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Significant improvement in cloning efficiency of an inbred miniature pig by Scriptaid treatment after somatic cell nuclear transfer

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Abstract

The NIH miniature pig was developed specifically for xenotransplantation and has been extensively used as a large animal model in many other biomedical experiments. However the cloning efficiency of this pig is very low (less than 0.2%) and this has been an obstacle to the promising application of these inbred swine genetics for biomedical research. It has been demonstrated that increased histone acetylation in somatic cell nuclear transfer (SCNT) embryos, by applying histone deacetylase inhibitors (HDACi) such as trichostatin A (TSA), significantly enhances the developmental competence in several species. However some researchers also reported that TSA treatment had various detrimental effects on the *in vitro* and *in vivo* development of the SCNT embryos. Here we report that treatment with 500 nM Scriptaid, a novel HDACi, significantly enhanced the development SCNT embryos to the blastocyst stage when NIH inbred fetal fibroblast cells (FFCs) (21% vs. 9%, $P < 0.05$) were used as donors compared to the untreated group. Scriptaid treatment resulted in 8 pregnancies from 10 embryo transfers (ET) and 14 healthy NIH miniature pigs from 8 litters while no viable piglets (only 3 mummies) were obtained from 9 ETs in the untreated group. Thus Scriptaid dramatically increased the cloning efficiency when using inbred genetics from zero to 1.3%. In contrast, Scriptaid treatment decreased the blastocyst rate in IVF embryos (from 37% to 26%, $P < 0.05$). In conclusion, the extreme low cloning efficiency in the NIH miniature pig may be caused by its inbred genetic background and can be improved by alteration of genomic histone acetylation patterns.

Keywords

SCNT; Histone acetylation; Scriptaid; inbred

Introduction

Due to their similar anatomy and physiology swine have been used in biomedical applications for decades as a model for human diseases, as a genetically defined model for surgery and xenotransplantation and as a source of human disease therapeutics [1]. By combining the technology for homologous recombination in somatic cells with that of SCNT, it is possible

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Summary sentence: Scriptaid, a histone deacetylase inhibitor, treatment after activation improves the in vitro and in vivo developmental competence of SCNT NIH miniature pig embryos, but not IVF embryos.

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to create specific modifications to the pig genome [2]. Several pig models that have potential applications in basic life science research or studying human diseases have been made, such as eGFP expressing pigs [3], alpha-1,3-Galactosyltransferase knockout pigs [4] and cystic fibrosis transmembrane conductance regulator knockout pigs [5,6]. However the widespread application of porcine SCNT to biomedical research is being hampered by the large adult size (300–600 lbs) of the commercial breeds commonly used for SCNT [7] as well as the low cloning efficiency. The miniature pig, also shares many physiological similarities with humans, offers several breeding and handling advantages (when compared to non-human primates), making it an optimal species for preclinical experimentation [8].

The NIH miniature pig was developed by David Sachs over 30 years ago [9] specifically for xenotransplantation. Three sub-lines with divergent genotypes at the swine leukocyte antigen (SLA^{aa}, SLA^{cc}, SLA^{dd}) were derived from two matings of the same two animals and are now highly inbred [10]. Possibly because these animals are highly inbred their reproductive characteristics are lower than domestic pigs [11,12]. Although several pig breeds have been successfully cloned, the remodeling and reprogramming of differentiated somatic nuclei into a totipotent embryonic state by SCNT is not efficient and the mechanism by which this remodeling occurs is not known. This results in the overall cloning efficiency to be low. Generally, in most mammalian species studied thus far, the survival rate to birth for cloned blastocysts is only about 1% – 5%, compared to a 30%–60% birth rate for IVF blastocysts [13]. Our inventory of NIH miniature pigs consisted of two SLA^{cc} animals (one male and one female). When we tried to clone them from adult ear fibroblast cells (EFCs) none of six embryo transfers established a pregnancy (unpublished data), and a previous reported showed a cloning efficiency of SLA^{dd} animals of only 0.2% [4]. The lower cloning efficiency has been an obstacle to the application of these inbred swine genetics for biomedical research. The HDAC inhibitor Trichostatin A (TSA) can improve the efficiency of cloning mice in both reproductive cloning [14,15] and SCNT embryonic stem cell derivation [14]. Adult male and female outbred mice, ICR, were successfully cloned only when TSA was applied [16]. SCNT embryos treated with TSA resulted in higher preimplantation embryonic development in pigs [17,18], cattle [19,20] and rabbits [21]. Although TSA application resulted in great improvement in somatic cloning in mice, the effects of TSA treatment on cloning efficiency are controversial in several species and some groups reported that TSA treatment had various detrimental effects on the *in vitro* and *in vivo* development of the SCNT embryos [22–25]. The effects of HDACi treatment on full term development have not been determined in any species other than in mice. Therefore we determined if HDACi treatment had beneficial effects on SCNT in the pig. Considering the effects of TSA treatment on SCNT, we investigated the effect of Scriptaid, a HDACi with low toxicity that enhances transcriptional activity and protein expression [26], on SCNT efficiency of NIH miniature pigs. The objective of this study was to: 1) investigate the effect of Scriptaid treatment on the developmental competence of somatic nuclei following SCNT using NIH miniature inbred donor cells *in vitro* and *in vivo*; and 2) to determine if Scriptaid affects development of *in vitro* produced embryos.

Materials and Methods

Media and reagents

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO USA) unless otherwise stated. All of the following solutions and media were filtered by 0.22 μm filter. The cell culture medium was Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% (v/v) fetal bovine serum (FBS, Hyclone, Logan, UT, Catalog: SH30071.03, Lot number: ASM31113) and antibiotics. The oocyte *in vitro* maturation (IVM) medium was TCM 199 (Gibco BRL Grand Island, NY) supplemented with 0.1% polyvinylalcohol (PVA) (w/v), 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 1 μg/mL Gentamicin, 0.57 mM cysteine, 0.5 μg/

mL luteinizing hormone, 0.5 μg/mL follicle stimulating hormone and 10 ng/mL epidermal growth factor. Medium used for IVF was a modified Tris-buffered medium (mTBM) containing 2 mg/mL BSA and 2 mM caffeine. Sperm washing medium was Dulbecco phosphate-buffered saline (DPBS, Gibco BRL Grand Island, NY) supplemented with 1 mg/mL BSA (pH 7.3). The embryo culture medium was Porcine Zygote Medium-3 (PZM3, pH 7.3) supplemented with 3 mg/mL BSA. Stock solutions of TSA and Scriptaid were dissolved in dimethyl sulfoxide (DMSO) at 100 nM and 1 mM, respectively, and stored at −20 °C. They were added to the embryo culture medium at specific amounts according to each experiment's protocol.

Primary cell establishment and donor cell preparation

Adult EFCs were established from our two NIH miniature SLA^{CC} animals and then used for SCNT and ET to produce pregnancies for making FFCs. At day 35 the female was sacrificed and FFCs were derived from two fetuses (one male and one female) and established as previously described [27]. Briefly, the fetuses were recovered and rinsed three times with DPBS. After removal of head, intestine, liver, limbs, and heart, the remaining tissues were finely minced into pieces (1 mm^3) using scissors in DPBS. Minced tissue pieces were digested with Collagenase (200 u/mL) and Dnase I (25K unit/mL) in cell culture medium for 4–5 hrs at 38.5 °C and 5% CO_2 in air. The digestion was rinsed in cell culture medium by centrifugation at 500 g for 5 min to remove the residual enzyme. The cell pellet was re-suspended in the cell culture medium, seeded in a 75 cm^2 culture flask and left to culture until confluent.

For SCNT the cells were thawed, cultured, and subsequently used between passages 3–5. A suspension of single cells was prepared by trypsinization of the cultured cells, followed by re-suspension in manipulation medium (25 mM Hepes-buffered TCM199 with 3 mg/mL BSA) prior to SCNT.

In Vitro Maturation

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the lab at 37 °C. Follicles between 3–6 mm in diameter were aspirated with an 18-gauge needle attached to a 10-cc syringe. The cumulus-oocyte complexes (COCs) in the follicular fluid were allowed to settle by gravity. COCs were rinsed in Hepes-buffered Tyrode's medium containing 0.01% PVA three times [28]. Only the COCs with multiple layers of intact cumulus cells and uniform ooplasm were selected for IVM. After washing three times in IVM medium, a group of 70–80 COCs were placed into each well of four well cell culture plate (Nunc, Denmark) containing 500 μL of IVM medium. COCs were matured for 44 hrs (if used for IVF) or 40 hrs (if used for SCNT) at 38.5°C and 5% CO_2 in air, 100% humidity. Matured COCs were then vortexed in 0.1% hyaluronidase in Hepes-buffered Tyrode's medium containing 0.01% PVA for 4 min to remove the cumulus cells. Only the matured oocytes having an extruded first polar body (PB) with uniform cytoplasm were used for the *in vitro* development of SCNT or IVF embryos. For *in vivo* development of SCNT embryos, maturing oocytes from sows were purchased from ART Inc. (Madison, WI) and shipped overnight in their commercial maturation medium #1[29]. Twenty-four hours after being placed in the maturation medium #1, the oocytes were moved to medium #2. After a total of 40 h of maturation, the cumulus cells were removed as described above and used for SCNT.

Somatic Cell Nuclear Transfer

Matured oocytes were used for SCNT in manipulation medium supplemented with 7.5 μg/mL cytochalasin B which was overlaid with warm mineral oil. Oocytes were enucleated by aspirating the PB and MII chromosomes and a small amount of surrounding cytoplasm using a beveled glass pipette with an inner diameter of 17–20 μm. A single intact donor cell was injected into the perivitelline space and placed adjacent to the recipient cytoplasm. Karyoplast

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cytoplast complexes (KCCs) were placed into embryo culture medium until fusion and activation. The fusion/activation of the KCCs was accomplished with 2 DC pulses (1 sec interval) of 1.2 kV/cm for 30 μsec provided by a BTX Electro-cell Manipulator 200 (BTX, San Diego, CA) in fusion medium (0.3 M mannitol, 1.0 mM $CaCl_2$, 0.1 mM $MgCl_2$, and 0.5 mM Hepes, pH adjusted to 7.0–7.4). Oocytes were then incubated for 20 minutes in PZM3 and evaluated for fusion under a stereomicroscope. Only the fused embryos were placed into four well cell culture plates (Nunc, Denmark) containing 500 μL of PZM3 at 38.5 °C and 5% CO_2 in humidified air.

In Vitro Fertilization

IVF was carried out as previously described [30]. Briefly, oocytes with a PB at 44 h of IVM were washed three times in mTBM medium. Approximately 30–35 oocytes were transferred into 50 μL droplets of IVF medium covered with mineral oil that had been equilibrated for 4 hrs at 38.5 °C in 5% CO_2 in air. A 0.1-mL frozen semen pellet was thawed at 38.5 °C in 10 mL sperm washing medium. After washing twice by centrifugation (1900×g, 4 min), cryopreserved ejaculated spermatozoa were resuspended with fertilization medium to a concentration of 1×10^6 cells/mL. Fifty microliters of the sperm sample was added to the fertilization droplets containing the oocytes, giving a final sperm concentration of 0.5×10^6 cells/mL. Oocytes were co-incubated with sperm for 4–6 hrs. After fertilization, oocytes were washed three times and cultured in 500 μL PZM3 in 4-well Nunclon dishes (Nunc, Denmark) at 38.5°C in 5% CO_2 in air.

Post-Activation Treatment and Embryo Culture

In a preliminary study we found that SCNT embryos derived from Landrace FFCs treated with 500 nM Scriptaid showed higher developmental competence than controls (unpublished data). Therefore following electrical activation or fertilization the SCNT (male were used as donor cells) or IVF embryos were treated with 500 nM Scriptaid for 14–16 hrs. After treatment, embryos were washed three times in fresh PZM-3 medium, transferred into a 4 well cell culture plate containing 500 μL PZM3 under mineral oil, and then cultured at 38.5 °C in 5% CO_2 in humidified air for 6 days. Cleavage and blastocyst formation were evaluated on Days 2 and 6, respectively, with the day of SCNT or IVF designated Day 0. Day 2 cleavage and day 6 blastocyst rates were used to evaluate *in vitro* development.

Embryo Transfer

Day 1 SCNT zygotes (more than 100) were transferred to the oviducts of surrogates on the day of, or one day after, the onset of estrus. Embryo transfers alternated between treated and control donor cells with the male donor cell ETs completed first and then about 6 months later the female cell ETs were completed. Pregnancy was diagnosed on Day 25 (Day 0 was the day of SCNT), and then was checked regularly at 2-week intervals by ultrasound examination. The cloned piglets were delivered by cesarean section on day 117 of gestation if they didn't start to farrow on their own and hand raised. Animals were treated according to preapproved institutional animal care and use guidelines.

Number of Nuclei in Blastocysts

For embryos derived from IVF, zonae pellucidae were removed by Pronase treatment to eliminate the attached sperm. Expanded day 6 blastocysts derived from IVF or SCNT were selected for cell number comparison. After being fixed in 4% paraformaldehyde in PBS for 15 min at RT, embryos were mounted on slides in mounting medium containing 4,6-diamidino-2-phenylindole (DAPI). At least ten oocytes/embryos were processed for each separate sample and the experiments were replicated three times. Slides were analyzed under an epifluorescent

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microscope (Nikon) equipped with a digital camera. Images were captured and processed using Nikon NIS element software.

Statistical analysis

Experiments were repeated at least 3 times and data expressed as proportions (percentages) were analyzed with chi-squared tests, and number of nuclei was analyzed by ANOVA using SAS 6.12.

Results

1. Effect of Scriptaid on the *in vitro* developmental competence of SCNT embryos reconstructed using NIH miniature inbred FFCs as donor cells

SCNT embryos derived from the NIH miniature inbred donor cells were treated with 500 nM Scriptaid for 14–16 hrs after activation. Development to the blastocyst stage was increased when SCNT embryos were treated with Scriptaid (21% vs. 9%, $P < 0.05$) (Table 1). However Scriptaid treatment had no effect on percentage cleavage at 24 and 48 hr and blastocyst quality as determined by total cell number.

2. Effect of Scriptaid on the full term developmental competence of SCNT embryos reconstructed using NIH miniature inbred FFCs as donor cells

SCNT embryos were transferred to the surrogates to test the effect of Scriptaid treatment on full term development. Scriptaid treatment resulted in a higher percentage of full term development in the treatment group compared to the untreated group. Eight of 10 surrogates (80%) became pregnant and went to term in the Scriptaid treatment group, while only one pregnancy was obtained in the untreated group from 9 ETs (Table 2). Twenty-one live piglets were born from 8 litters, accompanied with a total of 7 mummified fetuses. Two of the live births died soon after birth and 2 with deformed legs were euthanized. This resulted in an overall cloning efficiency 1.3%. However there was the only pregnancy in the untreated group, and it did not contain any viable piglets: only 3 mummies.

3. Effect of Scriptaid on the development of IVF derived embryos

IVF embryos were treated with 500 nM Scriptaid for 14–16 hrs after fertilization. Scriptaid treatment decreased development to the blastocyst stage (37% vs. 26%, $P < 0.01$) (Table 1), but had no effect on cleavage at 24 or 48 hr.

Discussion

Reprogramming events following the transfer of somatic nuclei into oocyte cytoplasm occurs at the epigenetic level [15]. Accumulating evidence suggests that epigenetic reprogramming in the SCNT embryo is defective and abnormal epigenetic modifications such as DNA methylation and histone modifications occur [31–34]. These abnormal epigenetic modifications are likely associated with the overall low success rate of cloning [14].

As described above, increased histone acetylation by TSA could dramatically enhance the developmental competence of SCNT embryos in mouse, pig, cattle and rabbit. However in contrast to these results, Meng et al [22] reported no differences in the cleavage and the blastocyst rates, and the blastocyst cell number between the TSA-treated and the untreated SCNT rabbit embryos; furthermore, both the TSA-treated and the untreated clones can develop to term in rabbits, but all of the offspring from TSA-treated embryos died within an hour to 19 days while four pups of the TSA-untreated group grew into adulthood, and three of them produced progeny. Wu et al [23] also reported that cells treated with 50 ng/ml of TSA resulted in significantly lower blastocyst development (9.9%) than the other experimental and the

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control groups (around 20%) in bovine. TSA is known to be teratogenic [24] and results in a significant reduction of the success rates of cloning when the concentration is high or exposure is long. It is possible that an overdose of TSA may cause developmental defects after implantation. SCNT zygotes treated with TSA for 14 h and 26 h reduced development to the blastocyst stage and fetuses were not obtained [25]. TSA-treatment of 500 nM for 10 h or 50 nM for 20 h to SCNT embryos resulted in more severe placentomegaly [14]. Considering the detrimental effects of the TSA on the cloning procedure, in the present study, we investigated the effect of an HDAC inhibitor, Scriptaid, on the *in vitro* and *in vivo* development potential of SCNT embryos using NIH miniature inbred somatic cells. We found that treatment with Scriptaid greatly enhanced the developmental potential of reconstructed NIH miniature inbred embryos *in vitro* and *in vivo*.

Inbred miniature pigs such as the NIH miniature have been extensively used as a large animal model in many biomedical experiments and may be a source of organs for xenotransplantation to humans. Since we had very low success when we tried to clone a NIH SLA^{cc} miniature pig by adult EFCs, we made FFCs from cloned fetus and then used them as donor cells. In order to test the effects of increased histone acetylation on the somatic cell cloning efficiency of NIH miniature inbred pig, we treated the reconstructed embryos with 500 nM Scriptaid after activation and found the development to the blastocyst stage was significantly improved as compared to the untreated group (23% vs. 9%); what's more, the pregnancy rate of the surrogates was also significantly improved (80% vs. 11%). And 14 healthy piglets were born from 8 litters; higher than the untreated group (1.3 % vs. 0%) ($P < 0.01$). Compared with the cloning efficiency of 0.2% a previous study [4], it is a great improvement in the cloning of this inbred pig.

Several researchers reported successful cloning of miniature pigs. Miyoshi et al. reported successful cloning of Clawn miniature pigs [35], while Li et al. cloned the Chinese Bana miniature pig at cloning efficiency of 0.1%[36]. Yucatan Miniature pigs were cloned successfully by Hao et al at cloning efficiency of 0.5% [37] and by Estrada et al. [7]; Kurome et al showed NIBS strain miniature pigs can be cloned and the cloning efficiency ranged from 0.9% to 7.8% when different donor cells and pig breeds were used as surrogates [38]. Wakai et al reported healthy cloned offspring of Gottingen miniature pigs [39]. However all the miniature pigs described above are outbred. In the mouse, the success of cloning depends on the genetic background [40,41]. To date many of the inbred mouse strains such as C57/B6 and C3H/He, have not been cloned, and while DBA/2 and 129/Sv strains have been cloned, the cloning efficiency of these two inbred strains is extremely low [40,41]. Therefore we assume the extreme low cloning efficiency in the NIH miniature pig may be caused by its inbred genetic background and might be improved by alteration of genomic acetylation patterns. In the present study, after the Scriptaid treatment to the reconstructed embryos, we made an improvement of cloning efficiency on the NIH miniature inbred pigs from zero to 1.3%, this is also higher than our previous results [4]. Combined with genetic manipulation, it will be a great improvement to facilitate the genetic modification of NIH miniature inbred pigs for biomedical research.

Although the mechanism underlying how the Scripatid treatment improves cloning efficiency remains unknown, it is thought that HDACi can induce the hyperacetylation of the core histones, resulting in structural changes in the chromatin that permit transcription as well as enhance DNA demethylation of somatic cell derived genome after nuclear transfer [14], which is a necessary part of genetic reprogramming [42,43]. Increased acetylation of histones leads to relaxed binding of the nucleosome to DNA and/or linker histones, relaxation of the chromatin structure, and formation of a transcriptionally permissive state [44–47]. Histone deacetylation, frequently followed by histone methylation, establishes a base for highly repressive chromatin structures, such as heterochromatin [48]. Scriptaid treatment induced hyperacetylation

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decreases DNA methylation and thus activation of genes which are key for development [49].

When we applied 500 nM Scriptaid to the IVF derived embryos after fertilization, we found this treatment decreased development to the blastocyst stage in IVF pre-implantation embryos. Scriptaid treatment of parthenogenetic activated (PA) embryos also numerically decreased the percentage blastocyst to 19% compared to 29% in the control group (Data not shown). Together, these results suggest that Scriptaid treatment negatively impacts the IVF embryos but is beneficial to SCNT embryos. These results are also similar to the previous report of TSA where it caused detrimental effects and teratogenicity on both pre-[50] and post-implantation development [50,51] in normal fertilized mouse embryos but not PA embryos. This may reflect the difference of chromatin structure between nuclei derived from SCNT, IVF and PA. Similarly, Kishigami et al. reported that TSA treatment inhibited preimplantation development of intracytoplasmic sperm injection (ICSI) embryos but not round spermatid injection (ROSI) or PA embryos and decreased the offspring rate for ICSI but not ROSI produced embryos [52]. Perhaps the negative effect of HDACi is detectable in IVF embryos because the chromatin is already in a state suitable for embryonic development and inhibiting histone deacetylation is detrimental. What's more, during early development of normal IVF embryos, it is believed that a transcriptionally repressive state is essential for the further development in IVF embryos, because relieving this state by inducing histone hyperacetylation with TSA inhibits cleavage of two-cell stage embryos to the four-cell stage [50]. However the current study showed that although the Scriptaid treatment decreased blastocyst development, it did not affect cleavage. The disparate results may be a result of different durations of exposure between our study (14–16 hrs) and Ma et al. (50 hrs) [50], or species-specificity, or the advantages of Scriptaid over TSA.

Here we focused on the application and optimization of an HDACi, Scriptaid, on improving the cloning efficiency of an inbred miniature pig. However further studies are still needed to elucidate which cluster of genes are affected by Scriptaid treatment thus improving the cloning efficiency. In conclusion, the *in vitro* and *in vivo* development competence of SCNT inbred NIH miniature pigs were dramatically improved by Scriptaid treatment.

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Table 1

Scriptaid treatment on the developmental competence of SCNT embryos (male donor cells) using NIH mini FFCs as donors and IVF embryos

Treatment		No.	Reps.	Cleavage		Blastocyst	Total cell No. in blastocyst
				24h	48h	144h	
SCNT	0 nM	171	3	92 (54)[a]	148 (87)[a]	16 (9)[d]	36.8±4.4[a]
	500 nM	155	3	81 (52)[a]	134 (86)[a]	33 (21)[c]	31.8±2.3[a]
IVF	0 nM	169	3	110 (65)[a]	134 (79)[a]	63 (37)[a]	47.8±3.3[a]
	500 nM	183	3	120 (66)[a]	142 (78)[a]	47(26)[b]	55.5±4.6[a]

Values with different superscripts within a column are significantly different ([a–b] $P < 0.05$; [c–d] $P < 0.05$) (developmental competence of embryos from different concentration in NT and IVF groups were compared individually)

Cleavage percentage: No. of embryos cleaved/No. of embryos cultured

Blastocyst percentage: No. of blastocysts/No. of embryos cultured

Cell Number in blastocyst: Mean ± SEM

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Table 2

Full term development of NT derived embryos using NIH miniature FFCs as donors following Scriptaid treatment

ET No.	Donor Cells	Transferred Embryos No.	Scriptaid treatment	42d Pregnant	Piglets born	Cloning efficiency
1	Male	190	+	+	1+4 mummies	
2	Male	109	+	−	/	
3	Male	154	+	+	1+3 mummies	
4	Male	174	+	+	2 live + 2 died after birth	
5	Male	152	+	+	2	1.3%[a]
6	Male	190	+	+	2	
7	Female	136	+	+	2+1 died after birth	
8	Female	160	+	+	2	
9	Female	169	+	+	5+1 died after birth	
10	Female	176	+	−	/	
11	Male	140	−	−	/	
12	Male	163	−	−	/	
13	Male	161	−	−	/	
14	Male	151	−	−	/	
15	Male	151	−	−	/	0%[b]
16	Female	146	−	−	/	
17	Female	161	−	+	3 mummies	
18	Female	138	−	−	/	
19	Female	178	−	−	/	

+: Scriptaid treated or pregnant

−: Untreated or not pregnant

Cloning efficiency: No. of piglets /No. of embryos transferred

Values with different superscripts within a column are significantly different ($P<0.01$)