Original: Japanese
Provisional translation

Risk assessment report on foods derived from cloned cattle and pigs produced by somatic cell nuclear transfer (SCNT) and their offspring (Novel foods)

June 2009

Food Safety Commission

Table of contents

<Progress of Risk assessment>

April 1, 2008	The Ministry of Health, Labour and Welfare (MHLW) requests a risk assessment in line with "foods derived from cloned cattle and pigs produced by somatic cell nuclear transfer and their offspring" and MHLW's request and references are received
April 3, 2008	232nd meeting of the Food Safety Commission (explanation of MHLW's request outline)
April 11, 2008	52nd meeting of the Novel Foods Expert Committee
May 2, 2008	1st meeting of the Working Group
June 2, 2008	2nd meeting of the Working Group
July 25, 2008	3rd meeting of the Working Group
October 6, 2008	1st meeting of the Subgroup of the Working Group
November 21, 2008	2nd meeting of the Subgroup of the Working Group
December 8, 2008	4th meeting of the Working Group
January 19, 2009	5th meeting of the Working Group
February 24, 2009	55th meeting of the Novel Foods Expert Committee
March 12, 2009	277th meeting of the Food Safety Commission (reporting of the discussion results from the Novel Foods Expert Committee)
March 12 to April 10, 2009	Public comments and information
June 8, 2009	58th meeting of the Novel Foods Expert Committee
June 23, 2009	Reports to the Chairperson of the Food Safety Commission from the Chairperson of the Novel Foods Expert Committee
June 25, 2009	291st meeting of the Food Safety Commission (reporting of the results reported from the Novel Foods Expert Committee) (notice of the results to MHLW as of June 25)

<Food Safety Commission Members>

Takeshi Mikami (Chairperson)
Naoko Koizumi (Deputy Chairperson)
Taku Nagao
Kazumasa Nomura
Keiko Hatae
Masao Hirose
Seiichi Honma

<Food Safety Commission Novel Foods Expert Committee Members>

(Until June 7, 2009)		(From June 8, 2009)	
Shuichi Kaminogawa* (Chairperson)		Sachie Ikegami (Chairperson)	
Sachie Ikegami (Deputy Chairperson)		Yasushi Yamazoe (Deputy Chairperson)	
Yoshiko Ishimi	Naoko Tajima	Yoshiko Ishimi	Makoto Shimizu
Hiroyasu Iso	Masamitsu Honma	Hiroyasu Iso	Naoko Tajima
Tetsuro Urushidani	Teruaki Matsui	Tetsuro Urushidani	Masamitsu Honma
Shinichi Oikawa	Takeshi Yamazaki	Shinichi Oikawa	Teruaki Matsui
Hiroshi Ozaki	Yasushi Yamazoe	Hiroshi Ozaki	Takeshi Yamazaki
Jun Kanno	Seiichiro Yamamoto	Jun Kanno	Seiichiro Yamamoto
Masuko Kobori	Masako Waki	Masuko Kobori	Masako Waki
Makoto Shimizu			

* Until March 31, 2009

<Working Group Members of the Food Safety Commission Novel Foods Expert Committee>

(Experts)

Takao Hayakawa (Chairperson)	
Sachie Ikegami	Susumu Kumagai
Atsuo Urisu	Junichi Sawada
Hiroshi Ozaki	Reiko Teshima
Shuichi Kaminogawa*	Shin Wakui

(Expert advisers)

Atsuo Ogura	Kunio Shiota
Toshiyuki Kojima	

* Until March 31, 2009

<Subgroup Members of Working Group of Food Safety Commission Novel Foods Expert Committee>

(Experts)

Takao Hayakawa (Chairperson)	
Susumu Kumagai	Junichi Sawada

(Expert advisers)

Atsuo Ogura	Kunio Shiota
Toshiyuki Kojima	

Summary

The safety of "foods derived from cloned cattle and pigs produced by somatic cell nuclear transfer (SCNT) (hereinafter, referred as SCNT cloned cattle and pigs) and their offspring" was assessed on the basis of data and published scientific papers submitted by the Ministry of Health, Labour and Welfare.

Cloning by SCNT is a technique in which a somatic cell or a nucleus of a somatic cell is transferred into an enucleated ooplast by direct injection and electrofusion, and the obtained (reconstructed) embryo is transferred to a surrogate dam to produce a bovine clone. In the risk assessment for the foods from the clones and their offspring, it was basically evaluated, on the basis of the current scientific findings, whether foods derived from SCNT cloned cattle and pigs and their offspring have equivalent safety as those derived from cattle and pigs produced by the conventional assisted reproduction technology (ARTs: e.g., article insemination).

In SCNT cloned cattle and pigs have indicated increase mortality observed during the perinatal period compared with those produced by h the conventional ARTs. In SCNT cloned cattle, the mortality rate tended to be higher in juvenile period. However, this higher mortality rate may be attributed to the lost of their totipotency of reconstructed embryo made using a somatic cell, and their causes of death have been found in the conventional ARTs. Although physiological parameter values in the clones in perinatal and juvenile period differed from those in cattle and pigs produced by the conventional ARTs, these differences decreased with growth and the clones became healthy.

There was no difference in the health profiles between the offspring of SCNT cloned cattle and pigs and those produced by the conventional ARTs.

These findings showed that SCNT cloned cattle and pigs and their offspring, which can be edible animal products, did not show any difference in health profile when compared with cattle and pigs produced by the conventional ARTs.

The abnormalities found in SCNT cloned cattle and pigs during the perinatal and juvenile periods were considered to result mainly from the normal development and differentiation due to normal epigenetic modification in the clones.

Since the DNA sequence in the nucleus of the donor animal is theoretically identical to that of SCNT cloned cattle and pigs, any new biomolecules that do not exist in donor animals or cattle and pigs produced by the conventional ARTs cannot be produced in the clones.

There were no differences associated with food safety hazards in nutrient components, or the results of studies, including micronucleus test, subchronic/chronic toxicity study in rats and mice and allergenicity test, between meat and milk from SCNT cloned cattle and pigs and their offspring and those from cattle and pigs produced by the conventional ARTs.

Although no detailed data have been obtained on foods other than meat or milk from the

clones, any new biomolecules are not produced in SCNT cloned cattle and pigs as stated above, and there were no differences associated with food safety hazards in milk and meat from SCNT cloned cattle and pigs and their offspring and those from conventionally bred cattle and pigs. Considering these findings, foods other than meat and milk derived from SCNT cloned cattle and pigs and their offspring would not differ in safety from those from cattle and pigs produced by the conventional ARTs.

The risk assessment based on current scientific findings showed that foods derived from SCNT cloned cattle and pigs and their offspring would have equivalent safety as those derived from cattle and pigs produced by the conventional ARTs.
Since SCNT is a new technique, it is necessary to continue collecting data on the safety of foods derived from SCNT cloned cattle and pigs in the risk management institution.

I. Introduction

1. Background

Since the birth of "Dolly", a sheep produced by somatic cell nuclear transfer (SCNT), in the U.K. in 1996, studies in various animals such as cattle, goats, and pigs have been carried out and many SCNT animal clones have already been born. In Japan, the first cloned calf in the world was successfully produced from a somatic cell of an adult animal in 1998; subsequently, many SCNT cloned cattle and pigshave been produced.

In Japan, the safety of foods from SCNT cloned cattle has been investigated and evaluated in studies such as scientific studies of 'the Ministry of Health, Labour and Welfare' (MHLW), and the profiles of products from the SCNT cloned cattle have been studied such as a 'Research project for utilizing advanced technologies in agriculture, forestry and fisheries'. The safety of foods from SCNT cloned livestock and their offspring has been assessed by the U.S. Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA).

Because data on the safety of foods from SCNT cloned livestock in Japan and other countries have been accumulated, and the collection of related articles have been conducted, MHLW requested the Food Safety Commission to assess the safety of foods derived from cattle and pigs produced by SCNT (hereinafter, referred as SCNT cloned cattle and pigs) and their offspring, on the basis of the rules specified in Paragraph 3, Article 24 of the Food Safety Basic Law (No. 48, 2003).

2. Outline of the Foods for Risk Assessment

The target foods requested by MHLW to evaluate the food safety are those derived from SCNT cloned cattle and pigs and their offspring.

The cloning with SCNT will be described later. Progeny of SCNT cloned cattle and pigs are animals bred from SCNT cloned cattle and pigs via fertilization and offspring born from them via fertilization.

3. Outline of Data on Safety

The safety of the foods were assessed on the basis of findings on the safety of foods from livestock produced by SCNT in Japan and other countries and the published scientific papers, which were collected by MHLW.

II. Concept of Food Safety Assessment

1. Basic concept

The basic concept of assessment for foods from SCNT cloned cattle and pigs and their offspring was to evaluate whether these foods have equivalent safety when compared to those obtained from cattle and pigs produced by the conventional assisted reproduction technology (ARTs: e.g., artificial insemination).

In other words, on the basis of current scientific data, we assessed whether any elements/factors potentially harmful to human health could be added to the health profile of grown SCNT cloned cattle and pigs and their offspring when compared with those produced by the conventional ARTs and whether foods from SCNT cloned cattle and pigs could have any additional elements/factors potentially harmful to human health when compared with those from cattle and pigs produced by the conventional ARTs.

The food safety assessment would be conducted in an objective, neutral, and fair manner, and no discussions on environmental effects and problems on ethic, morals, social economy, etc. would be performed.

2. Risk assessment of animal health of SCNT cloned cattle and pigs (Chapters III to V)

The effects of cloning with SCNT on individual animals were examined according to developmental stages to evaluate whether SCNT cloned cattle and pigs and their offspring are healthy equivalent to animals produced by the conventional ARTs. The risk assessment was conducted considering the possibility of supplying the clones at each developmental stage as foods.

3. Risk assessment of foods derived from cattle and pigs produced by SCNT (Chapter VI)

On the basis of the risk assessment in 2, we assessed whether there are any differences in safety between foods from SCNT cloned cattle and pigs and their offspring and those from cattle and pigs produced by the conventional ARTs. Data used for the assessment as reference included comparison data on nutrients between foods from SCNT cloned cattle and pigs and their offspring and those from cattle and pigs produced by the conventional ARTs.

III. Outline of ARTs in Livestock

1. Major ARTs

In Japan, techniques for livestock reproduction have been advanced from the start of development to the present time with the aims of effectively breeding individuals or strains with good genetic characters and stably supplying animal proteins in Japan. Particularly in Japan where we have to effectively utilize limited resources and lands, various studies and technological developments have been actively conducted since the end of the Second World War.

With the development of techniques for livestock reproduction, the consolidation of the legal system has been promoted, such as the enactment/revision of the relevant laws (Act on Improvement and Increased Production of Livestock [No.209, 1950]).

The current major ARTs applied in livestock, particularly cattle are as follows.

1) Artificial insemination

Artificial insemination (AI) is a technique in which sperm with a good genetic character is artificially injected into the reproductive tract of a healthy female animal in a state of estrus to produce offspring. Currently, AI with frozen sperm is performed in almost 100% of cows bred in Japan. However, it is impossible to control the sex and ability of offspring by this technique.

In Japan, the number of AIs in Holstein cows (dairy breed) with frozen sperm of Japanese Black bulls (beef breed) accounts for 30% of the total number of AIs in cattle (the statistics obtained from the Domestic Animal Inseminator Association of Japan from January to March 2008, national average). Cattle bred by this method (F1; first filial generation) are mainly used for meat.

2) Transplantation of in vivo fertilized egg

Transplantation of *in vivo* fertilized eggs (Embryo Transfer: ET) is a technique in which a female animal, superovulated by injection of hormone preparations such as follicle-stimulating hormone, is artificially inseminated; the fertilized egg (embryo) is harvested by perfusing the washed solution of the reproductive tract, and another female animal (e.g., embryo recipient cow or egg recipient cow) gets pregnant by transferring the embryo into the reproductive tract to produce offspring. Unlike AI, in this technique, the traits can be improved both in male and female animals, but the genetic improvement rate is greatly inferior to that of AI (the efficiency is affected by the number of transplantable embryos collected per egg harvest). In addition, it is impossible to control the sex of offspring with the ET alone.

The 2005 statistics in Japan show that 16,155 of cattle offspring (about 0.6% of the total) were produced with the ET (Ref. 1). In the developmental stage, when the embryo is surrounded by the pellucid zone, the proper washing of the embryo enables a reduction in the number of pathogenic microorganisms on the surface of the embryo to the level where

embryo recipient cows and offspring are not infected, as well as the blocking of infectious disease transmission. Such characters cannot be obtained in AI using a sperm.

3) Transplantation of *in vitro* fertilized egg

Transplantation of *in vitro* fertilized egg is a technique in which an intrafollicular egg removed by aspiration from the ovary is *in vitro* matured and then subjected to *in vitro* fertilization (IVF) and *in vitro* culture, and an embryo recipient cow gets pregnant by injecting the normally grown embryo into the reproductive tract to produce offspring. This technique contributes to the advances and improvements in *in vitro* fertilized egg culture techniques and is the foundation for SCNT cloning to be developed subsequently. The 2005 statistics in Japan show that 2,308 of cattle offspring (about 0.08% of the total) were produced with this technique (Ref. 1).

2. Cloning techniques

The cloning techniques used for breeding of livestock such as cattle are based on the nuclear transfer techniques, including cell fusion technique, can produce several embryos and individuals genetically identical to the donor embryo or animal providing a cell (containing a 2n nucleus) and are classified into 2 groups: fertilized egg cloning using a blastomere cell and SCNT using a somatic cell.

In order to produce a SCNT cloned animal, an egg that was previously aspirated from the ovarian follicle is cultured until mature and enucleated; a somatic cell or a nucleus of a somatic cell (e.g., skin cells, muscle cells, fallopian tube epithelial cells of a nucleus donor animal) is then transferred to the perivitelline space of the enucleated mature egg and fused using electrical stimulation. The embryo recipient cow gets pregnant by transferring the obtained reconstructed embryo into the reproductive tract to produce a SCNT cloned animal.

In a SCNT cloned animal, good genotypes of the cell donor animal are inherited and the sex of the clone is naturally identical to the donor. Since cells maintained in the cell culture system are used, it is theoretically possible to produce an endless number of genetically identical clones. Since differentiated somatic cells are used in the production of SCNT cloned animals, the reconstructed embryo requires nuclear reprogramming to acquire totipotency; the acquisition of totipotency would be an important requirement in the normal embryonic development and normal clone production. The success rate of SCNT cloned animal production could be affected by the origins of donor or recipient cells, cell culture methods, and nuclear transfer methods, etc.

SCNT was developed on the basis of the conventional ARTs for livestock such as AI, ET and IVF and is an extension of the conventional ARTs for livestock.

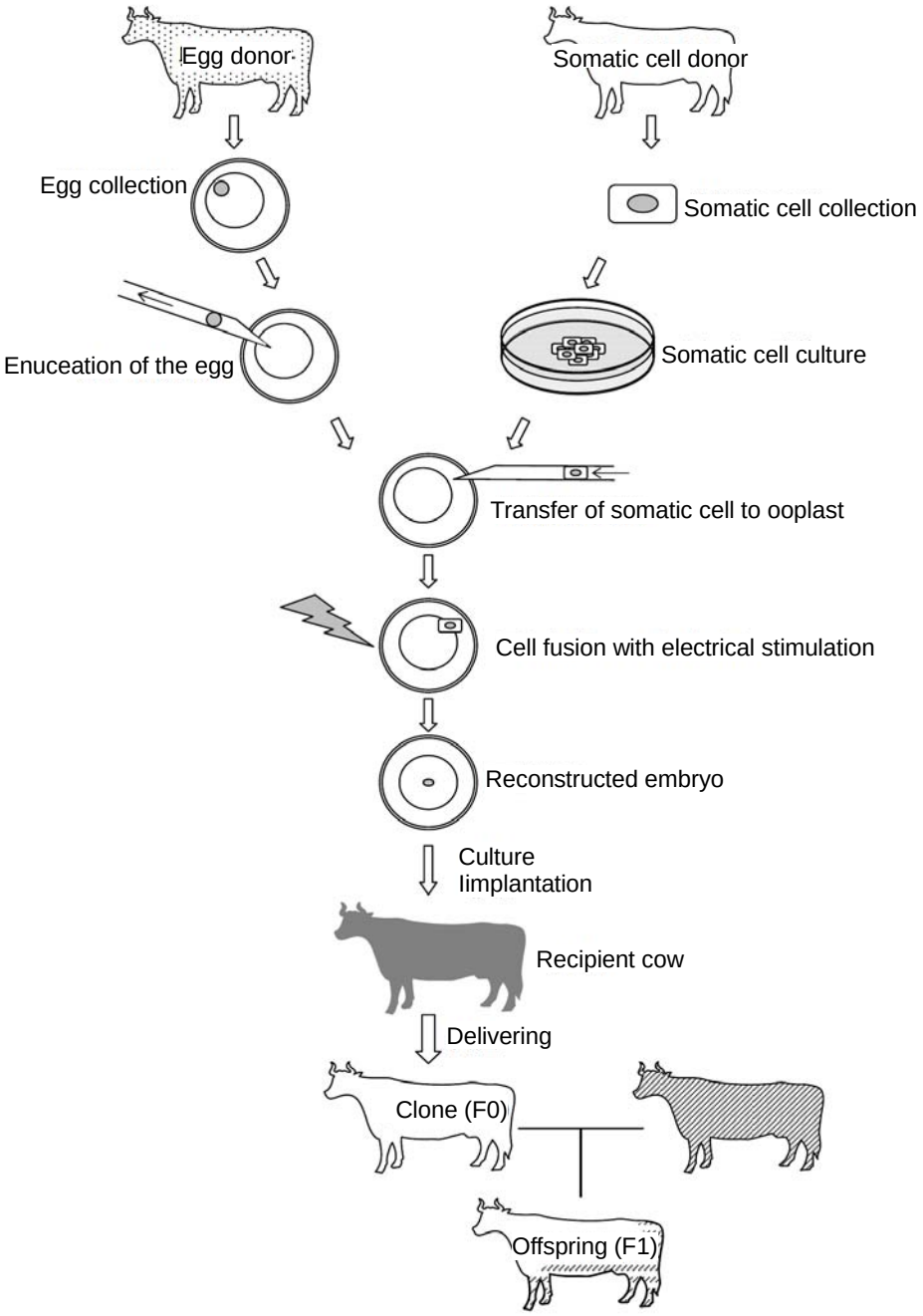

Figure 1. Production of SCNT cattle clone

3. Number and Efficiency of SCNT cloned animals

Since the birth of a SCNT cloned sheep "Dolly" in the U.K. in 1996, studies in various animals such as cattle, mice, goats, pigs, and rabbits have been carried out and many SCNT cloned animals have already been produced.

In Japan, against the background that various studies (e.g., studies on implantation of fertilized egg in cattle, IVF, and nuclear transfer using an early embryo (cloning by splitting a fertilized egg)) were performed with a high technological level in the world, studies have

been conducted mainly in cattle and the first SCNT cloned calf was successfully produced using an adult somatic cell in the world in 1998 (Ref. 2). The published data from MAFF show that the cumulative total number of live births of SCNT cloned animals and the cumulative total number of clones under breeding/study as of September 30, 2008 in Japan are 557 and 82 for cattle; and 335 and 35 for pigs, respectively.
In the U.S., the numbers of SCNT cloned cattle and pigs are estimated to be about 570 and 10, respectively. In the EU countries, the estimated number of SCNT cloned cattle is about 100, and the number of SCNT cloned pigs is estimated to be smaller than that of the cloned cattle. In other countries, including Argentina, Australia, China, and New Zealand, SCNT cloned livestock have been created (Ref. 3).

Although the overall rate of successful production of SCNT cloned livestock is lower than that of livestock produced by the conventional ARTs, it could vary according to animal species. In the study on the production rate of SCNT cloned cattle, 317 clones (9%) were born from 3,374 SCNT cloned embryos that were implanted into cows, 278 (8%) survived 24 h after birth and 225 (7%) survived 150 days or longer after birth (Ref. 4).
While a study reported that the success rate of SCNT in pigs was 5%, another study reported that the highest success rate was 17% (10 clones were obtained from 58 implanted embryos) (Ref. 5).
Abortion, fetal death, and large offspring syndrome (LOS) that were seen with other ARTs (e.g., AI, transplantation of fertilized egg) occurred with a higher frequency in SCNT cloned livestock when compared with animals produced by other ARTs. The study report on SCNT cloned cattle produced in Japan from the relevant institution shows that the rate of fetal death and died shortly after birth is 6.5% of cattle produced with the conventional ARTs and 30.8% of SCNT cloned cattle (Ref. 6).

IV. Cattle and pig clones produced by SCNT and their offspring

1. Cattle and pig clones produced by SCNT

The health profiles of SCNT cloned cattle and pigs were compared with those of cattle and pigs produced by the conventional ARTs according to the following developmental stages.

- Cell fusion - gestation (fetal growth)
- Perinatal period (before and after birth)
- Juvenile period
- Maturation and aging period after puberty (including reproduction)

Foods from animals in a stable health status are generally considered suitable for consumption by humans (Ref. 7), and we assessed whether cattle and pigs produced by SCNT that can be used as food have identical health profiles to those of cattle and pigs produced by the conventional ARTs.

Since the period at each developmental stage was not clearly defined in the zootechnical field, the developmental stages were classified in this assessment as follows: the period from nuclear transfer to the start of labor "cell fusion – gestation (fetal growth)"; a duration from the start of labor to about 1 week after birth "Perinatal period"; a subsequent duration until puberty onset "juvenile period" (generally, 6 to 13 months in cattle and 7 to 8 months in pigs (Ref. 8)); and a duration after puberty, including reproduction "maturation and aging stages after puberty".

(1) Cattle clones produced by SCNT

1) Cell fusion - gestation (fetal growth)

While a report said that the birth rate of offspring produced from SCNT cloned embryos was 10% or lower, another report has said that the formation rate of blastocyst, which are used for implantation and the fetal survival rate varied depending on the types of donor cells or recipient cells (oocytes).

Studies have reported that the blastocyst formation rate is higher when a ovarian cumulus cells or oviduct epithelial cells is used as a donor cell, than when a skin fibroblast cells, mammary gland epithelial cells, or follicular granulosa cells is used (Refs. 9, 10). It was also reported that in cattle, when a donor cell or recipient cell was used from a different breed of cattle, the fetal survival rate varied (Ref. 11).

The effects of the cell cycle of the donor cell used on the development of blastocyst have been examined. When fetal fibroblasts at the G1 or M phases were used as donor cells, the blastocyst formation rate was higher using the fibroblasts at the G1 phase than using those at the M phase (31% vs. 16%)(Ref. 12). Whereas embryo death was found early after implantation using cells at the G0 or G1 phases as donor cells, pregnancy was maintained until Day 120 or later after implantation, and no hydrops was also found when cells at the G0 phase were used as donor cells. When cells at the G1 phase were used as donor cells, the pregnancy rate continued to

decrease until delivery (21/43 Day 120 or later after implantation) and the incidence of hydrops was also high (18/43 [42%]) (Ref. 13).

The comparison of blastocyst development between IVF and SCNT cloned embryos reported that when bovine serum or defatted bovine serum albumin (BSA) was added to synthetic oviductal fluid (SOF) medium, the blastocyst formation rate in IVF embryo was similar both in SOF + bovine serum and SOF + BSA, and the blastocyst formation in SCNT cloned embryo was higher in SOF + bovine serum than in SOF + BSA (Ref. 14). In addition, findings obtained in the IVF have suggested that many combined factors, including the medium used, could have causal influences on highly frequent non-conception, abortion, and placental abnormality in SCNT cloned embryos (Refs. 15, 16, 17).

As in other ARTs, the pregnancy loss was frequently seen until Day 60 after implantation in SCNT cloned embryos of cattle. However, unlike AI and ET, the pregnancy loss was found throughout the entire period of gestation in IVF and SCNT cloned embryos (Refs. 18, 19).
The pregnancy rate in SCNT cloned embryos of cattle Day 50 after implantation was similar to those in controls (AI and IVF), but the pregnancy rate decreased in SCNT cloned embryos after Day 50. In the controls, such decrease was not found after Day 50. The pregnancy rate reduced to 40% in recipient cows to which SCNT cloned embryos were implanted Day 150 after implantation. There were no differences in the mean body weight of fetuses among the 3 groups Day 100 after implantation; 5 of 6 cloned cattle fetuses and 1 of 4 fetuses with IVF had body weights that were higher than two standard deviations from the mean body weight in fetuses with AI. A similar trend was observed in fetuses Day 150 after implantation. The liver and kidney were larger in cloned cattle fetuses than in fetuses with AI and IVF, and 1 of 3 cloned cattle fetuses had fatty infiltrations of the liver and the kidney (Ref. 20).

In the placentas of SCNT cloned embryo recipient cows Day 60 after implantation, the cotyledon and caruncle were poorly developed when compared with those of cows with AI, and the cotyledon was flat and the number of placentomes was approximately half of that of cows with AI (Ref. 21).
Another study reported that the mean number of caruncles and the number of placentomes Day 100 after implantation of SCNT cloned embryo were fewer in SCNT cloned cattle than in cattle with AI and IVF, but the total caruncle tissue weight was significantly higher in SCNT cloned cattle, and their larger cotyledons were thick and had a fist-shaped structures (Ref. 20).
Other studies have reported many cases of abnormal cotyledons observed in the placentas of SCNT cloned cattle and sheep (Refs. 22, 23, 24, 25). Some studies have

also reported that abortion in late gestation occurred with hydrops, enlarged umbilical cords, and abnormal placentas and mainly resulted from abnormal placenta growth (Refs. 22, 23, 26).
The normal placenta has important roles in the fetal growth, such as mother-to-fetus nutrient absorption, gas exchanges and excretion of waste products, and therefore the abnormal growth in the placenta, which has been seen with the conventional ARTs, has been regarded as a cause for not maintaining gestation.

Hydrops, which has been observed in animals produced by the conventional ARTs, was frequently found in the fetal membrane of SCNT cloned cattle. Hydroallantois, accounting for 85 to 90% of hydrops of the fetal membrane, is accompanied by a abnormalities of the placenta such as a hypertrophic placenta and is considered to be caused by allantois-chorion or the placental dysfunction.
In cattle, hydrops generally occurs in 1 in 7500 pregnancies. A study reported that the incidence of hydrops is 0.05 to 4% in the pregnancy with the IVF (Ref. 27). In the pregnancy of SCNT cloned cows, the pregnancy losses by Day 120 of gestation at a high rate and 58 to 86% of the pregnancies loss were accompanied by hydrops (Refs. 13, 28).
In SCNT cloned embryo recipients with hydroallantois, placental overgrowth was found before fetal overgrowth and the weights of placentomes Day 220 after implantation were higher than in the control fetuses. The number of placentomes tended to increase in late gestation. Some SCNT cloned embryo recipients had the normal weights and number of placentomes (Ref. 29).
In 3 of 20 (15%) recipients receiving SCNT cloned embryos, severe hydroallantois and enlarged placentome were found from 6 months gestation to term (Ref. 24).
The incidences of hydrops fetalis (13/37), placental edema (21/37), and hydroallantois (5/37) were high Day 100 or later after implantation of SCNT cloned cloned embryos. The majority of cases of hydrops fetalis (12/13) had placental edema (Ref. 4).
In SCNT cloned embryo recipients, the total fluid volume of the fetal membrane on Day 150 after implantation was significantly higher as compared with IVF, and 2 of 8 recipients had high allantoic fluid volumes (20 and 12 L) (Ref. 20).
The results of the pathological examination of the placentas at birth in 7 SCNT cloned calves showed that there were abnormalities in all placentas (moderate to severe edema, enlarged vessels, and other abnormal placentaion), the number of placentomes was fewer, the weight and surface area of placentome were heavier and larger, respectively, as compared with ET (Ref. 30).
The study suggested that the placental overgrowth could cause an increase in the level of fructose supplied to a fetus, leading to hypoglycemia and hyperfructosemia that affect the functions of muscles such as the cardiac muscle (Ref. 30).

There was no difference in the blood level of pregnancy-associated glycoprotein (PAG) between SCNT cloned calves born alive and those dead in late gestation, but the PAG level was obviously low in embryo recipient cows aborting SCNT cloned fetuses at the early stage of gestation (Days 35 to 90 of gestation) (Ref. 31).

2) Perinatal period (before and after birth)

The research reports on SCNT cloned calves created in Japan from the institutions concerned indicated that 451 SCNT cloned calves included 74 (16.4%) stillbirths and 65 (14.1%) died shortly after birth, and there was a significant difference in the mortality rate between the clones and cattle produced by the conventional ARTs (n = 566, stillbirths: 4.6%, died shortly after birth: 1.9%). The major causes of stillbirth were respiratory disorders, including dystocia, asphyxia and inhalation of amniotic fluid, and the major causes of died shorty after birth were also respiratory disorders such as asphyxia and inhalatuin of amniotic fluid. SCNT cloned calves that died shortly after birth tended to have LOS (Ref. 6).

While the occurrence of LOS was high in the gestation of SCNT cloned calves, LOS was found in gestation at a low frequency in embryonic cloning and AI. The incidence of LOS was 13.3% in SCNT cloned calves, 8.6% in embryonic cloning, and 9.5% in IVF (Ref. 24).

Many reports in Japan showed that the body weight at birth tended to be heavier in SCNT cloned calves (Refs. 32, 33, 34, 35, 36, 37).

Six SCNT cloned calves were born from cells of Japanese Black bulls, but 2 of them died shortly after birth. Four clones were healthy and normal and the mean body weight at birth was 36 kg (30.7 to 42.5 kg), which was higher than the mean body weight of Japanese Black calves (30 kg) by 20%. One of 2 clones that died shortly after birth had a diagnosis of having Akabane virus and another clone was dead from multiple causes in association with dystocia (Ref. 38).

It was also reported that the percentage of calves with the body weight of 50 kg or higher at birth was 10% in AI, 31.7% in IVF, and 15% (n = 126) in embryonic cloning (Ref. 39).

The occurrence of LOS often causes dystocia as well as disease and death associated with dystocia (Refs. 40, 41). A study reported that 6% of 2,191 natural and AI bred calves required some help in delivering (Ref. 41). USDA estimated the risk of developing dystocia in the general cattle population at 4% of pregnancies (Ref. 42).

The body weight at birth of offspring and the parity (number of previous deliveries) of cows are important factors for dystocia; particularly in nulliparous cows, the risk of dystocia is higher than in parous cow. Abnormal labor causes an increase in the number of deaths of neonatal calves, and the high morbidity rate, hypothermia,

respiratory and digestion failures, and other factors also increase the death risk. Damages due to difficult delivery or caesarean section (C-section) cause death of calves (Refs. 41, 43, 44).

The SCNT cloned calf delivered by C-section died of pneumonia aspiration 45 h after birth. The autopsy results revealed no abnormalities in the organs (Ref. 45).
The autopsy results showed that 2 SCNT cloned calves (twins), which died shortly after birth or stillbirth, died mainly because of inhalation of amniotic fluid in the accident in delivering (Ref. 46).
The SCNT cloned calf with a body weight of 71.0 kg at birth were delivered by C-section, but died on Day 3 after birth. In this case, the autopsy results showed abnormalities in several organs (Refs. 30, 47).
Twenty-four SCNT cloned calves were produced from donor cells from Japanese beef cattle and Holstein cow, and 13 calves had suevived. Seven SCNT cloned calves were stillborn or dead shortly after birth, 2 dead during C-section, but had no abnormalities. One clone appeared normal at birth but died of sepsis on Day 19 after birth. Six dead clones had significant morphological abnormalities in the kidney or hind legs. The mean body weight at birth in SCNT cloned calves was higher than that in calves produced by the conventional ARTs; the mean body weight at birth in 9 SCNT cloned calves was heavier by 40% (Ref. 48).
LOS occurred in SCNT cloned cattle and sheep in late gestation and is considered to cause increased perinatal mortality, abnormal placental development (e.g., increased incidence of fetal hydrops), enlarged internal organs, enhanced disease susceptibility, sudden death, reluctance to suckle, and difficulties in breathing and standing (2, 26, 49, 50).

A study has reported that the incidence of LOS varies according to the types of donor cells used (Ref. 48).
Whereas 1 out of 8 calves derived from embryos maintained in the sheep oviduct after IVF was dead, 7 out of 8 calves derived from embryos cultured *in vitro* with oviducal epithelial cells were dead (Ref. 40). This suggests that the *in vitro* culture conditions may also affect the development of LOS (Refs. 51, 52). When IVF embryos and SCNT cloned embryos were cultured under the same conditions, the development of LOS was higher in calves derived from SCNT cloned embryos than in those derived from IVF embryos (Refs. 23, 24, 53).

In addition to the occurrence of LOS, the results of the pathological examination in SCNT cloned cattle that died shortly after birth showed cardiac structural abnormalities as well as abnormalities in the myocardial tissue, connective tissue in the kidney, and soft tissue in the tendon (Ref. 54). In another study, the results of the pathological

finding in 9 calves that died by Week 1 after birth, showed hyperthermia, umbilical hernia, dyspnoea, ascites, fatty liver, limb malformation, gastrointestinal abnormalities, and other abnormalities (Ref. 55).

The hematological analyses in SCNT cloned calves showed that the red blood cell (RBC) and the white blood cell (WBC) counts were significantly low from immediately after birth to 24 h, indicating low hematologic function. However, there were no significant differences in any items other than glucose in blood biochemical analyses (Refs. 35, 36, 56). The levels of serum protein, magnesium, and inorganic phosphorus immediately after birth were significantly higher in SCNT cloned calves as compared with those in calves with AI (Ref. 33). The data in the blood biochemical analyses from immediately after birth to 2 months were within the normal range and there were no large differences between SCNT cloned calves and calves produced by the conventional ARTs (Ref. 57).
In SCNT cloned calves immediately after birth, the hemoglobin level was slightly low but was within the normal range. The low level continued for 65 days after birth and subsequently recovered to the same level as that in calves with AI (Ref. 55).

While 5 of 13 SCNT cloned calves required delivery by C-section, 7 control calves (produced with AI or IVF) were delivered by spontaneous vaginal delivery. Two of 5 C-section delivered clones had hydroallantois. Birth weight, plasma concentrations of cortisol, adrenocorticotropic hormone (ACTH), and components of the insulin-like growth factor (IGF) system were examined in 13 SCNT cloned calves and 7 controls. The C-section delivered clones were the heaviest at birth, followed by vaginally delivered clones and AI controls. The plasma concentrations of cortisol and IGF-I were lower in SCNT cloned calves than in control calves; the ACTH level was similar in the 2 groups, and the level of IGF binding protein-2 (IGFBP-2) was higher in SCNT cloned calves than in controls. The authors considered that those results were attributable to the C-section (Ref. 53).

In 8 SCNT cloned calves, the RBC count and hematocrit level were low at birth and 1 h after birth, but did not significantly differ from those in control calves (produced with AI and ET) thereafter. The mean body temperature in the clones decreased 1 h after birth, which was similar to that in control calves. The plasma concentrations of glucose and lactate were lower until 24 h after birth as in the controls, but these levels at 48 h did not differ between the 2 groups. The plasma concentrations of fructose in the clones were high immediately after birth but did not differ from the controls 6 h after that. There was no difference in the WBC count between the 2 groups (Refs. 30, 58).

In SCNT cloned calves, the body temperature was higher than in control calves with AI for 1 week after birth, and several temporal increases in the body temperature were observed. In the measurement of thyroxine (T4) level, the T4 level was lower for 2 weeks after birth in the clones than in the controls. The IGF-2 level was high at birth and low Day 15 after birth in the clones. These levels recovered to normal levels by Day 50 after birth. The authors considered that the reduced T4 level was associated with the increased body temperature and that as for differences in physiological parameters, these parameters recovered to almost normal levels by 2 months after birth (Ref. 23).

3) Juvenile period

In cows produced with the conventional ARTs, the mortality rate Week 1 after birth is the highest with 1.8 ± 0.3% (except for stillbirths). The most common diseases reported in these cows are respiratory disorders and diarrhea, whose incidences achieved peaks Week 2 after birth (Ref. 59).

The annual mortality rate from weaning to 4 years of age in SCNT cloned calves was 8% (7 of 59 clones died at 1 to 2 years of age; 3 of 36 clones died at 2 to 3 years of age; and 1 of 12 clones died at 3 to 4 years of age). The major cause of death was euthanasia because of poor prognosis due to musculoskeletal abnormalities (Ref. 60).

The research reports on SCNT cloned cattle produced in Japan from the institutions concerned said that 94 (19.5%) of the 482 clones investigated died of disease.

The age in days when a calf died of disease was investigated in Japanese Black cattle and Holstein cattle, whose data were collected for comparison (216 SCNT cloned calves and 991 control calves). The results indicated that the mortality rate until about 200 days of age tended to be higher in the clones as compared with control cattle produced by the conventional ARTs, but the mortality rate after about 200 days of age fluctuated at extremely low levels as in the controls. Of the identified death causes, respiratory disorder and malformation of the heart were found from Days 2 to 3 after birth, and the most common cause after that was pneumonia. In the pathological examination of 3 dead cloned calves, the identified causes of death were pulmonary congestion, immunodeficiency, and abnormal structure of the heart, which were found in cattle produced by the conventional ARTs.

In the surviving Japanese Black and Holstein clones, the hematological examination (e.g., RBC, WBC, serum protein, serum urea nitrogen, serum cholesterol, blood sugar) showed that these parameter values were within the variations of the parameters in cattle produced by the conventional ARTs.

The growth of SCNT cloned cattle was within the range of the breed traits or individual differences and did not largely depart from the growth in the controls or the standard growth curve. When donor cattle had better growth capability than the standard growth, their SCNT cloned calves tended to have better growth capability (Refs. 6, 61).

The pathological examination of SCNT cloned calf that died at 147 days of age indicated that they could have died of puerility due to abnormalities in the endocrine system (growth hormone) as well as nutritional deficiency due to insufficient nutrient digestion/absorption, and decreased resistance to infections due to immunodeficiency (Ref. 57).

In the SCNT cloned cattle that died at 25 months of age, the death cause was deficiencies in trace minerals (selenium and copper). However, there was no problem in other cattle raised on the same grass. The SCNT cloned cattle frequently had mild signs of bloating from a child (Ref. 47).

A SCNT cloned bull needed a lot of support from veterinarians immediately after birth since the calf had dyspnoea (the immature lung and pulmonary hypertension), insufficient sucking reflex, symptoms suggestive of type I diabetes and health problems. This calf soon recovered and diabetes was cured by 2 months of age (the calf could maintain the normal levels of blood glucose and insulin) and grew into a healthy adult (Ref. 62).

A SCNT cloned calf in which echocardiography revealed enlargement of the right ventricle was cured by treatment with medication. The examination of blood samples collected every other day after birth showed increased blood reticulocyte count and immature blood cell count over 3 weeks. The lymphocyte (WBC) count was normal until 1 month of age, but subsequently decreased rapidly. The hemoglobin level also reduced after about 40 days of age and the cloned calf died of anemia at 51 days of age. The pathological examination in the dead calf revealed dysplasia (hypoplasia) of the thymus, spleen, and lymph node as well as systemic aplasia of the lymphoid tissue. No synthesis of endogenous IgG was found. There was no involvement by virus causing thymic atrophy (Ref. 63).

Some studies reported that an average 30% of SCNT cloned cattle died from various causes before 6 months of age; the causes included respiratory failure, abnormal developments of the kidney, and hepatic steatosis; however, most SCNT cloned cattle normally grow after several months from birth and reach the maturation stage (Refs. 55, 60, 64).

There were no significant differences in parameters after the perinatal period, including hematological parameters and hormones, between SCNT cloned cattle and control cattle (produced with the conventional ARTs). In a study comparing SCNT cloned cattle at 6 months of age with age-matched control cattle, there were no differences in any blood biochemical parameters, urinary parameters, immune status, growth parameters, and reproductive parameters. Similarly, many studies reported that there were no differences in many physiological parameters (hematological profile) between

SCNT cloned cattle and age-matched control cattle (Refs. 4, 61, 65, 66, 67).

4) Maturation and aging period after puberty

In the research reports on SCNT cloned cattle produced in Japan from the institutions concerned, the analyses for clinical/pathological data and data on blood property, growth, reproductive fanction, and production of milk and meat revealed that SCNT cloned cattle living for 200 days after birth or later similarly grew and had similar physiological function as compared with cattle produced by the conventional ARTs.

The results of the fattening trials for 19 SCNT cloned Japanese Black cattle showed that the body weight gain per day and the carcass quality in this breed may slightly differ from that in cattle produced by the conventional ARTs, depending on the animal condition in fattening; however, the similarity was high as compared with cattle produced by the conventional ARTs (Ref. 6).

Several studies have reported that the growth rate in SCNT cloned cattle was similar to that in cattle produced by the conventional ARTs or was within the range of standard levels in donor cattle (Refs. 60, 68, 69, 70). Since SCNT cloned cattle inherited the desirable traits of donor cattle, the clones may have better growth levels than control cattle. In addition, SCNT cloned cattle from Japanese Black or Holstein cattle may show better growth profiles such as body weight and height that are over the upper limit of the standard growth levels (Refs. 37, 71).

Slaughter inspection and pathological findings in SCNT cloned cattle are ordinarily confirmed in fattening cattle. Particularly, the pathological examination for collectable organs (liver, kidney, heart, lung, skeletal muscle, and skin) showed no abnormal structural findings (Ref. 72).

In the pathological examination in 1 SCNT cloned cow at 61 months of age that had no pregnance experience the discoloration/hypoplasia of the uterine horn and ovarian quiescence were found. Many calcareous substances were deposited in the arterial medium of the uterus, and there were abnormally many arteries in the endometrium (Ref. 73).

As for the life spans of SCNT cloned cattle, healthy cloned cattle at 10 years of age are currently raised in Japan and there is a report mentioning cloned animals at 6 to 7 years of age (Ref. 55). However, to evaluate the life span of somatic cell clones animals, enough data have not been obtained.

A study reported that the life spans of SCNT cloned mice are short (Ref. 74) and another study reported that there are no differences in the life spans between cloned mice and control mice (Ref. 75).

The immune function was evaluated in 17 SCNT cloned cattle at 2 months to 5 years of age, which received rotavirus and ovalbumin vaccination. There were no differences in antibody productivity between the clones and control cattle produced by the conventional ARTs. The proliferation of antigen-specific cells after vaccination with rotavirus did not differ between the clones and the controls, but proliferation of antigen-specific cells after vaccination with ovalbumin was weaker in the clones. The same study was conducted again in the same control cattle and other SCNT cloned cattle, and the results indicated that the immune function was normal in the clones (Refs. 64, 67).

The proportions of γδ cells and WC1+γδ T cells in the blood temporarily declined in SCNT cloned cows aged 2 to 4 years in the early lactation stage as compared with the cows produced by the conventional ARTs, suggesting that the immune function may weaken in the cloned cows in the early lactation stage (Ref. 76).

(Reproductive function)
The research reports on SCNT cloned cattle produced in Japan from the institutions concerned have described the reproductive function of 52 SCNT cloned cattle (18 bulls and 34 cows).
The semen of the cloned bulls was normally produced and the property was similar to that of bulls produced by the conventional ARTs. The studies on reproductive function with ARTs such as IVF and AI using the semen obtained from SCNT cloned bulls confirmed that the SCNT cloned bulls are available as stud bulls. The pregnancy rate with AI using the semen from the cloned bulls was similar to that obtained with the conventional ARTs.
SCNT cloned cows after puberty had a normal estrous cycle and no abnormality in the blood progesterone concentration. The results in AI using the cloned cows and the transplantation of embryos to other cows cloned confirmed their reproductive function (Ref. 6).

As for sperms of SCNT cloned bulls, there were no differences in the proportion of normal sperms, the cleavage rate after IVF, and the blastocyst development rate between the clones and conventionally bred bulls, and the fertility test showed no abnormal findings (Refs. 69, 77, 78, 79, 80, 81, 82). The sperm from freeze-thawed semen did not have abnormal property and no abnormal findings in the insemination test, indicating no effects of freezing on the sperm (Refs. 78, 83, 84, 85).
In SCNT cloned bulls derived from an aged and infertile bull, the body weights at birth were high but they survived. The risk assessment of the semen property and the fecundity were normal (Ref. 86). In the fertility test using the semen of recloned

cattle, offspring were produced (Ref. 87).

Many SCNT cloned cows had normal first estrus and estrus cycle. However, a study reported that the onset of puberty was late, and the body weight at the first estrus was high in SCNT cloned cows. There were no differences in estrus cycle length, ovulatory follicule diameter or profiles of hormonal changes between the clones and conventionally bred cows. The diurnal variation patterns of the levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol, and progesterone were similar between SCNT cloned cows and cows with AI (Refs. 79, 88).
SCNT cloned cows reached puberty later than cows with conventional ARTs that were reared under the same conditions. However, there were no significant differences in the gestation period or survived offspring between the 2 groups (Ref. 64).

The blood progesterone concentration after first ovulation was low both in SCNT cloned cows and cows with AI. The cows with shorter estrus cycle tended to have a lower concentration of blood progesterone. There were no large differences in changes in blood progesterone concentration after second ovulation or later between the 2 groups (Ref. 89).

Two SCNT cloned cows at 26 months of age were artificially inseminated. One of 2 cloned cows became pregnant and delivered a cow with a body weight of 22.5 kg by spontaneous vaginal delivery Day 285 after conception. Another cloned cow that did not become pregnant was artificially inseminated again later and became pregnant. However, she had an abortion Day 252 after conception (Ref. 57).
There were no large differences in the gestation period between SCNT cloned cows and conventionally bred cows (Refs. 46, 54, 89, 90, 91, 92).

The reproductive function did not significantly differ between SCNT cloned cattle and cattle produced with the conventional ARTs, and the fetal growth was normal in the cloned cows (Refs. 60, 68, 86, 88).

The 305-day milk yield (observed value) in a donor cow was 10,722kg and that (expected value) in a SCNT cloned cow was 8,500kg. The 305-day milk yield in first calving differed between the donor cow and the cloned cow, but such difference would be affected by the feeding system (Ref. 92).
The lactational performance at primiparity tended to be lower in SCNT cloned cows as compared with cows with AI, but that at second calving was similar between the 2 groups (Refs. 36). On the other hand, a study reported that the milk yield even at second calving was low (Ref. 93).

(2) Pig clones produced by SCNT

1) Cell fusion – gestation (fetal growth)

Pigs are prolific animals and need multiple embryos that can grow in the uterus in early gestation and minimal embryos that can grow to maintain the gestation after the start of gestation. The number of embryos needed is considered to be about 4 (Refs. 94, 95). Unlike cattle, so many embryos have to be implanted in an embryo recipient pig because the death rate of SCNT cloned embryos is high in pigs. In a study where about 150 SCNT cloned embryos per pig were implanted, 4 living pigs were born from 2 embryo recipient pigs (Ref. 96). Some studies also reported that several dozen to about 100 SCNT cloned embryos per pig were implanted to produce SCNT cloned pigs (Refs. 5, 97, 98).

Research on the conventional ARTs indicated that the fetal mortality rate became higher on Day 35 of gestation and later (the placental development stage) with 9.2% (Ref. 99).

2) Perinatal period (before and after birth)

The research reports on SCNT cloned pig produced in Japan from the institutions concerned said that 90 SCNT cloned pigs included 22 (24.4%) stillbirths and 8 (8.9%) died shortly after birth. In SCNT cloned pigs, the proportion of stillbirths was higher and the proportion of died shortly after birth tended to be lower as compared with SCNT cloned cattle. In the SCNT cloned pigs, stillborn and died shortly after birth, no extremely LOS was found. Twenty-five SCNT cloned pigs (27.8%) died of disease (their death causes not described), and the number of deaths due to disease tended to be higher as compared with SCNT cloned cattle (Ref. 6).

Fetal fibroblasts of Duroc pigs were used as donor cells and 59 to 128 SCNT cloned embryos per pig (a total of 511 embryos) were implanted in 5 doms. The echography confirmed that all 5 cloned embryo recipients became pregnant Days 28 to 40 after implantation. Four of 5 recipients went to term and gave 5 to 9 SCNT cloned piglets, respectively (a total of 28 piglets). Three of 4 recipients were induced and delivered on Day 115 of gestation. The remaining 1 recipient delivered by spontaneous vaginal delivery on Day 117 of gestation. One of 28 SCNT cloned piglets was stillborn without any pathologically abnormal findings. One of SCNT cloned piglets born alive had anal atresia and was the smallest. Anal atresia is an abnormality found at a low frequency in piglets produced with the conventional ARTs. The survived cloned piglets had slightly lower body weights at birth than piglets of the same breed produced with the conventional ARTs. There were almost no influences on litter size, placenta weights, and fetal weights (Ref. 5).

Although the number of surrogate dams having SCNT cloned pigs is limited, the

gestation period was reported to be 114 to 120 days (the average gestation period in pigs produced by the conventional ARTs is 110 to 120 days) (Refs. 5, 100, 101, 102, 103).

Some studies reported that the body weight at birth and the placental weight in SCNT cloned piglets were within the normal ranges in pigs produced by the conventional ARTs (Refs. 97, 104). A study conducted under a controlled breeding environment showed that the average birth weight was significantly lower in SCNT cloned pigs as compared with pigs with AI, but the weaning period did not differ between the 2 groups. The study reported that the rates of stillbirths and postnatal mortality tended to be higher in SCNT cloned pigs (Ref. 105).

In contrast to the occurrence of LOS in SCNT cloned cattle, the occurrence of intrauterine growth retardation (IUGR) is higher in pigs produced with SCNT. Granulosa cells were used as donor cells, and 5 survived SCNT cloned piglets were delivered by C-section on Day 116 of gestation. The average birth weight of the clones was 2.72 pounds, which was about 25% lighter than offspring produced by natural mating of pigs, from which donor cells were obtained (Ref. 98). Twenty-three litters of SCNT cloned pigs (143 individuals) were compared to 112 litters of pigs with AI (1300 individuals). The SCNT cloned pigs had an increase in the number of IUGRs per litter (Ref. 105).

Pathological examination was performed in 2 SCNT cloned pigs that died shortly after birth, and that died at 139 days of age. One of 2 clones that died shortly after birth had abnormalities in the limbs and hernia; another clone had no significant changes in the organs, but showed hemorrhage in the abdominal cavity and in the brain. The SCNT cloned pig that died at 139 days of age had diagnoses of pleuropneumonia and systemic infection with corynebacterium. These observations were similar to those known in pigs produced by the conventional ARTs (Ref. 57).

Of 40 SCNT cloned pigs, 5 were stillborn, 22 were dead within first week after birth, and 1 died Day 40 after birth, due to various causes, including diarrhea, encephalomeningitis, and cardiac abnormal function. Twelve survived to reach adulthood (Ref. 101).

The morphological abnormality of the placenta in nonviable SCNT cloned pigs may be due to apoptosis of placental cells (Ref. 106).

The acute phase response (cortisol, TNF-α and IL-6) follows a lipopolysaccharide challenge at about Day 30 after birth was evaluated in 9 SCNT cloned piglets with donors of 2 different embryo-derived fibroblasts. As compared with piglets produced by the conventional ARTs, some cloned had lower acute response and other clones had similar acute response (Ref. 100).

3) Juvenile peiod

The body weights of SCNT cloned pigs showed similar patterns of variation to the standard values, and there was no large difference among the cloned pigs (Ref. 57).

The body weights of SCNT cloned pigs aged 3 to 5 weeks varied at significantly higher levels than those of pigs produced by the conventional ARTs. Differences in the body weights between the 2 groups were not seen after 6 weeks of age, and the body weights at 8 weeks of age in the clones were almost the same as in conventionally bred pigs (Ref. 104).

It was indicated that there were no differences in growth, physical condition, biochemical parameters, or immune function between SCNT cloned pigs at 15 and 27 weeks of age and pigs produced by natural mating (Refs. 107, 108). The hyperkeratosis was observed in 1 of SCNT cloned pigs. However, it could not be determined whether hyperkeratosis resulted from SCNT, because it was found in pigs raised with the conventional breeding methods (Ref. 107).

The blood test was performed for 17 hematological parameters, including RBC, and 24 blood biochemical parameters, including LDH, in 3 SCNT cloned pigs at about 18 months of age. The results showed no significantly difference between the clones and pigs produced with conventional ARTs (Ref. 6).

As compared with pigs produced by natural mating, SCNT cloned pigs had similar behavioral patterns or large individual differences in behavioral patterns (Ref. 109).

4) Maturation and aging period after puberty

The evaluation of semen from SCNT cloned boars showed similar ejaculate volume, sperm concentration, and mortality as compared with control pigs (Refs. 102, 110).

Forty-nine sows produced by the conventional ARTs were artificially inseminated with semen from 4 SCNT cloned boars, and 293 offspring survived to the weaning period (Ref. 102).

The age at first estrus was 97 to 125 days old in SCNT cloned sows other than 1 clone. The mean litter size, number of surviving clones, and the number of weaned clones were 11.7, 10.3 and 9, respectively, indicating no differences between the clones and control pigs (produced with the conventional ARTs). However, the mean body weights of piglets at birth and 3 weeks of age were lower in the clones than in the controls (Ref. 104).

SCNT cloned sows artificially inseminated with the semen from SCNT cloned boars delivered in the normal gestation period. Except for 1 cloned piglet with contracted flexor tendons of the hind limb, 62 were normal at birth. The incidence of abnormalities in the cloned piglets was 1.6%, which was similar to the estimated

incidence (1.2%) in the Australian pig industry. Whereas the stillbirth rate was 8% in control piglets (produced with the conventional ARTs), it was 4.5% in piglets produced from SCNT cloned pigs (Ref. 110).

Only 1 of 5 SCNT cloned pigs had lower IGF-I levels after birth and before slaughter as compared with control pigs (produced with the conventional ARTs), but the IGF-I levels in other clones were within the variation range in the controls. The 17β-estradiol levels were also low in the clones, but were within the variation range in the controls (Ref. 111).

(3) Summary of cattle and pigs produced by SCNT

The health profiles were compared between cattle and pigs produced with SCNT and those produced with the conventional ARTs, according to the developmental stages.

1) Cattle produced by SCNT

There were many stillbirths in the perinatal period and many died shortly after birth. The major causes were dystocia, asphyxia, and inhalation of amniotic fluid due to the occurrence of LOS. Although LOS occurred in cattle produced by the conventional ARTs, it frequently occurred in SCNT cloned cattle. The blood test for hematological parameters and other parameters immediately after birth showed differences between the clones and cattle produced by the conventional ARTs, but the values of those parameters returned normal within 2 months after birth.

It was reported that the mortality rate during the juvenile period was higher in the cloned cattle than in cattle produced by the conventional ARTs. The death causes included pneumonia, which was known as a death cause in cattle produced by the conventional ARTs. The decreased resistance to infections was also reported. The blood test for various parameters showed no significant difference between the clones and cattle produced by the conventional ARTs. The high mortality rate during the juvenile priod would be caused by factors common to abnormalities frequently found in the perinatal period. In the cloned cattle surviving until almost 6 months after birth or later, the mortality rate due to abnormalities seen during the juvenile period did not differ from that in cattle produced by the conventional ARTs.

The SCNT cloned cattle surviving after the juvenile period had no differences in the parameters, including clinical/pathological parameters and hematological parameters, as compared with cattle produced by the conventional ARTs. The growth, reproductive function, and production of milk and meat did not differ between the cloned cattle and cattle produced by the conventional ARTs. The pathological examination in SCNT cloned cattle, from which samples can be collected, showed no abnormal findings.

Although it was reported that the immune dysfunction was found in some SCNT

cloned cattle, many reports also said that there was no difference in the immune function between the clones and cattle produced by the conventional ARTs. Generally, it has not been reported that SCNT cloned cattle have particularly higher sensitivity to diseases, including infections, as compared with cattle produced by the conventional ARTs.

The success rate of SCNT cloned animal production could be affected by various factors such as origins of donor or recipient cells, cell culture methods, and nuclear transfer methods. Further study on these factors would improve the success rate of SCNT cloned animal production.

2) Pigs produced by SCNT

There were many stillbirths in the perinatal period and died shortly after birth. The death causes were found even in pigs produced by the conventional ARTs.

Although some reports said that the body weights at birth were low in the clones, their body weights recovered in the weaning period.

After the juvenile period, there were no differences in the clinical/pathological parameters, hematological parameters, growth, or reproductive function between SCNT cloned pigs and pigs produced by the conventional ARTs.

3) Summary of cattle and pigs produced with SCNT

Stillbirths and died shortly after birth were mainly due to the developmental abnormalities found in SCNT cloned cattle and pigs after and before birth. Although the mortality rate tended to be higher even during the juvenile period in the cloned cattle, they grew healthily after 6 months age as in cattle produced with the conventional ARTs. The death causes in the clones were also found in cattle produced by the conventional ARTs.

Although the physiological parameter values differed between SCNT cloned cattle and pigs and those produced by the conventional ARTs after birth and during the juvenile period, these differences decreased with growth and the clones became healthy.

Considering these findings, there was no differences in the health profiles between cattle and pigs produced with SCNT that could be used for food and those produced by the conventional ARTs.

When animals have or are suspected to have diseases specified in the Food Sanitation Law on the common infections in humans and animals (No.233, 1947), or when animals have abnormalities specified in the Abattoir Law (No. 114, 1953), necessary measures are taken in the conventional inspection before and after slaughter for such animals, irrespective of animals produced with SCNT.

2. Progeny of cattle and pigs produced by SCNT

The health profile of progeny (F1) was compared between SCNT cloned cattle and pigs and those produced by the conventional ARTs.

The progeny of SCNT cloned cattle and pigs is calves and piglets (F1) produced from SCNT cloned cattle and pigs via fertilization and their offspring produced via fertilization. Since offspring in all generations are produced via fertilization, we evaluated whether offspring in the first generation, F1, have health profiles similar to that in cattle and pigs produced by the conventional ARTs.

(1) Progeny (F1) of cattle produced by SCNT

The research reports on SCNT cloned cattle created in Japan from the institutions concerned indicatedthat 124 offspring of SCNT cloned cattle included 11 stillbirths (8.9%) and 1 died shortly after birth (0.8%), showing no significant difference in the mortality rate from that in cattle produced by the conventional ARTs (n = 566) (stillbirths, 4.6%; died shortly after birth, 1.9%). As 14 (6.9%) of 202 offspring of SCNT cloned cattle also died of diseases, the age (days) when offspring of clones died of disease was investigated in Japanese Black cattle and Holstein cattle whose data were collected for comparison (216 SCNT cloned cattle and 991 control cattle). The results revealed that the mortality rates in the clones Day 2 after birth or later were almost the same as those in the controls. These results showed that there were no significant differences in the incidences of stillbirths, died shortly after birth, or deaths due to diseases between progeny of SCNT cloned cattle and cattle produced by the conventional ARTs.

The pathological examination in 1 stillborn offspring of SCNT cloned cattle revealed that stillbirth resulted from the disease (immunodeficiency), which is found in cattle produced by the conventional ARTs. In addition, the pathological examination in 2 offspring of SCNT cloned cattle under breeding that appeared healthy showed no significant changes.

In the blood test in 18 offspring of SCNT cloned cattle, some variations in the values were seen among the offspring, but no values in any items largely departed from the variation range in the control cattle.

As for the growth, 16 offspring of SCNT cloned cattle investigated showed growth profiles that were within the breed traits or individual differences and did not largely depart from the standard growth curve in the control cattle.

The reproductive function was examined in 5 female offspring of SCNT cloned cattle. There were no differences in the gestation period with AI and body weights at birth of their calves between the offspring and the control cattle.

The study on milk yield in 5 offspring of SCNT cloned cattle indicated that the mean 305-day adjusted milk yield was lower in the offspring than in the control cattle. In the fattening experiment, the body weight gain, carcass quality as well as nutritional compositions, including amino acid composition and fatty acid composition, were analyzed. In the offspring, these values may vary slightly depending on the fattening

conditions, but the offspring had the standard growth performance (Ref. 6).

Fifty-two offspring of SCNT cloned cattle that were delivered by spontaneous vaginal delivery, 85% survival was observed until 24 h after birth, indicating that there was almost no difference in the survival rate between the offspring and cattle with AI (Ref. 60).
Twenty-one offspring of SCNT cloned cattle were delivered by spontaneous vaginal delivery and 20 of them survived after birth (Ref. 67).
In the pathological examination in fetal offspring of SCNT cloned cattle that was stillborn Day 252 after implantation, the findings in the spleen and the thymus suggested immunodeficiency, and the findings in the thyroid gland indicated abnormalities relevant to hormones (Ref. 90).

In 3 offspring of SCNT cloned cattle, LOS that was seen in SCNT cloned cattle did not occur, and the body weights at birth did not differ from those in the offspring of cattle with AI. All the offspring were delivered by spontaneous vaginal delivery. As for the blood profile, values in some parameters differed between offspring of SCNT cloned cattle and those with AI during a period, but were almost within the normal ranges. There were no differences in many parameters between the 2 groups (Ref. 112).
Offspring of SCNT cloned cattle that had delivered showed no abnormal appearance, stood up by themselves, and suckled. They did not differ from the offspring of cattle with AI and/or IVF (Ref. 113).

The semen profile in offspring (males) of SCNT cloned cattle was normal. AI and IVF were performed using this semen in 2 cows, both of which became pregnant on the first attempt and delivered normal calves (Ref. 80).
Offspring (females) produced from SCNT cloned cows with IVF were delivered calves (grandchildren) with AI, which normally grew (Refs. 73, 93).
The physiological function and genetic conditions were examined in offspring of SCNT cloned cattle (19 females and 11 males). The heart rate, respiratory rate, and body temperature were low for a while after birth. Chromosomal stability, growth parameters, physical parameters, hematological parameters, and reproductive parameters were at standard levels as compared with normal animals at 1 age of years. The offspring had also proper stress response (Ref. 114).

Many reports said that the body weights and heights in offspring of SCNT cloned cattle varied in a range of the standard values in the control cattle produced by the conventional ARTs. A study showed that the weight of the gastrointestinal tract and the weight of each organ such as the stomach and the intestine in the offspring of clones were within the normal range. The composition rates of muscle, fat, and bone and the proportion of

each muscle in carcass meat in the offspring of clones were within ranges of the normal values (Ref. 115).

(2) Progeny (F1) of pigs produced by SCNT

The research reports on SCNT cloned pigs produced in Japan from the institutions concerned said that 143 offspring of SCNT cloned pigs included 8 stillbirths (5.6%) and 2 died shortly after birth (1.4%). All offspring were delivered by spontaneous vaginal delivery and did not have LOS. The blood test in offspring of SCNT cloned pigs revealed that the range of measurement values were almost similar to that in the control pigs (produced with the conventional ARTs), and the body weight gain was almost similar to that of the control pigs and the standard growth curve (Ref. 6).

Six SCNT cloned sows were delivered 44 offspring with AI. Although the offspring had significantly lower body weights at birth than control piglets produced by the conventional ARTs, the number of fetuses born, the mean litter size, and the number of piglets surviving to the weaning period were similar to those in the controls (Ref. 116).

Nine SCNT cloned sows were produced by the conventional ARTs and delivered offspring. There were no differences in the mean litter size between litters from cloned gilts and naturally bred control pigs. In the blood test (10 items) in the offspring Weeks 15 (n = 14) and 27 (n = 8) after birth, there were slight differences in the mean levels of blood urea nitrogen (BUN) at Week 15 and alkaline phosphatase at Week 27, which were within the normal variation as compared with the control pigs (Ref. 108).

In 242 offspring of SCNT cloned pigs raised under the commercial settings, there were no differences in the health condition or mortality rate as compared with pigs produced by the conventional ARTs (Refs. 111, 117).

As for the growth status, 10 litters included deaths from reasons such as debilitation before weaning and some piglets in 3 litters also died of disease. The death causes were diarrhea and multiple serositis, which were found in conventionally bred pigs. Piglets in litters, which did not include deceased piglets due to disease, had a similar growth to that of pigs produced by the conventional ARTs (Ref. 118).

The blood biochemistry examination in offspring of SCNT cloned pigs showed that the values in some items such as the hemoglobin amount and WBC count significantly differed from those in control pigs produced by the conventional ARTs. However, most of these differences were temporal, and the ranges of values were almost similar to those in the controls (Ref. 118).

Offspring of SCNT cloned pigs had lower body weights at birth, and no difference in the

body weight gain per day to 30 kg as compared with pigs produced by the conventional ARTs. However, the body weight gain from 30 to 70 kg was higher in the offspring than in conventionally bred pigs (Ref. 119).

(3) Summary of progeny of cattle and pigs produced by SCNT

Offspring (F1) of SCNT cloned cattle and pigs had no abnormalities found in SCNT cloned cattle and pigs during the prenatal or juvenile period and did not differ in health profile from cattle and pigs produced by the conventional ARTs.

Offspring of SCNT cloned cattle and pigs are produced via fertilization with the conventional ARTs such as AI. Considering that the first generation offspring (F1) have equal health profile as compared with cattle and pigs produced by the conventional ARTs, there would be no differences in health profiles between offspring of clones in the second and later generations produced via fertilization and cattle and pigs produced by the conventional ARTs.

V. Epigenetics and other genetic properties for SCNT cloned animals

1. Development and differentiation of SCNT cloned animals

The zygote obtained after fertilization by natural mating becomes an embryo with "totipotency" and undergoes some cell divisions to differentiate into many different cells (e.g., muscle cells, fat cells, and brain cells). Some cells of them are actually used as embryonic stem (ES) cells. In other words, genetically identical cells with the same can differentiate into somatic cells with different roles or characters by normal expression regulation for essential genes. This concept has been established with the advances in embryology researches, including epigenetics, in recent years and is a basic concept of the embryology irrespective of the technique, whether natural mating, AI or cloning technology.

In SCNT, a nucleus of somatic cell is transferred into an enucleate unfertilized egg (recipient), or a somatic cell from a donor animal (cells other than germ cells including eggs and sperms, e.g., skin cells and muscle cells) is fused with an enucleated unfertilized egg using electrical stimulation, and then the egg is activated to begin development. After several cleavages in culture, the embryo reaches blastocyst stage (called SCNT cloned embryo or reconstructed embryo). Another female animal (embryo recipient animal or surrogate dam) gets pregnant by implanting the blastocyst into the uterus to deliver the mature fetus as a cloned neonate animal.

Since a nucleus of a differentiated somatic cell is used in the production of a SCNT cloned animal without the normal processes of fertilization, it is said that a SCNT cloned embryo needs to be reprogrammed to be at a "totipotency" state.

When a SCNT cloned embryo does not appropriately acquire "totipotency" in the processes of somatic cell clone production, it is expected that the subsequent cell differentiation and formation of organs are not appropriately performed. In recent years, in order to determine the causes of abnormalities in development, differentiation and growth of SCNT cloned animals, epigenetics research in the related fields have been actively conducted. This chapter summarizes the current findings on correlations between abnormalities in development or growth seen in the processes of SCNT cloned animal production and epigenetic modification.

2. What is epigenetics?

Epigenetics is defined as "the study of heritable changes in gene function that occur after cell divisions without a change in DNA sequence" (Ref. 120).

The known epigenetic controls include DNA methylation (Refs. 121, 122, 123, 124, 125) and histone modification (methylation, acetylation, phosphorylation and ubiquitination) (Refs. 126, 127, 128, 129, 130). In particular, DNA methylation is mainly studied in epigenetics research together with gene expression analysis and would play an important role in the epigenetic control of gene expression.

DNA methylation is the transfer of a methyl group to the number 5 of the cytosine base by

methyltransferase to form 5-methyl citosine. Cytosine bases of cytosine-guanine (CG) sequences in the gene control region are methylated to often suppress the gene expression.

DNA methylation patterns (differences in the cytosine methylation level among genes) vary according to the types of cells comprising the organs or tissues such as muscle, fat, and brain. Generally, the DNA methylation pattern is not lost by cell divisions and can be maintained. In mammals at least, appropriate methylation and demethylation of each gene would be important in the life support.

For example, in a study where the methylated state and gene expression of *Oct-4* gene (encoding a factor that regulates the transcription of other genes) were investigated in the preimplantation embryo (blastocyst) of a mouse, the inner cell mass had little methylation of *Oct-4* gene with *Oct-4* gene expression, and the trophoblast had highly methylated *Oct-4* genes without *Oct-4* gene expression (Ref. 131).

Genome-wide epigenetic modification occurs in development and differentiation processes of the mammalian embryo. The epigenetic modification immediately after fertilization is called "preimplantation reprogramming." The epigenetic modification at gametogenesis is called "gametogenesis reprogramming." Therefore, "reprogramming" is regarded as "global epigenetic modification" (Refs. 122, 132).
Differences in reprogramming between embryos created with SCNT and normal embryos attract attention. Many studies on the differences have been reported, but most of them involved preimplantation reprogramming (Refs. 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143). Such incomplete epigenetic regulation is not always limited in SCNT cloned animals, but is found in embryos obtained with other ARTs (Refs. 144, 145, 146).

3. Epigenetics for SCNT cloned animals

Findings on epigenetics for SCNT cloned animals are outlined as follows.

(1) DNA methylation

(Cell fusion to preimplantation/blastocyst stage)

A study reported that nuclei from the sperm were demethylated in zygotes (fertilized eggs) immediately after fertilization in mice, rats, pigs, and cattle. In cattle, methylation was reduced during cleavage from the 2-8 cell stage, but de novo methylation occurred from the 8-16 cell stage. In mice, de novo methylation was also reported to occur notably in the inner cell mass of blastocyst after 4 cleavages (Ref. 147). Thus, there are differences in the timing of remethylation among animal species.

It was reported that in SCNT cloned embryos in cattle, remethylation occurred earlier than in normal embryos because demethylation occurred in the cell fusion stage but was

incomplete. The morulae of SCNT cloned cattle, therefore, had more highly methylated nuclei (Ref. 147).
On the other hand, a study reported that methylation patterns were maintained until the 4-cell stage, and no demethylation occurred in the embryos of SCNT cloned cattle (Ref. 148).
Another study reported that in preimplantation embryos of SCNT cloned cattle, methylation did not appropriately occur in the genomic satellite region and the methylation level, which was very similar to that of the donor cell, was maintained (Ref. 149).

The demethylation status was compared between the satellite DNA sequence of centromere (a region where the long arm and the short arm of chromosome cross) and the PRE-1 sequence of euchromatin in germ cells and somatic cells of pigs. The oöcytes had negligibly methylation, and the sperms and somatic cells (fibroblasts) had hypermethylation. In SCNT cloned embryos, the hypermethylation status was maintained until 4-8 cell stages. In subsequent stages, demethylation started in the cloned embryos as in embryos with *in vitro* and *in vivo* fertilization and methylation sites were reduced obviously in the blastocysts (Ref. 150). Considering these results, in SCNT cloned embryos of pigs, demethylation would occur as in normal fertilized embryos.

(Placentas and fetuses)
The hypertrophic placenta in SCNT cloned cattle and mice are considered to result from differences in reprogramming in trophoblasts.

A study showed that in SCNT cloned mice, hypertrophic placenta and reduced body weights of fetuses were found and the placental overgrowth mainly resulted from the expansion of the spongiotrophoblast layer (Ref. 151).
The methylation status of CpG islands in a genome was investigated using cells from the placenta of SCNT cloned mice and the skin of cloned fetuses. The methylation patterns in most of the regions in the clones (99.5% in the placenta, 99.8% in the skin) were the same as in controls with the sexual reproduction, but different methylation patterns were also found. The regions with different methylation patterns corresponded to those for the tissue-specific gene expression (Ref. 152).
In addition, the relationship between the placenta overgrowth in SCNT cloned mice and DNA methylation/gene expression of a specific gene was investigated, and the *Sall3* locus was identified as a hypermethylated region in the placental genome of the cloned mice. Considering that the methylation level was correlated with the placenta hypertrophy level, it was concluded that the *Sall3* locus is the area with frequent epigenetic modification due to nuclear transfer (Ref. 153).

There were also differences in the methylation status between fetuses of SCNT cloned cattle and those produced from fertilization, suggesting a potential relationship between the methylation status and abnormal development.

The 5-methylcytosine content of DNA was measured in fetuses of SCNT cloned cattle, spontaneously aborted cloned fetuses and adult clones. The results showed that the cloned fetuses had low methylation and the spontaneously aborted cloned fetuses had remarkably low methylation. However, the adult clones had methylation levels similar to those in control cattle. On the basis of these results, it was concluded that the global DNA methylation losses may contribute to the developmental failure of the cloned fetuses (Ref. 154).

In the placentas of SCNT cloned fetuses Day 40 after implantation, there were no developments in the placental cotyledon, and abnormal imprinting of *Xist* gene locus and hypermethylation of satellite DNA sequences and epidermal cytokeratin promoter region in the chorion were found. These results indicate that differences in reprogramming affect gene expression patterns in the trophectoderm-derived tissues (Ref. 155).

The methylation status of a repeated sequence (satellite I) and the promoter regions of IL-3 and cytokeratin genes was analyzed in aborted fetuses of SCNT cloned cattle and derived from AI (Days 60 to 90 of gestation) as well as cloned adults and AI adults (18 to 24 months after birth). No difference in methylation level was observed in any loci between the cloned adults and AI adults. However, in aborted cloned fetuses, some aborted cloned fetuses showed very low methylation levels, and some had similar methylation patterns to those of aborted AI fetuses. These results suggested that appropriate methylation in some regions may be correlated with the normal development of the cloned fetuses (Ref. 156).

The intercotyledonal fetal membranes (ICFM) and the brain were compared in fetuses of SCNT cloned cattle and fetuses derived from AI Days 48 or 59 after implantation, and differences in DNA methylation were detected in specific gene regions (Ref. 157).

The analysis of the methylation status of *Igf-2r* imprinted gene in the liver, brain, heart and lung tissues of fetuses of SCNT cloned cattle also showed differences in the methylation status between the cloned fetuses and normal fetuses, indicating a potential relationship between methylation status and abnormal tissues in the cloned fetuses (Ref. 158).

(After birth)

A study investigating the methylation status of *Xist* gene in tissues (heart, liver and spleen) of SCNT cloned cattle reported that low methylation was found in some cloned cattle that died shortly after birth (Ref. 9).

The methylation status of genome was examined at 2,000 points in newborn, adult (8 to 11 months old) and aged (23 to 27 months old) SCNT cloned mice using kidney cells.

Differences in methylation patterns were detected at only 3 of 2,000 points between SCNT cloned mice and mice derived from IVF. These results indicated that the methylation status would be almost similar between SCNT cloned mice and mice derived from IVF. Difference in the methylation status detected between SCNT cloned mice and mice produced by sexual reproduction were observed at 1 point 11 months after birth and disappeared by 23 to 27 months after birth. These results indicated that errors in DNA methylation could disappear with advancement of the animals' age (Ref. 159).

A study indicated that healthy SCNT cloned pigs (15 or 27 weeks age) did not significantly differ in the body weight and any hematological parameters from control pigs. However, some hematological parameters largely varied and regional differences in the methylation status of nontranscribed regions of dermal DNA (PRE-1 SINE and centromeric satellite sequences) were observed (Ref. 107).

(2) Gene expression analysis

(Until preimplantation/blastocyst stage)

A study reported that the expression levels of the X-linked transcript genes (*G6PD, Xist*) in morulae and blastocysts of SCNT cloned cattle differed from embryos with IVF (Ref. 160).

The comprehensive gene expression analysis with the microarray technology indicated that the gene expression profiles of blastocysts of SCNT cloned cattle were drastically different from those of their donor cells and closely resembled those of the fertilized embryos. These results indicated that reprogramming was conducted to some degree. Thus, it is expected that there may be reprogramming errors that prevent differentiation in embryos after the blastocyst stage (Ref. 161).

A study where mRNA expression levels of IGF-1 receptors and IGF binding proteins were determined in blastocysts from SCNT cloned cattle, cattle produced by fertilized egg clones, cattle with IVF and cattle with ET, showed decreased mRNA expression levels of IGF-2 receptor and IGFBP-3 in embryos from SCNT cloned cattle (Ref. 162). Another study reported that embryos of SCNT cloned cattle showed no significant difference in the expression level of IGF-2 receptor gene (Ref. 163).

The gene expression analysis was carried out for 11 genes (including *Oct-4* gene) in embryos from SCNT cloned cattle at the 8-cell stage. The results indicated that no large differences in the expression patterns of 11 genes between embryos with IVF and those from SCNT cloned cattle. The expression levels of all the genes other than lactate dehydrogenase gene were higher in embryos with IVF and those from SCNT cloned cattle as compared with ET (Ref. 164).

Embryos of SCNT cloned cattle had changes in the expression levels of histone deacetylase, DNA methyltransferase (DNMT3) and transcription factor (Oct-4) depending on the types of donor cells and the cell stages (e.g., morula and blastocyst)

(Ref. 165).

A study indicated that in embryos of SCNT cloned female mice, 2 X chromosomes were active, as in normal embryos, during cleavage stages (by 8-cell stage) and random X inactivation occurred at the blastocyst stage or later. However, in the trophectoderm (TE), allele-specific inactivation of donor cells was kept (Ref. 166).
The expression level of *Oct-4* was determined using expression of mRNA and Oct-4-GFP in blastocysts of SCNT cloned mice. Abnormal *Oct-4* expression was detected in the blastocysts of clones as compared with IVF blastocysts, suggesting that abnormal *Oct-4* expression may cause failures in somatic cell cloning (Ref. 167).
The 10 candidate genes having a similar expression pattern to that of *Oct-4* of mice were identified, and their expressions were analyzed in SCNT cloned preimplantation embryos. All embryos of somatic cell clones derived from ES cells expressed all 10 candidate genes and *Oct-4* gene, but only 62% of SCNT cloned embryos derived from cumulus cells expressed all tested genes (Ref. 168).
In mouse embryos created with SCNT and IVF, the transcription of *Hprt*, *Tsx*, *Bex1*, *Bax*, *Cpt2* and *Oct-4* genes started at almost the same time irrespective of the techniques, but their expression levels differed as the cleavage stages progressed; particularly in the blastocyst stage, there was a variation in the ratio of gene expression level among the cloned embryos. These results suggested that reprogramming is incomplete at the preimplantation development stages (Ref. 169).

In embryos of SCNT cloned pigs and embryos produced by intracytoplasmic sperm injection (ICSI) at 2-4 cell stages and blastocyst stage, the expression profiles of *FGFr*2III*c*, *FGFr*72III*b*, *Xist*, *IL*6, *IL6r* and c-kit ligand genes were compared. The donor cells of SCNT cloned embryos were derived from 2 cell lines and the 2 activation protocols were used. The gene expression levels were similar between SCNT cloned embryos and ICSI embryos, but a reduced level of *FGFr*72III*b* expression and an increased level of *IL6r* were found in SCNT cloned blastocysts. The *FGFr*72III*b* and *Xist* genes showed different expression depending on the activation method at 2-4 cell stages. There was no significant difference in the expression profiles at 2-4 cell stages between 2 donor cells of somatic cell clones (Ref. 170).

(Placentas and fetuses)
In the placentas of SCNT cloned mice derived from mouse ES cell donors, the placental weight significantly increased, and there were very large variations in the expression levels of imprinted genes (*H19*, *Igf2*, *Peg1*/*Mest and Meg1*/*Grb10*) among cloned mice (Ref. 171).
The histological change and the expression of placenta-specific genes and imprinted genes were examined in the placentas of SCNT cloned mice in late gestation. The

placentas of the clones were larger than those of controls. There were histological changes in all layers of the placenta (trophoblast giant cell, spongiotrophoblast and labyrinth layer) particularly in the spongiotrophoblast layer, prominent expansion was seen. No significant change was found in the gene expression in the placenta. Although SCNT cloned fetuses tended to be larger in cattle and sheep, the average weight of mouse SCNT cloned fetuses were lower than that of controls, suggesting that morphological abnormality in the placentas due to SCNT may negatively affect the growth of cloned fetuses (Ref. 151).

The microarray analysis was used to assess the expression of more than 10,000 genes in the placentas of SCNT cloned mice (derived from ES cells or cumulus cells). The expression levels in about 4% of genes expressing in the placentas of the clones were different from those of the controls. The gene expression amounts of 286 genes varied similarly both in the cloned from cumulus cells and those from ES cells. Therefore, abnormalities in gene expression in the placentas of the cloned mice may commonly develop in the placentas of normal SCNT cloned mice. The placental size was not related to the abnormalities in gene expression (Ref. 172).

The placentas of SCNT cloned mice in late pregnancy were 2 to 3 times larger than those of control mice, irrespective of types of donor cells. There were abnormal decreases in the expression levels of many genes, including imprinted genes. Therefore, it was concluded that the gene expression of placenta cells were regulated independently of the mechanism of genomic imprinting (Ref. 173).

The expression patterns analysis with complementary microarray indicated that differences in the expression patterns in the liver between fetuses of SCNT cloned cattle and normal fetuses were detected in about 5% of the target genes, but the gene expression patterns largely varied among individuals (Ref. 174).

(After birth)

The expression of 10 genes on the X chromosome was examined in SCNT cloned cattle, and the aberrant expression patterns in the 9 genes were found in some organs of the clones that died shortly after birth (Ref. 9).

The gene expression levels of 3 genes (*Igf2*, *Igf2r* and *H19*) were examined in bovine SCNT cloned neonates that died shortly after birth and healthy adult cloned cattle, which were produced from the same donor cell. The results indicated that deceased cloned neonates exhibited aberrant expression of all genes and large variations in the expression levels of the genes among these clones (Ref. 175).

The expression levels of 8 developmentally important genes (*PCAF*, *Xist*, *FGFR2*, *PDGFRa*, *FGF10*, *BMP4*, *Hsp70.1* and *VEGF*) were compared in 6 organs (heart, liver, kidney, spleen, lung and brain) between SCNT cloned calves dead shortly after birth and control calves. Aberrant tissue-specific expression was found in the deceased clones depending on the genes. Such aberrant expression was most frequently detected in the

heart (5 of 8 genes) and was least frequently found in the kidney (2 of 8) (Ref. 176).

A study indicated that X chromosome inactivation was skewed in SCNT cloned female mice (Ref. 177).

The microarray analysis of genome-wide gene expression profiles in SCNT cloned mice showed that the brain, liver and kidney in the neonatal cloned mice with normal appearance had remarkable variations in the gene expression, which were not found in offspring produced via fertilization, and there are the chromosome regions in which expression of several genes was aberrantly controlled in SCNT clones (Ref. 178).

(3) Factors involved in epigenetic modification

The epigenetic situation is reported to be affected by differences in culture conditions or types of donor cells. Many studies have been performed to increase the successes rate of producing cloned animals with SCNT (see IV. (1). 1)). The important cloning points include types and traits (Refs. 165, 179); cell cycle (Refs. 163); culture conditions (Refs. 163, 180); chromosomal stability (Refs. 181, 182) of donor cells, age in month (Ref. 183), removed timing (Ref. 184); cell cycle (Ref. 185); timing of activation at cell fusion (Ref. 163) of recipient oocyte, and culture conditions of cloned embryos (Ref. 163).

(4) Progeny

It has been reported that some epigenetic modification in mammalian fetuses may be transmitted to progeny (Refs. 186, 187). However, abnormal phenotypes found in SCNT cloned mice (e.g., placenta overgrowth, fetal overweight, open eyelids, age-related obesity) were not transmitted to their offspring (F1) (Refs. 75, 188, 189). In addition, in offspring (11 females and 19 males) produced using the sperm of the same SCNT cloned bull, any abnormalities found in the father bull at birth and after birth were not seen in any of their offspring (Ref. 114). These findings suggested that abnormalities found in SCNT clones may be removed by gametogenesis reprogramming in the following generations (Refs. 189, 190). Therefore, the progeny of SCNT cloned animals would have health profiles similar to that of animals produced with the conventional ARTs (Ref. 191).

4. Others

(1) DNA mutation and chromosomal abnormalities

In SCNT, a somatic cell or nucleus of somatic cell is transferred into an enucleated mature egg and fused with electrical stimulation, but genes are not modified.

Spontaneous DNA mutations may occur in SCNT cloned animals after nuclear transfer, but can occur even in animals produced with the conventional ARTs.

The genotype assay using polymorphic microsatellite markers for genes of donor cattle,

donor cultured cells, SCNT cloned cattle revealed that the identical genotype were found in all groups (Refs. 45, 84, 192, 193, 194, 195) and recloned calves produced from SCNT cloned cattle also had identical genotypes (Ref. 192).
In preimplantation blastocysts of SCNT cloned cattle, karyotypic abnormalities (except for diplont) were frequently found (Ref. 196). The cytogenetic analysis using peripheral lymphocytes from SCNT cloned cattle indicated that about 20% of cells had numerical chromosome aberrations (except for diplont) in 2 of 20 cloned cattle (around 5% in controls) (Ref. 197).
The nuclear distribution patterns were examined using peripheral lymphocytes from 30 offspring produced from the same SCNT cloned cattle, and there was no difference in the nuclear distribution patterns between the offspring and control cattle (Ref. 114).

It has been reported that 90% or more of embryos of SCNT cloned mice (derived from cumulus cells) showed normal chromosomal constitution (Ref. 198).
In embryos of SCNT cloned mice and mouse embryos produced with IVF (ICSI), chromosomal segregation was examined until the 8-cell stage. Abnormalities were found in some cloned embryos, but in the blastocyst stage, fewer numerical chromosome aberrations were seen in the cloned embryos than in embryos with IVF. Considering these findings, defects in SCNT cloned embryos were expected to be caused mainly by epigenetic errors. However, the chromosomal abnormality may be rarely transmitted in SCNT cloned mice (Ref. 199).

(2) Mitochondrial DNA

The causes of low success rate of producing SCNT cloned animals include incomplete or improper reprogramming as well as changes in the transmission pattern of somatic-cell cloned mitochondrial DNA (mtDNA) after transfer. The sperm does not bring few mitochondria. The general fertilized egg has a single identical population of mtDNA derived from an egg (homoplasmy), and the SCNT cloned embryo had mixed populations of mtDNA genomes derived from a donor cell and a recipient egg (heteroplasmy). Some abnormalities observed in SCNT embryos, fetuses and offspring may be caused by heteroplasmic mtDNA populations (Refs. 200, 201, 202).
Although factors involved in the nuclear transfer (e.g., culture conditions of embryos, types of donor cells, quality of recipient oocyte) may affect the heteroplasmy level, most SCNT cloned animals (cattle, pigs and sheep) have homoplastic mtDNA (elimination of mtDNA derived from a donor cell) or heteroplastic mtDNA at a mild level (mix of mtDNA derived from a donor cell). The heteroplasmy level depends on tissues (Ref. 203).
It is difficult to determine the effects of heteroplasmy on health in SCNT cloned animals or consumption risks, even if heteroplasmic mtDNA is found in SCNT cloned animals. The phenotype could be normal irrespective of the heteroplasmy levels in SCNT cloned

animals. Currently, there is no obvious evidence suggesting that heteroplasmy due to nuclear transfer is harmful in individual development (Ref. 204).

(3) Telomere length

A telomere is DNA (5-20 kb) containing guanine-rich repetitive short sequences at the end of a chromosome and contributes to the chromosomal stability. As the telomere contains a single strand DNA at the end, it shortens by about 50-200 bases per cell division at DNA replication. The telomere, therefore, is also called "mitotic clock". In addition, the telomere is considered to be involved in DNA replication and chromosomal segregation, etc.

In a SCNT cloned sheep (Dolly), the telomere length was significantly shorter than in sheep at the same age. It was reported that the telomere length of the cloned sheep (Dolly) was consistent with the length at the age of the animal providing a donor cell and telomere erosion during cell culture before nuclear transfer (Ref. 205). Another study reported that the telomere length of SCNT cloned sheep was shorter than that of sheep at the same age (Ref. 206).

However, some studies reported that the telomere lengths of SCNT cloned cattle, pigs and goats were equal or longer as compared with age-matched control animals, even when aged cells were used as donor cells (Refs. 206, 207, 208, 209, 210, 211, 212).

The telomere length of 30 offspring born from the same SCNT cloned cow (a donor cell is a fibroblast) was the same in length as age-matched cattle (Ref. 114).

There are large differences in the telomere length of SCNT cloned animals depending on the animal species, types of somatic cells used and culture conditions, but the relationship between the telomere length and the life span of SCNT cloned animals has not been demonstrated (Refs. 205, 210, 213, 214).

Telomerase exists in cells, including germ cells, embryonic cells and immortalized cells and has the ability of elongating a telomere and maintaining the telomere length after several cell divisions. Telomerase is an enzyme that reconstructs and elongates a telomere and is activated during the embryonic stage. The telomerase activity is suppressed in most somatic cells after birth but is maintained in germ cells, tumor cells and stem cells (Refs. 212, 215, 216).

The telomerase activity was much higher in bovine fetal fibroblasts than in adult fibroblasts, but was significantly lower as compared with ES cell-like cells. The telomerase activity in bovine fetal fibroblasts was diminished by cell passaging and culture periods under serum starvation conditions. In embryos of SCNT cloned cows, the telomerase activity increased as in control embryos. The telomere length in SCNT cloned cattle obtained was longer than or almost similar to that of age-matched control cattle (Ref. 206).

In cattle and mice, a telomerase-dependent telomere elongation was found at the transition from morula to blastocyst (Ref. 217).

Cultured primary fibroblasts from SCNT cloned fetal sheep exhibited the same proliferative capacity and rate of telomere shortening as cultured primary fetal donor fibroblasts (Ref. 218). This suggests that aging of the cell division capacity is genetically determined and is not dependent on the telomere length (Ref. 124).

5. Summary of epigenetics and other properties for SCNT cloned animals

Genetically identical cells can adequately regulate the expression of essential genes and non-essential genes on the basis of epigenetic modification to differentiate somatic cells with various functions and traits.

As stated in 1 to 4, epigenetic studies on various processes in the production of SCNT cloned animals have reported that there are differences in epigenetic modification and gene expression profiles between SCNT cloned animals and animals produced via general fertilization.

The cells passing some differentiation stages are generally regulated not to differentiate into cells other than designated ones. In the production of SCNT cloned animals using somatic cells, the normal reprogramming in reconstructed embryos would be important for the subsequent development of SCNT cloned embryos and normal growth of cloned fetuses. However, in many cases of SCNT cloned animals, the epigenetic regulation is not completely performed unlike normal fertilized eggs (i.e., normal epigenetic regulation in somatic cells is carried over) and the cloned embryos are not well developed to fail to normally produce a cloned animal. One cause of the low success rate of producing SCNT cloned animals is that the reprogramming of nucleus of a donor cell is not well performed unlike the reprogramming of the nucleus from a sperm in a fertilized egg.

However, there are few differences in the epigenetic modification between SCNT cloned animals that showed healthy growth and normal animals. Although some different epigenetic modification remains in such cloned animals, these changes differed among individuals and most phenotypes are normal. In this case, even if different epigenetic modification remains, such changes could occur in the genome region, which has no effect on gene expression or which includes genes that do not affect survivals or phenotype.

Thus, inadequate development and differentiation in SCNT cloned animals would be mainly caused by improper regulation of epigenetic modification.

Abnormal regulation of epigenetic modification is not limited to SCNT cloned animals, but is found in all reproductive processes, including natural mating and AI. In particular, poor regulation of epigenetic modification frequently occurs in the ARTs using the assisted reproduction technology with many *in vitro* procedures.

SCNT cloned animals have the nuclear DNA sequences identical to that of donor animals. Although DNA mutations may occur even in SCNT cloned animals, spontaneous DNA mutations would occur as seen in animals produced by the conventional ARTs because the recombinant DNA technique is not used in the processes of producing SCNT cloned animals. Some reports said that DNA mutation and chromosomal abnormalities in SCNT cloned animals were not detected or were not different from those in conventionally bred animals via fertilization.

SCNT cloned animals theoretically have mixed populations of mtDNA genomes derived from a nuclear donor cell and ooplasm (heteroplasmy). Currently, there is no obvious evidence suggesting that heteroplasmy due to the nuclear transfer is harmful in individual development.

Many studies indicated that the telomere length varies among individuals and recovers in some cells. Thus, although there was a concern that "telomeres are very short in SCNT cloned animals" at the early stage of developing SCNT techniques, this phenomenon is not considered to occur in the cloned animals.

In progeny of SCNT cloned animals, the nuclei having the potential of different epigenetic modification, which may remain in parents (cloned animals), would be reprogrammed via germ cells, as in animals conventionally produced via fertilization. Therefore, the epigenetic regulation in progeny of SCNT cloned animals would be similar to that in offspring produced with the conventional ARTs via fertilization.

VI. Safety of foods derived from cattle and pigs produced by SCNT and their offspring

Generally, some proteins contained in foods derived from mammalian livestock may cause allergy in humans, but components of the foods are not known to be toxic or pathogenic to humans when the foods are taken.

Since genes are not modified in SCNT cloned cattle and pigs, new biomolecules are not produced in the clones as compared with cattle and pigs produced by the conventional ARTs.

The nuclear DNA sequences of animals produced with SCNT are theoretically identical to those of donor animals, and therefore proteins produced using SCNT cloned animals that grew healthily would be identical to those produced using donor animals.

Therefore, there would be no differences in safety between foods from healthy SCNT cloned cattle and pigs and their offspring, and those from animals conventionally bred.

On the basis of current data, we compared meat and milk from SCNT cloned cattle and pigs and their offspring with those from cattle and pigs produced by the conventional ARTs and evaluated potential differences in nutrient components, the results of studies (including micronucleus test), subchronic/chronic toxicity study in rats and mice, and allergenicity test.

1. Comparison of meat and milk components

It is said that components of milk and meat from cattle are affected by feed or environments to vary food composition (Refs. 108, 219).

(1) Beef

The general components (water, protein, fat, carbohydrate, ash, calcium and cholesterol), 18 amino acids, and 17 fatty acids were analyzed in meat samples (9 parts: shoulder, chuck eye roll, rib roast, sirloin, rib, round, bottom round, rump and tenderloin) from 1 Japanese Black bull (28 months of age). There were no substantial differences in them between samples of the clone and those of bulls produced by the conventional ARTs (Ref. 220).

In 2 SCNT cloned Japanese Black cattle (26 months of age), water, protein, fat, 18 amino acids in muscle samples (infraspinatus muscle, longissimus thoracis muscle, latissimus dorsi muscle, adductor muscle, biceps femoris muscle and semitendinosus muscle), 8 fatty acids of subcutaneous, intermuscular, coelomic and renal adipose tissues were analyzed, and the organs (e.g., liver, kidney and spleen) were pathologically examined. There were significant differences in water, proteins and fatty acids (olein acid, palmitic acid, palmitoleic acid, linoleic acid and linolenic acid) of the semitendinosus muscle, and linoleic acid of the longissimus thoracis muscle and renal adipose tissue, between the cloned cattle and cattle produced with the conventional ARTs. The differences in fatty acids would be derived from donor cattle with high marbling score and other parameter values were within the ranges of general values. There were no abnormalities in the pathological examination (Ref. 221).

Meat samples of 11 SCNT cloned cattle aged 12 to 43 months (6 females and 5 males; Angus, Brangus, Holstein and cross breeds) were analyzed for water, fat, proteins, carbohydrate, cholesterol, 19 amino acids, 7 fatty acids, 6 vitamins and 4 minerals. There were no significant differences in them between samples of clones and those of cattle produced by the conventional ARTs (Ref. 222).

A study has reported the semitendinosus muscle of 9 SCNT cloned cattle aged 8, 12, 18, and 24 months. This study did not represent actual data, but indicated that the percentages of myosin heavy chains (MyHC) I and IIa increased and the percentage of MyHC IIb decreased in the cloned cattle aged 12 months as compared with non-cloned cattle. In the clones aged 8 months, the percentage of polyunsaturated fatty acid increased and the percentage of monounsaturated fatty acid decreased. The percentages of trans-vaccenic acid (C18:1-*t*11) and cis-vaccenic acid (C18:1-*c*11) were lower in the clones aged 24 months and the delta-9 desaturase activity against C16 fatty acid was higher in the clones aged 18 and 24 months (Ref. 64).

The general components (water, protein, fat, carbohydrate, ash, and cholesterol), 18 amino acids and 17 fatty acids were analyzed in meat samples (3 parts: shoulder, sirloin and round) from 3 offspring aged 28 months of Japanese Black SCNT cloned cattle. There were no significant differences in them between samples of the offspring and those of cattle with conventional ARTs (Ref. 223).

(2) Pork

Meat samples of 4 Hamline SCNT cloned pigs aged 6 to 8 months showed similar qualitative traits, including carcass grading, color, hardness, and marbling score, to those of pigs produced by the conventional ARTs. Cholesterol, 17 amino acids, 32 fatty acids, 3 vitamins and 4 minerals were analyzed in meat samples of 5 SCNT cloned pigs aged 6 to 8 months (4 Hamline and 1 Duroc pigs). Meat of the clones showed very small differences in these parameters from meat of pigs conventionally bred and had similar values to the USDA's standard values (Refs. 47, 111).

Loin samples from 242 offspring of SCNT cloned pigs were analyzed for 58 different parameters: cholesterol, 17 amino acids, 33 fatty acids, 3 vitamins and 4 minerals. A difference was found only in eicosadienoic acid (C20:2) (0.04% in the clones vs. 0.01 to 0.03% in control pigs), but all the other parameter values in the samples of the offspring of clones were similar to the USDA's standard values (Ref. 117).

Meat samples from 44 offspring of SCNT cloned Jin Hua pigs (23 males and 21 females) were analyzed for parameters, including pH, water and fat, and showed similar values to those from pigs produced by the conventional ARTs (Ref. 116).

In 11 offspring of SCNT cloned Jin Hua pigs (5 males and 6 females), longissimus thoracis muscle, biceps femoris muscle, liver and heart samples were analyzed for water,

protein, fat and ash; and longissimus thoracis muscle and biceps femoris muscle samples were analyzed for 18 amino acids, 6 nucleic acids and 6 fatty acids of adipose tissue. Mean values for some parameters in samples of the offspring from the clones differed from those from control pigs produced by the conventional ARTs, but were within the variation ranges in control pigs (Ref. 118).

(3) Milk

Milk samples from 15 SCNT cloned cows (Holstein and cross breeds) were analyzed for fat, protein, lactose, pH, nitrogen, solid content, somatic cell count, acid level, 14 fatty acids, 8 minerals and 6 protein compositions. Small differences were found in 2 fatty acids (palmitic acid (C16:0) and linolenic acid (C18:3), potassium, zinc, strontium and phosphorus between milk samples from the clones and those from cows produced by the conventional ARTs. However, the differences were considered to result from different feed ingredients. There were no significant differences in other parameters (Ref. 224).

Milk samples from 6 SCNT cloned Holstein cows were compared for fat, protein, lactose, solid content, 14 fatty acids, 4 minerals and 7 protein compositions with those from donor cows. The values in serum albumin and 2 fatty acids (linoleic acid (C18:2) and linolenic acid (C18:3)) differed between the clones and donors, but were within ranges of values generally found in Holstein cows (Ref. 60).

Milk samples from 9 SCNT cloned cows were also compared for 18 amino acids, 3 vitamins and 8 minerals with those from cows produced by the conventional ARTs and no difference was found in any parameters (Ref. 77).

Fat, protein, lactose, solid content, BUN, somatic cell count and 7 protein compositions were analyzed in milk samples from 10 SCNT cloned Holstein cows, and there were no differences in any parameters between milk samples from the clones and those from cows produced by the conventional ARTs (Ref. 221).

Fat, protein and milk solid nonfat content were analyzed using milk samples from SCNT cloned cows (6 Holstein and 4 Jersey cows). There were differences due to the breeding environment and feed in the parameters between the clones and donors, which were within the range found in cows produced by the conventional ARTs (Ref. 68)

Milk samples from 5 SCNT cloned Holstein cows were analyzed for fat and 6 fatty acids. As compared with milk samples from control cows produced by the conventional ARTs, the values of stearic acid (C18:0), trans-vaccenic acid (C18:1-*t*11) and linolenic acid (C18:3) were lower in those from the clones (clones: 7.3% vs. controls: 10.5%, 0.94% vs. 1.30% and 0.21% vs. 0.34%, respectively) (Ref. 64).

Milk samples from SCNT cloned cows (6 Holstein and 3 Jersey cows) were analyzed for fat, protein, lactose, casein, pH, 18 amino acids, 23 fatty acids, 3 vitamins and 7 minerals; there were no differences in any parameters between milk samples from the clones and those from cows produced by the conventional ARTs (Ref. 65).

The general components (water, protein, fat, carbohydrate, ash, calcium and cholesterol),

18 amino acids and 21 fatty acids were analyzed in milk samples from 3 SCNT cloned Holstein cows, and there were no substantial differences in any parameters between milk samples from the clones and those from cows produced by the conventional ARTs (Ref. 220).

Fat, proteins, lactose, casein, pH, fatty acid, mineral and 8 protein compositions were analyzed in milk samples from SCNT cloned cows, and these values were within the range of the values in milk from cows produced by the conventional ARTs (Refs. 225, 226, 227).

The general components (water, protein, fat, carbohydrate, ash, calcium and cholesterol), 18 amino acids and 21 fatty acids were analyzed in milk samples from 3 offspring of SCNT cloned Holstein cows, and there were no differences in any parameters between milk samples from the offspring and those from cows produced by the conventional ARTs (Ref. 223).

2. Micronucleus test

In a micronucleus test in ICR mice aged 8 weeks fed diets containing freeze-dried meat powder (0, 1, 2.5 or 5%) or milk powder (0, 2.5, 5 or 10%) derived from SCNT cloned cattle aged 28 months, micronucleus was not observed in mouse bone marrow cells (Ref. 220).

In a micronucleus test in ICR mice aged 8 weeks fed diets containing freeze-dried meat powder (0, 1 or 5%) or milk powder (0, 2 or 10%) derived from progeny aged 28 months of SCNT cloned cattle, micronucleus was not observed in mouse bone marrow cells (Ref. 223).

A micronucleus test was carried out in ddY mice aged 8 weeks receiving freeze-dried meat powder derived from progeny of SCNT cloned Jin Hua pigs at an oral gavage dose of 0, 250, 1,000 or 2,000 mg/kg bw, and micronucleus was not induced in mouse bone marrow cells (Ref. 118).

3. Subchronic/chronic toxicity studies in rats and mice

(1) Beef and pork

A repeated dose study was conducted in SD rats aged 5 weeks (10 males and 10 females in a group) receiving diets containing freeze-dried meat powder derived from SCNT cloned cattle aged 28 month at a dose of 0, 1, 2.5 or 5% for 14 weeks. No abnormal findings due to administration of the meat powder from the clones were seen in the systemic condition, body weight, food consumption, functional observational battery, sensory response function, landing foot splay, grip strength, estrus cycle, urinalysis, blood test, organ weights and pathological examination (Refs. 66, 220).

A repeated dose study was carried out in Wistar rats (8 animals in a group, gender unknown) receiving meat-based diets prepared from SCNT cloned cattle for 21 days. No abnormal findings due to administration of the meat from the clones were observed in food consumption, body weight gain, organ weights, fasting blood sugar levels and levels of IgG, IgA, IgM and IgE (Ref. 228).

A 12-month repeated dose/reproduction test was conducted in SD rats aged 5 weeks (12 males and 12 females in a group) receiving freeze-dried meat powder derived from progeny aged 28 months of SCNT cloned cattle at a dose of 1 or 5%. The repeated dose study had no abnormal findings due to administration of the meat powder from the progeny of clones in the systemic condition, body weight, food consumption, functional observational battery, sensory response function, landing foot splay, grip strength, ophthalmologic examination, urinalysis, blood test, organ weights and pathological examination. The reproduction test showed that administration of the meat powder had no abnormal effect on the fecundity of parental animals or on the development of offspring (Refs. 223, 229).

A repeated dose study was carried out in ddY mice aged 5 weeks (6 males and 6 females in a group) receiving diets containing freeze-dried meat powder derived from SCNT cloned Jin Hua pigs at a dose of 0, 1, 2.5 or 5% for 28 days. No abnormal findings due to administration of the meat powder from the clones were observed in the performance status, body weight, organ weights and pathological examination (Ref. 118).

(2) Milk

A repeated dose study was conducted in SD rats aged 5 weeks (10 males and 10 females in a group) receiving diets containing freeze-dried milk powder derived from SCNT cloned cows at a dose of 0, 2.5, 5 or 10% for 14 weeks. No abnormal findings due to feeding with the milk powder from the clones were seen in the systemimc condition, body weight, food consumption, functional observational battery, sensory reflex function, landing foot splay, grip strength, estrus cycle, urinalysis, blood test, organ weights and pathological examination (Refs. 66, 220).

A repeated dose study was carried out in Wistar rats (8 animals in a group, gender unknown) receiving milk-based diets prepared from SCNT cloned cows for 21 days. No abnormal findings due to administration of the milk from clones were observed in food consumption, body weight gain, organ weights, fasting blood sugar level and levels of IgG, IgA, IgM and IgE (Ref. 228).

A 12-month repeated dose/reproduction test was conducted in SD rats aged 5 weeks (12 males and 12 females in a group) receiving freeze-dried milk powder derived from progeny of SCNT cloned cows at a dose of 2 or 10%. The repeated dose study had no

abnormal findings due to feeding with the milk powder from the progeny of clones in the systemic condition, body weight, food consumption, functional observational battery, sensory response function, landing foot splay, grip strength, ophthalmologic examination, urinalysis, blood test, organ weights and pathological examination. The reproductive and developmental test showed that the administration of the milk powder had no abnormal effect on the fecundity of parental animals or development of offspring (Refs. 223, 229).

4. Allergenicity tests

(1) 21-day multiple dose study

The repeated dose study was carried out in Wistar rats (8 animals in a group, gender unknown) receiving meat-based diets prepared from SCNT cloned cattle for 21 days. The blood levels of IgG, IgA, IgM and IgE as well as food consumption, body weight gain and organ weights were examined, and there were no abnormal increases in any antibody levels due to the administration of meat from the clones (Ref. 228).

The repeated dose study was carried out in Wistar rats (8 animals in a group, gender unknown) receiving milk-based diets prepared from SCNT cloned cows for 21 days. The blood levels of IgG, IgA, IgM and IgE as well as food consumption, body weight gain and organ weights were examined, and there were no abnormal increases in any antibody levels due to the administration of milk from the clones (Ref. 228).

(2) Intraperitoneal administration studies in mice

1) Beef and pork

ddY male mice aged 5 weeks were sensitized to a saline extract of freeze-dried meat powder derived from SCNT cloned cattle aged 28 months by intraperitoneal injection. They received a challenge dose of meat extract to the abdominal wall after intravenous injection of Evans blue solution Day 14 after sensitization. The diameter of the dye leakage spot at the injection site was measured, and there were no significant differences in the diameter between meat from the clones and that from cattle produced by the conventional ARTs (Ref. 220).

ddY male mice aged 5 weeks were sensitized to a saline extract of freeze-dried meat powder derived from progeny aged 28 months of SCNT cloned cattle by intraperitoneal injection. They received a challenge dose of meat extract to the abdominal wall after intravenous injection of Evans blue solution Day 14 after sensitization. The diameter of dye leakage spot at the injection site was measured, and there were no significant differences in the diameter between meat from the progeny of clones and that obtained from cattle produced by the conventional ARTs (Ref. 223).

ddY male mice aged 5 weeks were sensitized to a saline extract of freeze-dried meat powder derived from SCNT cloned Jin Hua pigs by intraperitoneal injection. They received a challenge dose of meat extract to the abdominal wall after intravenous injection of Evans blue solution Day 14 after sensitization. The diameter of the dye leakage spot at the injection site was measured, and there were no significant differences in the diameter between meat from the clones and that obtained from Jin Hua pigs produced by the conventional ARTs (Ref. 118).

2) Milk

ddY male mice aged 5 weeks were sensitized to saline extract of freeze-dried milk powder derived from 3 SCNT cloned cows by intraperitoneal injection. They received a challenge dose of milk extract to the abdominal wall after intravenous injection of Evans blue solution Day 14 after sensitization. The diameter of dye leakage spot at the injection site was measured, and there were no significant differences in the diameter between milk from the clones and that from cows produced by the conventional ARTs (Ref. 220).

ddY male mice aged 5 weeks were sensitized to saline extract of freeze-dried milk powder derived from 3 offspring of SCNT cloned cows by intraperitoneal injection. They received a challenge dose of milk extract to the abdominal wall after intravenous injection of Evans blue solution Day 14 after sensitization. The diameter of the dye leakage spot at the injection site was measured, and no significant differences were found in the diameter between milk from the offspring of clones and that from cows produced by the conventional ARTs (Ref. 223).

The method of Kataoka et al. used above (Ref. 230) does not assess IgE-mediated allergenicity by oral sensitization and evaluates the antigenic level of foreign protein.

Since the nuclear DNA sequences of SCNT cloned cattle and pigs are theoretically identical to those of donor animals, new proteins becoming allergens could not be produced. There have been no reports indicating significant differences in allergenicity between foods from SCNT cloned cattle and pigs and those from cattle and pigs produced by the conventional ARTs.

5. Protein digestability tests

(1) Beef

An *in vitro* digestion test was carried out in simulated gastric juice for 0, 0.75, 1.5, 6 and 12 h and in simulated intestinal juice for 0, 1.5, 3 and 6 h using meat from SCNT cloned calves (4 days after birth). As compared with meat from calves produced by the

conventional ARTs, the digestibility of meat from the clones was slightly lower in the simulated gastric juice for 0.75 h and slightly higher in the simulated intestinal juice for 1.5 h. There were no differences in the digestibility for other incubation times (Ref. 220).

SD male rats aged 26 weeks received diets containing freeze-dried meat powder derived from SCNT cloned cattle aged 28 months (protein content: 13.09%). The total nitrogen level in feces for 3 days was measured to compare the digestibility. There was no significant difference in the digestibility between the meat from the clones and that obtained from cattle produced by the conventional ARTs (Ref. 220).

SD male rats, aged 39 weeks, received diets containing freeze-dried meat powder derived from progeny, aged 28 months, of SCNT cloned cattle (protein content: 13.09%). The total nitrogen level in feces for 3 days was measured to compare the digestibility. There was no significant difference in the digestibility between the meat from the progeny of clones and that obtained from cattle produced by the conventional ARTs (Ref. 223).

(2) Milk

SD male rats, aged 26 weeks, received diets containing freeze-dried milk powder derived from SCNT cloned cows (protein content: 13.09%). The total nitrogen level in feces for 3 days was measured to compare the digestibility. There was no significant difference in the digestibility between the milk from the clones and that from cattle produced by the conventional ARTs (Ref. 220).

SD male rats aged 39 weeks received diets containing freeze-dried milk powder derived from progeny of SCNT cloned cows (protein content: 13.09%). The total nitrogen level in feces for 3 days was measured to compare the digestibility. No significant difference was found in the digestibility between the milk from the progeny of clones and that from cows produced by the conventional ARTs (Ref. 223).

6. Summary of foods derived from cattle and pigs produced with SCNT and their offspring

Generally, some proteins contained in foods derived from mammalian livestock may cause allergy in humans, but components of the foods are not known to be toxic or pathogenic to humans when the foods are ingested.

Since genes are not modified in SCNT cloned cattle and pigs, new biomolecules that do not exist in conventionally bred cattle and pigs are not produced in the clones.

The nuclear DNA sequences of animals produced with SCNT are theoretically identical to those of donor animals, and therefore proteins produced form SCNT cloned animals that grew healthily would be identical to those from donor animals.

On the basis of current data on meat and milk from SCNT cloned cattle and pigs and their

offspring, we compared meat and milk from SCNT cloned cattle and pigs and their offspring with those from cattle and pigs produced by the conventional ARTs, and evaluated potential differences in nutrient components and the results of studies, including micronucleus test, subchronic/chronic toxicity study in rats and mice, and allergenicity test. The obtained results showed no differences causing safety problems.

Although no detailed data on foods other than meat or milk from the clones have been obtained, new biomolecules are not produced in SCNT cloned cattle and pigs as stated above. It is unknown that when foods derived from cloned mammalian livestock are consumed, the components have toxicity and pathogenicity to humans. There were no differences causing safety problems between milk and meat from SCNT cloned cattle and pigs and their offspring and those from conventionally bred cattle and pigs. Considering these findings, foods other than meat and milk derived from healthy SCNT cloned cattle and pigs and their offspring would not differ in safety from those from cattle and pigs produced by the conventional ARTs.

VII. Safety assessment of food

The safety of foods derived from SCNT cloned cattle and pigs and their offspring were evaluated on the basis of data and published scientific papers provided by MHLW.

In the safety assessment of the foods from the clones and their offspring, it was basically evaluated, on the basis of current scientific findings, whether foods derived form SCNT cloned cattle and pigs and their offspring have equivalent safety as those from cattle and pigs produced by the conventional ARTs (e.g., AI).

In SCNT cloned cattle and pigs, the stillbirth rate and the rate of died shortly after birth were higher before and after birth, as compared with those produced by the conventional ARTs. The mortality rate in SCNT cloned cattle tended to be higher during the juvenile period, but the cloned cattle healthily grew up after about 6 months as in cattle produced by the conventional ARTs. These results may be attributed to the iintegrity level of totipotency of reconstructed embryo made using somatic cells, and the causes of death themselves have been found in the conventional ARTs. Although the physiological parameter values after birth and during the juvenile period may be different between the cloned cattle and pigs and pigs and cattle produced by the conventional ARTs, but the values recovered with growth and the clones became healthy.

Progeny of SCNT cloned cattle and pigs are produced via fertilization with the conventional ARTs such as AI. F1, offspring of clones at the first generation, had no abnormalities found in SCNT cloned cattle and pigs during the perinatal and juvenile periods and the health profile of F1 did not differ from that of cattle and pigs produced by the conventional ARTs. Therefore, there would be no differences in health profile between offspring of the clones in the second and later generations produced via fertilization and cattle and pigs produced by the conventional ARTs.

These results showed that the health profile of cattle and pigs produced with SCNT and their offspring, which could be used as food, is not different from that of cattle and pigs produced by the conventional ARTs.

With recent advances in the embryologic studies, including epigenetics, it has been demonstrated that genetically identical cells can differentiate into somatic cells with different roles and traits by normal expression regulation of essential genes.

Several studies on various processes involved in the production of SCNT cloned animals indicated that there were differences in epigenetic modification and gene expression profiles between SCNT cloned animals and animals produced via normal fertilization. Normal epigenetic modification would be a main cause of abnormal development and differentiation in SCNT cloned animals.

In progeny of SCNT cloned animals, the cell nucleus would be reprogrammed via germ cells, as in animals conventionally produced via fertilization. Therefore, the regulation of epigenetic modification in progeny of the clones would be similar to that in offspring produced via fertilization with the conventional ARTs.

Since the nuclear DNA sequences of SCNT cloned cattle and pigs are theoretically identical to those of donor animals, any new biomolecules that do not exist in donor animals and cattle and pigs produced by the conventional ARTs are not produced in the clones. Generally, some proteins contained in foods derived from mammalian livestock may cause allergy in humans, but components of the foods are not known to be toxic or pathogenic to humans when the foods are ingested.
Meat and milk from SCNT cloned cattle and pigs and their offspring were compared with those from conventionally bred cattle and pigs. There were no differences causing safety problems in nutrient components, and the results of studies, including micronucleus test, subchronic/chronic toxicity study in rats and mice, and allergenicity test.

No detailed data on foods other than meat or milk from clones have been obtained. However, any new biomolecules are not produced in SCNT cloned cattle and pigs as stated above, and there were no differences causing safety problems between foods from SCNT cloned cattle and pigs and those from conventionally bred cattle and pigs. Considering these findings, foods derived from SCNT cloned cattle and pigs would not differ in safety from those from cattle and pigs produced by the conventional ARTs.

The risk assessment based on current scientific findings showed that foods derived from SCNT cloned cattle and pigs and their offspring would have equivalent safety as those derived from cattle and pigs produced by the conventional ARTs.
Since SCNT is a new technique, it is necessary to continue to collect data on the safety of foods derived from SCNT cloned cattle and pigs in the risk management institution.

<References>

1 Newsletter, Livestock Improvement Association of Japan, 2008; 32

2 Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, Yasue H, Tsunoda Y. Eight calves cloned from somatic cells of a single adult. *Science* 1998; 282: 2095-2098

3 Scientific Opinion of the Scientific Committee. Food Safety, Animal Health and Welfare and Environmental Impact of Animals1 derived from Cloning by Somatic Cell Nucleus Transfer (SCNT) and their Offspring and Products Obtained from those Animals, *The EFSA Journal* 2008; 767: 1-49

4 Panarace M, Aguero JI, Garrote M, Jauregui G, Segovia A, Cane L, Gutierrez J, Marfil M, Rigali F, Pugliese M, Young S, Lagioia J, Garnil C, Forte Pontes JE, Ereno Junio JC, Mower S, Medina M. How healthy are clones and their progeny: 5 years of field experience. *Theriogenology* 2007; 67: 142-151

5 Walker SC, Shin T, Zaunbrecher GM, Romano JE, Johnson GA, Bazer FW, Piedrahita JA. A highly efficient method for porcine cloning by nuclear transfer using in vitro-matured oocytes. *Cloning Stem Cells* 2002; 4: 105-112

6 National Institute of Livestock and Grassland Science, National Agriculture and Food Research Organization, Somatic Cell Cloned Cattle and their Progeny Produced in Japan : A report for Animal Health and Attributes of Animal Products 2008

7 Codex Alimentarius, Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Animals, 2008; CAC/GL 68

8 Domestic Animal Inseminator Association of Japan, Textbook for seminor of artificial insemination in livestock (livestock artificial insemination edition),1980

9 Xue F, Tian XC, Du F, Kubota C, Taneja M, Dinnyes A, Dai Y, Levine H, Pereira LV, Yang X. Aberrant patterns of X chromosome inactivation in bovine clones. *Nat Genet* 2002; 31: 216-220

10 Gong G, Dai Y, Zhu H, Wang H, Wang L, Li R, Wan R, Liu Y, Li N. Generation of cloned calves from different types of somatic cells. *Sci China C Life Sci* 2004; 47: 470-476

11 Hiendleder S, Prelle K, Bruggerhoff K, Reichenbach HD, Wenigerkind H, Bebbere D, Stojkovic M, Muller S, Brem G, Zakhartchenko V, Wolf E. Nuclear-cytoplasmic interactions affect in utero developmental capacity, phenotype, and cellular metabolism of bovine nuclear transfer fetuses. *Biol Reprod* 2004; 70: 1196-1205

12 Ideta A, Urakawa M, Aoyagi Y, Saeki K. Early morphological nuclear events and developmental capacity of embryos reconstructed with fetal fibroblasts at the M or G1 phase after intracytoplasmic nuclear injection in cattle. *J Reprod Dev* 2005; 51: 187-194

13 Wells DN, Laible G, Tucker FC, Miller AL, Oliver JE, Xiang T, Forsyth JT, Berg MC, Cockrem K, L'Huillier PJ, Tervit HR, Oback B. Coordination between donor cell type and cell cycle stage improves nuclear cloning efficiency in cattle. *Theriogenology* 2003; 59: 45-59

14 Mastromonaco GF, Semple E, Robert C, Rho GJ, Betts DH, King WA. Different culture media requirements of IVF and nuclear transfer bovine embryos. *Reprod Domest Anim*

2004; 39: 462-467
15 Thompson JG. Comparison between in vivo-derived and in vitro-produced pre-elongation embryos from domestic ruminants. *Reprod Fertil Dev* 1997; 9: 341-354
16 van Wagtendonk-de Leeuw AM, Mullaart E, de Roos AP, Merton JS, den Daas JH, Kemp B, de Ruigh L. Effects of different reproduction techniques: AI MOET or IVP, on health and welfare of bovine offspring. *Theriogenology* 2000; 53: 575-597
17 Gardner DK, Lane M. Ex vivo early embryo development and effects on gene expression and imprinting. *Reprod Fertil Dev* 2005; 17: 361-370
18 Farin PW, Piedrahita JA, Farin CE. Errors in development of fetuses and placentas from in vitro-produced bovine embryos. *Theriogenology* 2006; 65: 178-191
19 Le Bourhis D, Chesne P, Nibart M, Marchal J, Humblot P, Renard JP, Heyman Y. Nuclear transfer from sexed parent embryos in cattle: efficiency and birth of offspring. *J Reprod Fertil* 1998; 113: 343-348
20 Lee RS, Peterson AJ, Donnison MJ, Ravelich S, Ledgard AM, Li N, Oliver JE, Miller AL, Tucker FC, Breier B, Wells DN. Cloned cattle fetuses with the same nuclear genetics are more variable than contemporary half-siblings resulting from artificial insemination and exhibit fetal and placental growth deregulation even in the first trimester. *Biol Reprod* 2004; 70: 1-11
21 Hashizume K, Ishiwata H, Kizaki K, Yamada O, Takahashi T, Imai K, Patel OV, Akagi S, Shimizu M, Takahashi S, Katsuma S, Shiojima S, Hirasawa A, Tsujimoto G, Todoroki J, Izaike Y. Implantation and placental development in somatic cell clone recipient cows. *Cloning Stem Cells* 2002; 4: 197-209
22 Farin PW, Crosier AE, Farin CE. Influence of in vitro systems on embryo survival and fetal development in cattle. *Theriogenology* 2001; 55: 151-170
23 Chavatte-Palmer P, Heyman Y, Richard C, Monget P, LeBourhis D, Kann G, Chillard Y, Vignon X, Renard JP. Clinical, hormonal, and hematologic characteristics of bovine calves derived from nuclei from somatic cells. *Biol Reprod* 2002; 66: 1596-1603
24 Heyman Y, Chavatte-Palmer P, LeBourhis D, Camous S, Vignon X, Renard JP. Frequency and occurrence of late-gestation losses from cattle cloned embryos. *Biol Reprod* 2002; 66: 6-13
25 Batchelder CA, Hoffert KA, Bertolini M, Moyer AL, Mason JB, Petkov SG, Famula TR, Anderson GB. Effect of the nuclear-donor cell lineage, type, and cell donor on development of somatic cell nuclear transfer embryos in cattle. *Cloning Stem Cells* 2005; 7: 238-254
26 Wells DN, Misica PM, Tervit HR. Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. *Biol Reprod* 1999; 60: 996-1005
27 Hasler JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, Neely B, Shuey LS, Stokes JE, Trimmer SA. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology* 1995; 43: 141-152
28 Forsyth JT, Wells DN. Health and neonatal care of bovine clones. *Methods Mol Biol* 2006;

348: 91-108
29 Constant F, Guillomot M, Heyman Y, Vignon X, Laigre P, Servely JL, Renard JP, Chavatte-Palmer P. Large offspring or large placenta syndrome? Morphometric analysis of late gestation bovine placentomes from somatic nuclear transfer pregnancies complicated by hydrallantois. *Biol Reprod* 2006; 75: 122-130
30 Batchelder CA, Bertolini M, Mason JB, Moyer AL, Hoffert KA, Petkov SG, Famula TR, Angelos J, George LW, Anderson GB. Perinatal physiology in cloned and normal calves: physical and clinical characteristics. *Cloning Stem Cells* 2007; 9: 63-82
31 Chavatte-Palmer P, de SN, Laigre P, Camous S, Ponter AA, Beckers JF, Heyman Y. Ultrasound fetal measurements and pregnancy associated glycoprotein secretion in early pregnancy in cattle recipients carrying somatic clones. *Theriogenology* 2006; 66: 829-840
32 Higa N, Yamashiro T, Chiba Y. Establishment of technology producing cloned cattle. *Bull Okinawa Pref Livestock Exp Stn* 2002; 40: 5-10
33 Taniyama A, Nakasato S, Hirokawa J, Ogasawara S, Matsuo N. Birth weights, hematology and clinical chemistry of cloned calves derived from somatic cells. *Bull Nagasaki Pref Livestock Exp Stn* 2006; 12: 4-5
34 Mori K, Kubota C, Kojima H, Terawaki S, Todoroki J, Ota H, Sato M, Kamimiyata M, Yamashita M. Present status of producing cloned cows derived from somatic cells. *Bull Kagoshima Pref Livestock Exp Stn* 2002; 35: 52-57
35 Nagano K, Mori K, Kubota C, Okamoto K, Terawaki S, Kojima H, Kamimiyata M, Yamashita M. Development of cloned Holstein cows derived fromsomatic cells. *Bull Kagoshima Pref Livestock Exp Stn* 2002; 35: 83-88
36 Hasegawa K, Sasaki E, Abe A, Murao K, Takani T. Physiological and pathological observations of cloned cows derived from cumulus cells and their offspring. *Bull Shimane Anim Hus Exp Stn* 2005; 38: 1-8
37 Hasegawa K, Sasaki E, Abe A, Takani T. Studies on production of somatic cell cloned bulls from candidates of sire. *Bull Shimane Anim Hus Exp Stn* 2004; 37: 1-5
38 Kubota C, Yamakuchi H, Todoroki J, Mizoshita K, Tabara N, Barber M, Yang X. Six cloned calves produced from adult fibroblast cells after long-term culture. *Proc Natl Acad Sci USA* 2000; 97: 990-995
39 Kruip T, den Daas JHG. In vitro produced and cloned embryos: Effects on pregnancy, parturition and offspring. *Theriogenology* 1997; 47: 43-52
40 Behboodi E, Anderson GB, BonDurant RH, Cargill SL, Kreuscher BR, Medrano JF, Murray JD. Birth of large calves that developed from in-vitro derived bovine embryos. *Theriogenology* 1995; 44: 227-232
41 Nix JM, Spitzer JC, Grimes LW, Burns GL, Plyler BB. A retrospective analysis of factors contributing to calf mortality and dystocia in beef cattle. *Theriogenology* 1998; 49: 1515-1523
42 USDA/NAHMS 1997
43 Lombard JE, Garry FB, Tomlinson SM, Garber LP. Impacts of dystocia on health and

survival of dairy calves. *J Dairy Sci* 2007; 90: 1751-1760
44 Sanderson MW, Dargatz DA. Risk factors for high herd level calf morbidity risk from birth to weaning in beef herds in the USA. *Prev Vet Med* 2000; 44: 97-106
45 Ueda J, Kobayashi S, Takei M, Kato Y. Production of cloned calf from a cumulus cell collected with trans-vaginal aspiration. *Res Bull Aichi Agri Res Ctr* 2000; 32: 197-202
46 Jindo M, Oomachi M, Kikushima K, Takahashi T, Shimizu K, Obi K, Koshiba T, Takagi Y. Growth and reproductive ability of clone cattle obtained after nuclear transfer of embryos and somatic cells. *Bull Yamanashi Pref Dairy Exp Stn* 2005; 16: 1-8
47 Center for Veterinary Medicine U. S. Food and Drug Administration Department of Health and Human Services 7500 Standish Place Rockville, MD 20855, Animal Cloning: A Risk Assessment, 2008
48 Kato Y, Tani T, Tsunoda Y. Cloning of calves from various somatic cell types of male and female adult, newborn and fetal cows. *J Reprod Fertil* 2000; 120: 231-237
49 Galli C, Duchi R, Moor R. M, Lazzari G. Mammalian leukocytes contain all the genetic information necessary for the development of a new individual. *Cloning* 1999; 1 (3): 161-170
50 Young LE, Fairburn HR. Improving the safety of embryo technologies: possible role of genomic imprinting. *Theriogenology* 2000; 53: 627-648
51 Bertolini M, Anderson GB. The placenta as a contributor to production of large calves. *Theriogenology* 2002; 57: 181-187
52 Bertolini M, Mason JB, Beam SW, Carneiro GF, Sween ML, Kominek DJ, Moyer AL, Famula TR, Sainz RD, Anderson GB. Morphology and morphometry of in vivo- and in vitro-produced bovine concepti from early pregnancy to term and association with high birth weights. *Theriogenology* 2002; 58: 973-994
53 Matsuzaki M, Shiga K. Endocrine characteristics of cloned calves. *Cloning Stem Cells* 2002; 4: 261-267
54 Livestock Improvement Association of Japan, MIC Co. Ltd. Studies on nuclear transfer for producing cloned cattle using embryo and somatic cells: Part 1 (FY2001). *In*: Research Project Report by the Japanese Research Association for Animal Embryo Transfer Technology (FY2001); 2002: 83-93
55 Chavatte-Palmer P, Remy D, Cordonnier N, Richard C, Issenman H, Laigre P, Heyman Y, Mialot JP. Health status of cloned cattle at different ages. *Cloning Stem Cells* 2004; 6: 94-100
56 Kubota C, Okamoto K, Todoroki J, Mizoshita K, Yamaguchi H, Tabara N. Blood properties of cloned bulls derived from somatic cells: Clinical chemistry of calves within one month after birth. *Bull Kagoshima Cattle Breeding Dev Inst* 2001; 6: 32-41
57 Yamaguchi D, Tozuka Y, Watanabe A, Adachi N, Akagi S, Takahashi S, Kubo M. Production of excellent livestock animals by somatic cell nuclear transfer. *Bull Ibaraki Pref Livestock Res Stn* 2005; 38: 5-12
58 Batchelder CA, Bertolini M, Mason JB, Moyer AL, Hoffert KA, Petkov SG, Famula TR,

Angelos J, George LW, Anderson GB Perinatal physiology in cloned and normal calves: hematologic and biochemical profiles. *Cloning Stem Cells* 2007; 9: 83-96
59 USDA/NAHMS 1994
60 Wells DN, Forsyth JT, McMillan V, Oback B. The health of somatic cell cloned cattle and their offspring. *Cloning Stem Cells* 2004; 6: 101-110
61 Watanabe S, Nagai T. Health status and productive performance of somatic cell cloned cattle and their offspring produced in Japan. *J Reprod Dev* 2008; 54: 6-17
62 Hill JR, Winger QA, Long CR, Looney CR, Thompson JA, Westhusin ME. Development rates of male bovine nuclear transfer embryos derived from adult and fetal cells. *Biol Reprod* 2000; 62: 1135-1140
63 Renard JP, Chastant S, Chesne P, Richard C, Marchal J, Cordonnier N, Chavatte P, Vignon X. Lymphoid hypoplasia and somatic cloning. *Lancet* 1999; 353: 1489-1491
64 Heyman Y, Chavatte-Palmer P, Berthelot V, Fromentin G, Hocquette JF, Martignat L, Renard JP. Assessing the quality of products from cloned cattle: an integrative approach. *Theriogenology* 2007; 67: 134-141
65 Laible G, Brophy B, Knighton D, Wells DN. Compositional analysis of dairy products derived from clones and cloned transgenic cattle. *Theriogenology* 2007; 67: 166-177
66 Yamaguchi M, Ito Y, Takahashi S. Fourteen-week feeding test of meat and milk derived from cloned cattle in the rat. *Theriogenology* 2007; 67: 152-165
67 Heyman Y, Chavatte-Palmer P, Fromentin G, Berthelot V, Jurie C, Bas P, Dubarry M, Mialot J. P, Remy D, Richard C, Martignat L, Vignon X, Renard J. P, Quality and safety of bovine clones and their products. *Animal* 2007; (1): 963-972
68 Yonai M, Kaneyama K, Miyashita N, Kobayashi S, Goto Y, Bettpu T, Nagai T. Growth, reproduction, and lactation in somatic cell cloned cows with short telomeres. *J Dairy Sci* 2005; 88: 4097-4110
69 Taniyama A, Nakasato S, Hirokawa J, Ogasawara S, Matsuo N. Development and semen properties of cloned bulls derived from somatic cells. *Bull Nagasaki Pref Livestock Exp Stn* 2006; 12: 6-7
70 Nakasato S, Inoue T, Taniyama A, Kiyomatsu K. *In vitro* growth and embryo transfer of somatic cell cloned embryos in cattle. *Bull Nagasaki Pref Livestock Exp Stn* 2001; 10: 4-6
71 Inoue K, Saito T, Abe Y, Yoshida S, Takagki K, Shibuya K, Hirai T. Investigation on homology of somatic-cloned dairy cattle. *Bull Oita Pref Anim Ind Exp Stn* 2002; 31: 69-71
72 Hasegawa K, Sasaki E, Abe A, Nakamura R, Takahito T. Studies on production of somatic cell cloned bulls from candidates of sire (2nd report). *Bull Shimane Livestock Tech Ctr* 2006; 39: 1-6
73 Koiwai-Farm Co. Ltd. Studies on milk yield and reproduction in cloned cows (FY2004). *In*: Research Project Report by Livestock Improvement Association of Japan (FY2004); 2005: 105-118
74 Ogonuki N, Inoue K, Yamamoto Y, Noguchi Y, Tanemura K, Suzuki O, Nakayama H, Doi K, Ohtomo Y, Satoh M, Nishida A, Ogura A. Early death of mice cloned from somatic

75 Tamashiro KL, Wakayama T, Yamazaki Y, Akutsu H, Woods SC, Kondo S, Yanagimachi R, Sakai RR. Phenotype of cloned mice: development, behavior, and physiology. *Exp Biol Med (Maywood)* 2003; 228: 1193-1200
76 Tanaka S, Miyazawa K, Watanabe K, Ohwada S, Aso H, Yonai M, Saito N, Yamaguchi T. Comparison of T cell subsets between somatic cloned and normal cow. *Am J Reprod Immunol* 2006; 55: 28-35
77 Wells DN. Animal cloning: problems and prospects. *Rev Sci Tech* 2005; 24: 251-264
78 Kubota C, Nozaki S, Nishi K, Niipuku Y, Kawakubo K, Todoroki J, Mizoshita K, Yamaguchi H, Tabara N, Onizuka T, Sato M. Reproductive performance of cloned bulls derived from somatic cells. *Bull Kagoshima Cattle Breeding Dev Inst* 2001; 6: 42-45
79 Heyman Y, Richard C, Rodriguez-Martinez H, Lazzari G, Chavatte-Palmer P, Vignon X, Galli C. Zootechnical performance of cloned cattle and offspring: preliminary results. *Cloning Stem Cells* 2004; 6: 111-120
80 Taniguchi M, Sumio Y. Establishment of technology of producing cloned cattle derived from somatic cells. *Bull Kumamoto Pref Agri Res Ct*r (FY2004) 2005: 84-89
81 Honta I, Sakamoto H, Tanji T, Hara M, Ishikawa Y, Shiga Y, Sugano M. Reproduction of bull by nuclear transfer of somatic cells. *Bull Fukushima Anim Hus Exp Stn* 2003; 10: 17-19
82 Hayasaka S, Takada N. Establishment of technology producing somatic cell cloned cattle. *Bull Miyagi Pref Livestock Exp Stn* (FY2002) 2002: 56-58
83 Sato W, Umeki H, Shiga K, Yamaguchi H. Test for reproductive ability of cloned cattle produced by transfer of nuclei from somatic cells. *Bull Oita Pref Anim Ind Exp Stn* 2000; 29: 108-109
84 Shiga K, Umeki H, Shimura H, Fujita T, Akamine M. Investigation on genetic homology the somatic- cloned Japanese Black cattle. *Bull Oita Pref Anim Ind Exp Stn* 2001; 30: 55-61
85 Sato W, Yoshida H, Umeki H, Shiga K. Test for individual performance and reproductive ability of cloned cattle produced by transfer of nuclei from somatic cells. *Bull Oita Pref Anim Ind Exp Stn* 2001; 30: 62-64
86 Shiga K, Umeki H, Shimura H, Fujita T, Watanabe S, Nagai T. Growth and fertility of bulls cloned from the somatic cells of an aged and infertile bull. *Theriogenology* 64 2005; 334-343
87 Tsuruta Y, Nozaki S, Kubota C, Uemura T, Nishi K, Shinpuku U, Uchiyama S, Yokoyama K. Studies for substantiating sire performance test using clones (7th report): Development and semen properties of somatic cell cloned bulls. *Bull Kagoshima Cattle Breeding Dev Inst* 2003; 8: 1-5
88 Enright BP, Taneja M, Schreiber D, Riesen J, Tian XC, Fortune JE, Yang X. Reproductive characteristics of cloned heifers derived from adult somatic cells. *Biol Reprod* 2002; 66: 291-296

89 Mori K, Nagano K, Kubota C, Okamoto K, Terawaki S, Kojima H, Kamimiyata M, Uehara S, Takahashi S, Tokunaga T. Reproductive performance of somatic cell cloned cows until first parturition. *Bull Kagoshima Pref Livestock Exp Stn* 2002; 36: 34-40
90 Yamaguchi D, Nemoto S, Watanabe A, Nirasawa K, Adachi N, Akagi S, Takahashi S, Kubo M. Production of excellent cattle by somatic cell nuclear transfer (4th report): Investigation of the breeding ability of somatic cell-cloned cattle and normality of its progeny calves. *Bull Ibaraki Pref Livestock Res Stn* 2004; 37: 79-83
91 Kasai H, Fukumi Y, Watanabe Y, Tachikawa S. Studies on superovulation treatment and offspring production of cloned Holstein cows derived from somatic cells. *Bull Tokushima Pref Livestock Res Ins* 2003; 3: 14-19
92 Zen-Noh. Studies reproduction of offspring produced from clones (FY2005). *In*: Research Project Report by Livestock Improvement Association of Japan (FY2005); 2006: 163-173
93 Koiwai-Farm Co. Ltd. Studies on milk yield and reproduction of cloned cows (FY2003). *In*: Research Project Report by Livestock Improvement Association of Japan (FY2003); 2004: 111-118
94 Polge C, Rowson LE, Chang MC. The effect of reducing the number of embryos during early stages of gestation on the maintenance of pregnancy in the pig. *J Reprod Fertil* 1966; 12: 395-397
95 Dziuk P. Effect of migration, distribution and spacing of pig embryos on pregnancy and fetal survival. *J Reprod Fertil* Suppl 1985; 33: 57-63
96 Betthauser J, Forsberg E, Augenstein M, Childs L, Eilertsen K, Enos J, Forsythe T, Golueke P, Jurgella G, Koppang R, Lesmeister T, Mallon K, Mell G, Misica P, Pace M, Pfister-Genskow M, Strelchenko N, Voelker G, Watt S, Thompson S, Bishop M. Production of cloned pigs from in vitro systems. *Nat Biotechnol* 2000; 18: 1055-1059
97 Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata T, Hanada H, Perry AC. Pig cloning by microinjection of fetal fibroblast nuclei. *Science* 2000; 289: 1188-1190
98 Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL, Colman A, Campbell KH. Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature* 2000; 407: 86-90
99 van der Lende T, van Rens BTTM. Critical periods for foetal mortality in gilts identified by analysing the length distribution of mummified foetuses and frequency of non-fresh stillborn piglets. *Anim Reprod Sci* 2003; 75: 141-150
100 Carroll JA, Carter DB, Korte SW, Prather RS. Evaluation of the acute phase response in cloned pigs following a lipopolysaccharide challenge. *Domest Anim Endocrinol* 2005; 29: 564-572
101 Park MR, Cho SK, Lee SY, Choi YJ, Park JY, Kwon DN, Son WJ, Paik SS, Kim T, Han YM, Kim JH. A rare and often unrecognized cerebromeningitis and hemodynamic disorder: a major cause of sudden death in somatic cell cloned piglets. *Proteomics* 2005; 5: 1928-1939
102 Williams NE, Walker SC, Reeves DE, Sherrer E, Galvin JM, Polejaeva I, Rampacek G,

Benyshek L, Christenson RK, Graves WM, Pratt SL. A comparison of reproductive characteristics of boars generated by somatic cell nuclear transfer to highly related conventionally produced boars. *Cloning Stem Cells* 2006; 8: 130-139

103 Du Y, Kragh PM, Zhang Y, Li J, Schmidt M, Bogh IB, Zhang X, Purup S, Jorgensen AL, Pedersen AM, Villemoes K, Yang H, Bolund L, Vajta G. Piglets born from handmade cloning, an innovative cloning method without micromanipulation. *Theriogenology* 2007; 68 (8): 1104-1110

104 Shibata M, Tsuchiya S, Ohtake M, Kawarasaki T. Growth and reproductive performance of Jin Hua pig cloned from somatic cell nuclei. *Shizuoka Swine and Poultry Exp Stn* 2003; 14: 13-16

105 Estrada J, Sommer J, Collins B, Mir B, Martin A, York A, Petters RM, Piedrahita JA. Swine generated by somatic cell nuclear transfer have increased incidence of intrauterine growth restriction (IUGR). *Cloning Stem Cells* 2007; 9: 229-236

106 Lee SY, Park JY, Choi YJ, Cho SK, Ahn JD, Kwon DN, Hwang KC, Kang SJ, Paik SS, Seo HG, Lee HT, Kim JH. Comparative proteomic analysis associated with term placental insufficiency in cloned pig. *Proteomics* 2007; 7(8): 1303-1315

107 Archer GS, Dindot S, Friend TH, Walker S, Zaunbrecher G, Lawhorn B, Piedrahita JA. Hierarchical phenotypic and epigenetic variation in cloned swine. *Biol Reprod* 2003; 069: 430-436

108 Mir B, Zaunbrecher G, Archer GS, Friend TH, Piedrahita JA. Progeny of somatic cell nuclear transfer (SCNT) pig clones are phenotypically similar to non-cloned pigs. *Cloning Stem Cells* 2005; 7: 119-125

109 Archer GS, Friend TH, Piedrahita J, Nevill CH, Walker S. Behavioral variation among cloned pigs. *Applied Animal Behaviour Science* 2003; 82: 151-161

110 Martin M, Adams C, Wiseman B. Pre-weaning performance and health of pigs born to cloned (fetal cell derived) swine versus non-cloned swine. *Theriogenology* 2004; 62: 113-122

111 Center for Veterinary Medicine U. S. Food and Drug Administration Department of Health and Human Services 7500 Standish Place Rockville, MD 20855, Animal Cloning: A Risk Assessment, Appendix F The ViaGen Dataset, 2008

112 Nagano K, Mori K, Kubota C, Imamura M, Terawaki S, Uehara S. Development of offspring of cloned Holstein cows derived from somatic cells. *Bull Kagoshima Pref Livestock Exp Stn* 2005; 39: 53-58

113 MIC Co. Ltd. Studies on growth and reproduction of cloned cattle (FY2004). *In*: Research Project Report by Livestock Improvement Association of Japan (FY2004); 2005: 119-125

114 Ortegon H, Betts DH, Lin L, Coppola G, Perrault SD, Blondin P, King WA. Genomic stability and physiological assessments of live offspring sired by a bull clone, Starbuck II. *Theriogenology* 2007; 67: 116-126

115 Sakashita K, Kubota C, Nishi H, Tabara N, Beppu A, Okano R. Studies on fattening trials in offspring of somatic cell cloned cattle. *Bull Kagoshima Pref Livestock Exp Stn* 2003;

37: 34-40
116 Shibata M, Otake M, Tsuchiya S, Chikyu M, Horiuchi A, Kawarasaki T. Reproductive and growth performance in Jin Hua pigs cloned from somatic cell nuclei and the meat quality of their offspring. *J Reprod Dev* 2006; 52: 583-590
117 Walker SC, Christenson RK, Ruiz RP, Reeves DE, Pratt SL, Arenivas F, Williams NE, Bruner BL, Polejaeva IA. Comparison of meat composition from offspring of cloned and conventionally produced boars. *Theriogenology* 2007; 67: 178-184
118 Shibata M, Ohtake M, Tsuchiya S, Kawarasaki T. Safety as food in offspring of cloned Jin Hua pig from somatic cells. *Shizuoka Swine and Poultry Exp Stn* 2007; 17: 13-23
119 Shibata M, Tsuchiya S, Ohtake M, Kawarasaki T. Productivity and quality of meat in offspring of cloned Jin Hua pig from somatic cells I (Growth and carcass characteristics in offspring of cloned pig). *Shizuoka Swine and Poultry Exp Stn* 2004; 15: 35-38
120 Riggs AD, Martienssen RA, Russo VEA, In introduction, Riggs AD, Martienssen RA, Russo VEA, (eds), Epigenetic mechanisms of gene regulation, Cold Spring Harbor Laboratory Press Cold Spring Harbor, NY, 1996; 1-4
121 Bird A. DNA methylation patterns and epigenetic memory. *Genes Dev* 2002; 16: 6-21
122 Shiota K, Yanagimachi R. Epigenetics by DNA methylation for development of normal and cloned animals. *Differentiation* 2002; 69 (4-5): 162-166
123 Jaenisch R. Human cloning-the science and ethics of nuclear transplantation. *N Engl J Med* 2004; 351: 2787-2791
124 Holliday R. DNA methylation and epigenotypes. *Biochemistry (Mosc)* 2005; 70: 500-504
125 Scarano MI, Strazzullo M, Matarazzo MR, D'Esposito M. DNA methylation 40 years later: Its role in human health and disease. *J Cell Physiol* 2005; 204: 21-35
126 Kanka J. Gene expression and chromatin structure in the pre-implantation embryo. *Theriogenology* 2003; 59: 3-19
127 Quivy V, Calomme C, Dekoninck A, Demonte D, Bex F, Lamsoul I, Vanhulle C, Burny A, Van Lint C. Gene activation and gene silencing: a subtle equilibrium. *Cloning Stem Cells* 2004; 6: 140-149
128 Cheung P, Lau P. Epigenetic regulation by histone methylation and histone variants. *Mol Endocrinol* 2005; 19: 563-573
129 Fuks F. DNA methylation and histone modifications: teaming up to silence genes. *Curr Opin Genet Dev* 2005; 15: 490-495
130 Verschure PJ, van der KI, de Leeuw W, van der VJ, Carpenter AE, Belmont AS, van Driel R. In vivo HP1 targeting causes large-scale chromatin condensation and enhanced histone lysine methylation. *Mol Cell Biol* 2005; 25: 4552-4564
131 Hattori N, Nishino K, Ko YG, Hattori N, Ohgane J, Tanaka S, Shiota K. Epigenetic control of mouse Oct-4 gene expression in embryonic stem cells and trophoblast stem cells. *J Biol Chem* 2004; 279 (17): 17063-17069
132 Morgan HD, Santos F, Green K, Dean W, Reik W. Epigenetic reprogramming in mammals. *Hum Mol Genet* 2005; 14 Spec No 1: R47-R58

133 Rideout WM III, Eggan K, Jaenisch R. Nuclear cloning and epigenetic reprogramming of the genome. *Science* 2001; 293: 1093-1098
134 Jaenisch R, Eggan K, Humpherys D, Rideout W, Hochedlinger K. Nuclear cloning, stem cells, and genomic reprogramming. *Cloning Stem Cells* 2002; 4: 389-396
135 Mann MR, Bartolomei MS. Epigenetic reprogramming in the mammalian embryo: struggle of the clones. *Genome Biol* 2002; 3: REVIEWS1003
136 Cezar GG. Epigenetic reprogramming of cloned animals. *Cloning Stem Cells* 2003; 5: 165-180
137 Han YM, Kang YK, Koo DB, Lee KK. Nuclear reprogramming of cloned embryos produced in vitro. *Theriogenology* 2003; 59: 33-44
138 Jouneau A, Renard JP. Reprogramming in nuclear transfer. *Curr Opin Genet Dev* 2003; 13: 486-491
139 Smith LC, Murphy BD. Genetic and epigenetic aspects of cloning and potential effects on offspring of cloned mammals. *Cloning Stem Cells* 2004; 6: 126-132
140 Young LE, Beaujean N. DNA methylation in the preimplantation embryo: the differing stories of the mouse and sheep. *Anim Reprod Sci* 2004; 82-83: 61-78
141 Wrenzycki C, Herrmann D, Lucas-Hahn A, Korsawe K, Lemme E, Niemann H. Messenger RNA expression patterns in bovine embryos derived from in vitro procedures and their implications for development. *Reprod Fertil Dev* 2005; 17: 23-35
142 Armstrong L, Lako M, Dean W, Stojkovic M. Epigenetic modification is central to genome reprogramming in somatic cell nuclear transfer. *Stem Cells* 2006; 24: 805-814
143 Eilertsen KJ, Power RA, Harkins LL, Misica P. Targeting cellular memory to reprogram the epigenome, restore potential, and improve somatic cell nuclear transfer. *Anim Reprod Sci* 2007; 98: 129-146
144 De Rycke M, Liebaers I, Van Steirteghem A. Epigenetic risks related to assisted reproductive technologies: risk analysis and epigenetic inheritance. *Hum Reprod* 2002 17 (10): 2487-2494
145 Niemann H, Wrenzycki C, Lucas-Hahn A, Brambrink T, Kues WA, Carnwath JW. Gene expression patterns in bovine in vitro-produced and nuclear transfer-derived embryos and their implications for early development. *Cloning Stem Cells*. 2002; 4 (1): 29-38
146 Powell K. Fertility treatments: Seeds of doubt.*Nature*. 2003; 422 (6933): 656-658
147 Dean W, Santos F, Stojkovic M, Zakhartchenko V, Walter J, Wolf E, Reik W. Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. *Proc Natl Acad Sci USA* 2001; 98: 13734-13738
148 Bourc'his D, Le Bourhis D, Patin D, Niveleau A, Comizzoli P, Renard JP, Viegas-Pequignot E. Delayed and incomplete reprogramming of chromosome methylation patterns in bovine cloned embryos. *Curr Biol* 2001; 11: 1542-1546
149 Kang YK, Koo DB, Park JS, Choi YH, Chung AS, Lee KK, Han YM. Aberrant methylation of donor genome in cloned bovine embryos. *Nat Genet* 2001; 28: 173-177
150 Kang YK, Koo DB, Park JS, Choi YH, Kim HN, Chang WK, Lee KK, Han YM. Typical

demethylation events in cloned pig embryos.Clues on species-specific differences in epigenetic reprogramming of a cloned donor genome. *J Biol Chem* 2001; 276: 39980-39984

151 Tanaka S, Oda M, Toyoshima Y, Wakayama T, Tanaka M, Yoshida N, Hattori N, Ohgane J, Yanagimachi R, Shiota K. Placentomegaly in cloned mouse concepti caused by expansion of the spongiotrophoblast layer. *Biol Reprod* 2001; 65: 1813-1821

152 Ohgane J, Wakayama T, Kogo Y, Senda S, Hattori N, Tanaka S, Yanagimachi R, Shiota K. DNA methylation variation in cloned mice. *Genesis* 2001; 30: 45-50

153 Ohgane J, Wakayama T, Senda S, Yamazaki Y, Inoue K, Ogura A, Marh J, Tanaka S, Yanagimachi R, Shiota K. The *Sall3* locus is an epigenetic hotspot of aberrant DNA methylation associated with placentomegaly of cloned mice. *Genes Cells* 2004; 9: 253-260

154 Cezar GG, Bartolomei MS, Forsberg EJ, First NL, Bishop MD, Eilertsen KJ. Genome-wide epigenetic alterations in cloned bovine fetuses. *Biol Reprod* 2003; 68: 1009-1014

155 Dindot SV, Farin PW, Farin CE, Romano J, Walker S, Long C, Piedrahita JA. Epigenetic and genomic imprinting analysis in nuclear transfer derived Bos gaurus/Bos taurus hybrid fetuses. *Biol Reprod* 2004; 71: 470-478

156 Chen T, Jiang Y, Zhang YL, Liu JH, Hou Y, Schatten H, Chen DY, Sun QY. DNA hypomethylation of individual sequences in aborted cloned bovine fetuses. *Frontiers in Bioscience* 2005; 10: 3002-3008

157 Kremenskoy M, Kremenska Y, Suzuki M, Imai K, Takahashi S, Hashizume K, Yagi S, Shiota K. DNA methylation profiles of donor nuclei cells and tissues of cloned bovine fetuses. *J Reprod Dev* 2006; 52: 259-266

158 Long JE, Cai X. Igf-2r expression regulated by epigenetic modification and the locus of gene imprinting disrupted in cloned cattle. *Gene* 2007; 388: 125-134

159 Senda S, Wakayama T, Arai Y, Yamazaki Y, Ohgane J, Tanaka S, Hattori N, Yanagimachi R, Shiota K. DNA methylation errors in cloned mice disappear with advancement of aging. *Cloning Stem Cells* 2007; 9: 293-302

160 Wrenzycki C, Lucas-Hahn A, Herrmann D, Lemme E, Korsawe K, Niemann H. In vitro production and nuclear transfer affect dosage compensation of the X-linked gene transcripts G6PD, PGK, and Xist in preimplantation bovine embryos. *Biol Reprod* 2002; 66 (1): 127-134

161 Smith SL, Everts RE, Tian XC, Du F, Sung LY, Rodriguez-Zas SL, Jeong BS, Renard JP, Lewin HA, Yang X. Global gene expression profiles reveal significant nuclear reprogramming by the blastocyst stage after cloning. *Proc Natl Acad Sci USA* 2005; 102: 17582-17587

162 Sawai K, Kageyama S, Moriyasu S, Hirayama H, Minamihashi A, Onoe S. Analysis of mRNA transcripts for insulin-like growth factor receptors and binding proteins in bovine embryos derived from somatic cell nuclear transfer. *Cloning Stem Cells* 2005; 7: 189-198

163 Wrenzycki C, Wells D, Herrmann D, Miller A, Oliver J, Tervit R, Niemann H. Nuclear transfer protocol affects messenger RNA expression patterns in cloned bovine blastocysts. *Biol Reprod* 2001; 65: 309-317

164 Camargo LS, Powell AM, Filho VR, Wall RJ. Comparison of gene expression in individual preimplantation bovine embryos produced by in vitro fertilisation or somatic cell nuclear transfer. *Reprod Fertil Dev* 2005; 17: 487-496

165 Beyhan Z, Forsberg EJ, Eilertsen KJ, Kent-First M, First NL. Gene expression in bovine nuclear transfer embryos in relation to donor cell efficiency in producing live offspring. *Mol Reprod Dev* 2007; 74: 18-27

166 Eggan K, Akutsu H, Hochedlinger K, Rideout W 3rd, Yanagimachi R, Jaenisch R. X-Chromosome inactivation in cloned mouse embryos. *Science* 2000; 290 (5496): 1578-1581

167 Boiani M, Eckardt S, Scholer HR, McLaughlin KJ. Oct4 distribution and level in mouse clones: consequences for pluripotency. *Genes Dev* 2002; 16: 1209-1219

168 Bortvin A, Eggan K, Skaletsky H, Akutsu H, Berry DL, Yanagimachi R, Page DC, Jaenisch R. Incomplete reactivation of Oct4-related genes in mouse embryos cloned from somatic nuclei. Development 2003; 130: 1673-1680

169 Sebastiano V, Gentile L, Garagna S, Redi CA, Zuccotti M. Cloned pre-implantation mouse embryos show correct timing but altered levels of gene expression. *Mol Reprod Dev* 2005; 70: 146-154

170 Miyazaki K, Tomii R, Kurome M, Ueda H, Hirakawa K, Ueno S, Hiruma K, Nagashima H. Evaluation of the quality of porcine somatic cell nuclear transfer embryo by gene transcription profiles. *J Reprod Dev* 2005; 51: 123-131

171 Humpherys D, Eggan K, Akutsu H, Hochedlinger K, Rideout WM, III, Biniszkiewicz D, Yanagimachi R, Jaenisch R. Epigenetic instability in ES cells and cloned mice. *Science* 2001; 293: 95-97

172 Humpherys D, Eggan K, Akutsu H, Friedman A, Hochedlinger K, Yanagimachi R, Lander ES, Golub TR, Jaenisch R. Abnormal gene expression in cloned mice derived from embryonic stem cell and cumulus cell nuclei. *Proc Natl Acad Sci USA* 2002; 99: 12889-12894

173 Inoue K, Kohda T, Lee J, Ogonuki N, Mochida K, Noguchi Y, Tanemura K, Kaneko-Ishino T, Ishino F, Ogura A. Faithful expression of imprinted genes in cloned mice. *Science* 2002; 295: 297

174 Herath CB, Ishiwata H, Shiojima S, Kadowaki T, Katsuma S, Ushizawa K, Imai K, Takahashi T, Hirasawa A, Takahashi S, Izaike Y, Tsujimoto G, Hashizume K. Developmental aberrations of liver gene expression in bovine fetuses derived from somatic cell nuclear transplantation. *Cloning Stem Cells* 2006; 8: 79-95

175 Yang L, Chavatte-Palmer P, Kubota C, O'neill M, Hoagland T, Renard JP, Taneja M, Yang X, Tian XC. Expression of imprinted genes is aberrant in deceased newborn cloned calves and relatively normal in surviving adult clones. *Mol Reprod Dev* 2005; 71: 431-438

176 Li S, Li Y, Du W, Zhang L, Yu S, Dai Y, Zhao C, Li N. Aberrant gene expression in organs of bovine clones that die within two days after birth. *Biol Reprod* 2005; 72: 258-265
177 Senda S, Wakayama T, Yamazaki Y, Ohgane J, Hattori N, Tanaka S, Yanagimachi R, Shiota K. Skewed X-inactivation in cloned mice. *Biochem Biophys Res Commun* 2004; 321 (1): 38-44
178 Kohda T, Inoue K, Ogonuki N, Miki H, Naruse M, Kaneko-Ishino T, Ogura A, Ishino F. Variation in gene expression and aberrantly regulated chromosome regions in cloned mice. *Biol Reprod*. 2005; 73 (6): 1302-1311
179 Inoue F, Matsuda J, Ohkoshi K, Furusawa T, Takahashi S, Sasada H, Sato E, Tokunaga T. Differences in gene expression patterns between somatic cell nuclear transfer embryos constructed with either rabbit granulosa cells or their derivatives. *Anim Reprod Sci* 2006; 93: 76-87
180 Bosch P, Pratt SL, Stice SL. Isolation, characterization, gene modification, and nuclear reprogramming of porcine mesenchymal stem cells. *Biol Reprod* 2006; 74: 46-57
181 Giraldo AM, Lynn JW, Godke RA, Bondioli KR. Proliferative characteristics and chromosomal stability of bovine donor cells for nuclear transfer. *Mol Reprod Dev* 2006; 73: 1230-1238
182 Mastromonaco GF, Perrault SD, Betts DH, King WA. Role of chromosome stability and telomere length in the production of viable cell lines for somatic cell nuclear transfer. *BMC Dev Biol* 2006; 6: 41
183 Camargo LS, Viana JH, Sa WF, Ferreira AM, Vale Filho VR. Developmental competence of oocytes from prepubertal Bos indicus crossbred cattle. *Anim Reprod Sci* 2005; 85 (1-2): 53-59
184 Liu G, Kato Y, Tsunoda Y. Aging of recipient oocytes reduces the development of cloned embryos receiving cumulus cells. *J Reprod Dev* 2007
185 Bordignon V, Smith LC. Telophase-stage host ooplasts support complete reprogramming of roscovitine-treated somatic cell nuclei in cattle. *Cloning Stem Cells* 2006; 8: 305-317
186 Gluckman PD, Hanson MA, Beedle AS. Early life events and their consequences for later disease: a life history and evolutionary perspective. *Am J Hum Biol* 2007; 19 (1): 1-19
187 Gluckman PD, Hanson MA, Beedle AS. Non-genomic transgenerational inheritance of disease risk. *Bioessays* 2007; 29 (2): 145-154
188 Shimozawa N, Ono Y, Kimoto S, Hioki K, Araki Y, Shinkai Y, Kono T, Ito M. Abnormalities in cloned mice are not transmitted to the progeny. *Genesis* 2002; 34 (3): 203-207
189 Tamashiro KL, Wakayama T, Akutsu H, Yamazaki Y, Lachey JL, Wortman MD, Seeley RJ, D'Alessio DA, Woods SC, Yanagimachi R, Sakai RR. Cloned mice have an obese phenotype not transmitted to their offspring. *Nat Med* 2002; 8: 262-267
190 Fulka J Jr., Miyashita N, Nagai T, Ogura A. Do cloned mammals skip a reprogramming step? *Nat Biotechnol* 2004; 22: 25-26
191 Committee on Defining Science-Based Concerns Associated with Products of Animal

Biotechnology, Committee on Agricultural Biotechnology, Health, and the Environment, National Research Council, Animal Biotechnology: Science-Based Concerns (NAS), 2002
192 Yamaguchi H, Kubota C, Mizoshita K, Todoroki J, Tabara N. Development of nuclear transfer in cattle: Individual identification. *Bull Kagoshima Pref Cattle Breeding Dev Inst* 2000; 5: 27-30
193 Honta I, Shinogi T, Hara M, Ishikawa Y, Shiga Y, Sugano M. Genetic similarity and growth of calves by nuclear transfer of somatic cells. *Bull Fukushima Anim Hus Exp Stn* 2003; 10: 13-16
194 Taniguchi S, Kashiwagi T, Noguchi H, Yamamoto Y. Production of somatic cell clone calf and study of similarity. *Bull Wakayama Res Ctr Agri Forestry Fisheries* 2002; 4: 57-61
195 Kato S, Hayashi N, Hayashi H, Hirao I, Soejima H, Kobayashi N, Otani K. Normality of cloned derived from somatic cells (1st report). *Bull Gifu Pref Livestock Res Inst* 2003; 3: 27-36
196 Booth PJ, Viuff D, Tan S, Holm P, Greve T, Callesen H. Numerical chromosome errors in day 7 somatic nuclear transfer bovine blastocysts. *Biol Reprod* 2003; 68 (3): 922-928
197 Hanada H, Takeda K, Tagami T, Nirasawa K, Akagi S, Adachi N, Takahashi S, Izaike Y, Iwamoto M, Fuchimoto D, Miyashita N, Kubo M, Onishi A, King WA. Chromosomal instability in the cattle clones derived by somatic cell nuclear-transfer. *Mol Reprod Dev* 2005; 71: 36-44
198 Yanagimachi R. Cloning: experience from the mouse and other animals. *Mol Cell Endocrinol* 2002; 187: 241-248
199 Balbach ST, Jauch A, Bohm-Steuer B, Cavaleri FM, Han YM, Boiani M. Chromosome stability differs in cloned mouse embryos and derivative ES cells. *Dev Biol* 2007; 308 (2): 309-321
200 Hiendleder S, Mund C, Reichenbach HD, Wenigerkind H, Brem G, Zakhartchenko V, Lyko F, Wolf E. Tissue-specific elevated genomic cytosine methylation levels are associated with an overgrowth phenotype of bovine fetuses derived by in vitro techniques. *Biol Reprod* 2004; 71: 217-223
201 St John JC, Moffatt O, D'Souza N. Aberrant heteroplasmic transmission of mtDNA in cloned pigs arising from double nuclear transfer. *Mol Reprod Dev* 2005; 72: 450-460
202 Bowles EJ, Campbell KH, St John JC. Nuclear transfer: preservation of a nuclear genome at the expense of its associated mtDNA genome(s). *Curr Top Dev Biol* 2007; 77: 251-290
203 Hiendleder S. Mitochondrial DNA inheritance after SCNT. *Adv Exp Med Biol* 2007; 591: 103-116
204 Smith LC, Thundathil J, Filion F. Role of the mitochondrial genome in preimplantation development and assisted reproductive technologies. *Reprod Fertil Dev* 2005; 17: 15-22
205 Shiels PG, Kind AJ, Campbell KH, Waddington D, Wilmut I, Colman A, Schnieke AE. Analysis of telomere lengths in cloned sheep. *Nature* 1999; 399: 316-317
206 Betts D, Bordignon V, Hill J, Winger Q, Westhusin M, Smith L, King W. Reprogramming of telomerase activity and rebuilding of telomere length in cloned cattle. *Proc Natl Acad*

Sci USA 2001; 98: 1077-1082
207 Lanza RP, Cibelli JB, Blackwell C, Cristofalo VJ, Francis MK, Baerlocher GM, Mak J, Schertzer M, Chavez EA, Sawyer N, Lansdorp PM, West MD. Extension of cell life-span and telomere length in animals cloned from senescent somatic cells. *Science* 2000; 288: 665-669
208 Tian XC, Xu J, Yang X. Normal telomere lengths found in cloned cattle. *Nat Genet* 2000; 26: 272-273
209 Jiang L, Carter DB, Xu J, Yang X, Prather RS, Tian XC. Telomere lengths in cloned transgenic pigs. *Biol Reprod* 2004; 70: 1589-1593
210 Betts DH, Perrault SD, Petrik J, Lin L, Favetta LA, Keefer CL, King WA. Telomere length analysis in goat clones and their offspring. *Mol Reprod Dev* 2005; 72: 461-470
211 Jeon HY, Hyun SH, Lee GS, Kim HS, Kim S, Jeong YW, Kang SK, Lee BC, Han JY, Ahn C, Hwang WS. The analysis of telomere length and telomerase activity in cloned pigs and cows. *Mol Reprod Dev* 2005; 71: 315-320
212 Schaetzlein S, Rudolph KL. Telomere length regulation during cloning, embryogenesis and ageing. *Reprod Fertil Dev* 2005; 17: 85-96
213 Kuhholzer-Cabot B, Brem G. Aging of animals produced by somatic cell nuclear transfer. *Exp Gerontol* 2002; 37: 1317-1323
214 Miyashita N, Shiga K, Yonai M, Kaneyama K, Kobayashi S, Kojima T, Goto Y, Kishi M, Aso H, Suzuki T, Sakaguchi M, Nagai T. Remarkable differences in telomere lengths among cloned cattle derived from different cell types. *Biol Reprod* 2002; 66: 1649-1655
215 Xu J, Yang X. Will cloned animals suffer premature aging-the story at the end of clones' chromosomes. *Reprod Biol Endocrinol* 2003; 1: 105
216 Betts DH, King WA. Genetic regulation of embryo death and senescence. *Theriogenology* 2001; 55: 171-191
217 Schaetzlein S, Lucas-Hahn A, Lemme E, Kues WA, Dorsch M, Manns MP, Niemann H, Rudolph KL. Telomere length is reset during early mammalian embryogenesis. *Proc Natl Acad Sci USA* 2004; 101: 8034-8038
218 Clark AJ, Ferrier P, Aslam S, Burl S, Denning C, Wylie D, Ross A, de SP, Wilmut I, Cui W. Proliferative lifespan is conserved after nuclear transfer. *Nat Cell Biol* 2003; 5: 535-538
219 Palmquist DL, Beaulieu AD, Barbano DM. Feed and animal factors influencing milk fat composition. *J Dairy Sci* 1993; 76 (6): 1753-1771
220 Research Institute for Animal Scicence in Biochemistry & Technology, Outline of studies on productive performance of somatic cell cloned cattle (FY1999 to 2001); 2002
221 Tian XC, Kubota C, Sakashita K, Izaike Y, Okano R, Tabara N, Curchoe C, Jacob L, Zhang Y, Smith S, Bormann C, Xu J, Sato M, Andrew S, Yang X. Meat and milk compositions of bovine clones. *Proc Natl Acad Sci USA* 2005; 102: 6261-6266
222 Yang X, Tian XC, Kubota C, Page R, Xu J, Cibelli J, Seidel G Jr. Risk assessment of meat and milk from cloned animals. *Nat Biotechnol* 2007; 25: 77-83
223 Research Institute for Animal Scicence in Biochemistry & Technology, Outline of studies

on productive performance derived from progeny of cloned cattle produced by SCNT, 2008

224 Walsh MK, Lucey JA, Govindasamy-Lucey S, Pace MM, Bishop MD. Comparison of milk produced by cows cloned by nuclear transfer with milk from non-cloned cows. *Cloning Stem Cells* 2003; 5: 213-219

225 Mackle TR, Petch SF, Bryant AM, Auldist MJ. Variation in the characteristics of milkfat from pasture-fed dairy cows during spring and the effects of grain supplementation. *NZ J Agic Res* 1997; 40: 349-359

226 Mackle TR, Bryant AM, Petch SF, Hooper RJ, Auldist MJ. Variation in the composition of milk protein from pasture-fed dairy cows in late lactation and the effect of grain and silage supplementation. *NZ J Agic Res* 1999; 42: 154

227 Auldist MJ, Johnston KA, White NJ, Fitzsimons WP, Boland MJ. A comparison of the composition, coagulation characteristics and cheesemaking capacity of milk from Friesian and Jersey dairy cows. *J Dairy Res* 2004; 71: 51-57

228 Tome D, Dubarry M, Fromentin G. Nutritional value of milk and meat products derived from cloning. *Cloning Stem Cells* 2004; 6: 172-177

229 Yamaguchi M, Itoh M, Ito Y, Watanabe S. A 12-month feeding study of reproduction/development in rats fed meat/milk powder supplemented diets derived from the progeny of cloned cattle produced by somatic cell nuclear transfer. *J Reprod Dev*. 2008; 54(5): 321-334

230 Kataoka H, Tsuda A, Tsuda Y, Baba A, Yoshida H, Hirasawa R, Tobimatsu Y, Nishiguchi M, Semma M, Ito Y. A novel method for induction and detection of anaphylactic reaction using the mouse abdomonal wall (AW method). *Biol Pharm Bull* 1997; 20(6): 714-716