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—Letter to Editor—

A New Frontier of Animal Biotechnology Research with the Birth of the Cloned Sheep

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The "cloned sheep" produced at the Losline Institute, UK has become a hot topic. The production of a "somatic cell clone" by fusing a cell from the embryo or fetus with an ovum was an event with the greatest impact in the recent development of animal biotechnology. The year 1997 will be long remembered as a special year in research on animal biotechnology.

This "cloned sheep" was produced with the aim in animal husbandry of more efficient production of good domestic animals. In the field of animal husbandry, animal biotechnology is often considered to consist of 2 categories. One is techniques such as animal rearing, animal breeding, animal feeding, animal hygiene, and animal management, which have developed on the basis of "traditional technology" and have already yielded a number of practical techniques that are contributing to the industry. The other is the technology that has emerged with the development of modern biology and consists primarily of cell manipulation such as gene recombination and cell fusion. Techniques represented by "cloned sheep" have been developed on the basis of both of these categories, and techniques such as artificial insemination, microscopic insemination, selective production of males and females, extracorporeal fertilization and embryonic transplantation, and storage of sperm and embryos by freezing have been developed until they now play important roles in medicine as well as animal industry.

The birth of the "cloned sheep" is influencing the frontier of research on animal biotechnology. It has effects on research on animal biotechnology itself by necessitating the establishment of new research subjects, modification of the strategy of present research projects, and challenges to attempt things formerly considered impossible.

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1. Studies of ova make clones more genuine clones

The ovum is needed for the production of a clone by nuclear transplantation. Clones are being made by nuclear transplantation of blastomeres from early embryos, but the appearance of the resultant clones is so different from the original animals that they hardly look like clones. This difference may be explained by the effects of the microenvironment, but I personally suspect that it is caused by the fact that the ova (denucleated unfertilized ova) used as recipients were not from the same animals. Mitochondrial DNA inherited via the ovum are involved also in the morphogenesis and functional expression, and their abnormality causes a variety of diseases. Therefore, ova from the same animal must be used as recipients to produce genuine clones by nuclear transplantation.

In the cow, 100–200 thousand ova are produced in the ovary and are potentially available. Many of them die in the course of oogenesis, and usually only 1 of them is ovulated in each estrus cycle, and only 100–200 of them are ovulated in a lifetime. There is therefore a mechanism in the mammalian ovary to selectively ovulate a small number of ova and induce death in most of them. I have started experimenting on the assumption that selective oogenesis can be avoided, and ovular death can be prevented, by releasing ova from the ovary. Collection of more ova from one animal is expected to become possible as a result of such investigation of oogenesis, and the availability of more ova from the same animal will make clones produced by nuclear transplantation more like clones.

This research on ova may lead to the establishment of new biology or medical technology. Cells constituting the body are conditioned to perform specific

functions. Genes of these cells have been considered to be irreversibly modified to perform only specific functions, but the birth of the "cloned sheep" demonstrated that the nucleus of a cell that performs a specific function reclaims the "omnipotential of differentiation" as it is transplanted to an ovum. Can such a function of the ovum be explained from the knowledge accumulated to date?

2. Strategy to produce gene-knock-out pigs modified due to the birth of the "cloned sheep"

Organ transplantation has been recognized as a treatment for patients with terminal organ failure, but the number of cases receiving organ transplantation has not increased recently due to difficulty in securing donors, so that research on heterologous organ transplantation is being advanced in earnest. The pig has emerged as the most promising organ producing animal, and this perception is achieving a global consensus.

In heterologous organ transplantation, the complement and antibodies of the recipient react violently to the grafted organ and cause "ultra-acute rejection". The mechanism of such "ultra-acute rejection" is considered to be as follows. Antigen-antibody reaction occurs between porcine antigens and inborn natural human antibodies to the pig, and the human complement system is activated. As a result, blood vessels in the grafted organ are destroyed, and the blood flow to the organ is terminated, causing death.

Therefore, (1) elimination of heteroantibodies, (2) enhancement of the activities of anti-complement factors, and (3) suppression of the antigenicity of the grafted organ have been attempted as methods to control of "ultra-acute rejection". Concerning (2), the heart of a transgenic pig was transplanted into a crab-eating monkey. Whereas an ordinary porcine heart is rejected after a mean of 5 days, the time until the occurrence of rejection was greatly prolonged when the heart of a transgenic pig was used.

(3), which is to destroy the gene of the enzyme that produces the antigenic determinant in the pig (gene knock-out), is the most promising strategy, and competition in producing a gene knock-out pig has already begun. The research strategy to produce a gene knock-out pig has changed greatly since the birth of the "cloned sheep". My strategy is based on nuclear transplantation. Oocytes collected from the ovary are matured in vitro to prepare denucleated unfertilized ova. In addition, ova matured in vitro are inseminated and developed in vitro into blastocysts, and embryo-derived cell lines are established from these blastocysts. Gene knock-out embryo-derived cells are prepared by manipulation of the target gene, and they are transplanted into denucleated unfertilized ova. Such nuclear transplanted embryos are activated, developed and transplanted to temporary parents. By such a strategy, organ-donor pigs can be produced quickly, cheaply and without large animal facilities compared with the conventional strategy with chimera pigs.

3. The somatic cell cloning technique saves endangered species

The number of existing species has decreased markedly in this century. Mammals that have ever appeared on the earth are classified into 35 orders by phylogenetic taxonomy, but 17 of them have been extinguished to date. On the species level, there were 4,226 mammalian species in the year 1600, when taxonomy was established, but 36 of them have been extirpated, and 120 are on the verge of extinction. The situation with birds, amphibians and reptiles is similar. Whether such a state is a result of the prosperity of mankind or is an outcome of the natural life span of species is an open question, but everyone would agree that aggressive measures to protect endangered species must be considered today. Concerning endangered species, not only conservation of their habitat but also technical intervention to avoid extirpation has become necessary. Furthermore, in species the extinction of which has not been avoided (or has been judged to be unavoidable), consideration of preservation of genes and analysis of the causes of extinction as well as measures for future restoration may be needed.

In such a trend, gene preservation projects have been initiated for some of the species (including the Japanese crested ibis). Discussion as to how such genes should be utilized and whether individuals of the species should be restored by nuclear transplantation technique or not has begun, and it has gained greater prominence with the birth of the "cloned sheep".

4. The "serial culturing" technique for germ cell series may circumvent the disadvantages of somatic cell clones

In animals having male and female sexes, the production of the next generation begins with fertilization. An embryo is made by fusion of a sperm and an ovum,

and primordial germ cells are then produced. Primordial germ cells migrate to the primordial gonad (which later develops into the testis or ovary), perform reduction divisions, and produce sperms and ova. Sperms and ova fuse and produce the next generation. This series of events, which is called the germ cell series, occurs presently only *in vivo*, but methods for extracorporeal insemination and culturing of early embryos and primordial germ cells have been established, and attempts at extracorporeal spermatogenesis and oogenesis have partially succeeded; and establishment of conditions for the vitro maintenance of germ cell series are in progress.

An increase in "clones" may jeopardize genetic diversity of species, but storage of sperms, embryos, and primordial germ cells by freezing is already possible, so that the establishment of a "serial culturing system for germ cell series" is expected to dissipate such anxiety. This technique is considered to be useful also for after-generation testing (examination of the ability of the offspring) of domestic animals and for examination of the effects of drugs and environmental factors on the offspring after several generations. For these reasons, the development of technique for "complete serial culturing of germ cell series" is considered to be vital for the survival of mankind.

5. Clone technology also changes genome science

If a cell in which a gene has been replaced (destroyed) by homologous recombination can be transplanted to a denucleated unfertilized ovum, an embryo can be developed, and it can be cultured in vitro until near completion of organogenesis (whole embryo culturing), the role of the gene in organogenesis can be observed directly. We have succeeded in observing mouse embryos by means of an *in vivo* microimaging system (magnetic resonance imaging; MRI) and expect to observe the internal structure of the embryo (fetus) in culture non-invasively and serially by further developing this method.

Genome science projects to clarify the entire structures of the genomes of higher animals, which consist of 3 billion base pairs, have been started in mankind, mouse, and domestic animals (cow, pig). A strategy to first prepare the genome map, to isolate and arrange genome DNA clones, and to identify the genes has been adopted, but the focus of the study will shift to determination of the base sequences of genes and, then, to functional analysis of genes in such animals as the mouse.

Such genome science will lead to identification of a large number of genes with unknown functions. The combination of homologous gene recombination, nuclear transplantation to denucleated unfertilized ova, extracorporeal development, whole embryo culturing, and MRI is expected to be a powerful method for analysis of the effects of such genes on organogenesis.

6. I object to restrictions on clone research

Discussion of restriction of clone research has become heated since the birth of the "cloned sheep". I agree that application of the cloning technique to humans must be made carefully, because it may shake the basis of the being of higher animals: Individuals die and they produce the next generation by sexual reproduction. Nevertheless, I object to the contention raised by some people that clone research of even domestic animals should be restricted. As mentioned in this article, clone research has a major impact on various fields of animal biotechnology, and restriction of research would cause great loss to society. Also, formal restriction is applied, it may lead to restriction of animal biotechnology research itself, which would have immense negative effects on society. I personally think that restriction of clone research on domestic animals should go no further than to have the association formulate independent guidelines and have investigators respect them.