Factors Determining the Efficiency of Porcine Somatic Cell Nuclear Transfer: Data Analysis with Over 200,000 Reconstructed Embryos

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Abstract

Data analysis in somatic cell nuclear transfer (SCNT) research is usually limited to several hundreds or thousands of reconstructed embryos. Here, we report mass results obtained with an established and consistent porcine SCNT system (handmade cloning [HMC]). During the experimental period, 228,230 reconstructed embryos and 82,969 blastocysts were produced. After being transferred into 656 recipients, 1070 piglets were obtained. First, the effects of different types of donor cells, including fetal fibroblasts (FFs), adult fibroblasts (AFs), adult preadipocytes (APs), and adult blood mesenchymal (BM) cells, were investigated on the further *in vitro* and *in vivo* development. Compared to adult donor cells (AFs, APs, BM cells, respectively), FF cells resulted in a lower blastocyst/reconstructed embryo rate (30.38% vs. 37.94%, 34.65%, and 34.87%, respectively), but a higher overall efficiency on the number of piglets born alive per total blastocysts transferred (1.50% vs. 0.86%, 1.03%, and 0.91%, respectively) and a lower rate of developmental abnormalities (10.87% vs. 56.57%, 24.39%, and 51.85%, respectively). Second, recloning was performed with cloned adult fibroblasts (CAFs) and cloned fetal fibroblasts (CFFs). When CAFs were used as the nuclear donor, fewer developmental abnormalities and higher overall efficiency were observed compared to AFs (56.57% vs. 28.13% and 0.86% vs. 1.59%, respectively). However, CFFs had an opposite effect on these parameters when compared with CAFs (94.12% vs. 10.87% and 0.31% vs. 1.50%, respectively). Third, effects of genetic modification on the efficiency of SCNT were investigated with transgenic fetal fibroblasts (TFFs) and gene knockout fetal fibroblasts (KOFFs). Genetic modification of FFs increased developmental abnormalities (38.96% and 25.24% vs. 10.87% for KOFFs, TFFs, and FFs, respectively). KOFFs resulted in lower overall efficiency compared to TFFs and FFs (0.68% vs. 1.62% and 1.50%, respectively). In conclusion, this is the first report of large-scale analysis of porcine cell nuclear transfer that provides important data for potential industrialization of HMC technology.

Introduction

PRODUCTION OF TRANSGENIC DOMESTIC pigs for biomedical purposes offers unique possibilities for biomedical research and applications (Lind et al., 2007; Vajta et al., 2007). Due to similarities in organ size, physiology, metabolism, and genetics, the pig can be an alternative source of organs for xenotransplantation and a feasible model for studying various human diseases and pharmaceutical effects.

In spite of various alternative attempts, somatic cell nuclear transfer (SCNT) is the most efficient and reliable way for genetic modification in domestic animals. Since the first report of successful porcine SCNT in 2000 (Onishi et al., 2000), thousands of cloned pigs have been produced. However, the low efficiency and required sophisticated procedure decelerate advancement to exploit these possibilities. Compared to traditional cloning (TC), handmade cloning (HMC) is an alternative, simpler, and quicker procedure with comparable

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efficiencies (Du et al., 2007). The main feature of HMC is that the zona pellucida is removed prior to enucleation and fusion. The whole process can be performed under a normal stereomicroscope; therefore, an expensive micromanipulator is not needed, reducing the costs of laboratory equipment and highly skilled workforce for operation (Vajta, 2007). Also, standardization is easier, with the possibility for future automation. So far, HMC has been successfully established in cattle (Vajta et al., 2004), pig (Du et al., 2007), horse (Lagutina et al., 2007), goat (Nasr-Esfahani et al., 2011), sheep (Zhang et al., 2013), and water buffalo (Saha et al., 2013).

The donor cell type maybe one of the most crucial factors that affect the overall efficiency of cloning. Theoretically nuclei of less differentiated cell types, such as embryonic stem cells (ESCs), are easier to reprogram compared to those of terminally differentiated cell types (Rideout et al., 2000). Epigenetic reprogramming is crucial for the early development of the embryo, and the process is similar among various mammals like mouse, rat, pig, and cattle (Dean et al., 2001). In porcine preimplantation embryos, paternal pronuclei undergo active and rapid demethylation, whereas the maternal genome is passively demethylated during early cell cycles (Deshmukh et al., 2011). Subsequently cells undergo *de novo* remethylation during blastocyst formation and postimplantation development.

In cloned embryos, however, the genome undergoes incomplete epigenetic reprogramming (Blelloch et al., 2006; Bourc'his et al., 2001; Huan et al., 2015; Kang et al., 2001; Lee et al., 2006; Morgan et al., 2005; Santos et al., 2003), which is considered to be a potential contributor to the overall low cloning efficiency (Dean et al., 2001; Li et al., 2008; Peat and Reik, 2012). In recent studies, to correct or relieve the incomplete epigenetic reprogramming of cloned embryos, different cell types were used as the nuclear donor for pig SCNT, such as fetal fibroblasts (FFs; Onishi et al., 2000), preadipocytes (Tomii et al., 2005), adult mesenchymal stem cells (MSCs; Faast et al., 2006), recloned pig somatic cells (Cho et al., 2007), and induced pluripotent stem cells (iPSCs; Fan et al., 2013).

Until now, more than 200 types of cells were used as nuclei donor and resulted in live offspring (Vajta and Gjerris, 2006). However, in spite of these advances in extending donor cell types for pig cloning, few of these studies give us an explicit answer for which cell type could result in higher overall cloning efficiency. The experimental data sizes are usually limited by the scale of cloning activity. Although some reviews attempted to provide a large-scale scene of porcine SCNT research by summarizing the results of different laboratories, due to the variance of oocyte sources, chemicals, and embryo production systems, it is difficult to summarize data (Vajta et al., 2007) and draw a reliable conclusion.

During the last few years' work on pig cloning, 18 standard cloning stations were well established as well as a professional embryo transplantation theatre with up-to-date surgical equipment (BAB Huidong branch, Guangdong, China). Over 20 researchers performed porcine cloning three times a week recovering 200 to ~400 ovaries each time, from January, 2010, to December, 2013; 72,241 ovaries were collected and 228,230 cloned embryos were constructed with HMC. The overall blastocyst rate on day 6 was 36.68% (82,969/228,230). A total of 407 of 656 recipients became pregnant after transfer of 64,557 blastocysts. In all, 1070 piglets (with or without genetic modification) were delivered by 266 sur-

rogates, among which 813 piglets were born alive. The overall efficiency was 1.25% (piglets born alive per transferred embryos). To our knowledge, so far these are the largest data collection derived from a single laboratory with an established and consistent porcine SCNT system.

The purpose of this study is to investigate the effects of (1) donor cells resources on the *in vitro* and *in vivo* developmental competence of HMC embryos, (2) recloning on HMC efficiency, and (3) genetic modification on developmental competence of HMC embryos.

Materials and Methods

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA), except where otherwise indicated.

Ethics statement

Animal experimental procedures were approved by the Life Ethics and Biological Safety Review Committee of BGI-Research.

Primary cell lines establishment and donor cells preparation

First, to compare the effects of different donor cells on the *in vitro* and *in vivo* developmental competence of HMC embryos, we chose four commonly used cell types as donors for HMC, including FFs, adult fibroblasts (AFs), adult preadipocytes (APs), and adult blood mesenchymal (BM) cells. FFs were established as previously described (Kragh et al., 2005; Onishi et al., 2000; Zhao et al., 2009). Briefly, fetuses obtained 40 days after insemination were decapitated, eviscerated, and cut into small pieces with fine scissors in phosphate-buffered saline (PBS). Minced tissue pieces were digested with collagenase type IV (Gibco Life Technologies Inc., Paisley, UK) and cultured in Dulbecco's Minimum Essential Medium (DMEM; Gibco) supplemented with 15% (vol/vol) fetal bovine serum (FBS; Gibco), 1% nonessential amino acid (NEAA; Invitrogen, Carlsbad, CA, USA), and 1% L-glutamine (Invitrogen) for 4–5 h at 38°C in 5% CO₂ in air. The digested sample was flushed with cell culture medium and centrifuged at 500×g for 5 min to remove the residual enzyme. The cell pellets were resuspended in the cell culture medium and seeded in a 6-cm cell culture dish until confluent.

AFs were established as reported by Giraldo et al. (2012) with slight modification. Briefly, tissues 1 cm in diameter, were collected from the ears of adult pigs using a biopsy punch. The inner layers between the dermis of the ear samples were separated carefully with sterile tweezers and a scalpel after rinsed three to five times with PBS containing 1×penicillin/streptomycin (P/S). Then samples were transported to the laboratory in PBS containing 1×P/S at room temperature and cut into ~1-mm³ cubes with scissors and scalpels within 10 h. The cubes were placed into a 10-cm cell culture dish filled with high-glucose DMEM (H-DMEM; Invitrogen) supplemented with 15% (vol/vol) FBS (Gibco), 1% NEAA (Invitrogen), 1% L-glutamine (Invitrogen), and 1×P/S. The cells were cultured at 38°C in a humidified atmosphere of 5% CO₂ in air (vol/vol) until confluent.

BM cells were isolated from adult pig peripheral blood as reported by Faast et al. (2006). Briefly, 5–10 mL of whole blood was collected from adult pigs by auricular venipuncture

into heparin-contained tubes and centrifuged in a HistoPaque-1077 density gradient at $400\times g$ for 30 min. Then mononuclear cells were collected from the opaque interface and washed with PBS for three times. Subsequently, isolated cells were re-suspended in low-glucose DMEM (L-DMEM; Invitrogen) supplemented with 15% (vol/vol) FBS (Gibco), 1% NEAA (Invitrogen), 10 ng/ μ L recombinant human fibroblast growth factor basic (bFGF; Invitrogen), and 30 IU/mL heparin in a gelatin-coated dish (BD, Becton Dickinson, Lincoln Park, NJ, USA). Culture medium was replaced every 3 days until the monolayers became confluent.

APs were obtained as reported by Tomii et al. (2005). Fat tissue samples were collected from the anterior abdominal wall of pigs by surgery, washed with PBS three times, and cut into small pieces (approximate 2-mm³ cubes) with fine scissors and scalpels. Subsequently, the tissue mass was digested with 200 IU/mL collagenase type IV for 1 h at 38.5°C in a water bath and centrifuged at $300\times g$ for 10 min. Pellets were washed for 1 h with PBS and resuspended in L-DMEM supplemented with 10% (vol/vol) FBS. Cells were cultured under conditions as for AFs until confluent.

Second, to investigate the effects of recloning on the efficiency of SCNT, fibroblast cell lines were established from cloned fetal fibroblasts (CFFs) in the same way as FFs and cloned piglets [cloned adult fibroblasts (CAFs)] from cloned pigs in the same way as AFs. Both of them were used as nuclear donors. The tissues from which the cells originated could impact the efficiency of SCNT. We only compared the *in vitro* and *in vivo* developmental competence of HMC embryos between the same cell types (FFs vs. CFFs, AFs vs. CAFs).

Third, the effects of genetic modification on the development of SCNT embryo were investigated by inserting a gene into the FFs genome [transgenic fetal fibroblasts (TFFs)] or knocking a endogenous gene out of FFs genome [gene knockout fetal fibroblasts, (KOFFs)]. The *in vitro* and *in vivo* developmental competence of HMC embryos was compared. TFFs and KOFFs were established as previously described (Lai et al., 2002, 2006; Liu et al., 2013a). Briefly, plasmids containing the target DNA fragments were transfected into FFs via liposome or electroporation. Subsequently, cells were selected by 500 μ g/mL Geneticin (G418, Gibco) in DMEM supplemented with 15% (vol/vol) FBS, 1% NEAA, and 1% L-glutamine until the positive cell clones were selected and used as nuclear donors for HMC. The transgenes were *BMP-15*, *BMPR-IB*, *FSH $\alpha\beta$* , *DGAT*, *PPAR γ* , *PGC-1 α* , *eGFP*, *Antimorphic Human Cryptochrome 1*, and *nematode fat-1*. The knockout (KO) genes included *MSTN* and *GHR*.

Cells between passages 3–10 (total passage) were used as nuclear transfer donors. Confluent monolayers were harvested by digestion with 0.05% (wt/vol) trypsin-EDTA (Gibco). The cell suspension was stored at room temperature for 0.5–1 h before fusion.

Oocyte maturation

Slaughterhouse-derived sow ovaries were collected in physiological saline solution and transported back to laboratory within 4 h at 32°C. Cumulus–oocyte complexes (COCs) were recovered from 3- to 5-mm follicles. The compact COCs were selected in groups of 50. Each group was matured in 400 μ L of bicarbonate buffered Tissue Culture Medium-199 (TCM-199; Gibco) supplemented with 10% (vol/vol) pig follicular fluid,

10% cattle serum (CS; Gibco), 5 IU/mL human chorionic gonadotropin (hCG), and 10 IU/mL equine chorionic gonadotropin (eCG) at 38.5°C in humidified atmosphere of 5% (vol/vol) CO₂ for 41–43 h. Subsequently, COCs were treated with 1 mg/mL hyaluronidase dissolved in HEPES-buffered TCM-199 to remove the cumulus cells. For the present study, 72,241 ovaries were collected and 1,111,400 COCs were incubated.

Handmade cloning

HMC was performed as previously described (Du et al., 2007). Briefly, the zona pellucidae of oocytes were partially digested by 3 mg/mL Pronase dissolved in T33 [T is HEPES-buffered TCM-199 medium; the number is the percentage (vol/vol) of calf serum supplementation]. Enucleation was performed with a microblade (AB Technology, Pullman, WA, USA) under a stereomicroscope. The cytoplasm were washed in T2 and T20 drops, and collected in a T10 drop.

Fusion was performed in two steps where the second one included the initiation of activation. For the first step, each cytoplasm was transferred to 20 μ L of T0 containing 1 mg/mL of phytohemagglutinin (PHA; ICN Pharmaceuticals, Australia) for 1–3 sec, and subsequently attached to a single donor cell. Then cytoplasm–fibroblast pairs were fused with a single 100 V direct current (DC) impulse of 2.0 kV/cm for 9 μ sec (BLS CF-150/B fusion machine, Budapest, Hungary) in fusion medium [0.3 M mannitol and 0.01% polyvinyl alcohol (PVA)]. About 1 h later, each fused pair was fused with another putative cytoplasm in activation medium [0.3 M mannitol, 0.1 mM MgSO₄, 0.1 mM CaCl₂, and 0.01% PVA (wt/vol)] with a single DC pulse of 0.86 kV/cm for 80 μ sec. Reconstructed embryos were treated with 10 mg/mL cycloheximide and 5 mg/mL cytochalasin B dissolved in PZM-3 at 38.5°C in 5% CO₂, 5% O₂ for 4–6 h. A total of 228,230 embryos were reconstructed for the study.

Embryo culture and transfer

Zona-free embryos produced from HMC were cultured in groups of 2–30 in 400 μ L of PZM-3 supplemented with 4 mg/mL bovine serum albumin (BSA) in a modified well-of-the-well (WOW) system (Vajta et al., 2000). Day 5 or day 6 blastocysts produced from HMC were surgically transferred to the uterine horns of a surrogate. Pregnancies were diagnosed by B ultrasound scanning on day 28 and monitored every 2 weeks afterward.

Statistical analysis

All data were analyzed by SPSS 10.0. A probability of $p < 0.05$ was regarded as significant. The chi-squared test was used to analyze pregnancy, farrowing, developmental, survival, developmental abnormalities (deformities, mummies, and stillbirths), and efficiency rates. Day 6 blastocyst rates and mean piglets born/litter were evaluated by an independent Student *t*-test, and results are expressed as the mean $\pm$ standard error of mean (SEM).

Results

Comparison of the *in vitro* and *in vivo* development of embryos derived from AFs, APs, BMs, and FFs

Compared to adult donor cells (AFs, APs, and BM cells, respectively), FFs resulted in a lower blastocyst rate

(30.38% vs. 37.94%, 34.65% and 34.87%, respectively; $p < 0.05$). No difference in blastocyst rate was found between AFs, APs, and BM cells (Fig. 1A). The pregnancy rate with BM cells was lower than with APs and FFs (43.00% vs. 76.00% and 74.19%, respectively; $p < 0.05$), whereas there was no difference between AFs, APs, and FFs (Fig. 1B). A higher live birth rate was obtained with FFs and APs when compared with AFs and BMs (91.30% and 82.93% vs. 66.67% and 66.67%, respectively; $p < 0.05$). No differences in live birth rates were found between AFs and BM cells or APs and FFs, respectively (Fig. 1F). When APs and FFs were used as donor cells, the ratio of developmental abnormalities (deformities, mummies, and stillbirths) was lower than those with AFs and BM cells (56.57% and 51.85% vs. 24.39% and 10.87%, respectively; $p < 0.05$ for both), but there was no difference between AFs and BMs or APs and FFs (Fig. 1G). In comparison to AFs, higher overall efficiency was obtained with FFs (1.50% vs. 0.86%, respectively; $p < 0.01$). However, no difference in overall efficiency was observed between AFs, APs, and BMs, or APs, BMs, and FFs (Table 1).

Comparison of the in vitro and in vivo development of embryos derived from AFs and CAFs

No difference was observed in blastocyst, pregnancy, farrowing, and live birth rates as well as litter sizes between AFs and CAFs (37.94% vs. 35.25% and 57.86% vs. 52.94% and 58.70% vs. 77.78% and 66.67% vs. 71.87% and 3.67% vs. 4.57, respectively; $p > 0.05$ for all) (Fig. 1 A–F). Compared to CAFs, AFs resulted in a lower piglet/transferred blastocyst rate (1.29% vs. 2.21%, respectively; $p < 0.01$) (Fig. 1D), but a higher ratio of developmental abnormalities (56.57% vs. 28.13%, respectively; $p < 0.01$) (Fig. 1G). The overall efficiency obtained with CAFs was higher than that obtained with AFs (1.59% vs. 0.89%; respectively; $p < 0.01$) (Table 1).

Comparison of the in vitro and in vivo development of embryos derived from FFs and CFFs

Similar blastocyst, pregnancy, farrowing, and developmental abnormalities rates as well as litter sizes were obtained with FFs and CFFs (30.38% vs. 28.22% and 74.19% vs. 72.73% and 73.91% vs. 75.00% and 1.64% vs. 1.75% and 2.71% vs. 2.83, respectively; $p > 0.05$ for all) (Fig. 1 A–E). However, when CFFs were used, the rate of developmental abnormalities was higher and live birth rate lower than those obtained with FFs (10.87% vs. 94.12% and 91.30% vs. 27.65%, respectively; $p < 0.001$ for both) (Fig. 1F, G). Accordingly, CFFs resulted in lower overall efficiency when compared with FFs (0.31% vs. 1.50%; $p < 0.001$) (Table 1).

Comparison of the in vitro and in vivo development of embryos derived from TFFs, KOFFs, and FFs

KOFFs resulted in a lower blastocyst rate than that obtained with TFFs (33.77% vs. 37.02%, $p < 0.05$), but higher than that obtained with FFs (33.77% vs. 30.38%, $p < 0.05$) (Fig. 1A). There was no difference in pregnancy (63.99% vs. 57.53% vs. 74.19%, $p > 0.05$ for all) (Fig. 1B) and farrowing rates (69.85% vs. 54.76% vs. 73.91%, $p > 0.05$ for all) (Fig. 1C) among TFFs, KOFFs, and FFs. Similar litter sizes were observed between FFs and KOFFs, but both were

lower than that achieved with TFFs (2.71% and 3.35% vs. 4.50%, $p < 0.01$ for FFs vs. TFFs and $p < 0.05$ for KOFFs vs. TFFs) (Fig. 1E). Compared to FFs and TFFs, KOFFs resulted in a lower developmental rate (1.07% vs. 1.64% and 1.99%, $p < 0.05$ for KOFFs vs. FFs and $p < 0.01$ for KOFFs vs. TFFs) (Fig. 1D), lower live birth rate (63.64% vs. 91.30% and 81.31%, $p < 0.001$ for all) (Fig. 1F), and lower overall efficiency (0.68% vs. 1.50% and 1.62%, $p < 0.001$ for all) (Table 1), whereas no difference was observed between FFs and TFFs. The rate of developmental abnormalities obtained with TFFs was higher than that obtained with FFs (25.24% vs. 10.87%, $p < 0.05$), but lower than that obtained with KOFFs (25.24% vs. 38.96%, $p < 0.01$) (Fig. 1G).

Discussion

In the present study, the effects of donor cells on *in vitro* and *in vivo* development of embryos produced by SCNT were investigated. Factors including cell origins, recloning, and genetic modification significantly affected the cloning efficiency.

Three of the donor cell lines were derived from adult pigs (APs, BMs, AFs) and one was from a fetus (FFs). All of them were used for porcine HMC between passages 3 and 10. Compared to adult-derived donor cells, FFs resulted in a lower blastocyst rate. Our results were in agreement with the observation of Tomii et al. (2005), in which the blastocyst rate of AP-derived embryos was slightly higher than that of FF-derived embryos. However, it was reported by different laboratories that FFs were superior to adult-derived donor cells. Lee et al. (2003) reported that more blastocysts were derived from SCNT of FFs than from that of other cells (AFs, cumulus cells, and oviduct cells). Yin et al. (2002) reported that, by using FFs, the blastocyst rate was higher than those obtained with adult cells of various origin (cumulus, liver, heart, kidney, muscle, oviduct). The difference may be due mainly to different types of adult-derived donor cells used in our study and those papers. APs and BMs in the present study were two adult-derived donor cells that have been demonstrated to be multipotent by different reports (Faast et al., 2006; Tomii et al., 2005).

Adult-derived donor cells used in the referred studies were fully differentiated cells (AFs, cumulus, liver, heart, kidney, muscle, or oviduct epithelial cells). Another possible reason might be the different technique used for SNCT. In the present study, HMC was used, whereas micromanipulator-dependent TC was applied in all referenced publications. Different techniques could be the reason for the observed difference between our results and those of Lee et al. (2003) comparing the *in vitro* development of cloned embryos reconstructed with AFs versus FFs.

It should be emphasized that the even the lowest blastocyst rate obtained with HMC by us was still higher than all those reported with TC in above publications. We used two enucleated oocytes to create one embryo: The 150% volume used in HMC may support reprogramming and development better than the 70–80% used in TC. The efficiency of the individual steps (enucleation, fusion) is higher than that usually described for TC. Shorter time for manipulation in HMC than in TC could lessen the damage caused by exposure to suboptimal conditions. Those beneficial steps may result in better quality of the surviving cytoplasts and embryos with less damage. Although the zona-free situation

FIG. 1. Effects of donor cell type and gene modification on the *in vitro* and *in vivo* developmental competence of HMC embryos. (A) Blastocyst rate, mean $\pm$ SEM. (B) Pregnancy rate, number of pregnant recipients/number of recipients (%). (C) Farrowing rate, number of farrowed recipients/number of pregnant recipients (%). (D) Developmental rate, number of piglets born/number of total transferred blastocysts (%). (E) Litter size, mean $\pm$ SEM. (F) Live birth rate, number of piglets born alive/number of total piglets (%). (G) Abnormality rate, number of deformities and mummies and still births/number of total piglets (%). (H) Efficiency, number of piglets born alive/number of total transferred blastocysts (%). Within the same column, values with different superscript letters (a, b, c, d, e, and f) were significantly different ($p < 0.05$).

TABLE 1. EFFECT OF DONOR CELL TYPE AND GENE MODIFICATION ON THE OVERALL EFFICIENCY OF HANDMADE CLONING

Donor cell	No. of reconstructed embryos	No. of transferred blastocysts	No. of recipients	No. of pregnant recipients	No. of farrowed recipients	Litter size	No. of piglets	No. of piglets born alive	No. of abnormalities	Efficiency (%)
FF	14897	2799	31	23	17	1–5	46	42	5	1.50 ^a
AF	54892	15402	159	92	54	1–12	198	132	112	0.86 ^{b,c}
AP	12263	3304	33	25	13	1–7	41	34	10	1.03 ^{a,b}
BM	7617	1984	21	9	7	2–6	27	18	14	0.91 ^{a,b,c}
CAF	5279	1450	17	9	7	1–8	32	23	9	1.59 ^a
CFF	4322	970	11	8	6	1–4	17	3	16	0.31 ^c
TFF	104312	31456	311	199	139	1–13	626	509	158	1.62 ^a
KOFF	24648	7192	73	42	23	1–10	77	49	30	0.68 ^{b,c}

Efficiency was calculated by piglets born alive/transferred blastocyst; within the same column, values with different superscript letters (a, b, and c) were significantly different ($p < 0.05$).

FF, fetal fibroblast; AF, adult fibroblast; AP, adult preadipocyte; BM, adult blood mesenchymal cell; CAF, cloned adult fibroblast; CFF, cloned fetal fibroblast; TFF, transgenic fetal fibroblast; KOFF, gene knockout fetal fibroblast.

is generally regarded as a disadvantage, there are some potential benefits as well. With the proper culture system and WOW (Vajta et al., 2000), *in vitro* development may even be higher. Moreover, during *in vitro* culture, the zona becomes compromised regarding transport function and mechanical features, with a potential effect on metabolism and a definite effect on hatching (Vajta et al., 2011). The virtual slot at TC (a hole in zona resulted from TC) may not be enough to eliminate these problems. However, we have no specific evidence about the possible role of individual factors in the differences found between our results and those of others with TC.

Although lower *in vitro* developmental competence was observed, embryos reconstructed with FFs showed higher *in vivo* developmental competence that was evaluated by pregnancy, farrowing, live birth, and subsequent developmental rates as well as rates of developmental abnormalities when compared to donor cell lines derived from adult pigs. According to our observations, piglets derived from FFs were healthier and with less abnormalities than those from adult donor cells.

Most previous publications failed to investigate the *in vivo* development or data collected were insufficient for statistical calculations (Faast et al., 2006; Tomii et al., 2005; Uhm et al., 2000; Yin et al., 2002). Our results confirmed that the capacity of nucleus to be reprogrammed cannot be evaluated exclusively by *in vitro* development; in contrast, *in vivo* development may be a more important characteristic.

The present study confirmed that FFs are one of the best candidates for pig SCNT. However, establishment of FFs requires sacrificing a fetus, and FFs are not available for cloning an existing adult individual. Thus, adult-derived donor cells need to be established for SCNT in some cases. According to our results, APs might be a good choice for SCNT among adult donor cells, because this approach has resulted in comparable live birth rates to those achieved with of FFs and a lower rate of developmental abnormalities than those seen after cloning with AFs and BMs.

Recloning has been performed successfully in many mammals, including mice, bull, pig, cat, and dog (Cho et al., 2007; Kubota et al., 2004; Oh et al., 2011; Wakayama et al., 2000; Yin et al., 2008). However, whether serial cloning would increase reprogramming efficiency or just lead to the accumulation of abnormalities that prevent successful serial recloning

over many generations has been a longstanding question. The answers from different publications are often contradicting. There are some reports that serial somatic cell cloning could be performed without compromising production efficiency (Kurome et al., 2008; Wakayama et al., 2013) or even improved the developmental competence of SCNT embryos (Fujimura et al., 2008; Zakhartchenko et al., 1999). However, contradicting results were also reported that increasing the number of serial clonings would decrease efficiency (Cho et al., 2007; Kubota et al., 2004; Wakayama et al., 2000).

One of the possible reasons for opposite conclusions in previous studies is the variation of donor cells types between recloning generations in the same research group and between different groups. In previous reports, genetically modified cell lines, FFs, AFs, and salivary gland progenitor cells were used as donor cells, and the types of donor cells varied between generations. In our study, the same cell types between offspring generations were used to evaluate the effects of recloning on SCNT (CAFs vs. AFs, CFFs vs. FFs). Compared to AFs (offspring regarded as generation 0), CAFs (generation 1) resulted in a lower rate of developmental abnormalities and higher overall efficiency. On the other hand, when using FFs (offspring regarded as generation 0) and CFFs (generation 1), CFF-derived embryos had lower developmental competence with a higher rate of developmental abnormalities and lower overall efficiency. The reason why the efficiency of serial cloning was increased by using CAFs as donor cells but decreased by CFFs is still unclear. Further studies are required to investigate the underlying mechanism.

TFFs were usually established by introducing one or more DNA fragments into the FF genome, whereas KOFFs had a knock out of an endogenous gene that usually inactivated the corresponding functional protein. In the past 20 years, many genetically modified pigs have been obtained with TFFs or KOFFs (Kragh et al., 2009; Lai et al., 2002; Li et al., 2014; Liu et al., 2013b; Zhang et al., 2012). However, most of these studies focused on the phenotype of cloned pigs caused by gene overexpression or silencing. Few of them investigated the developmental competence of cloned embryos after gene insertion or deletion. Reasons may include the lack of a large database for comparison or the focus was only to study the function of the gene of interested and the future application of gene-modified pigs.

In the present study, both TFFs and KOFFs were used as donor cells to investigate the effects of the genetic modification of donor cell genomes on the development of SCNT embryos. Overexpressed genes in this study could be divided into three categories according to their functions: (1) fertility-related genes (*FSH α* , *BMP-15*, *BMPL-1B*); (2) lipometabolism-related genes (*DGAT*, *PPAR γ* , *PGC-1 α*); and (3) exogenous genes (*Antimorphic Human Cryptochrome 1*, *Nematode fat-1 gene*, *eGFP*). Because it was more difficult to delete a gene than to insert one before the introduction of transcription activator-like effector nucleases (TALENs) and CRISPR/Cas9 technology (Cho et al., 2013; Sander et al., 2011), we had only two KO genetic modifications including genes *MSTN* and *GHR* that were related to carbohydrate metabolism and body development. In spite of this, there were still over 7000 gene-KO blastocysts implanted into 73 recipients.

Compared to FFs, both TFFs and KOFFs resulted in higher blastocyst rates. Because the genes we inserted were not specifically designed to support reprogramming, screening and selection of gene-modified cell clones before HMC may be a possibility for improving the early development of reconstructed embryos. The selection procedure, which usually lasts for 10–15 days, may eliminate weak cells. However, higher rates of developmental abnormalities were obtained with both TFFs and KOFFs in comparison with FFs. The exogenous genes were inserted into the cell genome randomly; thus, there was a possibility that some endogenous genes were disrupted by the inserted DNA fragments, which may have influenced the *in vivo* development of SCNT embryos and led to more abnormalities (Schmidt et al., 2014). In addition, the effects of the genes could also be a reason.

To relieve the problem resulting from the locus, we suggest introducing the desired gene into a constitutive and ubiquitous gene expression site of the genome, such as the *ROSA26* locus, in which the inserted DNA fragments will not disturb the structure of other endogenous genes. Similarly, deletion of certain genes in KOFFs may disrupt the relative signaling pathway. For example, *GHR*-KO would disrupt normal carbohydrate metabolism, glucose homeostasis, and insulin action (Liu and Andrzej, 2014; Zhou et al., 1997), resulting in higher perinatal mortality compared to wild-type mice (Zhou et al., 1997).

Deletion of genes that are critical for embryonic development could even be lethal. Approximately 15% of KO mice have mutations that lead to death (National Human Genome Research Institute, www.genome.gov/12514551). Although some genetic functions in mammals are redundant and the targeted loss of genes sometimes was reported to have no effect (Joyner et al., 1991), absence of a gene may also impact expression of other genes (Fu et al., 2014). Compared to KOFFs, more live piglets could be obtained with lower incidence of abnormalities. That suggests that insertion of an exogenous gene may be more tolerable than silencing an endogenous one by an organism. Not all the functional deficiencies of endogenous genes can be compensated for by other genes, whereas transgene silencing is a prevalent problem, especially when genes integrate as multicopy transgene arrays (Garlick et al., 1998; McBurney et al., 2002; Muskens et al., 2000). Although having low efficiency, the live offspring resulting from KO can be bred with traditional reproduction. With methods such as adding epigenetic modification

inhibiting factor or *Xenopus* egg extracts, choosing a more efficient KO system, and shortening the time of screening donor cells, the low efficiency could be elevated. Also intense care could also improve piglet viability. Detailed analysis of the outcome of these genetic modifications in the present study has been/will be the subject of other publications (Li et al., 2014; Liu et al., 2013a; Zhang et al., 2011, 2012).

In conclusion, our study, which is based on more than 200,000 porcine somatic cell embryos from in a single laboratory, confirmed that FFs were the best donor cells. The developmental competence of SCNT embryos was increased with CAFs as donor cells and decreased with CFFs. Genetic modification increases the developmental abnormalities, and this effect was more marked with KOFFs. The present study also provides important data for the potential industrialization of the HMC technology used here and useful information for improving porcine SCNT efficiency.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

T.B.L., H.W.D., and X.X. conceived and designed the comparisons; T.B.L., H.W.D., Y.L., Y.C., X.L., and J.X.Z. performed the experiments; T.B.L., L.L., X.X., and H.W. analyzed the data; Z.Z.Y., W.X.Y., H.M.Y., L.B., and Y.D. contributed reagents, materials, and analysis tools; X.Z.P., L.L., J.L., Z.F.W., and Y.X. cloned the animals; T.B.L., X.X., and G.V. wrote the paper; T.B.L., L.L., Y.J.Z., and G.V., Y.D., and L.L. helped finalize the manuscript.

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