# Reproductive Efficiency and Characteristics of Cloned Miniature Piglets Produced from Domestic Commercial Gilts

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## ABSTRACT

The objective of this study was to examine the reproductive characteristics of cloned miniature piglets produced from surrogate domestic pigs. Somatic cell nuclear transfer (SCNT) miniature pig embryos were transferred into domestic pigs. As controls, domestic pigs of the same breed with surrogates for SCNT embryos and miniature pigs of the same breed with the somatic cell donor were bred by artificial insemination and natural mating, respectively. Surrogate domestic pigs that farrowed cloned miniature piglets had a significantly longer gestation length (118.1 days) than conventionally bred domestic (115.4 days) and miniature (115.5 days) pigs. Furthermore, the birth weight of cloned miniature piglets produced from domestic pigs (743 g) was significantly greater than that of miniature piglets produced by natural breeding (623 g). Also, cloned miniature piglets had a significantly lower weaning rate (49.7%) than conventionally produced domestic (91.5%) and miniature (100%) piglets. No differences were observed between female and male cloned piglets in gestation length, litter size, birth weight, or weaning rate. Our results demonstrate that gestation length is extended in domestic pigs that are transferred with SCNT miniature pig embryos and that cloned miniature piglets have increased birth weight and high pre-weaning mortality.

(Key words : birth weight, gestation length, somatic cell nuclear transfer)

## INTRODUCTION

Somatic cell nuclear transfer (SCNT) in combination with embryo transfer offers a new strategy for generating specific animals. Pigs are widely used as target animals for the production of transgenic animals for biomedical or agricultural purposes (Nottle *et al.*, 2007; Cho *et al.*, 2009). In particular, mature miniature pigs are frequently considered as a candidate for bio-organ donors for xenotransplantation because of their similar body weight and size with humans (Kolber-Simonds *et al.*, 2004).

To generate transgenic miniature pigs, it is common to produce SCNT embryos using miniature pig cells and then transfer these embryos to surrogate pigs of domestic breeds because there are limited resources for recipient miniature pigs. In this case, domestic pigs farrow miniature piglets upon pregnancy, which is uncommon in conventional breeding. The gestation length of pregnant pigs is influenced by various factors, including breed, the number of fetuses, fetal gender, and induction of farrowing (Kennnedy and Moxley, 1978; Sasaki and Kobetsu, 2007; Rydhmer *et al.*, 2008). Too early an induction of farrowing by prostaglandin administration or Cesarean section is detrimental to neonatal viability and increases pre-weaning mortality due to the immaturity of neonatal piglets. Therefore, it is very important to determine and predict a desirable farrowing date to improve SCNT efficiency by decreasing the neonatal mortality of SCNT piglets due to premature birth. However, limited information is available on the gestation length of surrogate domestic pigs that have been transferred with SCNT miniature pig embryos, the birth weight and weaning rate of cloned piglets derived from domestic pigs, and the differences in reproductive characteristics between female and male cloned piglets (Williams *et al.*, 2006; Walker *et al.*, 2007).

The objective of this study was to examine reproductive

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characteristics such as gestation length, birth weight, litter size, and weaning rate in cloned miniature piglets produced from domestic pigs and to compare these characteristics to those of domestic and miniature piglets produced by conventional breeding. Our findings demonstrate that gestation length is extended in domestic pigs that are transferred with SCNT miniature pig embryos and that cloned miniature piglets have increased birth weight and high pre-weaning mortality.

## MATERIALS AND METHODS

### 1. Culture Media

All chemicals used in this study were obtained from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA) unless otherwise stated. The medium for *in vitro* maturation (IVM) of oocytes was Tissue Culture Medium-199 (Invitrogen, Grand Island, NY, USA) supplemented with 0.6 mM cysteine, 0.91 mM pyruvate, 10 ng/ml epidermal growth factor, 75 $\mu$g/ml kanamycin, 1 $\mu$g/ml insulin, and 10% (v/v) porcine follicular fluid. The medium used for transient culture and transfer of embryos into recipient pigs was North Carolina State University-23 (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 sodium pyruvate and 5 mM sodium lactate (Park *et al.*, 2005).

### 2. Oocyte Collection and IVM

Ovaries of prepubertal gilts were collected at a local abattoir. Cumulus-oocyte complexes (COCs) were aspirated from superficial follicles (3~8 mm in diameter). COCs having multiple layers of compacted cumulus cells were cultured in a well of a four-well multi-dish (Nunc, Roskilde, Denmark) containing 500 $\mu$l of IVM medium with 10 IU/ml eCG (Intervet International BV, Boxmeer, Holland) and 10 IU/ml hCG (Intervet International BV). 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 for an additional 18~19 h for SCNT.

### 3. Preparation of Donor Cells and Nuclear Transfer

Skin fibroblasts from a miniature pig were seeded into four-well culture plates and grown in Dulbecco's modified Eagle medium with the nutrient mixture F-12 (Invitrogen) containing 15% (v/v) fetal bovine serum. Cells (< 8 passages) were synchronized at the G0/G1 stage of the cell cycle by contact inhibition for 48~72 h and used for SCNT. After IVM, metaphase II (MII) oocytes that were stained with 5 $\mu$g/ml Hoechst 33342 for 15 min were transferred into a drop of manipulation medium (calcium-free TLH-BSA) containing 5 $\mu$g/ml cytochalasin B overlaid with mineral oil. Oocytes were enucleated by aspirating the first polar body and MII chromosomes using a 17-$\mu$m (inner diameter) 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 perivitelline space of each oocyte.

### 4. Electrofusion and Activation

Cell-oocyte couplets were placed on a 1-mm fusion chamber overlaid with 1 ml of 280 mM mannitol solution containing 0.001 mM $CaCl_2$ and 0.05 mM $MgCl_2$. Membrane fusion was induced by applying an alternating current field of 2 V cycling at 1 MHz for 2 sec, followed by two pulses of 170 V/mm direct current (DC) for 25 $\mu$sec using a cell fusion generator (LF101; NepaGene, Chiba, Japan). Immediately after fusion, the oocytes were incubated for 1 h in TLH-BSA and evaluated for membrane fusion prior to activation. Reconstructed oocytes were activated with two pulses of 120 V/mm DC for 60 $\mu$sec in 280 mM mannitol solution containing 0.01 mM $CaCl_2$ and 0.05 mM $MgCl_2$. Following activation, the SCNT embryos were treated with 0.4 $\mu$g/ml demecolcine in IVC medium for 4 h (Song *et al.*, 2009).

### 5. Breeding, Embryo Transfer, and Farrowing of Gilts

Breeding and embryo transfer procedures were approved by the Institutional Animal Care and Use Committee of Kangwon National University in accordance with the Guiding Principles for the Care and Use of Research Animals. Embryo transfers were conducted at the research farm of Gyeonggido Veterinary Service, Korea. SCNT embryos previously treated with 0.4 $\mu$g/ml demecolcine were surgically transferred into naturally cycling Landrace × Duroc crossbreed gilts on the first day of standing estrus. As controls, domestic gilts of the same breed with surrogate pigs for SCNT embryo transfer were bred by artificial insemination, and miniature gilts of the same breed with the somatic cell donor for SCNT were naturally mated. Pregnancy was diagnosed on day 30 (day 0 was the day of SCNT, artificial insemination, or natural mating) and was checked regularly at 4-week intervals using ultrasonography. All pregnant pigs were allowed to farrow naturally. The gestation length

of dams, piglet birth weights, and litter size were recorded. The piglets were weaned at 20~28 days of age.

### 6. Data Analysis

Statistical analyses were performed using the Statistical Analysis System (version 9.1; SAS Institute, Cary, NC, USA). Data were analyzed using a general linear model procedure followed by the least significant difference mean separation procedure when the treatments differed at $p<0.05$. The results were expressed as mean ± standard error of the mean.

## RESULTS

In this study, a total of 104 cloned miniature piglets were produced from 18 out of 53 domestic surrogate pigs that were transferred with 6,050 SCNT embryos (114.2 embryos/recipient). Domestic and miniature piglets were produced from 5 and 2 dams out of 6 and 2 pigs, respectively, after conventional breeding. Surrogate domestic pigs that farrowed cloned miniature piglets had a significantly longer gestation length (118.1 days) than conventionally bred domestic (115.4 days) and miniature (115.5 days) pigs. In addition, the birth weight of cloned miniature piglets produced from domestic pigs (743 g) was lower than that of domestic piglets (1,277 g) but significantly greater than that of miniature piglets produced by natural breeding (623 g). Furthermore, cloned miniature piglets had a significantly lower weaning rate (49.7%) than conventionally produced domestic (91.5%) and miniature (100%) piglets (Table 1).

When the reproductive characteristics were compared for female and male cloned pigs, respectively, no significant differences were observed in gestation length (118.4 vs. 117.0 days), litter size (5.3 vs. 7.5), birth weight (741 vs. 771 g), or weaning rate (51.4 vs. 43.8%; Table 2).

## DISCUSSION

Predicting the farrowing date in pigs is helpful for managing the dam and neonatal piglets. Generally, pigs are bred using conventional breeding, such as artificial insemination or natural mating. However, recent progress in embryo transfer techniques has made it possible to produce specific animals that are different in breed and body size from surrogates but are not routinely used in conventional breeding. In such cases, reproductive characteristics such as gestation length, litter size, and birth weight of piglets can be altered. Gestation length is influenced by various factors, such as the number of fetuses

Table 1. Comparison of gestation length and neonatal viability of newborn miniature piglets produced by conventional breeding and transfer of somatic cell nuclear transfer (SCNT) embryos to domestic pigs

| Breed* | Type of breeding** | No. of pigs farrowed | Gestation length (days) | No. of piglets born (litter size) | Birth weight of newborn piglets (g) | % piglets weaned |
|---|---|---|---|---|---|---|
| Dom-Dom | AI | 5 | 115.4 ± 0.4[a] | 59 (11.8 ± 0.4)[a] | 1,277 ± 15[a] | 91.5 ± 2.9[a] |
| Mini-Mini | NB | 2 | 115.5 ± 0.5[ab] | 12 (6.0 ± 0.0)[b] | 623 ± 13[b] | 100 ± 0.0[a] |
| Mini-Dom | SCNT-ET | 18 | 118.1 ± 0.5[b] | 104 (5.8 ± 0.6)[b] | 743 ± 17[c] | 49.7 ± 5.0[b] |

* Dom-Dom, domestic pigs were bred by artificial insemination with semen from domestic boars; Mini-Mini, miniature pigs were bred by natural mating with miniature boars; Mini-Dom, SCNT embryos derived from miniature pig cells were transferred to surrogate domestic pigs.

** AI, artificial insemination; NB, natural mating; SCNT-ET, transfer of SCNT embryos.

[a~c] Different letters indicate significant differences within a column ($p<0.05$).

Table 2. Gestation length and neonatal viability by gender of cloned miniature piglets produced by somatic cell nuclear transfer and embryo transfer to domestic pigs

| Gender of cloned piglets | No. of litters | Gestation length (days) | No. of piglets born (litter size) | Birth weight of newborn piglets (g) | % piglets weaned |
|---|---|---|---|---|---|
| Female | 14 | 118.4 ± 6.4 | 74 (5.3 ± 0.8) | 741 ± 20 | 51.4 ± 6.2 |
| Male | 4 | 117.0 ± 0.4 | 30 (7.5 ± 0.3) | 771 ± 31 | 43.8 ± 4.6 |

in the uterus, piglet body weight, and dam breed (Sasaki and Kobetsu, 2007; Rydhmer *et al.,* 2008). Gestation length is prolonged as litter size decreases (Sasaki and Kobetsu, 2007). In this study, we found that gestation length was extended in surrogate domestic pigs that were pregnant with SCNT miniature pig embryos. Although it is difficult to conclude because of the limited number of farrowed pigs, the extended gestation length in surrogate domestic pigs might be attributable to the small litter size of cloned piglets. Another reason for the extended gestation length may be that endocrine signals from cloned miniature fetuses are insufficient to induce the timely initiation of farrowing in surrogate pigs because of the small litter size and low fetal weight of miniature piglets compared to normal domestic piglets.

The birth weight of neonatal cloned piglets was greater than that of miniature piglets derived from natural breeding. It is unclear what increased the birth weight of the cloned piglets in this study. There are two probable factors for the alteration in birth weight: fetal overgrowth in a relatively large-capacity domestic pig uterus and "large offspring syndrome", which is observed with embryos produced *in vitro* (Pere *et al.,* 1997; Park *et al.,* 2001). Considering the smaller litter size and birth weight of cloned piglets than domestic piglets, the relative uterine capacity for cloned miniature piglets would be larger than that for domestic piglets, which might have increased the birth weight of the cloned piglets. Large offspring syndrome has been reported in cows and sheep when offspring are produced from embryos produced *in vitro* (Young *et al.,* 1998). A similar result was reported in a previous study of pigs (Park *et al.,* 2001) in which some of the cloned piglets had increased birth weight.

Cloned piglets have a higher mortality than piglets produced by conventional breeding (Park *et al.,* 2005). In the present study, the weaning rate (49.7%) of the cloned miniature piglets was almost half that of the conventionally produced domestic and miniature piglets but was higher than that (30.0%) of the previous study (Park *et al.,* 2005) in which 22 cloned piglets out of 35 died within the first week after birth. We used domestic gilts as surrogates for SCNT embryo transfer. They showed less development of the mammary gland than conventionally bred gilts, probably because of the small litter size and low piglet body weight, which may have resulted in insufficient milk production and high mortality in the cloned piglets in this study. Hand feeding may be helpful to decrease the neonatal mortality of cloned miniature piglets that were produced from domestic surrogates with underdeveloped mammary glands at the time of farrowing. As another reason for the high neonatal mortality, high incidence of phenotypic malformation or functional abnormalities in critical organs such as brain and lung of cloned animals can be thought (Hiendleder *et al.,* 2004; Lee *et al.,* 2007). Park *et al.* (2005) reported that cerebromengitis was a critical factor for early death in cloned male piglets but death of cloned female piglets was due to mostly care problem. However, interestingly, we could not find any difference in weaning rate between male and female cloned piglets.

Pigs are a litter bearing species and it is common to conceive mixed litter of male and female fetuses. Thus, it is not possible to determine the difference in the gestation length between dams that conceive female and male fetuses. In cows, gestation length is decreased in dams pregnant with male calves, and male calves are heavier than female calves at birth (Norman *et al.,* 2009). However, in this study, no differences were observed between female and male cloned piglets in dam gestation length, birth weight, or weaning rate. Further study with more replications is needed to clarify the differences in reproductive characteristics between male and female cloned piglets. Our data on gestation length, birth weight, and weaning rate of neonatal cloned piglets can be used in surrogate pig farrowing management and care of neonatal cloned piglets.

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