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Identification of muscle and adipose gene expression patterns in lean and obese pigs

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Abstract

Obesity is a major risk factor of preventable deaths worldwide, with increasing rates being observed in adults and children. To understand the mechanisms of obesity development, genetically lean (Duroc strain) and obese (Rongchang strain) pigs were used to identify potential differences in muscle and adipose development patterns following consumption of an identical diet for 180 days. Lean pigs had a significantly higher lean percentage (67.79% versus 44.71%) and lower obesity index (0.68 versus 0.84) than obese pigs. They also exhibited significantly lower adipocyte volumes and higher myofibre cross-sectional areas. Quantitative polymerase chain reaction showed that lean pigs had a significantly higher expression of muscle growth-related genes and lower expression of lipogenesis-related genes. By contrast, obese pigs had higher expression of a myostatin-related gene and lower expression of lipolysis-related genes. Additionally, the mitochondrial DNA copy number was higher in the muscle and lower in adipose tissue in lean compared with obese pigs. These results indicate that lean pigs have a distinct development pattern from obese pigs, involving lipogenesis, muscle growth, and energy metabolism. This study provides a basis for exploring the mechanisms of adipose deposition and muscle growth in obesity.

Keywords: Obesity, mitochondrial DNA, *Sus scrofa*
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Introduction

Obesity and overweightness are prevalent in developed and developing countries, and are increasing at an alarming rate (Abelson & Kennedy, 2004; Haslam & James, 2005). It is estimated that the number of overweight adults was 2.1 billion in 2013 worldwide, compared with 857 million in 1980 (Ng *et al*., 2014). By 2030, up to 58% of the world's adult population are predicted to be obese or overweight (Kelly *et al*., 2008). These are major risk factors for type 2 diabetes, cardiovascular diseases, hypertension, stroke, certain types of cancer, osteoarthritis and associated metabolic syndromes (Caballero, 2007), and are recognized as major worldwide public health problems, which lead to reduced life expectancy, poor quality of life, depression, and premature death.

Obesity and overweightness are the result of excessive energy intake compared with energy expenditure (Sikaris, 2004), which leads to an excess accumulation of body fat (Després, 2012). Adipose tissue and muscle have been identified as endocrine and signalling organs in the past decades with the secretion of a large amount of adipokines and myokines. The major changes of adipokine and myokine production have a wide range of physiological functions, which result in the expansion of the adipose tissue and muscle mass that defines obesity and overweightness. Indeed, the interaction of myokines and adipokines is implicated in processes such as the control of lipolysis and insulin sensitivity (Trayhurn, 2011). Therefore, adipose tissue plays an important role in the regulation of energy metabolism, including storage and dissipation (Li *et al*., 2012), and skeletal muscle can have direct beneficial effects on the control of bodyweight and metabolism (Satoor *et al*., 2011).

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The pig *(Sus scrofa)* is emerging as an ideal biomedical model for studying obesity and energy metabolism in human beings because it is devoid of brown fat postnatally and because pigs are closely comparable with humans in terms of size, cardiovascular systems, metabolism features and pathology (Spurlock & Gabler, 2008). Pigs also offer the advantages of low genetic variance, homogeneous feeding regimens, and abundant tissue for analyses or repetitive sampling. In the modern pig industry, animals have undergone strong artificial selection to obtain relatively inbred commercial lines for lean meat or adipose production. This has resulted in remarkable phenotypic variations and genetic adaptations, which make these lines perfect for comparative studies (Rocha & Plastow, 2006). Above all, genetically lean and obese pigs are useful models to study obesity and provide a phenotypic extreme to explore the possible mechanisms responsible for the development of adiposity in an animal species reared for meat. Moreover, a better understanding of the mechanisms of adipose deposition and muscle growth in pigs would contribute to improved pork production efficiency. Previous studies have reported differences between extreme phenotype pig breeds from molecular level, such as mRNA (Zhang *et al*., 2013; Zhou *et al*., 2013), microRNA (Liu *et al*., 2013) and methylation (Li *et al*., 2012). However, systematic and targeted studies of molecular expression and phenotype and their interrelationships, especially the mtDNA copy number, are scarce.

The Duroc pig is a modern Western breed that is recognized as a genetically lean strain. By contrast, the Rongchang pig is a regional pig strain from China that demonstrates excessive fat deposition. The present study was designed to compare *longissimus dorsi* muscle (LDM) and subcutaneous adipose tissue (SAT) development patterns between the genetically lean and obese pigs at phenotypic and molecular levels.

Material and Methods

The experimental protocol was approved by the Animal Care and Ethics Committee of Southwest University, Chongqing, China, under permit No. DKY-20143003. Twelve castrated males of each of the Duroc (lean-type pig, Western breed) and Rongchang pigs (obese-type pig, Chinese breed) were used in this study. There was no direct and collateral blood relationship within the last three generations among the 24 pigs from each of the breeds. Animals were housed in individual pens (2 m^2) and had ad libitum access to feed and water (nipple drinkers) under normal conditions. Feed was a commercial diet based on corn and soybean according to the nutrition standards by National Research Council (NRC, 1998).

All animals were humanely slaughtered simultaneously at 180 days old, using low voltage (200 V) electrical stunning followed by exsanguination to ameliorate suffering and were not fed the night before, in compliance with regulations for experimental animals that were established by the Ministry of Agriculture of China. The LDM and SAT were dissected immediately after slaughter, frozen in liquid nitrogen, and stored at -80 °C.

The lean percentage (LP) was calculated using the following formula:

$$\text{LP (\%)} = \text{muscle weight (kg)} / \text{carcass weight (kg)} \times 100\%.$$

The obesity index (OI) was calculated as described (Sebert *et al.,* 2005). The pig body is likened to a truncated cone, where the base is represented by the abdomen (A), the top by the neck (N) and the length by the body size (BS). These anthropometric parameters are then combined into a single value representative of the genetic predisposition to obesity. Pig body volume was defined (l) as:

$$V\ (l) = (\pi\ (BS/3)\ (cm) \times \{(A)^2\ (cm) + (N)^2\ (cm) + (A\ (cm) \times N\ (cm)\ )\ \}\ ) \times 10^{-3}.$$

It was then possible to determine the OI in litres per centimetre as:

$$OI\ (l / cm) = V\ (l) / BS\ (cm).$$

The adipocyte volume (AV) and myofibre cross-sectional area (MCSA) were measured using the haematoxylin-eosin (H&E) staining method as described (Li *et al*., 2012). Briefly, all tissues were fixed in 10% neutral buffered formalin solution, embedded in paraffin using TP1020 semi-enclosed tissue processor (Leica), sliced at a thickness of 6 μm using an RM2135 rotary microtome (Leica) and stained with H&E. The mean diameter of an adipocyte was calculated as the geometric average of the maximum and minimum diameter, and 100 cells were measured for each sample in randomly selected fields using a TE2000 fluorescence microscope (Nikon) and Image Pro-Plus 7.0 software (Media-Cybernetics). The mean AV was obtained according to the following formula:

$$AV = \pi/\ 6\ \Sigma f_i\ D_i^3/\Sigma\ f_i,$$

where: D_i is the mean diameter; f_i denotes number of cells with that mean diameter D_i. The MCSA was measured as an average of 100 fibres in randomly selected fields.

Table 1 Primer sequences used for Q-PCR

Gene symbol	Primer sequences (5' to 3')	Amplicon size (bp)	GeneBank/Ensemble ID
*GAPDH**	F:CTGGGAAACTGRGGCGTGAT R:AAGTGGTCGTTGAGGGCAAT	342	NM_001256799 ENSSSCG00000000694
*GCG**	F: GAATCAACACCATCGGTCAAAT R: CTCCACCCATAGAATGCCCAGT	147	NM_002054 ENSSSCG00000015895
MSTN	F: GAGACCCGTCGAGACTCCTAC R: AGTGCCTGGGTTCATGTCAAG	121	NM_005259 ENSSSCG00000016047
FABP5	F: TGAAGGAGCTAGGAGTGGGAA R: TGCACCATCTGTAAAGTTGCAG	212	NM_001444 ENSSSCG00000006153
SCD	F: GCCCCTCTACTTGGAAGACGA R: AAGTGATCCCATACAGGGCTC	161	NM_005063 ENSSSCG00000010554
FTO	F: CAGCAGTGGCAGCTGAAATA R: TGACCAGGTCCCGAAATAAG	133	NM_001080432 ENSSSCG00000002832
UCP2	F: GGAGGTGGTCGGAGATACCAA R: ACAATGGCATTACGAGCAACAT	116	NM_003355 ENSSSCG00000014833
MYOG	F: GGGGAAAACTACCTGCCTGTC R: AGGCGCTCGATGTACTGGAT	341	NM_002479 ENSSSCG00000015475
MYOZ1	F: ACCCCGGCCCCTAATAAGAA R: GCAGCGACAGTTCCTCCAA	139	NM_021245 ENSSSCG00000010304
IGF1	F: GCTCTTCAGTTCGTGTGTGGA R: GCCTCCTTAGATCACAGCTCC	133	NM_001111283 ENSSSCG00000000857
MYOD1	F: CGGACGTGCCTTCTGAGTC R: AGCACCTGGTATATCGGGTTG	145	NM_002478 ENSSSCG00000013375
LPL	F: TCATTCCCGGAGTAGCAGAGT R: GGCCACAAGTTTTGGCACC	125	NM_000237 ENSSSCG00000027959
ADIPOR1	F:TCCTGCCAGTAACAGGGAAG R: GGTTGGCGATTACCCGTTTG	89	NM_015999 ENSSSCG00000010930
ADIPOR2	F: CCTCTTACAAGCCCACC R: AGTCAGGCAGCACATCG	107	NM_024551 ENSSSCG00000000757
TWINKLE	F: GGAAGGAGGATGATGATAAGG R: GGTATGGAGAAGGTAAGAGAGC	211	ENSSSCG00000030428
COX1	F: ACTACTGACAGACCGCAACC R: TCCAATGGACATTATGGCTC	220	ENSSSCG00000018075
ND1	F: AGCCACATCCTCAATCTCC R: CCCGATGAGTGCGTATTTT	205	ENSSSCG00000018065
ATP6	F: TATTTGCCTCTTTCATTGCCC R: GGATCGAGATTGTGCGGTTAT	123	ENSSSCG00000018081

**GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *GCG* (glucagon) are the endogenous control genes for mRNA level and mtDNA copy number, respectively. F indicates forward primers, and R indicates reverse primers. bp: base pairs.

Total RNA was extracted from frozen LDM and SAT using TRIzol regent (Invitrogen) and further purified using an RNeasy column (Qiagen) according to the manufacturer's protocol. RNA integrity and concentration were analysed with Bioanalyzer 2100 (Agilent Technologies). DNA was isolated from LDM and SAT using the DNeasy blood & tissue kit (Qiagen) following the manufacturer's instructions. The relative gene expression level and mitochondrial DNA (mtDNA) copy number were determined by Q-PCR. Q-PCR was performed using the SYBR Premix Ex Taq kit (TaKaRa, Dalian, China) on a CFX96 Real-Time PCR detection system (Bio-Rad, Richmond, CA). Primer sequences used for the Q-PCR are shown in Table 1. All reactions were performed in triplicate, and negative controls (without template) were always included.

Relative gene expression level and mtDNA copy number per diploid cell were calculated using the $2^{-\Delta\Delta Ct}$ method. All statistical analysis was conducted with SPSS Statistics 21.0 software (IBM, NY, USA). The significance of differences was determined by Student's *t*-test and a correlation was analysed by Pearson's process. Data are expressed as means ± standard deviation (SD).

Results and Discussion

In this study, the authors selected two pig breeds based on their history of breed characteristics. The Duroc breed has been selected for lean meat for more than 60 years in the USA, whereas the Rongchang breed has not been genetically improved and has a tendency towards obesity. To determine accurately whether Duroc and Rongchang pigs were lean or obese, the authors calculated the lean percentage (LP) and obesity index (OI). As expected, the LP, which correlates negatively with the percentage of fat, was significantly higher in Duroc pigs than in Rongchang pigs (Student's *t*-test, 67.8% versus 44.7%, $P = 2.05 \times 10^{-5}$, Figure 1A), which is consistent with the definition of lean (LP > 60%) and obese pigs (LP <45%) (Furnols & Gispert, 2009). the authors also observed a significantly higher OI in Rongchang pigs compared with Duroc pigs (Student's *t*-test, $P = 2.52 \times 10^{-5}$) (Figure 1B). Accordingly, the OI can be used to select animals that are genetically predisposed to being extremely lean or obese (Kogelman *et al*., 2014).

To study adipocyte and myofibre regulation in the development of Duroc and Rongchang pigs, the authors measured the adipocyte volume (AV) and myofibre cross-sectional area (MCSA). Duroc pigs exhibited a significantly lower AV and higher MCSA than Rongchang pigs (Student's *t*-test, $P = 1.76 \times 10^{-9}$ (Figure 1C) and $P = 1.81 \times 10^{-6}$ (Figure 1D), respectively), which again agreed with the known characteristics of the two breeds. Obesity can be characterized as hyperplastic, involving an increase in adipocyte number, and hypertrophic, resulting in an increased AV. Hypertrophy is largely characteristic of all overweight and obese individuals (Hirsch & Batchelor, 1976), and two independent studies showed that adipose hypertrophy is an independent risk factor for developing type 2 diabetes (Weyer *et al*., 2000; Lonn *et al*., 2010). Moreover, the MCSA is important in describing myofibre adaptations to physiological and pathological changes (Miller & Stauber, 1994). These observed phenotypic differences in LDM and SAT between Duroc and Rongchang pigs imply the possession of intrinsic molecular differences between the breeds.

To study the association of gene expression level with phenotypic divergence, the authors investigated the expression of known muscle and adipose development-related genes. The expression level of genes that inhibit muscle growth (*MSTN*) and lipogenesis (*FABP5*, *FTO*, *SCD*, and *UCP2*) was significantly higher in Rongchang pigs than in Duroc pigs (Student's *t*-test, P <0.01) (Figure 2). By contrast, those genes that promote muscle growth (*MYOG*, *MYOZ1*, *IGF1*, and *MYOD1*) and lipolysis (*ADIPOR1*, *ADIPOR2*, and *LPL*) were expressed at significantly higher levels in Duroc pigs than in Rongchang pigs (Student's *t*-test, P <0.05).

The *MSTN* encodes myostatin, which inhibits the differentiation and growth of muscle and Akt-induced protein synthesis (Trendelenburg *et al*., 2009), while *FABP*5 encodes the cytoplasmic fatty acid binding protein 5, which binds long-chain fatty acids and participates in fatty acid uptake, transport, and metabolism. Research in murine models revealed that *FABP5*-deficient mice were protected against diet-induced obesity, insulin resistance and type 2 diabetes (Shearer *et al*., 2005). *FTO* is unequivocally associated with obesity and encodes the ubiquitously expressed fat mass and obesity-associated protein (Church *et al*., 2010). Stearoyl coenzyme A desaturase (encoded by *SCD*) expression serves as a bovine post-natal marker of adipocyte differentiation, which strongly promotes adipocyte differentiation, lipogenesis and lipid filling (Smith *et al*., 2009), while uncoupling protein 2 (encoded by *UCP2*) is a mitochondrial transport protein that acts in the negative regulation of insulin secretion by *β*-cells and in fatty acid metabolism (Liu *et al*., 2013).

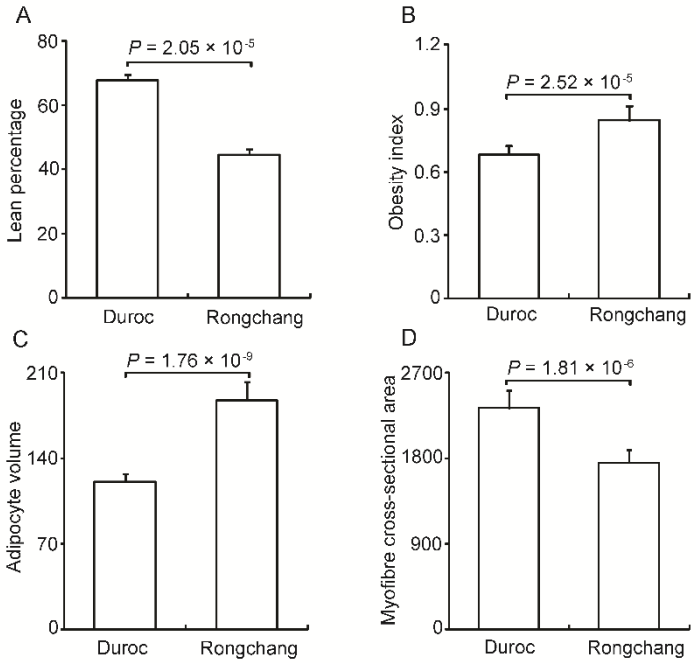

Figure 1 Characteristics of *longissimus dorsi* muscle and subcutaneous adipose tissue in lean and obese pigs. (A) lean percentage. (B) obesity index. (C) adipocyte volume. (D) myofibre cross-sectional area Student's *t*-test. Values are means ± SD

Encoding myogenin *(MYOG)* transforms potential mesoderm cells to sarcoblasts and has a critical role in the terminal differentiation of the specified muscle cells (Nabeshima *et al*., 1993). *MYOZ1* encodes myozenin 1, which is a structural component of skeletal muscle that binds calcineurin and is involved in the myocyte differentiation of skeletal muscle (Wan *et al*., 2013). Frey *et al*. (2008) reported that *MYOZ1*-deficient mice display a reduction in body weight and fast-twitch muscle mass. Insulin-like growth factor 1 (encoded by *IGF1*) acts as a positive regulator of muscle growth (Schiaffino *et al*., 2013). Mavalli *et al.* (2010) reported that muscle-specific inactivation of the IGF1 receptor impairs muscle growth because of a reduction in muscle fibre number and size. Conversely, elevated IGF1 has a hypertrophic effect on skeletal muscle (Shavlakadze *et al*., 2010). *MYOD1* is a master regulatory gene, which encodes myogenic differentiation antigen, which is involved in myogenesis and also converts fibroblasts to myoblasts (Van Neck *et al*., 1993), while *ADIPOR1* and *ADIPOR2* encode adiponectin receptor 1 and 2, respectively, which are receptors for adiponectin, a hormone secreted by adipocytes that regulates fatty acid catabolism and glucose levels (Kaser *et al*., 2005). Sadri *et al.* (2011) indicated a role for *ADIPOR1* and *ADIPOR2* in reducing insulin sensitivity at the level of the adipose tissue, leading to reduced glucose uptake and increased lipolysis. Finally, lipoprotein lipase (encoded by *LPL*) is an early marker of adipose cell differentiation and the rate-limiting enzyme in the lipolysis of triglyceride-rich lipoproteins (Spence *et al*., 2003). These results provide a molecular explanation for the differences between lean and obese pig development in muscle growth and adipose deposition.

To determine the relationship between development-related genes and phenotypic traits of SAT and LDM development, an association analysis of phenotypic parameters and gene expression using SPSS software was performed. Development-related gene expression was clearly shown to correlate with SAT and LDM phenotypic parameters (AV: $|r| \geqslant 0.75$, $P < 0.01$; OI: $|r| \geqslant 0.63$, $P < 0.01$; MCSA: $|r| \geqslant 0.75$, $P < 0.01$; LP: $|r| \geqslant 0.87$, $P < 0.001$) (Table 2), thus highlighting the critical roles of these genes in muscle growth and adipose deposition. Interestingly, the expression levels of development-related genes were also significantly correlated with each other (SAT: $|r| \geqslant 0.74$, $P < 0.01$; LDM: $|r| \geqslant 0.85$, $P < 0.001$), suggesting that these genes act synergistically in muscle growth and adipose deposition (Liu *et al*., 2012). However, the mechanism by which muscle and adipose development-related genes cooperate or interact with each other remains to be explored. Additionally, the gene variation in muscle and adipose development that reflects the dynamic equilibrium of metabolism should be studied further.

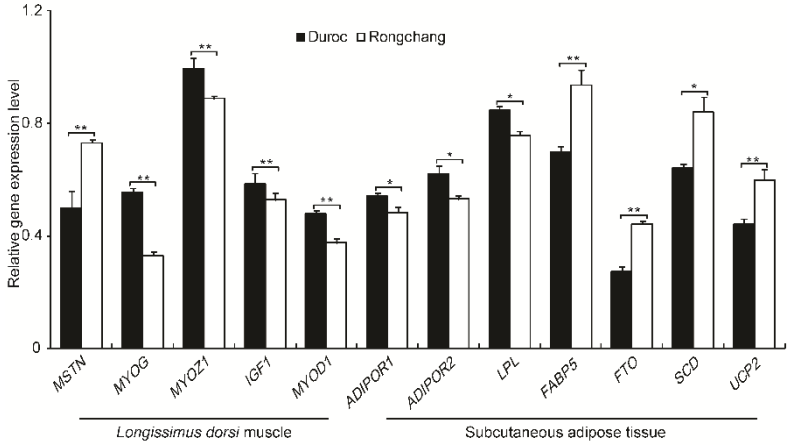

Figure 2 Development-related gene expression of *longissimus dorsi* muscle and subcutaneous adipose tissue in lean and obese pigs
Student's *t*-test. Values are means ± SD
**P* <0.01
***P* <0.001

Table 2 Correlation of phenotypic parameters and gene expression

		OI	***ADIPOR1***	***ADIPOR2***	***LPL***	***FABP5***	***FTO***	***SCD***	***UCP2***
SAT	AV	0.79**	-0.75*	-0.81**	-0.82**	0.85**	0.84**	0.82**	0.84**
	OI		-0.63*	-0.71*	-0.72*	0.73*	0.75**	0.74*	0.71*
	ADIPOR1			0.74*	0.76*	-0.79**	-0.81**	-0.76*	-0.79**
	ADIPOR2				0.88**	-0.87**	-0.86**	-0.88**	-0.87**
	LPL					-0.88**	-0.87**	-0.79**	-0.86**
	FABP5						0.89**	0.85**	0.89**
	FTO							0.87**	0.88**
	SCD								0.84**
		LP	***MSTN***	***MYOG***	***MYOZ1***	***IGF1***	***MYOD1***		
LDM	MCSA	0.77**	-0.77**	0.76*	0.78**	0.78**	0.75*		
	LP		-0.89**	0.88**	0.89**	0.87	0.88**		
	MSTN			-0.89**	-0.85**	-0.87**	-0.88**		
	MYOG				0.89**	0.88**	0.86**		
	MYOZ1					0.89**	0.88**		
	IGF1						0.87**		

SAT: subcutaneous adipose tissue; LDM: *longissimus dorsi* muscle
**P* <0.01
***P* <0.001

Adipose tissue and muscle are important energy metabolism organs that participate in the regulation of whole-body metabolism (Zhang *et al*., 2013). A previous study reported that regulation of the mtDNA copy number is essential for maintaining cellular energy requirements (Dickinson *et al*., 2013). The authors therefore determined the mtDNA copy number as a measure of energy metabolism. The LDM mtDNA copy number was significantly higher in Duroc pigs than in Rongchang pigs ($P = 9.57 \times 10^{-4}$)) Figure 3A), while the SAT mtDNA copy number was significantly higher in Rongchang pigs than in Duroc pigs ($P = 3.54 \times 10^{-3}$) (Figure 3A). This appears to be in accordance with the fact that high energy-requiring cells require high mtDNA copy numbers, while low energy-requiring cells maintain fewer copies (Moyes *et al*., 1998).

TWINKLE is the only replicative DNA helicase that is known to be required for complete mtDNA replication in mammalian mitochondria (Milenkovic *et al*., 2013). The authors therefore investigated the relationship between *TWINKLE* gene expression and mtDNA copy number in the two pig breeds. They found that the mtDNA copy number per cell was positively correlated with the expression of *TWINKLE* gene (LDM: $r = 0.96$, $P = 1.68 \times 10^{-10}$; SAT: $r = 0.87$, $P = 2.37 \times 10^{-6}$) (Figure 3B), so hypothesized that *TWINKLE* gene expression may serve as a candidate marker of porcine adipose deposition and muscle growth.

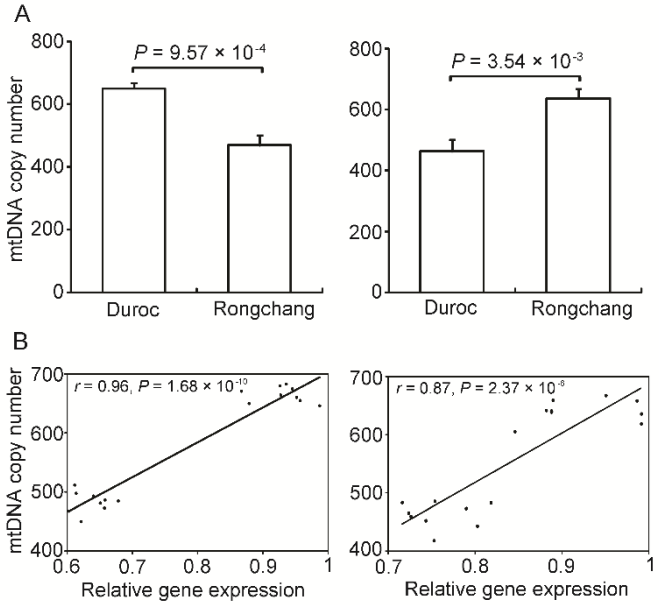

Figure 3 Analysis of mtDNA copy number. (A) *longissimus dorsi* muscle and subcutaneous adipose tissue mtDNA copy number in lean and obese pigs. Student's *t*-test. Values are means ± SD. (B) Pearson's correlation test comparing the mtDNA copy number and *TWINKLE* mRNA abundance

Conclusions

The findings of this study indicate that lean and obese pigs have distinct development patterns, including muscle growth and adipose deposition, as shown by the observed differences in lean percentage, obesity index, adipocyte volume, myofibre cross-sectional area, gene expression, and energy metabolism. However, this study investigated only one stage of porcine development, yet development is a process requiring long-term study. Therefore, future comparative studies should analyse multiple developmental time points.

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Authors' contributions

AFL and JZ conceived and designed the experiments. JZ and HH performed the experiment. JZ analysed the data. JZ and HH contributed reagents, materials and analysis tools. JZ contributed to the writing of the manuscript. AFL and JZ carried out critical reading and drafting of the manuscript. All the authors agreed with the final version to be submitted.

Conflict of interest declaration

The authors have no conflict of interest to declare.

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