Research in Veterinary Science 99 (2015) 99–104

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Differential miRNA expression profiles in the *longissimus dorsi* muscle between intact and castrated male pigs

Zhaowei Cai [a,b], Lifan Zhang [c], Xiaoling Jiang [a], Yifei Sheng [a], Ningying Xu [a,*]

[a] College of Animal Science, Zhejiang University, Hangzhou 310058, China
[b] Laboratory Animal Research Center, Zhejiang Chinese Medical University, Hangzhou 310053, China
[c] College of Animal Science and Technology, Nanjing Agricultural University, Nanjing 210095, China

ARTICLE INFO

Article history:
Received 11 April 2014
Accepted 17 December 2014

Keywords:
Castration
MicroRNA
Expression
Skeletal muscle
Pig

ABSTRACT

MicroRNAs (miRNAs) are important modulators of skeletal muscle development in multiple mammalian species, but their role in skeletal muscle growth in castrated male pigs has not been well studied. The aim of the present study was to determine the role of miRNAs in longissimus dorsi muscle under castration. Our results showed that castration caused a significant decrease in serum testosterone levels as well as carcass lean mass, but led to an increase in carcass fat mass. Moreover, miRNA expression profiles in skeletal muscle were significantly altered by castration, and seven differentially expressed miRNAs were discovered. More importantly, functional analysis suggested that these differentially expressed miRNAs and their targets are involved in the regulation of skeletal muscle contractile function and fat metabolism. Taken together, these results demonstrate altered miRNA expression in skeletal muscle of castrated male pigs, and suggest a potential mechanism underlying the effects of castration on porcine skeletal muscle growth.

1. Introduction

Castration of male pigs has been considered a traditional practice in most countries, and is performed to prevent boar taint produced and aggressive behavior shown by male animals (Sutherland et al., 2010). However, some disadvantages of castration, such as lower skeletal muscle mass and higher fat deposition, are also evident in the pig industry (Gispert et al., 2010). It is well known that castration of male pigs results in negative effects on skeletal muscle growth, but, thus far, the molecular basis for this defect is not clear.

MicroRNAs (miRNAs) are a class of small non-coding RNA transcripts that regulate gene expression by binding to genomic regions of specific target genes (von Deetzen et al., 2013). The role of miRNAs in the regulation of skeletal muscle growth and development has been previously demonstrated. For example, miR-26a is induced during skeletal muscle regeneration, and exogenous miR-26a promotes differentiation of myoblasts in humans (Dey et al., 2012); miR-27a increases muscle cell proliferation by directly inhibiting myostatin gene expression (Huang et al., 2012); miR-378 is considered a candidate for regulating myogenesis and participates in skeletal muscle development through the regulation of *BMP2* and *MAPK1* (Hou et al., 2012); and miR-155 affects porcine prenatal skeletal muscle development through the regulation of *OLFML3* (Zhao et al., 2012). Until now, little was known about the roles of miRNAs in the regulation of skeletal muscle growth and development in castrated male pigs. Specifically, it was not clear whether testosterone loss due to castration could influence miRNA expression patterns in porcine skeletal muscle. Previous studies have suggested that androgen treatment could alter miRNA expression levels in human skeletal muscle myoblasts. Androgen receptor (AR) regulates the downstream mRNA targets of these miRNAs specifically in skeletal muscle (Wyce et al., 2010). Moreover, androgen treatment upregulates the expression of a large set of miRNAs in the prostate and muscle in rats (Narayanan et al., 2010). We hypothesized that reduction of androgen levels due to castration may influence miRNA expression in skeletal muscle of male pigs. Therefore, we investigated the changes in miRNA expression profiles in the longissimus dorsi (LD) muscle between intact and castrated male pigs by using an miRNA microarray assay, thus providing a novel view of the role of miRNAs in castration-induced skeletal muscle growth and development.

2. Materials and methods

2.1. Animal experiments and sample collection

The experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee of

* Corresponding author. College of Animal Sciences, Zhejiang University, No. 388 Yuhangtang Road, Hangzhou 310058, China. Tel.: +86 571 88982089; fax: +86 571 88982089.
E-mail address: nyxu@zju.edu.cn (N. Xu).

http://dx.doi.org/10.1016/j.rvsc.2014.12.012
0034-5288/

Zhejiang University. A total of 14 male Large White pigs from seven pairs of full sibs were used in this experiment. On day 35, one pig from each pair was randomly selected to be castrated (CM) under anesthesia, and another one remained intact (IM). All animals were slaughtered at 210 days of age. The head, feet, and internal organs were removed, and individual carcass weight of both left and right sides were recorded. The left sides were physically dissected into bone, muscle, fat, and skin, which were also weighted individually. The LD muscle area was traced on an acetate film between the 10th and 11th ribs and subsequently determined by planimetry. Intramuscular fat content of the LD samples were determined with the Antaris meat analyzer (Thermo Electron Corporation, Waltham, MA, USA), working in the wavelength range of 780–2500 nm, based on the near infrared transmission (NIT) principle. The LD muscle samples were collected and frozen in liquid nitrogen, and then stored at –80 °C for subsequent experiments.

2.2. Measurement of serum testosterone levels

Blood samples were collected from male pigs at 35, 84, 147, and 210 days of age and were kept at room temperature for 2 h. Serum was collected by centrifugation (3000 × *g* for 15 min at 4 °C) and then stored at –80 °C until further use for the hormone assay. Serum testosterone levels were measured using a commercial radioimmunoassay kit (Beijing North Institute of Biotechnology, Beijing, China) according to the manufacturer's instructions.

2.3. Histological analysis

LD muscle 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). Then, the slides were viewed and photographed using a digital camera mounted on an Olympus Microscope (Olympus BX51, Tokyo, Japan). The resulting photos were analyzed with ImageJ software (NIH, Bethesda, MD) as previously described (Wu et al., 2009).

2.4. miRNA microarray assay

Total RNA was extracted from LD muscle samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA integrity was measured using the Agilent 2100 BIOANALYZER® (Agilent Technologies, Waldbronn, Germany). RNA integrity number (RIN) values of the samples ranged from 8.2 to 9.8. RNA from three pairs of LD muscle samples from intact and castrated male pigs were extracted and pooled, respectively. MiRNA microarray analysis was performed by LC Sciences (Houston, TX, USA). The chip contained 236 known *Sus scrofa* miRNAs corresponding to miRNA transcripts listed in Sanger miRBase release 16.0 (http://www.sanger.ac.uk/Software/Rfam/mirna/) (Appendix: Supplementary Table S1). Each probe was spotted in duplicate, and control probes used as quality controls in chip production, sample labeling, and assay conditions were also included on the chip. The microarray assay was performed as described by Wang et al. (2013).

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 (Pan, 2002). A transcript listed as detectable met the following conditions: signal intensity higher than 3 × (background standard deviation) and spot coefficient of variation (CV) less than 0.5. When repeating probes were present on an array, a transcript was listed as detectable only if the signals from at least 50% of the repeating probes were above the detection level (Li et al., 2012).

2.5. Stem-loop real-time RT-PCR

To validate the microarray results, stem-loop real-time RT-PCR was conducted to measure the expression levels of four selected miRNAs (Appendix: Supplementary Table S2) by using individual RNA samples that were pooled for use in the miRNA microarray assay. In brief, 1 μg total RNA was reverse transcribed using 0.5 μL M-MLV Reverse Transcriptase (200 U/μL) and 1 μL stem-loop RT primer (10 μM) in an Applied Biosystems 9700 Thermocycler with the following program: 42 °C for 60 min, followed by 70 °C for 15 min, and then held at 4 °C. Real-time PCR was performed using SYBR Green Master Mix (No. DRR041A, Takara, Japan) and the StepOne™ Software v2.0 (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. Porcine miR-24 was used as an internal control, and all PCR reactions were run in triplicate. The comparative $2^{-\Delta\Delta Ct}$ method was used to determine differences in the expression levels (Livak and Schmittgen, 2001).

2.6. Target prediction and functional enrichment of differentially expressed miRNAs

TargetScan (Lewis et al., 2005) and miRanda (John et al., 2004) were used to predict the potential targets of differentially expressed miRNAs. The prediction results are a combination of these two queries. GO enrichment analysis was performed using the Gorilla tool (http://cbl-gorilla.cs.technion.ac.il/) (Eden et al., 2009; Joly-Tonetti et al., 2013). Probability values were corrected through the Benjamini and Hochberg's false discovery rate (FDR) method and considered significant if the corrected *P*-value was less than 0.05 (Joly-Tonetti et al., 2013). In addition, Cytoscape software (Cline et al., 2007) was used to construct the miRNA-mRNA network based on regulatory interactions between differentially expressed miRNAs and target GO genes.

2.7. Statistical analysis

Statistical analyses were performed using SPSS 13.0 software (SPSS, Chicago, IL, USA). A two-tailed Student's *t*-test was used to evaluate the statistical significance of differences between groups. The results were expressed as mean values with standard errors. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Effects of castration on serum testosterone levels and carcass characteristics in male pigs

The effect of castration on serum testosterone levels are shown in Fig. 1. Serum testosterone levels decreased significantly with castration. Total serum testosterone levels in intact male pigs were significantly higher than those of castrated male pigs at 35–210 days of age.

Castrated male pigs had lower body weight, lean meat weight, lean meat percentage and loin eye area (LEA) than intact pigs ($P < 0.05$; Table 1). Moreover, castration caused a significant reduction in the mean cross-sectional area (CSA) of LD muscle fibers in male pigs ($P < 0.05$; Fig. 2), along with decreased LEA and skeletal muscle mass in castrated male pigs. In contrast, castration resulted in a significant increase in carcass fat weight, fat percentage ($P < 0.01$) and intramuscular fat content ($P < 0.05$) in male pigs (Table 1).

3.2. miRNA microarray analysis

To examine castration-induced miRNA expression changes in skeletal muscle of male pigs, we compared differential miRNA expression