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Production of Transgenic and Non-Transgenic Clones in Miniature Pigs by Somatic Cell Nuclear Transfer

Mayuko KUROME¹⁾, Takayuki ISHIKAWA²⁾, Ryo TOMII¹⁾, Satoshi UENO¹⁾, Arata SHIMADA¹⁾, Hajime YAZAWA²⁾ and Hiroshi NAGASHIMA¹⁾

¹⁾Laboratory of Developmental Engineering, Department of Life Science, Meiji University, Kawasaki 214-8571 and

²⁾Laboratory Animal Research Station, Nippon Institute for Biological Science, Hokuto 408-0041, Japan

Abstract. Miniature pigs have been recognized as valuable experimental animals in various fields such as medical and pharmaceutical research. However, the amount of information on somatic cell cloning in miniature pigs, as well as genetically modified miniature pigs, is much less than that available for common domestic pigs. The objective of the present study was to establish an efficient technique of cloning miniature pigs by somatic cell nuclear transfer. A high pregnancy rate was achieved following transfer of parthenogenetic (3/3) and cloned (5/6) embryos using female miniature pigs in the early pregnancy period as recipients after estrus synchronization with prostaglandin F2 alpha analog and gonadotrophins. The production efficiency of the cloned miniature pigs using male and female fetal fibroblasts as nucleus donors was 0.9% (2/215 and 3/331, respectively). Cloned miniature pigs were also produced efficiently (7.8%, 5/64) by transferring reconstructed embryos into the uteri of common domestic pigs. When donor cells transfected with the green fluorescent protein (GFP) gene were used in nuclear transfer, the production efficiency of the reconstructed embryos and rate of blastocyst development were comparable to those obtained by non-transfected cells. When transfected cell-derived reconstructed embryos were transferred to three common domestic pig recipients, all became pregnant, and a total of ten transgenic cloned miniature pigs were obtained (piglet production efficiency: 2.7%, 10/365). Hence, we were able to establish a practical system for producing cloned and transgenic-cloned miniature pigs with a syngeneic background.

Key words: Embryo transfer, *In vitro* matured oocyte, Miniature pig, Nuclear transfer, Transgenic

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In recent years, techniques for producing cloned pigs by somatic cell nuclear transfer (SCNT) have been actively utilized to produce genetically modified pigs. A number of cloned pigs have been produced, including those with genes for GFP [1–8], as well as alpha1,3-galactosyltransferase knockout pigs [9–16]. In this manner, genetically modified pigs are being used in a wide variety of biomedical fields, ranging from basic research to organ transplantation.

To date, more than ten breeds of miniature pigs have been established as experimental and companion animals [17]. As far as the use of pigs as experimental animals is concerned, miniature pigs are smaller and easier to handle than common domestic pigs, and they have been used in a variety of fields, such as medical and pharmacological research [10, 18–25]. However, compared with common domestic pigs, far less information regarding the production of cloned and genetically modified pigs is available for miniature pigs [10, 13, 14, 26–31].

In the present study, we conducted a series of experiments including (i) validation of an estrus synchronization procedure for miniature pig recipients, (ii) examination of the developmental competence of nuclear transfer embryos reconstructed with various nuclear donor cells and (iii) comparison of miniature pigs and common domestic pigs as recipients. The results indicate that it is possible to produce cloned miniature pigs using common domestic

pigs as the recipients and to produce genetically modified-cloned miniature pigs with a level of efficiency comparable to that for conventionally cloned miniature pigs.

Materials and Methods

Animal care

All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Meiji University (IACUC-03-005).

Chemicals

All chemicals were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA), unless otherwise indicated.

Preparation of donor cells

Primary culture fetal fibroblasts were used as nucleus donor cells in SCNT. Fetal fibroblasts were obtained from 30-day-old fetuses that were produced by mating NIBS strain miniature pigs (Laboratory Animal Research Station, Nippon Institute for Biological Sciences, Hokuto, Japan). The head, viscera and bones of each fetus were removed and washed three times using Dulbecco's phosphate buffered saline (D-PBS). The washed tissue was finely chopped into fragments smaller than 1 mm in size, and the tissue fragments were placed in 35-mm culture dishes (Falcon Primaria; Becton Dickinson, Franklin Lakes, NJ, USA) and incubated for 30 min in a humidified incubator at 38.5 C with 5% CO₂. After con-

firming that the tissue fragments adhered to the culture dish, a high-glucose Dulbecco's modified Eagle medium (GIBCO Invitrogen, Grand Island, NY, USA) containing 15% fetal calf serum (FCS; JRH Biosciences, Lenexa, KS, USA) was gently added. At 2–3 days later, the tissue fragments were removed, and the cells adhering to the bottom of the culture dish were cultured. After three to four passages, the cells were stored frozen according to the conventional methods.

Transfer of the GFP gene into miniature pig fetal fibroblasts

The GFP-neo fusion gene under control of the pgk promoter (pQBI-pgk, AFP2043; Qbiogene, CA, USA) was used for gene transfer. Using FuGENE-6 transfection reagent (1814443; Roche, Indianapolis, IN, USA), gene transfer was carried out according to the manufacturer's instructions. Briefly, a mixture of the transfection reagent and pQBI-pgk GFP vector, which was linearized using a restriction enzyme (*PvuI*; 3:1 mixture), was added to cells (1.0×10^5 cells/35-mm culture dish) and incubated for 24 h. According to the conventional methods, the cells recovered from the dish were seeded in 48-well plates ($2\text{--}3 \times 10^3$ cells/well). At 48 h after gene transfer, the cells were incubated in standard culture medium including 250 $\mu\text{g}/\text{ml}$ of G418 (Geneticin, 11811–023; GIBCO). Colonies resistant to G418 were subcultured in 4-well plates. Cells exhibiting GFP fluorescence under fluorescence microscopy were selected, and after two or three passages, the resulting cells were stored frozen in liquid nitrogen (6.0×10^5 cells/ml) until SCNT.

In vitro maturation of oocytes

Ovaries were collected at a local abattoir and transported to the laboratory in D-PBS containing 75 $\mu\text{g}/\text{ml}$ potassium penicillin G, 50 $\mu\text{g}/\text{ml}$ streptomycin sulfate and 0.1% (w/v) polyvinyl alcohol (PVA). Cumulus-oocyte complexes (COCs) were collected from the antral follicles (3.0–6.0 mm in diameter) by aspiration. COCs displaying at least three layers of compacted cumulus cells were selected and cultured in NCSU23 medium [32] supplemented with 0.6 mM cysteine, 10 ng/ml epidermal growth factor, 10% (v/v) porcine follicular fluid, 75 $\mu\text{g}/\text{ml}$ potassium penicillin G, 50 $\mu\text{g}/\text{ml}$ streptomycin sulfate, 10 IU/ml equine chorionic gonadotropin (eCG; Teikoku Hormone Medical, Tokyo, Japan) and 10 IU/ml human chorionic gonadotropin (hCG; Teikoku Hormone Medical). The COCs were cultured for 22 h with eCG and hCG and then for 20 h without these hormones in a humidified atmosphere of 5% CO_2 and 95% air at 38.5 C.

In vitro matured (IVM) oocytes with expanded cumulus cells were treated with 1 mg/ml hyaluronidase dissolved in Tyrode's lactose medium supplemented with 10 mM Hepes and 0.3% (w/v) polyvinylpyrrolidone (Hepes-TL-PVP) and were denuded of cumulus cells by gentle pipetting. Oocytes showing evenly granulated ooplasm and extrusion of the first polar body were selected for the subsequent experiments.

Induction of parthenogenetic development of oocytes

IVM oocytes were washed twice in an activation solution consisting of 0.3 M mannitol, 50 μM CaCl_2 , 100 μM MgSO_4 and 0.01% PVA (300 mOsm), placed between the two wire electrodes (1 mm apart) of a fusion chamber slide and overlaid with 0.1 ml of

the activation solution. A single direct current (DC) pulse of 150 V/mm was applied for 100 μsec using a somatic hybridizer (SSH-1; Shimadzu, Kyoto, Japan). The activated oocytes were treated with 5 $\mu\text{g}/\text{ml}$ cytochalasin B (CB) for 3 h to suppress extrusion of the pseudo-second polar body.

Somatic cell nuclear transfer

SCNT was performed using IVM oocytes as recipient cytoplasts, as described previously [7, 33]. Enucleation was performed using a chemically assisted method developed by Yin *et al.* [34]. Oocytes were cultured in NCSU23 medium supplemented with 0.1 $\mu\text{g}/\text{ml}$ demecolcine, 0.05 M sucrose (Nacalai Tesque, Tokyo, Japan) and 4 mg/ml bovine serum albumin for 0.5–1 h and then enucleated by aspirating the first polar body and adjacent cytoplasm using a bevelled pipette (30 μm in diameter) in Hepes-TL-PVP containing 0.1 $\mu\text{g}/\text{ml}$ demecolcine, 5 $\mu\text{g}/\text{ml}$ CB and 10% FCS. When a protrusion was observed on the surface of an oocyte, it was removed along with the polar body. Enucleation was confirmed by staining the cytoplasts with 5 $\mu\text{g}/\text{ml}$ bisbenzimidazole (Hoechst 33342).

The nuclear donor cells were used after cell cycle synchronization by serum starvation for 48 h. A single donor cell was inserted into the perivitelline space of each enucleated oocyte. Donor cell-oocyte complexes were placed in a 280 mM mannitol (Nacalai tesque) solution (pH 7.2) containing 0.15 mM MgSO_4 , 0.01% (w/v) PVA and 0.5 mM Hepes and held between two electrode needles. Membrane fusion was induced with a somatic hybridizer (SSH-1; Shimadzu) by applying a DC pulse (200 V/mm, 20 μsec) and a pre- and post-pulse alternating current (AC) field of 5 V, 1 MHz for 5 sec, respectively. The reconstructed embryos were cultured in NCSU23 for 1–1.5 h and then subjected to electrical activation. Activation of the reconstructed embryos was performed as described above.

Culture of reconstructed embryos

The developmental capacities of nuclear transfer embryos reconstructed with the non-transfected and transfected fetal fibroblast cells were first examined by *in vitro* culture. At least three independent experiments were replicated. *In vitro* culture of the embryos was performed in 20 μl droplets of Porcine zygote medium (PZM) [35] under paraffin oil in a plastic Petri dish maintained under a humidified atmosphere of 5% CO_2 , 5% O_2 and 90% N_2 at 38.5 C. For culturing embryos beyond the morula stage, 10% (v/v) FCS was added to the medium.

Cleavage and blastocyst formation of the reconstructed embryos were monitored during culture for 7 days. Blastocysts derived from the transfected cells were examined under fluorescent microscopy (TE300; Nikon, Tokyo, Japan) for GFP expression. On Day 7 the blastocyst cell number was also counted after fixation and staining.

Estrus synchronization of recipient pigs and embryo transfer

Sexually mature NIBS miniature pigs (aged 8–12 months with body weights of 28–32 kg) and prepubertal crossbred common domestic pigs (Large white/Landrace $\times$ Duroc aged 6.5–7 months with body weights of 100–105 kg) were used as embryo transfer recipients.

Pregnant miniature pigs were aborted by an i.m. injection of 0.3 mg of a prostaglandin F2 alpha analog (Cloprostenol; Estrumate, Schering-Plough Animal Health, Tokyo, Japan) between 25 and 40 days after mating followed by a second injection of 0.15 mg of Cloprostenol 24 h later. Two hundred and fifty international units (IU) of eCG were administered (i.m.) at the same time as the second Cloprostenol injection. In recipients with confirmed abortion, 120 IU of hCG was administered (i.m.) approximately 72 h after the eCG, and estrus was checked on the same or next day.

Prepubertal common domestic pigs were treated with a single i.m. injection of 1,000 IU eCG to induce estrus. Ovulation was induced by an i.m. injection of 1,500 IU hCG given 71 h after the injection of eCG.

Embryos were surgically transferred into the oviducts or uterine horns of the recipients after making a mid-ventral incision under general anesthesia.

Experiment 1: Confirmation of the estrus synchronization procedures for the miniature pig recipients

In order to identify a suitable time period for transferring the nuclear transfer embryos to the recipient miniature pigs, a preliminary experiment was conducted using parthenogenetic embryos prepared using IVM oocytes. Studies of cloned embryo transfer in the common domestic pigs have clarified that conception is successful when the age of the embryos is 1–2 days ahead of the number of days following ovulation [7, 15, 36, 37]. A preliminary experiment was conducted to ascertain whether this also applies to miniature pigs.

Parthenogenetic embryos approximately 24 (day 1) or 48 h (day 2) after activation were transferred to the oviducts of recipients 48 or 72 h after hCG administration. Based on ovulation in pigs occurring 40–42 h after hCG administration, the estrus cycle stage of the recipients was equivalent to the day of ovulation (immediately after ovulation) or the day after ovulation. At 22 days after transfer, a laparotomy was performed on the recipients to check for fetuses.

Experiment 2: In vitro assessment of the developmental capacity of cloned miniature pig embryos reconstructed with non-transfected or transfected fetal fibroblasts

The production efficiency of nuclear transfer embryos reconstructed using miniature pig fetal fibroblasts as nuclear donors and the developmental capacity of nuclear transfer embryos were investigated. Male and female fibroblasts (#3 and #5, respectively) confirmed to have normal chromosome compositions ($\geq 70\%$) were used as the nuclear donor cells.

Of these cells, female cells (#5) were transfected with the GFP gene, and the developmental capacities of the embryos reconstructed using the transfected cells were compared with those of embryos reconstructed using non-transfected cells.

Experiment 3: In vivo assessment of the developmental capacity of cloned miniature pig embryos

In this experiment, the developmental capacity of miniature pig embryos after transfer was investigated. The same three cells used in the *in vitro* experiment (Exp. 2) were used as nuclear donors.

Miniature pigs and common domestic pigs were used as recipients to ascertain the effects of different recipients on the production efficiency of cloned piglets.

Embryos reconstructed using non-transfected cells were cultured for 1 or 2 days, and morphologically normal embryos (1-cell stage on Day 1 and 2- to 8-cell stage on Day 2) were selected and transferred to the ampullae of the oviducts of the recipient miniature pigs. In addition, some reconstructed embryos were cultured *in vitro* for 3 days, and morphologically normal embryos in the 4-cell to early morula stages were transferred to the uterine horns of common domestic pigs approximately 96 h after hCG administration.

After 1 to 2 days of culture, morphologically normal embryos reconstructed using the transfected cells as the donor cells were selected and transferred to the ampullae of the oviducts of common domestic pigs about 48 h after hCG administration. At 113 days after embryo transfer, a caesarean section was performed to recover full term fetuses. The fetuses were sacrificed to ascertain systemic tissue GFP expression, and transgenic fetuses were identified.

Statistical analysis

For proportional data, differences between groups were analyzed using the χ^2 test. For blastocyst cell number data, differences between groups were determined using the Student's *t*-test. The level of significance was set at $P < 0.05$.

Results

Experiment 1: Confirmation of the estrus synchronization procedures for miniature pig recipients

Table 1 summarizes the transfer of parthenogenetic embryos to recipients in relation to estrus synchronization. When Day 1 or Day 2 embryos were transferred to recipients on the day of ovulation or one day after ovulation, conception was achieved with comparable efficiency. Based on this finding, either Day 1 embryos or Day 1 and Day 2 embryos together were transferred to recipient miniature pigs on the expected day of ovulation in the following cloned embryo transfer experiments.

Experiment 2: In vitro development of cloned miniature pig embryos reconstructed with non-transfected or transfected fetal fibroblasts

The production efficiency of nuclear transfer embryos reconstructed using miniature pig fetal fibroblasts as nuclear donors and the *in vitro* development of nuclear transfer embryos is summarized in Table 2.

Regardless of the donor cell type, i.e., non-transfected cells (male #3, female #5) and transfected cells (origin #5), the fusion rate of recipient oocytes and donor cells was high, ranging from 83.5% (66/79) to 90.7% (98/108). The rates of blastocyst formation were nearly equal in the three donor cell types and ranged from 13.6% (9/66) to 19.1% (9/47). However, the average number of blastocyst cells for the embryos reconstructed using transfected cells was significantly lower than that for the embryos reconstructed using non-transfected cells ($P < 0.05$).

Table 1. Development of IVM-derived parthenogenetic pig embryos after transfer into estrus synchronized miniature pig recipients

Age of embryos (day) ¹	Days after ovulation in recipients ²	Synchrony ³	No. of embryos transferred	No. of fetuses recovered (%)
1	0	+1	40	10 (25.0)
2	0	+2	40	6 (15.0)
2	1	+1	56	15 (27.0)

¹Days after activation. ²Ovulation was assumed to occur approximately 40–42 h after hCG injection. ³(Days after embryos activation)–(Days after ovulation in recipient).

Table 2. *In vitro* development of cloned embryos reconstructed with transfected or non-transfected donor cells in miniature pigs

Donor cells	Fusion rates	Normal cleavage (%)	Blastocyst formation (%)	Cell number in blastocysts (%)
#3 (male)	98/108 (90.7%)	68/98 (69.4) ^a	14/98 (14.3)	83.1 ± 15.2 ^a
#5 (female)	66/79 (83.5%)	56/66 (84.8) ^b	9/66 (13.6)	73.4 ± 8.7 ^a
#5 transfected with GFP gene	48/55 (87.3%)	22/47 (46.8) ^c	9/47 (19.1)	41.3 ± 5.1 ^b

^{abc}P<0.05.

Table 3. Production of cloned miniature pigs by nuclear transfer of fetal fibroblast cells

Donor cells	Recipients	No. of recipients	No. of embryos transferred	Pregnancy	Delivery	Offspring (stillborn)	No. offspring / No. embryos transferred ¹ (%)
#3 (male)	Mini-pig ²	3	314	2	1	2	2/215 (0.9)
#5 (female)	Mini-pig ²	3	331	3	2	3 (2)	3/331 (0.9)
	Common pig ^{3*}	2	131	1	1	5 (2)	5/64 (7.8)
#5 transfected with GFP gene	Common pig ^{2*}	3	365	3	3	10 (1)	10/365 (2.7)

¹No. of embryos transferred in the pregnant recipients. ²Oviductal transfer. ³Uterine transfer. *Common domestic pigs.

Experiment 3: Development of cloned miniature pig embryos after transfer

The results of transferring the cloned embryos into recipient pigs are shown in Table 3. Because influence from the recipient, such as luteal hypoplasia in pregnancy failure, can not be excluded, the calculations of cloning efficiency in Table 3 only considered the number of transferred embryos that led to a pregnancy.

Embryos that were reconstructed using male and female miniature pig fibroblasts were each transferred into three recipient miniature pigs. Following transfer of cloned embryos derived from male fibroblasts (#3), two recipients became pregnant, and while one miscarried (Day 86), the other delivered two live piglets (Fig. 1A). Following transfer of the cloned embryos reconstructed using female fibroblasts (#5), all recipients became pregnant, although

one miscarried (Day 78). The other two recipients delivered one live piglet and two stillborn piglets. Among the 5 recipients that became pregnant, the ratio of cloned piglets produced (including stillborn piglets) to the total number of transferred embryos for the two types of donors (#3, male, and #5, female) was 0.9% (2/215 and 3/331, respectively).

A microsatellite analysis was conducted by sending samples obtained from the cloned miniature pigs, donor cells and recipients to a company that specializes in porcine parentage verification (Prescribe Genomics, Ibaraki, Japan), and the results confirmed that the piglets were clones of the donor cells (data not shown).

A total of 131 embryos reconstructed using female donor cells (#5) were cultured for three days and then transferred to the uteri of two common domestic pigs. One recipient became pregnant and

Fig. 1. Production of cloned miniature pigs by nuclear transfer of fetal fibroblast cells. Newborn cloned miniature pigs delivered from a recipient of the same strain (A) or common domestic pig (B). C: Newborn transgenic cloned piglet obtained by caesarian section of a common domestic pig recipient. D: A pair of cloned miniature pigs grown from the piglets shown in A at 4 months of age. The scale bars in A, B and C represent 5 cm.

delivered three live and two stillborn piglets (Fig. 1B). For the recipient that delivered, the efficiency rate of piglet production in relation to the total number of transferred embryos was 7.8% (5/64).

Female fibroblasts (#5) were transfected with the GFP gene and then used as donor cells in SCNT. Following transfer of reconstructed embryos to the oviducts of three common domestic pigs, all the pigs became pregnant, and 10 live transgenic miniature piglets were obtained (including one stillborn piglet; Fig. 1C). The efficiency rate of piglet production in relation to the total number of transferred embryos was 2.7% (10/365).

The birth weights of live offspring, including full term fetuses obtained by caesarean section, were also compared. The average birth weights of the non-transgenic cloned piglets produced by transferring #3- and #5-derived embryos into recipient miniature pigs were 400.0 and 450.0 g (only one piglet for the #5-derived clones), respectively. The average body weight (465.0 g) of the non-transgenic cloned piglets produced by transferring #5-derived nuclear transfer embryos into the uterine horns of recipient common domestic pigs was comparable to that of piglets produced from recipient miniature pigs. Furthermore, the average birth weight (422.7 g) of the transgenic-cloned piglets originating from the #5 cells transfected with the GFP gene and produced by recipient common domestic pigs was comparable to that of the non-transgenic piglets.

Unfixed small tissue pieces were collected from the brains, hearts, livers, lungs, kidneys, intestines, peritonea and skeletal muscle of cloned piglets derived from GFP-transfected cells. Clear green fluorescence was observed in all tissues except for the brain. Of these tissues, primary cultured cells were established from the heart, lung, kidney, small intestine and skeletal muscle. Observa-

Fig. 2. Production of transgenic cloned miniature pigs from fetal fibroblast cells transfected with the GFP gene. A: Fetal fibroblast cells transfected with the GFP gene. B: Nuclear donor cells expressing GFP. C, D: A cloned blastocyst reconstructed with a transfected donor cell as observed under bright field (C) and fluorescence microscopy (B-excitation; D). E, F: Primary culture cells established from the heart (E) and lung (F) of a transgenic cloned miniature pig expressing GFP (A–F, $\times 200$ magnification).

tion of these cells under fluorescent microscopy confirmed clear green fluorescence in all samples. Figure 2 shows GFP-transfected nuclear donor cells, cloned blastocysts obtained by nuclear transfer and primary cultured cells originating from a cloned piglet.

Discussion

In recent years, a number of groups have reported successful SCNT using common domestic pigs [4, 7, 8, 15, 34, 36–46]. If SCNT became widely available for miniature pigs, the usefulness of miniature pigs as experimental animals would markedly improve. Since there is as yet no evident inbred strain of miniature pigs, cloned miniature pigs should allow highly accurate and precise studies using animals of the same genetic background.

The production of cloned piglets by SCNT requires several

steps, including preparation of donor cells and recipient oocytes along with artificial activation and culture of reconstructed embryos, and ultimately the cloned embryos are transferred to surrogates. While several groups have documented estrus cycle synchronization and embryo transfer in miniature pigs, there is no established method [23, 26, 47–52]. In our conventional embryo transfer efforts in common domestic pigs, eCG is administered to pigs just prior to puberty, and hCG is administered 72 h later. Stable results have been achieved using this technique [7, 15, 36, 37]. Similarly, several studies have reported estrus synchronization in miniature pigs by administering eCG and hCG just prior to puberty [47–49, 51], but the optimal time for gonadotropin administration in NIBS miniature pigs prior to puberty has not been established. Furthermore, NIBS miniature pigs reach puberty early at 3 to 4 months after birth, and it has not been clarified whether or not gilts at these ages are suitable as recipients of embryo transfer. Hence, in the present study, we synchronized estrus by artificially inducing abortion in pregnant miniature pigs. Estrus synchronization combined with induced abortion by administration of a prostaglandin F2 alpha analog and gonadotropin has been performed for years in common domestic pigs [53]. The present study suggests that this technique is also effective in miniature pigs and that estrus can be synchronized in a reliable manner.

In addition, as is the case with embryo transfer into common domestic pigs, the results of the present study confirmed that conception occurs when the embryonic age is 1 to 2 days ahead of the number of days after ovulation. Thus, the basic technique for using miniature pigs as recipients for cloned pig production has been established.

The fact that the present study showed that cloned miniature piglets could be successfully produced using common domestic pigs as recipients is significant for the utility of cloning miniature pigs. Compared with miniature pigs, common domestic pigs can accommodate more fetuses, and as a result, they are suitable for producing many piglets per pregnancy. The production efficiency of cloned piglets using common domestic pigs as recipients tended to be higher compared to that obtained using recipient miniature pigs, although there was no clear statistical difference due to the small number of transfer experiments.

When using common domestic pigs as recipients, management of labor and delivery is very important. Crossbred common domestic pigs weigh about three times as much as NIBS miniature pigs, and the average birth weight of miniature pigs is less than half that of common domestic pigs. If neonatal miniature pigs produced by surrogate common domestic pigs can be safely nursed using artificial milk, then producing cloned miniature pigs in this way is very practical. In fact, when producing transgenic miniature pigs expressing GFP from recipient common domestic pigs, the survival of full-term piglets obtained by a caesarean section was very high (9/10). These results indicate the advantage of using recipient common domestic pigs for producing value-added clones, such as, transgenic cloned miniature pigs.

Transfer of nuclear transfer embryos to the uterus is also effective for producing cloned miniature pigs. In uterine transfer, nuclear transfer embryos are cultured for three days; however, due to recent advances in porcine embryo culture media [35], this

degree of *in vitro* culture apparently does not compromise embryo survival. In recent years, Ueno *et al.* [54] have suggested the possibility of embryonic loss in transferring cloned embryos with damaged zona pellucida to the oviduct. In order to avoid such a latent risk, transferring cloned embryos to the uterus appears to be more practical.

In the present study, common domestic pig oocytes collected from an abattoir were used as the recipient cytoplasm for miniature pig nuclear transfer; however, the effect of utilizing common domestic pig oocytes on the physiological characteristics of cloned miniature pigs has not been clarified. The average birth weight of the cloned piglets produced by recipient miniature pigs was 425.5 g, which is comparable to the average birth weight of piglets produced by natural breeding (460 g). Furthermore, the growth curve of the live cloned piglets (data not shown) was comparable to that of naturally bred piglets, suggesting that the physical attributes of miniature pigs were maintained even when miniature pig clones were produced using common domestic pig oocytes. Compared with common domestic pigs, miniature pigs produce fewer piglets and the production costs are higher; as a result, establishing a mass production system for cloned miniature pigs by using IVM oocytes derived from abattoir ovaries will further accelerate usage of miniature pigs. However, use of abattoir-derived oocytes as recipient cytoplasts may have hygienic implications. The difference in health status between miniature and common domestic pigs, i.e., parvovirus, porcine reproductive and respiratory syndrome (PRRS) or other diseases widespread in agricultural animal husbandry has to be considered.

Transgenic cloned miniature pigs expressing GFP were also produced using common domestic pig recipients, and piglets were efficiently produced. In particular, five live piglets were obtained from one recipient, and the average birth weight of these piglets was 480 g, which is comparable to that of naturally bred NIBS miniature pigs. In the present study, we did not raise the piglets that were delivered by caesarean section, but we do believe it is feasible to raise these transgenic miniature pigs using foster mothers and artificial milk.

By simultaneously producing miniature pigs expressing a fluorescent marker, such as GFP, and syngeneic non-transgenic cloned pigs, it is possible to prepare highly valuable experimental models. For example, among syngeneic clones, because rejection is not an issue with organ or tissue transplantation [55, 56], transplantation-related experiments can be conducted with a very high level of accuracy and precision. As donors in transplantation experiments, a great many piglets and fetuses may be needed in certain studies. In such cases, clone production using common domestic pig recipients would be highly useful.

Southern analysis of the transgenic miniature pigs was not performed in this investigation, but the fluorescence of all eight organs examined and the primary cultured cells established from these organs, except for the brain, was clear. These findings together with the PCR analysis of the piglets (data not shown) indicated that the transfected gene had integrated into the donor cells used in nuclear transfer. The reason for the very mild fluorescence in the brain tissue was probably certain characteristics of the promoter.

In the present study, the SCNT technique that we had originally

developed for common domestic pigs was applied to miniature pigs, and we were able to confirm that reconstructed embryos could be efficiently produced. In addition, we demonstrated that both miniature pig and common domestic pig recipients can be used for production of both cloned and transgenic cloned miniature pigs. Hence, a practical system for cloning of miniature pigs has been developed.

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