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Castration-induced changes in microRNA expression profiles in subcutaneous adipose tissue of male pigs

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Abstract MicroRNAs (miRNAs) are class of molecular regulators found to participate in numerous biological processes, such as adipogenesis and obesity in mammals. To determine the roles of miRNAs involved in castration-induced body fatness, we investigated the different miRNA expression patterns in subcutaneous adipose tissue between intact and castrated male pigs. Our results showed that castration led to decrease serum testosterone but increase serum Leptin levels ($P< 0.01$). Moreover, castration also increased adipocyte size, body fat content and backfat thickness in male pigs ($P< 0.01$). Meanwhile, miRNA expression profiles in adipose tissue were changed by castration, and 18 miRNAs were considered as the differentially expressed candidates between intact and castrated male pigs. Furthermore, functional analysis indicated that the differential expressed miRNAs and their target genes are involved in the regulation of fatty acid metabolism. In brief, our present study provides a comprehensive view on how miRNAs works in subcutaneous adipose tissue with castration. These results suggested that miRNAs might play an important role in the castration-induced fat deposition in male pigs.

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Introduction

The metabolism of adipose tissue is known to be affected by gonadal steroid hormones such as testosterone (Andersen et al. 2010; Bélanger et al. 2006; Varlamov et al. 2012). For example, testosterone deficiency caused by castration increases the quantity of adipose tissue in male rat (Li and Bjorntorp 1995) and castration can also increase body fatness in male pigs (Christoffersen et al. 2010). On the other hand, obesity has usually been associated with reduced plasma testosterone levels, which play an important role in modulating of fat accumulation in both humans and animals, but unfortunately, the possible molecular mechanisms underlying this action are still not clear.

MicroRNAs (miRNAs) are endogenous small RNAs that regulate gene expression at the post-transcriptional level and have been shown to have important roles in numerous disease or physiological metabolism processes (Lhakhang and Chaudhry 2012). Many studies suggested that miRNAs contribute to the regulation of adipose deposition and adipogenesis (Esau et al. 2004; Kim et al. 2009; Gerin et al. 2010). Recently, there is growing evidence for an important role of miRNAs in the pathological development of obesity in mammals. For instance, miR-143 was up-regulated in mesenteric adipose tissue in high-fat diet induced obese mice (Takanabe et al. 2008), and was down-regulated in epididymal adipose tissue from ob/ob mice (Xie et al. 2009). Another pro-adipogenic miRNA is miR-21, which has been shown to enhance adipogenesis of hASCs (Kim et al. 2009) and is up-regulated in human obesity (Keller et al. 2011). Interestingly, miR-27a has been shown to be down-regulated during adipogenesis of 3 T3-L1 cells (Kim et al. 2010) but increased in

adipose tissue of genetically obese mice compared with lean mice (Lin et al. 2009). Another one, miR-130 also inhibits adipogenesis by targeting PPARγ mRNA, and it has been observed that adipose tissue from obese women had lower expression of miR-130 with higher expression of PPARγ mRNA than non-obese women (Lee et al. 2011).

But up to now, only a few studies have been conducted to investigate the role of miRNAs on regulation of fat deposition in domestic pigs, and most of them focused on identification of novel miRNAs in porcine adipose tissue (Li et al. 2011b; Chen et al. 2012). Therefore, it is not clear whether testosterone deficiency caused by castration can influence miRNA expression patterns in porcine adipose tissue or not. In order to clarify the molecular mechanism underlying the increased body fatness in testosterone deficiency pigs, we investigated the changes of miRNA expression in the subcutaneous adipose tissue between intact and castrated male pigs using miRNA microarray assay, and thus provide a novel view of the role of miRNAs in castration-induced metabolism.

Materials and methods

Animal experiment and sample collections

All experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee of Zhejiang University. Nine pairs of full sibs, a total of 18 male large white pigs were used in this experiment. On day 35, one of the pigs from each pair was randomly selected to be castrated under anesthesia, and the another one remained intact. All pigs were allowed ad libitum access to feed and water until slaughter. They were slaughtered at 210 days of age. The carcasses were eviscerated according to standard commercial procedures, and then the carcass fat weight and leaf fat weight were weighted. Back fat thickness (including shoulder fat thickness and 6~7th rib fat thicknesses) was measured using a ruler on the left side of carcass. Subcutaneous adipose tissue samples were collected from the left side of the carcass and were rapidly frozen in liquid nitrogen, then stored at −80 °C until analysis.

Serum hormones analysis

Blood samples were collected from each pig at slaughter during exsanguinations, and then kept at 37 °C for 2 h and centrifuged for 15 min at 3000×g at 4 °C. Serum was collected and stored at −70 °C for further analysis. Serum concentrations of testosterone and leptin were measured using commercial RIA kits (Beijing North Institute of Biotechnology, China) with a Gamma-counter (Packard 8500, USA).

Histological analysis

Subcutaneous adipose tissue samples were fixed in 10 % neutral formalin solution, embedded in paraffin blocks, and sectioned to 6 μm thickness. The sections were stained with hematoxylin and eosin (H&E) (Shanghai Chemical Co. Ltd., Shanghai, China). The slides were examined by an Olympus microscope (Olympus BX51) and photographed.

RNA extraction

Total RNA was extracted from adipose tissues using TRIzol® Regent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentrations of total RNA was measured using the NANODROP® spectrophotometer ND-1000 (Thermo Scientific, Waltham, MA, USA) and RNA integrity was measured using Agilent 2100 BIOANALYZER® (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). RNA with integrity (RIN) >7.8 was used for miRNA microarray and stem-loop real-time RT-PCR analysis.

miRNA microarray assay

The miRNA microarray analysis was carried out by LC Sciences (Houston, TX; http://www.lcsciences.com) as previously reported (Marsh et al. 2008). The microarray contains 236 known *Sus scrofa* miRNAs corresponding to miRNA transcripts listed in miRBase release 16.0. Briefly, RNAs from three pairs of adipose tissue samples from intact and castrated male pigs were extracted and mixed, respectively. Total RNA sample (2–5 μg) was size fractionated using a YM-100 microcon centrifugal filter (Millipore, Billerica, MA) and the isolated small RNAs (<300 nt) were 3'-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent dye staining. The RNA sequences were hybridized overnight on a μParaflo microfluidic miRAN microarray chip using a microcirculation pump (Atactic Technologies, Houston, TX). On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to target microRNA (from miRBase, Release 16.0, http://microrna.sanger.ac.uk/sequences/) or other RNA control sequences and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. The detection probes were made by in situ synthesis using photogenerated reagent (PGR) chemistry. The hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization was performed using 100 μL 6×SSPE buffer (0.90 M/L NaCl, 60 mM/L Na2HPO4, 6 mM/L EDTA, pH 6.8) containing 25 % formamide at 34 °C. After hybridization, miRNAs were detected by fluorescence labeling using tag-specific Cy3 dyes.

Hybridization images were collected using a GenePix 4000B laser scanner (Molecular Devices, Sunnyvale, CA) and digitized using Array-Pro image analysis software (Media Cybernetics, Sliver, Spring, MD).

Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression) (Bolstad et al. 2003). The ratio of the two sets of detected signals (Log2 transformed, balanced) and P-values of the *t*-test were calculated. Differentially expressed miRNAs with $P<0.01$ were selected for further analysis.

Stem-loop real-time RT- PCR

A miRNA quantification method described by Li et al. (2011a) was used, with some modification. For miRNA quantification, each RT reaction consisted of 1 μg of total RNA mixed with 2.0 μL of 5×RT buffer which included dNTPs (Takara), 1.0 μL of 10 μM stem-loop RT primer (Table 1), 0.5 μL RNase inhibitor (Takara), and 0.5 μL reverse transcriptase (Takara) in a final volume of 10 μL. The admixture was incubated at 42 °C for 60 min, 70 °C for 15 min, and then held at 4 °C. Real-time PCR was performed using SYBR Green Master Mix (Takara) on the StepOne™ Software v2.0 (Applied Biosystems) according to the manufacturer's instructions. The PCR volume was 20 μL, containing 1 μL diluted RT product, with the following cycling conditions: 95 °C for 30 s, followed by 40 cycles for 95 °C for 5 s and 60 °C for 30 s. Porcine miR-24 was used as an internal control and all PCR reactions were run in triplicate. The comparative $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen 2001) was employed to determine the expression level differences.

Table 1 Primers for qRT-PCR of miRNAs

miRNA		Primer sequences (5'-3')
ssc-miR-21	RT	GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACTCAACA
	Forward	GGCGGCGGTAGCTTATCAG
	Reversed	CGCAGGGTCCGAGGTATTC
ssc-miR-148a	RT	GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACACAAAG
	Forward	CCGCCGTCAGTGCACTACAG
	Reversed	CCAGTGCAGGGTCCGAGGT
ssc-miR-15a	RT	GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACACAAAC
	Forward	CCGCCGTAGCAGCACATAAT
	Reversed	CAGTGCAGGGTCCGAGGTA
ssc-miR-101	RT	GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACTTCAGT
	Forward	CCGCCGTACAGTACTGTGATAAC
	Reversed	CAGTGCAGGGTCCGAGGT
ssc-miR-320	RT	GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACTTCGCC
	Forward	CGGCTGAAAAGCTGGGTTG
	Reversed	CAGTGCAGGGTCCGAGGTAT
ssc-miR-423-5p	RT	GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACAAAGTC
	Forward	CCACTTGAGGGGCAGAGAGC
	Reversed	CGCAGGGTCCGAGGTATTC
ssc-miR-24	RT	GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACCTGTTC
	Forward	GCGGTGTTGGCTCAGTTCAG
	Reversed	CAGTGCAGGGTCCGAGGTATT

Target prediction and functional enrichment of differentially expressed miRNAs

TargetScan (Lewis et al. 2005) was used to predict the targets of differentially expressed miRNAs. TargetScan allows identification of target mRNAs for any specific microRNA based on the context score percentile (Grimson et al. 2007), and we set context score percentile of 50 as threshold value to filter the prediction results (Yehya et al. 2012). To discover the function of those differential expressed miRNAs, gene ontology (GO) analysis was applied to reveal the functions of the target genes of the differential expressed miRNAs. Fisher's exact test was used to classify the GO category, and the false discovery rate (FDR) was calculated to correct the P-value. Both P-value<0.05 and FDR<0.05 were used as a threshold to select significant GO categories (Wang et al. 2012). Afterwards, the network of miRNA-mRNA interaction, representing the critical miRNAs and their targets, was established according to the miRNA degree (Cline et al. 2007).

Statistical analysis

A two-tailed Student's *t* test was used to determine the significant differences between intact and castrated male pigs. Statistical analysis was performed using SPSS 13.0 software (SPSS, Chicago, IL), and $P< 0.05$ was considered to be a statistically significant difference.

Table 2 Effect of castration on serum hormones and body fatness traits in male pigs

Items	Castrate	Intact	*P*-value
Serum hormones			
Testosterone (ng/ml)	0.24±0.05	8.19±1.04	0.01
Leptin (ng/ml)	5.30±0.20	3.77±0.28	0.01
Body fatness traits			
Body weight (kg)	120.6±4.71	133.29±7.60	0.02
Carcass fat weight (kg)	6.89±0.07	4.41±0.07	0.01
Leaf fat weight (kg)	0.75±0.14	0.47±0.11	0.04
Shoulder fat thickness (cm)	3.78±0.24	3.14±0.39	0.01
6~7th rib fat thickness (cm)	2.48±0.25	1.98±0.34	0.01

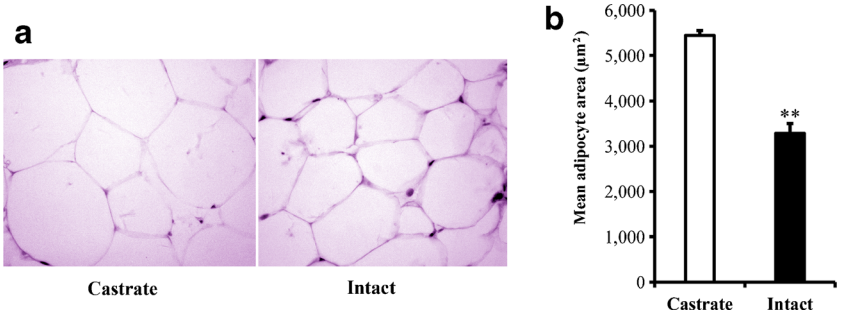

Fig. 1 Histological sections of subcutaneous adipose tissue from intact and castrated male pigs. **a** H&E staining of subcutaneous adipose tissue from intact and castrated male pigs (shown at×400 magnification); **b** The mean adipocyte size in intact and castrated male pigs. Adipocyte area was measured using Image J software from three different animals per group (60 fat cells for each individual). $^{**}P<0.01$

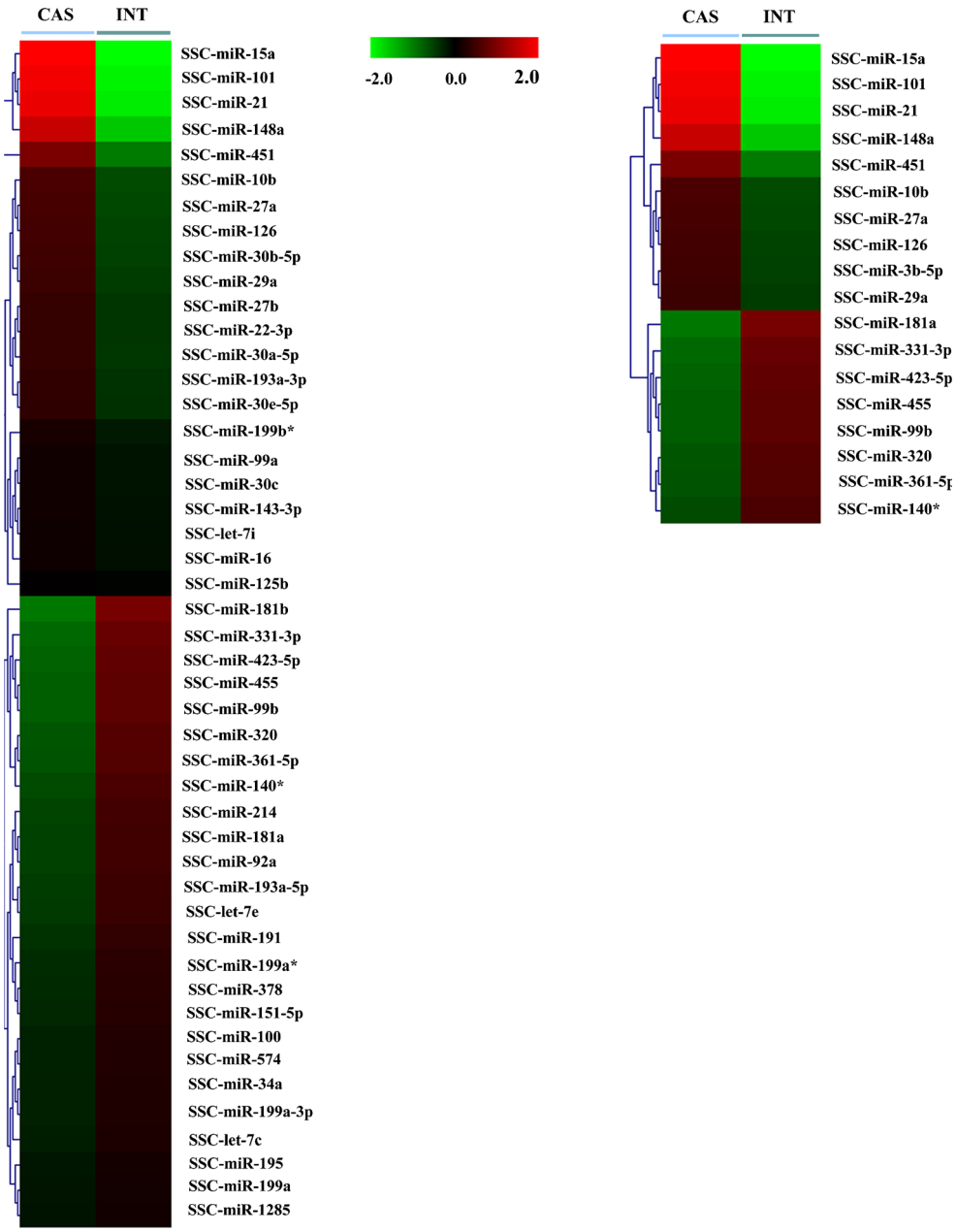

Fig. 2 Hierarchical cluster heat map for miRNAs expressed in subcutaneous adipose tissue from intact and castrated male pigs. **a** miRNAs with a signal value >500 and a *p*-value <0.01 (high signal group miRNAs); **b** miRNAs whose expression level showed a fold change ≥2 (differentially expressed miRNAs). "CAS" means castrated male pigs; "INT" means intact male pigs. The relative transcript abundance of each miRNA is color coded. The *red* or *green* color indicates relatively high or low expression, respectively

Results

Castration induces changes in serum hormones and body fatness

The effect of castration on serum hormones and body fatness traits are shown in Table 2. Castration can significantly reduce serum testosterone concentrations ($P<0.01$) and increase serum Leptin concentrations ($P<0.01$). The castrated male pigs had lower body weight but higher level of adiposity than that of intact pigs ($P<0.05$). In addition, castration caused a significant increase in carcass fat weight, leaf fat weight and back fat thickness in male pigs ($P<0.05$) (Table 2). These results corresponded to the higher adipocyte size observed in castrated male pigs compared with intact male pigs (Fig. 1). Histological examination revealed that adipocyte size was significantly increased by castration ($P<0.01$).

miRNA microarray analysis

miRNA microarray was performed to investigate miRNA expression changes in the subcutaneous adipose tissue between intact and castrated male pigs. Analysis of microarray data indicated that 146 miRNAs of the 236 probes were detected on the microarray. Moreover, 47 miRNAs among these 146 miRNAs were changed significantly between intact and castrated male pigs (signal value >500, $P< 0.01$) (Fig. 2a). Here we selected the miRNAs with a signal value>500 and a *P*-value <0.01 as the high signal group. Among the high signal group, miRNAs whose relative expression levels showed a fold change ≥2 were considered as the differentially expressed miRNAs. A total of 18 differentially expressed miRNAs were discovered in subcutaneous adipose tissue of castrated male pigs, including ten up-regulated and eight down-regulated miRNAs (Tables 3 and 4). As shown in Fig. 2, we can clearly see the distinguishable miRNA expression profiles in adipose tissue between intact and castrated male pigs.

Table 3 Up-regulated miRNAs in subcutaneous adipose tissue of castrated male pigs

Reporter name	Fold change	Regulation	*P*-value
ssc-miR-15a	19.97	Up	2.03E-04
ssc-miR-101	15.71	Up	1.05E-12
ssc-miR-21	14.25	Up	1.09E-14
ssc-miR-148a	9.58	Up	0.00E+00
ssc-miR-451	4.16	Up	8.88E-16
ssc-miR-10b	2.47	Up	2.69E-14
ssc-miR-27a	2.35	Up	3.79E-13
ssc-miR-126	2.25	Up	4.51E-14
ssc-miR-30b-5p	2.18	Up	9.37E-11
ssc-miR-29a	2.10	Up	5.00E-13

Table 4 Down-regulated miRNAs in subcutaneous adipose tissue of castrated male pigs

Reporter name	Fold change	Regulation	*P*-value
ssc-miR-181b	−3.40	Down	0.00E+00
ssc-miR-331-3p	−2.88	Down	6.66E-16
ssc-miR-423-5p	−2.71	Down	2.22E-16
ssc-miR-455	−2.55	Down	6.44E-15
ssc-miR-99b	−2.51	Down	2.29E-13
ssc-miR-320	−2.37	Down	6.66E-16
ssc-miR-361-5p	−2.30	Down	2.27E-13
ssc-miR-140*	−2.11	Down	5.77E-15

Validation of miRNA microarrays by stem-loop real-time RT-PCR

To validate the microarray results, real-time PCR was performed on six differentially expressed miRNAs (ssc-miR-148a, ssc-miR-21, ssc-miR-320, ssc-miR-423-5p, ssc-miR-15a and ssc-miR-101). As shown in Fig. 3, the expression of miR-15a, miR-101, miR-148a and miR-21 were up-regulated, while the expression levels of miR-320 and miR-423-5p were down-regulated in the adipose tissue. These results clearly demonstrate that the trend of miRNA changes was consistent with microarray analysis and real-time PCR.

Target prediction and GO analysis of targets of differentially expressed miRNAs

Potential mRNA targets of the 18 differentially expressed miRNAs confirmed by miRNA microarrays were discovered using TargetScan. In total, 7874 target genes were predicted by TargetScan, which were used for GO analysis. Functional analysis of these target genes revealed that 61 GO terms were significantly enriched for the 18 miRNAs ($P<0.05$) (Supplementary Table S1). The enriched GOs targeted of the up-regulated miRNAs were mainly involved in receptor activity, positive regulation of cell proliferation, negative regulation of apoptolic process, G-protein coupled receptor

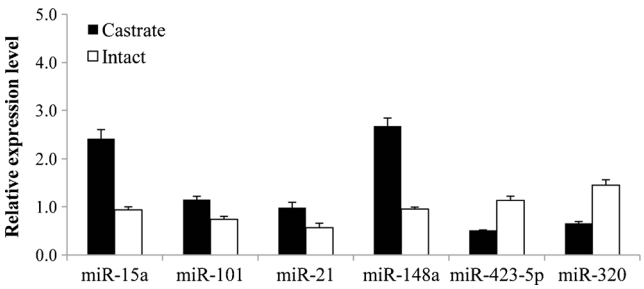

Fig. 3 Verification of miRNA microarray results by real-time PCR in the subcutaneous adipose tissue from intact and castrated male pigs. The *black bars* represent values from castrated male pigs; whereas the white bars represent values from intact male pigs

activity and lipid metabolism process while the specific GO of the target genes related to the down-regulated miRNAs were involved in protein binding, Golgi apparatus, anti-apoptosis and cell development (Fig. 4 and Supplementary Table S1). To further investigate the role of miRNAs in castration-induced fat deposition, a miRNA-mRNA regulatory network was subsequently established for the lipid metabolism process (Fig. 4c). Interestingly, miR-27a, miR-15a, miR-101, miR-30b-5p, miR-148a, miR-126 and miR-21 interacted with their target genes and were involved in lipid/fatty acid metabolism, suggesting that these miRNAs and their candidate targets might play important roles in castration-induced adiposity in male pigs.

Discussion

Castrated and intact male pigs demonstrate striking difference in body fatness (Mersmann 1984; Christoffersen et al. 2010). However, the molecular mechanisms that cause such differences are still not clear. In the past few years, many researchers have shown that miRNAs are involved in adipose tissue

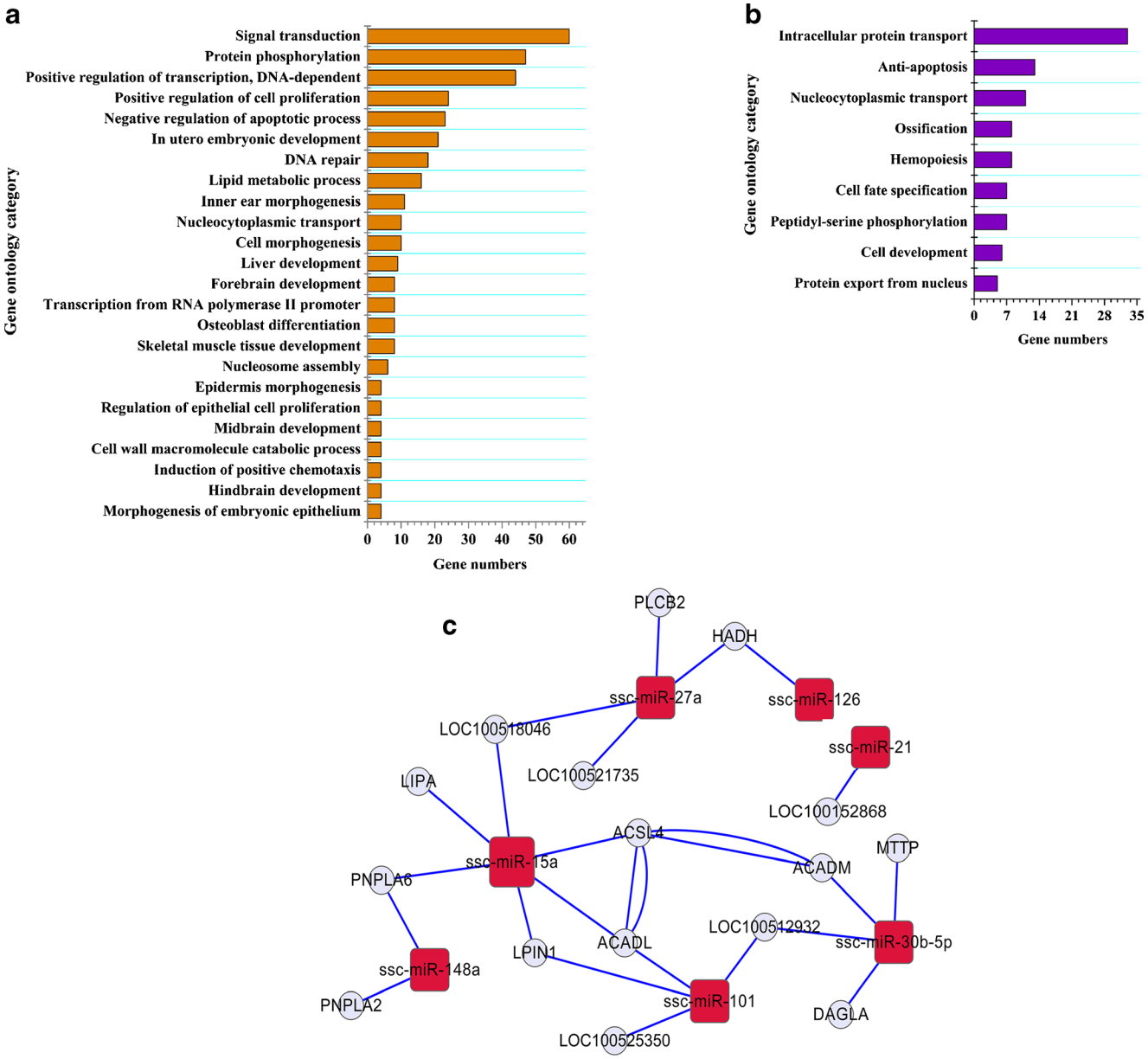

Fig. 4 Gene ontology (GO) analysis of genes targeted by differentially expressed miRNAs. **a** GOs targeted by up-expressed miRNAs. **b** GOs targeted by down-expressed miRNAs. The *vertical axis* is the GO category, and the *horizontal axis* is the enrichment of GO. Only the statistically significant GO terms in the biological process category are listed here. For other enriched GO terms, please see Supplementary Table S1. **c** miRNA-mRNA network. miRNAs are represented by *red box* and targets are represented by *blue nodes*. *Solid lines* show miRNA-target interactions

development and obesity (Takanabe et al. 2008; Xie et al. 2009; Gerin et al. 2010). Therefore, we used a miRNA microarray to investigate the effect of castration on adipose tissue miRNA expression profiles in male pigs. In this study, a total of 18 significantly differentially expressed miRNAs including 7874 of their unique target genes were determined in the subcutaneous adipose tissue between intact and castrated male pigs. Furthermore, GO functional analysis suggested that the widely biological processes are involved in castration-induced metabolism.

Previously, several miRNAs, such as miR-15a, miR-27a, miR-148a and miR-320, have been reported to perform very important functions in adipogenesis and obesity. For example, Kajimoto et al. (2006) found that miR-15a was significantly up-regulated during adipogenic differentiation in 3 T3-L1 preadipocytes, and overexpression miR-15a also increases 3 T3-L1 cell size (Andersen et al. 2010). MiR-27a was found to be up-regulated in adipose tissue of genetically obese mice (Lin et al. 2009). In the present study, we also found that miR-27a was significantly increased by castration in adipose tissue of male pigs. These results suggested that miR-27a is an important regulator involved in the obesity process. Besides, miR-320 had been reported to inhibit adipogenesis (Lee et al. 2011). This finding also accords with our observations, which showed that miR-320 was significantly down-regulated in adipose tissue of castrated male pigs. However, miR-148a was down-regulated in the mouse models of obesity (Xie et al. 2009), and the result is inconsistent with our study. One possible reason is this might be caused by the different experimental models of obesity. Up to now, there are no previous reports related to the effects of castration on miRNA expression in adipose tissue. In this study, these above adipogenesis-related miRNAs were found to be differentially expressed in adipose tissue between intact and castrated male pigs, suggesting that they can be regulated by sex hormones and may play important roles in the regulation of fat accumulation caused by castration-induced testosterone deficiency in male pigs. Our results might provide further evidence of a role for these miRNAs in the regulation of adipogenesis and obesity.

It is well known that miRNAs are involved in multiple biological processes through regulating gene expressions. In this study, TargetScan was used to predict potential targets of differentially expressed miRNAs. The predicted target genes were annotated by GO analysis, and thus explore the function of differentially expressed miRNAs. As expected, several GO terms including positive regulation of cell proliferation, G-protein coupled receptor activity and lipid metabolism process, were significantly enriched. Our findings coincide with the opinion that hormonal regulation of adipose tissue metabolism is a complex process and associated with many biological processes (Etherton and Walton 1986). Furthermore, we built a potential miRNA-mRNA interaction network for lipid metabolism process. In this network, several target genes for miR-15a and miR-101, such as ACADL (Gondret et al. 2012), ACSL4 (Mashek et al. 2006) and LPIN1 (Donkor et al. 2008), were reported to be involved in fatty acid oxidation. This observation suggested that fatty acid oxidation may play key roles in the regulation of fat storage in castrated male pigs. There has been evidence that lower fat oxidation in women than in men causes a higher fat storage (Levadoux et al. 2001). As such, our studies indicated that these miRNAs may affect fat accumulation in castrated male pigs by targeting genes involved in fatty acid oxidation. Collectively, these enrichment analysis illustrate the possible roles of the differentially expressed miRNAs in adipose tissue of castrated male pigs.

In conclusion, castration-induced testosterone deficiency in male pigs could increase body fat accumulation. Moreover, castration also resulted in significant changes in miRNAs expression profiles in subcutaneous adipose tissue. Our results found that specific miRNAs could be directly involved in the body fat deposition resulting from castration-induced testosterone deficiency in male pigs. Our study provides a new clue for understanding the changes of body fat deposition caused by castration-induced testosterone deficiency.

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Conflict of Interest The authors declare that they have no conflict of interest.

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