# Improved Enucleation Efficiency of Pig Somatic Cell Nuclear Transfer by Early Denudation of Oocytes at 30 Hours of *In Vitro* Maturation

**Kilyoung Song[1], Sang-Hwan Hyun[2] and Eunsong Lee[3,*]**

[1]*College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea*
[2]*College of Veterinary Medicine, Chungbuk National University, Cheongju 361-763, Korea*
[3]*School of Veterinary Medicine, Kangwon National University, Chunchon 200-701, Korea*

## ABSTRACT

Our goal was to examine the effects of early denudation on the enucleation efficiency and developmental competence of embryos following somatic cell nuclear transfer (SCNT) and parthenogenetic activation (PA). Oocytes were denuded following 30 h of *in vitro* maturation (IVM) and then cultured with (D+) or without (D−) their detached cumulus cells for additional 10~14 h. Control oocytes were denuded after 40~44 h of IVM. The size of the perivitelline space was larger at 40 h of IVM (11.7~11.8 $\mu$m) than at 30 h (8.9 $\mu$m; $p<0.01$). The distances between the metaphase II (M II) plates and the polar bodies (PBs) were shorter in D+ (19.4 $\mu$m) and D− oocytes (18.9 $\mu$m) than in control oocytes (25.5 $\mu$m; $p<0.01$). Enucleation rates following blind aspiration at 40 h of IVM were higher ($p<0.01$) in D+ (92%) and D− oocytes (93%) compared to controls (82%). Early denudation did not affect oocyte maturation or the *in vitro* development of SCNT and PA embryos. When SCNT embryos from D+ oocytes were transferred to four gilts, pregnancy was established in two pigs, and one of them farrowed three live piglets. In conclusion, early denudation of oocytes at 30 h of IVM could improve the enucleation efficiency by maintaining the M II plate and the PB within close proximity and support the *in vivo* development of SCNT embryos to term.

(Key words : cumulus cells, enucleation, perivitelline space, pigs, somatic cell nuclear transfer)

## INTRODUCTION

Somatic cell nuclear transfer (SCNT) is a routine method used to produce cloned animals for economic and pharmaceutical purposes. Cloned offspring have been successfully produced by SCNT in various species (Wilmut *et al.*, 1997; Cibelli *et al.*, 1998; Wakayama *et al.*, 1998; Polejaeva *et al.*, 2000); however, the production efficiency of cloned offspring by SCNT is still low. The *in vitro* and *in vivo* development of SCNT embryos is influenced by multiple factors, including oocyte quality, manipulation method, and reconstructed oocytes activation (Hyun *et al.*, 2003; Kurome *et al.*, 2003; Miyoshi *et al.*, 2006). Thus, modifications of these factors are necessary to improve SCNT efficiency.

The accurate preparation of high-quality cytoplasts is the critical step that determines whether SCNT is successful. In addition, the complete elimination of chromatic materials from recipient oocytes is a necessary prerequisite to avoid chromosomal aneuploidy. Mature oocytes are generally enclosed by expanded cumulus cells, and these cells are removed by enzymatic and/or mechanical treatment before enucleation. Vortexing (Walker *et al.*, 2002) or repeated pipetting (Hyun *et al.*, 2003) is often used to remove cumulus cells; however, these types of mechanical manipulations may put physical pressure on oocytes and cause changes in the relative location of the polar body (PB) and metaphase II (M II) plate. The perivitelline space (PVS) of oocytes is formed during oogenesis and changes in size during oocyte maturation (Talbot and Dandekar, 2003; Kafi *et al.*, 2005). The PVS is relatively narrow at the germinal vesicle stage, but becomes enlarged and asymmetrical following PB extrusion (Talbot and Dandekar, 2003). It is thus possible that the PB may become displaced by external pressure applied during the mechanical stripping of cumulus cells in oocytes with an enlarged PVS. In support of this hypothesis, significant positional shifts of the PB within the PVS are observed after mechanical denudation of oocytes in cattle, pigs, mice, and rhesus monkeys (Hewitson *et al.*, 1999; Atabay *et al.*, 2001; Miao *et al.*, 2004). The PB is commonly used as an indicator of the location of M II plates during the enucleation process. A PB that is displaced from the M II plate can cause the removal of

* Correspondence : E-mail : eslee@kangwon.ac.kr

metaphase chromosomes to become difficult, especially when small volumes of cytoplasm are removed together with the PB. Atabay *et al.* (2001) suggested that it is useful to remove a greater volume of cytoplasm to increase the enucleation rate. However, studies of bovine nuclear transfer embryos have found that both the developmental rate to the blastocyst stage and the number of embryonic cells are lower when cytoplasts of smaller volume are used (Westhusin *et al.*, 1996; Peura *et al.*, 1998). Many studies have attempted to improve enucleation efficiency. In mice, Kono *et al.* (1991) developed a method for the removal of oocyte chromosomes reliably and quickly by enucleating oocytes at the telophase stage of the first meiotic division. In addition, it has been reported in pigs that demecolcine was used successfully for the chemically assisted removal of maternal chromosomes (Kawakami *et al.*, 2003). Thus, it is generally accepted that both maintaining the PB near the M Ⅱ chromosomes and reducing the cytoplasmic loss during enucleation are helpful in improving the enucleation efficiency and later development of SCNT embryos.

We hypothesized that denuding oocytes at 30 h of IVM, at which point the PVS is comparatively narrow, would maintain the M Ⅱ plate and the PB within close proximity and would therefore make it easier to enucleate without impairing later development following SCNT and PA. To evaluate these ideas, we examined both PVS size and distances between the M Ⅱ plates and the PB after early denudation of oocytes at 30 h of IVM. In addition, we investigated the effects of early cumulus cell removal on the enucleation efficiency and *in vitro* development following SCNT and PA. Finally, the *in vivo* competency of SCNT embryos derived from oocytes denuded at 30 h of IVM was examined using an embryo transfer experiment.

## MATERIALS AND METHODS

### 1. Culture Media

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The base medium for IVM was tissue culture medium (TCM)-199 (Invitrogen, Grand Island, NY, USA), supplemented with 0.6 mM cysteine, 0.91 mM pyruvate, 10 ng/ml epidermal growth factor, 75 μg/ml kanamycin, 1 μg/ml insulin, and 10% (v/v) porcine follicular fluid. The same batch of pFF was used in all experiments. The *in vitro* culture (IVC) medium used for embryo development was North Carolina State University (NCSU)-23 medium, containing 0.4% (w/v) bovine serum albumin (BSA) (Petters and Wells, 1993), which was modified by replacing glucose with 0.5 mM pyruvate and 5.0 mM lactate (Park *et al.*, 2005).

### 2. Oocyte Collection and IVM

Ovaries were obtained from prepubertal gilts at a local abattoir. Follicular contents were aspirated from superficial follicles (3～8 mm in diameter), pooled into 15 ml conical tubes, and allowed to settle as sediment. The sediment was placed in HEPES-buffered Tyrode's medium (TLH) containing 0.05% (w/v) polyvinyl alcohol (TLH-PVA) (Bavister *et al.*, 1983) and observed under a stereomicroscope; only cumulus-oocyte complexes (COCs) with more than three layers of compact cumulus cells were selected. After washing twice in TLH-PVA and once in IVM medium, a group of 50～90 COCs was placed into each well of a four-well multi-dish (Nunc, Roskilde, Denmark) containing 500 μl of IVM medium with 10 IU/ml eCG (Intervet International BV, Boxmeer, The Netherlands) and 10 IU/ ml hCG (Intervet International BV). COCs were cultured at 39℃ in a humidified atmosphere of 5% $CO_2$. After 22 h in the maturation culture, the COCs were washed three times in fresh hormone-free IVM medium and then cultured in hormone-free IVM medium (Funahashi and Day, 1993) for an additional 18 h for SCNT and 22 h for PA in Experiment 3.

### 3. Preparation of Donor Cells

Ear skin fibroblasts from a newborn miniature piglet were seeded in a four-well plate and grown in Dulbecco's modified Eagle medium (DMEM) with the nutrient mixture F-12 (Invitrogen), supplemented with 15% (v/v) fetal bovine serum from the same batch (Sigma F9423, lot # 24k2409) until a complete monolayer formed. Donor cells were induced to synchronize at the G0/G1 stage of the cell cycle by contact inhibition for 24～72 h. Cells of the same passage (3～8 passages) were used in each replicate for the various treatments. A single cell suspension was prepared by trypsinization of cultured cells and resuspension in TLH containing 0.4% (w/v) BSA (TLH-BSA) prior to nuclear transfer.

### 4. Somatic Cell Nuclear Transfer

After 40 h of IVM, cumulus-cell-free oocytes were incubated for 15 min in manipulation medium (calcium-free TLH-BSA) containing 5 μg/ml Hoechst 33342. Following incubation, the oocytes were washed twice in a fresh manipulation medium and transferred into manipulation medium drops containing 5 μg/ml cytochalasin B overlaid with warm mineral oil. Oocytes

were enucleated by aspirating the PB and M II chromosomes using a 17-$\mu$m beveled glass pipette (Humagen, Charlottesville, VA, USA). Enucleation was confirmed under an epifluorescence microscope (TE300; Nikon, Tokyo, Japan). After enucleation, a single cell was inserted into the PVS of each oocyte. Oocyte-cell couplets were placed on a 1-mm fusion chamber overlaid with 1 ml of 280 mM mannitol, containing 0.001 mM $CaCl_2$ and 0.05 mM $MgCl_2$ as previously described (Walker *et al.*, 2002). Membrane fusion was induced by applying an alternating current field of 2 V, 1 MHz for 2 s, followed by two pulses of 160 V/mm direct current (DC) for 40 $\mu$s using a cell fusion generator (LF101; NepaGene, Chiba, Japan). Oocytes were then incubated for 1 h in TLH-BSA and evaluated for fusion under a stereomicroscope.

### 5. Activation and Embryo Culture

Reconstructed oocytes were activated immediately after incubation for 1 h in TLH-BSA by two pulses of 120 V/mm DC for 60 $\mu$s in 280 mM mannitol containing 0.01 mM $CaCl_2$ and 0.05 mM $MgCl_2$. For PA, oocytes with the PB at 44 h of IVM were activated by the same pulses used for activation of SCNT oocytes and were incubated for 4 h in IVC medium containing 5 $\mu$g/ml cycloheximide. SCNT and PA embryos were washed in fresh IVC medium, transferred into 30-$\mu$l IVC droplets under mineral oil, and cultured at 39℃ in a humidified atmosphere of 5% $CO_2$, 5% $O_2$, and 90% $N_2$ for 6 days. Cleavage and blastocyst formation was evaluated on Days 2 and 6, respectively (the day of SCNT was designated Day 0). Total blastocyst cell number was assessed using Hoechst 33342 staining under an epifluorescence microscope.

### 6. Calculation of PVS Size

PVS size was estimated by calculating the distance between the zona pellucida (ZP) and the oocyte cytoplasm (Fig. 1). Briefly, images of oocytes denuded at 30 h of IVM were recorded at 200× magnification using a digital camera (DS-5Mc-L1; Nikon) attached to an inverted microscope (TE-300; Nikon). After a line was drawn through the imaginary center of the oocyte and the PB using an image analysis program (Image J 1.36; NIH, Bethesda, MD, USA), PVS size was calculated by subtracting the diameter of the cytoplasm from the inner diameter of the ZP. In oocytes from which the PB was not extruded, the widest PVS was measured and calculated. Denuded oocytes that were photographed at 30 h of IVM were cultured in IVM medium for an additional 10 h and photographed again.

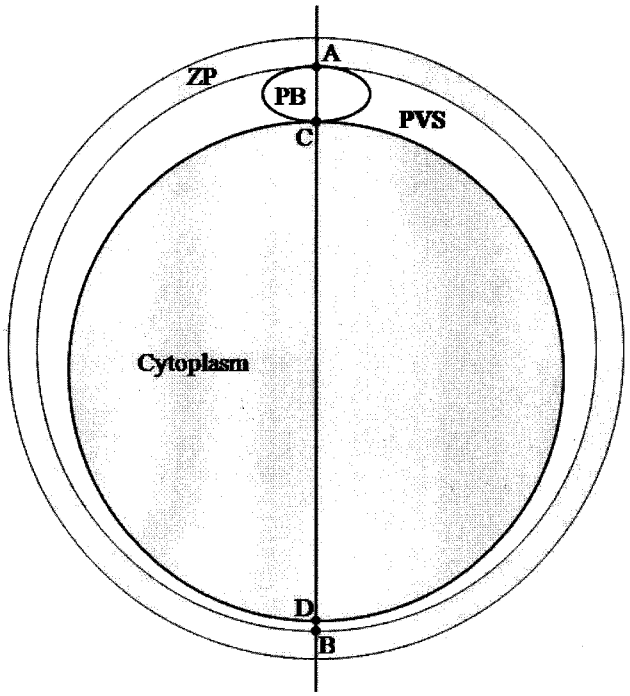


Fig. 1. Diagram used to calculate the size of the perivitelline space (PVS). A line was drawn through the imaginary center of the cytoplasm and the polar body (PB) of the oocyte. AB indicates the inner diameter of the zona pellucida (ZP), and CD indicates the diameter of the cytoplasm without the PB. PVS size was calculated using the equation: PVS = AB−CD.

Control oocytes were denuded at 40 h of IVM and PVS size was examined.

### 7. Distance Measurement between the PB and the M II Plate

After 40 h of maturation culture, control and denuded oocytes were stained with 5 $\mu$g/ml Hoechst 33342 and observed under an inverted microscope equipped with an epifluorescence detector. Oocytes were photographed using a digital camera at the point where the PB and the M II plate were focused on the same plane. The distance between the PB and the M II plate was measured using an image analysis program (Image J).

### 8. Transfer of SCNT Embryos Derived from Oocytes Denuded at 30 h of IVM

SCNT embryos in IVC medium containing 0.4 $\mu$g/ml demecolcine were loaded into a 0.25 ml plastic straw, transported to the farm, and transferred into the oviduct, without removing the demecolcine. Embryo transfer was carried out at the research farm of Gyeonggi Veterinary Service, Korea. Recipient gilts were

maintained at the farm using routine herd care protocols. SCNT embryos were surgically transferred to oviducts of naturally cycling gilts on the first day of estrus. Pregnancy was diagnosed by ultrasonography on Days 30 and 60 after transfer.

### 9. Experimental Design

In this study, ovaries of prepubertal gilts from various sources were used. To minimize the potential effect of the variability in oocyte sources, all oocytes used in these experiments were randomly assigned to each treatment, and a minimum of three replications was performed. At 30 h of IVM, some COCs were denuded by gentle pipetting in maturation medium, washed in fresh maturation medium, and then cultured with (D+) or without (D−) their detached cumulus cells. Control oocytes were denuded at 40 and 44 h of IVM for SCNT and PA, respectively.

#### Experiment 1: Size of PVS at 30 h and 40 h of *In Vitro* Maturation

Denuded oocytes were photographed to observe changes in PVS size. At 30 h of maturation, oocytes of D+ group were photographed at 200× magnification with digital camera, and then cultured for further 10 h. At 40 h of maturation, oocytes of D+ group were taken a photograph again without further denuding procedure. In case of control group, oocytes were denuded with 0.1% hyaluronidase at 40 h and then were photographed.

#### Experiment 2: Distance between the PB and the MⅡ Plate and Enucleation efficiency

After 40 h of maturation culture, control or denuded oocytes were photographed using a digital camera after staining with Hoechst 33342. The distance between the PB and the MⅡ plate was measured using an image analysis program. Blind enucleation was performed by a single person using the same enucleation pipette. The volume of aspirated cytoplasm was held constant by aspirating the cytoplasm of each oocyte to the same point on the enucleation pipette. Enucleation efficiency was examined after the removal of 7.7±0.4% or 15.0±0.9% cytoplasmic volume (mean±S.E.M.).

#### Experiment 3: *In Vitro* Development of PA Embryos

To determine the effect of early denudation on the developmental competence of oocytes, PA embryos derived from D+ and D− oocytes were compared with those derived from control oocytes. PA was induced with electric stimulation followed by cycloheximide treatment.

#### Experiment 4: *In Vitro* Development of SCNT Embryos

*In vitro* development to blastocyst was also examined by SCNT embryos. Reconstructed embryos derived from D+ and D− oocytes were cultured in IVC medium and compared with those derived from control oocytes.

#### Experiment 5: *In Vivo* Development of SCNT Embryos Derived from D+ Oocytes

SCNT embryos derived from D+ oocytes were transferred to recipient gilts to examine the effect of early denudation on the *in vivo* competency. In total, 576 embryos ($n$ = 146, 120, 150, and 160, respectively) were transferred to four recipients. The average number of transferred embryos per recipient was 144 (120～160 embryos/recipient).

### 10. Statistical Analysis

All statistical analyses were performed using the Statistical Analysis System (version 8.2; SAS Institute, Cary, NC, USA). Data were analyzed using a general linear model, followed by the least significant difference mean separation procedure when treatments differed at $p<0.05$. Percentage data were arcsine transformed prior to analysis to maintain homogeneity of variances. Results are expressed as means±S.E.M.

## RESULTS

#### Experiment 1: Size of PVS at 30 h and 40 h of *In Vitro* Maturation

Changes in PVS size at different times of IVM are summarized in Table 1. Irrespective of time point, oocytes that extruded the PB had a larger PVS than oocytes without the PB ($p<0.01$). In denuded oocytes, the PVS measured at 30 h of IVM (10.7 $\mu$m) was narrower than that measured at 40 h (12.7 $\mu$m; $p<0.01$). There was no significant difference in the PVS size of denuded and control oocytes measured at 40 h of IVM.

#### Experiment 2: Distance between the PB and the MⅡ Plate and Enucleation Efficiency

The distance between the PB and the MⅡ plate at the time of enucleation was shorter in D+ and D− oocytes (19.4 and 18.9 $\mu$m, respectively) compared to control oocytes (25.5 $\mu$m; $p< 0.05$). Enucleation rates after the removal of 7.7% cytoplasmic volume by blind aspiration were higher ($p<0.05$) in D+

and D− oocytes (75% and 68%, respectively) compared to controls (54%). Enucleation efficiency was further increased in all treatment groups (82%, 92%, and 93% in control, D+ and D−, respectively) when the removed cytoplasmic volume was in creased to 15% ($p<0.05$; Table 2).

### Experiment 3: *In Vitro* Development of PA Embryos

Both early denudation of oocytes at 30 h of IVM and coculture of denuded oocytes with their detached cumulus cells did not alter oocyte maturation (93%, 94%, and 91% for D+, D−, and control, respectively). Embryo cleavage (72～75% versus 70%), blastocyst formation (26～28% versus 26%) and embryo cell number (38～42 cells versus 41 cells) after PA was not affected by either early denudation or coculture with cumulus cells (Table 3).

### Experiment 4: *In Vitro* Development of SCNT Embryos

The effects of early denudation and coculture of denuded oocytes on oocyte maturation and *in vitro* development of SCNT embryos are summarized in Table 4. Compared with controls, oocytes denuded at 30 h of IVM and cocultured with detached cumulus cells did not display altered oocyte maturation (88～

Table 1. Size of the perivitelline space in oocytes denuded at 30 and 40 h of *in vitro* maturation

| Group | Time of measurement | *n* | Size of PVS ($\mu$m) | | |
|---|---|---|---|---|---|
| | | | PBO (*n*) | PBX (*n*) | Total |
| Control oocytes | 40 h of IVM | 75 | 12.7±0.3$^{ac}$ (63) | 6.6±0.7$^{d}$ (12) | 11.7±0.4$^{a}$ |
| Denuded oocytes | 30 h of IVM | 71 | 10.7±0.4$^{bc}$ (49) | 5.0±0.6$^{d}$ (22) | 8.9±0.4$^{b}$ |
| | 40 h of IVM | 71 | 12.7±0.3$^{ac}$ (60) | 6.8±0.7$^{d}$ (11) | 11.8±0.4$^{a}$ |

PVS, perivitelline space; PBO, oocytes with the first polar body; PBX, oocytes without the first polar body.
$^{a,b}$ Within a column, different superscripts are significantly different ($p<0.01$).
$^{c,d}$ Within a row, different superscripts are significantly different ($p<0.01$).

Table 2. Distance between the first polar body and the metaphase II plate and enucleation rates in control and denuded oocytes after the removal of 7.7% or 15% removal of cytoplasmic volume by blind aspiration

| Group | Distance | | 7.7% removal | | 15% removal | |
|---|---|---|---|---|---|---|
| | *n* | Mean ($\mu$m) | *n* | % enucleated | *n* | % enucleated |
| Control | 85 | 25.5±1.6$^{a}$ | 246 | 54±5$^{a}$ | 155 | 82±2$^{a}$ |
| D+ | 82 | 19.4±0.9$^{b}$ | 238 | 75±3$^{b}$ | 160 | 92±2$^{b}$ |
| D− | 87 | 18.9±0.9$^{b}$ | 228 | 68±5$^{b}$ | 161 | 93±2$^{b}$ |

D+, oocytes denuded at 30 h and cultured with cumulus cells; D−, oocytes denuded at 30 h and cultured without cumulus cells; Distance, distance between the first polar body and the metaphase plate at 40 h
$^{a,b}$ Within a column, different superscripts are significantly different ($p<0.05$).

Table 3. *In vitro* development of parthenogenetically activated oocytes denuded at 30 h of *in vitro* maturation

| Group | Maturation | | Embryo development | | | No. of cells/ blastocyst |
|---|---|---|---|---|---|---|
| | *n* | Matured (%) | *n* | ≥ 2-cell (%) | Blastocyst (%) | |
| Control | 386 | 91±2 | 338 | 70±4 | 27±5 | 41±2 |
| D+ | 384 | 93±1 | 344 | 72±4 | 26±3 | 42±2 |
| D− | 381 | 94±1 | 356 | 75±2 | 28±5 | 38±2 |

D+, oocytes denuded at 30 h and cultured with cumulus cells; D−, oocytes denuded at 30 h and cultured without cumulus cells. There were no significant differences in the oocyte maturation, embryo development, and cell number in blastocyst.

Table 4. *In vitro* development of somatic cell nuclear transfer embryos derived from oocytes denuded at 30 h after *in vitro* maturation

| Group | Maturation | | Reconstruction | | Embryo development | | | No. of cells/ blastocyst |
|---|---|---|---|---|---|---|---|---|
| | *n* | Metaphase II (%) | *n* | Fused (%) | *n* | ≥ 2-cell (%) | Blastocyst (%) | |
| Control | 839 | 89±2 | 693 | 79±4 | 530 | 65±3 | 4±1 | 43±4 |
| D+ | 810 | 90±2 | 684 | 77±4 | 512 | 67±3 | 4±1 | 37±3 |
| D− | 827 | 88±2 | 673 | 76±4 | 470 | 69±4 | 5±1 | 41±4 |

D+, oocytes denuded at 30 h and cultured with cumulus cells; D−, oocytes denuded at 30 h and cultured without cumulus cells. There were no significant differences in the oocyte maturation, cell fusion, embryo development, and cell number in blastocyst.

90% versus 89%), fusion rates (76~77% versus 79%), blastocyst formation (4~5% versus 4%), or embryo cell numbers (37~41 cells versus 43 cells) after SCNT.

### Experiment 5: *In Vivo* Development of SCNT Embryos Derived from D+ Oocytes

Pregnancy was established in two recipients following the transfer of 576 SCNT embryos derived from D+ oocytes into four recipient gilts. One of the gilts aborted at 57 days after embryo transfer and the other farrowed three live piglets at 115 days after transfer.

## DISCUSSION

We examined whether the early denudation of oocytes at 30 h of IVM affects both the enucleation efficiency in SCNT and the *in vitro* development of porcine embryos after SCNT and PA. In addition, the *in vivo* viability of SCNT embryos derived from early-denuded oocytes was determined following embryo transfer to recipient pigs. Our results suggest that the denudation of oocytes at 30 h of IVM may improve the enucleation efficiency of blind enucleation. These effects may be due to the short distance maintained between the first PB and the M Ⅱ plate. Furthermore, the birth of live piglets from the transfer of SCNT embryos derived from early-denuded oocytes indicated that removal of cumulus cells from recipient oocytes at 30 h of IVM was capable of supporting normal *in vivo* development to term of SCNT embryos.

The PVS changes in size according to the stage of oocyte maturation (Wang *et al.*, 1998; Hyun *et al.*, 2003; Kidson *et al.*, 2003; Talbot and Dandekar, 2003; Miao *et al.*, 2004; Kafi *et al.*, 2005). In general, the size of the PVS in immature oocytes is small, but becomes enlarged as maturation proceeds (Talbot and Dandekar, 2003; Miao *et al.*, 2004; Kafi *et al.*, 2005). In this study, the PVS of oocytes that extruded PB was larger than that of oocytes that did not. These results are in agreement with previous findings demonstrating that the PVS of mature porcine oocytes is wider than that of immature oocytes (Kidson *et al.*, 2003). These patterns were also observed in mice; PVS size increased gradually with time during maturation and aging *in vivo* and *in vitro* (Miao *et al.*, 2004).

PB displacement is a PVS-dependent process. Accordingly, mechanical denudation processes that are performed following PB extrusion significantly enhance the displacement of the PB within the enlarged PVS (Atabay *et al.*, 2001; Miao *et al.*, 2004). The PB was displaced from the M Ⅱ plate to a greater degree in control oocytes than in early-denuded oocytes. Although the PVS size of denuded oocytes was similar to that of control oocytes, the PB and the M Ⅱ plate of D+ and D− oocytes were located closer to each other compared to the controls. Cumulus cells were removed from D− and D+ oocytes at 30 h of IVM, and no mechanical denudation was applied before further use. Therefore, we hypothesize that the lack of mechanical denudation in D+ and D− oocytes may maintain the PB and the M Ⅱ plate in closer proximity. Collectively (Atabay *et al.*, 2001; Miao *et al.*, 2004), it is thought that the principal cause of PB displacement from the M Ⅱ plate is the mechanical denudation of mature oocytes with an enlarged PVS.

The PB position affects the enucleation efficiency in SCNT (Atabay *et al.*, 2001) because PBs are the only mature oocyte structures that can be used as indicators of the M Ⅱ plate position under a light microscope. Generally, enucleation is performed by aspirating the PB and its adjacent cytoplasm from the oocyte (Willadsen, 1986). Thus, it is difficult to remove the M Ⅱ plate completely using blind aspiration when the PB is severely displaced from the M Ⅱ plate (Atabay *et al.*, 2001). The enucleation efficiency significantly improved after removal

of 7.7% cytoplasmic from 54% in non-denuded control oocytes to between 68 and 75% in denuded oocytes when the PB was kept close to the M Ⅱ plate by early denudation of oocytes at 30 h of IVM. Removal of larger cytoplasmic volume (15%) further improved the enucleation efficiency (82% vs. 92~93%). This is consistent with a previous report that denudation by vortexing or mechanical stripping of oocytes can significantly move the PB and lead to reduced enucleation rates in bovine SCNT (Atabay *et al.*, 2001). Enucleation efficiency was closely related to the volume of cytoplasm removed during enucleation (Atabay *et al.*, 2001). In this study, 7.7~15% of the cytoplasm was removed during blind enucleation. Notwithstanding the smaller cytoplasmic loss, the enucleation efficiency (92~93% in early denuded oocytes) in our study was higher than the 64.3~84.2% in previous studies (Atabay *et al.*, 2001; Ikeda and Takahashi, 2001), in which 10~20% of the cytoplasm was removed during enucleation. Combined use of the early denudation method with demecolcine treatment may be a new strategy for further improvement of enucleation efficiency in pig SCNT.

Oocyte cumulus cells also play important roles in the oocyte maturation process by facilitating gap junction communication (Isobe *et al.*, 1998; Mori *et al.*, 2000). In pigs, intercellular coupling between oocytes and cumulus cells decreases 32 h after hCG injection when anaphase I or telophase I is predominant. In addition, partial uncoupling is observed in oocytes cultured *in vitro* for 16 h (Motlik *et al.*, 1986). Recently, Wongsrikeao *et al.* (2005) reported that the proportion of oocytes that reach M Ⅱ increases with the delayed removal of cumulus cells. However, contradictory results indicating that the removal of cumulus cells has no affect on PB expulsion have been reported in pigs (Yamauchi *et al.*, 1999), mice, and rats (Binor *et al.*, 1979; Magnusson, 1980). We tested whether the physical disruption of intercellular communication between the oocyte and cumulus cells by the early denudation of oocytes at 30 h of IVM would be detrimental to oocyte maturation and subsequent embryonic development. We found that the nuclear maturation of oocytes and the *in vitro* development of SCNT and PA embryos were not influenced by early denudation and the coculture with cumulus cells. This is consistent with previous findings showing that different time courses of cumulus-corona cell removal have no effect on oocyte maturation and embryo development following intracytoplasmic sperm injection in humans (Van de Velde *et al.*, 1998) and *in vitro* fertilization in bovines (Van der Westerlaken *et al.*, 1994). The rate of oocytes with extruded PBs at 30 h of IVM was about 69%, similar to the report of Miyoshi *et al.* (2002) stating that 71.4% of oocytes reached M Ⅱ stage at 30 h of IVM. When considering the relatively high rates of oocyte maturation at 30 h of IVM and the short duration (10 h) of coculture following denudation, it is likely that the beneficial effects of cumulus cells may not have been seen in the later stages of IVM. Our results indicate that early removal of cumulus cells from oocytes at 30 h of IVM can support oocyte maturation and subsequent development. These results were further verified by the birth of normal live piglets after transfer of SCNT embryos derived from early-denuded oocytes, although the cloning efficiency was low (3/576, 0.52%). Because of the limited number of embryo transfers in this study, it was not clear whether the low efficiency was attributable to the early denudation process itself. Extensive embryo transfer studies would be needed to clarify any detrimental effect of an early denudation process on SCNT embryo development to term and on postnatal viability of piglets. In conclusion, the early denudation of oocytes at 30 h of IVM is useful for maintaining the PB in close proximity to the M Ⅱ plate. In addition, this technique improves the enucleation efficiency in pig SCNT and can support the normal *in vitro* and *in vivo* viability of SCNT pig embryos.

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