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RESEARCH ARTICLE

Factors influencing the somatic cell nuclear transfer efficiency in pigs

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Abstract Using a data set from our laboratory, we assessed the effects of several factors on pig cloning efficiency. The results demonstrated that cells at high confluence (> 90%) used as donor cell resulted in higher pregnancy rate, delivery rate and overall cloning efficiency (number of live offspring born per reconstructed embryo transferred to recipients) compared with the cells at 60% to 79% confluence and 80% to 89% confluence. Cells with four, five and six passages compromised the pregnancy and delivery rates compared with first passage cells. The number of blastocysts transferred by somatic cell nuclear transfer (SCNT) did not significantly affect the cloning efficiency, but transfer of blastocyst derived from *in vitro* culture 5 d after SCNT achieved a significantly higher pregnancy rate compared with one to two cell SCNT embryos from overnight culture. The highest pregnancy rate, delivery rate and the largest litter size were obtained when Bama Miniature pig fibroblasts were used as donor cells and Landrace/Yorkshire hybrid gilts were used as recipients. Recipients treated with chemicals for estrus synchronization had higher pregnancy rates compared with untreated recipients. Our data might be helpful for improving SCNT efficiency in pigs.

Keywords blastocyst, donor cell, estrus synchronization, pregnancy rate, pig cloning, somatic cell nuclear transfer

1 Introduction

Pigs are not only considered to be valuable livestock animals but also an essential large animal model for biomedical research[1], and are even regarded as the primary species for xenotransplantation to humans because their anatomical and physiological characteristics are similar[2–5].

The successful cloning of pigs from somatic cells was first reported almost concurrently in 2000 by three independent groups[6–8]. Since then, somatic cell nuclear transfer (SCNT) has been widely used for the generation of different types of genetically-engineered and knockout pigs[9–12]. Although a large number of transgenic pigs have been produced by SCNT over the past 10 years, the efficiency of cloning (number of live offspring per reconstructed embryo transferred to recipients) is still low[13–15]. Moreover, the cloned animals usually suffer from various developmental defects.

To date, several studies had reported the key factors associated with the production of cloned pigs and have suggested a number of approaches to improve the cloning efficiency, for example, SCNT[16], donor cell type and preparation[17], method of genetic modification[18], oocyte and embryo culture systems[19], recipient breed selection[20] and season[21]. However, despite the above endeavors, there are still some factors, which might affect pig cloning efficiency, that have not been investigated. For example, donor cell passage and extended growth in culture, culture time of cloned embryos before transfer, number of transferred cloned embryos per recipient, donor cell and recipient breed, and estrus synchronization of recipients might affect the efficiency of porcine cloning. To our knowledge, a combined assessment of multiple factors and comparative analysis of their relative contribution to cloning efficiency has not been performed.

Here, we assessed the effects of donor cell characters (culture time, confluence degree and cell passage), the number of embryos transferred, the culture interval between nuclear transfer manipulation and embryo transfer surgery, donor cell and recipient breed selection, and chemical treatment-induced estrus synchronization on pig cloning efficiency. Using a data set for more than 37151 reconstructed embryos that were transferred to 263

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recipients generating 266 cloned piglets using different cells over the past 3 years, we investigate the impact of these factors on the pregnancy and delivery rates, as well as overall cloning efficiency. An improvement in pig cloning efficiency might be achieved by selecting donor cell, adjusting the quality of transferred embryos and *in vitro* culture time of reconstructed embryos before transfer, selecting the donor cell and recipient breed, and applying estrus synchronization treatment.

2 Materials and methods

2.1 Animals

All pig experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. Every effort was made to minimize any animal suffering.

2.2 Media and reagents

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

2.3 Donor cell preparation

Fetal fibroblast cells were derived from 35-d-old wild-type Landrace/Yorkshire hybrid (LY) fetuses or Bama Miniature pig (BM) fetuses. According to the experimental design, donor cells were thawed, and used immediately or cultured to different confluence degrees, and then used for SCNT. To evaluate the effect of the cell passage on pig cloning efficiency, cells with four to eight passages were cultured to the same confluence degree and then used for SCNT. First passage cells were established from already existing transgenic pigs and used as controls. Additionally, cloned embryos (at the blastocyst stage) were transferred into natural estrus recipient gilts in all of the above experiments.

Before SCNT, a suspension of single cells was prepared by trypsinization of the cultured cells, followed by resuspension in manipulation medium (GIBCO Media 199 supplemented with 0.75 $g \cdot L^{-1}$ HEPES, 0.05 $g \cdot L^{-1}$ penicillin, 0.06 $g \cdot L^{-1}$ streptomycin, 1.755 $g \cdot L^{-1}$ NaCl and 3.00 $g \cdot L^{-1}$ BSA).

2.4 Oocyte collection and *in vitro* maturation

Porcine ovaries derived from gilts were obtained from a local slaughterhouse, the Slaughterhouse of Meat Processing Factory, Nanjing, China, and incubated in 0.9% (w/v) NaCl at 37°C until use. The cumulus-oocyte complexes (COCs) and follicular fluid were aspirated using an 18 gauge disposable needle from mature follicles (3–6 mm diameter) and transferred into a 15 mL conical tube. The samples were rinsed three times with TL-HEPES containing 0.01% polyvinyl alcohol (PVA, w/v). COCs with uniform cytoplasm and several layers of cumulus cells were selected under a stereomicroscope and rinsed three times with *in vitro* maturation medium (GIBCO Media 199 supplemented with 0.1% PVA, 3.05 $mmol \cdot L^{-1}$ D-glucose, 0.91 $mmol \cdot L^{-1}$ sodium pyruvate, 0.57 $mmol \cdot L^{-1}$ cysteine, 0.5 $mg \cdot mL^{-1}$ luteinizing hormone, 0.5 $mg \cdot mL^{-1}$ follicle-stimulating hormone, 10 $ng \cdot mL^{-1}$ epidermal growth factor and 10 $mg \cdot mL^{-1}$ gentamicin). About 50–70 COCs were transferred into each well of four-well multidishes containing 500 μL of maturation medium covered with mineral oil and incubated at 38.5°C in an atmosphere of 5% CO_2 in air. After 42–44 h of maturation culture, COCs were transferred into denuding medium (100 mg hyaluronidase, 6 g mannitol, 0.001 g BSA, 5 mL PVA-TL-HEPES stock and 95 mL milli-Q water), vortexed for 1 min to remove the cumulus cells. Oocytes with the first polar body were selected for SCNT.

2.5 Somatic cell nuclear transfer

Matured oocytes with the first polar body were transferred into manipulation medium (GIBCO Media 199 supplemented with 0.75 $g \cdot L^{-1}$ HEPES, 0.05 $g \cdot L^{-1}$ penicillin, 0.06 $g \cdot L^{-1}$ streptomycin, 1.755 $g \cdot L^{-1}$ NaCl and 3.00 $g \cdot L^{-1}$ BSA) supplemented with 0.006 $g \cdot L^{-1}$ cytochalasin B, which was overlaid with warm mineral oil. The polar body along with a portion of the adjacent cytoplasm which presumably contained the metaphase II plate, was removed, and a donor cell was placed in the perivitelline space by using a beveled glass pipette with an inner diameter of 17–20 μm. Then the reconstructed embryos were placed into embryo culture medium until fusion and activation.

The reconstructed embryos were rinsed three times with activation medium (distilled water supplemented with 0.3 $mol \cdot L^{-1}$ mannitol, 1.0 $mmol \cdot L^{-1}$ $CaCl_2 \cdot 2H_2O$, 0.1 $mmol \cdot L^{-1}$ $MgCl_2 \cdot 6H_2O$ and 0.5 $mmol \cdot L^{-1}$ HEPES) and aligned within a chamber with two electrodes placed 0.5 mm apart, which was covered with activation medium. Two 30-ms electrical pulses of 1.2 $kV \cdot cm^{-1}$ were delivered. After fusion, embryos were then incubated for 20 min in PZM3 (108.0 $mmol \cdot L^{-1}$ NaCl, 10.0 $mmol \cdot L^{-1}$ KCl, 0.35 $mmol \cdot L^{-1}$ KH_2PO_4, 0.4 $mmol \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 25.07 $mmol \cdot L^{-1}$ $NaHCO_3$, 0.2 $mmol \cdot L^{-1}$ Na-pyruvate, 2.0 $mmol \cdot L^{-1}$ $Ca(lactate)_2 \cdot 5H_2O$, 1.0 $mmol \cdot L^{-1}$ glutamine, 5.0 $mmol \cdot L^{-1}$ hypotaurine, 20 $mL \cdot L^{-1}$ Eagle's basal medium amino acid solution, 10 $mL \cdot L^{-1}$ modified Eagle's medium amino acid solution, 0.05 $mg \cdot mL^{-1}$ gentamicin and 3 $mg \cdot mL^{-1}$ BSA) and evaluated for fusion. Only the fused embryos were cultured in PZM-3 covered with mineral oil, at 38.5°C, with 5% CO_2, 7% O_2, 88% N_2 and 100% RH.

2.6 Embryo culture and embryo transfer

The cloned embryos were cultured for 22–24 h (one- to two-cell stage), 44–48 h (four- to 16-cell stage) or 5 d (blastocyst stage) and transferred into recipient gilts which were in natural estrous. According to the experimental design, a different number of blastocysts were transferred into natural estrus recipient gilts or cloned embryos (at one- to two-cell stage) derived from different type of donor cell (LY or BM fibroblasts) were transferred into induced estrous LY gilts or a Chinese local pig breed (Meishan). The estrus synchronization procedure was as previously reported[22].

All of the recipient gilts were in standing estrus on the day of SCNT manipulation (day 0). The cloned embryos (at one- to two-cell stage or four- to 16-cell stage) were transferred into the oviduct of recipient gilts and the blastocysts were transferred into cornua uteri of recipient gilts. The surgical procedure was as previously reported[20]. Pregnancy was diagnosed on day 30 and then checked regularly by ultrasound examination every 2 weeks. The cloned piglets were born on days 117–120.

2.7 Statistical analysis

If one recipient became pregnant or farrowed, the percentage of pregnancy or delivery was 100%, otherwise it was 0%. Differences in data were analyzed with SPSS statistical software. All the data were analyzed using a general linear model. $P < 0.05$ were considered to indicate statistical significance.

3 Results

3.1 Effect of the culture time and confluence degrees of donor cells on pig cloning efficiency

Before SCNT, the donor cells were cultured *in vitro* for 0–4 d (day 0 was the day when the cells were thawed). The cells usually reached 60% to 79%, 80% to 89% and 90% to 100% confluence after culture for 1, 2, 3 and 4 d, respectively. The results demonstrated that the cells at high confluence ($>90\%$) after culture for 4 d or just after thawing resulted in higher pregnancy rates (44.4% and 42.9%), delivery rate (22.2% and 42.9%) and overall cloning efficiency (2.1% and 3.8%) compared with the fresh cells at 60% to 79% confluence after culture for 1–2 d (6.3%, 6.3% and 0.5%) and at 80% to 89% confluence after culture for 3 d (8.3%, 8.3% and 0.7%) (Table 1).

3.2 Effect of cell passage on transgenic pig production

After screening by antibiotic, the transgenetic cells were cultured *in vitro* from cells with four to eight passages. The effect of cell passage on the cloning outcome was investigated. Cells with four to six passages showed significantly compromised pregnancy (40.0%, 42.9% and 44.4%) and delivery rates (10.0%, 42.9% and 44.4%) compared with first passage cells established from already existing transgenic pig lines (both 66.7%). The cells with seven and eight passages did not result in piglets (Table 2).

3.3 Effect of cloned embryo culture time and transferred blastocyst number on pig cloning efficiency

SCNT embryos cultured for 22–24 h, 44–48 h or 5 d were transferred to recipients. The different ovulation status of recipients is shown in Fig. 1. The results demonstrated that transfer of cloned blastocysts cultured for 5 d resulted in a significantly higher pregnancy rate (81.8% versus 20.0%), compared to transfer of one- to two-cell stage embryos cultured for 22–24 h (Table 3. However the number of transferred SCNT blastocysts (30–49, 50–69 and 70–110) did not significantly affect the cloning efficiency (Table 4).

3.4 Effect of donor fibroblast cell and recipient's breeds on pig cloning efficiency

The cells derived from LY or BM fetuses were used as donor cells for SCNT, and then the cloned embryos were transferred into LY or Meishan gilts. There was no significant difference between pregnancy and delivery rates with LY fibroblasts in Meishan gilts (both 12.5%) and LY fibroblasts in LY gilts (both 22.2%). These values were significantly lower than those with BM fibroblasts in LY gilts (53.9% and 46.2%) (Table 5). The most noteworthy was the large litter size when BM fibroblasts were used as donor cells and the LY gilts were used as recipients. Three large litters with 10 piglets/litter were obtained from a total of 914 embryo transfers. The average was about 6 piglets/litter.

Table 1 The effect of the culture time and confluence degrees of donor cells on pig cloning efficiency

Cell culture time (confluence degree)	No. of transferred embryos	No. of recipients	No. of pregnant recipients/%	No. of farrowed recipients/%	No. of piglets/%	Litter size
0 d	340	7	3 (42.9)^{a}	3 (42.9)^{a}	13 (3.8)^{a}	4.3
1–2 d (60%–79%)	933	16	1 (6.3)^{b}	1 (6.3)^{b}	5 (0.5)^{b}	5.0
3 d (80%–89%)	757	12	1 (8.3)^{b}	1 (8.3)^{b}	5 (0.7)^{b}	5.0
4 d (90%–100%)	582	9	4 (44.4)^{a}	2 (22.2)^{ab}	12 (2.1)^{ab}	6.0

Note: Values with different superscripts within a column differ at $P < 0.05$.

Table 2 The effect of cell passage on transgenetic pigs production

Cell passage	No. of transferred embryos	No. of recipients	No. of pregnant recipients/%	No. of farrowed recipients/%	No. of piglets/%	Litter size
1	561	6	4 (66.7)[a]	4 (66.7)[a]	14 (2.5)[a]	3.5
4	773	11	2 (40.0)[b]	2 (10.0)[c]	8 (0.4)[b]	3.0
5	486	8	3 (42.9) [b]	3 (42.9)[b]	16 (4.1)[a]	5.7
6	546	9	3 (44.4)[b]	3 (44.4)[b]	13 (2.6)[a]	3.5
7	286	5	0	0	0	0
8	292	4	0	0	0	0

Note: Values with different superscripts within a column differ at $P < 0.05$.

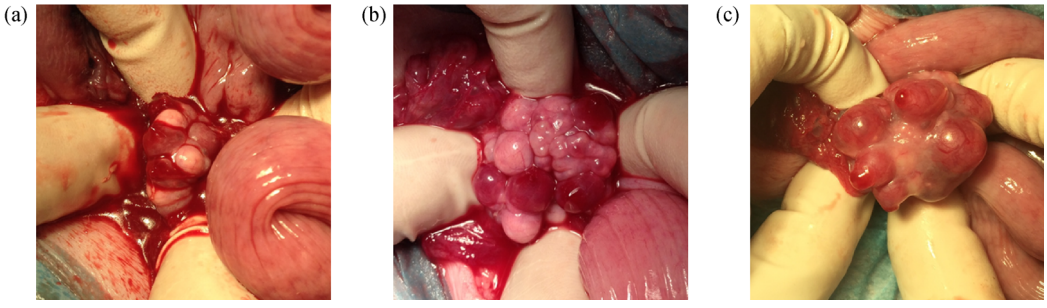

Fig. 1 The ovulation statuses of recipient. The SCNT embryos cultured for 22–24 h (a), 44–48 h (b) and 5 d (c) were transferred to recipients with different ovulation statuses, respectively.

Table 3 The effect of cloned embryo culture time on pig cloning efficiency

Embryo culture time	No. of transferred embryos	No. of recipients	No. of pregnant recipients/%	No. of farrowed recipients/%	Mean litter size
22–24 h	2993	10	2 (20.0)[a]	1 (10.0)	11.0
44–48 h	3719	12	6 (50.0)[ab]	2 (16.7)	6.5
5 d	704	11	9 (81.8)[b]	4 (36.4)	2.8

Note: Values with different superscripts within a column differ at $P < 0.05$.

Table 4 The effect of transferred blastocyst number on pig cloning efficiency

Transferred embryos per recipient	No. of transferred embryos	No. of recipients	No. of pregnant recipients/%	No. of farrowed recipients/%	No. of piglets/%	Litter size
30–49	421	12	4 (33.3)	2 (16.7)	8 (1.9)	4.0
50–69	1608	27	11 (40.7)	5 (18.5)	21 (1.3)	4.2
70–110	1745	23	8 (34.8)	5 (21.7)	19 (2.1)	3.8

Table 5 The effect of donor fibroblast cell and recipient's breeds on pig cloning efficiency

Donor fibroblast cell type	Recipient's breeds	No. of transferred embryos	No. of recipients	No. of pregnant recipients/%	No. of farrowed recipients/%	No. of piglets/%	Mean litter size
LY-FBs	Meishan	2289	16	2 (12.5)[a]	2 (12.5)[a]	8 (0.4)	4
LY-FBs	LY	3747	18	4 (22.2)[a]	4 (22.2)[ab]	14 (0.4)	3.5
BM-FBs	LY	3837	13	7 (53.9)[b]	6 (46.2)[b]	36 (0.9)	6

Note: LY-FBs, Landrace and Yorkshire hybrid fibroblasts; BM-FBs, Bama Miniature pig fibroblasts; Meishan, a Chinese pig breed of Meishan; LY, Landrace and Yorkshire hybrid gilt. Values with different superscripts within a column differ at $P < 0.05$.

3.5 Effect of recipient estrus synchronization treatment on pig cloning

To investigate whether treating recipients with chemicals for estrus synchronization improved the efficiency of pig cloning, pregnancy rate, delivery rate and cloning efficiency rate were compared with those of untreated recipients. A total of 10532 embryos (at one- to two-cell stage) were transferred into 17 treated gilts (treated group) and 17 untreated gilts (untreated group). Twelve treated gilts and six untreated gilts were found to be pregnant at day 30 by ultrasound examination, and eventually 26 piglets and 13 piglets were born from six treated gilts and four untreated gilts, respectively. The pregnancy rate (70.6%) in the treated group was markedly higher than in untreated group (35.3%).

4 Discussion

The outcome of SCNT is affected by complex interactions between multiple factors. Synchronization of the cell cycle between the donor nucleus and oocyte cytoplasm is a major factor that affects the success of SCNT. To maintain correct ploidy of reconstructed embryos for proper development after SCNT, the donor nucleus and oocyte cell cycle should be well coordinated throughout[23]. Previous reports had shown that the donor nuclei must be in the G0 or G1 phase of the cell cycle when oocytes with higher levels of maturation-promoting factors were used as recipients[24]. In porcine SCNT studies, contact inhibition[25–28] is most frequently used to synchronize cells to the G0/G1 phases for SCNT. Treatment with DMSO has been shown to effectively synchronize some cell populations in G0[27]. Some authors have claimed that the use of cells in G0/G1 was required for complete reprogramming[29,30], and that the use of donor cells in this phase results in the production of high-quality embryos. In our study, contact inhibition might have occurred when the cells were cultured for 4 d and reached high confluence ($>90\%$), as most of the cells might have been at the G0 or G1 phase of the cycle at this point. The cells at high confluence should still be in G0 or G1 phase after freezing and thawing with 10% DMSO. Thus, the cells at high confluence before and just after freezing/thawing resulted in higher pregnancy rate, delivery rate and overall cloning efficiency.

Donor cell passage had an effect on cloning efficiency in our data set. *In vitro* conditions may provoke changes of the epigenetic modifications that regulate chromatin compaction and gene expression in cultured cells[31]. Such changes will affect the ability of the cells to undergo reprogramming during SCNT. Shortly after the formation of the cloned embryos, the nucleus responds to the molecular factors in the cytoplast by the epigenetic reprogramming of genomic DNA modifications[32,33]. Various mechanisms that affect the proliferation of cultured cells, such as chromosomal abnormalities, damaged DNA and DNA methylation, have been reported between early and late passages[34–36]. As described above, our study showed that the efficient development of cloned embryo was achieved with first passage cells, compared with cells with four to six passages. Since cells transfection and screening requires time and cell passage, and in order to obtain sufficient genetically modified cells for SCNT, the earliest passage of cells we obtained was four passages. We kept the number of cell passages as low as possible–less than eight passages of *in vitro* culture, avoiding using cells with more passages because they do not produce piglets. The results indicated that the piglets can be obtained with donor cells with four to six passages without affecting pregnancy rate. Unfortunately, cells with seven and eight passages did not produce piglets. The higher number of cell passage might be the main reason for this.

The interval between SCNT manipulation and embryo transfer also might affect the efficiency of pig cloning. Reconstructed embryos have usually been transferred to the preovulatory recipient without *in vitro* culture[37]. Recently, some groups reported that transfer of mixed embryos cultured for 20 and 40 h led to a higher pregnancy rate than with embryos only cultured for 20 h, but the pregnancy rate was similar to that obtained with embryos cultured for 40 h[38]. Callesen reported that bicornual transfer of blastocysts gave rise to more efficient pregnancy and delivery than unicornual transfer in manual cloning[11]. So far, it is not clear whether *in vitro* culture of SCNT embryos affects pig cloning efficiency. In our data set, culture of SCNT embryos for 5 d and then transfer of blastocysts to recipients resulted in a higher pregnancy and delivery rates, as well as overall cloning efficiency, compared to transfer of one- to two-cell stage embryos after overnight culture. This suggests that SCNT embryos that undergo normal cleavage and then eventually develop into blastocysts *in vitro* within the expected time frame have a greater chance of full term development *in vivo*.

Embryonic genomic activation usually occurs at the eight-cell stage in cattle and at the four-cell stage in pigs. An obvious way to select the embryos with high developmental potency is to extend the duration of culture to the blastocyst stage — a process that allows identification of embryos in which the embryonic genome is activated[39]. Otherwise, transfer of the embryo to the uterine cavity after 5 d of estrus is thought to provide better embryo-endometrium synchrony, and therefore higher chances of implantation as it mimics more closely the sequence of events that occur in natural conception[40].

Unexpectedly, the number of transferred cloned blastocysts did not seem to influence recipient pregnancy and delivery rates since gilts that received 30–110 blastocysts exhibited similar pregnancy and delivery rates in our study. From this result, it can be hypothesized that the quality of blastocyst (the developmental potential of embryos, which

is positively correlated with the blastocyst formation rate) but not the number of blastocysts had a dominant impact on the embryo implantation. When the blastocyst formation rate was below 10%, even transfer of more than one hundred SCNT blastocysts did not produce piglets (data not shown) in our laboratory.

Cloning inefficiency associated with SCNT may be attributed to many factors that are not well understood, but the breed of the cloned embryos and their recipients has been suggested to be one of these factors. Researchers found that pregnancy and delivery rates were significantly increased if the transferred cloned embryos and their recipient were the same breed[11,20]. Our study found the opposite result; transfer of embryos cloned from BM fibroblasts to LY recipients resulted in a significantly higher pregnancy rate. However, the pregnancy rate was lower when cloned embryos derived from LY fibroblasts were transferred into the oviduct of Meishan. This suggests that embryos generated from BM fibroblasts allow their LY recipients to establish pregnancy and maintain pregnancy to term more easily, and Meishan is not a suitable recipient for embryos cloned from LY fibroblasts.

Recipient estrus synchronization is one more critical factor for the establishment of lines of genetically engineered pigs by SCNT. Synchronization between the reconstructed embryos and the recipient is critical to maintaining pregnancy[37]. Currently, the methods of estrus synchronization are categorized into two classes: chemical induced and natural estrus. Our data showed that recipients treated with chemicals to induce estrus can have an improved pregnancy rate, compared with recipients with the natural estrus. This must be because recipient estrus can be precisely controlled with chemicals, and close synchronization between embryos and recipients will increase the chance of full term development embryos *in vivo*.

5 Conclusions

Herein, we have focused on the factors influencing the SCNT efficiency in pigs, and tried to improve the cloning efficiency of pigs at different stages of the cloning process from donor cell to recipient. Although our results cannot be simply extrapolated to other cloning laboratories, the approach used in this study may help to improve the cloning efficiency of pigs by (1) using donor cells at 90% confluence after 4 d of culture before freezing or just after thawing, (2) culturing the SCNT embryos to blastocysts before embryo transfer, (3) using BM fibroblasts as donor cells and LY as recipients, and (4) chemically synchronizing estrus in recipient pigs. However, further studies are needed to elucidate other factors affecting cloning efficiency in pigs.

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All applicable institutional and national guidelines for the care and use of animals were followed.

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