



HOKKAIDO UNIVERSITY

Title	Difference in sensitivity to culture condition between In Vitro fertilized and somatic cell nuclear transfer embryos in pigs
Author(s)	Yamanaka, Ken-ichi; Sugimura, Satoshi; Wakai, Takuya et al.
Citation	Journal of Reproduction and Development, 55(3), 299-304 https://doi.org/10.1262/jrd.20174
Issue Date	2009-06
Doc URL	https://hdl.handle.net/2115/68131
Type	journal article
File Information	55_299_00.pdf



—Full Paper—

Difference in Sensitivity to Culture Condition Between *In Vitro* Fertilized and Somatic Cell Nuclear Transfer Embryos in Pigs

Ken-ichi YAMANAKA¹⁾, Satoshi SUGIMURA¹⁾, Takuya WAKAI^{1)†}, Manabu KAWAHARA^{1)##} and Eimei SATO¹⁾

¹⁾Laboratory of Animal Reproduction, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan

[†]Present: Department of Veterinary and Animal Sciences, University of Massachusetts, Massachusetts 01003, USA

^{##}Present: Laboratory of Animal Resource Development, Faculty of Agriculture, Saga University, Saga 840-8502, Japan

Abstract. We evaluated the developmental competence of somatic cell nuclear transfer (SCNT) embryos using *in vitro* embryo culture systems. Embryos were cultured in NCSU-23, NCSU-23 supplemented with essential and non-essential amino acids (NCSU-23aa) or modified PZM-5 supplemented with BSA instead of PVA (mPZM-5). The rates of blastocyst formation were significantly higher in the mPZM-5 group than in the other groups, regardless of the method of embryo production (38.0 *vs.* 25.3 or 29.1% for IVF, 18.2 *vs.* 8.7 or 9.4% for SCNT, respectively). The mean cell numbers of IVF and SCNT blastocysts were also significantly higher in mPZM-5 than in the other groups (62.0 *vs.* 42.3 or 43.0 for IVF, 46.5 *vs.* 29.4 or 31.3 for SCNT, respectively). Next, the embryos were cultured in mPZM-5 from days 0 to 4 and then in mPZM-5 (P/P), NCSU-23 (P/N) or NCSU-23aa (P/Naa) until day 6. The rates of blastocyst formation were similar among the 3 two-step culture systems in both embryo groups (36.2, 34.2, and 33.6% for IVF, 20.8, 14.1, and 17.2% for SCNT, respectively). The mean cell number in the IVF and SCNT blastocysts was significantly lower in P/N than in P/P and P/Naa (46.5 *vs.* 63.5 and 68.7 for IVF, 29.3 *vs.* 45.5 and 39.7 for SCNT, respectively). Next, we examined the effect of media on apoptosis in IVF and SCNT blastocysts. The apoptosis indices in the blastocysts derived from either NCSU-23 or mPZM-5 were analyzed by TUNEL assay. The apoptosis index of the SCNT blastocysts was significantly lower in mPZM-5 than in NCSU-23 (8.8 *vs.* 13.6%), whereas no such difference was observed between groups in the IVF embryos (5.1 *vs.* 4.4%). These data suggested that SCNT embryos were more easily affected by culture environment compared with IVF embryos, offering the possibility to further enhance the developmental competence of SCNT embryos by developing more appropriate culture conditions in pigs.

Key words: Apoptosis, *In vitro* embryo culture, Miniature pig, Nuclear transfer

(J. Reprod. Dev. 55: 299–304, 2009)

Cloned pigs by somatic cell nuclear transfer (SCNT) might be used as improved domestic livestock. However, their use is not limited to this. Owing to the many similarities in between the anatomy and physiology of pigs and humans, these cloned pigs can be used for xenotransplantation and as improved models for studying human physiology and disease [1, 2]. Moreover, in particular, miniature pigs offer several advantages: their small body size enables space-saving and facilitates better control of specific pathogens. They therefore represent attractive options for use in medical applications and related technologies [3]. Thus, miniature pig cloning by SCNT might also prove useful in many biomedical applications [4]. Although it has been successful recently, its efficiency is still low, as is the case in other animals [5]. One of the reasons for the very low success rate of miniature pig cloning is the limitations of the *in vitro* production (IVP) system for pigs, including *in vitro* maturation (IVM) and their subsequent *in vitro* culture (IVC). Therefore, the porcine IVP system has been modified by many researchers [6–10], but has not yet been optimized because of the low quality of porcine embryos produced *in vitro* compared

with that *in vivo*, resulting in low pregnancy rate and average litter size after transfer [11–14].

The IVC system is a fundamental procedure for improving the developmental competence of *in vitro* embryos produced by IVM-IVF as well as SCNT. Moreover, Heindryckx *et al.* demonstrated that mouse SCNT embryos are more sensitive to IVC conditions than parthenogenetic embryos and that the selection of culture medium can influence the preimplantation development of SCNT embryos [15]. For IVC of pigs, several media have been developed, including North Carolina State University (NCSU)-23 and NCSU-37 media [16], Beltsville Embryo Culture Medium (BECM)-3 [17] and Porcine Zygote Medium (PZM)-3 and PZM-4 [18]. Although NCSU-23 has been widely used and is one of the most successful media for the culture of porcine embryos after IVF or SCNT, in recent studies, it has reported that PZM-3 is superior to NCSU-23 in supporting development of the embryo to the blastocyst stage of IVF [18], parthenogenetic activation (PA) [19] or SCNT embryos [20]. Furthermore, Suzuki *et al.* developed a porcine *in vitro* culture medium, namely, PZM-5 [21, 22]. Thus, it is possible that PZM-5 also improves the developmental competence of SCNT embryos. However, no study has tested the superiority of PZM-5 with regard to facilitating *in vitro* development of SCNT embryos. A previous study has also reported that there are distinct changes in the utilization and preferences for energy substrates

Accepted for publication: February 11, 2009

Published online in J-STAGE: March 16, 2009

Correspondence: K Yamanaka (National Agricultural Research Center for Kyushu Okinawa Region (KONARC), 2241 Suya, Koshi, Kumamoto 861-1192, Japan) (e-mail: kenichi@affrc.go.jp)

depending on the developmental stages of embryos [23, 24]. Similarly, another study suggests that the IVC of viable embryos may require the use of 2 or more culture media, each designed to respond to the changing requirements of the embryo [25]. Hence, according to these reports, using different media at different stages may promote embryo development.

In the present study, in order to determine the optimal IVC conditions for IVF and SCNT embryos, 3 experiments were performed. To identify the most suitable culture media for IVF and SCNT embryos, first, the developmental competence of IVF and SCNT embryos cultured in different media were compared. Second, on the basis of the results of these experiments, we investigated the effect of two-step culture systems on *in vitro* development of IVF and SCNT embryos. Finally, we investigated whether culture media affected the incidence of apoptosis in IVF and SCNT embryos in the blastocyst stage.

Materials and Methods

Chemicals

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

In vitro maturation of oocytes

Pig cumulus-oocyte complexes (COCs) were isolated as described by Yamanaka *et al.* [26]. In brief, COCs were aspirated from nonatretic ovarian follicles (3–5 mm in diameter) of ovaries, which were obtained from prepubertal gilts at a local slaughterhouse. After washing in PB1, about 50 COCs were cultured in a 4-well multidish (Nunc, Roskilde, Denmark) containing 500 μ l of BSA-free NCSU-23 medium for 44 h at 38.5 C in a humidified atmosphere of 5% CO₂ in air. The culture medium used for the first 22 h of maturation contained 10 IU/ml pregnant mare serum gonadotrophin (PMSG; Serotropin, Teikokuzouki, Tokyo, Japan), 10 IU/ml human chorionic gonadotropin (hCG; Puberogen, Sankyo, Tokyo, Japan), 0.6 mM cysteine, 5 μ g/ml insulin (Gibco BRL Life Technologies, Grand Island, NY, USA), 20 μ M β -mercaptoethanol, 1 mM dibutyl cyclic adenosine monophosphate (dbcAMP) and 10% (v/v) porcine follicular fluid. For the subsequent 22 h, COCs were cultured in the same medium without dbcAMP and hormonal supplementation. After the cultivating the cells up to maturation, expanded cumulus cells were removed by vortexing in PB1 containing 1 mg/ml hyaluronidase. Oocytes were observed under a stereomicroscope, and mature oocytes, i.e., those containing the first polar body, were selected and used for the production of IVF and SCNT embryos. The mature oocytes were placed in PB1 until use.

In vitro fertilization of porcine oocytes

After *in vitro* maturation, cumulus-free mature oocytes were washed 3 times with TU medium (113.1 mM NaCl, 3.0 mM 10.0 mM CaCl₂·2H₂O, 25.07 mM NaHCO₃, 11.0 mM glucose, 5.0 mM Na-pyruvate, 2.0 mM caffeine-benzoate and 1 mg/ml BSA [27]), and 30–40 oocytes were transferred into 100- μ l drops of TU medium. Cryopreserved semen was thawed and spermatozoa were washed 2 times by centrifugation (at 1000 $\times$ g for 4 min) in Dulbecco's PBS (Nissui, Tokyo, Japan) supplemented with 1 mg/ml BSA. The sper-

matozoa were resuspended in the TU medium, and 20–30 μ l of this suspension was added to the fertilization drop containing IVF oocytes to yield a final concentration of 7.5×10^6 cells/ml. Coincubation of oocytes with sperm was carried out for 6 h post-insemination. The oocytes were then washed 3 times, and 20–30 oocytes were cultured in 100- μ l drops of culture medium for 6 days at 38.5 C in 5% CO₂ in air. The rates of cleavage and blastocyst development were assessed on days 2 and 6 of IVC, respectively.

Assessment of *in vitro* fertilization

To assess fertilization, oocytes were fixed for 48 h in 25% acetic acid (v/v) in ethanol at room temperature 6 h after fertilization, stained with 1% (w/v) orcein in 45% (v/v) acetic acid and then examined under a phase-contrast microscope.

Preparation of donor cells

Donor cells were obtained from miniature pig fetuses (Gotttingen; Chugai, Suwa, Japan) collected from a pregnant gilt on the 56th day of pregnancy. The cells from a single fetus were thawed and cultured in DMEM + 10% fetal bovine serum (FBS) and were passaged 4–9 times. The cells were cultured for 1 week after confluence and then used as donor nuclei.

Nuclear transfer

The oocytes were stained by 5 mg/ml Hoechst 33342 at 38.5 C for 5 to 10 min and manually enucleated in PB1 containing 7.5 μ g/ml cytochalasin D. Enucleation was performed by aspirating both the first polar body and adjacent cytoplasm using a beveled pipette driven by a piezo-actuated unit (Prime Tech, Ibaraki, Japan) and confirmed by visualizing the cytoplasm under UV light. A single donor cell was injected into the perivitelline space of each oocyte and electrically fused using 2 direct current pulses of 150 V/mm for 50 μ sec in 0.28 mol/L mannitol supplemented with 0.1 mM MgSO₄ and 0.01% polyvinyl alcohol (PVA). The fused oocytes were cultured in each of the media for 3 h before activation. For activation, oocytes were incubated in 15 μ M ionomycin for 20 min and then in 5 μ g/ml cycloheximide and 2.5 μ g/ml cytochalasin D for 5 h. We then cultured 20–30 embryos in 100 μ l of each culture medium separately under mineral oil for 6 days at 38.5 C in an atmosphere of 5% CO₂ in air.

Cytological evaluations

To count total cell number, blastocysts on day 6 of IVC were fixed in 3.7% paraformaldehyde (PFA) for 40 min and stained with 5 μ g/ml Hoechst 33342 for 10 min. After being mounted onto a precleaned microscope slide, the nuclei of the embryos were counted under a fluorescence microscope. Moreover, to detect apoptotic cells in blastocysts, we performed a terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick end labeling (TUNEL) assay using an ApopTag fluorescein *in situ* apoptosis detection kit (Chemicon International, USA) as per the manufacturer's instructions. Briefly, IVC blastocysts on day 6 were fixed and permeabilized in PBS supplemented with 2% PFA and 0.2% (v/v) Triton X-100 for 40 min at room temperature. After washing twice in PBS, the blastocysts were incubated with equilibration buffer for 10 sec at room temperature. After incubation, the

Table 1. Composition of culture media

Component	NCSU-23	NCSU-23aa ^a	Modified PZM-5
NaCl (mM)	108.73	108.73	108.00
KCl (mM)	4.78	4.78	10.0
CaCl ₂ ·2H ₂ O (mM)	1.70	1.70	—
KH ₂ PO ₄ (mM)	1.19	1.19	0.35
MgSO ₄ ·7H ₂ O (mM)	1.19	1.19	0.40
NaHCO ₃ (mM)	25.07	25.07	25.07
Glucose (mM)	5.55	5.55	—
Na-pyruvate (mM)	—	—	0.20
Ca-(lactate) ₂ ·5H ₂ O (mM)	—	—	2.00
L-Glutamine (mM)	1.00	1.00	2.00
Taurine (mM)	7.00	7.00	—
Hypotaurine (mM)	5.00	5.00	5.00
BME-EAA ^b (ml/l)	—	20.00	20.00
MEM-NEAA ^c (ml/l)	—	10.00	10.00
Bovine serum albumin (mg/ml)	4.00	4.00	4.00

^a NCSU-23aa: NCSU-23 supplemented with essential and non-essential amino acids. ^b Basal medium Eagle essential amino acids (Sigma, B6766). ^c Minimum essential medium non-essential amino acids (Sigma, M7145).

blastocysts were immediately transferred and incubated with 30% TdT enzyme (v/v) in a reaction buffer for 2 h at 37 °C and then with 3% stop/wash buffer for 10 min incubation at room temperature. After washing 3 times in PBS supplemented with 0.2% Triton X-100 and 0.1% PVA (1 min per wash), the blastocysts were incubated in the dark with 47% anti-digoxigenin conjugate (v/v) in blocking solution for 1 h at room temperature. After washing 4 times in PBS supplemented with 0.2% Triton X-100 and 0.1% PVA for 2 min per wash, blastocysts were stained with 10 µg/ml propidium iodide in PBS for 1 h at room temperature. All samples were examined under a laser-scanning confocal microscope (MCR-1024; BIO-RAD Hercules, CA, USA); those stained red were considered to be nuclei, and those stained green and yellow were apoptotic bodies. The total apoptotic index was calculated for each embryo as follows: apoptotic index=(number of TUNEL-positive nuclei/total number of nuclei in blastocyst) × 100.

Statistical analysis

All data were obtained from more than 4 replicates. Blastocyst cell number and apoptosis index were analyzed using ANOVA and then Fisher's protected least significant difference ($P < 0.05$). Other data were analyzed by chi-square tests ($P < 0.05$).

Experimental design

Experiments were designed to study the effects of culture media and two-step culture systems on the preimplantation development of IVF and SCNT embryos.

Experiment 1: We evaluated the effect of culture media on *in vitro* development and blastocyst cell numbers in IVF and SCNT embryos among the 3 treatment groups. The embryos were cultured in either NCSU-23 [16], NCSU-23 supplemented with amino acids (NCSU-23aa), or modified PZM-5 (mPZM-5), and the developmental rates of the groups were compared. The modified NCSU-23aa was prepared by adding 20.0 ml/L basal medium Eagle (BME) essential amino acids (EAAs) and 10.0 ml/L minimum essential medium (MEM) non-essential amino acids

(NEAAs) to NCSU-23. The mPZM-5 was obtained by adding 4 mg/ml BSA instead of 3 mg/ml PVA to PZM-5 [21] (Table 1).

Experiment 2: The effect of two-step culture systems on *in vitro* development of IVF and SCNT embryos was studied. Based on the results of experiment 1, all embryos were cultured in mPZM-5 from days 0 to 4, followed by culture in either mPZM-5 (P/P), NCSU-23 (P/N) or NCSU-23 supplemented with amino acids (P/Naa) until day 6.

Experiment 3: We examined the effect of media on the incidence of apoptosis in IVF and SCNT blastocysts. On the basis of the results of experiment 1 and 2, IVF and SCNT blastocysts were obtained by using the mPZM-5 and NCSU-23 media. We assessed the incidence of apoptosis in these blastocysts by TUNEL assay and examined whether culture media affect the incidence of apoptosis.

Results

Experiment 1: Effect of culture media on *in vitro* development of IVF and SCNT embryos

We examined the effect of culture media on *in vitro* development of IVF and SCNT embryos. In IVF embryos, some oocytes were fixed for evaluations of fertilization. The rate of penetration was 95.8% (91/95), and the rate of monospermic penetration was 61.5% (56/91) of penetrated oocytes. For comparison of the media, IVF and SCNT embryos were cultured in NCSU-23, NCSU-23aa and mPZM-5 (Table 1). In both types of embryo, the rates of blastocyst formation were significantly higher ($P < 0.05$) in mPZM-5 than in NCSU-23 and NCSU-23aa. The mean cell numbers of IVF and SCNT blastocysts were also significantly higher ($P < 0.05$) in mPZM-5 than in NCSU-23 and NCSU-23aa (Table 2).

Experiment 2: Effect of two-step culture systems on *in vitro* development of IVF and SCNT embryos

On the basis of the results of experiment 1 (Table 2), we prepared 3 two-step culture systems—P/P, P/N and P/Naa—and investigated their effect on *in vitro* development to the blastocyst

Table 2. Effects of culture media on *in vitro* development of IVF and SCNT embryos

Group	Medium ^a	No. of cultured embryos	No. (%) of cleaved embryos	No. (%) of blastocysts	No. of cells/blastocyst (mean $\pm$ S.E.)
IVF	NCSU-23	146	97 (66.4)	37 (25.3) ^b	42.3 $\pm$ 2.9 ^b
	NCSU-23aa	141	101 (71.6)	41 (29.1) ^b	43.0 $\pm$ 2.2 ^b
	mPZM-5	155	115 (74.2)	59 (38.0) ^c	62.0 $\pm$ 2.2 ^c
SCNT	NCSU-23	161	89 (55.3)	14 (8.7) ^b	29.4 $\pm$ 2.2 ^b
	NCSU-23aa	138	84 (60.9)	13 (9.4) ^b	31.3 $\pm$ 2.1 ^b
	mPZM-5	132	84 (63.3)	24 (18.2) ^c	46.5 $\pm$ 2.9 ^c

^a NCSU-23aa: NCSU-23 supplemented with essential amino and non-essential amino acids. ^{b,c} Values with different superscripts are significantly different for each medium in the same treatment group ($P < 0.05$).

Table 3. Effects of the two-step culture system on *in vitro* development of IVF and SCNT embryos

Group	Medium ^a	No. of cultured embryos	No. (%) of cleaved embryos	No. (%) of blastocysts	No. of cells/blastocyst (mean $\pm$ S.E.)
IVF	P/P	116	84 (72.4)	42 (36.2)	63.5 $\pm$ 3.2 ^b
	P/N	120	89 (74.2)	41 (34.2)	46.5 $\pm$ 2.6 ^c
	P/Naa	134	98 (73.1)	45 (33.6)	68.7 $\pm$ 3.1 ^b
SCNT	P/P	130	85 (65.4)	27 (20.8)	45.5 $\pm$ 2.7 ^b
	P/N	149	99 (66.4)	21 (14.1)	29.3 $\pm$ 1.9 ^c
	P/Naa	174	120 (69.0)	30 (17.2)	39.7 $\pm$ 2.5 ^b

^a Embryos were cultured in mPZM-5 from days 0 to 4, followed by mPZM-5 (P/P), NCSU-23 (P/N) or NCSU-23 supplemented with essential and non-essential amino acids (P/Naa) until day 6. ^{b,c} Values with different superscripts are significantly different for each medium in the same group ($P < 0.05$).

Table 4. Effects of media on apoptosis of IVF and SCNT blastocysts on day 6

Group	Medium	No. (%) of blastocysts treated	No. of cells/blastocyst (mean $\pm$ S.E.)	Apoptosis index (mean $\pm$ S.E.)
IVF	NCSU-23	12	44.8 $\pm$ 5.5 ^a	5.1 $\pm$ 0.8 ^a
	mPZM-5	10	67.4 $\pm$ 6.2 ^b	4.4 $\pm$ 0.7 ^a
SCNT	NCS-23	10	29.4 $\pm$ 2.7 ^c	13.6 $\pm$ 1.8 ^b
	mPZM-5	10	47.6 $\pm$ 3.8 ^a	8.8 $\pm$ 0.4 ^c

^{a-c} Values with different superscripts within the same column are significantly different ($P < 0.05$).

stage and blastocyst cell numbers in IVF and SCNT embryos (Table 3). In both embryo groups, the rates of embryos developed to the blastocyst stage were similar among the 3 two-step culture systems, indicating that the two-step culture system did not improve the developmental competence to the blastocyst stage in both IVF and SCNT embryos. Meanwhile, the mean cell numbers of IVF and SCNT blastocysts were significantly lower ($P < 0.05$) in P/N than in P/P and P/Naa.

Experiment 3: Effect of media on incidence of apoptosis in IVF and SCNT blastocysts

To investigate whether the difference of culture medium—mPZM-5 and NCSU-23—affects the incidence of apoptosis in blastocysts, we detected apoptotic cells in IVF and SCNT blastocysts by using a TUNEL assay (Table 4). There was no significant difference between the apoptosis indices for IVF blastocysts cultured in mPZM-5 or NCSU-23. However, the apoptosis index of SCNT blastocysts was significantly lower ($P < 0.05$) when they

were cultured in mPZM-5 than when they were cultured in NCSU-23. However, the apoptosis indices of SCNT blastocysts were significantly higher ($P < 0.05$) than those of IVF blastocysts regardless of the type of culture medium.

Discussion

In the present study, we demonstrated that mPZM-5 improved the blastocyst formation rates of not only IVF embryos but also SCNT embryos and that mPZM-5 could decrease the incidence of apoptosis in SCNT blastocysts. In Experiment 1, we investigated the *in vitro* development of IVF and SCNT embryos cultured in different media, namely, NCSU-23, NCSU-23aa and mPZM-5 (Table 1). The rate of development to blastocysts and mean cell numbers in IVF and SCNT embryos were significantly higher ($P < 0.05$) in mPZM-5 than in the other culture media (Table 2). This difference in the rate of development to blastocysts may be due to the differences in the energy substrates and concentration of phosphate

present in the media. NCSU-23 and NCSU-23aa contain glucose as an energy substrate and high concentrations of phosphate, whereas mPZM-5 contains lactate, pyruvate and a low concentration of phosphate. Glucose is known as an energy substrate that is important for IVF embryos during the late stages of *in vitro* development. In contrast, pyruvate and lactate are important during the early stages in mice [32], cattle [33] and pigs [6, 18]. Furthermore, it has been suggested that glucose blocks development to the two-cell stage in hamster embryos [34]. Likewise, Seshagiri and Bavisser reported that glucose and phosphate inhibit respiration and oxidative metabolism in hamster embryos, which is known as “Crabtree effect” [35]. Moreover, exposing embryos to high concentrations of glucose during early stages causes developmental retardation in mice [36], cattle [33] and humans [37]. These detrimental effects of glucose are caused by the production of methylglyoxal—a metabolic by-product of glycolysis [38]. It also causes inactivation of intracellular glutathione peroxidase, which plays an important role in scavenging oxygen free radicals [39]. Therefore, for porcine embryos at the early developmental stage, medium containing glucose might be a harmful condition inducing oxidative stress. On the other hand, pyruvate and lactate have important roles in maintenance of an appropriate NADH:NAD ratio and redox equilibrium [40]. Our data also indicated that mPZM-5 containing pyruvate and lactate as energy substrates improved the developmental competence of porcine embryos regardless of the embryo production techniques, i.e., IVF or SCNT. This result is consistent with those of previous studies on porcine IVF embryos [18, 41]. In the present study, addition of EAAs and NEAAs to NCSU-23 had no effect on the *in vitro* development of IVF and SCNT embryos. However, previous studies have suggested that addition of EAAs and NEAAs to the culture medium promotes the development of embryos in mice [42], cows [43, 44] and pigs [22, 45]. These previous studies reported that EAAs and NEAAs are components that promote embryo development when cultured in media containing lactate and pyruvate as energy substrates. Contrastively, in our study, addition of EAAs and NEAAs to NCSU-23, which contained glucose as the energy substrate, had no effect on the development of IVF and SCNT embryos in pigs. Taken together, our data suggested that glucose was not necessary to promote the developmental competence of embryos, and the combination of energy substrates and amino acids is important for supporting the development of viable embryos. This finding also indicates that the effects of EAAs and NEAAs depend on the energy substrate in the culture medium. Our data clearly demonstrates that mPZM-5 is a suitable medium for supporting the *in vitro* development of not only porcine IVF embryos but also miniature pig SCNT embryos.

In Experiment 2, we investigated the effect of two-step culture systems on the *in vitro* development of IVF and SCNT embryos (Table 3). The previous studies have reported that the glucose uptake of porcine embryos was increased at the morula and blastocyst stage [23, 24]. Based on these findings and the results of experiment 1, we cultured embryos in mPZM-5 from days 0 to 4, followed by the culture in mPZM-5 (P/P), NCSU-23 (P/N) or NCSU-23aa (P/Naa) until day 6. In both groups—IVF and SCNT

embryos—the mean cell number of blastocysts obtained by the P/P or P/Naa culture protocols was significantly higher ($P < 0.05$) than that of blastocysts obtained by the P/N culture protocol (Table 3). However, no significant increase in the rate of blastocyst formation or mean cell numbers in blastocysts was observed among the two-step culture groups as compared with the mPZM-5 group (Tables 2 and 3). This result is consistent with that of a previous study, which demonstrated that two-step culture protocols supported the *in vitro* development of embryos in mice, but that they are not necessary [46]. In contrast, Kikuchi et al. have reported that the mean cell number in blastocysts derived from porcine IVF embryos increases when the embryos are cultured by two-step protocols in which the energy substrate is changed from pyruvate/lactate to glucose on day 2 of culture [6]. This discrepancy might be due to the timing of exchange of the medium because we exchanged the medium at day 4 of culture. Thus, in the present study, the two-step culture was not necessary for *in vitro* development of porcine IVF and miniature SCNT embryos.

In Experiment 3, we investigated the relationship between culture media and the incidence of apoptosis in IVF and SCNT embryos. In general, the indicators for evaluating embryonic quality are total cell number and incidence of apoptosis in blastocysts [14]. It is known that the incidence of apoptosis in SCNT embryos is higher than that in IVF embryos [47]. Likewise, in the comparison with IVF and SCNT embryos, the incidence of apoptosis in SCNT blastocysts was significantly higher than that in IVF blastocysts regardless of the type of culture media in the present study. There was no difference in the incidence of apoptosis in IVF blastocysts cultured in NCSU-23 and mPZM-5, whereas the incidence of apoptosis in SCNT blastocysts cultured in mPZM-5 was significantly lower than that of blastocysts cultured in NCSU-23. This difference between IVF and SCNT embryos is consistent with a previous study, which stated that the unusual responses of SCNT embryos to the changes in the culture environment distinguishes these embryos from IVF, PA and tetraploid embryos produced by somatic cell nuclear injection [48]. Moreover, the rate of blastocyst formation of SCNT embryos and the distribution of the mRNA coding for the pluripotent stem cell marker *Oct4* in SCNT blastocysts is highly dependent on the culture environment after cumulus cell nuclear transfer [49]. These findings, together with our data, indicate that SCNT embryos are more easily affected by the culture environment than IVF embryos. In other words, the developmental competence of SCNT embryos may be potentially enhanced by establishing an optimal culture system for SCNT embryos.

On the basis of the results of the present study, we concluded that mPZM-5 was an efficient medium not only for porcine IVF embryos but also for miniature pig SCNT embryos. In addition, mPZM-5 decreased the incidence of apoptosis in SCNT blastocysts but not in IVF blastocysts. Hence, it is possible to enhance the developmental competence of the SCNT embryos by optimizing culture conditions. Further research is necessary to develop a specific *in vitro* culture system that can optimize the developmental competence of SCNT embryos, which is different from that of IVF embryos in terms of its sensitivity to the culture environment.

Acknowledgments

We are grateful to the staff of the meat inspection office, Sendai City, for supplying porcine ovaries. This study was supported by a Research Fellowship for Young Scientists to KY from the Japan Society for the Promotion of Science.

References

- Prather RS, Tao T, Machaty Z. Development of the techniques for nuclear transfer in pigs. *Theriogenology* 1999; 51: 487–498.
- Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, Samuel M, Bonk A, Rieke A, Day BN, Murphy CN, Carter DB, Hawley RJ, Prather RS. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. *Science* 2002; 295: 1089–1092.
- Bollen P, Ellegaard L. The Gottingen minipig in pharmacology and toxicology. *Pharmacol Toxicol* 1997; 80 (Suppl 2): 3–4.
- Prather RS, Hawley RJ, Carter DB, Lai L, Greenstein JL. Transgenic swine for biomedicine and agriculture. *Theriogenology* 2003; 59: 115–123.
- Wakai T, Sugimura S, Yamanaka K, Kawahara M, Sasada H, Tanaka H, Ando A, Kobayashi E, Sato E. Production of viable cloned miniature pig embryos using oocytes derived from domestic pig ovaries. *Cloning Stem Cells* 2008; 10: 249–262.
- Kikuchi K, Onishi A, Kashiwazaki N, Iwamoto M, Noguchi J, Kaneko H, Akita T, Nagai T. Successful piglet production after transfer of blastocysts produced by a modified *in vitro* system. *Biol Reprod* 2002; 66: 1033–1041.
- Yoshioka K, Suzuki C, Itoh S, Kikuchi K, Iwamura S, Rodriguez-Martinez H. Production of piglets derived from *in vitro*-produced blastocysts fertilized and cultured in chemically defined media: effects of theophylline, adenosine, and cysteine during *in vitro* fertilization. *Biol Reprod* 2003; 69: 2092–2099.
- Coy P, Romar R. *In vitro* production of pig embryos: a point of view. *Reprod Fertil Dev* 2002; 14: 275–286.
- Marchal R, Feugang JM, Perreau C, Venturi E, Terqui M, Mermillod P. Meiotic and developmental competence of prepubertal and adult swine oocytes. *Theriogenology* 2001; 56: 17–29.
- Ozawa M, Nagai T, Fahrudin M, Karja NW, Kaneko H, Noguchi J, Ohnuma K, Kikuchi K. Addition of glutathione or thioredoxin to culture medium reduces intracellular redox status of porcine IVM/IVF embryos, resulting in improved development to the blastocyst stage. *Mol Reprod Dev* 2006; 73: 998–1007.
- Day BW. Mutants yield a pharmacophore model for the tubulin-paclitaxel binding site. *Trends Pharmacol Sci* 2000; 21: 321–324.
- Kikuchi K, Kashiwazaki N, Noguchi J, Shimada A, Takahashi R, Hirabayashi M, Shino M, Ueda M, Kaneko H. Developmental competence, after transfer to recipients, of porcine oocytes matured, fertilized, and cultured *in vitro*. *Biol Reprod* 1999; 60: 336–340.
- Machaty Z, Day BN, Prather RS. Development of early porcine embryos *in vitro* and *in vivo*. *Biol Reprod* 1998; 59: 451–455.
- Pomar FJ, Teerds KJ, Kidson A, Colenbrander B, Tharasanit T, Aguilar B, Roelen BA. Differences in the incidence of apoptosis between *in vivo* and *in vitro* produced blastocysts of farm animal species: a comparative study. *Theriogenology* 2005; 63: 2254–2268.
- Heindryckx B, Rybouchkin A, Van Der Elst J, Dhont M. Effect of culture media on *in vitro* development of cloned mouse embryos. *Cloning* 2001; 3: 41–50.
- Petters RM, Wells KD. Culture of pig embryos. *J Reprod Fertil Suppl* 1993; 48: 61–73.
- Dobrinsky JR, Johnson LA, Rath D. Development of a culture medium (BECM-3) for porcine embryos: effects of bovine serum albumin and fetal bovine serum on embryo development. *Biol Reprod* 1996; 55: 1069–1074.
- Yoshioka 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.
- Nanassy L, Lee K, Javor A, Machaty Z. Effects of activation methods and culture conditions on development of parthenogenetic porcine embryos. *Anim Reprod Sci* 2008; 104: 264–274.
- Im GS, Lai L, Liu Z, Hao Y, Wax D, Bonk A, Prather RS. *In vitro* development of preimplantation porcine nuclear transfer embryos cultured in different media and gas atmospheres. *Theriogenology* 2004; 61: 1125–1135.
- Suzuki C, Iwamura S, Yoshioka K. Birth of piglets through the non-surgical transfer of blastocysts produced *in vitro*. *J Reprod Dev* 2004; 50: 487–491.
- Suzuki C, Yoshioka K. Effects of amino acid supplements and replacement of polyvinyl alcohol with bovine serum albumin in porcine zygote medium. *Reprod Fertil Dev* 2006; 18: 789–795.
- Sturmeier RG, Leese HJ. Energy metabolism in pig oocytes and early embryos. *Reproduction* 2003; 126: 197–204.
- Gandhi AP, Lane M, Gardner DK, Krisher RL. Substrate utilization in porcine embryos cultured in NCSU23 and G1.2/G2.2 sequential culture media. *Mol Reprod Dev* 2001; 58: 269–275.
- Gardner DK. Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. *Theriogenology* 1998; 49: 83–102.
- Yamanaka K, Sugimura S, Wakai T, Shoji T, Kobayashi J, Sasada H, Sato E. Effect of activation treatments on actin filament distribution and *in vitro* development of miniature pig somatic cell nuclear transfer embryos. *J Reprod Dev* 2007; 53: 791–800.
- Miyoshi K, Umez M, Sato E. Effect of hyaluronic acid on the development of porcine 1-cell embryos produced by a conventional or new *in vitro* maturation/fertilization system. *Theriogenology* 1999; 51: 777–784.
- Enright BP, Lonergan P, Dinnyes A, Fair T, Ward FA, Yang X, Boland MP. Culture of *in vitro* produced bovine zygotes *in vitro* vs *in vivo*: implications for early embryo development and quality. *Theriogenology* 2000; 54: 659–673.
- Drost M, Ambrose JD, Thatcher MJ, Cantrell CK, Wolfsdorf KE, Hasler JF, Thatcher WW. Conception rates after artificial insemination or embryo transfer in lactating dairy cows during summer in Florida. *Theriogenology* 1999; 52: 1161–1167.
- Farin PW, Piedrahita JA, Farin CE. Errors in development of fetuses and placentas from *in vitro*-produced bovine embryos. *Theriogenology* 2006; 65: 178–191.
- Lazzari G, Wrenzycki C, Hermann D, Duchi R, Krup T, Niemann H, Galli C. Cellular and molecular deviations in bovine *in vitro*-produced embryos are related to the large offspring syndrome. *Biol Reprod* 2002; 67: 767–775.
- Gardner DK, Leese HJ. The role of glucose and pyruvate transport in regulating nutrient utilization by preimplantation mouse embryos. *Development* 1988; 104: 423–429.
- Kim JH, Funahashi H, Niwa K, Okuda K. Glucose requirement at different developmental stages of *in vitro* fertilized bovine embryos cultured in semi-defined medium. *Theriogenology* 1993; 39: 875–886.
- Schini SA, Bavister BD. Two-cell block to development of cultured hamster embryos is caused by phosphate and glucose. *Biol Reprod* 1988; 39: 1183–1192.
- Seshagiri PB, Bavister BD. Glucose and phosphate inhibit respiration and oxidative metabolism in cultured hamster eight-cell embryos: evidence for the “crabtree effect”. *Mol Reprod Dev* 1991; 30: 105–111.
- Scott L, Whittingham DG. Influence of genetic background and media components on the development of mouse embryos *in vitro*. *Mol Reprod Dev* 1996; 43: 336–346.
- Quinn P. Enhanced results in mouse and human embryo culture using a modified human tubal fluid medium lacking glucose and phosphate. *J Assist Reprod Genet* 1995; 12: 97–105.
- Ankrah NA, Appiah-Openg R. Toxicity of low levels of methylglyoxal: depletion of blood glutathione and adverse effect on glucose tolerance in mice. *Toxicol Lett* 1999; 109: 61–67.
- Park YS, Suzuki K, Taniguchi N, Gutteridge JM. Glutathione peroxidase-like activity of caeruloplasmin as an important lung antioxidant. *FEBS Lett* 1999; 458: 133–136.
- Thompson JG, Partridge RJ, Houghton FD, Cox CI, Leese HJ. Oxygen uptake and carbohydrate metabolism by *in vitro* derived bovine embryos. *J Reprod Fertil* 1996; 106: 299–306.
- Iwasaki T, Kimura E, Totsukawa K. Studies on a chemically defined medium for *in vitro* culture of *in vitro* matured and fertilized porcine oocytes. *Theriogenology* 1999; 51: 709–720.
- Gardner DK, Lane M. Amino acids and ammonium regulate mouse embryo development in culture. *Biol Reprod* 1993; 48: 377–385.
- Takahashi Y, First NL. *In vitro* development of bovine one-cell embryos: Influence of glucose, lactate, pyruvate, amino acids and vitamins. *Theriogenology* 1992; 37: 963–978.
- Liu Z, Foote RH. Effects of amino acids on the development of *in vitro* matured/*in vitro* fertilization bovine embryos in a simple protein-free medium. *Hum Reprod* 1995; 10: 2985–2991.
- Van Thuan N, Harayama H, Miyake M. Characteristics of preimplantational development of porcine parthenogenetic diploids relative to the existence of amino acids *in vitro*. *Biol Reprod* 2002; 67: 1688–1698.
- Biggers JD, McGinnis LK, Lawitts JA. One-step versus two-step culture of mouse preimplantation embryos: is there a difference? *Hum Reprod* 2005; 20: 3376–3384.
- Hao Y, Lai L, Mao J, Im GS, Bonk A, Prather RS. Apoptosis and *in vitro* development of preimplantation porcine embryos derived *in vitro* or by nuclear transfer. *Biol Reprod* 2003; 69: 501–507.
- Chung YG, Mann MR, Bartolomei MS, Latham KE. Nuclear-cytoplasmic “tug of war” during cloning: effects of somatic cell nuclei on culture medium preferences of preimplantation cloned mouse embryos. *Biol Reprod* 2002; 66: 1178–1184.
- Boiani M, Gentile L, Gambles VV, Cavaleri F, Redi CA, Scholer HR. Variable reprogramming of the pluripotent stem cell marker Oct4 in mouse clones: distinct developmental potentials in different culture environments. *Stem Cells* 2005; 23: 1089–1104.