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Effects of Synchronization of Donor Cell Cycle on Embryonic Development and DNA Synthesis in Porcine Nuclear Transfer Embryos

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Abstract. The relationship between donor cell cycle and the developmental ability of somatic cell nuclear transfer (SCNT) embryos has not fully been elucidated. Donor cells that are usually prepared by serum starvation or confluent-cell culture for SCNT represent a heterogeneous population that includes mainly G0 phase cells, other cells in different phases of the cell cycle and apoptotic cells. In this study, we compared the developmental ability of porcine SCNT embryos reconstructed from G0 phase cells (G0-SCNT embryos) and strictly synchronized-G1 phase cells (G1-SCNT embryos), and examined the developmental rates and timing of first DNA synthesis. The G0 phase cells were synchronized by confluent culture, and the G1 phase cells were prepared from actively dividing M phase cells. The G1-SCNT embryos showed a significantly higher ($P<0.05$) developmental rate to the blastocyst stage per cleaved embryo (59%) than the G0-SCNT embryos (43%). Moreover, initiation of first DNA synthesis and cleavage occurred significantly earlier in the G1-SCNT embryos than in the G0-SCNT embryos. Delay of initiation of first DNA synthesis in the SCNT embryos by aphidicolin resulted in decreased developmental rates to the blastocyst stage without any effect on cleavage rates. Our data demonstrates that synchronized-G1 phase cells can be used as donor cells for SCNT embryos and that earlier initiation of first DNA synthesis may be important for subsequent development of SCNT embryos. The SCNT system using G1-synchronized cells, in terms of their highly uniform and viable cell states, can be useful for studying the reprogramming processes and embryonic development of SCNT embryos.

Key words: Cell cycle synchronization, DNA synthesis, Nuclear transfer, Reprogramming
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Somatic cell-derived cloned animals from many species have been reported [1–9] since the first successful cloning from somatic cells was accomplished in sheep [1]. However, the efficiency of cloning is very low, and the appropriate conditions for reprogramming somatic cells are still unclear.

The cell cycle of donor cells is a major factor that affects the efficiency of producing somatic cell nuclear transfer (SCNT) embryos [10]. Wilmut *et al.* [1] reported that G0 phase donor cells prepared by serum starvation can be used for SCNT and may facilitate nuclear reprogramming in unfertilized oocytes. Therefore, G0 cells synchronized by serum starvation or a confluent cell culture system have been widely used as donor cells for SCNT [11]. However, serum starvation causes reduced cell

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survival and increased DNA fragmentation [12] and apoptosis [13] in porcine cells. The confluent cell culture system is as effective for cell cycle synchronization as serum starvation, especially for arresting cells in the G0/G1 phase [14–16]. Moreover, SCNT embryos reconstructed with donor cells obtained by confluent cell culture or serum starvation do not show different developmental rates during *in vitro* development [17]. Donor cells prepared by serum starvation or confluent culture consist of a mixture of cell cycles [12, 14, 16]. It is unclear which cell cycle of donor cells is effective for nuclear reprogramming and production of cloned animals, and successful cloning has been achieved with cell types other than the G0 phase [3, 18, 19]. Because unfertilized oocytes quickly move to the G1 phase after activation and/or nuclear transfer, donor cells in the early G1 phase are considered to be synchronous with the cell cycle of recipient oocytes. In fact, high developmental ability has been reported for SCNT embryos using G1 cells as donor cells [15, 20]. Although G1 phase cells have many common features with G0 phase cells, they are definitely distinct from G0 cells in that G1 cells are in a cell cycling state. Synchronized G1 phase cells are easily collected by treatment with a mitosis inhibitor, 2-methoxyestradiol, followed by the shake-off method [20], and they have been shown to effectively support development of SCNT embryos as donor cells to term [20]. Although both G0 and G1 cells can support high developmental ability for SCNT embryos in terms of developmental rates to term and/or the blastocyst stage, there is little information concerning the differences in nuclear reprogramming and early embryonic development between G0-SCNT and G1-SCNT embryos.

The onset of DNA synthesis in embryos is one of the most important steps in initiation of early preimplantation development. The duration of the first DNA replication positively correlates with high developmental rates to the blastocyst stage for *in vitro*-fertilized eggs in mice [21] and cattle [22]. Earlier onset of DNA synthesis in embryos correlates with a longer period of the first S phase [23]. The timing of DNA synthesis initiation depends on the donor cell cycle in bovine SCNT embryos [24]. However, there is little information about the relationship between first DNA synthesis and developmental rates in SCNT embryos.

In this study, we reconstructed SCNT embryos from synchronized donor cells at the G0 phase and G1 phase, and compared the timing of first DNA synthesis and developmental ability of SCNT embryos to the blastocyst stage to elucidate the critical steps of nuclear reprogramming for support of early embryonic development in pig SCNT embryos.

Materials and Methods

In vitro maturation of oocytes

Ovaries from domestic gilts (6 months old) were obtained at a local slaughterhouse. Follicles 3–6 mm in diameter were dissected with two scalpels as described by Hoshino *et al.* [25]. Briefly, 20–50 cumulus-oocyte complexes were collected and cultured in HEPES-buffered TCM-199 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 2.2 mg/ml NaHCO₃, 100 mg/l pyruvic acid (Sigma-Aldrich), 10% fetal bovine serum (FBS; JRH Bioscience, Inc., Kansas City, KS, USA), 50 mg/ml penicillin G (Sigma-Aldrich) and 50 mg/ml streptomycin (Meiji, Tokyo, Japan) (Medium A). They were then cultured with two inverted follicle shells in maturation medium, which consisted of TCM-199 medium (Sigma-Aldrich) containing 2.2 mg/ml NaHCO₃, 100 mg/l pyruvic acid, 0.1 IU/ml human menopausal gonadotropin (Teikoku Zoki, Co., Tokyo, Japan), 10% FBS, 50 mg/ml penicillin G and 50 mg/ml streptomycin, at 38.5 °C under humidified air containing 5% CO₂ for 44–45 h.

Cell cycle synchronization of donor cells

Donor fibroblast cells were collected from the kidney of a male miniature Potbelly pig (10 months old). Small pieces of tissue from the kidney were placed in 3.5 cm culture dishes and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% FBS, 50 mg/ml penicillin G and 50 mg/ml streptomycin (Medium B) at 37 °C in 5% CO₂ in air. After removal of the explants, growing fibroblast cells were further cultured in Medium B. These porcine fibroblast cells were used at passages 2 to 10. The cells were synchronized at the G0 phase of the cell cycle by an additional 3 days of culture after reaching confluency in Medium B (confluent culture). Synchronized cells at the G1 phase were prepared as described by Urakawa *et al.* [20] with a

Fig. 1. Synchronized cells at the G1 phase. Cells just after dividing equally (arrows) were used as donor cells. Scale bar is 10 μ m.

minor modification. Briefly, the growing cell culture in Medium B was changed to the Minimum Essential Medium without calcium ion (Invitrogen) supplemented with 10% FBS, 2 mM glutamine (Wako, Osaka, Japan) and 2-methoxyestradiol (Sigma-Aldrich), a mitotic inhibitor, at 1 μ M to synchronize cells to the M phase. After 30 min of culture at 37 C in 5% CO₂ in air, the culture dish was shaken for 1 min to detach the M phase cells, and the supernatant was discarded. This treatment was repeated once, and finally the supernatant was collected and centrifuged at 200 $\times$ g for 5 min. The M phase cells were resuspended in Medium A and cultured for 25 min. Cells that had just cleaved equally were used for SCNT as G1-synchronized cells (Fig. 1).

Somatic cell nuclear transfer (SCNT)

In vitro-matured pig oocytes were used as recipient oocytes for SCNT. After 44–45 h of maturation culture, the oocytes were treated with 1 mg/ml hyaluronidase (Sigma-Aldrich) to remove the surrounding granulosa and cumulus cells. Oocytes that clearly extruded a first polar body were selected as recipient cytoplasts. The first polar body and adjacent cytoplasm, presumably including the metaphase chromosomes, were

extruded by squeezing with a glass needle and were stained with Hoechst 33342 (Sigma-Aldrich). Successful enucleation was confirmed by the appearance of a metaphase plate in the removed karyoplast when illuminated by ultraviolet light. Donor cells that were synchronized at the G0 or G1 phase were injected into the perivitelline space of enucleated oocytes. These couplets were settled between two needle electrodes (444–100; Meiwa Shoji, Osaka, Japan) attached to a micro-manipulator (Narishige, Tokyo, Japan) and were fused by two direct current pulses of 30 V for 30 μ sec using an ECM 2001M (BTX, San Diego, CA, USA) in 0.3 M mannitol solution containing 0.05 mM CaCl₂ and 0.1 mM MgSO₄.

The reconstructed embryos were cultured in porcine zygote medium-3 (PZM-3; [26]) at 38.5 C in 5% CO₂ in air for 144 h. The developmental rates of the reconstructed embryos to each embryonic stage were examined every 24 h until 144 h after activation, and cleavage rates were assessed at 48 h of culture. The developmental rates are the proportions of embryos that developed to each stage. To compare the developmental rates of SCNT embryos to parthenogenetically activated (PA) embryos, matured oocytes were electronically activated by the same procedure.

Control of DNA synthesis in SCNT embryos

To delay the initiation of DNA synthesis during the first cleavage of SCNT and PA embryos, we added 1 μ g/ml aphidicolin (Sigma-Aldrich), a reversible inhibitor of DNA polymerase α , σ and ϵ [27], to PZM-3. After activation, G0-SCNT embryos, G1-SCNT embryos and PA embryos were randomly divided into two groups. The duration of aphidicolin treatment required to delay initiation of DNA synthesis was determined in a preliminary experiment. At 7 h after activation, only few embryos entered the S phase; however, all embryos entered the S phase at 14 h after activation (data not shown). Therefore, one group of embryos was treated with aphidicolin 7–14 h after activation, and the other group served as a control (no addition of aphidicolin).

Detection of DNA synthesis of SCNT embryos

DNA synthesis of SCNT and PA embryos was detected by incorporation of 5-bromo-2'-deoxyuridine (BrdU) into embryonic DNA and immunofluorescence assay using a BrdU Labeling

and Detection Kit I (1 296 7361; Roche Diagnostics, Indianapolis, IN, USA). Embryos were cultured in 100 μ M BrdU-containing medium 3–6, 6–9, 9–10.5 h or 10.5–12 h after fusion and activation of SCNT embryos or activation for PA embryos at 38.5 C in 5% CO₂ in air. After culture, the zona pellucida was removed with 0.05% Actinase E (Kaken, Tokyo, Japan), and the embryos were fixed and treated with anti-BrdU and anti-mouse immunoglobulin-fluorescein solution. Finally, the embryos were stained with propidium iodide (Sigma-Aldrich) to confirm whether a somatic cell nucleus successfully entered into the recipient cytoplasm. The BrdU signals of the embryos were examined using fluorescent microscopy, and the onset of DNA synthesis was compared among the G0-SCNT, G1-SCNT and PA embryos.

Differential staining of ICM and trophectoderm cells in blastocysts

The number of cells at the blastocyst stage was counted at 150 h. After removal of the zona pellucida, blastocysts were incubated in PBS with 10% rabbit anti-pig antiserum for 15 min at 37 C. Then, they were incubated in PBS with 5% guinea pig complement (Sigma-Aldrich) for 15 min at 37 C. Subsequently, the embryos were washed in PBS (4 C) with 10 μ g/ml propidium iodide and were fixed in ethanol containing 10 μ g/ml Hoechst 33342 overnight at –20 C. Trophectoderm cells were recognized by the red fluorescence of propidium iodide under a fluorescence microscope, and inner cell mass (ICM) cells were identified by blue fluorescence.

Semiquantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) of SCNT embryos

Purification of total RNA for RT-PCR was carried out on a single SCNT and PA embryo at the blastocyst stage using a ToTally RNA kit (Ambion, Austin, TX, USA). Briefly, the total RNAs were extracted with acid-phenol/chloroform, precipitated by ethanol with glycogen and treated with DNase for 20 min at room temperature. For reverse transcription, SuperScript III (Invitrogen) was added to the total RNA solution and incubated at 25 C for 10 min, 50 C for 55 min and at 70 C for 15 min. PCR amplification was carried out on 1 μ l of RT product from an embryo per 20 μ l of PCR reaction mixture comprised of 13.3 μ l of water, 2 μ l of 10 $\times$ PCR buffer (TaKaRa, Kyoto, Japan), 1.6 μ l of

2.5 mM dNTP (TaKaRa), 2 μ l of each sense and antisense primer and 0.1 μ l of ExTaq (TaKaRa). The PCR cycles for detection of *Oct4* (*Pou5f1*) and β -actin (*Actb*) gene expression were conducted as follows: preheating reaction at 94 C for 2 min for denaturation and 32 cycles of denaturation at 94 C for 30 sec, annealing at 64 C for 30 sec and extension at 72 C for 30 sec. The last cycles were followed by a 7 min extension reaction at 72 C. The PCR primers were designed for *Oct4* (GenBank accession number, AJ251914; sense, 5'-CGAGTGAGAGGCAACCTGGA-3'; antisense, 5'-AGCCCAGAGGGGTGACAGAC-3' for a 346 base pairs [bp] product) and for β -actin (GenBank accession number, AJ312193; sense, 5'-GCCCATCTACGAGGGGTACG-3'; antisense, 5'-CACGTCGCACTTCATGATCG-3' for a 374 bp product). Ten μ l of the PCR products was visualized on 2% agarose gel under ultraviolet light. Relative band intensities were determined using a densitograph (model 4.0A; Atto, Tokyo, Japan) during the exponential stage of PCR amplification. The exponential stage was confirmed by three different PCR cycles. The intensities of *Oct4* expression were presented relative to the intensity of the band for β -actin expression.

Statistical analysis

The developmental rates (Tables 1 and 4), cell numbers (Table 5), and expression levels of *Oct4* gene at the blastocyst stage (Fig. 3B) were analyzed using analysis of variance (ANOVA). Differences in developmental progressions of SCNT embryos (Table 2) and initiation of DNA synthesis (Table 3) were analyzed by the Tukey method using the general linear model procedure of Statistical Analysis System (SAS Institute, Cary, NC, USA). Statistical significance was determined when the P value was less than 0.05.

Ethical approval

All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee of Kyoto University.

Results

Developmental ability of SCNT embryos

The developmental rates of the SCNT embryos

Table 1. Developmental ability of SCNT embryos reconstructed from G0 or G1 cells

Groups	No. of trials	No. of embryos examined	No. of embryos cleaved (mean $\pm$ SD%)	No. of embryos to blastocysts (mean $\pm$ SD%)	% of blastocysts/cleaved embryos (mean $\pm$ SD%)
G0-SCNT	6	89	62 (69 $\pm$ 7%)	27 (30 $\pm$ 9%)	43 $\pm$ 12%
G1-SCNT	6	103	63 (63 $\pm$ 10%)	37 (37 $\pm$ 7%)	59 $\pm$ 3% ^a

^aValues differ significantly within the same column ($P < 0.05$).

reconstructed from G0 and G1 cells and cultured in PMZ-3 for 144 h are shown in Table 1. There were no significant differences in cleavage rates and developmental rates to the blastocyst stage between the G0-SCNT and G1-SCNT embryos, but the developmental rate to the blastocyst stage per cleaved embryo was significantly higher in the G1-SCNT embryos.

Developmental progression of SCNT embryos

The developmental stages of the embryos were examined every 24 h after nuclear transfer. The G1-SCNT embryos developed faster at every developmental stage compared with the G0-SCNT embryos (Fig. 2). A distinct difference was observed from the first cleavage stage after 24 h (G0-SCNT, 44%; G1-SCNT, 70%). In contrast, most PA embryos (94%) cleaved 24 h after culture. Additionally, compared with PA embryos, SCNT embryos exhibited a characteristic developmental pattern at the end of culture (Table 2); that is, a significantly higher population of G0-SCNT embryos were arrested at the 2-cell stage than G1-SCNT embryos.

Initiation of first DNA synthesis and the effect of delay of first DNA synthesis on embryonic development in SCNT embryos

The timing of DNA synthesis of SCNT embryos was measured by the incorporation of BrdU 3–6, 6–9, 9–10.5 and 10.5–12 h after nuclear transfer (Table 3). Most PA embryos initiated DNA synthesis 6–9 h after nuclear transfer (83%). In contrast, only a few SCNT embryos initiated DNA synthesis at 9 h after nuclear transfer, and most of them started synthesis 9–10.5 h after nuclear transfer. At 10.5–12 h after nuclear transfer, embryos in all experimental groups began to synthesize DNA. Therefore, DNA synthesis of the PA embryos was initiated significantly earlier than that of the G1-SCNT and G0-SCNT embryos. Moreover, initiation of DNA synthesis 9–10.5 h after nuclear transfer was significantly earlier in the G1-SCNT embryos

(86%) than in the G0-SCNT embryos (63%).

To confirm whether delay of first DNA synthesis affects the developmental rates, the timing of first DNA synthesis of SCNT and PA embryos was delayed by treatment with aphidicolin. As shown in Table 4, the developmental rate to blastocyst stage of the G0-SCNT embryos was significantly decreased compared to the non-treatment controls (3 and 31%, respectively). However, aphidicolin treatment did not have any effect on the cleavage rates. A similar result was obtained in the G1-SCNT embryos (aphidicolin treatment and control; 3 and 31%, respectively) and PA embryos (23 and 64%). Delay of first cleavage was observed in the aphidicolin-treated embryos. Most of the aphidicolin-treated G0-SCNT embryos did not cleave 24 h after activation (16%), although 44% of the non-treated embryos cleaved. At 27 h after activation, 55% of the aphidicolin-treated embryos cleaved, suggesting that the delay of first DNA synthesis was within 3 h in length. At 144 h after activation, 16% of the aphidicolin-treated and 4% of the non-treated embryos arrested at the morula stage.

Cell number and Oct4 gene expression of SCNT embryos at the blastocyst stage

The quality of SCNT embryos at the blastocyst stage was assessed by differential staining of ICM and trophectoderm cells and *Oct4* gene expression. There were no significant differences in the total cell numbers of blastocysts or cell numbers of ICM and trophectoderm cells among the PA, G0-SCNT and G1-SCNT embryos (Table 5). Furthermore, there were no significant differences in the ratios of ICM cell numbers to total cell numbers of blastocysts among the different types of embryo (Table 5).

We conducted semiquantitative RT-PCR analysis of the SCNT and PA embryos. The relative amount of *Oct4* transcripts in an individual blastocyst was calculated by the intensity of β -actin gene expression. Lower expression was observed for the

Fig. 2. Developmental progression of G0-SCNT, G1-SCNT and PA embryos. The developmental stages of embryos were examined every 24 h. The proportion of embryos at each stage was represented as developed embryos per number of cleaved embryos from 48 to 144 h after activation, and the proportion of 1-, 2- and 3-cell stage embryos to total embryos (abnormally divided embryos were excluded) was examined after 24 h. The data in Fig. 2 came from a portion of the data in Table 1 (5 trials in Table 1).

Table 2. Differences in the developmental progression of G0-SCNT, G1-SCNT and PA embryos*

Groups	2- and 3-cell stages at 24 h	4- and 5-cell stages at 48 h	Embryos arrested at the 2-cell stage
G0-SCNT	20/45 (44%) ^c	11/45 (24%) ^f	16/45 (36%) ^g
G1-SCNT	44/63 (70%) ^b	27/63 (43%) ^e	10/63 (16%) ^h
PA [#]	30/32 (94%) ^a	31/32 (97%) ^d	0/32 (0%) ⁱ

^{a-i}Different superscripts indicate significant differences within the same column ($P < 0.05$).

[#]PA: Embryos produced by parthenogenetic activation.

*Tables in each column represent the number of developed embryos per the number of cleaved embryos. Values in parentheses indicate percentages.

Table 3. Initiation of DNA synthesis in SCNT embryos reconstructed from G0 or G1 cells[#]

Groups	Duration (h) and incorporation (%) of BrdU after nuclear transfer			
	3–6 h	6–9 h	9–10.5 h	10.5–12 h
G0-SCNT	1/11 (9%)	2/10 (20%) ^b	17/27 (63%) ^d	14/15 (93%)
G1-SCNT	1/16 (6%)	4/18 (22%) ^b	24/28 (86%) ^c	7/7 (100%)
PA	0/16 (0%)	15/18 (83%) ^a	17/17 (100%) ^c	10/10 (100%)

^{a-d}Different superscripts indicate significant differences within the same column ($P < 0.05$).

[#]Tables in each column represent the no. of BrdU positive embryos per the no. of embryos examined.

Oct4 gene in the SCNT embryos compared with the PA embryos (Fig. 3). However, no significant differences were observed between the G0-SCNT and G1-SCNT embryos.

Discussion

Several reports refer to differences in the developmental ability of SCNT embryos

Table 4. Developmental rates of G0-SCNT embryos treated with aphidicolin during 7–14 h after nuclear transfer

Groups	Treatment	No. of trials	No. of embryos examined	No. of embryos cleaved (mean $\pm$ SD%)	No. of embryos to blastocysts (mean $\pm$ SD%)
G0-SCNT	Aphidicolin	6	84	54 (64 $\pm$ 12%)	4 (3 $\pm$ 4%)
	Control	6	81	55 (67 $\pm$ 16%)	26 (31 $\pm$ 17%) ^a
G1-SCNT	Aphidicolin	5	66	34 (53 $\pm$ 13%)	2 (3 $\pm$ 4%)
	Control	5	66	39 (58 $\pm$ 6%)	20 (31 $\pm$ 8%) ^b

^{a,b}Values differ significantly within the same column of the same donor cell cycle stage ($P < 0.05$).

Table 5. Cell numbers and proportions of ICM cells in SCNT and PA blastocysts

Groups	No. of embryos examined	Total cell number (mean $\pm$ SE)	ICM cell number (mean $\pm$ SE)	TE cell number (mean $\pm$ SE)	ICM/TE (mean $\pm$ SE%)
G0-SCNT	9	44.2 $\pm$ 2.0	13.7 $\pm$ 0.8	30.6 $\pm$ 1.6	31.0 $\pm$ 1.6
G1-SCNT	11	43.4 $\pm$ 2.0	13.5 $\pm$ 1.2	29.8 $\pm$ 1.5	31.0 $\pm$ 2.2
PA*	8	48.5 $\pm$ 2.2	13.5 $\pm$ 0.9	34.8 $\pm$ 2.3	28.2 $\pm$ 2.2

*PA: Embryos produced by parthenogenetic activation.

Fig. 3. Comparison of the expression of *Oct4* transcripts in SCNT and PA embryos. (A) Gel electrophoresis of a semiquantitative RT-PCR product from a single G0-SCNT (G0), G1-SCNT (G1) and PA embryo. P represents the positive control (*Oct4* in oocytes and β -actin in fibroblasts) and N represents the negative control (*Oct4* in fibroblasts and β -actin with no template DNA added). (B) Relative amount of *Oct4* transcripts to β -actin transcripts in each blastocyst. *Value differs significantly from the other groups ($P < 0.05$).

reconstructed from G0 or G1 cells. Wells *et al.* [28] showed that for non-transgenic fibroblasts, synchronization in the G0 phase results in a higher percentage of viable calves to term than synchronization in the G1 phase, suggesting that G0 cells are superior to G1 cells as donor cells in cattle [10]. In contrast, G1 cells support a higher developmental rate to term than G0 cells in cattle [15] and exhibit higher developmental rates to term in cattle [20]. In this study, the G1-SCNT embryos had a significantly higher developmental rate to the blastocyst stage per cleaved embryo than the G0-SCNT embryos (59% for G1 and 43% for G0; $P < 0.05$). This indicates that G1-synchronized cells can support preimplantation development of SCNT embryos and may be more effectively reprogrammed than G0 cells in porcine SCNT. Disadvantages of G0 cells may be explained by their heterogeneous cell population. G0-synchronized cells obtained using the confluent cell culture system contain cells of other cell cycles or apoptotic cells [13, 14]. Contamination of these cells may cause a lower developmental rate.

The timing of first cleavage of embryos correlates with subsequent development in some species [29–33]. Duration of the 2-cell stage increased as the time before cleavage to the 2-cell stage increased in porcine embryos [29]. Early onset of first cleavage of embryos is associated with increased blastocyst formation in bovine [31] and human embryos [33] and is also related to a high pregnancy rate in

humans [30, 32]. In the present study, G1-SCNT embryos with high developmental rates cleaved faster 24 h after nuclear transfer and reached the blastocyst stage earlier than the G0-SCNT embryos (Fig. 2). This suggests that the difference in donor cell cycle affects developmental progression of SCNT embryos and that earlier development of SCNT embryos may correlate with higher developmental potential. Interestingly, a considerable number of the G0-SCNT embryos (16/45=36%) remained at the 2-cell stage even at the end of culture, while only 16% of the G1-SCNT embryos and 0% of PA embryos were at the 2-cell stage (Table 2). Failure of embryonic development *in vitro*, caused by insufficient culture conditions or a deficient developmental program, may result in developmental arrest at around embryonic genome activation [34]. Since embryonic genome activation occurs at the 4-cell stage in domestic pigs [35], developmental arrest at the 2-cell stage in this experiment was a unique phenomenon in SCNT embryos and may have been caused by the failure of nuclear reprogramming.

The timing of onset of DNA synthesis in the SCNT embryos varied and was dependent on the donor cell cycle (Table 3). When G0 cells were used as donor cells, the onset of DNA synthesis was delayed compared with that of G1 cells. Kurosaka *et al.* [24] also reported that donor cells at the G1/S phase initiate DNA synthesis earlier than G0-phase cells, which is in agreement with our results. This may result from the characteristics of G0 cells. Because G0 cells exit the normal cell division cycle and enter a quiescent state, much more time may be required to enter the S phase. *In vitro* fertilized bovine embryos, which have high developmental ability to the blastocyst stage, initiate DNA synthesis earlier [22, 23] and have a longer S phase than embryos with low developmental ability [23]. In this study, the G1-SCNT embryos entered into the S phase earlier, and this resulted in a higher developmental rate. Moreover, delay of initiation of the first S phase dramatically reduced the developmental rates to the blastocyst stage in the SCNT and PA embryos in this experiment. These results suggest that earlier initiation of DNA synthesis may be one of the indicators of developmental competence for SCNT embryos. The fact that a higher proportion of the G1-SCNT embryos cleaved and developed into blastocysts may be because they entered the S-phase earlier

than the G0-SCNT embryos. An essential process for nuclear reprogramming and/or development of SCNT embryos may exist during the phase between first DNA synthesis and nuclear transfer into oocytes.

The effect of the cell cycle of donor cells on the morphological and biochemical aspects of SCNT embryos was examined at the blastocyst stage. The total cell numbers and the ratios of ICM cell numbers to total cell numbers in the SCNT embryos did not differ significantly between the SCNT and PA embryos (Table 5). The cell numbers and ratios of ICM cells were similar to those of *in vivo* porcine embryos [26], suggesting that obvious defects in cell morphology were not observed in the SCNT embryos reconstructed from G0 or G1 cells. Gene expression of undifferentiated stem cell marker *Oct4* was examined at the blastocyst stage by semiquantitative RT-PCR analysis. *Oct4* is a gene that encodes for a transcriptional factor required for early embryonic development [36]. The *Oct4* gene is not expressed in differentiated somatic cells, and abnormal distribution of *Oct4* mRNA and low levels of *Oct4* expression in mouse SCNT embryos have been reported [37]. In the present study, down-regulation of *Oct4* gene expression was observed in the SCNT embryos compared with the PA embryos. PA embryos obtained by the same method can be efficiently implanted until day 29 of pregnancy [38]. Taking this high implantation rate, high developmental rate to the blastocyst stage, and normal cell numbers at the blastocyst stage into consideration, the PA embryos in our experiment may have properties that are close to normal embryos, at least during preimplantation development. Therefore, the *Oct4* gene in porcine SCNT embryos may be incompletely reactivated as in mouse SCNT embryos [37]. No significant difference, however, was observed in gene expression between the G0-SCNT and G1-SCNT embryos. In porcine blastocysts, Oct4 protein is detected in both the ICM and trophoctoderm [39]. This is in contrast to the mouse [39], in which Oct4 is only expressed in ICM cells. Further investigation is needed to characterize the expression of *Oct4* in porcine SCNT embryos.

The differences between the developmental patterns of the SCNT embryos from the two different donors and the PA embryos became apparent during the first cell cycle (Fig. 2 and Table 3). Many events that are associated with nuclear

reprogramming in SCNT embryos, including replacement of somatic linker histones with oocyte-specific linker histone H1FOO [40, 41], changes in the composition of nuclear lamins and nucleolar changes [42, 43] and formation of premature chromosome condensation [44], occur at a very early developmental stage or at least before first cleavage. Failure of these events to occur may

result in abnormal loss of DNA synthesis or embryo cleavage. More studies are needed to clarify the early reprogramming events of SCNT embryos. The present experiment provides a model system for reprogramming analysis of SCNT embryos using synchronized G1 donor cells with high cellular viability and a homogeneous cell state.

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