O-9

Cloning of Farm Animals in Japan; The Present and the Future

Yasuo Shioya

Department of Animal Breeding and Reproduction
National Institute of Livestock and Grassland Science

Summary

1. About fifty thousand of cattle embryos were transferred and 16000 ET-calves were born in 1999. Eighty percents of embryos were collected from Japanese Black beef donors and transferred to dairy Holstein heifers and cows.

   Since 1985, we have achieved in bovine *in vitro* fertilization using immature oocytes collected from ovaries of slaughterhouse. Now over 8000 embryos fertilized by Japanese Black bull, as Kitaguni 7～8 or Mitsufuku, famousbulls as high marbling score of progeny tests were sold to dairy farmers and transferred to their dairy cattle every year.
2. Embryo splitting for identical twins is demonstrated an useful tool to supply a bull for semen collection and a steer for beef performance test. According to the data of Dr.Hashiyada (2001), 296 pairs of split-half-embryos were transferred to recipients and 98 gave births of 112 calves (23 pairs of identical twins and 66 singletons).
3. A blastomere-nuclear-transferred cloned calf was born in 1990 by a joint research with Drs.Tsunoda, National Institute of Animal Industry (NIAI) and Ushijima, Chiba Prefectural Farm Animal Center. The fruits of this technology were applied to the production of a calf from a cell of long-term-cultured inner cell mass (1998, Itoh *et al*,ZEN-NOH Central Research Institute for Feed and Livestock) and a cloned calf from three-successive-cloning (1997, Tsunoda *et al.*).

   According to the survey of MAFF of Japan, over 500 calves were born until this year and a half of them were already brought to the market for beef.
4. After the report of "Dolly", in February 1997, the first somatic cell clone female calves were born in July 1998 as the fruits of the joint research organized by Dr.

   Tsunoda in Kinki University (Kato *et al*, 2000). The male calves were born in August and September 1998 by the collaboration with NIAI and Kagoshima Prefecture.

   Then 244 calves, four pigs and a kid of goat were now born in 36 institutes of Japan.
5. Somatic cell cloning in farm animal production will bring us an effective reproductive

method of elite-dairy- cows , super-cows and excellent bulls. The effect of making copy farm animal is also related to the reservation of genetic resources and re-creation of a male bull from a castrated steer of excellent marbling beef. Cloning of genetically modified animals is most promising to making pig organs transplant to people and providing protein drugs in milk of pig, goat and cattle.

6. Farm animal cloning is one of the most dreamful technologies of 21th century. It is necessary to develop this technology more efficient and stable as realistic technology of the farm animal production. We are making researches related to the best condition of donor cells for high productivity of cloning, genetic analysis of cloned animals, growth and performance abilities of clone cattle and pathological and genetical analysis of high rates of abortion and stillbirth of clone calves (about 30% of periparutum mortality).
7. It is requested in the report of Ministry of Health, Labor and Welfare to make clear that carbon-copy cattle (somatic cell clone cattle) are safe and healthy for a commercial market since the somatic cell cloning is a completely new technology. Fattened beef steers (well -proved normal growth) and milking cows (shown a good fertility) are now provided for the assessment of food safety.

Introduction

In February 1997, the sheep "Dolly" revealed to the public as the first somatic cell clone from adult cells and the world of scientific novels is now realistic. The success of Dolly is great and has buried the knowledge that completely differentiated somatic cells of mammals no more had totipotency, the ability to develop individuals in a full term.

Many people are afraid of this technology to be applied to human and ethical discussion has been ending to prohibition of human cloning.

The cloning research has a long history in farm animal production and embryo micromanipulation producing twins orquadruplets were reported in 1979 and nuclear transfer technology using embryos are also developed to make many identical multiplets of cattle.

I would like to explain the present condition of embryo transfer and development of related technology and the recent research of cattle clone in Japan for the future orientaion.

1. Embryo Transfer

I am proud of working with Dr.Sugie in NIAI for several years in 1980s. He was succeeded in non-surgical embryo collection and transfer of cattle in 1964 and his report with high and stable success rates had stimulated the researchers of embryo transfer in the world. According to the contribution of Dr. Sugie and the governmental support, embryo transfer technology has developed and became popular in Japan(Fig. 1).

The Ministry of Agriculture, Forestry and Fishery (MAFF) has amended the Law of Livestock Improvement and Breeding related ET in 1983 and special artificial insemination technicians can

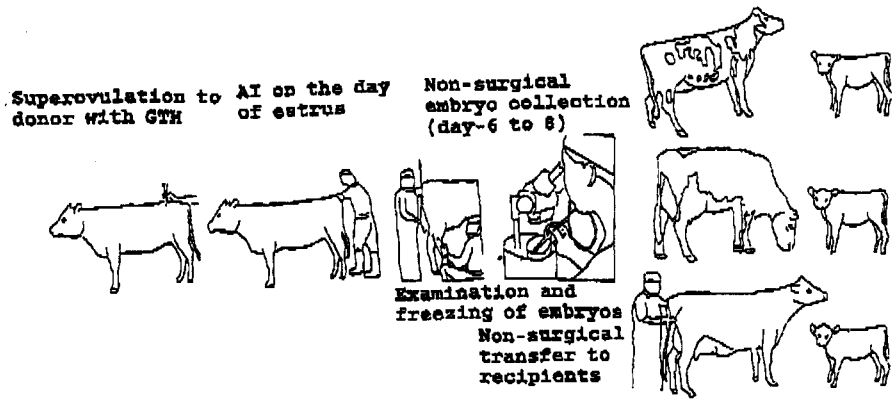

Fig. 1. Embryo Transfer of cattle.

handle and transfer embryos under the law.

1) About fifty thousand of cattle embryos were transferred and 16000 ET-calves were born in 1999. Eighty percents of embryos were collected from Japanese Black (JB)beef donors and transferred to dairy Holstein heifers and cows.

The JB beef cattle produce high quality meat with high marbling score called "Sashi". The beef price difference between JB and dairy cattle has urged the dairy farmers to transfer JB embryos to their milking cattle. In fact, dairy calves (1 to 3 months old) are usually sold for 30,000 to 50,000 yen and JB calves (8 to 10 months old) for over 250,000 yen.

The figures as follows have demonstrated that ET is used for improvement of cattle. About 40% of registered Holstein heifers (62,000/157,000), top Holstein bulls from the firstto 15th and about 30% of JB young bulls for direct performance tests are born by embryo transfer.

Frozen embryos in glycerol after transfer show high fertility rate. This procedure needs to de-cryoprotectant under a stereomicroscope. The group of National Livestock Breeding Center, led by Dr. Dochi demonstrated good pregnancy rate of direct transfer without de-cryoprotectant

Table 1. Numbers of embryo transfer in Japan (data; MAFF)

Year	No. of donors	No. of transfer	No. of offspring	No. of IVF embryos transferred
1975	32	10	1	-
1980	317	498	73	-
1985	2724	5034	887	(Calves born)
1990	7704	19865	5912	3916
1995	11079	40742	11322	4642
1999	14722	51827	16467	8821

Table 2. The price list of beef carcass in Japan (Yen/1kg of carcass weight)
(data; newspaper Sept. 2001)

Breed		Carcass Grade 5	4	3	2	(Carcass weight)
JB	Female	2,643	1,720			
JB	Steer	2,201	1,669	1,352		(445Kg)
JBxHol.	Steer	–	1,261	1,066	893	(450KG)
Hol.	Steer	–	–	804	712	(462KG)

using ethylene glycol. Now so called direct transfer is increasing because of easiness and good conception rate.

2) *In Vitro* Fertilization

Since 1985, we have achieved in bovine *in vitro* fertilization using immature oocytes collected from ovaries of slaughterhouse (Fig. 2). Now over 8000 embryos fertilized by Japanese Black bull, as Kitaguni 7~8 or Mitsufuku, famous bulls as high marbling score of progeny test were sold to dairy farmers and transferred to their dairy cattle every year.

The development of ultrasonography is remarkable and ovum-pick-up (OPU) using ultrasonography and aspiration apparatus is increasing to collect oocytes from heifers or cows to make up the defects of ET with super-ovulation.

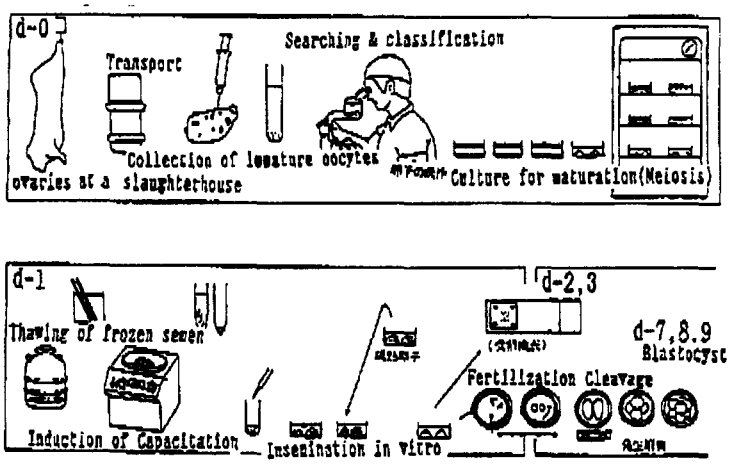

Fig. 2. *In Vitro* Maturation & *In vitro* Fertilization.

2. Embryo Splitting

Embryo splitting for identical twins is demonstrated useful tools to supply a bull for semen collection and a steer for beef performance test. According to the data of Dr. Hashiyada (2001), 296 pairs of split-half-embryos were transferred to recipients and 98 gave births of 112 calves (23 pairs of identical twins and 66 singletons).

Micro knives or needles are used for dissecting embryos into halves. Polish group reported a new method to make half embryos utilizing the hatching ability of embryo itself. Just before hatching, a small hole near Inner cell mass (ICM)was drilled through the zona pellucida and then a half of the ICM would hatch out in the shape of the figure 8. Using a micro-glass-needle embryo was easily cut into halves. This method is relatively easy and useful. Accordingto our experiments 13 out of 26 blastocysts hatched into 8- shaped and were split into halves (Katska *et al*, 1999).

Table 3. Production of identical twins by embryo splitting

(Data; Takakura *et al.* 1989 and Hashiyada, 2001)

Researcher	No. of Recipients	Pregnant	Calving	Twin	Single	Alive
Takakura	78	43(55.1%)	39	18	21	57(73.1%)
Hashiyada	296	128(43.2%)	98	23	66	112(37.8%)

3. Nuclear transfer

Nuclear transfer is a method to drive totipotency of nuclear by injecting into or fusing with ooplasma. The blastomeres of two- or 4-cell stage embryos can develop solely into individuals to identical twins or quadruplets if cultured well. But the blasomeres of 8-cell stage or further embryos have not totipotency solely because cleavage decreases the size of each cell and the material for development is in shortage. However the nuclear has total set of genes, it can regain the development ability when provided the material for development(Fig. 3). Nuclear transfer is the resolution of this shortage.

Nuclear transfer consists enucleation of recipient oocytes,injection of nuclear of blastomere, activation and fusion of cells and oocyte and in vitro culture to blastocyst for transfer to recipient animals.

A blastomere-nuclear-transferred cloned calf was born in 1990 by a joint research with Drs.Tsunoda, National Institute of Animal Industry (NIAI) and Ushijima, Chiba Prefectural Farm Animal Center. The fruits of this technology was applied to the production of a calf from a cell of long-term-cultured inner cell mass (1998, Itoh *et al*, ZEN-NOH Central Research Institute for Feed and Livestock) and a cloned calf from three-successive-cloning (1997, Tsunoda *et al.*). According to the survey of MAFF of Japan, over 500 calves were born until this year and a half of them were already brought to the market for beef.

4. Somatic cell cloning

After the report of "Dolly", in February 1997, the Japanese Government ceased the cloning research until the conclusion discussed in the Council for Science and Technology Policy, Cabinet Office and in August 1997 the Prime Minister has announced that human cloning should be prohibited but the cloning technology of farm animals was important research and should

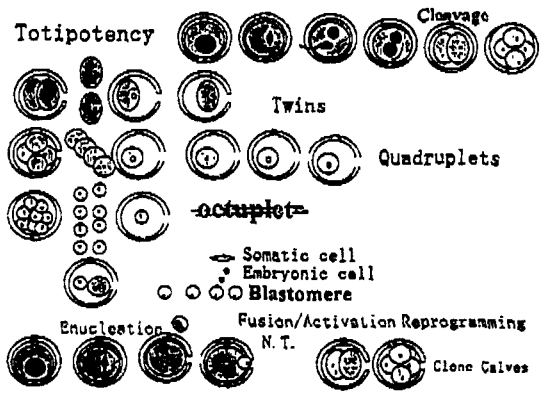

Fig. 3. Totipotency and Nuclear Transfer.

Table 4. Clone calf production in Japan (MAFF)

	Blastomere	Somatic cell
No. of institutes	38	36
No. of calves born	594	244
Stillbirth, death	86(14.5%)	74(30.3%)
Alive (Growing)	116	116
Death by diseases	70(11.8%)	31(12.7%)
Death by accidents and experiments	59	23
Sold out	263	-

be developed opening the research information to the public.

The first somatic cell clone female calves were born in July 1998 as the fruits of the joint research organized by Dr. Tsunoda in Kinki University (Kato *et al*, 2000). The male calves were born in August and September 1998 by the collaboration with NIAI and Kagoshima Prefecture. Then 244 calves were now born in 36 institutes of Japan.

Many calves were born but the accompaning problems are critical in high mortality around peripartum period; high incidence rates of abortion, stillbirth and death within 2 or 3days of birth, large offspring, immune defects etc.

It is also important that many calves are growing well and healthy and the grownup heifers have already given births of own calves and bulls have shown good fertility after insemination of their semen.

5. Advantage of cloning and transgenic animals

Somatic cell cloning in farm animal production will bring us an effective reproductive method of elite-dairy-cows, super-cows and excellent bulls.

The effect of making copies of farm animals is also relatedto the reservation of genetic resources and re-creation of amale bull from a castrated steer of excellent marbling beef.

Cloning of genetically modified animals is most promising to making pig organs transplant to people and providing protein drugs in milk of pig, goat and cattle.

6. Research

Farm animal cloning is one of the most dreamful technologies of 21th century. It is necessary to develop this technology more efficient and stable as realistic technology of the farm animal production. We are making researches related to the best condition of donor cells for high productivity of cloning, genetic analysis of cloned animals, growth and performance abilities of clone cattle and pathological and genetical analysis of high rates of abortion and stillbirth of clone calves (about 30% of periparutum mortality).

1) Mixing of Mitchondoria DNA of donor cell and oocyte.
2) The length of Telomere
3) Mechanizms of Reprogramming
4) Genomic imprinting

7. Public acceptance and Safety

In April 1999 the opinion magazine reported that "experimental" beef cattle were shipped to consumers without any notice. The "experimental" beef was blastomere-nuclear-transfer cattle and the MAFF considered them without any problem. But the mass media reported that clone beef cattle were dangerous for human health and many people were anxious about the safety of the new technology. The MAFF has decided to distinguish clone of somatic cells from that of blastomere. Finally clone cattle by blastomere-nuclear-transfer should be sold to the market with notice, but somatic cell clone cattle should be asked not to be brought to the market until the final result of special investigations of the Minister of Health, Labor and Welfare(MHLW).

In May 2000 the intermediate report was issued that the cloning technology is not related to production of dangerous food from scientific standpoints. But, it is also requested to make clear that carbon-copy cattle(somatic cell clone cattle) are safe and healthy for a commercial market since the somatic cell cloning is a completely new technology. The MAFF is now supporting researches for safety of milk and beef from somatic cell clone cattle. Fattened beef steers (well-proved normal growth) and milking cows (shown a good fertility) are now provided for the assessment of food safety. It takes a year to get conclusions.