Head title: Identification of novel MEF2A transcripts

Novel Alternatively Spliced Isoforms of MEF2A and Their mRNA Expression Patterns in Pig

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Abstract

The present study aimed to identify alternatively spliced isoforms of pig MEF2A gene and determine their mRNA expression patterns. Four alternatively spliced isoforms of pig MEF2A gene (i.e., MEF2A1, MEF2A2, MEF2A3, and MEF2A4) were cloned according to the results of transcriptome sequencing. The fifth to eight exons of MEF2A1 were normally spliced. In MEF2A2, the fifth exon was missing; the sixth exon had an extra 138 bp at its 5' end, and the seventh exon had extra 102 bp at its 3' end. In MEF2A3, the fifth exon was missing, and the sixth exon had an additional 138 bp at its 5' end. In MEF2A4, the seventh exon had an extra 102 bp at its 3' end. Quantitative real-time PCR analysis indicated that the expression profiles of the four alternatively spliced transcripts in the longissimus dorsi differed between Mashen and Large White pigs. MEF2A1 and MEF2A2 expression was the highest at 90 days of age and the lowest at 180 days of age. MEF2A3 and MEF2A4 expression increased with age (in days). The four alternatively spliced isoforms of MEF2A were also expressed in the small intestine, cerebellum, pancreas, heart, and lung. The discovery of new alternatively spliced transcripts of MEF2A gene may be utilized in better understanding its biological functions.

Keywords: pig; MEF2A gene; alternative splicing; mRNA expression

Introduction

Myocyte enhancer factor 2 (MEF2) is a member of the MADS-box (MCMI, agamous, deficiens and serum response factor) family. The N-terminal of MEF2 contains one MADS family member-specific MADS-box domain (Linseman et al. 2003) and one MEF2-specific MEF2 domain. The MADS-box domain is composed of 57 amino acids and is highly conserved among different species. The MEF2 domain is composed of 29 amino acids that are situated adjacent to the MADS-box, and its primary function is to increase binding affinity to the A/T sequence the
regulatory region of the target DNA (Martin et al. 1994). It is also a place for the interaction between MEF2 and other factors (Molkentin et al. 1996) to mediate MADS-box protein dimerization and transcriptional activation of the expression of muscle-specific genes. The C-terminal of MEF2 is a transcriptional activation region containing a potential phosphorylation site in the conserved region of the terminal and is the target site of relevant kinases that are involved in the initiation of gene expression. The differences in the selection of splicing methods result in the formation of different isoforms of MEF2 proteins. With a wide range of functions, MEF2 is a transcription factor that plays a key role in muscle development through the mutual recognition and regulation of bHLH and the MADs domain with myogenic regulatory factors (MRFs) (Molkentin et al. 1995; Wang DZ et al. 2001), as well as in muscle formation, neurodevelopment (Akhtar et al. 2012), and liver fibrosis.

MEF2 is a multi-gene family consisting of the MEF2A, MEF2B, MEF2C, and MEF2D genes. These four transcription factors have different and overlapped expression patterns in developing embryos and adult animal tissues (McKinsey et al. 2002). MEF2A promotes the regeneration of adult rat skeletal muscle by regulating the microRNA (miRNA)-mediated Wnt signaling pathway (Snyder et al. 2013). MEF2A-knockout mice have increased mortality within the first week after birth, and the dead mice exhibit severely damaged cardiomyocytes, significant dilatation of the right ventricles, and mitochondrial derangement. Only a few MEF2A-knockout mice can survive to adulthood yet show poor mitochondrial function (Naya et al. 2002). Neonatal cardiomyocytes deficient in MEF2A result in myofibrils and plaque deformities associated with anchorage-dependent programmed cell death (Ewen et al. 2011). The above studies have shown that MEF2A maintains the structural integrity and proper mitochondrial content of postnatal cardiomyocytes, whereas other MEF subtypes do not possess these functions.

Mashen pig is an excellent local breed pig in Shanxi Province, China, with strong fecundity, good adaptability, stress tolerance, strong disease resistance and good meat quality, but low feed conversion ratio (FCR) and slow growth rate. Compared to the Mashen pig, Large White pig has a high FCR, rapid growth rate, and high carcass lean rate. Mashen and Large White pigs are ideal animal models for studying pig muscle growth and developmental patterns. The present study used the prediction results of transcriptome sequencing to predict two potential alternative splice sites via in vitro amplification and cloning to obtain four alternatively spliced isoforms of the pig MEF2A gene. Quantitative real-time PCR (qPCR) was used to detect the expression patterns of the four alternatively spliced isoforms of the MEF2A gene in 13 different tissues and in the longissimus dorsi during development to identify the roles of MEF2A gene in muscle formation and development.

**Materials and Methods**

All of the animal procedures were as per the Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm). The methods were performed in accordance with the Good Experimental Practices adopted by the College of Animal Science and Veterinary Medicine, Shanxi Agricultural University (Shanxi, China). Moreover, the local animal welfare laws, guidelines, and policies were strictly followed for the feed and use of experimental animals.

**Laboratory Animals and Tissue Collection**

Nine Mashen and nine Large White pigs were purchased from the Datong Pig Breeding Farm (Shanxi, China) to collect tissue samples on postnatal 1, 90, and 180 days (three pigs per each of postnatal stages; male pigs underwent castration at weaning. Cerebellum, lung, spleen, small intestine, pancreas, fat, kidney, stomach, liver, and heart tissues of 90 day as well as longissimus dorsi, biceps femoris and psoas of Mashen and Large White pigs at the three postnatal stages were collected, followed by wrapping in aluminum foil, placing in centrifuge tubes, snap-freezing in
liquid nitrogen, and preserving in a −80°C freezer for later use. Among all collected tissues, longissimus dorsi, biceps femoris and psoas were tested by RNA sequencing (RNA-seq) in Shanghai Biotechnology Corporation.

**Total RNA Extraction and Purification**

TRIzol reagent (Life Technologies, Carlsbad, CA, USA) was used for total RNA extraction from the samples in accordance to the standard operating procedures provided by the manufacturer. Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) was used to confirm the quality of the extracted total RNA, followed by using RNaseasy micro kit and RNase-Free DNase Set (QIAGEN, GmBH, Germany) to purify the total RNA. NanoDrop ND-2000 spectrophotometer and Agilent Bioanalyzer 2100 was used to test the quality of purified RNA (RIN ≥7.0, 28S/18S ≥0.7).

**RNA-Seq and Alternative Splice Prediction**

Total RNA extracts underwent rRNA removal, fragmentation, first-strand cDNA synthesis, second-strand cDNA synthesis, terminal repairing, polyadenylation at 3’ end, linker ligation, and enrichment to generate a sequencing library of the sample. An Illumina HiSeqTM 2500 system was used for sequencing, with 125 bp of double-ended sequences. Sequencing results were filtered to remove low-quality sequence and linkers to obtain clean reads. Spliced mapping algorithm of Tophat (version 2.0.9) was used for genome mapping, and cufflinks (version 2.1.1) was used for gene quantification of the Tophat alignment results (Ghosh et al. 2016). Most of the occasional events caused by noise from all splice site information of the Tophat alignment results (≥ five reads) was filtered out, followed by a comparison with the known or reference splice sites (allowing 1 bp error) to find the known splice sites and to perform classification of alternative splicing from the remaining new splice sites.

**PCR Amplification of Alternatively Spliced Pig MEF2A Transcripts**

The extracted total RNA was reversed transcribed to synthesize cDNA according to the manufacturer’s instructions of PrimeScriptTM RT-PCR reagent Kit with gDNA Eraser (TAKARA, China). According to the predicted alternative splicing and porcine MEF2A gene sequence from NCBI, we designed the primers using NCBI Primer-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Oligo 7 software. P1 primer was used to amplify MEF2A gene predicted alternative splicing region (CDS region between exon 5 to 8). The P2–P5 primers amplified the four alternative splice forms of MEF2A gene separately (Table 1). The cDNA pool of the three different types of muscle tissues from different individuals at different postnatal age in days was used as PCR template. Ten microliters of each PCR system contained 0.4 µL of cDNA, 5 µL of 2×Taq PCR MasterMix (TIANGEN, Beijing, China), 0.2 µL of the upstream primer, 0.2 µL of the downstream primer, and the remaining volume was filled with RNase-free water. PCR conditions included 95°C for five min denaturation; 35 cycles of 95°C denaturation for 30 s, 56°C–64°C annealing for 30 s, and 72°C elongation for 40 s; and final elongation at 72°C for 5 min. PCR products were separated using 1.5% agarose gel electrophoresis.

**Cloning and Sequencing for the Identification of Alternatively Spliced Transcripts of MEF2A**

After purifying the PCR products using OMEGA Gel Extraction Kit (OMEGA, Shanghai, China), the clones were ligated to a pMD-19T vector (Takara, Dalian, China). The positive clones were sequenced by BGI’s genome sequencing (Guangdong, China), and the sequencing results were aligned using DNAMAN software (Lynnon Corp., Quebec, Canada).

**mRNA Expression Levels of the Four Alternatively Spliced Transcripts of MEF2A Using qPCR**
To investigate the expression of MEF2A in the skeletal muscle tissues of Mashen and Large White pigs, qPCR was used to detect the mRNA expression of four alternatively spliced transcripts of MEF2A in 13 different tissues, including cerebellum, lung, spleen, small intestine, pancreas, fat, kidney, stomach, liver, and heart tissues as well as the longissimus dorsi, biceps femoris, and psoas of Mashen and Large White pigs on postnatal 1, 90, and 180 days. 18S rRNA was used as reference to design the specific primers for the four alternative splice forms of MEF2A (Table 1). SYBR PrimeScript™ RT-PCR Kit (Takara, China) was used to run the qPCR in ABI-7500 system (Life Technologies) according to the manufacturer’s instructions. Each qPCR reaction system contained 10 μL of SYBR®Premix Ex Taq™ II, 0.8 μL of 10 μmol/L of the upstream primer, 0.8 μL of the 10 μmol/L downstream primer, 0.4 μL of ROX, 2 μL of the cDNA (5 times dilution), and 6 μL of double distilled water. PCR amplification conditions were denaturation at 95°C for 5 min; followed by 45 cycles of denaturation at 95°C for 30 s and 60°C for 34 s; and by 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s.

Statistical Analysis

The 2^−ΔΔCT method was used to calculate the relative mRNA expression of the four alternatively spliced transcripts of MEF2A. SPSS 17.0 software (SPSS Inc., Chicago, IL) were used for variance analysis of expression differences in different tissues and at different postnatal stages. Duncan method was used for the analysis of significant differences between multiple groups of samples. P<0.05 (*) was considered as significantly different, and P<0.01 (**) was considered to show highly significant differences.

Results

Prediction of Transcriptome Sequencing of Alternatively Spliced Transcripts

Approximately 272 Gb data were generated after RNA-seq, with an average of 15 Gb sequencing quantity per tissue. Tophat alignment showed that >73% of the data matched with the pig genome (genome version: USCS susScr3). All splice site information in the Tophat alignment results was analyzed to exclude low-quality alignment results and obtain splice information of 11,500 genes, which was divided into eight kinds of alternative splicing events, including exon skipping, alternative 5’ end splicing, alternative 3’ end splicing, intron detention, mutually exclusive exons, alternative promoters, alternative polyadenylation, and various combinations of splicing (see results in Figure 1).

Amplification of Alternatively Splicing CDS Region of the MEF2A Gene

The amplification products were detected by 1.5% agarose gel electrophoresis, which showed two bands between 500 bp and 700 bp in size that were in line with the size of the target fragments (Figure 2). These two bands were not separated and collected for DNA gel extraction for subsequent cloning and sequencing.

Identification of the New Alternatively Spliced Transcripts of the Porcine MEF2A Gene

Sequences of positive bacterial culture were aligned using DNAMAN software, and the results showed four alternatively spliced isoforms of MEF2A gene, namely MEF2A1 (GenBank accession number: KY313741), MEF2A2 (GenBank accession number: KY305402), MEF2A3 (GenBank accession number: KY313742), and MEF2A4 (GenBank accession number: KY313743). Figure 3 showed that the two alternative splice sites were found in the four MEF2A splice forms, including a 138 bp hypervariable region between 28 bp and 165 bp and a 102 bp insertion region between 447 bp and 548 bp. MEF2A1 was the normal mRNA splicing product. The 102 bp insertion region was found in MEF2A4 but not MEF2A1. The 138 bp hypervariable region and 102 bp insertion regions were
found in MEF2A2 but not in MEF2A1. The 138 bp hypervariable region was found in MEF2A3 but not in MEF2A1. Despite a presence of T-to-C mutation site in the 102 bp insertion region of MEF2A2 and MEF2A4, the remaining sequences of MEF2A2 and MEF2A4 were consistent.

Further analysis showed that the 138 bp hypervariable region was located at the 5’ end of the sixth exon, and the 102 bp insertion region was located at the 3’ end of the seventh exon (Figure 4a). The MEF2A mRNA precursor underwent normal splicing at the fifth to eighth exons to form MEF2A1 during the process of mRNA maturation. MEF2A2 had a missing fifth exon, a 138 bp extension at the 5’ end of the sixth exon, and a 102 bp extension at the 3’ end of the seventh exon. MEF2A3 had a missing fifth exon and a 138 bp extension at the 5’ end of the sixth exon. MEF2A4 had a 102 bp extension at the 3’ end of the seventh exon (Figure 4b). Protein sequences alignments of MEF2A1, MEF2A2, MEF2A3 and MEF2A4 were shown in Figure 5.

The prediction of function domain indicated that those four MEF2A isoforms (MEF2A1, MEF2A2, MEF2A3 and MEF2A4) all had a HJURP_C conserved domain (Figure 5). HJURP_C was a repetitive sequences of the C terminal of the Holliday junction regulator protein family, and was also a common protein region of MEF2 protein. This protein family was a new chaperone which can promote the attachment of centromere protein A (CENP-A) at the centromere.

The four alternatively spliced isoforms of the MEF2A gene were used to design the specific primers (Table 1) and amplified by PCR. PCR products were detected by 1.5% agarose gel electrophoresis (Figure 6), and the results showed a clear single band. DNA gel extraction, cloning, PCR amplification of the bacterial culture, and sequencing of the positive bacterial culture were performed to further validate the results. The sequencing results were consistent with the first sequencing data.

**Developmental mRNA Expression of the Four Alternative Splice Forms of MEF2A in the Longissimus Dorsi**

The mRNA expression of MEF2A1 and MEF2A2 gene in the longissimus dorsi of Large White pigs was first increased and subsequently decreased with increasing postnatal age in days; whereas MEF2A3 and MEF2A4 mRNA expression increased with increasing postnatal age in days. The mRNA expression of the MEF2A1, MEF2A2, and MEF2A4 genes in the longissimus dorsi of Mashen pig was optimized on the 90 postnatal day and remained stable afterwards. MEF2A3 mRNA expression in the longissimus dorsi of Mashen pig initially increased and then decreased with increasing postnatal age in days. Comparison of Mashen and Large White pigs showed that the MEF2A1 and MEF2A2 mRNA expression of the Mashen pig was significantly higher than that of the Large White pig on postnatal 180 day (P<0.01); however, at postnatal 1 day, MEF2A3 mRNA expression in the Mashen pig was significantly higher than that of the Large White pigs on postnatal 90 and 180 days (P<0.01); finally, MEF2A4 mRNA expression of the Large White pigs was significantly higher than that of the Mashen pigs at the same postnatal stages (P<0.05, Figure 7).

**mRNA Expression Profiles of the Four Alternatively Spliced Transcripts of MEF2A in 13 Different Tissues**

In the Large White pigs, MEF2A1 mRNA expression was maximized in the longissimus dorsi and almost absent in the small intestine and pancreas; MEF2A2 mRNA expression was maximized in the cerebellum and heart; MEF2A3 mRNA expression was relatively high in the pancreas, psoas, and longissimus dorsi but almost undetectable in other tissues, and despite the lack of expression in fat, and MEF2A4 mRNA expressed in the remaining tissues. In Mashen pigs, MEF2A1 mRNA expression was highest in the small intestine; MEF2A2 mRNA was expressed in all 13 tissues; MEF2A3 mRNA expression was highest in the longissimus dorsi; MEF2A4 mRNA expression in the lung, pancreas, and biceps femoris was higher than in the remaining tissues; and MEF2A1, MEF2A2, MEF2A3, and MEF2A4
mRNAs were highly expressed in skeletal muscles (Figure 8).

**Discussion**

Alternative splicing was first discovered in 1978 (Gilbert 1978), it is the process where an mRNA precursor is spliced in different ways to form different mRNA splice isoforms. Alternative splicing is a type of post-transcriptional regulatory event and an important mechanism for the formation of transcriptome structures and abundant protein function diversities in higher eukaryotes (Excoffon et al. 2014; Hamid et al. 2014). Bioinformatics provide essential data for alternative splicing studies but require the complementarity with high-throughput detection techniques to achieve a comprehensive mechanism study of alternative splicing (Wang J et al. 2017). RNA-Seq using a new generation of high-throughput detection techniques that accurately quantifies the gene expression in tissue and recognizes gene sequencing, and is thus used in the discovery and identification of alternative splicing events in animal and plant genes (Graveley et al. 2011; Marquez et al. 2012; de Klerk et al. 2015). Mortazavi et al. (Mortazavi et al. 2008) utilized RNA-Seq to detect 3,500 mouse genes with alternative splicing and found 145,000 mRNA splicing patterns. Graveley et al. (Trapnell et al. 2009) used RNA-Seq to detect transcriptomes at multiple developmental stages in fruit flies and showed that 12,295 exons of genes contain 22,965 new splice sites. More than half of the genes undergo alternative splicing. This study also based on RNA-Seq prediction results to study the alternative splice forms of pig MEF2A in Mashen pigs and showed that the alternative splice sites of the four MEF2A alternative splice forms coincided with the prediction results. Identification of alternative splice forms is beneficial to the comprehensive study of gene function. A previous study has shown that Mnk2 gene contains two alternative splice forms. Mnk2a inhibits tumor cell activities in p38-MAPK pathway and is an in vitro tumor promoter (Maimon et al. 2014). MEF2Cα2 regulates myogenic differentiation, and MEF2Cα1 inhibits MEF2C-promoted differentiation, mainly playing the role in myocyte formation (Zhang M et al. 2015).

Chen et al. (Chen FY et al. 2013) cloned the CDS regions of cattle MEF2A gene and did not find any new splice isoforms. Zhang et al. (Zhang DJ et al. 2009) identified two mutants of the porcine MEF2A gene. This study was the first to discover four alternatively spliced transcripts of the porcine MEF2A, namely MEF2A1, MEF2A2, MEF2A3, and MEF2A4. Sequence alignment showed that MEF2A3 and MEF2A4 were the new alternatively spliced transcripts of the porcine MEF2A gene. MEF2A1 and MEF2A2 were homologous to the two mutants of porcine MEF2A gene obtained by Zhang et al. MEF2A3 and MEF2A4 are thus novel alternatively spliced transcripts that have been characterized in this study.

In the longissimus dorsi, MEF2A1 and MEF2A expression patterns were similar, and MEF2A1 and MEF2A expression was highest on postnatal 90 day and reached the lowest level on postnatal 180 day. Qi et al. (Yan-Xia et al. 2012) studied the MEF2A mRNA expression of the longissimus dorsi of Nanyang cattle and showed that MEF2A reached the lowest expression at three months old, suggesting different MEF2A expression among different species. MEF2A3 and MEF2A4 expression increased with increasing postnatal age in days, indicating that MEF2A expression level was directly correlated to the fiber diameter of the longissimus dorsi. This result was consistent with the findings of Chen et al. (Chen L et al. 2015), suggesting that the MEF2 gene family might play an important role in increasing muscle fiber area and maintaining the muscle function integrity afterbirth. On postnatal 180 day, MEF2A1 and MEF2A2 mRNA expression levels in Mashen pigs were significantly higher than those of the Large White pigs at the same postnatal stage, which may be due to the slow growth of Mashen. The muscle fiber diameter of the Mashen pigs was lower than the Large White pigs, but the muscle fiber density of the Mashen pigs was higher than the Large White pigs.
In the present study, the four MEF2A splice forms had different expression levels in different tissues. MEF2A1 was mainly expressed in the small intestine and longissimus dorsi. MEF2A2 was highly expressed in the cerebellum and heart than the other tissues. MEF2A3 was highly expressed in the pancreas, psoas, and longissimus dorsi. MEF2A4 was highly expressed in the lungs, pancreas, and biceps femoris. These results suggest that different splice forms of the same gene may play different biological functions.

Conclusions

Because MEF2A is the key gene for the regulation of skeletal muscle development and has been considered as one of the candidate genes for livestock and poultry traits, this study used transcriptome sequencing prediction to identify four alternatively spliced transcripts of MEF2A, i.e., MEF2A1, MEF2A2, MEF2A3, and MEF2A4, which were highly expressed in skeletal muscles and expressed in small intestine, cerebellum, pancreas, heart, and lung. Our findings may improve our understanding of the role of the MEF2A gene.

Acknowledgements

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Reference


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Table 1 Primers used for cloning and qPCR of pig MEF2A and 18S rRNA genes

<table>
<thead>
<tr>
<th>Names</th>
<th>Primer sequences (5’&gt;3’)</th>
<th>Product sizes (bp)</th>
<th>Notes</th>
</tr>
</thead>
</table>
| P1    | F1: AGCAGAACCAACTCGGATATTG  
    | R1: TCAACTCCAATTCTCTTCCCTCC | 550 | clone for MEF2A |
| P2    | F2: GGCCTTCAACTCCAGAAAC  
    | R2: CTITGATCCAGCTCTTTCCCA | 333 | clone and qPCR for MEF2A1 |
| P3    | F3: ACAGTGCAATATCCCAGTGACA  
    | R3: AGCTGCTTGCTTCTGAAAAAC | 361 | clone and qPCR for MEF2A2 |
| P4    | F4: GCCCAGACCTGATCACTTCCCT | 399 | clone and qPCR for MEF2A3 |
| P5    | F5: TTGAGCACAGTGACTCTTCG  
    | R5: AATCCATTCCCCACTGGGCT | 367 | clone and qPCR for MEF2A4 |
| 18S rRNA | F: CCCACGGAATCGGAAAGAG  
    | R: TTGACCGGAAGGGCAACCA | 122 | qPCR for 18S rRNA |
Figure 1 Alternative splicing statistics.

Note: (1) Skipped exon (SE); (2) Alternative 5’ splicing site (A5SS); (3) Alternative 3’ splicing site (A3SS); (4) Retained intron (RI); (5) Mutually exclusive exon (MEX); (6) Alternative promoters (AP); (7) Alternative poly(A) (APA)
Figure 2 PCR result of *MEF2A* in Mashen pig.
Figure 3 Nucleotide sequence alignments of MEF2A1, MEF2A2, MEF2A3 and MEF2A4 in Mashen pig
Figure 4 The genomic structure diagram of MEF2A1, MEF2A2, MEF2A3 and MEF2A4 in Mashen pig.

Note: the blue bar means normal exons, purple bar means insert exons, white bar means deletion exons, arrows means the positions of the primers.
Figure 5 Protein sequences alignments and conserved domain analysis of MEF2A1, MEF2A2, MEF2A3 and MEF2A4 in Mashen pig
Figure 6 PCR result of MEF2A1, MEF2A2, MEF2A3 and MEF2A4 in Mashen pig
Figure 7 The mRNA relative expression of MEF2A1, MEF2A2, MEF2A3, and MEF2A4 in longissimus dorsi tissue at different stages of Mashen and Large White pigs.

Note: Capital letter and small letter represent different levels of difference in different stages. Values with different small letter superscripts mean significant difference ($P<0.05$); with different capital letter superscripts mean extremely significant difference ($P<0.01$). “*” mean the difference between Mashen and Large White is significant difference ($P<0.05$); “**” mean the difference between Mashen and Large White is significant difference ($P<0.01$); “ns” represent no significant difference.
Figure 8 The mRNA relative expression of MEF2A1, MEF2A2, MEF2A3 and MEF2A4 in 13 tissues of Mashen and Large White pigs (90d).

Note: Ce, Cerebellum; Lu, Lung; Sp, Spleen; Si, Small intestine; Pa, pancreas; Ad, adipose; K, Kidney; St, Stomach; Li, liver; H, Heart; Pm, Psoas major; Bf, Biceps femoris; Ld, longissimus dorsi