGURA[1–3]

[1] *RIKEN BioResource Center, Tsukuba, Ibaraki 305-0074, Japan*
[2] *Graduate School of Life and Environmental Science, University of Tsukuba, Ibaraki 305-8572, Japan*
[3] *The Center for Disease Biology and Integrative Medicine, Faculty of Medicine, The University of Tokyo, Tokyo 113-0033, Japan*

Abstract: Reproductive engineering techniques are essential for assisted reproduction of animals and generation of genetically modified animals. They may also provide invaluable research models for understanding the mechanisms involved in the developmental and reproductive processes. At the RIKEN BioResource Center (BRC), I have sought to develop new reproductive engineering techniques, especially those related to cryopreservation, microinsemination (sperm injection), nuclear transfer, and generation of new stem cell lines and animals, hoping that they will support the present and future projects at BRC. I also want to combine our techniques with genetic and biochemical analyses to solve important biological questions. We expect that this strategy makes our research more unique and refined by providing deeper insights into the mechanisms that govern the reproductive and developmental systems in mammals. To make this strategy more effective, it is critical to work with experts in different scientific fields. I have enjoyed collaborations with about 100 world-recognized laboratories, and all our collaborations have been successful and fruitful. This review summarizes development of reproductive engineering techniques at BRC during these 15 years.
Key words: cryopreservation, ICSI, mouse, nuclear transfer, stem cell

Introduction

RIKEN BioResource Center (BRC) was founded at the RIKEN Tsukuba Institute as one of the infrastructure centers of RIKEN in 2001. The mission of RIKEN BRC is to collect, maintain, and distribute bioresources including experimental animals (mice), experimental plants, cells, genes, and microbes. The late Dr. Kazuo Moriwaki was the founding Director. My division, the Bioresource Engineering Division, is the sole technology division at BRC and I was appointed Head of this division in 2002. Since then I have been in charge of the development of bioresource-related techniques, especially reproductive engineering techniques applied to mice and stem cell technologies, and of maintaining and distributing these bioresources at a high quality. The specific functions of the division are: (1) cryopreservation of embryos and gametes; (2) microinsemination (sperm injection); (3) nuclear transfer; and (4) the establishment of new stem cell lines and generation of new animal models. All these functions belong to or are closely related to reproductive engineering techniques. Reproductive engineering techniques are developed to reproduce the reproductive/ developmental phenomena experimentally by manipulating germ cells, embryos and, if necessary, stem cells outside the body (*in vitro*). They also include techniques for genetic modification of gametes or embryos to generate transgenic or knockout/knockin animals. A more commonly used term, "assisted reproductive technology (ART)", constitutes a basic part of reproductive engi-
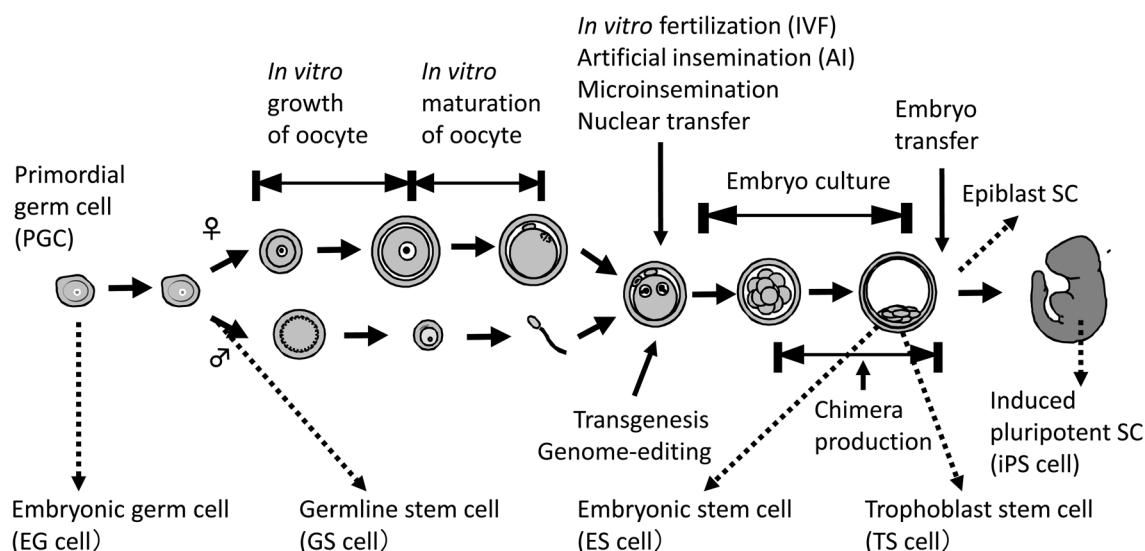
Fig. 1. Current basic reproductive engineering techniques (red) and stem cells generated *in vitro* (blue). Additionally, cryopreservation techniques constitute the integral parts of efficient use and preservation of germ cells, embryos and stem cells. These techniques and stem cells are key players in studies on reproductive and developmental biology as well as generation of new bioresources.

neering techniques, such as superovulation, *in vitro* fertilization (IVF) and embryo transfer. The major reproductive engineering techniques used in mammals are presented in Fig. 1. The mission of my division is both to develop new techniques and to keep the existing routine technologies at the highest standards so that BRC's project (the National BioResource Project [NBRP]) can progress most effectively. To my knowledge, our laboratory is the only one in the world that uses all four technologies at the highest level. In this review, I summarize the major achievements of these four specific functions at my division.

Cryopreservation

Development of a broadly applicable cryopreservation protocols for a large mouse strain stock

When I came to the RIKEN BRC in 2002, my first aim was to devise a cryopreservation system for two-cell mouse embryos. At that time, the RIKEN Tsukuba Institute maintained a number of inbred strains of live mice, many of which were unique to the institute and not available in any other laboratories. However, their maintenance as live mice incurred very high costs. Therefore, we attempted to develop a cryopreservation system that could be applied to a broad range of mouse strains and simplified as a routine system for use at the

BRC. My colleagues, Mr. Keiji Mochida and Ms. Ayumi Hasegawa, devoted themselves to this technology development in collaboration with members of Experimental Animal Division at BRC [87].

The cryopreservation technique that we finally selected is based on the use of cryosolutions containing ethylene glycol as a cryoprotectant and cryotubes as containers. Using this technique, more than 90% of the mouse strains, which differ greatly in their sensitivity to *in vitro* handling and cryopreservation, can be maintained successfully in liquid nitrogen and shipped to other laboratories using dry shippers [53, 54]. Thus, our cryopreservation technique formed the technical basis for the mouse archives at the BRC. Our protocol for mouse embryo cryopreservation is available at: www.jove.com/video/3155/.

In a technical sense, this embryo cryopreservation protocol is not a "freezing" method, but a "vitrification" method. In general, vitrification requires neither slow cooling nor a programmable freezer, so the procedure is very rapid [3, 28]. The survival rates of embryos after thawing (warming) are consistently high under optimal experimental conditions because there is no ice formation within embryos [3]. However, to avoid cryodamage to embryos, they should be kept supercooled below −130°C during cryopreservation and need to be warmed rapidly at recovery. We could overcome this problem by

Microdroplet IVF
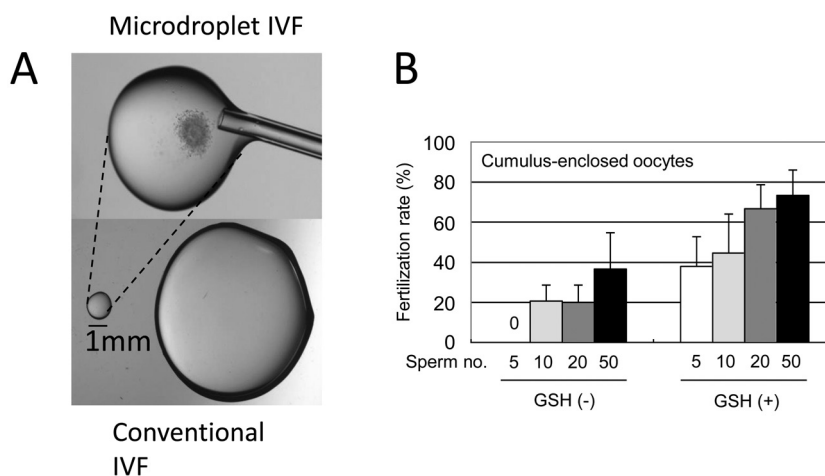
Conventional
IVF

Fig. 2. Microdroplet IVF reduces the number of spermatozoa necessary for fertilizing oocytes in mice [8]. (A) A microdroplet contains 1 μl IVF medium (upper) and is much smaller than a conventional IVF droplet (80 μl, lower). (B) Effects of the presence of reduced glutathione (GSH) on the microdroplet IVF. There was an interaction between the presence/absence of GSH and the group of GSH (+) had a significantly better fertilization rate ($P<0.05$).

using a high osmolality vitrification (HOV) solution containing 42.5% (v/v) ethylene glycol, 17.3% (w/v) Ficoll and 1.0 M sucrose [51]. In the HOV solution, embryos are highly dehydrated and ice crystals do not form even at −80°C [28]. This solution is more viscous than other cryopreservation solutions, but easy handling of embryos was assured by employing a less viscous equilibration solution before vitrification. Most (>80%) embryos cryopreserved in this solution survived at −80°C for at least 30 days. Normal mice were recovered even after intercontinental transportation in a conventional dry-ice package for 2–3 days, indicating that special containers such as dry shippers with liquid nitrogen vapor are unnecessary [51].

We also develop techniques for cryopreservation of spermatozoa. First, we attempted to develop a new protocol for sperm cryopreservation using cryotubes as the containers. Use of cryotubes instead of plastic straws increased the accessibility of mouse sperm cryopreservation and decreased the possibility of the fracture of the containers during handling or transportation [9]. This technique is now routinely used at BRC. Furthermore, we sought to minimize the number of spermatozoa necessary for IVF by decreasing the size of IVF droplets. The sperm number could be reduced to 1/240, suggesting that we may recover mice even when only poorly motile spermatozoa or a limited number of motile spermatozoa are available due to inadequate freezing or genetic rea-

sons [8] (Fig. 2).

Development of a new superovulation protocol using anti-inhibin serum (AIS)

Recently, we have developed reproductive engineering techniques for wild-derived strains of mice. These strains are genetically different from the laboratory mouse strains and are expected to increase the likelihood of finding polymorphisms or novel modifier genes that are responsible for disease-resistant or disease-prone characteristics. At RIKEN BRC, the large set of wild-derived strains from four *Mus* species makes the center a unique and invaluable core facility for mouse genetic resources, many of which are valuable gifts from the late Dr. Kazuo Moriwaki [87]. However, the small number of oocytes ovulated by a single female and the frequent prenatal death of fetuses after embryo transfer hampered their safe cryopreservation and broader use. We found that superovulation based on treatment with AIS was very effective at least some of the wild-derived strains, in collaboration with Drs. Kazuyoshi Taya and Gen Watanabe, the pioneers of AIS development [7]. The intra-uterine deaths of fetuses could be avoided by injection of cyclosporin A to recipient females and co-transfer of embryos from laboratory mice [7]. As a result, the efficient production of cryopreserved embryos from these wild-derived strains has facilitated safer and cheaper strain preservation and transportation without the risk of
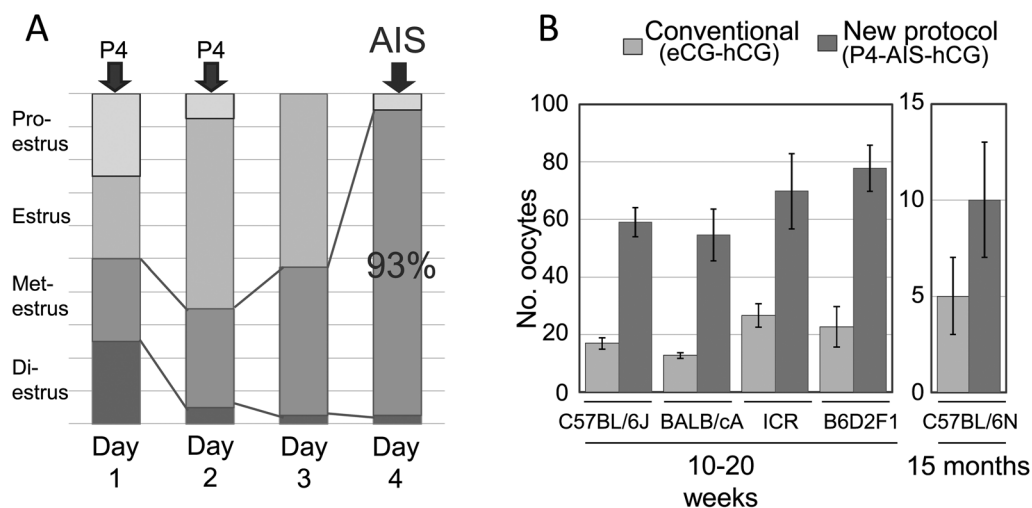
Fig. 3. High-yield superovulation in adult mice by anti-inhibin serum (AIS) treatment combined with estrous cycle synchronization [6]. (A) After two daily injections of progesterone, the estrous cycle is synchronized to metestrus on Day 4. On this day, AIS is injected to superovulate the females. (B) This superovulation protocol results in two- to four-fold increases of the number of oocytes ovulated, irrespective of the strain and age of females.

escapes [7, 52]. Furthermore, we successfully applied the AIS treatment to different laboratory mouse strains by synchronizing the estrous cycle by progesterone treatment. Importantly, this protocol is highly effective irrespective of the mouse strain or the age of females [6] (Fig. 3).

Microinsemination (sperm injection)

Historically, microinsemination (sperm microinjection) was developed after IVF as an assisted fertilization technology. The initial successful attempts used mature spermatozoa in rabbits and bovines in the 1980s. Since then, the technique has been known as intracytoplasmic sperm injection (ICSI) (for review, see [68]). In mice, live offspring were produced using round spermatids before development of mouse ICSI [36, 67]. At present, secondary spermatocytes [35], and primary spermatocytes [34, 69] can be used for production of mouse offspring. These techniques significantly expand the applicability of microinsemination techniques to fertilization research and production of offspring using gametes generated *in vitro* or associated with abnormal phenotypes. The latter experiments were performed to ascertain the genomic integrity of the haploid gametes (spermatids and spermatozoa) by the requests of collaborators (for review, see [68]). ICSI also can be used for production of offspring from male germ cells cryo-

preserved by simple freezing. We confirmed that mice were born using testicular spermatozoa retrieved from the mouse bodies frozen for 15 years [64] (Fig. 4). Ms. Narumi Ogonuki has been involved in most of the projects related to microinsemination since the establishment of my laboratory in 2002.

Factors that affect the efficiency of microinsemination

To define the factors that might affect these outcomes, we performed a large-scale series of microinsemination experiments in mice. We used a $5 \times 3 \times 2$ factorial design with the following factors: mouse genotype (ICR, C57BL/6, DBA/2, C3H/He, and 129/Sv strains), male germ cell type (epididymal spermatozoa and elongated or round spermatids), and freeze–thawing treatment. The male germ cell type affected all four of the outcome parameters: oocyte survival after injection, cleavage of oocytes, implantation, and birth of offspring [65]. More oocytes tended to survive after round spermatid injection (ROSI) than after ICSI or elongated spermatid injection (ELSI). Overall, spermatozoa and elongated spermatids yielded better rates of embryo development than round spermatids (Fig. 5A). This indicates that ROSI-generated embryos may have specific difficulties with postimplantation development compared with ICSI- or ELSI-generated embryos.

The poor developmental ability of embryos following ROSI may have genetic or epigenetic causes, or both.

Fig. 4. Birth of mice from spermatozoa retrieved from testes of male mice frozen for 15 years [64]. (A) Dr. Iwaki kindly provided us with frozen mice stored for 15 years in a conventional freezer. (B) The spermatozoa retrieved were heavily damaged (arrows in the lower photo). The plasma membrane and other structures were disrupted. (C) Pups born after injection with these damaged spermatozoa. (D) All of them grew into normal adults.
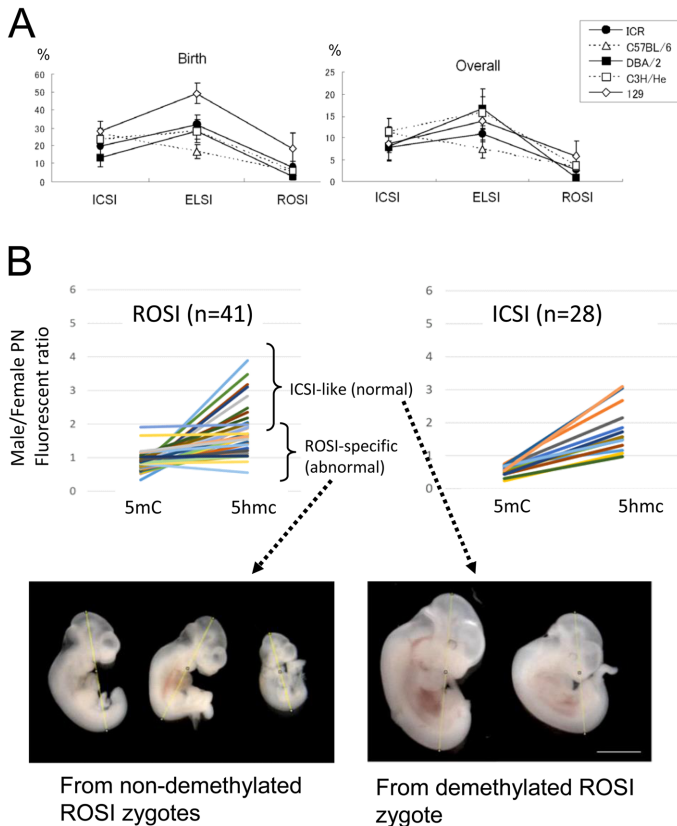
Fig. 5. Microinsemination in mice. (A) Multiple comparisons revealed that spermatozoa (ICSI) and elongated spermatids (ELSI) gave better birth rates than did round spermatids (ROSI), while spermatozoa and elongated spermatozoa were indistinguishable in their ability to support embryonic development. "Overall" means the birth rates per oocytes used [65]. (B) Active DNA demethylation is impaired in a significant proportion of ROSI zygotes (upper). Our live-cell imaging experiments demonstrated that this impaired DNA demethylation was due to the presence of histone in the round spermatid nuclei. The embryos with impaired DNA demethylation were often associated with retarded development after implantation (lower) [40].
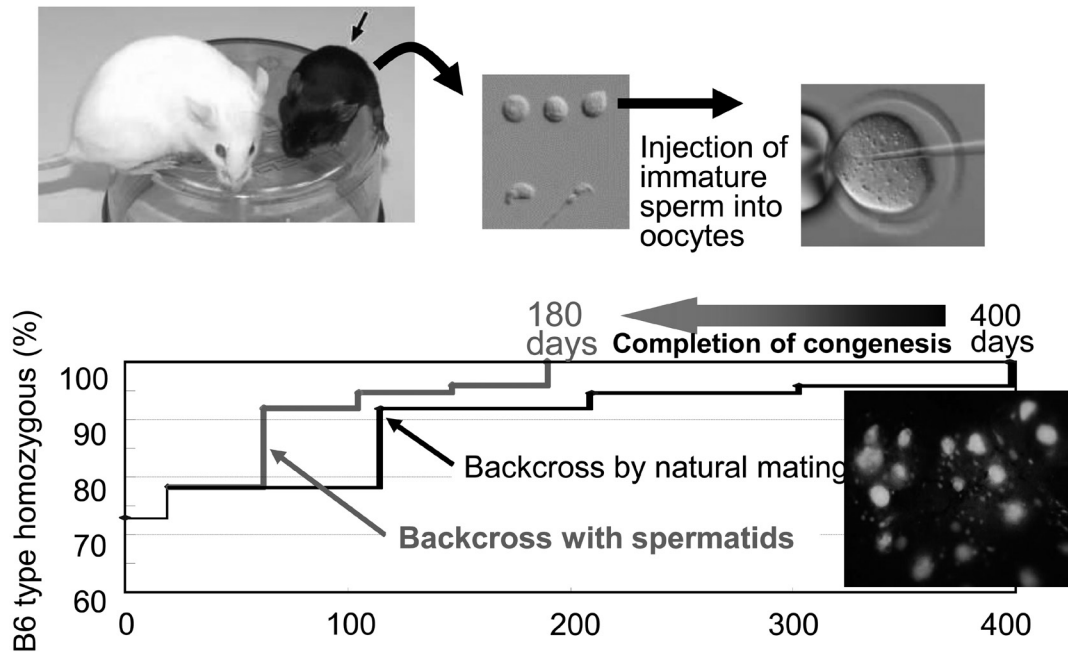
Fig. 6. A high-speed congenic strategy using first-wave spermatids from immature males at about 20 days of age (arrow in the upper left panel). Accelerated generation of a congenic strain from a genetically modified male (MVH-Venus transgenic) with a mixed genetic background through rapid generation turnover between 41 and 44 days (including a 19-day pregnancy period). This new high-speed breeding strategy enables us to produce congenic strains within about half a year [60].

We found that rabbit embryos produced by ROSI frequently exhibited aneuploidy (abnormal numbers of chromosomes), which was probably due to impaired formation of the microtubule-organizing center [62, 80]. These findings strongly suggest that the poor development of ROSI-derived embryos might be attributable at least partly to chromosomal aberrations during early development. We also examined whether ROSI-derived embryos carried epigenetic abnormalities, especially at the DNA methylation (5-methylcytosine, 5mC) level. Mammalian zygotic genomes undergo global DNA demethylation via active and passive mechanisms [47, 59]. Active demethylation proceeds in a more pronounced manner in the paternal genome compared with the maternal genome at the pronuclear stage, so the fully developed male pronucleus has a lower level of 5mC and a higher level of 5-hydroxycytosine (5hmC, an intermediate in the demethylation process) than the female pronucleus. We found that a significant proportion of ROSI-derived embryos had unchanged 5mC and 5hmC levels, and they did not exhibit preferential localization of Tet3 (5mC → 5hmC conversion enzyme) to the male pronucleus [40] (Fig. 5B). Our live-cell imaging experi-

ments demonstrated that this impaired DNA demethylation was due to the presence of histone in the round spermatid nuclei, whereas most of the histones are replaced with protamine in the mature sperm nuclei. Furthermore, the embryos with impaired DNA demethylation were often associated with retarded development after implantation. Thus, we concluded that ROSI embryos are prone to epigenetic abnormalities caused by DNA demethylation failure [40].

Therefore, elongated spermatids can be used as substitute gametes in an efficient manner in the mouse, and round spermatids may also be employed to produce healthy offspring if necessary [61]. As an application of ROSI, we are performing speed congenic experiments (repeated backcross breeding) using round spermatids from immature males that carry modified genes of interest. In mammals, the first wave of spermatogenic cells (immature sperm cells) undergoes meiosis before puberty; therefore, microinsemination using first-wave spermatogenic cells is expected to be useful for shortening the generation turnover time. Using round spermatids taken from mice at 22–25 days of age, we can shorten one-generation turnover to 41–44 days (including a 19-

day pregnancy period). This is less than half the normal generation time in mice (ca 3–4 months at the shortest) [41, 60] (Fig. 6). We have created several congenic lines in the standard C57BL/6 background or nonobese/severe combined immunodeficiency (NOD/SCID) background. The latter are expected to serve as the source of immunologically "humanized" mice.

Use of in vitro-produced gametes to generate offspring

Germ cell development to functional gametes is a highly complex process where genetic and epigenetic modifications of the genome are critical. Consequently, in germ cell culture experiments, it is very common to employ microinsemination techniques using *in vitro*-derived spermatids or immature spermatozoa to produce embryos or full-term offspring.

We undertook a series of experiments to produce functional gametes from PGCs to determine the extent to which their development depends on the specific context. Mouse PGCs collected from male and female gonads at E12.5 were transplanted under the kidney capsule of adult mice together with gonadal somatic cells, and the transplanted cells constructed testis-like and ovary-like tissues, respectively, within 4 weeks. Normal-looking round spermatids and fully grown germinal vesicle (GV) oocytes developed within these tissues. The round spermatids supported full-term development after conventional ROSI. The GV oocytes were also shown to be functional by maturation *in vitro* followed by ICSI using normal spermatozoa. Thus, PGCs exhibit remarkable flexibility in their developmental capacity under spatially and temporally "foreign" conditions [46]. This transplantation system may provide a unique technical basis for inducing the development of early germ cells of exogenous origins, including ES and iPS cells.

To circumvent the inherent obstacles associated with germ cell development *in vitro*, several attempts have been made to exploit the advantages of tissue culture systems to mimic the environment surrounding germ cells *in vivo*. In particular, Dr. Takehiko Ogawa and his colleagues at Yokohama City University developed a gas–liquid interphase culture system for tissue fragments of neonatal mouse testes, which only contained gonocytes and spermatogonia. Under these culture conditions, spermatogenesis continued for more than 2 months, whereupon spermatids and spermatozoa appeared in the tissue fragments. We injected them into oocytes and normal pups were born, thereby indicating that normal

spermatogenesis was completed in their culture system [75–77, 86]. Subsequently, they have made further improvements to their techniques and published important papers in collaboration with our group.

We have also collaborated with Dr. Takashi Shinohara's group at Kyoto University for many years. In the 1990s, Dr. Shinohara learned the intra-seminiferous tubule transplantation technique at Dr. R.L. Brinster's laboratory in the USA. This technique allows the transplanted cells (usually spermatogenic cells) to develop into spermatozoa in the host animal. In 2003, Dr. Shinohara first generated male germline stem (GS) cells, which are derived from neonatal spermatogonial stem cells, and confirmed their capacity for full differentiation *in vivo* using his transplantation technique [32]. However, the number of spermatozoa obtained is often too low to fertilize oocytes by natural mating, so a microinsemination technique is necessary in these cases. Dr. Shinohara published the first GS cell paper in collaboration with us, and our collaboration has continued since then. In a series of studies, Dr. Shinohara proposed new mechanisms for the renewal of spermatogonial stem cells and spermatogenic cell differentiation [31, 81]. A recent study investigated the requirement for reactive oxygen species in spermatogonial cell renewal [55]. As of August 2016, there are 30 papers published by collaborations of Dr. Shinohara's laboratory and my laboratory.

Nuclear Transfer

Nuclear transfer using unfertilized mature oocytes is the sole reproductive engineering technique that endows the donor cell genome with totipotency. Somatic cell nuclear transfer (SCNT) is a technique used to produce a live animal from a single somatic cell nucleus. Because somatic cells can be proliferated and gene-modified *in vitro*, this technique is expected to have major effects in the farm animal production industry, drug production, regenerative medicine, and conservation of invaluable genetic resources. In addition to its broad practical applications, SCNT can provide unique and interesting experimental systems for genomic research, especially epigenetics, aimed at learning how the somatic cell genome is reprogrammed into a state equivalent to that of the fertilized oocyte, that is, the totipotent state (for see, review [43, 66]). Dr. Kimiko Inoue and Dr. Shogo Matoba played the major roles in the advancements of nuclear transfer technology in my laboratory.
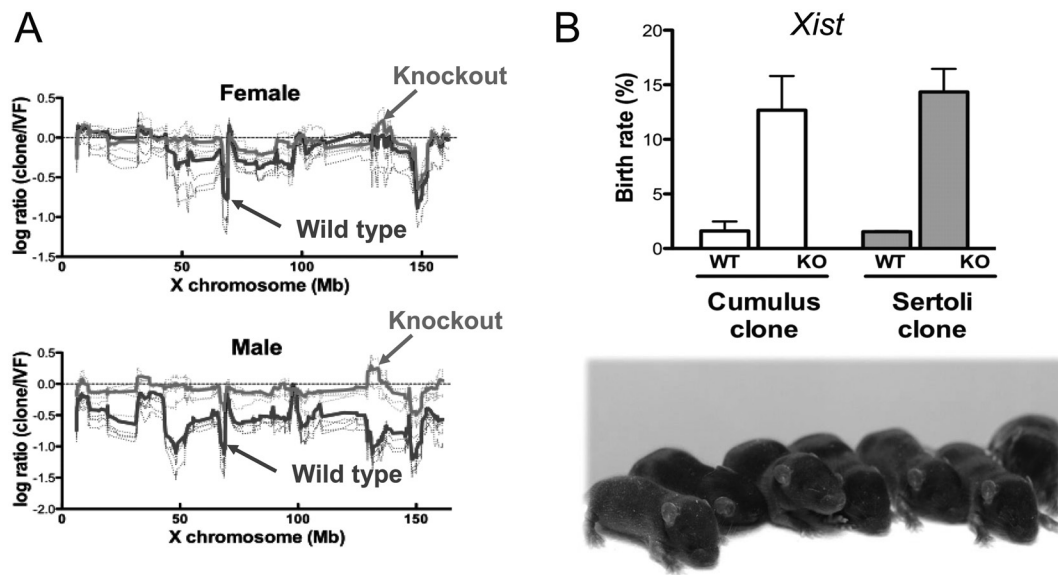
Fig. 7. Effects of *Xist* knockout on the gene expressions and embryonic development in SCNT cloned embryos [21]. (A) The expression levels of X-linked genes are plotted according to the position on the X chromosome. The repressed pattern of wild-type clones (red line) was normalized by *Xist* knockout (green line). (B) Use of *Xist* knockout donor cells resulted in about 8 to 9 fold increase of the birth rates in both sexes. Many cloned pups were born in a single cloning experiment. These results indicated that ectopic *Xist* expression in SCNT embryos critically affected the efficiency of full-term development.

Nuclear transfer for the study of X chromosome inactivation

In mammals, female cells have two X chromosomes whereas male cells have only one X chromosome. Therefore, dosage compensation of X-linked products between females and males is achieved by the epigenetic repression of one of the two X chromosomes in females cells, a process called X chromosome inactivation (XCI). XCI is primarily dependent on the expression of a noncoding RNA gene, *Xist*, from the future inactive X [2]. While searching for SCNT-specific abnormalities during pre-implantation embryonic development, we found that X-linked genes were downregulated in a chromosome-wide manner in SCNT blastocysts of both sexes. Consistent with this phenomenon, *Xist* was overexpressed in these embryos as evidenced by quantitative RT-PCR and RNA-FISH [21].

To examine how much this X-linked gene dysregulation affects the development of SCNT embryos, we cloned donor cells derived from *Xist* knockout (KO) mice, expecting that the ectopically inactivated X could be reactivated in SCNT embryos. The effect was remarkable. The number of downregulated genes was reduced to 20% in both X-linked genes and autosomal genes. Transferring these embryos into recipient females in-

creased the birth rates of cloned offspring nearly 10-fold [21] (Fig. 7). These results unequivocally demonstrated that ectopic *Xist* expression has a critically adverse effect on the development of SCNT embryos.

We next examined the feasibility of injecting small interfering RNA, the so-called knockdown (KD) strategy. We first used male SCNT embryos, which carry only one X chromosome in their nuclei (Fig. 6). We found that its effect was similar to that of the KO experiments, and the birth rate increased about 10-fold [44] (Fig. 7). Combining this with trichostatin A treatment increased the rate to 20% of transferred embryos that reached term. Importantly, this study demonstrated that SCNT-specific ectopic *Xist* expression was autonomously corrected after implantation. We concluded that the fate of cloned embryos is determined almost exclusively before implantation by their XCI status [44]. Next, we examined whether the same KD strategy could be used to rescue female SCNT embryos, but we found that it did not have any beneficial effect, probably because of an inability to mimic consistent monoallelic *Xist* expression in these embryos in the simple KD strategy [71].

One of the questions that remained to be answered was why ectopic *Xist* expression occurred in SCNT embryos. To obtain clues to understanding the mechanisms

underlying SCNT-associated XCI perturbation, we performed a large-scale nuclear transfer study using different donor cell types throughout the life cycle. We examined which genomes repress or allow the expression of *Xist* in the reconstructed embryos. The genomes from all germ cell types (primordial germ cells [PGCs] spermatogonia, spermatids, and growing oocytes), with the exception of fully grown oocytes, equally allowed *Xist* to be expressed in reconstructed embryos at the morula stage [70].

These findings suggested that the nature of imprinted XCI is the maternal *Xist*-repressing imprint established at the last stage of oogenesis. Then we understood that expression of *Xist* by zygotic gene activation is its default mode and that this *Xist* expression can be repressed only by some maternal imprint established at the very last stage of oogenesis [70]. This explains why *Xist* is ectopically expressed in SCNT-derived embryos and provides a comprehensive view of the XCI cycle in mice, which is essential information for future investigations of XCI mechanisms.

Nuclear transfer for the study of genomic plasticity

Unlike farm animals, the laboratory mouse provides a highly reproducible experimental system because of the availability of defined genetic backgrounds and well-established protocols for superovulation, embryo culture, and embryo transfer. This enables the researcher to identify the effects of the cell type and genotype on cloning efficiency. Following the first birth of cloned mice by Dr. Wakayama [83], we cloned 13 cell types for the production of cloned mice [50, 66]. The conclusions drawn from our research are as follows. The genome of fully differentiated cells can be reprogrammed to support full-term development. This was evidenced by the birth of cloned mice from lymphocytes with DNA rearrangement, a genetic differentiation marker [27, 30] (Fig. 8). We first generated cloned mice using adult brain neurons from the hippocampus and cerebral cortex [50]. These cells are known to be unreprogrammable cells for iPS cell generation, which indicates that genomic reprogramming using enucleated oocytes has a greater potential than the forced expression of reprogramming transcription factors [84].

We are also interested in the effects of the genetic background (mouse strain) on the efficiency of cloning by SCNT. For mouse cloning, F_1 hybrid mice such as B6D2F1 (a cross of a C57BL/6 female mouse and a DBA/2 male mouse) are the most frequently used donors due to the so-called "hybrid vigor." By contrast, cloning mice from inbred strains is extremely difficult. The only exception is the 129 strain, which is a superior source of ES cells. We have generated a number of cloned mice using several somatic cell types with the 129 genetic background [22–24, 27]. For example, hematopoietic cells are not normally capable of being cloned by nuclear transfer, but those from the 129 strain can be reprogrammed easily [23]. It is well known that cloned placentas show hyperplastic enlargement with abnormal histology but those with the 129 genetic background do not. These findings indicate that some factor or factors conferring genomic plasticity must exist in the 129 genome but remain unidentified.

To identify the genes or the regions responsible for this genomic plasticity, we used the so-called "forward genetics" strategy using nine recombinant inbred (RI) strains between 129 and C57BL/6 RI strains. We examined the birth rates of clones and the morphology of placentas in all RI strains. Based on the birth rates, the placental weights, and the genomic data of the RI strains, we finally identified four candidate genomic regions that might be responsible for the plasticity of the 129 genome [25].

Nuclear transfer for the study of genomic imprinting

Premeiotic germ cells can also be used for constructing diploid embryos by nuclear transfer, and this can work as a powerful tool to identify the dynamics of epigenetic changes during germ cell development. One important type of study is the analysis of the genomic imprinting status of germ cells, such as PGCs and prospermatogonia (early male germ cells before meiosis). Genomic imprinting involves an epigenetic "memory" for parental allele-specific expression in about 100 genes in eutherian mammals [74]. In our early nuclear transfer experiments, we showed that reprogramming in the mature ooplasm does not alter the genomic imprinting [20]. Therefore, cloned fetuses generated from germ cells are expected to reflect the donor's genomic imprinting status faithfully, and analysis of fetuses and placentas reconstructed from germ cells might provide invaluable information about their genomic imprinting status. Following our previous analysis of the erasure process of genomic imprinting in PGCs [42], we started to analyze the establishment process of genomic imprinting in prospermatogonia. We concluded that establishment of the pa-

ternal genomic imprinting is complete by E17.5 following a short intermediate period at E16.5 and that the DNA methylation patterns of the differentially methylated regions (DMRs) do not always correlate with the gene expression patterns [29]. We also found that the methylation status of the *Gtl2*-DMR, the secondary DMR that acquires DNA methylation after fertilization, probably follows the methylation status of the upstream IG-DMR, the primary DMR [29].

We have also found loss of imprinting of three placenta-specific imprinted genes (*Sfmbt2*, *Gab1*, and *Slc38a4*) in SCNT placentas in collaboration with Dr. Arima's group at Tohoku University [72].

What hampered the normal embryonic development of SCNT embryos?

There are at least two major stages of developmental arrest in SCNT embryos; the first occurs in the two-cell stage and the second in the early postimplantation stage (around E6.5–8.5) [66]. During mouse development, the two-cell stage is the point at which major zygotic gene activation (ZGA) occurs and the early postimplantation stage is when the first differentiation of placental tissues occurs. Once clones are born and weaned normally, they usually follow a normal life span, except for cases with immunological deficiency [63].

For normal ZGA, the paternal and maternal genomes must have been fully reprogrammed so that they are ready for *de novo* RNA synthesis in response to maternal transcription factors. By analyzing the gene expression profiles of SCNT embryos treated with trichostatin A (TSA), a potent histone deacetylase inhibitor, we found that TSA treatment specifically improved the expression of a small subset of genes encoding transcription factors and their regulatory factors [26]. This may explain why TSA treatment improves the development of SCNT embryos [37, 38]. Matoba *et al.* [45] reported that the reprogramming resistant regions (RRRs) identified by SCNT experiments were associated with a repressive histone mark, histone H3 lysine 9 trimethylation (H3K-9me3). Interestingly, there are overlaps between the genes responsive to TSA and those repressed by H3K-9me3 in SCNT embryos. Therefore, it is very likely that TSA-induced hyperacetylation promotes the removal of repressive epigenetic marks from these histones, thereby enhancing the expression patterns of transcription-related genes [26].

We also sought to identify the causes of loss of SCNT

embryos immediately after implantation. They start to exhibit developmental arrest, which is associated with a series of SCNT-specific abnormal phenotypes. Based on the genome-wide gene expression profiles of E6.5 mouse postimplantation SCNT embryos, we concluded that *Dlk1* might be one of the major direct causes of the lethality of SCNT embryos and the trophoblast cells in the SCNT placentas were precociously differentiated, leading to poor placental development. Thus, SCNT embryos have abnormal phenotypes in both embryonic and extraembryonic lineages [11]. We also found that the placental phenotype of cloned embryos might be closely related to the factors from the embryonic side [49].
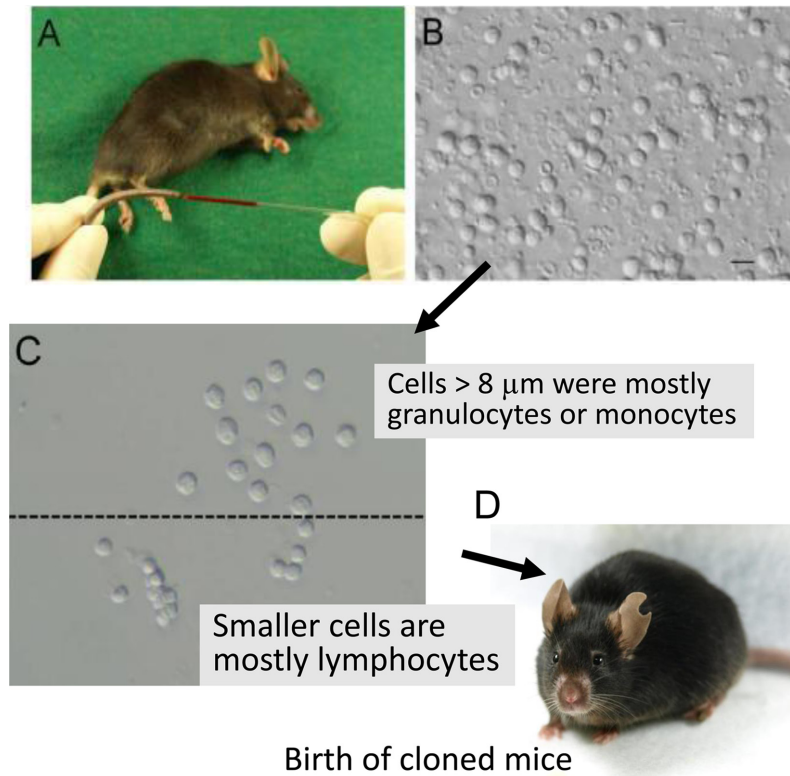
Therefore, for further technical improvements of somatic cell nuclear transfer, we should understand the molecular mechanisms underlying the epigenetic changes in the embryonic genome around the time of implantation and in the somatic genome at the germline specification [5]. As the first step, we sought to identify the histone modifications that are enriched in the genome of late preimplantation embryos using the retrotransposon regions as models. Retrotransposons comprise substantial proportions of mammalian genomes and can be harmful when activated ectopically. DNA methylation is the major mechanism for retrotransposon silencing, but we do not know how late preimplantation embryos—exceptionally hypomethylated—are protected from retrotransposons. We found that CAF-1 was responsible for the deposition of histone variant H3.1/3.2, and of repressive histone marks including H4K20me3 and H3K9me3 at retrotransposon regions [10]. It should be important to know whether the some genes other than the retrotransposon regions are also repressed by the same mechanism.

Establishment of New Stem Cell Lines and Animal Models

Stem cells are characterized by their ability to self-renew and differentiate into defined cell lineages. Stem cells exist in many organs and tissues, such as the intestine epithelium, seminiferous epithelium, and hair follicles, where differentiated cells are produced continuously. Stem cells can also be artificially established from developing embryos, germ cells, or even differentiated somatic cells. The major stem cell types artificially established so far are shown together with their source cell types in the bottom of Fig. 1. Stem cells provide an in-

Cells > 8 μm were mostly granulocytes or monocytes

Smaller cells are mostly lymphocytes

Birth of cloned mice

Fig. 8. Mouse cloning using a drop of peripheral blood [30]. (A) Collection of peripheral blood from the tail. A small incision was made at the tip of the tail, and approximately 15–45 μl of blood was collected from the incision by using a capillary tube. (B) Peripheral blood cells after treatment with erythrocyte-lysing buffer. Intact cells in the suspension were mostly leukocytes. (C) Isolated leukocytes. A separate FACS analysis revealed that Based cell populations with a diameter>8 μm were mostly granulocytes/ monocytes while those with a diameter<6 μm were lymphocytes. (D) Either cell population gave rise to normal-looking cloned mice, but use of lymphocytes as donors resulted in generation of mice carrying rearranged DNA in their whole body.

#1

All black

8 bp
allele A: aagaagatcagcagaaaagaag--------cggaagaggtcttccaagg

8 bp
allele B: aagaagatcagcagaaaagaagcc-------gaattaggtcttccaagg

#2

Black, a white spot

8 bp
allele A: aagaagatcagcagaaaagaag-------cggaagaggtcttccaagg

8 bp
allele A: aagaagatcagcagaaaagaag-------cggaagaggtcttccaagg

#3

Black, gray-color belly

33 bp
allele C: gaaaaagg--------------------------------cttcgccctgcgtgg

13 bp deletion +2 bp insertion
allele D: gaaaaaggcttcgaaaaa-----------aaggcccccgccaccttcgccctgcgtgg

Fig. 9. The a (nonagouti) mutation in wild-derived MSM/Ms mice. The founder (F_0) mice had black coats, indicating loss of the agouti protein, while they showed variations in their belly color. DNA sequencing analysis revealed that some indels (insertions and deletions) were identified at the sgRNA targeted loci. Four mutated alleles (A to D) were observed in the F_0 KO mice.

valuable research tool for both basic biology and regenerative medicine.

In 2002, when we started our mission on new stem cell lines at BRC, mouse ES cells were the most widely used because they provided a unique system for the study of pluripotency and *in vitro* differentiation as well as the materials available for practical gene targeting. Therefore, our primary aim was the establishment of new mouse ES cell lines from different types of *in vitro* manipulated embryos. Those include feeder-free C57BL/6 [58], ICSI-derived (C57BL/6 × MSM) F$_1$ [39, 78], SCNT-derived [27], parthenogenetic [4], and androgenetic lines [48]. All were established by Michiko Hirose who joined my laboratory in 2003. Many of them have been deposited to the Cell Bank at BRC and are available for researchers worldwide. We also succeeded in isolating thecal stem cells, which could differentiate into mature thecal cells (steroidogenic cells surrounding follicles)*in vitro* and *in vivo*(within the ovaries) [13, 15]. Our more recent attempts focused on generating pluripotent stem cells in rabbits, expecting that they might be used as models for human regenerative medicine. Dr. Arata Honda (Miyazaki University at present) and Ms. Michiko Hirose played leading roles in this project. We first generated rabbit ES cells from rabbit *in vivo*-derived embryos [16] and confirmed that they resembled human ES cells in terms of colony morphology and the intracellular signaling required for maintaining their undifferentiated status: thus, Activin/Nodal signaling through Smad2/3 activation is necessary [17]. Following Dr. Yamanaka's successful generation of iPS cells in mice and humans, we next attempted to generate rabbit iPS cells. We reported the first generation of rabbit iPS cell lines in 2010 [14] and their conversion to a naïve-like less differentiated status [12, 19].

In 2008, it was reported that blockade of Erk pathway and suppression of glycogen synthase kinase-3 (Gsk3) with selective small molecule inhibitors (the so-called 3i or 2i) could effectively stabilize and sustain ES cells with full pluripotency [85]. This was a big breakthrough for practical applications of mouse ES cells, especially those from the C57BL/6 strain, because they were the essential tools for generation of gene-targeted mice at that time. By the use of these inhibitors combined with leukemia inhibitory factor (LIF) the quality of ES cells reached the ultimate level. Therefore, we shifted our target to another stem cell type, trophoblast stem cell (TS cell). TS cells were first established from the polar trophoectoderm of blastocysts in the presence of fibroblast growth factor (FGF)-4, heparin, and mouse embryonic fibroblasts as feeder cells or in MEF-conditioned medium [82]. They retain the capacity to differentiate into all lineages of the placenta *in vivo*, as evidenced by chimera embryo experiments. However, TS cells consist of heterogeneous cell populations and are easily differentiate *in vitro*. We then aimed at characterization of TS cell colony types for a better understanding of how TSCs are maintained over multiple passages. We found that colonies of TSCs could be classified into four major types which contained different cell types. We identified the most undifferentiated colony type and cell type by a series of morphological and biochemical analyses [56, 57]. We expect that they may provide key information for future improvements in the quality of TS cell lines.

Besides new stem cell lines, we also generated new animal models for the study of developmental biology or human diseases. Initially, we produced transgenic mice for the purpose of gene-compensation experiments by request of collaborators [1, 33, 79]. More recently, we have introduced the CRISPR/Cas9 gene-targeting system [73] for generation of knockout animals. The first attempt was made using rabbits, as a continuation of the rabbit stem cell project. We successfully deleted the rabbit tyrosinase gene by simple pronuclear injection of a circular plasmid and a heterozygous knockout rabbit was born [18].

Next, we sought to determine whether the genomes of wild-derived mice can be modified by combining the reproductive engineering techniques we devised [7]. It is assumed that wild-derived strains, such as MSM/Ms, carry many unique genes, including disease-resistant genes. Therefore, they may provide a new set of model strains that cannot be obtained using conventional laboratory mice. We targeted the nonagouti (a) gene encoding the agouti protein localized in hair and the brain. We obtained three homozygous knockout mice as founders, all showing black coat color (Fig. 9). Interestingly, mice from the black MSM strain showed specific domesticated behaviors: hypoactivity in the dark phase and a decline in the avoidance of a human hand. This may be the first experimental evidence for the empirical hypothesis that nonagouti is a domestication-linked gene, the loss of which might repress aggressive behavior.

Importance of Collaborations with Experts Inside and Outside Japan

Because we routinely use reproductive engineering techniques, we want to combine them with genetic and biochemical analyses to solve important biological questions. We expect that this strategy makes our research more unique and refined by providing deeper insights into the mechanisms that govern the reproductive and developmental systems in mammals. To make this strategy more effective, it is critical to work with experts in different scientific fields. Fortunately, we have enjoyed collaborations with about 100 world-recognized scientists inside and outside of Japan, and all our collaborations have been successful and fruitful. Besides technical development related to bioresources, I may find new approaches to know the molecular mechanisms that govern epigenetic turning points during mammalian development. The outcome of these researches may facilitate not only our understanding of mammalian evolution but also development of treatments for epigenetic disorders during our life span.

Acknowledgments

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