

Increasing the Number of Transferred Embryos Results in Delivery of Viable Transgenically Cloned Guangxi Bama Mini-pigs

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XIANGXING ZHU^{1,2,3}, SHOUNENG QUAN^{1,2,3#}, YONGLIAN ZENG^{1,4##}, JUNYU NIE^{1,2,3}, HUIYAN XU^{1,2,3}, XIAOGAN YANG^{1,2,3}, YANGQING LU^{1,2,3}, KEHUAN LU^{1,2,3}, SHENGSHENG LU^{1,2,3*}

¹State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources; ²Guangxi High Education Key Laboratory for Animal Reproduction and Biotechnology; ³College of Animal Science and Technology; ⁴College of Life Science and Technology; Guangxi University, Nanning 530004, China; [#]Present address: Assisted Reproductive Centre, People's Hospital, Guigang 537100, Guangxi, China; ^{##}Present address: Guangxi Key Laboratory of Molecular Medicine in Liver Injury and Repair, Guilin Medical University, Guilin 541004, Guangxi, China.

*Corresponding author: S.S. Lu (E-mail: shengshenglu@sina.com).

Abstract

The somatic cell nuclear transfer (SCNT) technique is the most popular method for producing genetically modified pigs, but its efficiency depends upon a number of factors. In the present study, we attempted to generate transgenically cloned Guangxi Bama mini-pigs--which possess great potential for modeling human diseases--through genetic modification of fibroblasts, followed by SCNT and embryo transfer (ET). However, the first batch of five recipients transferred achieved a low number of cloned embryos (148.2 ± 13.0) and failed to become pregnant. We then changed the ET strategy (significantly increasing the number of transferred embryos [greater than 200, $n = 7$]), resulting in the delivery of three litters and a total of sixteen live-born cloned Guangxi Bama mini-pigs in which the integrated transgene was confirmed by genotyping. Thus, such a practical strategy can now be employed in our future work to create transgenic Guangxi Bama mini-pigs more efficiently.

Keywords: Guangxi Bama-mini pig; Genetic modification; Somatic cell nuclear transfer (SCNT); Embryo transfer (ET); Cloning.

1. Introduction

Guangxi Bama mini-pigs are a unique local miniature swine species with a very long breeding history in Bama County, China (LIU & al. [1]; [2]; [3]), and are characterized by small body size, with an adult body mass of approximately 50~60 kg. Moreover, the majority of their body surfaces are covered with white skin, whereas their heads and haunches are generally black (as is shown in Figs. 1B~F). This unique "top-end black" appearance makes them significantly different from other miniature swine species in China, such as the Wuzhishan and Tibetan mini-pigs, which are generally black over their entire bodies (FANG & al. [4]). Their small body size results in significant breeding and handling advantages (SMITH & SWINDLE [5]); and their white skin may facilitate experimental manipulations, e.g., blood sampling and skin transplantation. Therefore, we hypothesize that Guangxi Bama mini-pigs can serve as a novel preclinical large-animal model for investigating human diseases. Although pigs in general are widely used as models in biomedical research, their value in modeling human diseases could be significantly enhanced by genetic modification (AIGNER & al. [6]; WHYTE & PRATHER [7]; NAGASHIMA & al. [8]; PRATHER & al.

[9]). Somatic cell nuclear transfer (SCNT), also called classical “cloning”, is considered to be the most popular method for producing genetically modified pigs since the beginning of the new millennium. Using this method, a large number of genetically modified pigs have been generated and used widely in agriculture and biomedical research (AIGNER & al. [6]; WHYTE & PRATHER [7]; NAGASHIMA & al. [8]; PRATHER & al. [9]). Our laboratory has focused on cloning and genetically modifying Guangxi Bama mini-pigs for several years, and, to date, we have generated cloned (LIU & al. [1]) and transgenically cloned (LIU & al. [3]) Guangxi Bama mini-pig embryos as well as live-born cloned Guangxi Bama mini-pigs (LIU & al. [2]); however, the delivery of viable transgenic Guangxi Bama mini-pigs has not yet been achieved. Several key factors influencing the production efficiency of genetically modified pigs via SCNT have been investigated by several groups, and a number of approaches for improving efficiency have already been provided. These include optimizing the SCNT procedure (MIYOSHI & al. [10]; WHITWORTH & al. [11]), in-vitro embryonic culture (YOSHIOKA & al. [12]; DANG-NGUYEN & al. [13]; YOSHIOKA [14]), and genetic modification methodology (NAKAYAMA & al. [15]; RICHTER & al. [16]); as well as selecting the most suitable donor cell types (RICHTER & al. [16]; HAO & al. [17]; LEE & al. [18]). And in accordance with these effective approaches, we have now modified our laboratory protocols. In this rapid communication, therefore, we report for the first time our generation of viable transgenically cloned Guangxi Bama mini-pigs through a practical strategy where we significantly increased the number of transferred embryos.

2. Materials and Methods

2.1. Animals, reagents and medium

All animal protocols used in this study complied with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Guangxi University, China. Unless otherwise stated, all organic and inorganic reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All self-made media and solutions were filtered using a 0.22- μ m filter (Millipore, Bedford, MA, USA), followed by storage at 4 or -20°C.

2.2. Preparation of transgenic fibroblasts

A recombinant plasmid referred to as hGFAP-DsRed (a generous gift from Dr. Lang Zhuo at Guangxi Medicinal Herb Garden, Nanning, China; its schematic representation is shown in Fig. 1G) was used for introducing astrocyte-specific expression of a DsRed reporter gene under the control of a 2.2-kb human glial fibrillary acidic protein (GFAP) promoter (DING & al. [19]). The procedures used for preparation of transgenic Guangxi Bama mini-pig fibroblasts were based on those described previously (ZHU & al. [20]). Briefly, thawed fibroblasts were seeded onto one well of a 6-well cell culture cluster (NUNC, Shanghai, China), and cultured in antibiotic-free Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 15% (v/v) newborn cattle serum (NBGS; Gibco) until reaching almost 50% confluence. We replaced the medium with fresh cell culture medium, and then added the polyplexes prepared with 1.5 μ L Xfect transfection reagent (Clontech, Palo Alto, USA) plus 5 μ g plasmid DNA, and incubated these overnight at 37°C. Polyplexes were then removed and fresh cell culture medium (supplemented with antibiotics) added. At 24 h post-transfection, cells were split into two 6-well cell culture clusters (NUNC) and cultured for 24 h, and 300 μ g/mL Zeocin (Invitrogen, Grand Island, NY, USA) was added for screening for 7 days. The surviving cell colonies were then picked up and seeded onto a 12-well cell culture cluster (NUNC) in fresh medium with 150 μ g/mL Zeocin. When the

cells achieved confluence, one third of the cells were genotyped for the presence of the hGFAP-DsRed fragment, and the rest were plated to expand the cell population and then frozen in liquid nitrogen. In order to prepare nuclear transfer donor cells, positive transgenic fibroblasts were thawed and cultured for 2 days, following synchronization by serum starvation (DMEM supplemented with 0.5% NBCS) for 48 h. The cells were then harvested and re-suspended with 1 mL micromanipulation medium (20 mM HEPES-buffered TCM-199 containing 0.3% [w/v] bovine serum albumin [BSA]), supplemented with 7.5 µg/mL cytochalasin B. This cell suspension was maintained at room temperature and used as nuclear transfer donor cells.

2.3. SCNT and embryo transfer (ET)

In vitro matured porcine oocytes were used as nuclear transfer recipients and prepared according to methods described previously (LIU & al. [1]; [2]; [3]). Briefly, cumulus-oocyte complexes (COCs) were aspirated from the follicles, and washed twice in PVA-TL-HEPES medium. The COCs were transferred into 200 µl drops of preheated maturation medium (bicarbonate-buffered TCM-199 supplemented with 0.1% [w/v] polyvinyl alcohol [PVA], 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 10 ng/mL epidermal growth factor [EGF], 0.5 µg/mL follicle-stimulating hormone [FSH], 0.5 µg/mL luteinizing hormone [LH], 0.0750 g/L penicillin G, 0.0500 g/L streptomycin and 10% [v/v] porcine follicular fluid [PFF]), covered with mineral oil, and then incubated for 20~22 h at 38.5°C in a humidified atmosphere of 5% (v/v) CO₂ in air. Then, the COCs were cultured for an additional 20 h in the same medium without the gonadotropins. Following maturation, expanded cumulus cells were removed from the oocytes by vigorous pipetting in the presence of 0.1% (w/v) hyaluronidase. Oocytes with an evenly granulated ooplasm and an extruded first polar body were selected and placed into the micromanipulation medium drop (containing donor cells and 7.5 µg/mL cytochalasin B) on a 60-mm cell culture dish (NUNC) covered with mineral oil. SCNT was performed as described previously (LIU & al. [3]) with minor modifications. Briefly, matured oocytes were enucleated by aspirating the first polar body plus a portion of the adjacent cytoplasm (presumably containing the metaphase II plate) by using a sharp-beveled glass pipette (WPI, Sarasota, Florida, USA) with a diameter of 20-25 µm. After enucleation, a donor cell was injected into the perivitelline space with care taken to maximize the amount of cell membrane contact between the donor cell and the oocyte. The fusion and activation of nuclear transferred embryos were performed simultaneously by using electrical pulses (2 successive DC pulses of 1.2 kV/cm for 30 µs; BTX2001, BTX Inc., San Diego, CA, USA) in a fusion medium (0.3 M mannitol, 1.0 mM CaCl₂•2H₂O, 0.1 mM MgCl₂•2H₂O, 0.5 mM HEPES plus 0.3% [w/v] BSA). After fusion and activation, reconstructed embryos were placed into PZM-3 containing 0.3% (w/v) BSA and cultured at 38.5°C in a humidified atmosphere of 5% (v/v) CO₂ in air. Fusion was checked 40~60 min later, and fused embryos were cultured until ET. For ET, several hundred cloned embryos were cultured 0-1 days *in vitro*, and then surgically transferred into the oviductal ampullary-isthmic junction of surrogates exhibiting natural estrus (within one day of the onset of estrus). Pregnancy was diagnosed, and surrogates were delivered by natural parturition on Day 114 to 116 of gestation (SCNT was performed on Day 0).

2.4. Genotyping

Genomic DNA was extracted from Zeocin-resistant fibroblasts and tail biopsies from newborn cloned piglets using a TIANamp Genomic DNA Kit (TIANGEN, Beijing, China). PCR reactions were conducted with 2 µL of genomic DNA, 1 µL forward primer (10 mM),

1 μ L reverse primer (10 mM), 10 μ L Premix Ex Taq™ Version 2.0 (TaKaRa, Dalian, China), and was added deionized water to a total volume of 20 μ L. Two pair of primers were designed to detect the presence of exogenous DsRed (F, ATCAAGGAGTTCATGCGCTT; R, CAACTAGAAGGCACAGTCGA) and endogenous porcine glyceraldehyde-phosphate dehydrogenase (GAPDH; F, TCTGCATCAGTGCTCCTTGA; R, AAGAGGTGATGAAGCTCCGA) fragments, resulting in 720-bp and 650-bp amplicons, respectively. PCR amplification conditions were as follows: one cycle at 95°C for 5 min; 35 cycles at 94°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec; followed by 72°C for 10 min. The PCR products were examined using 2% (w/v) agarose gel electrophoresis containing 0.01% (v/v) Andy Gold™ Nucleic Acid Gel Stain (Applied BioProbes, Davis, CA, USA). The bands were captured using a SYNGENE G:BOX (Syngene, Frederick, MD, USA) equipped with GeneSnap imaging software. Plasmid and genomic DNA from the surrogate sow were used as a positive and negative control, respectively. For Southern blotting, about 20 μ g of high molecular-weight genomic DNA isolated from the tissues of each PCR-positive cloned piglet was used. Genomic DNA was digested with AflIII and NotI restriction endonuclease (Takara, Dalian, China) at 37°C overnight and separated using 0.7% (w/v) agarose gel electrophoresis, followed by blotting onto a positively charged nylon membrane (Roche) using a capillary bridge. The spotted membrane was then hybridized with a digoxin-labeled probe, which was produced using a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany), targeting the DsRed sequence (marked in Fig. 1G), at 37°C overnight. After extensive washing, the hybridized membrane was detected by exposure to a piece of X-ray film (Kodak, USA) according to the manufacturer's instructions. Wild-type sample and linearized plasmid were used as a negative and positive control, respectively. Targeted bands (2640 bp) were captured and processed using Adobe Photoshop software (Adobe Inc., San Jose, USA).

2.5. Statistical Analysis

Cloning efficiency of transgenic Guangxi Bama mini-pigs expressed as percentages was analyzed by χ^2 test using SPSS 18.0 software (SPSS Inc., Chicago, Illinois, USA). Statistical significance was determined when a *P* value was less than 0.05.

3. Results

Transgenic kidney fibroblasts from Guangxi Bama mini-pigs were established via Xfect transfection reagent (data not shown), and used as donor cells to produce SCNT embryos. First, a total of 741 constructed embryos were surgically transferred into the oviducts of five synchronized recipients (148.2 ± 13.0 embryos per recipient; mean \pm standard deviation), but all recipients returned to estrus within 40 days (Table 1). We then changed the ET strategy so as to significantly increase the number of transferred embryos per recipient to more than 200, with a maximum of 400. With this new strategy, a total of 2,110 constructed embryos were transferred to seven recipients, with five recipients becoming pregnant. One litter of three fetal piglets from a recipient aborted at day 68 of gestation (Fig. 1A), while the remaining four recipients ultimately gave birth to 19 cloned piglets, including three cases of stillbirth (Fig. 1B~D). Overall cloning efficiency was approximately 0.9% (19/2110). The ET data from the second batch of seven recipients were separated into two groups: a group with 200~300 embryos and a group with more than 300 embryos. As is shown in Table 1, the cloning efficiency of the 200~300 embryo group (245.0 ± 22.1 , $n = 3$) was slightly higher than the latter group (343.8 ± 54.7 , $n = 4$; 0.95% vs. 0.87%), but this difference was not

statistically significant ($P > 0.05$). Four cloned piglets died several days after birth due to low body weight and poor vitality, whereas the remaining piglets were clinically healthy and developed normally (Fig. 1E,F). The exogenous transgene fragments that we expected to be integrated into the genome of these live-born cloned piglets were confirmed by PCR (Fig. 1H) and Southern blotting (Fig. 1I) assays.

Table 1. Indices of cloned Guangxi Bama mini-pigs delivered after transfer of different numbers of cloned embryos.

Group	Recipient No.	No. of embryos transferred	Day 40 pregnancy status*	No. of piglets born (alive/stillborn)	Cloning efficiency** (%)
< 200	1#	147	-		
	2#	150	-		
	3#	168	-		
	4#	132	-		
	5#	144	-		
	Sum	741		0	0 ^b
200~300	6#	243	+	Abortion	
	7#	224	+	0/1	
	8#	268	+	5/1	
	Sum	735		5/2	0.95 ^a
> 300	9#	425	+	8/1	
	10#	318	-		
	11#	307	+	3/0	
	12#	325	-		
	Sum	1375		11/1	0.87 ^a

*Pregnancy status: +, pregnant; -, not pregnant; **Cloning efficiency: No. of piglets born / No. of embryos transferred \times 100%; ^{a,b} Values with different superscripts indicate a significant difference ($P < 0.01$).

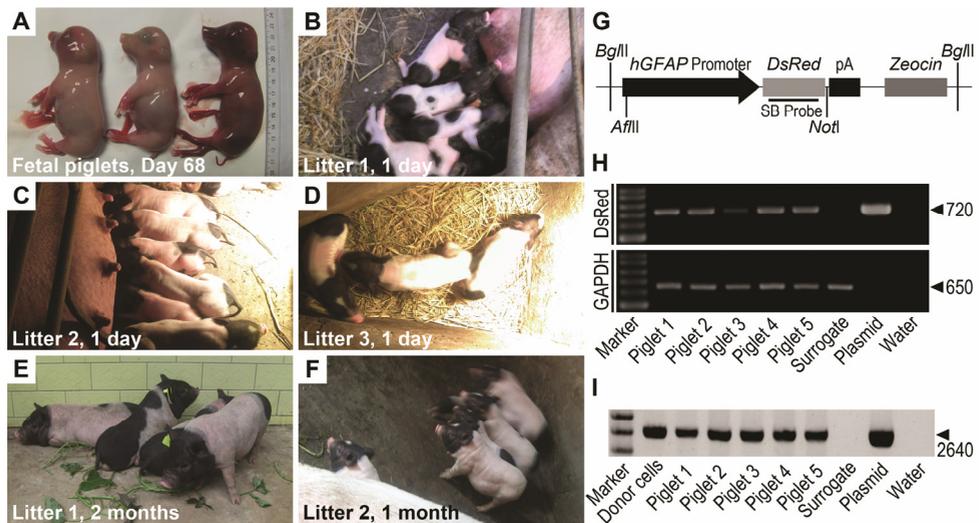


Figure 1. Generation of transgenically cloned Guangxi Bama mini-pigs via somatic cell nuclear transfer (SCNT) and embryo transfer (ET). (A) One litter of three fetal piglets from a surrogate sow that aborted at day 68 of gestation. (B~F) Three litters of viable cloned Guangxi Bama mini-pigs after full-term delivery, show no abnormalities at different periods after birth. (G) Schematic illustration depicts our transgenic plasmid. A 2.2-kb human glial fibrillary acidic protein (hGFAP) promoter was used to specifically express the *DsRed* in astrocytes.

Restriction endonuclease sites used for linearizing the plasmid (*BglII*) and for Southern blotting (SB) assay (*AflII* and *NotI*), as well as the probe for targeting *DsRed* sequence, are marked. The exogenous transgene fragments integrated into the genome of five live-born cloned piglets from litter 1 were confirmed by PCR (H) and SB (I) assays.

4. Discussion

As we described above, we generated live, transgenically cloned Guangxi Bama mini-pigs through genetic modification of fibroblasts followed by SCNT. Although the full-term delivery rate (57.1%, 4/7) in this study was comparable to those in previous studies carried out by other groups (WHITWORTH & al. [11]; RICHTER & al. [16]; HAO & al. [17]; LEE & al. [18]; MATSUNARI & al. [21]; ZHAO & al. [22]; [23]; KUROME & al. [24]), the cloning efficiency was largely compromised. There is a widespread consensus that porcine SCNT embryos are less ideal than embryos produced via in-vitro fertilization (IVF), owing to their lower developmental competence. In our laboratory, the blastocyst formation rate of porcine SCNT embryos was often 20%, while the data from IVF embryos is usually as high as 50% (YOSHIOKA & al. [12]; YOSHIOKA [14]). Therefore, considering the poor in-vitro developmental capacity of cloned porcine embryos and that a critical minimal signal from four good embryos is required in a sow in order to initiate and maintain pregnancy (POLGE & al. [25]; MISUMI & al. [26]), practical strategies such as reducing the in-vitro culture time and increasing the number of transferred embryos might be utilized to solve this issue. Such a practical strategy has been employed by several groups (SCHMIDT & al. [27]; LI & al. [28]; RIM & al. [29]; CALLESEN & al. [30]), and resulted in a significant increase in the delivery rate and cloning efficiency in pig cloning; and this effect was also reproduced in the present study. Other than the effect of the number of transferred embryos, pig cloning – especially cloning with genetically modified fibroblasts as donor cells – is complicated and depends upon many factors. 1) First, genetic modification of fibroblasts necessarily involves a series of procedures, such as transfection, resistance screening and extended cultivation, all of which could possibly affect the embryo's ability to support complete nuclear reprogramming (KUROME & al. [24]). 2) Second, the donor cell type also plays an important role in pig cloning. The generation of genetically modified mouse models requires that mouse embryonic stem (ES) cells produce the genetic modifications with high efficiency. Unfortunately, the lack of a stable source of true porcine ES cells has impeded the use of this method for creating transgenic pigs in an efficient manner (HAO & al. [17]; LEE & al. [18]; FAN & al. [31]). Porcine somatic cells, the cells routinely used as SCNT donor cells, exhibit limited proliferative capacity and have an extremely low frequency of genetic modification compared to mouse ES cells. Although several types of porcine adult stem cells (HAO & al. [17]; LEE & al. [18]) and even induced pluripotent stem (iPS) cells (FAN & al. [31]) have been examined and shown to be capable of generating full-term cloned piglets, the application of these emerging donor cell types for genetic modification is still under development. 3) Finally, increasing lines of evidence suggest that incompatible epigenetic modifications, (such as DNA methylation and histone acetylation), in SCNT embryos are closely associated with the low overall efficiency of pig cloning (ZHAO & al. [22]; [23]; LUO & al. [32]). Fortunately, several epigenetics-regulating drugs, such as trichomycin A (TSA), scriptaid and oxamflatin, have been developed and applied to pig cloning, resulting in significant enhancement of the developmental capacity of transgenic SCNT embryos (HIMAKI & al. [33]; MAO & al. [34]; ZHU & al. [35]), and the efficiency of generating viable cloned offspring (ZHAO & al. [22]; [23]). Such successful experiences can also then be used in our future work creating transgenic pigs with higher efficiency. At the

time we submitted this manuscript, our first litter of transgenically cloned Guangxi Bama mini-pigs almost reached sexual maturity (at about 5 months of age) and did not show any clinical abnormalities. Applications of these transgenic pig models to neuroscience research require phenotypic analysis and confirmation of stable germ-line transmission of transgenes through generations, and these validations are ongoing in our laboratory currently.

5. Conclusion

In this rapid communication, we report for the first time the generation of viable transgenically cloned Guangxi Bama mini-pigs using a practical strategy that significantly increases the number of transferred embryos during embryo transfer. Such a successful strategy can now be employed in our future work to create transgenic pigs with greater efficiency.

6. Acknowledgements

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7. Authors' contributions

S.S. Lu and K.H. Lu conceived the study and designed the experiments. X.X. Zhu, S.N. Quan, Y.L. Zeng and J.Y. Nie performed the experiments. S.S. Lu and X.X. Zhu drafted the manuscript. All authors participated in discussion of the results and read and approved the final manuscript. The manuscript was polished by the LetPub.

8. Conflict of interest disclosure

The authors declare that no conflicting financial interests exist.

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