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Birth of Somatic Cell Nuclear Transfer (SCNT)-Cloned Miniature Piglet Following Co-Transfer of Parthenogenetic Embryos

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Summary

In miniature pigs, establishment and maintenance of pregnancy to term is required to implant at least 4 or 5 viable embryos. However, most of the somatic cell nuclear transfer (SCNT)-derived miniature pig embryos are lost in the peri- or post-implantation stages. Thus, it is important to develop strategies for improving the chances of implantation and maintenance of pregnancy in order to produce viable SCNT cloned miniature piglets. In present study, we investigated the effect of co-transfer of parthenogenetic (PA) embryos with SCNT-derived embryos on the establishment and maintenance of pregnancy and production of SCNT-cloned miniature piglets. PA embryos exhibited higher *in vitro* developmental competence to blastocyst stage (PA : 31.2% vs. SCNT : 6.6%) and total number of cells at blastocyst stage (PA : 35.1±3.3 vs. SCNT : 22.9±1.0) as compared to the SCNT embryos. Further, SCNT embryos were transferred alone or co-transferred with PA embryos into recipient miniature gilts. On ultrasonography at day 50 after embryo transfer, no pregnancy was detected in the only-SCNT group, while 3 pregnancies were detected in the co-transfer group. One of the 3 successful pregnancies completed term and delivered a single, healthy SCNT-cloned miniature piglet. Furthermore, the weight increase in the SCNT-cloned miniature piglet was similar to that of the piglet derived from *in vivo* fertilized embryos. These data indicated that co-transfer PA embryos could support the establishment and maintenance of pregnancy and help achieve full-term development of SCNT

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embryos, which have a low developmental competence.

Key words: co-transfer, miniature pigs, parthenogenetic embryo, somatic cell nuclear transfer

Miniature pig cloning by somatic cell nuclear transfer (SCNT) has many potential biomedical applications such as xenotransplantation and the development of animal models for human diseases (Dai *et al.*, 2002; Lai *et al.*, 2002) because the characteristics of anatomy, physiology, etc., of miniature pigs are similar to those of humans (Tucker *et al.*, 2002). Despite recent developments in miniature pig cloning technology, the cloning efficiency is low (Lai and Prather 2003). One of the main obstacles to the successful production of SCNT-cloned miniature piglet is the difficulty of the establishment and maintenance of pregnancy.

In polytocous animals such as the pig, maintenance of pregnancy requires a minimum foetal occupancy within the uterus. It is critical to achieve this minimum level between approximately day 11 and day 12 of gestation, when conceptus-derived oestrogen is responsible for the maternal recognition of pregnancy (Geisert *et al.*, 1990). A minimum of 4 or 5 viable embryos (Polge *et al.*, 1966) or at least 50% uterine occupancy (Geisert *et al.*, 1990) is required for the maintenance of pregnancy. This requirement ceases to be relevant by day 14, after which the number of embryos remaining is less critical (Dziuk 1985). In addition, despite recent improvement in the porcine SCNT procedure, the developmental competence of SCNT embryos is still poor as compared to that of *in vitro* fertilized embryos, particularly when *in vitro* matured oocytes are used for recipient cytoplasms. Furthermore, the trophoblast linege and placental defects which may cause implantation and/or pregnancy failure are common problems in developing SCNT embryos (Yang *et al.*, 2007). Thus, the development of strategies focused on improving the chances of implantation and pregnancy is indispensable.

Thus far, although many efforts have been undertaken to improve the efficiency of the SCNT technique, relatively few approaches have focused on the improving the chances of implantation and pregnancy. Previous reports have indicated the development of techniques for the maintenance of pregnancy by novel approaches incorporating fertilized or parthenogenetic embryos as helper embryos for micromanipulated embryos (King *et al.*, 2002). In addition, a recent study on mice revealed that the co-transfer of parthenogenetic embryos improves the chances of pregnancy and implantation of SCNT embryos (Meng *et al.*, 2008).

In the present study, we evaluated the utility of parthenogenetic embryos as helper embryos for the production of SCNT-cloned miniature piglets and investigated its effect on pregnancy and full-term development of SCNT miniature pig

embryos.

Materials and Methods

Donor cells

Goettingen miniature pig foetuses (CSK, Suwa, Japan) were collected from a sow on day 56 of pregnancy. Each foetus was decapitated and eviscerated. The remaining tissues were washed in Dulbecco's phosphate-buffered saline (PBS, Sigma Chemical Co., St. Louis, MO) and then digested with 0.1% trypsin-ethylenediaminetetraacetic acid (EDTA, Sigma) for 45 min at 38.5°C. After digestion, the cells collected as miature pig foetal fibroblasts (mFFs) were cultured in Dulbecco's modified Eagle medium (DMEM, Sigma) supplemented with 10% foetal bovine serum (FBS, Sigma). The culture medium was replaced every 2 days until the cells reached confluence. Subsequently, the cells were harvested with 0.1% trypsin in PBS containing 0.5 mM EDTA for 5 min at 38.5°C, frozen with a cryoprotectant (Cellbanker, Zenyaku, Tokyo, Japan) and stored in liquid nitrogen (passage 0). Prior to the start of the experiments, the cells derived from a single foetus were thawed and cultured in DMEM plus 10% FBS and cells between passages 4 and 9 were used.

In vitro *maturation of oocytes*

Ovaries from prepubertal gilts were collected at a local slaughterhouse and transported to the laboratory within 2 h in a container containing warm saline. Porcine oocytes, 3 to 6 mm in diameter, were aspirated from the antral follicles using a 5-mL disposable syringe attached to an 18-gauge needle. Cumulus-oocyte complexes (COCs) with uniform ooplasm and compact cumulus cell mass were selected in modified Dulbecco's PBS medium containing 5.56 mM glucose (Wako, Osaka, Japan), 0.33 mM sodium pyruvate (Wako), 0.01 mL/mL antibiotic antimycotic solution (Sigma) and 4 mg/ml fatty acid-free BSA (Sigma) (PB1 medium) (Quinn *et al.*, 1982). After washing in PB1 medium, the COCs were cultured in bovine serum albumin-free (BSA-free) NCSU-23 medium (Miyoshi *et al.*, 2000) for 44 h at 39°C in a highly humidified atmosphere of 5% CO_2 in air. Culturing for the first 22 h of maturation was performed in a medium containing 10 IU/mL of equine chorionic gonadotropin (eCG; Serotropin, Teikokuzouki, Tokyo, Japan), 10 IU/mL of human chorionic gonadotropin (hCG; Puberogen, Sankyo, Tokyo, Japan), 0.1 mg/mL of cysteine (Sigma), 1 mM dibutyryl cyclic adenosine monophosphate (Sigma) and 10% porcine follicular fluid. Subsequent culturing for the remaining 22 h was carried out in the same medium without hormonal supplementation. After culturing, the expanded cumulus cells of the COCs were removed by vortexing in PB1 medium containing 1 mg/mL of hyaluronidase (Sigma), and under stereomicroscopy, the oocytes with the first polar body

were identified and selected as mature oocytes. The matured oocytes were transferred into PB1 medium and used for the further experiments.

Somatic cell nuclear transfer

The cumulus-free oocytes were stained with 5 μg/mL of Hoechst 33342 (Sigma) for 5 to 10 min at 39°C and transferred into PB1 medium containing 2.5 μg/mL of cytochalasin D (Sigma). Enucleation was performed by aspirating the first polar body and a small volume of the adjacent cytoplasm with a enucleation pipette of 20-μm diameter using a piezo-driven unit (Primetech Co., Tokyo, Japan) and was confirmed by visualizing the presence of the metaphase plates within the removed cytoplasm under ultraviolet (UV) light. The enucleated oocytes were washed 3 times with NCSU-23 medium and transferred to a 100-μL droplet of NCSU-23 medium for the subsequent microinjection of the donor nucleus. The prepared donor cells were transferred to a 50-μL droplet of PB1 medium, and their plasma membranes were broken by gently aspirating them in and out of the injection pipette of 10 μm. The denuded nucleus was microinjected into the cytoplasm of the enucleated oocyte.

In vitro *fertilization*

In vitro fertilization (IVF) was also performed with IVM oocytes. After the IVM, oocytes were washed 3 times with TU medium (Miyoshi *et al.*, 1999), and 30 to 40 oocytes were transferred to a 100-μL drop of IVF medium. Cryopreserved semen was thawed, and spermatozoa were centrifuged twice (1,500 $\times$ g for 5 min) in Dulbecco's PBS supplemented with 1 mg/mL of BSA (Sigma). Spermatozoa were resuspended in IVF medium, and 20 to 30 μL of the suspension was added to the drop containing oocytes to give a final concentration of 3×10^5 cells/mL. At 6 h post-insemination, oocytes were washed 3 times with NCSU-23 medium and cultured in NCSU-23 medium at 38.5°C in 5% CO_2 in air.

Activation and culture of embryos

The embryos were activated according to the method described by Yamanaka *et al.* (2007). To activate the SCNT embryos, 3 h after the injection or fusion of donor cells the reconstructed embryos were treated with 15 μM ionomycin (Sigma) in NCSU-23 medium containing fatty acid-free BSA (Yoshioka *et al.*, 2002) for 20 min at 38.5°C in 5% CO_2 in humidified air and then washed with NCSU-23 medium. Next, SCNT embryos were cultured in NCSU-23 medium containing 5 μg/mL of cycloheximide (Sigma) with cytochalasin D (Sigma) for 5 h at 38.5°C in 5% CO_2 in humidified air and then washed 5 times with cycloheximide-free NCSU-23 medium (Sugimura *et al.*, 2008). To produce parthenogenetic embryos, *in vitro* matured oocytes were stimulated by the same procedure. Lastly, these embryos were transferred to NCSU-23 medium and cultured at 38.5°C in an

atmosphere of 5% CO_2 in air for 7 days. At the end of the culture period, the number of nuclei in all the blastocysts was counted under UV light after staining with 5 μg/ml of Hoechst 33342.

Embryos transfer

Recipient pubertal miniature gilts were injected with 200 IU of eCG and 100 IU of hCG, followed by injection of 100 IU of hCG 72 h later. At 40 to 48 h after the last hCG injection, cleaved SCNT embryos (2–4 cell stage) were surgically transferred with or without parthenogenetic embryos into ampullar region of the oviduct of the recipients. The pregnancy status of the recipients was assessed both by daily checking for signs of estrus and ultrasonography performed between day 43 and day 50 of embryo transfer.

Statistical analysis

The data on the *in vitro* development competence were obtained from more than 3 replicates. Developmental rates were analysed by chi-square tests ($P<0.05$). Total cells number at blastocyst stage were analysed using analysis of variance (ANOVA) and then by Fisher's protected least significant difference ($P<0.05$).

Results

Evaluation of in vitro *developmental competence of parthenogenetic embryos*

In vitro developmental competence and total number of cells at the blastosyst stage of the IVF, PA, and SCNT embryos are shown in Table 1. PA embryos showed a significantly higher development competence at the blastocyst stage as compared to the SCNT embryos (31.2 and 6.6%, respectively) ($P<0.05$); the developmental competence of the former was comparable to that of the IVF embryos (34.5%). The mean total number of cells at the blastocyst stage of the PA embryos was significantly higher than that of the SCNT embryos (35.1±3.3

Table 1. Comparison between the in vitro development of parthenogenetic embryos and in vitro fertilization (IVF) or somatic cell nuclear transfer (SCNT)-embryos

Type of embryos	No. of cultured embryos	No. (%) of cleaved embryos	No. (%) of blastocysts	No. cells/blastocysts (Mean±S.E.)
IVF	148	102 (68.9)	49 (34.5)[a]	43.7±2.4[a]
PA	77	54 (68.8)	24 (31.2)[a]	35.1±3.3[a]
SCNT	76	42 (59.2)	5 (6.6)[b]	22.9±1.0[b]

[a,b]Values with different superscripts within the same column are significantly different ($P<0.05$).

and 22.9±1.0) ($P<0.05$), and the mean total number of cells was comparable to that of the IVF embryos (43.7±2.4).

In vivo *development competence of SCNT-derived miniature pig embryos*

The *in vivo* developmental competence of the SCNT-derived miniature pig embryos to the blastocyst stage is shown in Table 2. SCNT embryos alone were transferred into 4 recipient gilts; all the 4 recipients failed to become pregnant, with the return of the estrus 18-25 days after the last estrus. SCNT embryos were co-transferred with PA embryos in 6 recipient gilts. Although pregnancy was established in 2 of these 6 recipients, completion of term could not be achieved in these cases. Nevertheless, in 3 of these 6 recipient gilts, fluid filled areas containing a foetal mass each could be detected by ultrasonography between day 43 and day 50 of embryos transfer; in one of these 3 cases, a single, healthy SCNT-derived miniature piglet was delivered on day 115 of gestation. The cloned piglet still survives, although it is 3 years since its birth (Fig. 1).

Weight development of SCNT cloned miniature piglet

The weight development of the SCNT-cloned miniature piglet is shown in Figure 2. The birth weight was 0.36 kg. The weight at 1, 2, 3 and 4 weeks-the 4-week period that corresponds to the weaning stage-were 0.392, 0.734, 1.35 and 2.15 kg, respectively. The weight at 28 weeks which represents the stage of attaining sexual maturity was 20.2 kg. The weigh at 3 years after birth (present

Table 2. In vivo development of somatic nuclear cell transfer (SCNT)-cloned miniature

Co-transfer	Breed Surrogate	No. of transferred embryos		Pregnancy status (day for return of estrus)
		SCNT	PA	
Yes	Chinese*	89	44	Not pregnant (Day 42)
	Chinese	88	41	Not pregnant (Day 46)
	Chinese	85	46	Not pregnant (Day 21)
	Chinese	136	40	1 piglet born, Day 115
	Cross breed**	165	35	Lost, Day 50-63†
	Cross breed	143	32	Lost, Day 43-63
No	Chinese	229	0	Not pregnant (Day 25)
	Chinese	148	0	Not pregnant (Day 25)
	Cross breed	148	0	Not pregnant (Day 18)
	Cross breed	256	0	Not pregnant (Day 18)

*Chinese miniature pigs, **Cross-breed miniature pigs (Chinese×Clawm×Göttingen)

†The recipients were assessed for pregnancy by ultrasonography.

PA: parthogenetic embryos.

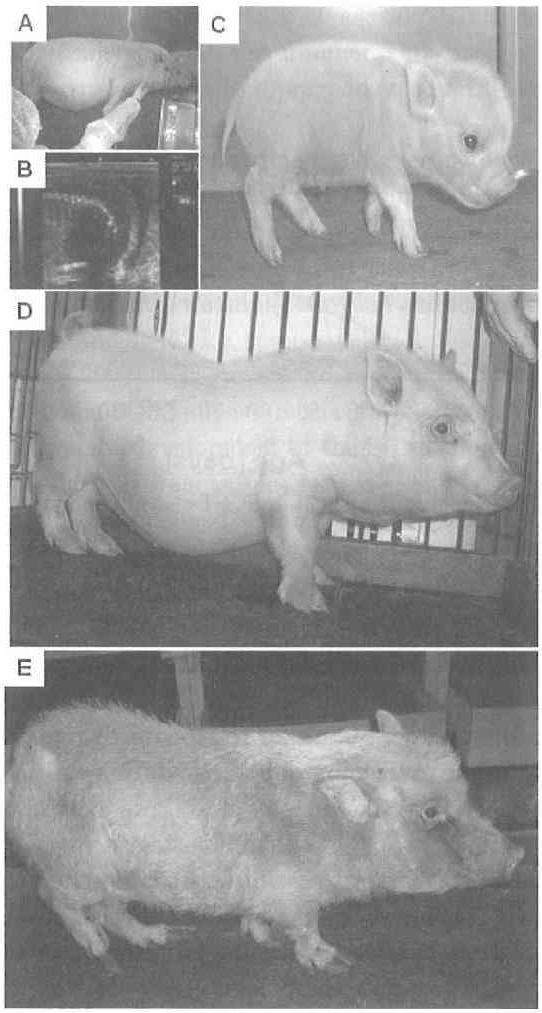

FIG. 1. SCNT-cloned miniature piglet derived from foetal fibroblasts. (A) Recipient miniature gilt at 105 days of embryos transfer. (B) Fluid filled area containing a foetal mass could be detected by ultrasonography in 91 days of embryos trnasfer. SCNT-cloned miniature piglet at (C) 2 days, (D) 115 days and (E) 3 years after birth.

weight) is 22.5 kg.

Discussion

The efficiency of SCNT in miniature pig cloning is strictly low, and most of the SCNT embryos are lost in the pre- or postimplantaion stages or do not survive to term. In present study, we demonstrated that co-transfer of parthenogenetic embryos could improve the chances of pregnancy and full-term development of

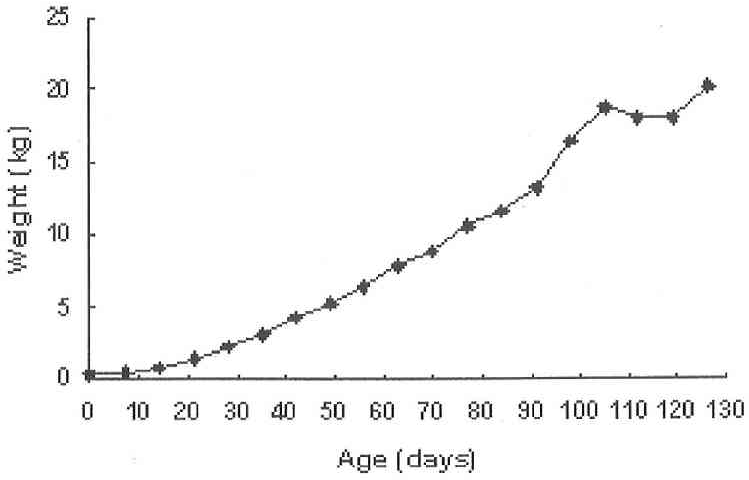

FIG. 2. Weight development of SCNT-cloned miniature piglet.

SCNT-derived miniature pig embryos. Although the mechanism by which the chances of pregnancy are improved is unclear, it has been reported that trophoblastic interferons acting on the endometrium are required for the implantation and establishment of pregnancy in pigs (Spencer *et al.*, 2004). In a previous study, it was demonstrated that a major reason for the low birth rate of cloned pigs is the abnormal apoptosis in the extraembryonic tissue during early pregnancy (Chae *et al.*, 2006). Furthermore, in the present study, we demonstrated that SCNT embryos have a lower number of cells at the blastocyst stage as compared to IVF and PA embryos. In SCNT porcine embryos, it is reported that low number of cells at the blastocyst stage is due to the low proliferation of trophoectoderm cells (Koo *et al.*, 2004). Thus, it is possible that parthenogenetic embryos which may have functional trophoblast cells could be implanted in the endometrium more effectively which may in turn improve the chances of establishing and maintaining pregnancy. A previous report also indicated that the maintenance of the pregnancy of SCNT embryos could be enhanced by co-transfer of parthenogenetic embryos (De Sousa *et al.*, 2002). Our present study strongly supports this report. In our study, 32–42 parthenogenetic embryos derived from domestic pig oocytes were co-transferred into recipient miniature gilts. It is possible that the co-transfer number or lineage of the helper embryos may influence the maintenance of pregnancy and the full-term development of the SCNT-derived miniature pig embryos. However, we observed that 3 of the 6 recipients in which the SCNT embryos were co-transferred with about 40 parthenogenetic embryos became pregnant, and none of the recipients in the group in which SCNT embryos were transferred alone became pregnant. Moreover, King *et al.*, showed that live piglets were delivered after co-transfer of 3 fertilized embryos with 55–60 parth-

enogenetic embryos into surrogates, (King *et al.*, 2002). Hence, in the present study, it could be considered that the number and lineage of co-transferred parthenogenetic embryos did not affect pregnancy and full-term development, negatively.

There have been a few reports regarding the higher incidence of abnormalities such as the large offspring syndrome observed among the neonatal SCNT-cloned domestic piglet as compared to the neonates of SCNT-cloned mouse, bovine and sheep (Onishi *et al.*, 2000 ; Yang *et al.*, 2007). Generally, in Göttingen minipigs, the average body weight at birth, weaning (4 weeks) and sexual maturity stage (4–5 mounth) are 0.45, 2-3, and 18–22 kg, respectively (Bollen *et al.*, 1998 ; Kohn *et al.*, 2007 ; Kohn *et al.*, 2008). There are no reports on the details about the weight development in the SCNT-cloned miniature piglets ; in the present study, the birth weight and weight development of the SCNT-cloned piglet was similar to those of the piglets derived from *in vivo* fertilized embryos. Furthermore, the SCNT piglet has grown to adulthood without any abnormalities after birth.

In summary, parthenogenetic embryos can sustain full-term development of SCNT embryos. Thus, the present study demonstrated that co-transfer with parthenogenetic embryos may be one of the strategies for the maintenance of pregnancy and the production of SCNT-cloned miniature pig clones. We believe that our procedure would be useful to produce genetically modified miniature pigs for xenotransplantation or animal models of human diseases.

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