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Selection of reference genes for gene expression studies in porcine skeletal muscle using SYBR green qPCR

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ABSTRACT

Quantitative PCR (qPCR) is a method for rapid and reliable quantification of mRNA. Internal controls such as reference genes are used to normalize mRNA levels between different samples for an exact comparison of gene transcription level. However, the expression levels of these reference genes may vary between cell types, developmental stages, species and experimental conditions, thus proper normalization strategy is an important precondition for reliable conclusions. In this study, we explored 10 commonly used reference genes in porcine skeletal muscle using SYBR green qPCR. We used both geNorm and NormFinder to analyze the expression stability and found that *PPIA*, *HPRT* and *eEF-1*γ were suitable internal controls for porcine skeletal muscle. However, *PPIA*, *HPRT* and *SDHA* were suitable for skeletal muscle of western pigs while *PPIA*, *eEF-1*γ and *HPRT* for indigenous Chinese pigs. Normalized qPCR data of *ROCK2* were compared with microarray data to evaluate our developed set of reference genes.

1. Introduction

Meat is a major part of human nutrition containing essential protein ingredients. Before muscle becomes meat, skeletal muscle fulfills the important task of maintaining body stature and locomotion and its growth and development is a major part of body growth. Anatomical, physiological, pathological and genomic likenesses between pig and human have suggested that pigs could be considered a model species for human health issues (Rothschild, 2004; Schook et al., 2005; Wernersson et al., 2005). So understanding the growth and development of porcine skeletal muscle is one of the most important goals in animal and meat science and the study of it is also related to human medicine. Many studies have examined the mechanisms underlying porcine skeletal muscle growth and development. Understanding changes of gene expression patterns that accompany the whole process may provide insight into complex regulatory networks and help to identify genes involved in the process (Li et al., 2008; Pan, 2003; Xu et al., 2009). QPCR is one of the most powerful quantification methods for gene expression analysis. Similar to other methods used in expression studies, qPCR data are usually required to be normalized against a set of data or references to correct for the differences in starting materials. The genes used as references are usually those which are assumed constantly expressed in certain tissues and under certain circumstances. However, increasing evidence shows that transcription levels of those commonly used reference genes vary between cell types (Fischer et al., 2005; Goidin et al., 2001; Li et al., 2005; Mogal and Abdulkadir, 2006; Ohl et al., 2006; Schmittgen and Zakrajsek, 2000), developmental stages (Goossens et al., 2005; Kuijk et al., 2007), species (Brinkhof et al., 2006; Nygard et al., 2007; Olsvik et al., 2005; Overbergh et al., 2005; Spinsanti et al., 2006) and experimental conditions (Tricarico et al., 2002; Zhang et al., 2005). Therefore, selection of a proper normalization strategy is of importance for data interpretation.

A few studies have examined reference genes in porcine cells, tissues and organs (Erkens et al., 2006; Foss et al., 1998; Kuijk et al., 2007; Nygard et al., 2007; Tramontana et al., 2008), and reveal that *GAPDH* and *ACTB*, two commonly used reference genes, are not necessarily constitutively expressed. Reference genes for skeletal muscle at different developmental stages in pigs have not yet been studied, however. We examined the expression of 10 commonly used control genes including *GPX1* (glutathione peroxidase 1), *PPIA* (peptidylprolyl isomerase A), *GAPDH* (glyceraldehydes 3-phosphate dehydrogenase), *SDHA* (succinate dehydrogenase complex subunit A), *eEF-1 r* (eukaryotic elongation factor 1 gamma-like protein), *ATP5G1* (ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C1 (subunit 9)), *SHAS2* (Sus scrofa hyaluronan synthase 2), *GPI* (glucose phosphate isomerase), *ACTB* (actin, beta) and *HPRT* (hypoxanthine phosphoribosyltrans-

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ferase) in longissimus dorsi (LD) muscles at four developmental stages in Yorkshire (a typical lean-type western breed) and Meishan (a typical indigenous Chinese breed) pigs.

Rho GTPases are a family of molecular switches that control various cell functions (Etienne-Manneville and Hall, 2002). As one of the major downstream effectors, rho-associated, coiled-coil containing protein kinase 2 (*ROCK2*) plays an important role in skeletal muscle development (Fortier et al., 2008; Pelosi et al., 2007), making it an ideal gene to evaluate our developed set of reference genes.

2. Materials and methods

2.1. Sample collection

All pigs were raised at the Jingpin Pig Station of Huazhong Agricultural University (Wuhan, China). And all animal procedures were performed according to protocols approved by Hubei Province, PR China for Biological Studies Animal Care and Use Committee. Yorkshire and Meishan sows were mated with the boars of the same breeds. We collected LD muscles from fetuses at 65 days post conception (dpc) and pigs at 3 days, 60 days, 120 days afterbirth with three biological repeats at each stage of either pig breed. The muscle tissues were sampled within 5 min after euthanasia and were immediately frozen in liquid nitrogen and stored at −80 °C after being collected.

2.2. RNA preparation

Total RNA was isolated using Trizol reagent (Invitrogen, USA). To remove the contaminating genomic DNA, we treated total RNA with RNase-free DNase I (Promega, USA) according to the ratio of 1 μg total RNA to 1 μL RQ1 RNase-Free DNase I (1 U/μl). Nucleic acid concentrations were measured at 260 nm with a BioPhotometer (Eppendorf, Germany). Purity of the total RNA was determined by the A260/280 and A260/230 ratio and its integrity was tested by electrophoresis using 1% formaldehyde denaturing agarose gel.

2.3. CDNA synthesis and quality confirmation

The first strand cDNA was synthesized using M-MLV reverse transcriptase (Promega, USA) in a 25 μL reaction mixture according to the manufacturer's instructions. Briefly, a mixture of 2 μg total RNA and 0.5 μL oligo (dT) was incubated at 70 °C for 5 min to break the secondary structure of RNA. After the mixture was chilled on ice for at least 2 min, 5 μL 5× RT buffer, 1.25 μL dNTPs (10 mM each), 25 U RNase Inhibitor and 200 U M-MLV reverse transcriptase were added with a total volume of 25 μL. The RT mixture was incubated at 42 °C for 60 min. Finally, the reverse transcriptase was inactivated by incubation at 72 °C for 5 min. The cDNA was diluted according to the ratio of 1 μL cDNA to 9 μL nuclease-free water. We tested the cDNA using the *ACTB* primers in a 20 μL reaction mixture, and at the same time we performed two contrast tests with genomic DNA and sterile water. The cycling conditions consisted of 1 cycle (94 °C for 4 min), followed by 40 cycles (94 °C for 50 s; 57 °C for 50 s; 72 °C for 30 s) and by 72 °C for 10 min.

2.4. Candidate reference gene selection and primer design

We selected 10 candidate reference genes from commonly used reference genes based on the principle that they did not belong to the same biological pathways that might be co-regulated (Erkens et al., 2006; Foss et al., 1998; Kuijk et al., 2007; Nygard et al., 2007; Tramontana et al., 2008). The porcine sequences of these genes were obtained from GenBank database. All the primer pairs were designed by Primer 3 software (Rozen and Skaletsky, 2000). Gene-specific amplifications were confirmed by electrophoresis in 1.5% agarose gel, sequencing and melt curve analysis.

2.5. QPCR with SYBR green

QPCR reactions were carried out in an IQ5 real-time cycler (BIO-RAD, USA) using the SYBR Green method. Each PCR (in 25 μL) contained 12.5 μL SYBR Green Real-time PCR Master Mix (TOYOBO, Japan), 0.6 μL primers (10 μM) (0.3 μL for each), plus 0.5 μL template cDNA. The cycling conditions consisted of an initial single cycle (95 °C for 3 min) followed by 40 cycles (95 °C for 15 s; 57 °C for 15 s; 72 °C for 20 s; plate reading at 80 °C). A final melting program ranging from 55 °C to 95 °C with a heating rate of 0.5 °C/10 s was carried out to create melt curves. PCRs were performed in quadruplicate for each cDNA sample. And negative controls with no template were included for each primer pair.

2.6. PCR efficiency and Ct value calculation

We calculated PCR efficiency and Ct value using the LinRegPCR software (version 11.0) following the instructions. First, we put the background corrected fluorescence data into the LinRegPCR software according to the appointed format. Then we corrected the baseline and set the Window-of-Linearity to gain the target mRNA quantity (R0) and the amplification efficiency (E) as well as the Ct value. The Ct value of every single reaction and the mean efficiency of each amplicon were used to calculate their relative expression levels.

2.7. Analysis of expression stability

The expression stability was evaluated by both geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004). GeNorm generates a stability measure (the M value) for every gene allowing ranking them according to their expression stability (with lower value indicating increased gene stability across samples). It also generates a pairwise stability measure to decide the benefit of adding extra reference genes for the normalization. NormFinder generates a stability measure and groups samples to allow direct estimation of expression variation, ranking genes according to their stability using a model-based approach.

2.8. Microarray data collection

The same RNA samples were treated and hybridized to Affe GeneChip® Porcine Genome Array (AFF-900623) (24 arrays in total) to analyze the genome-wide transcriptional profile of porcine skeletal muscle (not published). The normalized expression signals of *ROCK2* were collected.

2.9. ROCK2 mRNA expression and data processing

QPCR was conducted with the primers for *ROCK2* as described previously, after which the mRNA expression of *ROCK2* was calculated and normalized to the selected set of reference genes, and then the qPCR data and microarray data were converted into logarithmic values and plotted in graph.

3. Results

3.1. Total RNA extraction and purification

All the ratios of 28 S to 18 S were greater than 1:1 showing good integrity of RNA. The A260/280 and A260/230 values were respective 1.69–1.98 and 0.46–1.85 before RNase-free DNase I treatment