The pathophysiological changes associated with neonatal death of cloned pigs

Zheng Ao^{1,2,3,*}, Ting Gu^{1,3,*}, Huaxing Zhao^{1,3}, Junsong Shi⁴, Enqin Zheng^{1,3}, Gengyuan Cai^{1,3}, Zhenfang Wu^{1,3} and Zicong Li^{1,3}

¹National Engineering Research Center for Breeding Swine Industry, College of Animal Science, South China Agricultural University, Guangzhou, China, ²Key Laboratory of Animal Genetics, Breeding and Reproduction in the Plateau Mountainous Region, Ministry of Education, College of Animal Science, Guizhou University, Guizhou, China, ³Guangdong Provincial Key Laboratory of Agro-animal Genomics and Molecular Breeding, College of Animal Science, South China Agricultural University, Guangdong, China and ⁴Wen's Research Institute, Guangdong Wen's Foodstuff Group, Ltd., Guangdong, China

Correspondence should be addressed to Z Wu or Z Li; Email: wzfemail@163.com or lizicong@scau.edu.cn

Abstract

Cloned pigs generated by the somatic cell transfer nuclear (SCNT) technique are highly valuable for agriculture, biomedicine, and life sciences. However, the neonatal mortality rate of cloned pigs is very high. The reasons causing the massive loss of cloned pigs during their neonatal ages are unclear. In the present study, we found that the neonatal death of cloned pigs was associated with aberrant purine metabolism, impaired renal morphology and function, and decreased hepatic *Hprt1* expression. The downregulation of *Hprt1*, a key purine metabolism regulation gene, in the liver was responsible for the elevation of an important purine metabolite, uric acid, in the serum, causing abnormalities in kidney morphology and function and leading to death of neonatal cloned pigs. This study provided insights into the pathophysiological mechanisms underlying the neonatal death of clone pigs, and results will help improve their survival rate.

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Introduction

Cloned pigs, especially genetically modified cloned pigs that are generated by the somatic cell transfer nuclear (SCNT) technique, are highly valuable to the swine industry, biomedicine, and life sciences (Yang & Wu 2018). However, the neonatal death rate of cloned piglets is extremely high, which reaches 47.8–74.5% (Jiang *et al.* 2008, Kurome *et al.* 2013, Huan *et al.* 2015, Schmidt *et al.* 2015). The neonatal death of cloned pigs mainly occurs within 4 days after birth (Ao *et al.* 2017). Most of the cloned piglets that died shortly after birth had various malformations (Jiang *et al.* 2008, Park *et al.* 2010, Schmidt *et al.* 2015). The massive loss of cloned pigs during neonatal age severely limits their application. The mechanisms underlying the high mortality rate of neonatal cloned pigs remain unknown.

Serum contains a wide range of metabolites involved in numerous biochemical and biophysiological processes in the animal body (Psychogios *et al.* 2011). Analysis of dynamic alteration of serum metabolic profiling has become an important approach in monitoring the state of biological organisms and elucidates the physiological and pathological mechanisms related

to various diseases (Zhang *et al.* 2012, Mussap *et al.* 2013, Yi *et al.* 2016). Currently, metabolomics is a powerful tool for exploring global metabolite changes in biological systems by analyzing the metabolites with regard to various exposures or disease states (Johnson *et al.* 2016). Metabolomics analysis is particularly important in neonatal and pediatric studies for the early diagnosis and identification of a variety of neonatal complications, such as intrauterine growth retardation (IUGR) (Marincola *et al.* 2015), inborn errors of metabolism (Vernon 2015), perinatal asphyxia (Ahearne *et al.* 2016), kidney disease (Noto *et al.* 2016), and bronchopulmonary dysplasia (Rivera *et al.* 2016).

To investigate the pathophysiological mechanisms underlying the high neonatal death rate of cloned pigs, we compared the serum metabolomic differences among dead SCNT-derived, survived SCNT-derived, and survived artificial insemination (AI)-produced neonatal pigs, and explored the possible causes of neonatal loss of cloned pigs. We found that neonatally deceased cloned pigs exhibited abnormal purine metabolism and impaired renal morphology and function, which might be caused by decreased expression of hepatic *Hprt1*.

Materials and methods

Ethics statement

All animal procedures used in this study were conducted in accordance with "The Instructive Notions with Respect to Caring for Laboratory Animals," issued by the Ministry of Science and Technology of China and were approved by the Institutional Animal Care and Use Committee of South China Agricultural University (Animal protocol approval number: 2017K006). All efforts were made to minimize animal suffering.

Serum sample collection and preparation

SCNT-derived and AI-derived pigs were produced as described in a previous study (Ao *et al.* 2017). The semen used for AI and the donor cells used for SCNT were derived from a same Duroc boar. SCNT recipient sows and artificially inseminated sows were raised at a same pig farm under the same conditions. All the piglets were delivered by spontaneous farrowing at around 114 days of gestation. After birth, the piglets were dried by towel immediately and placed in an incubator with temperature of 30°C for 1 h, and then they were assisted to suck colostrum. Piglets were injected intramuscularly with 200 mg iron dextran at 24 h after birth to prevent anemia. At delivery, piglets are held firmly by an assistant to prevent umbilical cord rupture. Umbilical cord was bound with a numbered sterile thread to distinguish the placenta of each piglet. After expulsion of the placentas, the attached amniotic membrane, necrotic tips of avascularized chorion, and umbilical cord on placenta were removed. Then, placental weight and surface area were measured using a previously described method (Baxter *et al.* 2008). Fresh blood was collected from the anterior vein of live piglet at 36 h after birth. Anterior vein serum was prepared from blood by centrifugation at 1,600 *g* for 15 min and were immediately snap-frozen in liquid nitrogen and stored at −80°C. Survival and morbidity status of each piglet were consistently tracked and recorded every hour after blood collection. Piglets were divided into three groups according to their survival and morbidity status at 4 days after birth, as follows: the SCNT-derived male piglets that died within 4 days (SCNT-DW4); SCNT-derived male piglets that survived over 4 days (SCNT-SO4); and AI-generated male piglets that survived over 4 days (AI-SO4). Renal and liver tissues were immediately collected from the dead SCNT-DW4 piglets and the terminated AI-SO4 piglets at 4 days after birth.

Prior to metabolomics analysis, all serum samples were thawed at 4°C, and each serum sample (200 µL) was added to a 1.5 mL Eppendorf tube with 10 µL of 2-chloro-L-phenylalanine (0.3 mg/mL) dissolved in methanol as the internal standard, and the tube was vortexed for 10 s. Subsequently, the samples were mixed with 240 µL ice-cold mixture of methanol and acetonitrile (2/1, v/v), vortexed, and centrifuged (13,000 *g*, 15 min, 4°C). The supernatants (200 µL) from each tube were collected using crystal syringes, filtered through 0.22 µm microfilters, and transferred to liquid chromatography (LC) vials. The vials were stored at −80°C until LC-mass spectrometry (LC-MS) analysis. Quality control (QC) samples were prepared by mixing aliquots of the all samples to create the pooled sample.

LC-MS/MS analysis

The prepared samples were analyzed by an ACQUITY ultra-performance liquid chromatography (UPLC) I-Class system (Waters Corp., Milford, USA) coupled with QTOF Mass spectrometer (Waters Corp.) following the manufacturer's instructions. An ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 µm) maintained at 45 °C was used for the reversed phase separation with a mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) under the following elution gradient: 0 min, 1% B; 1 min, 30% B; 2.5 min, 60% B; 6.5 min, 90% B; 8.5 min, 100% B; 10.7 min, 100% B; 10.8 min, 1% B and 13 min, 1% B. The flow rate was 0.4 mL/min, and the injection volume per sample was 2 µL. The autosampler temperature was set at 4°C. A high-resolution tandem mass spectrometer Xevo G2-XS QToF (Waters Corp.) was used to detect the compounds eluted from the column using electrospray ionization (ESI) in both positive and negative modes. Data acquisition was performed in full scan mode (*m/z* ranges from 50 to 1000 Da) with a scanning time of 0.1 s. The optimized conditions of QTOF/MS were as follows: capillary voltage, 3 kV/−2 kV; source temperature, 120°C; sampling cone, 40 V; and cone gas flow, 50 L/h. The desolvation temperature and flow rate were 450°C and 800 L/h, respectively. The mean square error (MSE) model was selected for acquisition. The lower collision energy was 6 V, and the higher collision ramp energy was 20–35 V. During the acquisition, the leucine-enkephalin (LE) signal was acquired every 3 s to calibrate the mass accuracy. Furthermore, QC sample (from the pool of all samples) was injected after every eight samples to evaluate the stability of the LC-MS during the entire acquisition.

Data processing and metabolite identification

The acquired LC-MS raw data were analyzed by the Progenesis QI 2.3 software (Waters) for data processing. Data pretreatment of raw UPLC-MS data included peak picking, peak grouping, retention time (RT) correction, second peak grouping, and annotation of isotopes and adducts. The Excel file was obtained with three-dimensional data sets, including *m/z*, peak RT, peak intensities, and RT-*m/z* pairs, which were used as the identifier for each ion. The resulting matrix was further reduced by removing peaks with more than 80% missing values (ion intensity=0) and those with isotope ions from each group. The adduct ion forms, including [M+H-2H₂O]⁺, [M+H]⁺, [M+K]⁺, [M+Na]⁺, [M+HH₄]⁺, [2M+H]⁺, [2M+NH₄]⁺, [2M+Na]⁺, and [2M+k]⁺ in the positive ion mode and [M-H₂O-H][−], [M-H][−], [2M-H][−], and [2M+FA-H][−] in the negative ion mode, were selected based on their ionization behaviors. Each retained peak was normalized to the QC sample using robust Loess signal correction. A threshold of 40%, which was accepted as a standard in the assessment of repeatability in metabolomics data sets, was set for the relative standard deviation values of metabolites in the QC samples. The resulting data were imported into SIMCA 14.0 software (Umetrics, Sweden). Principle component analysis (PCA) and partial least-squares-discriminant analysis (PLS-DA) were carried out to visualize the metabolic alterations among the experimental groups

after mean centering (Ctr) and Pareto variance (Par) scaling, respectively. The reliability of the models was assessed with the relevant R^2 and Q^2 . The performance of the PLS-DA models was evaluated using permutation testing (200 permutations). The variable influence on projection (VIP) from the PLS-DA model with values >1.0 was set as the threshold for selecting statistically significant metabolites. Then, *t*-test and fold change (FC) analysis were performed to measure the significance of metabolic features from the preprocessed UPLC-MS datasets. Our screening requirement for differential metabolites was $FC > 1.2$ or < 0.8333 , P value < 0.05 and $VIP > 1.0$. Afterward, Human Metabolome Databases (HMDB) (<http://www.hmdb.ca/>) and the Lipid Maps Database (<http://www.lipidmaps.org/tools/>) were used for serum metabolite identification. A combination of database queries was performed using the exact molecular masses and fragmentation traits.

Serum biochemical analysis

Serum uric acid, creatinine, and urea nitrogen levels were analyzed by HITACHI 7020 automatic chemistry analyzer (Hitachi). Test reagent kits were used according to the manufacturer's instructions (Kehua, Shanghai, China).

Enzymatic determination in serum

Hypoxanthine phosphoribosyltransferase 1 (HPRT1) and phosphoribosyl pyrophosphate synthetase 1 (PRPS1) levels in the serum samples were measured using commercially available enzyme immunoassay kits (Jianglai, Shanghai, China) according to the manufacturer's instructions.

Histopathological analysis of kidney

The renal tissues were fixed with 4% paraformaldehyde and was embedded in paraffin for histological analysis. The embedded sections were stained with hematoxylin & eosin (HE) based on standard histological procedures (Hong *et al.* 2014). The morphological changes in the renal tissue were observed using a Nikon 80i light microscope (200× magnification) fitted with a Nikon (DS-Fi1) digital camera (Nikon).

Quantitative real-time PCR

Total RNA was extracted from liver tissues using Total RNA Kit (Omega Bio-tek, GA, USA). The cDNA was synthesized from 1 µg of total RNA using the PrimeScript RT Reagent Kit (Takara). Quantitative real-time PCR was performed using the SYBR Select Master Mix Kit (Thermo Fisher Scientific) and QuantStudio™ 7 Flex Real-Time PCR System (Thermo Fisher Scientific). Primer information for target genes is presented

in Table 1. Each reaction mixture (10 µL) contained 1 µL of the cDNA solution, 0.3 µL of 10 mM of each specific primer, 5 µL of SYBR Select Master Mix, and 3.4 µL of ddH₂O. The reactions were run as follows: initial denaturation at 95°C for 5 min; 40 cycles of denaturation at 95°C for 10 s; annealing at 60°C for 15 s with an extension at 72°C for 20 s; and finally, a melting cycle consisting of 95°C for 15 s, 55°C for 15 s, and 60°C for 15 s. The relative expression levels of target genes were calculated with $2^{-\Delta\Delta C_t}$ method and normalized to β -Actin. All gene expression experiments were conducted in triplicate. Each pair of primers was confirmed for equal amplification efficiency for serial dilutions of cDNA sample in accordance to a previously reported method (Dhanasekaran *et al.* 2010).

DNA preparation and DNA methylation measurements

Genomic DNA was extracted from liver tissues using the DNA extraction kit (Bio-tek Corporation, Beijing, China) according to the manufacturer's instruction. A Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific) was used to measure DNA concentration of each liver sample. The CpG island of the promoter region (between -1500 and +1000 bp from transcription start site, chromosome X:110319704-110322204) was analyzed through the website (http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/) (Fig. 6A). Then, the upstream and downstream primers for quantitative methylation analysis were designed by using the EpiDesigner (Agena Bioscience, San Diego, CA, USA). The primer information was as follows: forward, 5'-TTTTTTTGTGGTTTAAATTGGG-3' and reverse, 5'-AAATAAAAAACAATTAAATATCCCC

TC-3'. Quantitative methylation analysis was performed using the MassARRAY platform (Agena Bioscience, San Diego, CA, USA), which relies on uracil-specific cleavage followed by matrix-assisted laser desorption/ionization-time of flight-mass spectrometer (MALDI-TOF-MS). Genomic DNA was treated by NaHSO₃ (Zymo, Orange, CA, USA) through the following reactions: PCR amplification (94°C for 4 min; 45 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 1 min; then 72°C for 3 min); shrimp alkaline phosphatase (SAP) reaction (37°C for 20 min, 85°C for 5 min); and finally base specific cleavage reaction (37°C for 3 h). Subsequently, the resins were used to purify the abovementioned products that were then spotted on a 384-well SpectroCHIP® bioarray (Agena Bioscience). The SpectroCHIP were analyzed using MassARRAY system, and methylation levels of each CpG site were obtained using the EpiTYPER software (Agena Bioscience).

Statistical analyses

The differences in birth weight, placental traits, serum biochemical indexes, serum enzymatic levels, mRNA

Table 1 Primers used for qPCR.

Genes	Primer sequences (5' to 3')		Product size (bp)	GenBank accession no.
	Forward	Reverse		
<i>Hprt1</i>	CCCAGCGTCGTGATTAGTGATG	CAAGCCGTTCACTCTGTCCAT	133	NM_001032376
<i>Prps1</i>	CCGATGCTGGAGGAGCTAAGAG	TCCGTGAGTCAGGATTGCGTAA	245	XM_003135301
<i>Actb</i>	CCACGAGACCACCTCAACTC	TGATCTCCTTCTGCATCCTGT	131	DQ845171

expression levels, and methylation levels among the different groups were statistically analyzed by one-way ANOVA using the SPSS 19.0 software (IBM Corp.). All data are presented as mean $\pm$ S.E.M.. Significant difference of means between two different groups was determined at $P < 0.05$.

Results

Differences in malformation rate, neonatal survival rate, birth weight, and placental parameters between SCNT and AI piglets

We obtained 12 AI-derived male piglets farrowed by 3 sows and 18 SCNT-derived male piglets delivered from 5 recipient sows (Table 2). All 12 AI piglets survived over 4 days (SO4) after birth. Among the 18 cloned piglets, 12 died within 4 days (DW4) after birth, and 6 survived over 4 days after birth. No malformation was observed in all 12 AI piglets, but phenotypic malformations, such as macroglossia, umbilical hernia, limb hyperflexion, hypoplastic testis, were found in 7 newborn SCNT piglets, which all died within 4 days after birth (Table 2). The birth weight, placental surface area, placental weight, and placental efficiency were similar between AI-SO4 and SCNT-SO4 piglets, but these developmental indexes were significantly higher in AI-SO4 and SCNT-SO4 piglets than in SCNT-DW4 piglets (Table 3).

Differences in serum metabolite profiles among SCNT-DW4, SCNT-SO4 and AI-SO4 piglets

As shown in Supplementary Figs 1 and 2 (see section on supplementary data given at the end of this article), the total ion chromatograms (TIC) and PCA score plot of QC samples revealed that the chromatographic separation had acceptable stability and reproducibility. A total of 15,588 ions (9586 in positive and 6002 in negative ion mode) were selected for the subsequent screening process. The PCA and PLS-DA score plots of acquired normalized data showed that the SCNT-SO4 group was closer to the AI-SO4 group than to the SCNT-DW4 group (Fig. 1). This suggested that the SCNT-SO4 and AI-SO4 groups had a more similar serum metabolite profile compared with the SCNT-DW4 group. The PLS-DA model demonstrated a more explicit separation among the three piglet groups than the PCA model (Fig. 1). Therefore, significantly different ions between SCNT-DW4 and AI-SO4 piglets and those between SCNT-DW4 and SCNT-SO4 piglets were analyzed by the PLS-DA model and subjected to subsequent analyses.

By comparing the exact ion masses and their fragmentation patterns with those from the HMDB and LipidMaps database, 95 and 68 differential serum metabolites were identified between SCNT-DW4 and AI-SO4 piglets, and between SCNT-DW4 and SCNT-SO4 piglets, respectively. Among them, 31 differential serum metabolites were common between AI-SO4 and SCNT-SO4 piglets compared with SCNT-DW4 piglets (Table 4). Among these 31 common differential serum metabolites, 12 were upregulated and 19 were downregulated in SCNT-DW4 piglets, compared with SCNT-SO4 and AI-SO4 piglets (Table 4). Pathway analysis of the 31 common differential serum metabolites showed that three and two common differential serum metabolites were significantly enriched in the purine metabolism and the steroid hormone biosynthesis pathways, respectively (Fig. 2). The serum levels of all five common differential serum metabolites enriched in two pathways, including uric acid, xanthosine, deoxyinosine, cortol, and tetrahydrocortisone, were dramatically elevated in SCNT-DW4 piglets compared with those in SCNT-SO4 and AI-SO4 piglets (the FC numbers are shown in Table 4). The abnormal steroid hormone biosynthesis found in SCNT-DW4 piglets could be related to the dysregulation of the expression of some steroid hormone biosynthesis enzyme genes in the placenta and the level of some steroid hormones in the amniotic fluid of cloned pig fetuses, as we previously reported (Ao *et al.* 2019). Therefore, we subsequently focused on exploring the causes of the aberrant purine metabolism in SCNT-DW4 piglets.

Differences in serum uric acid, HPRT1, and PRPS1 levels among SCNT-DW4, SCNT-SO4 and AI-SO4 piglets

Our serum biochemical analysis confirmed that the serum uric acid level of SCNT-DW4 piglets was significantly higher than those of AI-SO4 and SCNT-SO4 piglets (Fig. 3). This was consistent with the metabolomic results shown in Table 4. Uric acid is a waste product of purine metabolism, which is regulated by two key enzymes encoded by *Hprt1* and *Prps1* genes (Maiuolo *et al.* 2016). Downregulation of *Hprt1* expression or upregulation of *Prps1* expression usually results in the elevation of serum uric acid level (Ahmed *et al.* 1999, Ceballos-Picot *et al.* 2009, Riches *et al.* 2009). Therefore, we measured the serum HPRT1 and PRPS1 enzyme levels to determine whether they are related to the serum uric acid level in three groups of piglets. Our results showed

Table 2 Comparison of malformation rate and neonatal survival rate between SCNT and AI piglets.

Group	Litters	Piglets born			DW4/Malformed	SO4/Malformed
		Total	Stillborn	Malformed		
AI	3	12*	0	0	0/0	12/0
SCNT	5	18	0	7	12/7	6/0

*Only male fetuses from the AI group were used as sex-matched controls, because SCNT-derived fetuses were cloned from male donor cells.

Table 3 Comparison of birth weight and placental parameters between SCNT and AI piglets.

Item	AI-SO4 (n = 12)	SCNT-SO4 (n = 6)	SCNT-DW4 (n = 12)
Birth weight (g)	1702 ± 240.3 [*]	1793 ± 338.4 [*]	1316 ± 199.5 [†]
Placental weight (g)	205.4 ± 32.4 [*]	212.2 ± 28.3 [*]	174.6 ± 19.6 [†]
Placental surface area (cm ²)	2755 ± 424.3 [*]	2824 ± 648.1 [*]	1899 ± 298.6 [†]
Placental efficiency (g/g) [*]	8.34 ± 0.76 [*]	8.41 ± 0.59 [*]	7.53 ± 0.76 [†]

^{*}†Values labeled with different superscript symbols within the same row mean significant difference from one another at $P < 0.05$.

^{*}Placental efficiency = birth weight/placental weight.

that SCNT-DW4 piglets had a significantly lower serum HPRT1 enzyme level than AI-SO4 piglets and an almost significantly lower ($P = 0.069$) serum HPRT1 enzyme level than SCNT-SO4 piglets but had a similar serum PRPS1 enzyme level with SCNT-SO4 and AI-SO4 piglets (Fig. 3). These results suggested that the increased serum uric acid level in SCNT-DW4 piglets was related to the lower serum HPRT1 enzyme level but not the serum PRPS1 enzyme level.

Figure 1 Multivariate analysis of the serum metabolic profiles of AI-SO4, SCNT-SO4, and SCNT-DW4 piglets. (A) PCA score plot. (B) PLS-DA score plot ($R^2X = 0.503$, $R^2Y = 0.89$, and $Q^2 = 0.676$).

Differences in kidney function and morphology between AI-SO4 and SCNT-DW4 piglets

Elevated uric acid level contributes to the development and progression of renal diseases (Siu *et al.* 2006, Hobbs *et al.* 2010, Kanbay *et al.* 2011). Therefore, we investigated whether the abnormal increase of serum uric acid level in SCNT-DW4 piglets impairs kidney function and morphology. We found that the serum levels of two clinical indices of renal function, namely creatinine and urea nitrogen, were significantly increased in SCNT-DW4 piglets compared with AI-SO4 piglets (Fig. 4A). Histopathological analysis also showed that kidney tissues from SCNT-DW4 piglets exhibited tubule abnormalities (Fig. 4B). These data suggested that the elevated serum level of uric acid caused injury to the kidney morphology and function of SCNT-DW4 piglets.

Differences in hepatic Hprt1 and Prps1 mRNA levels between SCNT-DW4 and AI-SO4 piglets

We measured the hepatic mRNA level of two key purine metabolism regulation genes, *Hprt1* and *Prps1*, to see if they are related to the erroneously increased serum uric acid level in SCNT-DW4 piglets due to the fact that purine metabolism mainly occurs in the liver. The qPCR results showed that SCNT-DW4 piglets had a significantly lower hepatic *Hprt1* transcript level than AI-SO4 piglets; yet, their hepatic *Prps1* mRNA abundance was similar to that of SCNT-DW4 piglets (Fig. 5). These results demonstrated that downregulation of *Hprt1* transcription level in the liver tissue was associated with decreased serum HPRT1 level and elevated serum uric acid level in SCNT-DW4 piglets.

Differences in hepatic Hprt1 gene promoter DNA methylation level between SCNT-DW4 and AI-SO4 piglets

To investigate whether the decreased expression of *Hprt1* gene in the liver of SCNT-DW4 piglets was linked to its promoter DNA methylation level, we subsequently determined the *Hprt1* gene promoter DNA methylation degree in the liver tissues of AI-SO4 and SCNT-DW4 piglets. Our results indicated that AI-SO4 and SCNT-DW4 piglets showed no difference in the average hepatic *Hprt1* gene promoter DNA methylation level, but two piglets in the SCNT-DW4 group had a higher hepatic *Hprt1* gene promoter DNA methylation level than the AI-SO4 piglets (Fig. 6)

Discussion

We found that cloned piglets born with malformation, low birth weight, and low placental indexes, like those of SCNT-DW4 piglets, have an extremely high risk of dying during the neonatal age. These findings were

Table 4 Common serum differential metabolites between AI-SO4 and SCNT-SO4 in comparison with SCNT-DW4 piglets.

Metabolites	Metabolic pathways	SCNT-DW4/AI-SO4			SCNT-DW4/SCNT-SO4		
		VIP	P value	FC	VIP	P value	FC
Uric acid	Purine metabolism	2.04	0.004	3.43	1.81	0.048	2.22
Xanthosine	Purine metabolism	1.65	0.001	2.08	1.48	0.023	1.63
Deoxyinosine	Purine metabolism	3.18	0.003	11.61	2.9	0.024	4.86
Cortol	Steroid hormone biosynthesis	2.73	0.001	7.11	1.95	0.041	3.00
Tetrahydrocortisone	Steroid hormone biosynthesis	2.31	0.001	3.98	1.94	0.015	2.33
Carnosine	Histidine metabolism	1.61	0.001	1.97	1.27	0.032	1.46
L-Methionine	Methionine biosynthesis	1.93	0.019	2.27	2.67	0.003	3.14
Glycolic acid	Chlorocyclohexane and chlorobenzene degradation	2.37	0.033	9.48	2.67	0.044	9.03
Phosphoenolpyruvic acid	Glycolysis/Gluconeogenesis; Citrate cycle (TCA cycle)	1.10	0.000	1.35	1.65	0.002	1.54
Aminoadipic acid	Lysine biosynthesis	3.56	0.000	12.34	3.11	0.005	4.30
Linoleic acid	Linoleic acid metabolism; Biosynthesis of unsaturated fatty acids	1.27	0.014	0.66	1.74	0.007	0.60
Retinal	Retinol metabolism	2.02	0.032	0.42	2.67	0.018	0.35
Arginyl-Histidine	Null	1.11	0.037	0.70	2.33	0.001	0.45
Aspartyl-Glutamine	Null	1.94	0.002	0.32	1.45	0.031	0.66
LysoPC(18:2(9Z,12Z))	Null	1.14	0.007	0.71	1.53	0.004	0.68
LysoPC(18:3(6Z,9Z,12Z))	Null	1.62	0.002	0.55	1.99	0.008	0.52
LysoPE(0:0/16:0)	Null	1.16	0.038	0.68	1.45	0.032	0.67
LysoPE(0:0/18:3(6Z,9Z,12Z))	Null	2.01	0.003	0.40	1.83	0.016	0.54
LysoPE(0:0/20:2(11Z,14Z))	Null	1.95	0.009	0.40	2.42	0.010	0.41
LysoPE(0:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	Null	1.67	0.017	0.59	1.75	0.033	0.54
LysoPE(18:2(9Z,12Z)/0:0)	Null	2.02	0.000	0.47	2.54	0.001	0.39
LysoPE(20:4(5Z,8Z,11Z,14Z)/0:0)	Null	1.50	0.010	0.56	1.44	0.032	0.66
LysoSM(d18:1)	Null	1.58	0.011	0.55	2.27	0.003	0.45
MG(15:0/0:0/0:0)	Null	1.68	0.002	0.42	2.12	0.007	0.4
PC(17:2(9Z,12Z)/0:0)	Null	1.06	0.030	0.73	1.50	0.011	0.67
PE(16:0/22:5(4Z,7Z,10Z,13Z,16Z))	Null	1.56	0.003	0.50	1.64	0.011	0.61
PE(10:0/10:0)	Null	1.82	0.004	0.47	2.69	0.001	0.36
PS(12:0/15:1(9Z))	Null	1.55	0.010	0.47	2.78	0.008	0.24
PS(20:4(5Z,8Z,11Z,14Z)/0:0)	Null	1.80	0.002	0.48	2.02	0.003	0.52
PC(18:3(9Z,12Z,15Z)/22:1(13Z))	Null	2.15	0.000	3.04	1.66	0.019	1.77
PS(19:0/20:2(11Z,14Z))	Null	2.17	0.010	4.45	2.57	0.015	4.47

similar to those reported previously (Estrada *et al.* 2007, Schmidt *et al.* 2015, Ao *et al.* 2017). We further demonstrated that neonatally deceased cloned piglets exhibited abnormal purine metabolism at birth. Three purine metabolites, including uric acid, xanthosine and deoxyinosine, had a significant higher level in SCNT-DW4 piglets than in AI-SO4 and SCNT-SO4 piglets. This is the first study showing that purine metabolism problem is associated with the neonatal death of cloned piglets.

Under normal physiological conditions, the enzymes involved in purine metabolism can maintain the balance between the synthesis and degradation of purine nucleosides (Maiuolo *et al.* 2016). Uric acid is the end product of the metabolism of purine, which was proposed to be one of the major antioxidants of the plasma that prevent oxidative damage to cells (Sautin & Johnson 2008). However, a high level of uric acid is a key risk factor for the pathogenesis of many diseases, such as gout (Maiuolo *et al.* 2016), hypertension (Perlstein *et al.* 2006), diabetes (Dehghan *et al.* 2008), and cardiovascular disease (Borghi *et al.* 2015).

Elevated uric acid is also relevant to the progression and development of nephropathy (Kang *et al.* 2002, Zhou *et al.* 2012, Ryu *et al.* 2013). Hyperuricemia rat models were subjected to severe renal dysfunction, including thickening of the preglomerular arteries with smooth muscle cell proliferation, renal hypertrophy, and glomerulosclerosis, which may be associated with

Figure 2 Pathway enrichment analysis of common serum differential metabolites between AI-SO4 and SCNTSO4 in comparison with SCNT-DW4 piglets.

Figure 3 Comparison of serum uric acid, HPRT1, and PRPS1 levels among AI-SO4, SCNT-SO4, and SCNT-DW4 piglets. *, $P < 0.05$; **, $P < 0.01$.

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Figure 4 Comparison of renal function and morphology between AI-SO4 and SCNT-DW4 piglets. (A) Comparison of serum creatinine and urea nitrogen levels, which are two clinical indices for renal function, between AI-SO4 and SCNT-DW4 piglets. **, $P < 0.01$. (B) Comparison of renal morphology between AI-SO4 and SCNT-DW4 piglets by hematoxylin and eosin staining of kidney sections. The kidneys of SCNT-DW4 piglets exhibited several hemorrhagic foci (blue arrow), extensive edema of renal tubular epithelial cells (red arrow), cytoplasm loosening (green arrow), and partial tubular necrosis (black arrow), bar = 100 μm .

vascular disease linked to increased uric acid level (Kang *et al.* 2002). Hyperuricemia in experimental animals and humans leads to the worsening of proteinuria and renal failure along with associated

Figure 5 Comparison of Hprt1 and Prps1 mRNA expressions in the liver tissues of AI-SO4 and SCNT-DW4 piglets. * means significant difference at $P < 0.05$.

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Figure 6 Comparison of the Hprt1 promoter DNA methylation levels in the liver tissues of AI-SO4 and SCNTDW4 piglets. (A) Diagram of the tested CpG units within the Hprt1 promoter. (B) Hepatic DNA methylation levels of the tested CpG sites within the Hprt1 promoter. Yellow and blue circle mean hypomethylation and hypermethylation, respectively. (C) Comparison of hepatic DNA methylation levels of the Hprt1 promoter between SCNT-DW4 and AI-SO4 piglets.

glomerular sclerosis and tubulointerstitial fibrosis (Lapsia *et al.* 2012, Xu *et al.* 2017). Previous studies had found that SCNT-derived piglets were accompanied by some renal abnormalities, such as dilated renal pelvis, hydronephrosis, misshapen kidney, severe calcification, tubular degeneration and increased apoptosis (Park *et al.* 2009, Schmidt *et al.* 2015). Moreover, deceased neonatal cloned pigs were associated with diabetic nephropathy (Park *et al.* 2010). In this study, SCNT-DW4 piglets showed abnormal renal pathological characteristics, including extensive hydropic swelling and cytoplasmic rarefaction of tubular cells and partial tubular necrosis and significantly increased levels of serum creatinine and urea nitrogen. These results suggested that the SCNT-DW4 piglets had renal dysfunction. Uric acid could cause abnormal morphology and function of renal tubular cells by decreasing E-cadherin synthesis through

the activation of Snail and Slug and by enhancing the degradation of E-cadherin to induce the epithelial-to-mesenchymal transition of renal tubular cells (Ryu *et al.* 2013). Therefore, increased uric acid level might serve as a potential risk factor that contributes to the morbidity and mortality of neonatal cloned pigs.

Uric acid formation during purine catabolism is mainly catalyzed by an enzyme called HPRT1, which is an essential enzyme responsible for the metabolic salvage of purines in mammalian cells (Ceballos-Picot *et al.* 2009). SCNT-DW4 piglets had a lower serum HPRT1 level than AI-SO4 piglets. In humans, HPRT1 deficiency causes clinical disorders, such as Lesch-Nyhan disease (LND), which is characterized by the overproduction of uric acid and characteristic neurobehavioral abnormalities, including self-injurious behavior, dystonia, and cognitive disability (Jinnah *et al.* 2006,

Figure 7 Schematic representation of a possible mechanism underlying neonatal death of cloned pigs by abnormal purine metabolism. Red downward and upward arrows represent decrease and increase, respectively.

Ceballos-Picot *et al.* 2009). Some clinical case reports showed that patients with LND suffered from sudden and unexpected death (Neychev & Jinnah 2006). Cloned pigs are likely to have neurological problems; sudden death is also a frequent phenomenon in cloned pigs (Park *et al.* 2005). The liver is the main organ that produces endogenous uric acid (Maiuolo *et al.* 2016). Hepatic *Hprt1* mRNA expression level was significantly downregulated in SCNT-DW4 piglets, which is consistent with the findings of a previous study (Jiang *et al.* 2008). Thus, the reduced hepatic expression of *Hprt1* may be responsible for the elevated uric acid level in SCNT-DW4 piglets.

Hprt1 is an X chromosome-linked gene. Aberrant expression of X-linked genes due to erroneous epigenetic reprogramming of donor cells have been found in cloned mammalian embryos (Jiang *et al.* 2008, Inoue *et al.* 2010, Ruan *et al.* 2018). Thus, the abnormal expression pattern of X-linked genes was likely to persist in cloned neonates (Jiang *et al.* 2008). The DNA methylation pattern is the main way by which to regulate gene expression (Cedar & Bergman 2009). However, our results showed that there was no significant correlation between *Hprt1* expression and promoter DNA methylation level, indicating that the reduced expression of *Hprt1* may be regulated by other epigenetic modifications, such as histone modification, which were related to the erroneous gene expression and low developmental competence of cloned pig embryos or piglets (Yamanaka *et al.* 2009, Zhou *et al.* 2014, Cao *et al.* 2015, Xie *et al.* 2016).

Given that the increased serum uric acid level could be the cause of neonatal death of cloned pigs, reducing serum uric acid level may be an effective way to improve the survival rate of cloned pigs during the neonatal period. Several uric acid-reducing drugs have been clinically used to treat hyperuricemia. These medicines include Allopurinol (Yelken *et al.* 2012), Febuxostat (Kataoka *et al.* 2015) and Benzbromarone (Ogino *et al.* 2010). Therefore, treating newborn cloned piglets with these drugs in the future may be an attractive attempt to improve their neonatal survival rate.

In summary, as shown in Fig. 7, neonatally deceased cloned pigs displayed renal dysfunction and purine metabolism abnormalities, which might be caused by the SCNT-induced downregulation of hepatic *Hprt1* expression.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-20-0143>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Zheng Ao, Zicong Li, and Zhenfang Wu conceived and designed the experiments. Zheng Ao, Ting Gu, Junsong Shi performed the experiments. Zheng Ao and Ting Gu analyzed the data. Ting Gu, Junsong Shi, Enqin Zheng, and Gengyuan Cai contributed materials. Zheng Ao, Zicong Li, and Zhenfang Wu wrote and revised the paper.

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