# Selection of Early Cleaved Embryos and Optimal Recipients to Improve Efficiency of Pig Cloning

**Ok Jae Koo, Dong Won Lee, Jung Taek Kang, Dae Kee Kwon, Hee Jung Park, Sol Ji Park, Su Jin Kim, Goo Jang and Byeong-Chun Lee***

*Department of Theriogenology and Biotechnology, College of Veterinary Medicine and the Research Institute for Veterinary Science, Seoul National University, Seoul 151-742, Korea*

## ABSTRACT

Early cleavage is a reliable prognostic tool for successful embryo transfer in assisted reproduction because early cleaved embryo show better pregnancy rate after transfer. There for, preparation of good embryo recipient is important factor to optimize efficiency of pig cloning. The present study was performed to evaluate the effect of early cleavage on the *in vivo* development of cloned embryos and to analyze breed, parity and estrous synchrony to optimize recipient for pig cloning. *In vitro* matured porcine oocytes derived from local slaughterhouse and fibroblasts derived from miniature pig fetuses were used for somatic cell nuclear transfer (SCNT). Reconstructed embryos were transferred to recipient pigs on the same day of SCNT or after 1~2 days of *in vitro* culture for selecting early cleaved embryos. Breed, parity and date of standing estrous of recipients were recorded for analysis. After 25~35 days after embryo transfer pregnancy was diagnosed using ultrasonography, and pregnant recipients were monitored till delivery. Between purebred and crossbred, no significant difference was founded in both pregnancy and delivery rates. However, early cleaved embryos showed significantly higher pregnancy (46.2%) and delivery (12.8%) rates compared to non-selectively transferred group (24.8% and 4.5%, respectively). The results also showed that the recipients showing standing estrous on the same day of SCNT and less than 4 parities were most suitable for pig cloning.

(Key words : breed, early cleavage, parity, pig cloning, recipient)

## INTRODUCTION

Pork industry is becoming huge today. The scale of Korean pork production and processing set to reach more than 9 billion dollars in 2009, the largest in agriculture and livestock industry (http://www.chukkyung.co.kr/news/article.html?no=21754). To maximize the profit, many studies are also performed to understand reproductive physiology and improve productivity of the pigs. It is well reported that reproductive performance of the gilts and sows is influenced by many factors including age (Brooker and Smith, 1980), breed (de Fredrick and Osborne, 1977), body condition (Esbenshade *et al.*, 1986), parities (Tummaruk *et al.*, 2001) and season (Love, 1981).

Reproductive performance of the pig is also important in biomedical research field because it's closely related with production of transgenic or disease-model pigs. Because of physiologically and anatomically similar to humans many scientists regarded pigs as a good source of organ donors for xenotransplantation and human disease model animal (Prather *et al.*, 2003). For these purposes various transgenic pigs have been produced (Oropeza *et al.*, 2009; Petersen *et al.*, 2009; Phelps *et al.*, 2009; Umeyama *et al.*, 2009) by pig cloning technique (Polejaeva *et al.*, 2000) called somatic cell nuclear transfer (SCNT). To produce cloned pigs, preparation of good embryo recipient is one of the important factors. In the previous reports, we already reported that factors influencing reproductive performance also affect on efficiency of pig cloning (Koo *et al.*, 2009a; Koo *et al.*, 2009b). However, we also found that effect of some factors in pig cloning are different from the condition of normal breeding (Koo *et al.*, 2009a). Thus, to improve efficiency, it will be important to investigate effect of many factors for reproduction performance on efficiency of pig cloning.

On the other hand, human embryo transfer study shows that

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* Correspondence : E-mail : bclee@snu.ac.kr

selection of early cleavage embryos is a reliable prognostic tool for successful embryo transfer (Bos-Mikich *et al.*, 2001) because early cleaved embryo shows better pregnancy rate after transfer. However, in many cases of pig cloning, cloned embryos were transferred to recipient at the same day of SCNT without *in vitro* culture (Walker *et al.*, 2002; Hyun *et al.*, 2003; Lee *et al.*, 2005; Phelps *et al.*, 2009) because *in vitro* culture condition for porcine embryos is not well established yet (McElroy *et al.*, 2008) and the development competence of embryos may be reduced after *in vitro* culture. Thus it can be controversial for improving efficiency of pig cloning if one or two days of *in vitro* culture was performed to collect early cleaved embryos.

Therefore the present study was performed to compare the efficiency of two embryo transfer strategies (short culture without selection or selection of early cleaved embryos after 1~2 days of culture) and evaluate effect of several factors (breed and parity of recipient and synchrony between cloned embryos and recipient) related with reproduction performance of pigs on the development of cloned pig embryos.

## MATERIALS AND METHODS

### 1. Chemicals

All chemicals were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA) unless otherwise stated.

### 2. Preparation of Porcine Fetal Fibroblasts

Porcine fetal fibroblast was isolated from 35-day old miniature-pig fetuses. Briefly, the fetuses were washed three times in $Ca^{2+}$- and $Mg^{2+}$-free Dulbecco's phosphate buffered saline (DPBS; Invitrogen, Carlsbad, CA, USA) and embryonic internal organs were removed from the abdominal cavity using dissecting forceps and minced with a surgical blade. The minced tissues were dissociated in TrypLE Express (Invitrogen) for 10 min at 37℃. Trypsinized cells were washed three times in DPBS by centrifugation at 1,500 RPM for 2 min, and seeded onto 100-mm plastic culture dishes (Becton Dickinson, Lincoln Park, NJ, USA). Subsequently, cells were cultured for 3~7 days in Dulbecco's modified Eagle's/Nutrient Mixture F-12 medium (DMEM/F12; Invitrogen) supplemented with 10% (v/v) FBS (Invitrogen), 1 mM Glutamax I (Invitrogen), 25 mM $NaHCO_3$, 1% (v/v) minimal essential medium (Invitrogen), non-essential amino acid solution (Invitrogen) and 1% (v/v) Antibiotic-Antimycotics (Invitrogen) at 39℃ in a humidified atmosphere of 5% $CO_2$ and 95% air. After removal of unattached clumps of cells or explants, attached cells were further cultured to confluence. The cells were subcultured (at intervals of 3~5 days) by trypsinization for 1 min. Trypsinized cells were allocated to three new dishes for further passaging, or stored in liquid nitrogen at −196℃. The freezing medium consisted of 70% (v/v) DMEM/F12, 10% (v/v) dimethylsulfoxide and 20% (v/v) FBS.

### 3. Preparation of Recipient Oocytes

Ovaries were collected at a local abattoir and stored in sterile physical saline at 30~35℃ during transportation. Cumulus-oocyte complexes (COCs) were aspirated from antral follicles (3~6 mm) with 18-gauge needle attached to a 10 ml disposable syringe. COCs with several layers of cumulus cells and uniform cytoplasm were chosen and cultured in tissue culture medium (TCM)-199 (Invitrogen) supplemented with 10 ng/ml EGF, 0.57 mM cysteine, 0.91 mM sodium pyruvate, 5 μg/ml insulin, 1% (v/v) Pen-Strep (Invitrogen), 0.5 μg/ml follicle stimulating hormone (FSH), 0.5 μg/ml luteinizing hormone (LH) and 10% porcine follicular fluid at 39℃ in a humidified atmosphere of 5% $CO_2$, first, with FSH and LH for 22 hr and then without them for a further 18~20 hr. COCs were washed at each steps.

### 4 Somatic Cell Nuclear Transfer

After a maturation culture, oocytes were denuded by pipetting with 0.1% hyaluronidase in DPBS (Invitrogen) supplemented with 0.1% polyvinyl alcohol (PVA). Denuded oocytes with evenly-granulated and homogeneous cytoplasm were selected and then utilized for SCNT. For SCNT, a micromanipulator (NT-88-V3, Nikon-Narishige, Tokyo, Japan) attached to an inverted microscope (TE2000, Nikon Instrument, Tokyo, Japan) was used. Cumulus-free oocytes were placed in TALP media supplemented with 5 μg/ml of cytochalasin B. The first polar body and adjacent cytoplasm, presumably containing the metaphase-II chromosomes, were removed with beveled micro-pipette (Humagen, Charlottesville, VA, USA). Enucleation was confirmed by staining the cytoplasm with 0.5 μg/ml bisbenzimide (Hoechst 33342) during manipulation. Single transgenic fibroblast cell with smooth surface was selected under a microscope and transferred into the perivitelline space of enucleated oocytes. Membrane fusion was followed. Cell-oocyte complexes were placed in a 280 mM mannitol solution (pH 7.2) containing 0.1 mM $MgSO_4$, 0.01% (w/v) PVA and 0.5 mM HEPES

and held between two electrode needles. Membrane fusion was induced with an electro cell fusion generator (LF101, Nepagene, Ichikawa, Japan) by applying a single direct current (DC) pulse (2.0 kV/cm for 30 $\mu$sec) and a pre- and post-pulse alterating current field of 5 V, 1 MHz, for 5 sec, respectively. The reconstructed embryos were cultured in porcine zygote medium-5 (PZM-5) (Funakoshi, Tokyo, Japan) for 1 to 1.5 hr and then subjected to electrical activation. Reconstructed embryos were washed three times in an activation solution comprising 280 mM mannitol, 0.1 mM $CaCl_2$, 0.1 mM $MgSO_4$ and 0.01% (w/v) PVA and were activated by with a single 1.5 kV/cm DC pulse for 60 $\mu$sec using a BTX Electro-Cell Manipulator 2001 (BTX Inc., Holliston, MA, USA). Activated oocytes were washed then loaded into straw as described later for Direct group or transferred into 450 $\mu$l of PZM-5 covered with mineral oil for in Selective group. In Selective group, embryos were maintained under a humidified atmosphere of 5% $CO_2$, 5% $O_2$ and 90% $N_2$ at 38.5℃ for 1~2 days until embryo transfer.

### 5. Embryo Transfer and Pregnancy Diagnosis

Embryo transfer was followed as the previous report (Koo *et al.*, 2009b). Briefly, 90 to 150 of SCNT embryos were loaded into a sterilized 0.25 ml straw (Minitüb, Tiefenbach, Germany) and kept in a portable incubator (Minitüb) during transportation to the embryo transfer facility. Recipient pigs were selected 1~3 days after standing estrus up to experimental design. Breed and parity of the recipients were recorded for analysis. A recipient was anaesthetized by a combination of 1.13 mg/kg ketamine (Yuhan, Seoul, Korea) and 0.3 mg/kg xylazine (Bayer Animal Health, KS, USA) through IV for induction and 3% of isoflurane (Hana Pharm, Seoul, Korea) for maintenance. One oviduct was exposed by laparotomy and the straw containing the embryos was put directly into the oviduct of the recipient and embryos were expelled from the straw using 1 ml syringe (Becton Dickinson). Recipients were checked for pregnancy by transabdominal ultrasound (100 Falco; Pie Medical, Maastricht, Netherlands) examination in day 30 after embryo transfer and delivery were also recorded.

### 6. Statistical Analysis

All data were subjected to one-way ANOVA followed by Tukey's test or *t*-test using Prism software (Version 5.0, GraphPad Sofware, San Diego, CA, USA) to determine differences among experimental groups. Statistical significance was determined when the P value was less than 0.05.

## RESULTS

### 1. Effect of Recipient Pig Breeds on Development of Transferred Cloned Embryos

For analyzing effect of breed difference, pure Yorkshire and Landrace pigs and crossbred (Yorkshire × Landrace) pigs were used as recipient for embryo transfer. Cloned embryos were transferred to 33 pure breed and 80 crossbred recipients then pregnancy and delivery rates were monitored. As shown in Table 1, 7 recipients (21.2%) in pure breed group were pregnant and among them 2 recipients (6.1%) were delivered. In crossbred group, 25 recipients (31.3%) were pregnant and 4 recipients (5.0%) were delivered. Crossbred group shows slightly higher pregnancy rate, however, no significantly differences in pregnancy and delivery rates were founded between two groups.

### 2. Effect of Two Embryo Transfer Strategies on Development of Cloned Pig Fetuses

Pregnancy and delivery rate were compared between two embryo transfer strategies. For Direct strategy, cloned embryos were transferred to recipient pig in same day of SCNT without any selection. For Selective strategy, cloned embryos were cultured for 1~2 days and only early cleaved embryos were selected and transferred to recipient. As shown in Table 2, both pregnancy (46.2%) and delivery rates (12.8%) were significantly improve in Selective strategy compared to Direct strategy (24.8% and 4.5% for pregnancy and delivery rates, respectively). Since efficiency of Selective strategy was better, this method was adopted for further experiments.

### 3. Effect of Estrous Synchronization between Transferred Cloned Embryos and Recipient Pigs

Recipient pigs are classified into 3 groups. These groups are "−1" group that shown standing estrous one day before SCNT, "0" group that shown standing estrous on same day of SCNT

Table 1. Effect of recipient pig breeds on development of transferred cloned embryos

| Breed of surrogates* | Total No. of surrogates | Pregnancy (%) | Delivery (%) |
|---|---|---|---|
| Pure breed | 33 | 7 (21.2) | 2 (6.1) |
| Crossbred | 80 | 25 (31.3) | 4 (5.0) |

*Pure Breed : Yorkshire or Landrace; Crossbred: Yorkshire × Landrace.

Table 2. Comparison between two embryo transfer (ET) strategies for pig cloning

| ET strategies | Total No. of surrogates | Pregnancy (%) | Delivery (%) |
|---|---|---|---|
| Direct[1] | 133 | 33 (24.8)[a] | 6 ( 4.5)[c] |
| Selective[2] | 39 | 18 (46.2)[b] | 5 (12.8)[d] |

[1] ET was performed same day of somatic cell nuclear transfer without selection of embryos.
[2] Cloned embryos were cultured 1~2 days *in vitro* and only early cleaved embryos were selected for ET.
[a~d] Within columns, values with different superscripts are significantly different ($p<0.05$).

and "+1" group that shown standing estrous one day after SCNT. Cloned embryos were transferred to 7, 24 and 4 recipients for "−1", "0" and "+1" groups, respectively. Pregnancy rates for "−1", "0" and "+1" groups are 28.6%, 54.2% and 50.0%, respectively. Though there is no statistically significance among 3 groups, only "0" group can maintain pregnancy and 5 recipients (20.8%) were delivered cloned piglets.

### 4. Effect of Parity of RecipienT Pigs on Development of Transferred Cloned Embryos

To analyze effect of parity, recipient pigs were classified into 4 groups (parity 1, parity 2, parity 3 and more than 4). Gilts were used for parity 1 group. Cloned embryos were transferred to 9, 15, 6 and 4 recipients for each parity 1, parity 2, parity 3 and more than 4 groups, respectively. In parity 1 group, 6 recipients were pregnant (66.7%) and 2 were delivered. In parity 2 group, 8 recipients were pregnant (53.3%) and 2 were delivered. In parity 3 group, 2 recipients were pregnant and 1 were delivered. Though there is no statistically significance, more than 4 group show tendency of low pregnancy rate (25%) and cloned piglet was not produced.

Table 3. Effect of estrous synchronization between transferred cloned embryos and recipient pigs on development

| Synchronization* | Total No. of surrogates | Pregnancy (%) | Delivery (%) |
|---|---|---|---|
| −1 | 7 | 2 (28.6) | − |
| 0 | 24 | 13 (54.2) | 5 (20.8) |
| +1 | 4 | 2 (50.0) | − |

* −1: standing estrous was detected one day before SCNT; 0: standing estrous was detected on same day of SCNT; +1: standing estrous was detected one day after SCNT.

Table 4. Effect of parity of recipient pigs on development of transferred cloned embryos

| Parity of surrogates | Total No. of surrogates | Pregnancy (%) | Delivery (%) |
|---|---|---|---|
| 1 (gilt) | 9 | 6 (66.7) | 2 (22.2) |
| 2 | 15 | 8 (53.3) | 2 (13.3) |
| 3 | 6 | 2 (33.3) | 1 (16.7) |
| More than 4 | 4 | 1 (25.0) | − |

## DISCUSSION

In the present study, we show that using crossbred recipients cannot improve the efficiency of pig cloning, however, selection of early cleaved embryos before embryos transfer significantly improve pig cloning efficiency. Also, after selection of early cleaved embryos, recipients showing standing estrous at the same day of SCNT tend to be the most suitable for pig cloning. Furthermore, cloning efficiency seems to be improved using earlier parity recipients and no cloned piglets were produced when using more than 4 parities recipients.

Many previous reports shows crossbred female pigs have higher reproductive performance. Crossbred gilts maintain 6.3% more embryos at 30 days of gestation and delivered 8.7% more piglets compared to pure breed (Johnson and Omtvedt, 1973). Also, piglets from crossbred pigs show 5.56% greater survival rate and average 11.72 kg heavier at weaning compared to piglets from pure breed (Gaugler *et al.*, 1984). Interestingly, female of Yorkshire-Landrace crossbred pigs show largest litter size at birth and heaviest at weaning compared to other crossbreds of Duroc, Yorkshire, Landrace and Spotted breeds (Buchanan and Johnson, 1984). However, in the present study, crossbred recipients did not improve efficiency to produce cloned piglets even though we used Yorkshire-Landrace crossbred (the most productive breed) as the crossbred group. This result may reveal that higher efficiency of crossbred in the previous studies was up to the breed of fetuses rather than breed of sows. This idea is consistent with the previous study of reciprocal embryo transfer between Chinese Meishan and Landrace × York shire gilts(Ashworth *et al.*, 1990). In the report, the Meishan pig embryos show less tolerant to embryo transfer procedures but the breed of the recipient gilt does not affect on

surviving of transferred embryos. Also, it was very well know that only hybrid strain (crossbred) mice can be cloned and outbred or inbred strain mice were extremely hard to clone (Wakayama and Yanagimachi, 2001; Inoue *et al.*, 2003) regardless of recipient breed. In pig cloning, efficiency to clone inbred pig (NIH miniature pig) is also very low (Zhao *et al.*, 2009).

In the previous report using *in vitro* produced hamster embryos (McKiernan and Bavister, 1994), faster cleaving embryos show better viability (51%) compared to slower cleaving embryos (26%). Also it is very well known that early cleavage is a good and strong indicator of embryo quality in human assisted reproductive technology (Shoukir *et al.*, 1997; Sakkas *et al.*, 1998; Lundin *et al.*, 2001; Salumets *et al.*, 2003). Thus we hypothesized that early cleaved cloned porcine embryos have better developmental competence and selection of these embryos may improve the efficiency of pig cloning. However, as we mentioned before, *in vitro* culture condition may reduce competence of cloned embryos and elongated culture period due to selection of early cleaved embryos can be controversial. But, the present result clearly shows that selective transfer of early cleaved embryos significantly improves the efficiency of producing cloned piglets. Thus, culture of cloned embryos for 1~2 day after SCNT is not critically detrimental to development and discarding retarded bad quality embryos can be improve overall efficiency of pig cloning.

After transfer of selective embryos, "0" group seems to show better efficiency compared to "−1" and "+1" group in the present study. In pigs, ovulation occurs 38~42 h after standing estrous (Hughes and Varley, 1980). Thus, the recipients in "0" group are faster compared to the state of estrous cycle of cloned embryos. However, it is already shown that development of SCNT embryos is slower than *in vivo* embryos (Martin *et al.*, 2007), we conclude that using the faster recipient that showed standing estrous at the same day of SCNT is optimal procedure for preparing recipient. Also the present result are consistent to our previous report that pre-ovulated recipients, that faster than cloned embryos, show better developmental result after transfer of cloned embryos at the same day of SCNT (Koo *et al.*, 2009a) but in this time we used ovulated recipients and 1~2 days cultured embryos. Interestingly, no piglets were produced in "−1" and "+1" group and this result revealed that synchrony between recipients and cloned embryos is very critical to produce cloned pigs.

Parity of the recipients was also analyzed in the study. It is already reported that reproductive performance of sows is highest at parity 2 and parity 3 (Tantasuparuk *et al.*, 2000). In the present study, recipients of parity 2 and parity 3 groups show good results consistent to the previous studies. However, different from normal breeding condition that shows reduced reproductive performance, especially small litter size, in gilts (Brooker and Smith, 1980), it seems that gilts (parity 1) also show good result to develop cloned embryos compared to parity 2 and parity 3 groups in this study. In normal breeding condition, ovulation rate and litter size are dependent on the body weight and it's the one of limiting factor to reduce reproductive performance of gilts (Tummaruk *et al.*, 2001). However, in the pig cloning, litter size is much smaller (4 to 6) than normal breeding of gilts (about 10) and also independent to the number of ovulation of recipients. Thus, in the result of the present study, reproductive performance in gilts can be similar to parity 2 or parity 3 recipients. On the other hand, more than 4 group show reduced pregnancy rate and cannot produced cloned piglets in this study. In the normal breeding condition, high litter size are maintained until parity 6 (Tantasuparuk *et al.*, 2000). However, it was also reported that, although the reasons are still not clear, number of stillborn and number of piglets that died before weaning are increased after parity 4 (Schwarz *et al.*, 2009). Thus, same factors can be affected detrimentally on the development of cloned embryos transferred to recipient of more than 4 parities.

The limitation of the present study is we cannot found statistically difference in synchronization and parity analysis due to small number of recipients are used in the study. However, we still believe that the results were informative to reduce time and efforts producing cloned piglets because in both cases, no piglets were produced in poor developmental groups and showing clear tendency for optimization of preparing recipient following physiology of pigs and properties of cloned embryos.

In conclusion, procedure for pig cloning can be optimized using early cleaved embryos and recipients at 1~3 of parity number that showing standing estrous at the same day of SCNT. However, crossbred recipient did not improve the efficiency of pig cloning.

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