

Original Article

Effects of variation in the number and developmental stage of donor embryos and ovulation status of the surrogate mother on the efficiency of pig somatic cell cloning

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Received August 27, 2020
Revised September 16, 2020
Accepted September 18, 2020

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ABSTRACT This study investigated the effect of variation in the number of somatic-cell-cloned embryos and their developmental stage at transfer on pregnancy, as well as the influence of the estrus status of recipient pigs on *in vivo* development of cloned porcine embryos after embryo transfer. For somatic cell nuclear transfer (SCNT), fibroblast cells were obtained from a male porcine fetus. Recipient oocytes were collected from prepubertal gilts at a local abattoir and then cultured. After SCNT, reconstructed embryos of different numbers and developmental stages were transferred into recipient pigs. The developmental stage of the cloned embryos and the number of transferred embryos per surrogate showed no significant differences in terms of the resulting cloning efficiency. However, the pregnancy rate improved gradually as the number of transferred cloned embryos was increased from 100-150 or 151-200 to 201-300 per recipient. In pre-, peri-, and post-ovulation stages, pregnancy rates of 28.6%, 41.8%, and 67.6% and 16, 52, and 74 offspring were recorded, respectively. The number of cloned embryos and estrus status of the recipient pig at the time of transfer of the cloned embryo affect the efficiency of pig production; therefore, these variables should be particularly considered in order to increase the efficiency of somatic cell pig cloning.

Keywords: cloned embryo, embryo transfer, ovary, pig, recipient

INTRODUCTION

The technology of somatic cell nuclear transfer (SCNT) in mammals has progressed over the past few decades

since the production of the first cloned sheep from a mammary gland cell (Wilmut et al., 1997). This technology has been applied in many fields, such as the genetic improvement of farm animals, the rescue of endangered

species, and the production of transgenic animals for therapeutic applications. Pig SCNT has also been studied with the aim of improving the efficiency of production (Betthauser et al., 2000; Huang et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000; Li et al., 2013; Rim et al., 2013; Callesen et al., 2014; Huan et al., 2015; Yoo et al., 2017; Jeong et al., 2020).

Given the low efficiency of SCNT, many questions on the mechanisms involved remain unanswered in both basic and applied fields. The difficulty in porcine cloning has been attributed to multiple factors, including oocyte quality (*in vivo* matured vs. *in vitro* matured), donor cell type, inadequacies in the culture and media used during the micromanipulation process, lack of an efficient activation method, requirement for a minimum number of fetuses, and the appropriate recipient conditions to maintain a pregnancy (Bang et al., 2002; Yoo et al., 2017; Dang-Nguyen et al., 2020). Polejaeva et al. (2000) reported the first successful birth of five healthy cloned female piglets by SCNT using *in vivo*-matured oocytes. Around the same time, Onishi et al. (2000) reported the birth of a single, cloned female piglet from *in vivo*-matured oocytes using piezo-actuated microinjection. These studies used the difficult SCNT procedure and expensive *in vivo*-matured oocytes for the production of cloned piglets. In contrast, Betthauser et al. (2000) systematically optimized each step of SCNT procedure, including sourcing oocytes and *in vitro* oocyte maturation, culture of donor cells, activation of oocytes following fusion, *in vitro* culture of embryos, and transfer of embryos to recipient gilts. This led to a more reproducible methodology; however, the efficiency of production of normal, live, cloned offspring remained very low.

Different approaches to the improvement of pig SCNT efficiency have indicated that selection of a suitable donor cell type, ovulation status in recipient gilts, and transfer of an appropriate number of cloned embryos could increase the success rate of piglet cloning (Koo et al., 2010; Li et al., 2013; Rim et al., 2013; Wei et al., 2013; Huan et al., 2015; Shi et al., 2015).

Although most of these factors are important, optimization of the embryo transfer conditions is one of the key steps for producing cloned piglets. Studies have suggested factors affect the pregnancy rate of pig SCNT, such as ovulation status of surrogated gilts, and the number and duration of *in vitro* cloned embryos for transfer (Petersen

et al., 2008; Koo et al., 2010; Schmidt et al., 2010; Huang et al., 2013; Li et al., 2013; Rim et al., 2013; Liu et al., 2014). However, these previous studies were usually performed using an inaccurate synchronization program or a relatively small number of surrogate mothers. Therefore, the present study was conducted to investigate the effect of the following three factors on cloning efficiency using a more accurate synchronization program and a large number of surrogate mothers: the estrus status of the surrogate mother, cleavage stage, and the number of cloned embryos for transfer.

MATERIALS AND METHODS

Isolation and *in vitro* culture of porcine somatic cells

Fibroblast cells were obtained from a male porcine fetus. The cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum under 5% CO₂ at 37°C. When fetal fibroblast cells were confluent, the cells were passaged. Donor cells were used for nuclear transfer between passages 4 and 8 of *in vitro* culture. The cells were used for nuclear transfer within three days of reaching confluence.

In vitro maturation of oocytes

Ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory at 25–35°C. Antral follicles (2–6 mm in diameter) were aspirated with an 18-gauge needle. Aspirated oocytes, which had an evenly granulated cytoplasm and were surrounded by at least three uniform layers of compact cumulus cells, were selected for *in vitro* maturation. Approximately 100 oocytes were cultured for 40 h in four-well plates (Nunc, Roskilde, Denmark), each well of which contained 500 µL of TCM-199 (Invitrogen Carlsbad, CA, USA) medium, supplemented with 10% porcine follicular fluid, 0.6 mmol/L cysteine, 4 IU/mL FSH, LH, and 10 ng/mL EGF.

Somatic cell nuclear transfer (SCNT)

Nuclear transfer was carried out as described previously (Park et al., 2004). In brief, matured oocytes with the first polar body were treated in PZM-3 medium supplemented with 0.4 mg/mL demecolcine (Sigma) and 0.05 mol/L sucrose for 1 h. The treated oocytes were transferred to a medium supplemented with 5 µg/mL cytochalasin B and 0.4 mg/mL demecolcine, and the protrusion was removed

with a beveled pipette. A single donor cell was injected into the perivitelline space of each oocyte and was electrically fused by using two direct current pulses of 150 V/mm for 50 µsec in 0.3 M mannitol, supplemented with 0.1 mM MgSO₄ and 0.01% PVA and incubated at 39°C in 5% CO₂.

After 1h, the fused oocytes with donor cells that were judged under an inverted microscope were activated by two direct current pulses of 100 V/mm for 20 µsec in 0.3 M mannitol supplemented with 0.1 mM MgSO₄ and 0.05 mM CaCl₂ (Park et al., 2004). Activated embryos were transferred to PZM-3 medium at 39°C in 5% CO₂.

Estrus synchronization and embryo transfer

Eight-month-old gilts were used as the recipients for embryo transfer. Estrus synchronization of the recipient was established as described in previously studies (Onishi et al., 2000; Yoo et al., 2017). Briefly, an i.m. injection of 0.2 mg cloprostenol, a prostaglandin F2 alpha analogue (Planate; Sumitomo Seiyaku, Osaka, Japan), was administered to pregnant gilts (8 mo old, 120 to 130 kg) on days 33 to 53 of gestation, followed by a second injection of 0.2 mg cloprostenol 24 h later. One thousand international units of eCG (PMS 1000; Tani, NZ) was administrated i.m. at the same time as the second cloprostenol injection. Ovulation was induced by i.m. injection of 500 IU hCG (Puberogen; Sankyo, Tokyo, Japan) at 72 h after the eCG injection. At 41 to 42 h after the hCG injection, 1-4 cell SCNT embryos were then surgically transferred into the oviducts using a straw. The status of the recipient's estrus was divided into the following three groups based on the recipient's ovarian status: pre-ovulation stage group (Graafian follicles), peri-ovulation stage group (Graafian follicles and are bloody), and post-ovulation stage group (corpus hemorrhagicum). Ultrasound was used to test for pregnancy between days 25 and 30 after embryo transfer.

Experimental design

In Experiment 1, we transferred different numbers of reconstructed embryos into recipient pigs and compared the effect of embryo numbers on pregnancy. In Experiment 2, different developmental stages of reconstructed embryos were transferred into recipient pigs and the effect of the various embryo stages of pregnancy was determined. In the third experiment, we investigated the impact of the estrus status of the recipient pigs on pregnancy after reconstructed embryo transfer.

Statistical analysis

The data were subjected to arcsine transformation for each replication. The transformed values were analyzed using one-way ANOVA. A value of $p < 0.05$ was used to determine statistical significance.

RESULTS

Experiment 1: Effects of transferred somatic-cell-cloned embryo number on pregnancy

The effects of the number of somatic-cell-cloned embryos on *in vivo* development to term were evaluated. After nuclear transfer, embryos were cultured under *in vitro* conditions and embryos that developed to the one- to four-cell stage were transferred randomly to recipient pigs in three groups with different numbers of transferred embryos (Group 1: 100-150 embryos, Group 2: 151-200 embryos, and Group 3: 201-300 embryos). As shown in Table 1, Group 1 (10.0%) had a significantly lower pregnancy rate than Group 2 (37.9%) and Group 3 (29.2%). The farrowing rates were 6.7% in Group 1, 20.7% in Group 2, and 25% in Group 3, but these were not significantly different. In addition, the rate of cloned piglet production did not differ among the three groups.

Table 1. Effect of the number of cloned embryos transferred on pig cloning efficiency

Range of the number of embryos transferred	No. of recipients used	No. of embryos transferred (Mean No./recipient)	No. of recipients pregnant (%)	No. of recipients farrowed (%)	No. of cloned piglets born (%)
100-150	30	3,537 (117.9)	3 (10.0) ^b	2 (6.7)	8 (0.22)
151-200	29	5,069 (174.8)	11 (37.9) ^a	6 (20.7)	16 (0.32)
201-300	24	5,895 (245.6)	7 (29.2) ^a	6 (25.0)	25 (0.42)

^{a,b}Values in the same column with different superscripts differ significantly ($p < 0.05$).

Experiment 2: Effect of embryo development stage for embryo transfer on the efficiency of pig cloning

To examine the effects of the developmental stage of somatic-cell-cloned embryos on cloned pig production at the time of embryo transfer to recipient pigs, the reconstructed embryos were cultured for 12–24 h and 150–300 embryos were transferred into recipient pigs in four different groups (Group A: one-cell-stage embryos, Group B: one- to two-cell-stage embryos, Group C: two- to four-cell-stage embryos, and Group D: one- to four-cell-stage embryos). As shown in Table 2, the pregnancy rates were 23.8% in Group A, 22.2% in Group B, 15.4% in Group C, and 30% in Group D. The farrowing rates were 14.3% in Group A, 22.2% in Group B, 11.5% in Group C, and 20% in Group D. These two variables did not show significant differences among the groups. In addition, the rate of

production of cloned piglets did not differ among the four groups.

Experiment 3: Effect of ovulation status of surrogate gilts on pig cloning efficiency

To determine the effect of estrus status of recipient gilts on the successful generation of somatic cell nuclear transfer (SCNT) pigs, one- to four-cell-stage embryos were transferred into the oviducts of recipient gilts with three different ovarian statuses in Table 3 (Type A: Pre-ovulation, Type B: Peri-ovulation, Type C: Post-ovulation; Fig. 1). Type C showed significantly higher pregnancy and farrow rates than the other types. However, there was no significant difference in the mean numbers of cloned piglets per recipient among the three types.

Table 2. Effect of embryo stages on pig cloning efficiency

Group (embryo stage)	No. of recipients used	No. of embryos transferred (Mean No./recipient)	No. of recipients pregnant (%)	No. of recipients farrowed (%)	No. of cloned piglets born (%)
Group A (1 cell)	21	3,202 (152.5)	5 (23.8)	3 (14.3)	12 (0.38)
Group B (1–2 cell)	9	2,401 (266.8)	2 (22.2)	2 (22.2)	6 (0.25)
Group C (2–4 cell)	26	4,411 (169.7)	4 (15.4)	3 (11.5)	10 (3.3)
Group D (1–4 cell)	10	1,521 (152.1)	3 (30.0)	2 (20.0)	9 (4.5)

Table 3. Effect of ovulation status of surrogate gilts on pig cloning efficiency

Recipient condition*	No. of recipients used	No. of embryos transferred (Mean No./recipient)	No. of recipients pregnant (%)	No. of recipients farrowed (%)	No. of cloned piglets born (Mean \pm SD/recipient)
Type A	21	7,528 (358.4)	6 (28.6) ^b	4 (19.0) ^b	16 (4.00 \pm 2.45)
Type B	55	18,737 (340.7)	23 (41.8) ^b	16 (29.1) ^b	52 (3.25 \pm 1.95)
Type C	37	12,944 (349.8)	25 (67.6) ^a	20 (54.1) ^a	74 (3.70 \pm 2.15)

*A: Pre-ovulation, B: Peri-ovulation, C: Post-ovulation.

^{a,b}Values in the same column with different superscripts differ significantly ($p < 0.05$).



Fig. 1. Recipient ovulation status before the transfer of cloned embryos. (A) Pre-ovulation, follicles large and developed. (B) Peri-ovulation, follicles partly ovulated. (C) Post-ovulation, follicles all ovulated.

DISCUSSION

Because somatic cell animal cloning is very complicated, many factors affect its efficiency. In this study, the effects of major factors, including the number and developmental stage of cloned embryos for embryo transfer, and the ovulation status of recipient gilts on the overall cloning efficiency were examined, in order to improve the generation of cloned pigs.

In pigs, signals from three or more embryos are required to maintain pregnancy (King et al., 2002). The pregnancy and delivery rates of surrogate females receiving SCNT embryos are usually lower than those resulting from the transfer of fertilized embryos (Hornen et al., 2007; Kurume et al., 2008; Salilew-Wondim et al., 2013). One possible reason for this is that the low developmental ability of cloned embryos significantly reduces their signaling to the recipient mother after embryo transfer. Therefore, they are unable to cause the establishment of pregnancy in the recipients or help in maintaining pregnancy to term. If this is correct, it may be possible to minimize this adverse effect by increasing the number of cloned embryos transferred to an individual surrogate. However, in our study, the transfer of more than 100 cloned embryos did not appear to markedly influence the pregnancy and delivery rates of the recipients.

It has been shown that *in vitro* culture cannot provide porcine embryos with a developmental environment that is comparable to *in vivo* conditions (Macháty et al., 1998; Nakamura et al., 2017). In many pig SCNT studies, short *in vitro* culture times for cloned embryos before embryo transfer were used instead of longer ones (Liu et al., 2014; Shi et al., 2015). Our study used SCNT embryos cultured both for a shorter time (one-cell-stage embryos for 16 h) and for a longer time (one-cell, one- to two-cell, two- to four-cell, and one- to four-cell embryos for 16–40 h) and found that culture time prior to transfer did not significantly affect the rates of pregnancy and delivery.

The estrus status of recipient gilts has been considered important for the outcome of SCNT in pig (Koo et al., 2010; Huang et al., 2013; Huan et al., 2015). It is known that synchronization between cloned embryos and surrogates determines the subsequent development of pregnancy, and that the primary cause of failure in the development of cloned embryos, transferred 14-h post-activation into surrogates with different estrus stages,

is related to the coordination of synchronization. The results of the present study show that surrogates on Day 2 of estrus were more suitable for the implantation and full-term development of cloned embryos. One possible explanation for this is that the cloned embryos develop at a slower rate than their *in vivo* counterparts and are synchronized with the surrogates on Day 2 of estrus when cultured 14-h post-activation (Martin et al., 2007). In the present study, nearly all of the surrogates in the post-ovulation group carried the cloned embryos to full term and gave birth to cloned piglets.

In conclusion, we have successfully cloned pigs under conditions of varying embryo numbers and developmental stages, and different estrus statuses of recipient gilts. It is acknowledged that animal cloning is a complicated process, and many other potential factors such as embryo quality should be investigated. Our results should help improve pigs SCNT and other important animals and should provide insights into the factors that affect cloning in animals.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGEMENTS

This work was supported by a 2-Year Research Grant of Pusan National University.

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