—Mini Review—

Reproductive Technologies and Related Studies in the Cynomolgus Monkey

Nobuhiro Shimozawa[1]*, Tadashi Sankai[1] and Atsuo Ogura[2–4]

[1]Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Ibaraki 305-0843, Japan
[2]Bioresource Center, RIKEN, Tsukuba, Ibaraki 305-0074, Japan
[3]Graduate School of Life and Environmental Science, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan
[4]The Center for Disease Biology and Integrative Medicine, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan

Abstract: *In mice, basic reproductive technologies, such as oocyte/sperm collection, embryo production, micromanipulation, and embryo transfer, have been established. With these technologies, production of transgenic mice has become routine. The cynomolgus monkey, which is one of the laboratory animals closest to human beings, has been used to obtain vaccine approval and for medical research. Therefore, production of transgenic animals in the monkey is a very significant subject. We describe herein the current state of related studies in addition to current findings regarding reproductive technologies.*

Key words: *Cynomolgus monkey, Non-human primate, Gamete, Micromanipulation, ES cells*

Introduction

The cynomolgus monkey (*Macaca fascicularis*) belongs to the group of catarrhine primates, the same as humans (*Homo sapiens*). Like humans, non-human primates such as cynomolgus, rhesus (*Macaca mulatta*) and Japanese (*Macaca fuscata*) monkeys, specifically Old World Macaques, have menstruation cycles, and the number of young delivered in these monkeys is usually one. Unlike rhesus and Japanese monkeys, which are seasonal breeders, the cynomolgus monkey and human are annual breeders, and the similarity between the two is very high. In many cases, microbiologically clean cynomolgus monkeys are used in various types of biomedical research, however, unlike mice, there are hardly any animal models for specific diseases in the monkey. In some cases, spontaneous, familial animals with the diseases under investigation are available.

Reproductive technologies, such as sperm/oocyte collection, embryo production, micromanipulation and embryo transfer, are necessary to propagate non-human primates with human pathology and to establish transgenic non-human primates. In view of the low production efficiency of transgenic animals, many oocytes or embryos and some recipients must be prepared for *in vitro* manipulations and for embryo transfer, respectively. Current reproductive technologies must be further modified and improved in the cynomolgus monkey. Of course, researchers must wait approximately 3 years for transgenic cynomolgus monkeys to be able to propagate after they are produced. However, such monkeys offer many benefits that more than offset the drawback of waiting such a long time until biomedical research can be carried out. In this review, we describe the current state of reproductive technologies and related studies (Fig. 1) that will greatly contribute to the development of biomedical research with regard to the cynomolgus monkey.

Basal Techniques for Manipulating Gametes

Many areas remain undeveloped with regard to basic reproductive technology for the monkey. Technological developments in these areas will greatly benefit researchers working in this field. Herein, we provide outlines of egg collection, sperm collection, *in vitro* fertilization (IVF) and embryo transfer.

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*To whom correspondence should be addressed.
e-mail: shimo@nibio.go.jp

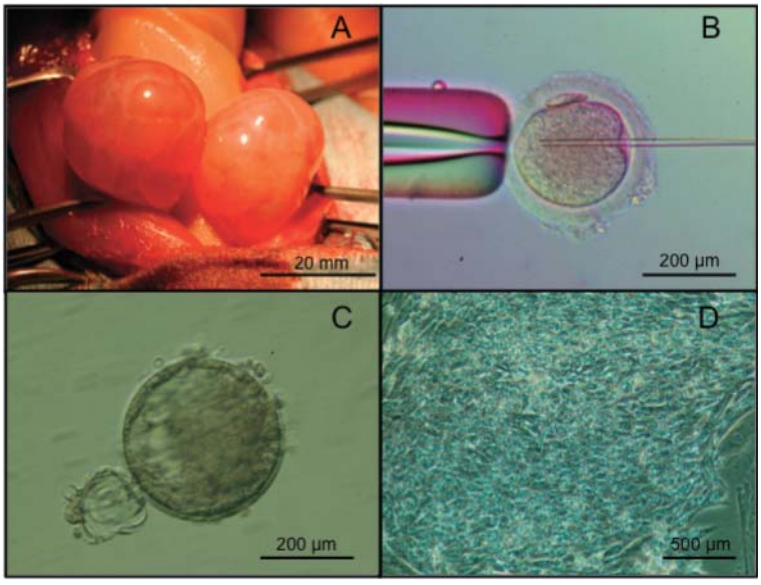

Fig. 1. Ovaries with developed follicles (A), ICSI (B), an ICSI-derived blastocyst stage embryo (C) and ES cells (D).
(A) Oocytes are collected from ovaries with developed follicles after hormone treatment. (B, C) Mature oocytes subjected to ICSI develop to blastocyst stage embryos. (D) ES cell lines are established from blastocyst stage embryos.

1) Collection of gametes and sperm cryopreservation

A newborn non-human primate was obtained by IVF and embryo transplantation in 1984. The technology for this procedure, however, remains unreliable, and overall, the results have been unsatisfactory. An important drawback of this technology is that the reproducibility and stability of the results for collecting good quality eggs is not perfect. Therefore, the uneven quality of the obtained eggs negatively impacts research results. Even if the same hormone processing is utilized, there are wide individual differences in the reactivity of the ovaries of monkeys. It is important in carrying out research to use animals with good-quality eggs that can be reliably obtained from hormone processing techniques. Although data concerning hormone processing have been reported since the 1980s, it is difficult to identify a particular technique that provides consistently reliable results.

The purposes of the hormone treatment are to induce follicle development and to mature it with regard to the egg in the follicle. We have executed some methods that use of variety of hormones such as FSH and recombinant FSH. It is possible that the effects of FSH are different in each lot because FSH is an animal extract that is subsequently refined. Moreover, it is thought that the molecular configuration of FSH differs in each type of animal, and it is uncertain whether or not the ovary reacts with FSH. Naturally, we agree that the reactivity is different in each type of monkey [1]. It has been confirmed that the ovaries of the cynomolgus monkey react to FSH very often and that FSH administration generates the growth of many ovarian follicles. Although only one ovarian follicle develops in a normal menstrual cycle, nearly 100 ovarian follicles develop with FSH administration. However, when we calculate the average number of eggs collected, the standard deviation is quite large, meaning that the individual differences are quite large. It is thought that the reason for this difference corresponds to the differences in the reactivity to FSH of each individual because individual differences are also observed when recombinant FSH is used. Moreover, the hCG is administered instead of endogenous LH, and it is necessary to determine the appropriate dosage of hCG for each type of animal. The appropriate hCG dosage for the monkey has not yet been determined, and the dosage used has generally been different for each researcher. Moreover, determining the timing of hCG administration is extremely difficult. The best time for hCG administration cannot be reliably determined

because the growth situation of the ovarian follicle is different in each individual even if the hormone administration method is the same. It is assumed that the eggs in the follicle develop from the GV stage to the MII stage in response to hCG administration. However, eggs can often be collected from other stages, such as the GV, MI and MII stages. These results indicate that there is a difference in the quality of granulosa cells even when they are within the ovary. Many problems continue to arise in hormone processing, which is a basic technology for egg collection crucial to executing the latest research.

The technology for sperm collection is reliable compared with that for egg collection. The animal is approached from the rectum, and electrical stimulation is given to the erection central nerve and ejaculation central nerve. This method is also effective under anesthesia, and it seems applicable to animals other than the monkey. It is also possible that the monkey can be trained to masturbate, as it has relatively high brain function. We gather sperm from cynomolgus monkeys by electrical stimulation of the rectum. The collected sperm is then used in reproductive experiments for IVF and intracytoplasmic sperm injection. In addition, cryopreservation technology is being used for monkey sperm development. Almost complete revitalization of sperm movement can be achieved after thawing, and IVF can be carried out successfully with these sperm [2].

This cryopreservation method is currently being used by various research laboratories. However, damage to the cell membrane of the sperm head has been observed by electron microscope after freezing and thawing [3]. It has been confirmed that this damage to the sperm head is similar to that occurring with the acrosome reaction, which is an indispensable phenomenon for fertilization. It therefore must be considered that the properties of the sperm head before and after freeze-thawing are different. However, it is possible to apply this method to various areas of research by providing accurate basal information for the sperm.

2) IVF and embryo transfer

It is well known that phenomena related to the acrosome reaction and capacitation of sperm are different in each type of animal, and it is unknown whether the present method used is the most suitable for the monkey. Furthermore, embryo transfer is also likely to be difficult. In 1984, the first newborn non-human primate was obtained by IVF and embryo transfer in monkeys [4–6]. This occurred 6 years after the same achievement in humans. Since then, 25 years have passed, but the number of research laboratories using this technology to actually obtain newborn monkeys by embryo transplantation remains limited. Considering the above-mentioned constraints regarding the technology, it is necessary to carry out basic technological development concurrently with more advanced research.

We have successfully carried out IVF in the cynomolgus monkey, African green monkey, and Japanese monkey [7–9], and a newborn cynomolgus monkey was obtained after the transfer of fertilized eggs to the oviducts of females and synchronization of the growth stages of the eggs and menstrual cycles of the females [10]. This procedure also included successful intracytoplasmic sperm injection (description following). Currently, fertilized eggs are generally created by IVF and intracytoplasmic sperm injection. Even if the results are considerably influenced by the quality of the eggs used and fertilization is confirmed, not all of the eggs develop well. Recently, more research has been carried out utilizing fertilized eggs. For instance, fertilized eggs are used for research regarding embryonic stem cells (description following) and gene manipulation. It is necessary to conduct this research from the perspective of also performing basic research.

The importance of these problems must be clear to all experienced researchers. However, because multiple factors are involved, including egg quality, composition of the culture solution and temperature, the problems are quite complex. There might be many researchers who do not evaluate the basic research because of a brilliant result reported in the monkey. However, this area of research cannot be avoided if we wish to continuously develop reliable technologies.

Progress cannot be achieved without carrying out basic research. It is necessary to conduct basic research that also supports continuing advanced research. The researchers must recognize enough that monkeys have a differentia and the individual difference and the result of showing of each individual is all true. It is necessary to synthesize and evaluate these results. Monkeys are important laboratory animals in medical research related to humans. Therefore, if there are individual differences between monkeys that affect research results, learning how to develop procedures that can handle these individual differences is significant for development of similar procedures in humans.

Micromanipulating Embryos and Gametes

As in the case of other mammalian species, manipulating the embryos and gametes of non-human primates under a microscope has attracted many researchers in the biomedical field. This type of study allows for exploration of the cellular and molecular basis of various assisted reproductive technologies (ART) and regenerative medicine in humans, while avoiding the ethical and practical issues of working with human oocytes and embryos. In this section, we review the development and present status of two micromanipulating techniques, microinsemination (ICSI) and nuclear transfer cloning, in non-human primates.

1) Microinsemination (ICSI)

Mammalian ICSI (intracytoplasmic sperm injection) started with use of the golden hamster by Dr. Yanagimachi's group in the 1970s [11, 12]. This species had been extensively used in the field of fertilization study because of the ease of superovulation, the clear cytoplasm of their oocytes and the easily visible acrosome reaction [13]. Their group demonstrated that a directly injected sperm head could transform into a male pronucleus and undergo DNA synthesis. However, the very strong developmental arrest of hamster embryos in vitro hampered further analysis of the developmental ability of the resultant embryos. The first ICSI babies in mammals were obtained in the rabbit in 1988 [14] and in the bovine in 1990 [15]. The birth of human ICSI babies has been reported as early as 1992 [16]. In primates, however, the development of ICSI techniques has not gone smoothly, probably because of the limited availability of fresh oocytes and recipient females for embryo transfer, which requires a large cohort of females at the appropriate reproductive ages. Fortunately, primate oocytes share several cellular and morphological similarities with human oocytes, and primate ART procedures are almost identical to those in humans. Therefore, primate ICSI may provide the best experimental model for the study of human ICSI without the complicated ethical and moral issues. In 1996, the first primate ICSI trials were reported by Hewitson *et al.*, who demonstrated that although the cellular process after ICSI basically mimics that occurring after IVF, occasional fertilization failure, including a lack of pronuclear formation and abnormal recondensation of chromosomes, may occur specifically in ICSI oocytes [17]. Subsequently, normal conception by ICSI in rhesus monkeys was reported by the same group; out of 14 embryos transferred, 5 developed to term, and 4 were born normally [18]. They argued that this rate was greater or equal to that reported in human clinics, although they raised some concerns about the behavior of the injected sperm nucleus, including abnormal sperm decondensation and remnants of sperm components inside the ooplasm. Following this success, ICSI in primates was put into practice, and the birth of babies was reported for rhesus monkeys and cynomolgus monkeys in other laboratories [19, 20]. ICSI is a very convenient way to produce fertilized oocytes and embryos, especially when the number of oocytes used is very limited. At present, therefore, monkey embryonic stem (ES) cells are primarily generated from ICSI-derived embryos [21–23].

Due to the advent of micromanipulation techniques, not only mature spermatozoa but also immature sperm cells (spermatids and spermatocytes) can be used as male gametes for conception. Studies with mice have played a leading role in the development of such microinsemination technologies using elongated spermatids, round spermatids and even secondary and primary spermatocytes [24–26]. The production of mouse offspring by round spermatid injection is easier than previously expected, and the technique has been routinely used in laboratories specializing in mouse ICSI [24]. The success of this technique is probably due to maternal (oocyte) inheritance of the microtubule organizing center (MTOC) in the mouse, which is completely different from the paternal (sperm) inheritance that occurs in other species [27]. Round spermatids in these species have not acquired the ability to form MTOC, and as Schatten's group predicted, this deficiency may lead to abnormal segregation of chromosomes at mitotic divisions. In fact, in rhesus monkeys, healthy offspring have been produced by injection of testicular spermatozoa or elongated spermatids, but not with round spermatids [28]. Only a mid-gestation fetus has been obtained by round spermatid injection in the cynomolgus monkey, and this fetus aborted at 103 days of pregnancy for unknown reasons (average pregnancy period: 165 days) [29]. This type of complicated development following round spermatid injection has commonly been reported in several species thus far; for example, rabbit embryos derived from round spermatids are associated with high rates of aneuploidy due, at least in part, to the inability of round spermatids to form MTOC after incorporation into the ooplasm [30, 31]. The incomplete capacity of round spermatids to activate oocytes might contribute to the poor development of round spermatid-

derived embryos. It is also possible that the genome of the round spermatid itself is not identical to that of mature spermatozoa because the male pronucleus from a round spermatid is prone to quick remethylation after fertilization, in contrast to the gradual demethylation occurring in the sperm-derived male pronucleus [32]. A small proportion of male-factor infertility in humans is thought to be affected by spermatogenic arrest at the round spermatid stage, although controversy remains regarding the accuracy of the investigation of testicular biopsy specimens [33]. If there are really cases that require round spermatid injection as a treatment, the safety and efficiency of this technique should be thoroughly clarified using primate models before its broad application to human clinics.

Genetically engineered animals offer opportunities for understanding the function of genes of interest, studying the pathogenesis and treatment of diseases and screening new chemicals for pharmaceutical purposes. An overwhelming majority of these animals are produced in mice due to the ease and efficacy of producing transgenic and gene-targeted mice. However, mouse models do not always provide sufficient information to extrapolate the data obtained to humans because the mouse and human are very different in certain physiological characteristics. It seems that primate models provide the best data in this respect, but conventional genetic modification technologies in the mouse, which allow for pronuclear DNA injection and generation of chimeric animals with embryonic stem (ES) cells, have not been successfully applied to primates. To overcome the low transgenic efficiency associated with pronuclear DNA injection, a method of retroviral mediated transgenesis into unfertilized oocytes followed by ICSI was developed in rhesus monkeys. In 2001, birth of a male baby carrying the green fluorescent protein (GFP) gene, named ANDi, was reported, although he did not express the characteristic green fluorescence for unknown reasons [34]. More recently, the technique was improved by using a lentiviral vector instead of a retroviral vector, and transgenic rhesus monkeys carrying mutant human Huntington gene were successfully produced [35]. The resulting transgenic monkeys showed important clinical features of Huntington's disease, including dystonia and chorea, which did not appear in mouse models. Thus, monkey transgenesis has become practically available based on efficient ICSI techniques.

2) Nuclear transfer cloning

Cloning animals using somatic cells shows great promise in the field of basic biology as well as for industrial and clinical purposes. However, the early studies of animal cloning by nuclear transfer were carried out using nuclei from preimplantation embryos as donors, probably because the reprogramming event of the blastomere nuclei is less complicated than that of somatic cell nuclei. This is also the case with monkeys; the first cloned rhesus monkey was produced from a 16-cell embryonic nucleus [36], while embryos from somatic cells developed only up to the 8-cell stage. Until now, no cloned monkey has been obtained by somatic cell nuclear transfer. However, since mouse ES cell lines were generated from somatically cloned mouse embryos (ntES cells [37]), cloning researchers in primatology have shifted their interest from cloning monkeys to the generation of ntES cells from cloned blastocysts as an experimental model for human regenerative medicine. Cloned mouse embryos develop into blastocysts very efficiently (30–70%), and many of these blastocysts contribute to ES cell establishment [38, 39]. It has also been demonstrated in mice that severe immunodeficiency can be cured completely by transplantation of hematopoietic cells differentiated from gene-transfected ntES cells [40]. However, it is extremely difficult to culture cloned monkey embryos to the blastocyst stage because most of them exhibit developmental arrest at the 8–16 cell stages. As this type of developmental failure has never been reported for other mammalian species, it may be a feature unique of primates, including humans. Only one group has thus far succeeded in generating ntES cells in rhesus monkeys, and the efficiency is still very low (2 lines from 213 reconstructed embryos) [41]. The very poor development of cloned monkey embryos may be attributed to chromosomal instability due to the removal of NuMA, nuclear mitotic apparatus protein, at the time of enucleation [42]. However, their consistent developmental arrest at certain cleavage stages (8–16 cell stages) is reminiscent of the so-called "developmental block" at the maternal to zygotic transition. It is very probable that the cloned embryos fail to activate zygotic genes due to incomplete reprogramming of the somatic donor genome. Thus, cloned primate embryos may suffer from genetic as well as epigenetic insufficiencies. It has recently been reported that genomic reprogramming of reconstructed embryos can be enhanced by treatment with chromatin-modifying chemicals, including histone deacetylase inhibitors [43]. At present, there are many chromatin-modifying chemicals, and each has its own functional mechanisms; some, therefore, may have a significant

effect on primate nuclear transfer. Trials of these types of chemicals require further effort, but this kind of primate research may open up a new area of regenerative medicine in humans.

ES Cells

The ES cell line in mammals was established from mouse blastosyst embryos for the first time in 1981 [44, 45]. The somatic and germ cells of ES cell origin were included in progeny produced from mouse embryos into which ES cells were injected. Using this property, many transgenic mice, including gene-targeted mice, have begun to be produced. However, the introduction of ES cells in the human and monkey has attracted attention as a tool in medical applications not intended to produce transgenic animals. Moreover, many basic and application studies for regenerative medicine have begun to be carried out because induced pluripotent stem (iPS) cells similar to ES cells have recently been established in humans, but not monkeys [46]. It is important that the safety of this technique in monkeys be established before direct application to humans. Herein, we primarily review ES cells in the cynomolgus monkey.

1) Establishment and characteristics

Primate ES cell lines were established in the rhesus monkey for the first time in the United States in 1995 [47] and were subsequently established in humans in 1998 [48]. In Japan, they were established in the cynomolgus monkey in 2001 because the monkey is widely used for biomedical research [22]. Monkey ES cell lines are different from mouse ES cell lines but are very similar to human ES cell lines [49–51]. In addition, the diversity of the genetic background of the embryos that are the origins of the ES cell lines may create delicate differences in properties among primate ES cell lines [52–54].

Blastocyst stage embryos derived from *in vivo* or *in vitro* fertilization (including ICSI) are used to establish cynomolgus monkey ES cell lines. Each inner cell mass (ICM) is isolated by a method involving either a combination of antiserum and complement (immunosurgery) or injection needles. Isolated ICM is cultured on a mouse embryonic fibroblast (MEF) cell monolayer. The adherent ICM is gradually extended as a colony. By day 8–10 of each culture, the colony is passaged onto a fresh MEF cell monolayer. The passage is performed by dividing the colony into some clusters with collagenase-based solution and injection needles. Primate ES cells usually fail to form colonies following dissociation into single cells when using trypsin-based solution. Consequently, maintenance of cynomolgus monkey ES cell lines is performed by dividing the colony into small clusters with pipetting following dissociation with either collagenase-based solution or low concentrations of trypsin and collagenase-based solution. It has recently been reported that treatment of single human ES cells with the Rho-associated kinase inhibitor, Y-27632, greatly improves colony development [55]. This result is important for researchers attempting large-scale culture in primate ES cells because a large number of differentiated cells of ES cell origin are necessary for regenerative medicine.

Characterization of ES cells involves consideration of the following three key factors: self-renewal, pluripotency and normal karyotype. In particular, pluripotency, which means the ability to differentiate to various cells, is a very important property when considering medical applications. Pluripotent ES cells must be in an undifferentiated state, which can be shown by confirming expression such as for Oct3/4, Nanog, Sox2, SSEA1, SSEA3, SSEA4, TRA-1-60, TRA-1-81, and alkaline phosphatase. These expression patterns differ between primates and mice. In primates, all of the above except for SSEA1 are expressed, while in mice, SSEA3, SSEA4, TRA-1-60 and TRA-1-81 are not expressed. However, the expression of SSEA3 varies in primates. Both rhesus monkey and human ES cells express SSEA3, but cynomolgus monkeys do not. These differences may not be important because it has been reported that the expressions of SSEA3 and SSEA4 are not essential to maintaining the pluripotency of ES cells [56]. Mouse ES cell lines should differentiate into germ cells, but in primates, this differentiation is not essential. Examining whether ES cells differentiate into germ cells via chimeras is difficult in primates because they have a long life cycle and only delivery one baby per pregnancy. In particular, it is impossible to ethically clarify this issue in humans. Instead, it is necessary to be able to develop teratomas. A teratoma is a tumor consisting of tissues from three embryonic germ layers (mesoderm, endoderm and ectoderm) that develops by transplantation of ES cells into immunodeficient mice. Recently, stem cells (EpiS cells) from the epiblast of postimplantation mouse embryos have been established [57, 58]. The colony morphology of these cells resembles that of primates. EpiS cells also have the ability to develop teratomas, but not to form chimeras. These findings may suggest

Table 1. Comparison of reproductive technologies and related studies in monkeys (Macaques), mice and rabbits

	Monkeys (Macaques)	Mice	Rabbits
Oocyte collection	Anesthesia Follicular aspiration	Euthanasia Ovulation	Anesthesia or Euthanasia Ovulation
Good quality oocytes	A few	Many	Medium
Sperm collection	Anesthesia Electroejaculation	Euthanasia Cauda epididymis	Using an artificial vagina
IVF	Difficult	Easy	Easy
ICSI	Difficult	Easy	Easy
Embryo culture	Difficult	Easy	Easy
ES cells	A little difficult	Easy	A little difficult
Embryo transfer	Difficult	Easy	Easy
ntES cell	Very difficult	Relatively easy	Difficult
Transgenic animals	Very difficult	Relatively easy	Difficult
Cloned animals	Very difficult (no report)	Difficult	Difficult
Contribution to medicine	Very high	High	High
Ethical issues	Very high	High	High

that primate ES cells do not have the ability to differentiate into germ cells. Therefore, the strategy of producing transgenic cynomolgus monkeys via chimeras may not be a wise choice.

2) Application

Some clinical applications have been advanced by translational research using cynomolgus monkeys. Thus, the expectations for regenerative medicine using ES cells have risen rapidly, and results have been reported in not only mice but also in monkeys. Future developments could lead to the availability of cells differentiated *in vitro* from ES cells for regenerative medicine. However, the pluripotency of ES cells involves a large risk. Teratomas may develop after transplantation into the body if undifferentiated cells are present in the population of differentiated cells. To avoid this risk, a previous study used a cell sorter to remove cells found to be positive for an undifferentiated marker, SSEA4, and the population of selected differentiated cells was then transplanted into cynomolgus monkeys. It was then confirmed that teratomas did not develop in the monkeys [59].

Moreover, the function of the transplanted cells has also been investigated. A transplant experiment using dopaminergic precursor cells from ES cells was conducted in cynomolgus monkeys with artificially induced Parkinson's disease. Transplantation resulted in improvement of symptoms [60]. This demonstrates that transplanted cells engrafted and differentiated into dopaminergic neurons. In addition, research to induce differentiation into cells such as hematopoietic or retina pigment epithelial cells has also been conducted using cynomolgus monkey ES cells [61, 62].

Next, we look at regenerative medicine from the perspective of immunology. Although it is relatively difficult for immunorejection to occur in the brain, it is often necessary to consider avoidance of this type of immunorejection. The nuclear transfer cloning technique (ntES cells), which we have already described, will contribute greatly to this end. Effective treatment free from immunorejection can be expected if various cells derived from the somatic cells of individuals are transplanted into the body. An examination of therapeutic methods using ntES cells has already been performed in mice [40]. Establishment of somatic cell-cloned ES cell lines has recently been reported in rhesus monkeys for the first time [41]. This report leads us to expect that these cells lines could be established in the cynomolgus monkey. Further developments in embryo technology for the cynomolgus monkey are necessary to produce novel biological resources for the medical sciences.

Conclusion

We reviewed recent findings regarding collection of oocytes and sperm, production of fertilized embryos, micromanipulation, and ES cells in the cynomolgus monkey, which is an experimental animal closely related to humans. Comparison of items contained in this review among monkeys (Macaques), mice and

rabbits is summarized in Table 1. We believe that the technologies related to the monkey are immature. However, the monkey, with its history of use for official approval of vaccines and preclinical studies, is indispensable to biomedical research and safety testing. The expectation of producing transgenic individuals is great for these types of monkey. It is hoped that to achieve this purpose, the various techniques related to gametes, embryos, and ES cells in the cynomolgus monkey described in this review will be investigated in detail and will continue to be improved.

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