Successful cloning of pigs from somatic cells has been reported [1–3]. Because of the repeatable and predictable potential of somatic cell cloning in pigs, application of this procedure to the pork industry can be expected. Several studies on pig cloning, including those on genetically modified cloned pigs, have been performed, although the success rate of cloning in pigs has been extremely low [4–8]. Furthermore, this research has been propelled by the need for animals for biomedical or experimental purposes [9–11]. However, to apply cloning to the pork industry, we must examine the growth and health of pigs along with the safety of meat products not only of cloned pigs but also their progenies [12]. Although several studies have been reported on cloned pigs and their progenies bred with cloned boars [13, 14], little is known about cloned female pigs and their progenies [15]. Somatic cell cloning is expected to be successful in the production of superior commercial breeds and the conservation of superior economic traits. Clone-derived foods, such as meat or milk, have been analyzed in several nations, and their safety has been reported [16–18]. However, most nations still have restrictions on the entry of products from cloned animals into the food chain because little data exists on the safety of clone products. Thus, more research is required to apply somatic cell cloning for the conservation of superior genes and for pig meat production. To demonstrate an application of somatic cell cloning in conserving superior genes and pig meat production, we produced cloned pigs and compared the growth, reproductive performance, and meat quality characteristics of Landrace cloned female pigs with those of their progenies. In this paper, we discuss the normality of the clones and their progenies and note that abnormalities found in somatic cell clones do not transmit to their progenies.

Materials and Methods

Animals and cloning

Donor cell culture and nuclear transfer were carried out at PRIME
TECH (Tsuchiura, Japan), and each step of the nuclear transfer procedure was observed, as previously described [1]. A Landrace sow was used as a donor pig. We obtained somatic cells for nuclear transfer from the ear of a female purebred Landrace pig. The ear skin was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 20% fetal bovine serum to prepare the fibroblasts, which would be used as nucleus donors. Cultures were established by plating cells for five passages. Cell culture was continued for 14 days without media replacement until confluent in DMEM. Cells were then removed from the plate using 0.25% trypsin and resuspended in DMEM until nuclear transfer. Oocytes from cross-bred gilts were collected from a slaughterhouse and used as recipients of nuclear transfer. Following oocyte maturation, cumulus cells were removed in Hepes-buffered TCN-199 medium (Gibco, Grand Island, NY, USA) supplemented with 0.1% hyaluronidase. Enucleation was performed by micromanipulation. Donor nuclei were injected into enucleated oocytes using piezo-actuated microinjection (PRIME TECH) in porcine zygote medium 3 [19]. The reconstructed pre-embryos were incubated at 38.5°C for 3 h before electroactivation. The electroactivation was performed with a single current pulse of 150 kV/cm for 99 μsec in activation solution (0.28 M D-mannitol, 0.05 mM CaCl₂, 0.1 mM MgSO₄ and 0.01% (w/v) bovine serum albumin).

Experimental animals were kept at the Ibaraki Prefecture Livestock Experiment Center, and standard experimental procedures were performed with the approval of the Institutional Research Committee.

Embryo transfer and mating of offspring

At 8–10 months of age, the estrus cycles of the recipient gilts were synchronized with hormone injections. They received 2 ml of prostaglandin F₂α analogues containing 92 μg/ml of cloprostenol sodium (Planate, Intervet, Osaka, Japan) on the first day of the early pregnancy period between 21 and 45 days of gestation. They received the same dosage of PGF₂α analogues with 1,000 IU of equine chorionic gonadotropin (Serotropin, ASKA Pharmaceutical, Tokyo, Japan) on day 2 and 500 IU of human chorionic gonadotropin (Gonatropin, ASKA Pharmaceutical, Tokyo, Japan) on day 5. The onset of estrus was observed on day 6. Approximately 200 cloned embryos at the 2- to 16-cell stage were surgically transferred into the oviducts of seven anesthetized recipient gilts at 2 days after the onset of estrus. One recipient sow produced a litter of five cloned female piglets (clones A–E) via vaginal delivery. DNA microsatellite markers were used to confirm the genetic identity of the cloned piglets to the donor sow. The body weights from birth to 8 weeks of age of the clones and their progenies are presented in Table 1. We obtained five cloned piglets born alive from one litter. Three of the five cloned piglets were healthy. The cloned piglets grew similarly to the Landrace controls. The cloned pigs received the experimental barn’s normal feeding program; that is, the piglets were weaned at about 3 weeks of age. They received creep feed until weaning. The weaned pigs were fed feed in mash form ad libitum until 5 months of age. At 9–10 months of age, two of the cloned gilts were bred with a Landrace boar. Because Clone C did not express a distinct estrus, we performed an anatomy examination. Clone C did not show any phenotypic abnormalities in the ovaries or uterus, which grew normally, and was diagnosed instead with pituitary basophilism. The gilts consumed 2.5 to 3.0 kg of feed, which was restricted based on their live weight, until parturition. After parturition, F1 progenies were used for a progeny test. At 7–8 months of age, two of the F1 gilts were bred with a Duroc or Large White boar.

Progeny test, carcass analysis, pathological analysis and chromosomal analysis

We measured the body weight, growth rate and feed conversion and performed a pathological analysis of the anatomy for any abnormal pigs or pigs that died following birth. Three of the five cloned pigs were allocated to a growth test. F1 (8 males and 9 females) and F2 (9 males and 10 females) progenies were used for the growth test and a carcass quality test. All male piglets were castrated before the growth test. The progeny test was performed under the standard conditions for testing of pork products in pigs [20]. Pigs were slaughtered at the plant in the experimental center. Data collected included the carcass length, thickness of back fat and number of vertebrae. During carcass dissection into prime cuts, the ratio of the cuts and the loin eye area were measured. Dressing percentages were calculated from both the live and carcass measures of weight. The basic micronutrients in a loin sample were evaluated at the Tsukuba Food Evaluation Center (Tsuchiura, Japan), and the amino acid composition of the same sample was analyzed by ion-exchange chromatography by the Japan Frozen Foods Inspection Corporation (Tokyo, Japan). We compared the micronutrient and amino acid compositions of samples from clones and progenies to a national composite data source [21, 22]. Pathological analyses of all pigs were performed at the National Institute of Animal Health. Chromosome analysis was carried out on peripheral lymphocytes from cloned pigs (n = 2) and their offspring (6 F1s and 4 F2s) according to the method described previously [23].

Statistical analysis: The Student’s t-test was used for statistical analysis. A P value <0.05 was considered significant.

Results and Discussion

Pathological and chromosomal analyses

Five cloned female piglets (clones A–E) were born after 115 days of gestation from a recipient Landrace sow. Three of the five cloned piglets (clones A, B and C) were used for the growth test. No specific abnormalities of pathological status were observed in clones A or B or in the F2 progenies. Clone C was diagnosed with pituitary basophilism. The details regarding the pathological anatomy of clone C are described in the section concerning reproductive performance. Clone D died on the day of birth and had an umbilical hernia with a deformed leg. Beckwith-Wiedemann syndrome is an epigenetic disorder usually present at birth that commonly includes abdominal wall defects such as hernia in humans [24]. Umbilical hernia is also reported in cloned cattle but is not always caused by epigenetic abnormalities peculiar to cloned animals [25]. Of the congenital abnormalities in pigs, umbilical hernia or ruptures are the most common and are considered to have very low heritability. No umbilical hernia was observed in the progenies in this study. It was not clear whether the umbilical hernia in clone D was caused by epigenetic or genetic disorder. However, it should be noted that
the causes of death for the clones were widely known abnormalities that are common in conventionally bred pigs. Clone E died of pleuropneumonia 139 days after birth. Clone E was diagnosed as being infected with *Actinobacillus pleuropneumonia* and *Actinomyces pyogenes*. Park et al. suggested that cerebromeningitis and hemodynamic disorder are major risk factors for sudden death in cloned piglets [26], but the piglets that survived to adulthood did not show any abnormalities. Although we cannot exclude the possibility that somatic cell-cloned pigs are susceptible to respiratory infections, the pathological data suggested that the death of clone E was because of common diseases. Although all F1 progenies were phenotypically normal, six of the 20 F1 progenies had periarteritis in the heart or spleen. Periarteritis was not observed in the clones or F2 progenies. Periarteritis is an inflammation of the outer membrane of an artery, which results in a mild lesion and is occasionally seen in the organs of pigs. It may be induced by autoimmune inflammation. Porcine Reproductive & Respiratory Syndrome (PRRS) is associated with periarteritis of the lung, heart and kidney [27]. However, since pigs usually are not subjected to precise pathological analysis under the meat production system, little is known about the incidence of periarteritis in conventional pigs. In this case, the F1 progenies were usually not subjected to precise pathological analysis under the meat production system. In order to prevent periarteritis, it may be induced by the delayed growth in the LW controls caused the inferior daily gain to those of the F1 progenies (clone × Landrace). This age coincides with the lactation period, and the daily intake of milk in clones was considered to be superior to the controls because of the small litter size. However, this difference was not observed at 8 weeks of age, perhaps because the nursing advantage for the cloned piglets was limited to the early period of growth.

With regard to the progenies, there were no differences between F1 progenies and Landrace controls in body weight until 8 weeks of age. Since the litter size for cloned sows was within the normal range for the breed [29], which ranged from 7 to 11, the nursing conditions can be assumed to be the same for the F1 progenies as for the Landrace controls. Martin et al. reported that progeny derived from the mating of cloned parents show normal growth performance in the pre-weaning period [30]. The data from their study support those reported by the Japan Pork Producers Association [29]. F2 progenies [F1 (clone × Landrace) × Large White] and LW (Landrace × Large White) crossbred pigs. h, i Values without common characters in the same row differ significantly (P<0.05). h, i Values without common characters in the same row differ significantly (P<0.01).

### Growth performance

The clones were phenotypically normal and grew normally. The body weights of the clones were not different from those of the Landrace controls (Table 1). However, the body weights of the clones from 3 to 5 weeks of age were significantly greater than those of the F1 progenies (clone × Landrace). This age coincides with the lactation period, and the daily intake of milk in clones was considered to be superior to the controls because of the small litter size. However, this difference was not observed at 8 weeks of age, perhaps because the nursing advantage for the cloned piglets was limited to the early period of growth.

With regard to the progenies, there were no differences between F1 progenies and Landrace controls in body weight until 8 weeks of age. Since the litter size for cloned sows was within the normal range for the breed [29], which ranged from 7 to 11, the nursing conditions can be assumed to be the same for the F1 progenies as for the Landrace controls. Martin et al. reported that progeny derived from the mating of cloned parents show normal growth performance in the pre-weaning period [30]. The data from their study support those reported by the Japan Pork Producers Association [29]. F2 progenies [F1 (clone × Landrace) × Large White] and LW (Landrace × Large White) crossbred pigs. h, i Values without common characters in the same row differ significantly (P<0.05). h, i Values without common characters in the same row differ significantly (P<0.01).

### Table 1. Body weight from birth to 8 weeks of age in clones and their progenies

| Animal          | n  | Day 0 (kg)  | 1 week (kg) | 3 weeks (kg) | 5 weeks (kg) | 8 weeks (kg) |
|-----------------|----|-------------|-------------|--------------|--------------|-------------|
| Clone¹          | 3  | 1.34 ± 0.14 | 3.70 ± 0.34 | 8.30 ± 0.35H | 14.55 ± 0.45H | 26.37 ± 3.35 |
| F1 (clone × L)² | 17 | 1.48 ± 0.19 | 3.18 ± 0.40I | 6.18 ± 0.78I | 10.92 ± 1.41I | 23.03 ± 3.68 |
| Landrace³       | 100| 1.60 ± 0.31 | 2.94 ± 0.74 | 6.79 ± 1.40  | 11.91 ± 2.24  | 23.24 ± 4.34 |
| F2 (F1 × D)⁴    | 11 | 1.62 ± 0.16B | 2.57 ± 0.26H | 6.69 ± 0.26  | 11.01 ± 1.21B | 29.27 ± 2.56 |
| LD⁵             | 7  | 1.87 ± 0.27  | 4.65 ± 0.69H | 7.53 ± 0.99  | 12.66 ± 1.75  | 29.07 ± 4.94 |
| F2 (F1 × W)⁶    | 8  | 1.74 ± 0.18H | 3.77 ± 0.56H | 7.70 ± 1.03H | 13.64 ± 1.34H | 29.25 ± 2.58H |
| LW⁷             | 14 | 1.48 ± 0.19I | 2.51 ± 0.36  | 5.50 ± 0.93I | 8.95 ± 1.24  | 21.04 ± 2.31 |

Values are expressed as means ± SD. ~ Cloned females derived from Landrace donor cells. ~ F1 pigs from the cloned sows mated with a Landrace (L) boar. ~ Purebred Landrace pigs. ~ F2 pigs from the F1 sows mated with a Duroc (D) boar. ~ Landrace × Duroc crossbred pigs. ~ F2 pigs from the F1 sows mated with a Large White (W) boar. ~ Landrace × Large White crossbred pigs. h, i Values without common characters in the same row differ significantly (P<0.05). h, i Values without common characters in the same row differ significantly (P<0.01).
Compositions

Carcass characteristics and micronutrient and amino acid compositions of the loin samples of the clones and their progenies were examined for safety. In a risk assessment of the products derived from cloned animals, the FDA and EFSA reported that edible products from healthy clones pose no increased food consumption risks or the progenies. The compositions showed no unusual nutrient values in either the clones and progenies compared with the controls. These values depend on the sampling regions, and the sample sites may have been different in the controls. With regard to the amino acid composition, no abnormal values were observed in the clones or their progenies. The compositions of essential amino acids were within the normal range in both clones and progenies [16, 17]. Although only a very small number of animals were examined, the basic micronutrient and amino acid compositions showed no unusual nutrient values in either the clones or the progenies.

To facilitate the integration of cloning techniques into the livestock industry, the food products from these cloned animals were closely examined for safety. In a risk assessment of the products derived from cloned animals, the FDA and EFSA reported that edible products from healthy clones pose no increased food consumption risks relative to comparable products from non-cloned animals [16, 17]. A similar assessment was reported for the progeny of cloned cattle in Japan [18]. However, most nations still restrict entry of cloned pigs from healthy clones and progenies because the data are insufficient and the experiments have only been conducted over a short term.

Reproductive performance

Clones A and B reached puberty at 4–5 months of age, and they conceived by natural breeding with a Landrace boar at 9–10 months of age. The gestation periods and litter sizes of the clones and the F1 progenies are presented in Table 3. The clones and F1 progenies were born after 115–116 and 113–116 days of gestation, respectively. Their litter sizes and numbers of progenies born alive ranged from 8 to 11 and were similar to those of Landrace controls. This result supports observations from the literature in which gestation period and average litter size were similar to those of non-cloned pigs [15, 33]. Basophilism is a syndrome caused by increased production of ACTH from a tumor of the adrenal cortex or the anterior lobe of the pituitary gland. In the present study, no microscopic lesions or tumors were observed. It was unclear as to whether the basophilism in clone C was related to epigenetic abnormalities of cloned animals. However, we may conclude that cloned female pigs and their F1 progenies are capable of normal reproductive performance.

Carcass characteristics and micronutrient and amino acid compositions

The carcass characteristics of the progenies are presented in Table 4. There were no significant differences in carcass traits between F1 or F2 progenies and their controls, with the exception of dressing percentage (P<0.01), back fat thickness at the shoulder (P<0.05) and loin length (P<0.01). The reasons for these differences were unclear, but the differences have no biological significance and resulted in pigs with values in the normal range. The micronutrient and amino acid compositions of the loin samples of the clones and progenies are presented in Table 5. There were no differences in basic micronutrients between clones and their progenies. However, the levels of energy and fat tended to be lower both in the clones and the progenies compared with the controls. These values depend on the sampling regions, and the sample sites may have been different in the controls.

With regard to the amino acid composition, no abnormal values were observed in the clones or their progenies. The compositions of essential amino acids were within the normal range in both clones and progenies [21, 22]. Although only a very small number of animals were examined, the basic micronutrient and amino acid compositions showed no unusual nutrient values in either the clones or the progenies.
Foot-and-mouth disease (FMD) has recently appeared in many countries, particularly in East Asia. FMD is an extremely contagious disease of cloven-hoofed animals, notably in pigs. The disease is dreadful, causing economic disaster, including loss of superior genetic resources, and thus FMD is the target of international regulations and tightened trade restrictions. Cloning technology has the potential for conserving superior genetic traits, which are beneficial to the pig industry, including provision against FMD. Thus, further long-term study or more data will be required for the application of cloning to the pig industry. It is expected that, in future years, additional analysis may be conducted to elucidate the safety of products from cloned animals given the importance of cloning technology for food production.

Acknowledgments

The authors wish to thank the staff at the Ibaraki Prefecture Livestock Research Center for animal care and technical help throughout the experiment.

References

1. Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata H, Hanada H, Perry ACF. Pig cloning by microinjection of fetal fibroblast nuclei. Science 2000; 289: 1188–1190. [Medline] [CrossRef]
2. Polejaeva IA, Chen SH, Vaugh TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayars DL, Colman A, Campbell KH. Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 2000; 407: 86–90. [Medline] [CrossRef]
3. Bethanhauser J, Forbsberg E, Augenstein M, Betthauser P, Golueke M, Koppang K, Leistner NA, Mack P, Misica M, Mullins Y, Ziegenhock M, Voelker DB. Production of nuclear transfer-derived swine that express the enhanced green fluorescent protein. Anim Biotechnol 2001; 12: 173–181. [Medline] [CrossRef]
nuclear transfer (SCNT) pig clones are phenotypically similar to non-cloned pigs. Nat Biotechnol 2005; 23: 437–445. [Medline] [CrossRef]

19. Yoshikawa K, Suzuki C, Tanaka A, Anas IM, Iwamura S. Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. Biol Reprod 2002; 66: 112–119. [Medline] [CrossRef]

20. Japan pork producers association. Standard pork products test. 2005; Japan (In Japanese).

21. U. S. Food and Drug Administration. National Nutrient Data Base for Standard Reference, Release 22, 2009; USA.

22. Ministry of Education Culture, Sports, Science and Technology-JAPAN. Standard Tables of Food Composition in JAPAN. 2010; Japan (In Japanese).

23. Hanada H, Takeda K, Tagami T, Nirasawa K, Akagi S, Adachi N, Takahashi S, Iizake Y, Iwamoto M, Fuchimoto D, Miyashita N, Kubo M, Onishi A, King WA. Chromosome stability in the cattle clones derived by somatic cell nuclear transfer. Mol Reprod Dev 2005; 71: 36–44. [Medline] [CrossRef]

24. Delhaan MR, Niemitz EL, McNeil DE, Brandenburg SA, Lee MP, Feinberg AP. Epigenetic alterations of HIV and LT1 distinguish patients with Beckwith-Wiedemann syndrome with cancer and birth defects. Am J Hum Genet 2002; 70: 604–611. [Medline] [CrossRef]

25. Chavatte-Palmer P, Remy D, Cordonnier N, Richard C, Issenman H, Laigre F, Heyman Y, Mililot JP. Health status of cloned cattle at different ages. Cloning Stem Cells 2004; 6: 94–100. [Medline] [CrossRef]

26. 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 cerebroembolism and hemorrhagic disorder: a major cause of sudden death in somatic cell cloned piglets. Proteomics 2005; 5: 1928–1939. [Medline] [CrossRef]

27. Straw BE, Zimmerman JJ, D’Allaire SD, Taylor DJ. Disease of Swine. 9th ed. Blackwell Publishing 2006; 397.

28. Tamashiro KL, Fukayama T, Akihisa H, Yamazaki Y, Lacey 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. [Medline] [CrossRef]

29. Japan Pork Producers Association. Results of Pig Pregnies Test in Japan. 2007 (In Japanese).

30. 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. [Medline] [CrossRef]

31. 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. [Medline] [CrossRef]

32. Onishi A. Cloning of pigs from somatic cells and its prospects. Cloning Stem Cells 2002; 4: 233–249. [Medline] [CrossRef]

33. 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. [Medline] [CrossRef]