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RESEARCH ARTICLE

Comparison of birth weight and umbilical and placental characteristics of cloned and artificial insemination-derived piglets

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Abstract Somatic cell nuclear transfer (SCNT)-derived piglets have significantly higher stillbirth rate and postnatal mortality rate than artificial insemination (AI)-generated piglets. The question whether the low survival rate of SCNT piglets was related to birth weight, umbilical cord or placenta development was investigated. In this study, stillbirth rate, neonatal death rate, birth weight, umbilical cord status, placental parameters and placental gene expression patterns were compared between SCNT and AI piglets. Results showed that mortality rates at birth and during the neonatal stage of SCNT piglets were significantly higher than those of AI piglets. The incidence of abnormal umbilical cord in SCNT and SCNT-liveborn (SCNT-LB) piglets was significantly higher than in AI and AI-liveborn (AI-LB) piglets. Birth weight, placental weight, placental surface area and placental efficiency in SCNT and SCNT-LB piglets were significantly lower than those of AI and AI-LB piglets. Placental expression profiles of imprinting, angiopoiesis and nutrient transport-related genes were defective in SCNT-LB piglets compared with those in AI-LB piglets. Thus, the low survival rate of SCNT piglets may be associated with abnormal umbilical cord and placenta development. These characteristics may have resulted from aberrant expression of angiogenesis, nutrient transport, and imprinting-related genes in the placentas.

Keywords cloned, pig, death, placenta, SCNT, umbilical cord

1 Introduction

Pig somatic cell nuclear transfer (SCNT) technique have valuable applications in agriculture, bioscience, and biomedicine[1–5]. However, SCNT-generated cloned porcine embryos only have a full-term developmental rate of approximately 1%[6], which is considerably lower than the developmental efficiency of *in vivo* fertilized pig embryos[7]. Furthermore, previous reports indicated that the cloned pigs were had stillborn rates of 17%–32.8%[8–12] and 48%–74.5% mortality from birth to weaning[12–14], which is much higher than in vivo fertilization-derived piglets (3%–8% and 10%–18%, respectively)[15–17].

Low birth weight, which parallels intrauterine growth retardation (IUGR), is believed to be related to the survival ability of the fetus in the uterus and/or after birth[18–20]. Placental dysfunction is also considered the major cause of IUGR, late fetal loss, and postnatal death[14,21–24]. In addition, umbilical cord malformation is associated with fetal death and neonatal mortality[12,14,25]. Therefore, the high stillbirth rate and postnatal death rate observed in SCNT piglets could be related to retarded intrauterine growth and abnormal placenta and umbilical cord development.

Relatively limited information is available in the literature, however, on the assessment of birth weight and placenta and umbilical cord development in cloned piglets. In this study, we compared the birth weight, umbilical cord status, placental traits and placental gene expression patternss between SCNT and artificial insemination (AI)-derived piglets.

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2 Materials and methods

2.1 Production of piglets by SCNT and AI

Piglets were produced following the protocols described in a previous report[14].

2.2 Placenta and umbilical cord collection and analysis

At birth, placental umbilical cord with a number representing the birth order of a piglet was used to distinguish individual placentas. This method was adapted from Wilson et al.[26]. Briefly, one assistant gently held the piglets to prevent umbilical breakdown caused by the movement of the piglets at birth. A prenumbered piece of sterile surgical thread was tied to the umbilical cord end close to the placenta, while a second assistant tied a sterile surgical thread round the cord at about 4 cm from the umbilicus of piglets to prevent blood loss. The cord was then cut between two ligatures, and the tagged umbilical cord was allowed to retract back into the uterus. After placental expulsion, the placentas were dissected and analyzed. The piglets were immediately dried and weighed, and birth order was recorded corresponding to the tagged umbilical cord on the placenta. After expulsion, individual placentas were dissected from secundines, and placental tissue samples were immediately collected from the center of individual placenta, washed with PBS and stored in liquid nitrogen. Each placenta was weighed after the attached amniotic membrane, necrotic tips of a vascularized chorion and umbilical cord were removed. Each placenta was then spread unwrinkled on paper, and the placental surface area was estimated by doubling the area on the paper bounded by a line traced around the placenta. Placental efficiency was defined as the ratio of piglet birth weight to placental weight. Placental tissue samples were used to analyze gene expression. Additionally, the umbilical cord status of piglets during farrowing was recorded to obtain the proportion of abnormal umbilical cords in piglets.

2.3 Quantitative real-time PCR

Total RNA was extracted from placenta tissue using Total RNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA). The RNA concentration was measured by Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). RNA quality was analyzed using nondenaturing agarose gel electrophoresis. Only RNA samples that did not show signs of degradation were used and stored at –80°C. Subsequently, cDNA was synthesized from 1 μg of total RNA using PrimeScript RT Reagent Kit (Takara Bio, Otsu, Shiga, Japan). The cDNA was stored at –20°C for further analysis. Quantitative real-time PCR was performed using SYBR Select Master Mix Kit (Thermo Fisher Scientific, Waltham, MA, USA) and Eco™ Real-Time PCR System (Illumina, San Diego, CA, USA). The primer information for target genes is presented in Table 1. Each reaction mixture (10 μL) contained 1 μL of cDNA solution, 0.3 μL of 10 mmol·L^{-1} of each specific primer, 5 μL of SYBR Select Master Mix, and 3.4 μL of ddH_2O. The reactions were run as follows: initial denaturation at 95°C for 5 min; followed by 40 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s; and finally a melting cycle at 95°C for 15 s, 55°C for 15 s, and 60°C for 15 s. The transcripts of all genes were quantified in triplicates. Specificity of the PCR reaction was confirmed through a single peak in the melting curve. *β-actin* was used as an endogenous control to normalize gene expression throughout this study. The relative gene expression level of the target gene was calculated through the $2^{-\Delta\Delta C_T}$ method.

2.4 Statistical analyses

Differences in the survival rate and incidence of abnormal umbilical cord (AUC) between SCNT and AI groups were analyzed by χ^2 tests. Differences in birth weight, placental traits, and placental gene expression levels between the SCNT and AI groups were analyzed by one-way ANOVA using the SPSS 19.0 software (IBM Corp., Armonk, NY, USA). Values are presented as mean±SEM. Significant difference of means between two different groups was determined at $P < 0.05$.

3 Results

A total of 111 male piglets were delivered by 20 artificially inseminated sows, and 58 male cloned piglets were farrowed from 13 recipient sows (Table 2). The stillbirth rate and postnatal death rate of SCNT piglets were significantly higher than those of AI piglets (stillbirth rate, 20.7% vs. 5.4%, $P = 0.002$; postnatal death rate, 65.2% vs. 5.7%, $P < 0.001$) (Table 2).

The umbilical cords of newborn piglets were divided into normal umbilical cord and AUC according to their characteristics (Fig. 1). The incidence of AUC in SCNT piglets was significantly higher than that in AI piglets (39.7% vs. 10.8%, $P < 0.001$). SCNT-liveborn (SCNT-LB) piglets also exhibited a significantly higher AUC frequency than AI-liveborn (AI-LB) piglets (32.6% vs. 14.2%, $P = 0.009$). However, the SCNT-stillborn (SCNT-SB) group and AI-stillborn (AI-SB) group showed no significant differences in the incidence of AUC (66.7% vs. 33.3%, $P = 0.18$) (Table 3).

Birth weight, placental surface area, placental weight, and placental efficiency of the AI and AI-LB groups were significantly higher than those of the SCNT and SCNT-LB groups ($P < 0.001$) (Table 4). Birth weight, placental surface area, and placental weight of AI-SB piglets were

Table 1 Sequences of primers used for the analysis of gene expression in the placenta

Gene	Sequence (5′–3′)	Product size/bp	GenBank accession no.
BCL-2	F: TTGCCGAGATGTCCAGCCA	255	XM_003121700.4
	R: CATCCCAGCCTCCGTTATCCT		
BAX	F: AAGCGCATTGGAGATGAACT	251	XM_013998624.1
	R: CGATCTCGAAGGAAGTCCAG		
VEGFA	F: GCCTTGCTGCTCTACCTCCA	271	NM_214084
	R: TGGCGATGTTGAACTCCTCAGT		
VEGFR2	F: GAGTGGCTCTGAGGAACGAG	209	BQ603967
	R: ACACAACTCCATGCTGGTCA		
PHLDA2	F: TCAAGGTGGACTGCGTGGAG	147	NM_001174057
	R: GGCGGTTCTGGAAGTCGATGA		
CDKN1C	F: TGGACCACGAGGAGCTGAGT	100	HQ679903
	R: GGCACGTCCTGCTGGAAGTT		
IGF2	F: CGTGGCATCGTGGAAGAGTG	168	X56094
	R: CCAGGTGTCATAGCGGAAGAAC		
H19	F: GGCCGGAGAATGGGAAAGAAGG	148	AY044827
	R: CGCAGTGCTGCGTGGGAACG		
PEG3	F: GGAGTGTGCGGAGACCTTCA	118	EF619475
	R: CTCGGTGGGATGGGAGTTCT		
GRB10	F: GGTCCGTGCATCGTTCAGA	101	NM_001134965
	R: TCCAACAAACCAGCCAACCT		
SLC2A1	F: GCAGGAGATGAAGGAGGAGAGC	258	EU012358
	R: ACGAACAGCGACACGACAGT		
SLC2A3	F: GCCCTGAAAGTCCTCGGTTCCT	252	XM_003355585
	R: ACACGGCGTTGATGCCAGAGA		
SLC38A4	F: CGTGGTCATGGTGCCCAACAAC	118	XM_021092582
	R: ACTGCCGTGAAGAGAGCCCTTG		
β-actin	F: CCACGAGACCACCTTCAACTC	131	DQ845171
	R: TGATCTCCTTCTGCATCCTGT		

Table 2 Comparison of stillbirth rate and postnatal death rate between SCNT-derived and AI-derived piglets

Item	No. of litters	Total piglets born	Stillborn	Postnatal death
AI	20	111[#]	6 (5.4/%)	6 (5.7/%)[*]
SCNT	13	58	12 (20.7/%)	30 (65.2/%)
P-value	–	–	0.002	< 0.001

Note: [#]Only male AI-derived piglets were included in this table, because SCNT-derived piglets were cloned from male donor cells. [*]Postnatal death and survival were only recorded four days after birth.

also significantly higher than those of SCNT-SB piglets ($P < 0.05$), but placental efficiency was not different between the two groups ($P > 0.05$) (Table 4)

Placental mRNA expression levels of six imprinted genes and seven non-imprinted genes related to angiogenesis, nutrient transport, and apoptosis were examined and compared between the AI-LB and SCNT-LB groups. Expression levels of angiogenesis-related vascular endothelial growth factor receptor 2 (*VEGFR2*), nutrient transport-related solute carrier family 2 member 3 (*SLC2A3*) and solute carrier family 38 member 4 (*SLC38A4*) in SCNT-LB placentas were significantly lower than those in AI-LB placentas ($P < 0.05$). However, expression levels of angiogenesis-related vascular

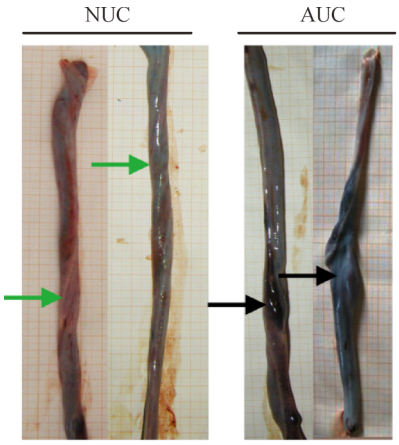

Fig. 1 Phenotype of normal umbilical cord (NUC) and abnormal umbilical cord (AUC) in newborn piglets. NUCs are light red and spiraled (green arrow). AUCs are dark, are not spiraled, and have severe occlusive thrombus (black arrow).

endothelial growth factor A (*VEGFA*) and nutrient transport-related solute carrier family 2 member 1 (*SLC2A1*) were not different between the two groups ($P > 0.05$). Expression levels of apoptosis regulator *BCL-2* and BCL2 associated X (*BAX*) were also not different between the two groups ($P > 0.05$). Among the six investigated imprinted genes, *H19*, growth factor receptor bound protein 10 (*GRB10*) and pleckstrin homology-like domain family A member 2 (*PHLDA2*) had significantly lower mRNA expression levels, whereas paternally expressed 3 (*PEG3*) had significantly higher mRNA expression levels in SCNT-LB placentas than in AI-LB placentas ($P < 0.05$). However, no difference was found in the mRNA expression level of insulin-like growth factor 2 (*IGF2*) and cyclin-dependent kinase inhibitor 1C (*CDKN1C*) between the SCNT-LB and AI-LB placentas ($P > 0.05$) (Fig. 2).

4 Discussion

In this study, the SCNT piglets had 20.7% stillbirth rate, which is similar to that (17%–24%) reported by Estrada et al.[10], Kurome et al.[11], and Park et al.[12]. Moreover, the neonatal mortality rate of SCNT-LB piglets reached 65.2%. This remarkably high death rate of neonatal cloned

Table 3 Comparison of the frequency of abnormal umbilical cords (AUC) between AI and SCNT piglets

Item	Total piglets	Piglets with AUC
AI	111	17 (10.8%)
SCNT	58	23 (39.7%)
P-value	–	< 0.001
AI-LB	105	15 (14.2%)
SCNT-LB	46	15 (32.6%)
P-value	–	0.009
AI-SB	6	2 (33.3%)
SCNT-SB	12	8 (66.7%)
P-value	–	0.18

Table 4 Comparison of birth weight and placental parameters between AI and SCNT piglets

Item	Birth weight/g	Placental weight/g	Placental surface area/cm^2	Placental efficiency/(g·g^{-1})$^{\#}$
AI (n = 111)	1430±35	177±5	2530±69	8.41±0.16
SCNT (n = 58)	1040±47	147±6	1850±76	7.17±0.21
P-value	< 0.001	< 0.001	< 0.001	< 0.001
AI-LB (n = 105)	1440±36	177±6	2530±71	8.43±0.17
SCNT-LB (n = 46)	1130±41	157±6	1950±74	7.36±0.24
P-value	< 0.001	0.036	< 0.001	< 0.001
AI-SB (n = 6)	1400±127	180±20	2500±356	7.79±0.29
SCNT-SB (n = 12)	714±127	107±13	1450±203	6.42±0.49
P-value	0.004	0.007	0.012	0.082

Note: Values are presented as mean±SEM. $^{\#}$Placental efficiency represents the ratio of birthweight to placental weight

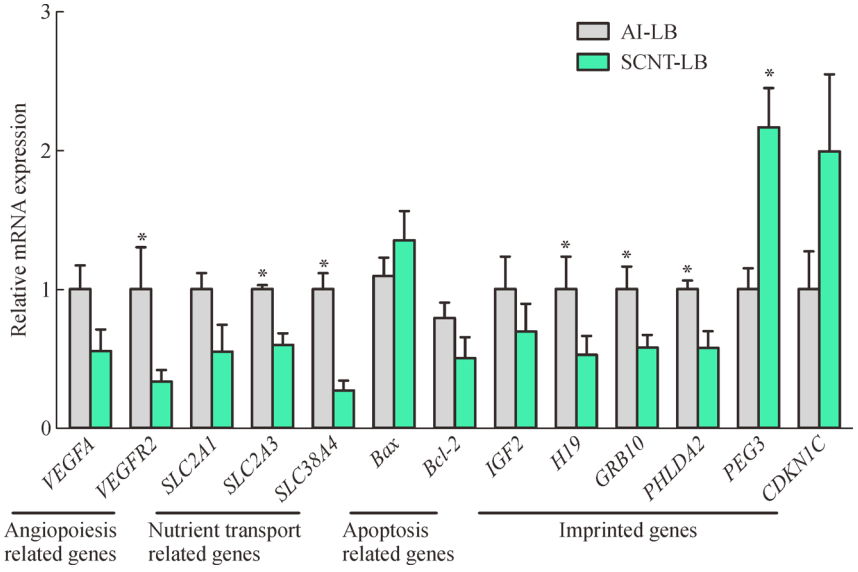

Fig. 2 Comparison of placental gene expression levels between artificially insemination-derived and somatic cell nuclear transfer-derived liveborn piglets (AI-LB and SCNT-LB, respectively). The expression levels were normalized against *β-actin*. Values are presented as mean±SEM. Values labeled with asterisk (*) were considered significantly different between two groups ($P < 0.05$).

pigs was also consistent with previous studies[12,13,27]. These data indicated that a large portion of cloned piglets die during late gestation period, at birth, and the during neonatal stage.

The high incidence of AUC could be one of the factors responsible for the high stillbirth rate and neonatal death rate in SCNT piglets. AUC would restrict blood flow or oxygen transport to the fetuses, leading to intrauterine asphyxia or hypoxic injury which could result in stillbirth and/or postnatal mortality[28–30].

Placental deficiency is also a significant cause of stillbirth and even postnatal death[21,31,32]. In this study, placental surface area, placental weight and placental efficiency of SCNT piglets were significantly lower than those of AI piglets. These results suggest that the high death rate at birth and during neonatal stage of SCNT piglets could be attributed to abnormal placenta development.

Imprinted genes play critical roles in the regulation of placenta development[33–35]. The results showed that four examined imprinted genes (*H19*, *GRB10*, *PHLDA2* and *PEG3*) exhibited aberrant expression patterns in the placentas of the SCNT piglets. Defective expression of imprinted genes could be related to the abnormal placenta development observed in the SCNT piglets. In addition, the expression levels of angiopoiesis-related *VEGFR* and nutrient transport-related *SLC2A3* and *SLC38A4* in the placentas of SCNT piglets were significantly reduced. This phenomenon implies that the SCNT-derived fetuses in the uterus were in a state of malnutrition, which not only causes low birthweight but also leads to stillbirth and/or postnatal mortality.

5 Conclusions

In summary, SCNT piglets exhibited high stillbirth rate and neonatal death rate. The low survival rate of SCNT piglets was associated with AUC and abnormal placenta development. These anomalies could have resulted from the aberrant expression of angiogenesis, nutrient transport and imprinting-related genes in the placentas.

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Compliance with ethics guidelines Zheng Ao, Chengfa Zhao, Yanmin Gan, Xiao Wu, Junsong Shi, Enqin Zheng, Dewu Liu, Gengyuan Cai, Zhenfang Wu, and Zicong Li declare that they have no conflicts of interest or financial conflicts to disclose.

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

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