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Cloning Efficiency Evaluation Based on the Production of Cloned Diannan Miniature Pigs

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ABSTRACT

The currently observed cloning efficiency is low and can be calculated using different methods. Therefore, a reasonable evaluation method for calculating the cloning efficiency in pigs is important for promoting the application of gene-modified miniature pigs in biomedical fields. In this study, we proposed a method for the scientific evaluation of cloning efficiency in pigs based on the production of cloned Diannan miniature pigs. Of 9 recipient gilts that were transferred with cloned embryos, 8 (88.9%) gilts became pregnant, and 7 (87.5%) of these delivered 37 piglets, 22 (59.5%) of which were alive and 17 survived to adulthood (over 1 year of age). Among the 15 stillborn piglets, 11 piglets experienced intrapartum death due to dystocia, and the other 4 died due to blastocolysis at the late stage of pregnancy. The greatest number of littermates was 11 piglets. The 3 piglets delivered by Caesarean section were alive and healthy. The procedure used for cloning pigs is a costly process in terms of both time and money; specifically, it requires 677.7-1265.4 min and $715.8-764.5 to transfer 50-400 cloned embryosto a recipient. The cloning efficiency defined as the total number of cloned piglets (including dead and mother piglets) divided by the total number of transferred embryos was 0.93%, whereas the cloning efficiency obtained based on the total number of live piglets divided by the total number of recipients was 2.44 piglets. The value is higher than those previously obtained using 19 different types of donor cells. These findings suggest that donor cells derived from Diannan miniature pigs are a valuable cell source for somatic cell nuclear transfer and the definition of cloning efficiency in pigs as “the total number of live cloned piglets divided by the total number of transferred recipient gilts” may be a reasonable scientific strategy.

INTRODUCTION

Somatic cell nuclear transfer (SCNT) has provided a powerful tool for creating copies of selected individuals of different species since 1997, when the first mammal cloned from adult somatic cells, the sheep “Dolly”, was born [1]. Feasible SCNT procedures have also been established in pigs [2-4]. Although SCNT has also been successfully applied to the cloning of several miniature pigs, including potbelly miniature pigs [5], Clawn miniature pigs [6], Gottingen pigs [7], Yucatan minipigs [8], NIBS strain

miniature pigs [9], National Institutes of Health miniature pigs [10], Bama miniature pigs [11] and Banna miniature inbred pigs [12], the widespread application of pig SCNT in biomedical research has been hampered by the low cloning efficiency obtained [8,13]. The type of nuclear donor cell used is a vitally important factor that influences the outcomes of SCNT [14,15].

The Diannan miniature pig is well known as an exclusive native breed that is conserved in Yunnan Province in China. This pig breed has a suitable body weight and genetic diversity [16]. Moreover, it is easy to handle due to its docile character and strong disease-resistant nature as well as its ability to subsist on roughage. The time to sexual maturity of this breed is shorter, and mating can occur when males are three months old and females are four months old. The numbers of piglets per litter in primiparous and multiparous sows are approximately 7 to 10, respectively. These characteristics make the Diannan miniature pig an ideal model for various types of research studies [17,18]. Thus, clones of these pigs are very important for their further application. However, the cloning process and efficiency in Diannan miniature pigs remain unclear.

The pig SCNT procedure has been in development for 15 years, specifically since the births of the first cloned pigs were reported. The cloning efficiency in pigs has been calculated by different laboratories using various methods, such as “the total number of cloned piglets (including dead and mummified) divided by the total number of transferred embryos” [10,19-21], “the total number of live cloned piglets divided by the total number of transferred embryos”[12], “the total number of healthy cloned piglets divided by the total number of transferred embryos”[21], “the number of piglets born (including dead and mummified) divided by the number of cloned embryos transferred from a recipient”[22] and “the number of offspring divided by the number of embryos transferred that led to a pregnancy”[9]. Because of these differences, we endeavored to determine a more scientific method for evaluating cloning efficiency in pigs.

In the present study, we showed that donor cells derived from Diannan miniature pigs are a valuable cell source for the generation of pig biomedical models by SCNT. Furthermore, the method used in this study to calculate the cloning efficiency in pigs based on the production of cloned Diannan miniature pigs and its comparison with the methods detailed in previously published papers are also discussed.

MATERIAL AND METHODS

All of the animal experiments were performed with approval from the Animal Care and Use Committee of Yunnan Agricultural University in China.

Chemicals

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

Preparation of Donor Cells

Fibroblast cell lines were established as donor cells from male Diannan miniature pig fetuses at 30 days of gestation. The fetuses without heads, limbs or internal organs were minced and digested in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) containing 20% fetal bovine serum (FBS; Hyclone), 1% penicillin-streptomycin, and 1 mg/ml collagenase IV for 4 h at 37°C. The cells were centrifuged at 1000 rpm for 5 min, resuspended in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin, and then cultured in a flask until grown to 90% confluence. The cells were further passaged and frozen in DMEM containing 20% FBS and 10% dimethyl sulfoxide for future use.

In vitro Maturation (IVM) of Oocytes

Porcine ovaries were collected from the Hongteng abattoir (Chenggong Ruide Food Co., Ltd., Kunming, Yunnan Province, China) with the permission to use animal parts for this study, and the ovaries were transported to the laboratory in 0.9% (w/v) NaCl solution at 25-30°C. The oocytes were obtained and cultured using the same method as previously described [12].

Somatic Cell Nuclear Transfer

SCNT was performed using IVM oocytes as recipient cytoplasts, as previously described [12]. After culturing for 38-42 h, oocytes with expanded cumulus cells were briefly treated with 0.1% (w/v) hyaluronidase and were denuded of cumulus cells using a finely drawn glass capillary pipette. Oocytes extruding the first polar body with uniform cytoplasms were cultured in NCSU23 medium supplemented with 0.1 μg/ml demecolcine, 0.05 M sucrose, and 4 mg/ml BSA for 0.5-1 h. Each reconstructed embryo formed from a single donor cell was inserted into the perivitelline space of an enucleated oocyte through micromanipulation. The reconstructed embryos were fused with a single direct current pulse of 200 V/mm for 20 μs using an LF201 Electro Cell Fusion Generator (NEPA GENE Co., Ltd., Japan) in fusion medium (0.25 M D-sorbic alcohol, 0.05 mM $Mg(C_2H_3O_2)_2$, 20 mg/ml BSA, and 0.5 mM HEPES [acid-free]). The reconstructed embryos were then cultured for 2 h in PZM-3 and were activated with a single pulse of 150 V/mm for 100 ms in activation medium containing 0.25 M D-sorbic alcohol, 0.01 mM $Ca(C_2H_3O_2)_2$, 0.05 mM $Mg(C_2H_3O_2)_2$, and 0.1 mg/ml BSA. The reconstructed embryos were equilibrated in PZM-3 supplemented with 5 μg/mL cytochalasin B for 2 h at 38.5°C in a humidified atmosphere with 5% CO_2, 5% O_2, and 90% N_2 (APM-30D, ASTEC, Japan).

The Reconstructed Embryo Culture

The reconstructed embryos were cultured in PZM-3 medium and were placed in a humidified incubator supplied with 5%

CO_2, 5% O_2, and 90% N_2 at 38.5°C. Cleavage and blastocyst formation were monitored on days 2 and 7, respectively. The number of blastocysts was counted after fixation and Hoechst33342 staining under an ultraviolet light microscope.

Embryo Transfer

Crossbred (Large White/Landrace Duroc) gilts were used as the surrogate mothers of the cloned embryos, as previously described [12]. Pregnancy was detected approximately 23 days after surgical transfer using an ultrasound scanner (HS-101V, Honda Electronics Co., Ltd., Yamazuki, Japan).

Microsatellite Analysis

Parentage analysis was performed in the piglets produced by SCNT and the surrogate recipients to confirm the genetic identity of the piglets from donor cells. The isolated genomic DNA samples obtained from each piglet (ear tissue) and recipient (ear tissue) were used for microsatellite analysis and were sent to a company that specializes in parentage verification for swine (Shanghai GeneCore BioTechnologies Co., Ltd., China). The microsatellite analysis of genomic DNA was performed using 10 porcine-specific microsatellite markers (S0026, S0070, S0155, S0226, SW122, SW72, SW830, SW840, SW857 and SW936) labeled with the fluorescent dye FAM.

Data for the Times and Costs of SCNT in Pigs

The total time required from ovarian collection to transplantation can be divided as follows: (1) The time for the *in vitro* maturation of oocytes includes ovarian collection and culture medium preparation, the aspiration of cumulus-oocyte complexes (COCs) from the antral follicles of the abattoir-derived ovaries, and the preparation of donor cells; (2) The time for construction of the cloned embryos includes their preparation in medium, the insertion, of the enucleated oocyte inserted into a single donor cell, electro-fusion and activation of the oocyte, and equilibration of the reconstructed embryos in PZM-3 medium. The time for SCNT is the round-trip time from the lab to the swine farm plus the time required for 6-7 male laborers to move the recipient gilts on to/off the operation table.

The costs can be calculated as follows. It costs $ 20.60 per day for ovarian collection and $ 20.60 or $ 41.20 to cultivate 50-200 or 250-400 clone embryos, respectively. In addition, its cost $ 56.00 to construct 200-400 clone embryos and $ 69.30 for embryo transfer. In addition, a cost of approximately $ 577.30 is required to raise recipient gilt. We ignored the costs of low-value consumables when calculating these costs.

Statistical Analysis

For the proportional data, the differences between groups were analyzed using a t-test (Statview® software package). The level of significance was set to $P<0.05$.

RESULTS

In vitro and *in vivo* Development Competence of Cloned Embryos

We investigated the *in vitro* and *in vivo* developmental abilities of cloned embryos derived from fetal fibroblast cells (FFCs) of a Diannan miniature pig. The results showed that the cleavage rate and blastocyst formation rate were 80.3% and 22.8%, respectively **(Table 1).**

Table 1. Development competence of *in vitro* cloned embryos using Diannan miniature FFCs as donors.

No. of embryos (Repeat)	Cleavage rate (%)	Blastocyst formation rate (%)	No. of cells in blastocyst
1626 (6)	1305 (80.3 ± 2.7)	371 (22.8 ± 2.3)	47.2 ± 9.7

The percentage is expressed as the mean ± SD.

The cloned embryos were each transferred into 9 recipient gilts, 8 of which became pregnant and 7 of which carried the pregnancy to full term. Finally, 19 live piglets and 15 stillborn piglets were recovered from 6 recipient gilts by spontaneous delivery. In addition, 3 live piglets (and no stillborn piglets) were obtained from 1 recipient gilt by Caesarean delivery **(Table 2).**

In total, 37 piglets were born from the 7 recipient gilts, and 17 of these piglets survived to adulthood and mated with Diannan miniature pigs to produce descendants **(Figure 1).** One other recipient did not return to heat, and surgery showed no fetal contents in the uterus at 114 days of gestation. The causes of death of the 15 stillborn piglets were determined by necropsy. Among these, 11 stillborn piglets had normal phenotypes and scarlet blood after exploratory thoracotomy, and fibroblast cell lines were derived and established from their ear tissues. Thus, we inferred that these piglets suffered intrapartum death due to dystocia. For the other four piglets, which were underweight and had soft bodies, fibroblast cell lines could not be established, and the cause of death was determined to be blastocolysis closely before full term. Thus, the stillbirth rate reached 29.7% (11/37), with only 10.8% (4/37) dying from blastocolysis **(Table 2).** The highest number of littermates was 11 piglets. The overall pregnancy rate of the recipients was 88.9%, the delivery rate was 87.5%, the average litter size was 5.3 piglets, and the cloning efficiency was 0.93% **(Table 3).**

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Table 2. Summary of the cloning outcomes at different stages using Diannan miniature FFCs as donors.

Recipients	No. of transferred embryos	Pregnancy	Days of pregnancy (d)	Offspring (stillborn/ aborted)	Stillborn offspring's		Cloning efficiency (%)
					Scarlet blood	Establishment of fibroblast cell line	
1	447	+	121	6 (3)	3	3	1.34
2	409	+	128	1 (1)			0.14
3	285	+	115	7 (3)	3	3	2.46
4	594	+	115	2 (1)	1	1	0.34
5	419	+	113	3 (cesarean)			0.72
6	376	+	—	—	—	—	—
7	512	+	116	7 (3)	2	2	1.37
8	477	—	—	—	—	—	—
9	460	+	115	11 (4)	2	2	2.39
Total	3977	+ (8)		37 (15)	11	11	0.93

Cloning efficiency (%) = No. of cloned piglets / No. of transferred embryos × 100.

Figure 1. Obtained partly piglets derived from the fibroblasts of Diannan miniature pig.

Table 3. Comparison of previously used methods for evaluating cloning efficiency in pigs.

Breeding[a]	Donor cell type	No. of recipient	Transferred embryos		No. of pregnancy	No. of delivery	Cloned piglets		Cloned efficiency			A	F
			Total	Time of culture			Total	Live piglets	Method of calculate[b]	cloning efficiency	References		
Diannan	Fetal fibroblasts	9	3977	6 h	9	6	37	22	A	0.93%	-	0.93%	2.44
NIBS	Fetal fibroblasts	11	1312	-	5	5	13	8	A	0.99%	[20]	0.99%	0.73
MGH	Lung fibroblasts from neonatal GalT-KO	12	1953	-	5	5	6	2	A	0.10%		0.10%	0.17
MGH	GT KO/hCD46 KI	19	2990	After fusion	6	3	4	1	-	-	[22]	0.13%	0.05
	Post-thawed GT KO/hCD46 KI	12	2350		8	6	12	8	D	0.74%-2.54%		0.51%	0.67
PDLY and LW	Fetal fibroblasts and adult fibroblasts	328	92005	20 h	189	147	488	389 (291)	B (C)	0.42% (0.32%)	[21]	0.53%	1.2
Banna	Fetal fibroblasts	5	1230	6-30 h	3	3	8	8	A	0.65%	[12]	0.65%	1.6
	Newborn fibroblasts	5	740	6-30 h	4	1	3	1	A	0.4%		0.40%	0.2
	Adult fibroblasts	3	914	6-30 h	1	1	1	1	A	0.1%		0.10%	0.33
Bama	Fetal fibroblasts	5	870	48 h	-	1	1	1	-	-	[11]	0.11%	0.2
NIH	Fetal fibroblasts	10	1610	Day 1	8	8	21	17	A	1.30%	[10]	1.30%	1.7
Yucatan	Fetal fibroblasts (1# round of SCNT)	6	631	1 h	3	3	8	7	-	-	[8]	0.13%	1.17
	Fetal fibroblasts (2# round of SCNT)	3	315	1 h	1	1	4	3	-	-		1.27%	1.00

NIBS	Fetal fibroblasts (♂)	3	314	1 or 2 day	2	1	2	2	E	0.93%	[9]	0.64%	0.67
	Fetal fibroblasts (♀)	5	462	1 or 2 day	4	3	8	4		2.02%		1.73%	0.80
	Fetal fibroblasts (♀) GFP gene	3	365	1 or 2 day	3	3	10	9		2.74%		2.74%	3.00
LD	Salivary gland	4	615	1 or 2 day	4	4	12	6	A	1.95%	[19]	1.95%	1.5
Clawn	Fetal fibroblast	2	146	6-40 h	2	1	2	1	-	-	[6]	1.37%	0.5
Potbelly	Lung cells	9	191	48 h	1	1	3	3	-	-	[5]	1.57%	0.33
	Kidney cells	9	196	48 h	1	1	2	2	-	-		1.02%	0.22

[a]Diannan: Diannan miniature pig; NIBS: NIBS strain miniature pig; MGH: MGH miniature pig; PDLY and LW: similar Western swine breeds (Pietrain, Duroc, Landrace, and Yorkshire) and Chinese pig breeds of Laiwu; Banna: Banna miniature inbred pig; Bama: Bama miniature pig; NIH: NIH miniature pig; Yucatan: Yucatan minipig; LD: Landrace-Duroc swine; Clawn: Clawn miniature pig; Potbelly: Potbelly miniature pig

[b]A: Total number of cloned piglets (including dead and mummified)/total number of transferred embryos; B: Total number of live cloned piglets/total number of transferred embryos; C: Total number of healthy cloned piglets/total number of transferred embryos; D: Number of piglets born (including dead and mummified)/number of cloned embryos transferred from a recipient; E: Number of offspring/number of embryos transferred resulting in pregnancy; F: Total number of live cloned piglets/total number of recipients.

In contrast, the cloning efficiency defined as the total number of live piglets divided by the total number of recipients was 2.44 **Table 3.** Moreover, we compared the cloning efficiencies obtained from 19 different types of donor cells, as detailed in previously published papers, with the total number of live piglets divided by the total number of recipients, which ranged from 0.05 to 1.7. Only 1 donor cell type attained an efficiency of 3.0, but the total number of recipients was 3.0. For Diannan miniature pigs, the total number of live piglets divided by the total number of recipients was 2.44, which is higher compared with the values obtained for 19 different donor cell types **Table 3.** In addition, a parentage analysis was performed on the cloned piglets, donor cells, and surrogate mothers. The genotype of each piglet was identical to that of the donor cell but different from that of its surrogate mother. Due to sequencing errors, cloned piglets #24, #31 and #36 had microsatellites that differed from those of the donor cells **Table 1.** These data confirmed that the piglets obtained were derived from the cell lines used for SCNT. Together, these findings suggest that donor cells derived from Diannan miniature pigs constitute a valuable cell source for the generation of pig biomedical models by SCNT. Moreover, it is necessary to propose a reasonable scientific method for evaluating the cloning efficiency in pigs.

The body weights at birth of the cloned and artificially inseminated male Diannan piglets are shown in **Table 2.** The average body weight (713 g) of the cloned group was significantly greater than that of the non-cloned group (566 g) ($P<0.01$). Although the average body weight of the live cloned group (738 g) was slightly less than that of the intrapartum death cloned group (795 g), there was no significant difference between the two groups ($P>0.05$). Moreover, the heaviest pig in the intrapartum death cloned group was 1397 g. The average body weight of the blastocolysis death cloned group (350 g) was significantly less than those of the live cloned group and the intrapartum death cloned group ($P<0.01$).

These data suggest that the 11 stillborn piglets would have survived had Caesarean section been performed prior to full gestation. If events had occurred in this manner, the cloning outcomes and efficiency obtained in our study would have been better than the above-described results.

Times and Costs of SCNT in Pigs

The times and costs for SCNT in pigs are summarized in **Tables 4 and 5**, respectively. Specifically, 59.0-238.9 min are required to generate 100-800 *in vitro* maturated oocytes, and 141.7-549.5 min are needed to reconstruct 50-400 clone embryos. In addition, 7 male laborers are needed to move the recipients onto the operating table before embryo transfer could begin, requiring a total of 477.0 min. Thus, the total sum of the times described above, from *in vitro* maturated oocytesto embryo transfer, is 677.7-1265.4 min **(Table 4).** In terms of costs, $20.60 is the cost for ovary collection, and it costs $ 20.60 or $ 41.20 to cultivate 50-200 or 250-400 clone embryos, respectively. In addition, it costs $ 28.00 or $ 56.10 for 50-150 or 200-400 construction clone embryos, respectively, and $69.30 for embryo transfer. In addition, an approximately $ 577.30 are required for each recipient gilt. Altogether, payments of $ 715.80, $ 743.90 and $ 764.50 are needed to transfer 50-150, 200 and 250-400 cloned embryos, respectively **(Table 5).** Notably, the cost only increased by 6.8% when the number of transplanted clone embryos was increased from 50 to 400. These results suggest that the times and costs for SCNT in pigs mainly depend on the cost of the surrogate sows when transferring a certain number of cloned embryos.

*No. of *in vitro* matured oocytes = No. of cloned embryos × 2.

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Table 4. Times requires for the construction and cultivation of cloned embryos and their transplantation.

Category	No. of cloned embryos							
	50	100	150	200	250	300	350	400
In vitro maturation of oocyte (min, n=12)*	59.0 ± 1.5	84.7 ± 2.2	110.4 ± 3.2	136.1 ± 4.3	161.8 ± 5.4	187.5 ± 6.6	213.2 ± 7.7	238.9 ± 8.9
Reconstruction of cloned embryos (min, n=6)	141.7 ± 9.0	182.3 ± 10.4	240.3 ± 9.9	296.3 ± 14.0	360.3 ± 3.3	419.3 ± 15.0	480.3 ± 13.3	549.5 ± 27.6
Embryos transfer (min, n=7)	477.0±72.8 (7 peoples)							
Total (min)	677.7	744	827	909.4	999.1	1083.8	1170.5	1265.4

Table 5. Costs of SCNT in pigs.

Category	No. of cloned embryos							
	50	100	150	200	250	300	350	400
Collected ovaries ($)	20.6	20.6	20.6	20.6	20.6	20.6	20.6	20.6
In vitro maturation of oocyte ($)	20.6	20.6	20.6	20.6	41.2	41.2	41.2	41.2
Reconstruction of cloned embryos ($)	28.0	28.0	28.0	56.1	56.1	56.1	56.1	56.1
Embryos transfer ($)	69.3	69.3	69.3	69.3	69.3	69.3	69.3	69.3
Recipient ($)	577.3	577.3	577.3	577.3	577.3	577.3	577.3	577.3
Total ($)	715.8	715.8	715.8	743.9	764.5	764.5	764.5	764.5

$ 1 (USD) is equal to 6.063 Yuan (RMB).

DISCUSSION

The cloning efficiencies in large animals and mice, which are usually defined as the total number of live cloned animals divided by the total number of transferred embryos, can range from 0 to 20%, and efficiency rates just under 3% are typical in pigs [13,23-25]. Moreover, recent studies have shown that the cloning efficiency in pigs has been calculated using various methods, as described in **Table 3**, which makes the assessment of the cloning efficiency in different pig breeds confusing. Although calculating the cloning efficiency based on the total number of live cloned animals divided by the total number of transferred embryos is objective and reasonable for mice, the entire cloning procedure for pigs requires several steps, including ovary collection, *in vitro* maturation of oocytes, reconstruction of cloned embryos, embryo transfer, and breeding the recipient gilts. This entire procedure requires more than 114 days completing, although it only takes 2.5 days from ovary collection to embryo transfer **(Figure 2 and Table 4).** Moreover, the total cost of a transfer is explained by the cost of the recipient gilt, which is more than 80% of the total cost **(Table 5).** Hence, we believe that the method of calculating the cloning efficiency in pigs should consider the number of recipient gilts. In addition, some studies have shown that the transfer of an excessive number of cloned embryos into individual surrogates does not increase the cloning efficiency in pigs [21]. Thus, under this condition, the calculation of cloning efficiency as the "total number of cloned or live cloned piglets divided by the total number of recipients" is objective and reasonable for pigs. However, SCNT for pig is a complex multistep procedure described above. Moreover, we should consider the cost and time for a large animal such as pig. Therefore, many factors are believed to contribute to the outcome of cloning efficiency. Thus, if define the cloning efficiency as "total number of live cloned piglets divided by the total number of transferred recipient gilts", we need to try to improve the quality of cloned embryos, the number of cloned embryos transferred in a recipient gilt, the time of ovulation and embryo transfer, the operation skills of embryo transfer, the feeding management of recipient gilts as well as the survival rate of the cloned individual in the birth of a recipient gilt. To solve these problems, we can improve the cloning efficiency in pig.

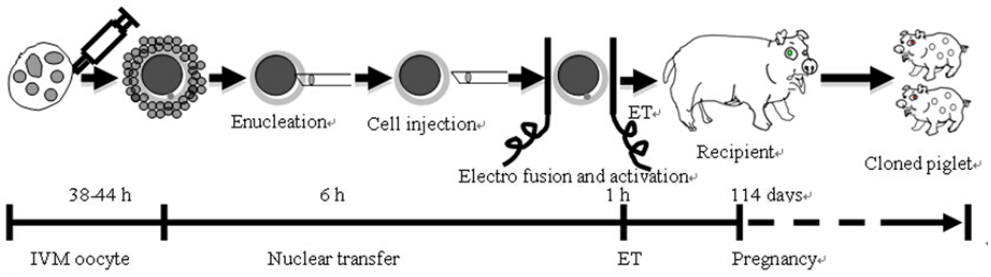

Figure 2. Schematic of somatic cell nuclear transfer in pigs. In this model, the whole procedure, including the in vitro maturation of oocytes, nuclear transfer, embryo transfer and pregnancy period, is shown. A total of 114 days are required for the gilts to give birth to piglets after embryo transfer, although it takes only 2.5 days from ovary collection to embryo transfer.

The number of cloned piglets is also a major factor in determining the level of cloning efficiency, as demonstrated by the previously used methods for the calculation of cloning efficiency. It is well known that the mortality of cloned piglets produced by SCNT is a major problem [26]. Our findings showed that 11 stillborn piglets died intrapartum, 3 additional stillborn piglets died due to blastocolysis at nearly full term **(Table 2).** Previous results have indicated a significant association between prolonged birth intervals and stillbirth rates, indicating that the stillbirth rate increases markedly when the individual piglet birth interval extends

beyond 1 h [27]. Our results also show that the length of gestation and the birth intervals are significantly longer than those of artificially inseminated piglets **Table 3.** This association may explain the mortality of the cloned piglets, which should be associated with the negative motility and expulsion of porcine fetuses and with insufficient fetal-maternal interaction [28]. In addition, we also observed cornu uteri adhesion of the recipients resulting from the transfer of the cloned embryos to the uterus (data not shown), which may serve as another explanation for the mortality resulting from maternity. One method used to increase the survival rate of cloned piglets at birth has been to deliver the piglets by Caesarean section [29,30]. Our results showed that three live healthy piglets were recovered by Caesarean section **Table 2.** Furthermore, data obtained from the 23 recipient gilts also confirmed that the survival rate of the cloned piglets would have been significantly increased by Caesarean section (data not shown).

The outcomes of SCNT can be affected by complex interactions between multiple factors. Although some of these factors are difficult to control, others, such as the choice of nuclear donor cell source, can help increase the efficiency of cloning [21]. Our results showed that 8 recipients (88.9%) became pregnant, and 7 (87.5%) of these gave birth to 37 offspring, with an average litter size per recipient of 5.3 piglets and a maximum litter size of 11 piglets **Table 2.** Moreover, we compared the cloning efficiencies obtained from 19 different types of donor cells utilized in previously published studies, and the cloning efficiency ranged from 0.05 to 1.7. Although, 1 type of donor cell attained an efficiency of 3.0, the total number of recipients was only 3.0. The cloning efficiency of Diannan miniature pigs was 2.44, which is higher than that obtained with the 19 different donor cell types **Table 3.** Our findings suggest that donor cells derived from Diannan miniature pigs are a valuable cell source for the generation of pig biomedical models by SCNT. Nevertheless, the efficiency of pig cloning remains low, and its applications are limited, indicating that further improvement is needed.

In conclusion, our results demonstrate that donor cells derived from Diannan miniature pigs constitute a valuable cell source for the generation of pig biomedical models by SCNT. Furthermore, we suggest that the definition of cloning efficiency in pigs as “the total number of live cloned piglets divided by the total number of transferred recipient gilts” may be a reasonable and scientific strategy.

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