in vivo *30*: 617-622 (2016)

Preservation and Reproduction of Microminipigs by Cloning Technology

SATOKO ENYA[1], TATSUO KAWARASAKI[2], MASAYOSHI OTAKE[1], AKIHISA KANGAWA[1], HIROHIDE UENISHI[3], SATOSHI MIKAWA[4], TAKASHI NISHIMURA[5], YASUSHI KUWAHAWA[5] and MASATOSHI SHIBATA[1]

[1]*Swine and Poultry Department, Shizuoka Prefectural Research Institute of Animal Industry, Swine and Poultry Research Center, Shizuoka, Japan;*
[2]*School of Agriculture, Tokai University, Kumamoto, Japan;*
[3]*Animal Bioregulation Unit, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Ibaraki, Japan;*
[4]*Animal Genome Unit, Division of Animal Breeding and Reproduction, Institute of Livestock and Glassland Science, National Agriculture and Food Research Organization, Ibaraki, Japan;*
[5]*Fuji Micra Inc., Fujinomiya, Shizuoka, Japan*

Abstract. *Background/Aim: Microminipigs have been maintained in small populations of closed colonies, involving risks of inbreeding depression and genetic drift. In order to avoid these risks, we assessed the applicability of cloning technology. Materials and Methods: Male and female clones were produced from a stock of cryopreserved somatic cells, obtaining offspring by means of natural mating. Phenotypic and genotypic characteristics of original microminipigs, clones and their offspring were analyzed and recorded. Results and Conclusion: Clones presented characteristics similar to those of the cell-stock data. Although the body weight of clones tended to be heavier than that of the cell-stock data, body weights of their offspring were similar to those of previous reports. Thus, cloned microminipigs have the potential to be a valuable genetic resource for reproduction and breeding. Our proposed methodology might be useful to provide a large number of animals with adequate quality from a limited population with sufficient genetic diversity.*

Microminipigs were produced as experimental animals by Fuji Micra Inc. in Shizuoka, Japan (1). At 6 months, their body weight reaches about 10 kg (2), which is less than that of conventional miniature pig breeds, such as the Göttingen miniature pig (1). Some Japanese researchers have already used them in pharmaceutical and toxicological studies (3-8).

Microminipigs resulted from breeding between pot-bellied pigs and other types of miniature pigs (1), which might have favoured genetic diversity. In fact, Ando *et al*. (9) described 11 haplotypes of swine leukocyte antigen (SLA) class I and II in a resource herd of microminipigs. These pigs have been suggested as a potential genetic resource to develop animals for experimental use.

Microminipigs have been maintained in small populations, increasing the risk for inbreeding depression and progressive genetic drift. With a constant genetic background, even though microminipigs may have a population with relevant genetic diversity, they cannot be supplied in large numbers for commercial use.

On the other hand, few reports have described successful cloning of pigs from somatic cells (10-12). Therefore, microminipig cloning may be used to preserve an invaluable genetic resource and provide a large number of full-sib groups from multiple cloned-parents.

In this study, we collected and cryopreserved somatic cells from individual microminipigs; we recorded some of their particular phenotypic characteristics and determined their genotypes associated with coat-colour genotypes and SLA haplotypes. Subsequently, we produced pig clones and enable their natural mating, registering the same characteristics explored in the original sample of microminipigs (*i.e.* phenotypic characteristics, coat colour genotypes, SLA haplotypes), in addition to biochemical parameters. Based on this, we assessed the applicability of the cloning technology for microminipig reproduction and breeding.

Correspondence to: Satoko Enya, Swine and Poultry Department, Shizuoka Prefectural Research Institute of Animal Industry, Swine and Poultry Research Center, 2780 Nishikata, Kikugawa, Shizuoka 439-0037, Japan. Tel/Fax: +81 537352291, e-mail: fujiyork@sp-exp.pref.shizuoka.jp

Key Words: Miniature pig, experimental animal, SCNT, clone, offspring, full-sib, cryopreservation, risk, inbreeding, genetic drift.

Materials and Methods

Characterization of microminipigs and cell stock. Sixty microminipigs were used in this study, obtained from a closed herd produced by Fuji Micra Inc. (Shizuoka, Japan). We recorded their weights at birth, coat colour and snout characteristics; snouts were classified as either straight (Figure 1A and C) or undershot-jaws snouts (Figure 1B).

Somatic cells from microminipigs were obtained by means of ear biopsy. After quick rinses in 75% ethanol and PBS, ear tissues were minced into pieces of 2-3 mm. Six to eight ear samples of individual pigs were cultured with 10 μl Cellmatrix Type I-P drop (Nitta Gelatin Inc., Osaka, Japan) in 35-mm cell-culture dishes and incubated in 2 ml of Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Paisley, UK) with 10% embryonic stem cell foetal bovine serum (ES Cell FBS; Invitrogen, Grand lsland, NY, USA) and antibiotics. Dishes were then placed in a humidified incubator at 37˚C with 5% CO_2, changing the culture medium every other day. Fibroblast outgrowth was collected from the dishes with 0.25% trypsin-EDTA and passaged at 1:2 split ratios. Somatic cells obtained at passages 1 to 3 were frozen in CELLBANKER (Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan).

Genotype analysis of extension locus and dominant white locus. To detect mutations in the Extension (*E*) locus, which encodes the melanocortin receptor 1 (MC1R) (13) and in the Dominant white (*W*) locus, associated with the *Kit* genes (14), we used polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) method, a technique previously described by Okumura (15).

Haplotype analysis of the SLA. Alike previous studies (16), SLA haplotypes were explored. The amplification of 11 microsatellite markers was performed *via* PCR using selected primers (*Slams064, Slams073, SlamsA00, SlamsA16, SlamsA05, SlamsA14, SlamsA13, Slams044, Slams043, Slams095* and *Slams047*).

Somatic cell cloning. From the somatic cells of donor microminipigs, we selected homozygous SLA haplotype and silver coat colour from one male and one female. Ovaries from prepubertal gilts were obtained at a local abattoir. Cumulus-oocyte complexes (COCs) were collected from follicles (3-6 mm in diameter) in Medium 199 (Sigma-Aldrich, St. Louis, MO, USA) with 1 mg/ml BSA, 20 mM Hepes and 100 U/ml penicillin G potassium. About 50 COCs were cultured in 500 μl of maturation medium HP-POM (Research Institute for the Functional Peptides, Yamagata, Japan), adding 1 mM dibutyryl cAMP, 10 IU/ml equine chorionic gonadotropin (eCG) and 10 IU/ml human chorionic gonadotropin (hCG), in four-well dishes for 20-22 h. The COCs were subsequently cultured for 24 h with the same maturation medium (HP-POM) but without the dibutyryl cAMP and the hormones. This latter cultivation was carried out at 39˚C under conditions of 5% O_2, 5% CO_2 and 90% N_2. Matured oocytes with the first polar body were cultured in PZM5 (Research Institute for the Functional Peptides) supplemented with 0.1 μg/ml demecolcine, 35 mM sucrose, 25 mM Hepes and 5% FBS for 10-30 min. A first polar body and cytoplasm, presumptively containing nucleus, were pushed out through a slit on the zona pellucida using a fine glass needle. Fibroblasts synchronized by serum starvation for 72 h were used as nuclear donors. A single donor cell was inserted into the perivitelline space of an enucleated oocyte. Donor cell-oocyte complexes were then placed in a 0.28 M mannitol solution containing 100 μM $MgSO_4$ and 0.01% (w/v) polyvinyl alcohol (PVA) and held between two electrode needles. Membrane fusion was induced by applying a single direct current pulse (200 V/mm, 20 μs) and a pre- and post-pulse alternating current field of 3 V and 1 MHz for 3 s, respectively. Enucleated oocytes, reconstructed with fibroblasts, were then activated by a single direct current pulse of 150 kV/cm for 99 μs in 0.28 M mannitol solution (50 μM $CaCl_2$, 100 μM $MgSO_4$ and 0.01% (w/v) PVA). Activated oocytes were cultured in PZM5 supplemented with 4 μg/ml cytochalasin B and 1 mM valproic acid ((VPA); Wako, Osaka, Japan) under paraffin oil in a plastic Petri dish for 2 h at 39˚C, with an atmosphere of 5% CO_2 and humidified air. The embryos were subsequently cultured in PZM5 with 1 mM VPA for 18 h and, again, cultured in PZM5 without the additive agent until transfer. On day two, after nuclear transfer, the reconstructed embryos were transferred to the oviducts of prepubertal gilts, which weighted 100 to 120 kg. Recipient gilts were Duroc, Large White or crossbreeds. All recipients were in oestrus, induced by an intramuscular injection of 1,000 IU eCG and 750 IU hCG.

Offspring of the cloned microminipigs. Offspring was obtained by natural mating of cloned microminipigs (5 females, 5 males). We recorded their coat colour and snout characteristics. Genotype and haplotype analysis were performed using the aforementioned methods. For biochemical analysis of the offspring, serum samples were obtained *via* jugular venipuncture of 6-month-old microminipigs; 13 blood parameters were analysed in the Health Science Research Institute (Shizuoka, Japan).

Management of cloned microminipigs and their offspring. Cloned microminipigs and their offspring were housed individually or in groups in pens of at least 0.54 m^2/head, at temperatures ranging from 16˚C to 36˚C (30˚C to 36˚C until they reached the age of 2 weeks), with humidity ranging from 60% to 90% and in constant 12/12 light/dark cycles. The animals were weaned at the age of 4 weeks and body weights were measured once a week (0-26 weeks of age). Feeding management was set according to their age: 4- to 7-week-old animals were provided with 3-6% of their body weight of a weaning diet (>81.0% total digestible nutrients (TDN), >18.0% crude protein (CP); JA Higashinihon Kumiai Shiryou Co., Ltd, Gunma, Japan); animals between 8 and 12 weeks of age were provided with 3-6% of their body weight of a piglet diet (>77.0% TDN, >16.0% CP, JA Higashinihon Kumiai Shiryou Co., Ltd); and, finally, pigs aged 13 weeks and above were provided with 2-5% of their body weight of a laboratory miniature pig diet (mean=318 kcal/100 g, mean=15.7% CP; Oriental Yeast Co., Ltd., Tokyo, Japan). They had free access to tap water at all ages. All procedures were approved by the Animal Care and Use Committee of Shizuoka Prefectural Research Institute of Animal Industry, Swine and Poultry Research Centre, which comply with the Guidelines for Proper Conduct of Animal Experiments from the Science Council of Japan (2006).

Results

Characterization of microminipigs and cell stock. From the 60 microminipigs included in this study, most of them presented straight snouts (70.0%) and only a 30.0% presented undershot-jaw snouts. We recorded three coat

Figure 1. *Microminipig clones and their offspring. (A) Cloned male with silver coat colour and straight snout, similar to that from the cell-stock data. The SLA haplotype A/A, Extension locus E/E and Dominant white locus i/i, were also identical to the types from their cell-stock data. (B) Cloned female with silver coat colour and undershot-jaw snout, similar to that from cell-stock data. The SLA haplotype A/A, the Extension locus E/E and the Dominant white locus i/i were also identical to the types from cell-stock data. (C) Offspring with silver coat colour and straight snout, with SLA haplotype A/A, Extension locus E/E and the Dominant white locus i/i.*

colours: 33.3% of the microminipigs were described as white, 23.3% as black and 43.3% presented a silver coat colour.

Genotype analysis of Extension locus and Dominant white locus. Considering the genotypes associated with coat colour, all microminipigs studied presented an *E/E* genotype in the Extension locus. Microminipigs with black or silver coat colour had an *i/i* genotype in the Dominant white locus and white coloured microminipigs presented *I/i* or *I/I* genotypes.

Haplotype analysis of the SLA. Based on the combination of the 11 microsatellite markers for the SLA locus, we described nine SLA haplotypes, classified from A to I (Table I). Nine (15.0%) microminipigs showed homozygous genotype (A/A) and 32 (53.3%) showed heterozygous genotype (A/-) for haplotype A, totalling in a frequency of 41.7% for the haplotype A. On the other hand, homozygous genotypes of C and D summed 1.7%, with haplotype frequencies of 26.7% and 6.7%, respectively. Haplotype frequencies of B, E, F, G, H and I are 5.8%, 1.7%, 3.3%, 10.8%, 1.7% and 1.7%, respectively.

Microminipig clones. Five clones were reproduced using the cryopreserved donor somatic cell-stock from one of the microminipig males (Figure 1A). All male microminipig clones presented a silver coat and straight snout, similar to the characteristics registered in their microminipig donors. Their SLA haplotype was A/A, the Extension locus *E/E* and the Dominant white locus *i/i*, identical to the genotypes registered in their microminipig donors. The average body weight at birth of male clones was 594 g, which is 1.5-times heavier than that of the data from the cell-stock (400 g) (Table II).

Five clones were reproduced using the cryopreserved donor somatic cell-stock from one of the microminipig females (Figure 1B). All female microminipig clones presented a silver coat and undershot-jaw snout, similar to the characteristics from the cell-stock data. Their SLA haplotype was A/A, the Extension locus *E/E* and the Dominant white locus *i/i*, identical to the genotypes from the microminipig cell-stock data. The average body weight at birth of the female clones was 549 g, which is 1.2-times heavier than that of their microminipig cell-stock data (460 g) (Table II). We did not observe any external abnormality in any of the microminipig clones.

Offspring derived from microminipig clones. Sperm mobility of the cloned males (>6 months old) was above 70%. Cloned females presented clear signs of oestrus after 8 months of age. The behavioural oestrus continued for 2.16±0.47 days and the length of oestrous cycle was estimated to be 21.9±0.3 days. By natural mating of five male and five female clones, we obtained 50 males and 55 females in 21 parturitions. Their gestation period was 112.8±1.7 days; total and alive litter sizes were 6.39±0.99 and 4.57±1.26, respectively. Offspring presented silver or black coat colour, straight snout and no observable abnormalities (Figure 1C). Their SLA haplotype was A/A, the Extension locus *E/E* and the Dominant white locus *i/i*.

The body weight at birth of the offspring was significantly lower compared with that of male ($p<0.01$) and female clones ($p<0.05$). The body weight of the offspring at 6 months was significantly lower than that in male clones ($p<0.01$). The difference of that with female clones was not statistically significant (Table II). Concerning blood biochemical analysis, we randomly chose seven males and nine females (Table III). Most of the parameters observed showed lesser differences between male and female offsprings, except for total cholesterol, triglycerides, calcium and potassium, that showed higher levels in females.

Table I. *SLA: Estimated haplotypes (A∼I) and amplification fragment lengths of 11 microsatellite markers (Slams).*

Haplotype	Microsatellite markers: Slams										
	064	073	A00	A16	A05	A14	A13	044	043	095	047
A	271	229	212	325	256	257	215	284	225	230	237
B	271	229	212	325	256	257	215	280	247	230	237
C	267	241	212	301	246	261	203	276	247	230	237
D	271	241	210	319	239	263	203	280	247	230	237
E	256	241	210	335	250	261	200	272	237	228	228
F	266	243	212	325	252	259	197	266	229	236	231
G	261	243	210	325	238	257	221	280	249	230	229
H	271	243	212	325	252	259	197	266	229	236	231
I	251	241	210	325	238	273	201	276	253	232	237

Data correspond to the cell-stock obtained from 60 microminipigs. Values are presented in PCR size product (bp).

Table II. *Average body weight of microminipig clones and their offspring at birth and at 6 months.*

Animal	Gender	Birth				6 months			
		Weight (g)	CV (%)	Range (g)	n	Weight (kg)	CV (%)	Range (kg)	n
Clone	Male	594±75**	12.6	455-652	5	14.0±1.8**	12.9	11.1-15.5	5
Clone	Female	549±152*	27.8	319-763	5	14.6±4.5	31.1	8.0-20.5	5
Offspring	Male	313±61	19.6	177-429	50	8.3±1.6	19.3	5.1-11.7	22
Offspring	Female	308±61	19.8	166-471	55	10.7±2.0	18.6	5.9-14.5	20

Weight values are presented as mean±standard deviation. Statistical analysis of the differences were assessed using *F*-test, Student's *t*-test or Welch's *t*-test, considering significance at $p<0.05$. Offspring (50 males and 55 females) were obtained from 21 parturitions after natural mating between five male and five female clones. Data of 6-month-old offspring were obtained from 20 males and 22 females chosen randomly. *$p<0.05$; **$p<0.01$; both * and **represent significant differences between parent-clones and their offspring.

Table III. *Serum biochemical parameters analysed in the offspring of microminipig clones.*

Parameter		Male (n=7)				Female (n=9)			
				Range				Range	
	Unit	Mean	SD	Min	Max	Mean	SD	Min	Max
Alkaline phosphatase	U/ l	471	162	246	704	432	108	309	708
Total protein	g/dl	6.9	0.4	6.3	7.5	7.0	0.3	6.6	7.5
Albumin	g/dl	4.8	0.2	4.5	5.2	4.6	0.2	4.3	5.0
Albumin-globin ratio	ratio	2.3	0.4	1.7	2.8	2.0	0.2	1.7	2.4
Total cholesterol	mg/dl	76	11	61	94	115***	13	92	142
Triglycerides	mg/dl	28	7	18	38	43**	9	29	58
Urea nitrogen	mg/dl	8	2	4	10	9	2	7	11
Creatinine	mg/dl	0.7	0.2	0.5	1.0	0.8	0.1	0.6	1.0
Phosphorus	mg/dl	7.1	0.9	5.9	8.2	7.5	0.6	6.6	8.4
Calcium	mg/dl	10.5	0.3	10.0	11.1	10.9*	0.4	10.3	11.5
Sodium	mEq/l	147	4	141	154	146	1	144	147
Potassium	mEq/l	5.1	0.6	4.2	6.3	6.1**	0.4	5.2	6.6
Chloride	mEq/l	100	2	97	103	99	2	96	103

Statistical analysis of the differences between males and females were assessed using *F*-test and Student's *t*-test or Welch's *t*-test, considering significance at $p<0.05$. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ all represent values with significant difference.

Discussion

There are two main strategies to preserve pig genetic resources. The first one is the maintenance of live animals, which is the most certain method, but requires high costs associated with space, facilities and management. The second strategy is the cryopreservation of germ cells and embryos. Currently, preservation of frozen semen is the most popular and useful method to conserve swine genetic resources. The cryopreservation of oocytes or embryos is still being studied, exploring the potential applications for pig reproduction (17, 18). These aforementioned two strategies are not adequate for the maintenance of genetic resources from a limited population, even though it presents significant genetic diversity. In order to avoid inbreeding depression and to ensure the phenotype and genotype traits of the offspring, here we proposed a new method for the production of clonal microminipigs as experimental animals. This new method consists in the cryopreservation of somatic cells, with the registration of the corresponding phenotype and genotype, followed by the production of offspring from somatic-cell cloned parents. This method can preserve the genetic resource of microminipigs *per se*.

In order to confirm the diversity of the original microminipigs, we observed phenotypic characteristics and genotypes associated with SLA and coat colours. The phenotypic characteristics, such as coat colour and snout type, differed among individuals; genotypes also presented relevant diversity. SLA is one of the histocompatibility antigens and has important roles associated with sensitivity to various infectious pathogens. In our study, we described nine SLA haplotypes (Table I), similar to a previous study that described 11 SLA haplotypes (9). Concerning coat-colour genotypes, the original microminipigs presented two types of Dominant white locus. Thus, the studied cell-stock of original microminipigs showed relevant genetic diversity and turned out to be an important genetic resource.

Phenotypic characteristics and genetic outcomes of cloned microminipigs reflected those of the donor microminipigs, as previously described in a study with Landrace pigs (19). On the other hand, the body weights at birth of cloned microminipigs were heavier compared with that from the donor microminipigs (Table II). Although we did not measure the body weights of the donor pigs at the age of 6 months, we assume that they would be lower compared with that in clones, considering that Kawaguchi *et al*. (2) reported the average body weight of 6-month-old pigs kept at the Fuji Micra Inc.to be below 10 kg. The explanation for this body weight differences between microminipig clones and donors could be related with several factors. Uterine, environmental and/or epigenetic factors can produce different maternal effects on genetically identical animals (20). Previous studies have reported that miniature pig clones present greater body weights at birth when the recipients are conventional breeds (21, 22). In these cases, the differences in body weights could be attributed to the different body sizes (21) or small litter sizes (22) of the recipients.

Based on natural mating, we obtained 105 individuals, which presented no apparent abnormalities. The body weights of the offspring produced *via* natural mating in our study were similar to those reported by Kawaguchi *et al*. (2). In cloned mice, it was suggested that epigenetic effects tended to disappear in the offspring produced by natural mating of the clones (23). This seems to be replicated in our outcomes; in the cloned-microminipig offspring we also described a normal growth performance, which had also been reported previously for swine species (24-27). Finally, as shown in Table III, the serum biochemical parameters of the offspring were similar to that from previously described parameters of microminipigs (2).

In conclusion, cloned microminipigs have the potential to be valuable genetic resources for reproduction and breeding. By applying the presented methodology, particular desired characteristics from microminipigs can be selected and propagated from a variety of heterogeneous populations. Furthermore, this methodology might be useful to provide a large number of animals with adequate quality from a limited population with sufficient genetic diversity.

Acknowledgements

The Authors gratefully acknowledge Drs. Takato Nakamura, Kimio Bamba and Makoto Mori in Shizuoka University and Dr. Naohiko Okumura for their valuable suggestions in preparing this manuscript. They appreciate the technical support of Ms. Yukiko Otsu (Shizuoka Perfectural Toubu Public Center) during early days of the study. They are also indebted to all staffs in Shizuoka Prefectural Research Institute of Animal Industry, Swine and Poultry Research Center for taking good care of animals.

References

1 Kaneko N, Itoh K, Sugiyama A and Izumi Y: Microminipig, a non-rodent experimental animal optimized for life science research: Preface. J Pharmacol Sci *115*: 112-114, 2011.

2 Kawaguchi H, Yamada T, Miura N, Takahashi Y, Yoshikawa T, Izumi H, Kawarasaki T, Miyoshi N and Tanimoto A: Reference values of hematological and biochemical parameters for the world smallest microminipigs. J Vet Med Sci *74*: 933-936, 2012.

3 Murayama N, Kaneko N, Horiuchi K, Ohyama K, Shimizu M, Ito K and Yamazaki H: Cytochrome P450-depedent drug oxidation activity of liver microsomes from Microminipigs, a possible new animal model for humans in non-clinical studies. Drug Metab Pharmacokinet *24*: 404-408, 2009.

4 Mogi M, Toda A, Iwasaki K, Kusumoto S, Takehara H, Shimizu M, Murayama, Izumi H, Utoh M and Yamazaki H: Simultaneous pharmacokinetics assessment of caffeine, warfarin, omeprazole, metoprolol, and midazolam intravenously or orally administered to microminipigs. J Toxicol Sci *37*: 1157-1164, 2012.

5 Yoshikawa T, Takahashi Y, Kawaguchi H, Utsunomiya S, Miura N, Izumi H, Miyoshi N and Tanimoto A: A dermal phototoxicity study following intravenous infusion administration of ciprofloxacin hydrochloride in the novel microminipigs. Toxicol Pathol *41*: 109-113, 2013.

6 Higashi E, Ando A, Iwano S, Murayama N, Yamazaki H and Miyamoto Y: Hepatic microsomal UDP-glucuronosyltransferase (UGT) activities in the microminipig. Biopharm Drug Dispos *35*: 313-320, 2014.

7 Ohno K, Tomizawa A, Jakubowski J.A, Mizuno M and Sugidachi A: Characterization of platelet aggregation responses in microminipigs: Comparison with miniature pigs and the influence of dual antiplatelet administration of aspirin plus prasugrel. Thromb Res *135*: 933-938, 2015.

8 Guruge KS, Noguchi M, Yoshioka K, Yamazaki E, Taniyasu S, Yoshioka M, Yamanaka N, Ikezawa M, Tanimura N and Sato M: Microminipigs as a new experimental animal model for toxicological studies: comparative pharmacokinetics of perfluoroalkyl acids. J App Toxicol *36*: 68-75, 2016.

9 Ando A, Imaeda N, Ohshima S, Miyamoto A, Kaneko N, Takasu M, Shiina T, Kulski JK, Inoko H and Kitagawa H: Characterization of swine leukocyte antigen alleles and haplotypes on a novel miniature pig line, Microminipig. Anim Genet *45*: 791-798, 2014.

10 Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata T, Hanada H and Perry AC: Pig cloning by microinjection of fetal fibroblast nuclei. Science *289*: 1188-1190, 2000.

11 Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S and Ayares DL: Cloned pigs produced by nuclear transfer from adult somatic cells. Nature *407*: 86-90, 2000.

12 Betthauser J, Forsberg E, Augenstein M, Childs L, Eilertsen K, Enos J, Forsythe T, Golueke P, Jurgella G and Koppang R: Production of cloned pigs from *in vitro* systems. Nat Biotechnol *18*: 1055-1059, 2000.

13 Kijas J, Wales R, Törnsten A, Chardon P, Moller M and Andersson L: Melanocortin receptor 1 (MC1R) mutations and coat color in pigs. Genetics *150*: 1177-1185, 1998.

14 Marklund S, Kijas J, Rodriguez-Martinez H, Rönnstrand L, Funa K, Moller M, Lange D, Edfors-Lilja I and Andersson L: Molecular basis for the dominant white phenotype in the domestic pig. Genome Res *8*: 826-833, 1998.

15 Okumura N: Shin shokuhinbunsekihou. 1 ed. Kourin. Tokyo. 342-355, 2006.

16 Tanaka M, Ando A, Renard C, Chardon P, Domukai M, Okumura N, Awata T and Uenishi H: Development of dense microsatellite markers in the entire SLA region and evaluation of their polymorphisms in porcine breeds. Immunogenetics *57*: 690-696, 2005.

17 Misumi K, Hirayama Y, Egawa S, Yamashita S, Hoshi H and Imai K: Successful production of piglets derived from expanded blastocysts vitrified using a micro volume air cooling method without direct exposure to liquid nitrogen. J Rep Dev *59*: 520-524, 2013.

18 Somfai T, Yoshioka K, Tanihara F, Kaneko H, Noguchi J, Kashiwazaki N, Nagai T and Kikuchi K: Generation of live piglets from cryopreserved oocytes for the first time using a defined system for *in vitro* embryo production. PLoS One *9*: e97731, 2014.

19 Chen CH, Jiang BH, Huang SY, Yang TS, Lee KH, Tu CF and Wu SC; Genetic polymorphisms, growth performance, hematological parameters, serum enzymes, and reproductive characteristics in phenotypically normal Landrace boars produced by somatic cell nuclear transfer. Theriogenology *80*: 1088-1096, 2013.

20 Archer GS, Dindot S, Friend TH, Walker S, Zaunbrecher G, Lawhorn B and Piedrahita JA: Hierarchical phenotypic and epigenetic variation in cloned swine. Biol Reprod *69*: 430-436, 2003.

21 Wei H, Qing Y, Pan W, Zhao H, Li H, Cheng W, Zhao L, Xu C, Li H, Li S, Ye L, Wei T, Li X, Fu G, Li W, Xin J and Zeng Y: Comparison of the efficiency of Banna miniature inbred pig somatic cell nuclear transfer among different donor cells. PLoS One *8*: e57728, 2013.

22 Schmidt M, Kragh PM, Li J, Du Y, Lin L, Liu Y, Bogh IB, Winther KD, Vajta G and Callesen H: Pregnancies and piglets from large white sow recipients after two transfer methods of cloned and transgenic embryos of different pig breeds. Theriogenology *74*: 1233-1240, 2010.

23 Tamashiro KL, Wakayama T, Akutsu H, Yamazaki Y, Lachey JL, Wortman MD, Seeley RJ, D'Alessio DA, Woods SC, Yanagimachi R and Sakai RR: Cloned mice have an obese phenotype not transmitted to their offspring. Nat Med *8*: 262-267, 2002.

24 Adachi N, Yamaguchi D, Watanabe A, Miura N, Sunaga S, Oishi H, Hashimoto M, Oishi T, Iwamoto M, Kubo M and Orishi A: Growth, reproductive performance, carcass characteristics and meat quality in f1 and f2 progenies of somatic cell-cloned pigs. J Reprod Dev *60*: 100-105, 2014.

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

26 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 *52*: 583-590, 2006.

27 Hu K, Kong Q, Zhao Z, Lu X, Liu B, Li Y, Wang H and Liu Z: Assessment of reproduction and growth performance of offspring derived from somatic cell cloned pigs. Anim Sci J *83*: 639-643, 2012.

Received June 20, 2016
Revised July 11, 2016
Accepted July 12, 2016