Cloning and functional characterization of porcine Sox6

Wanxue WEN*, Xiaoling CHEN*, Daiwen CHEN, Bing YU, Junqiu LUO, Zhiqing HUANG**

Key Laboratory for Animal Disease-Resistance Nutrition of China Ministry of Education, Institute of Animal Nutrition, Sichuan Agricultural University, Chengdu, Sichuan, P. R. China

1. Introduction

Mammalian skeletal muscle comprises different types of muscle fibers (Schiaffino and Reggiani, 2011). Postnatal skeletal muscle fibers are generally classified as fast- or slow-twitch subtypes based on their contractile and metabolic properties (Schiaffino and Reggiani, 2011). Fast-twitch fibers (also called white muscle fibers) specialized for phasic activity exhibit a glycolytic metabolism, whereas slow-twitch fibers (also called red muscle fibers) specialized for more continuous activity are rich in myoglobin and predominantly oxidative (Naya et al., 2000). According to the major myosin heavy chain (MyHC) isoforms, postnatal skeletal muscle fibers can be accurately classified into four types: slow type I with MyHC I, fast type IIA with MyHC IIA, fast type IIX with MyHC IIX, and fast type IIB with MyHC IIB (Pette and Staron, 1990; Schiaffino and Reggiani, 1994). Although the total number of skeletal muscle fibers is fixed before birth in most mammalian species, transition of skeletal muscle fiber types occurs frequently in response to changes in internal and external cues during postnatal development. Changes in MyHC isoforms follow a general scheme of sequential and reversible transitions from fast-to-slow and slow-to-fast in the rank order: MyHC I ↔ MyHC IIA ↔ MyHC IIX ↔ MyHC IIB (Pette and Staron, 2000). Previous studies have implicated various transcription factors in the control of skeletal muscle fiber-type transitions including peroxisome-proliferation–activated receptor-γ coactivator-1α (PGC-1α), nuclear factor of activated T-cells (NFAT), and estrogen receptor-related receptor γ (ERRγ) (Lin et al., 2002; McCullagh et al., 2004; Narkar et al., 2011).

Sox6, a member of the Sox [Sry-related high mobility group (HMG) box] family of transcription factors, is highly expressed in skeletal muscle (Hagiwara et al., 2000; Cohen-Barak et al., 2001). Overexpression of Sox6 in mice has been shown to repress transcript levels of slow-twitch fiber specific genes and to cause a concomitant decrease in slow-twitch fibers (van Rooij et al., 2009). Consistently, lack of a functional Sox6 in mice results in a significant increase in the expression of slow-twitch fiber specific genes and leads to conversion of muscle to a slow-twitch fiber phenotype (Hagiwara et al., 2005; Quiat et al., 2011). Together, these findings suggest that Sox6 plays a critical role in the control of skeletal muscle fiber-type transition.

The pig is evolutionarily related to humans and is considered to share many physiological, biochemical, and anatomical features with humans. To the best of our knowledge, there is no report of porcine Sox6 (pSox6) in the literature. In this study, we cloned pSox6 cDNA.

Abstract: Sox6, a fast myofiber-enriched repressor of slow muscle gene expression, plays an important role in the control of skeletal muscle fiber-type transition. Mammalian Sox6 has been isolated and characterized from mice, rats, and humans. In this study, the porcine Sox6 (pSox6) cDNA was cloned by degenerate PCR. The entire open reading frame (ORF) of pSox6 is 2406 bp. The predicted protein is composed of 801 amino acids and contains the HMG box, leucine-zipper motif, and glutamine-rich Q-box. The amino acid similarities between the Sox6 from pig and other mammals range from 90% to 95%. Real-time quantitative PCR analysis indicated that pSox6 mRNA was most abundant in the heart and skeletal muscles. Overexpression of pSox6 led to downregulation of myosin heavy chain (MyHC) I expression and upregulation of MyHC IIA, MyHC IIX, and MyHC IIB expressions. This is the first report on molecular cloning and characterization of porcine Sox6.

Key words: Porcine Sox6, cloning, myosin heavy chain

Received: 18.03.2015 • Accepted/Published Online: 08.06.2015 • Final Version: 05.01.2016

* These authors contributed equally to this work.
** Correspondence: zqhuang@sicau.edu.cn
and examined its tissue distribution. We also examined the effect of pSox6 overexpression on MyHC isoform expression at the mRNA level in vivo.

2. Materials and methods

2.1. RNA isolation and reverse transcription
Total RNA was isolated from the longissimus lumborum (LL) muscle of a 3-day-old male Duroc × Landrace × Yorkshire (DLY) pig using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the recommendations of the manufacturer. The concentration of total RNA was determined spectrophotometrically using a Beckman Coulter DU730 (Beckman Coulter, Fullerton, CA, USA). Reverse transcription was performed with 1 µg of total RNA using a PrimeScript 1st Strand cDNA Synthesis kit (TaKaRa, Dalian, China) as recommended by the manufacturer.

2.2. Cloning of pSox6
The full coding region of pSox6 was PCR amplified from the cDNA prepared above. The degenerate primers used were 5’-ATGTCTTCCAAGCAAGCCACCTCTCC-3’ (forward) including initiation codon and 5’-TCAGTTGGCACTGACAG(C/G)(T/C)TC(T/C)GGG-3’ (reverse) including termination codon and were designed based on regions of high homology among the sequences of human, mouse, and rat Sox6. The purified PCR product was cloned into pMD19-T vector (TaKaRa) to generate pMD19-T-pSox6. The procedure for pMD19-T-pSox6 plasmid construction is illustrated in Figure 1.

2.3. Tissue sample collection
All animal procedures were performed according to protocols approved by the Animal Care Advisory Committee of Sichuan Agricultural University. Three 10-week-old female DLY pigs (body weight 31–31.6 kg) were slaughtered in a humane manner. The heart, liver, spleen, lung, kidney, fat, LL muscle, extensor digitorum longus (EDL) muscle, and soleus (SOL) muscle were removed and immediately snap frozen in liquid nitrogen prior to storage at −80 °C until use.

2.4. Plasmid construction
For the expression of pSox6, the entire open reading frame (ORF) of pSox6 was PCR amplified using the specific primers [pcDNA3.1(+)-pSox6-F: 5’-CGCGAATTCTCATGTCTCTAGAGCAAAGCCAC-3’ and pcDNA3.1(+)-pSox6-R: 5’-TTACTCGAGTTCAGTTGCCAGTGCAGCC-3’] and the plasmid pMD19-T-pSox6 as a template. Primer pcDNA3.1(+)-pSox6-F introduced an EcoRI site, and primer pcDNA3.1(+)-pSox6-R contained an XhoI site (underlined). After digestion with EcoRI and XhoI, the PCR product was cloned into pcDNA3.1(+) plasmid (Invitrogen). Proper construction was confirmed by DNA sequencing and was defined as pcDNA3.1(+)-pSox6.

2.5. Intramuscular injection
In vivo experiments were carried out on 6-to-8-week-old male BALB/c mice. Animals were housed in a facility with food and water ad libitum. Plasmid DNA (50 µL, 1 µg/µL) in normal saline was injected into the tibialis anterior (TA) muscles using a 50-µL syringe. On day 7, the mice were euthanized by cervical dislocation, and the TA muscles were collected.

2.6. Real-time quantitative PCR
Real-time quantitative PCR was performed using SYBR Select Master Mix (Applied Biosystems, Foster City, CA, USA) in a 7900HT real-time PCR system (384-cell standard block) (Applied Biosystems) in a final volume of 10 µL. The gene-specific primers used are listed in the Table. The PCR cycling conditions used were 45 cycles at 95 °C for 15 s and 58 °C for 30 s. Melting curve analysis was performed at the end of each PCR run to confirm amplicon specificity. Relative mRNA expression was quantified using the comparative Ct method (Livak and Shmittgen, 2001) with GAPDH or β-actin as an endogenous control.

2.7. Statistical analysis
Data presented as mean ± SE were compared by one-way ANOVA followed by Tukey’s test (SPSS Inc., Chicago, IL, USA). Statistical significance was set at P < 0.05.

3. Results and discussion

3.1. Cloning and sequence analysis of pSox6
Sox6 was initially cloned from an adult mouse testis cDNA library (Connor et al., 1995). Mammalian Sox6s have also been isolated and characterized from rats (Narahara et al., 2002) and humans (Cohen-Barak et al., 2001). In this study, molecular cloning of the full coding region of pSox6 was carried out by degenerate PCR. A full-length 2406-bp pSox6 gene fragment was amplified from skeletal muscle of a DLY pig (Figure 2). The pSox6 gene was successfully inserted into pMD19-T vector, and the resulting plasmid pMD19-T-pSox6 was confirmed by colony PCR (Figure 3) and DNA sequencing. The nucleotide sequence of pSox6 was deposited in GenBank with accession number KF933861. This nucleotide sequence (Figure 4) shares

![Figure 1. Map of the pMD19-T-pSox6 plasmid.](image)
Table. Primers used in real-time quantitative PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5’ → 3’)</th>
<th>GenBank ID</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MyHC I</td>
<td>Forward</td>
<td>CTTCTACAGGCCTGGGCTTAC</td>
<td>NM_080728</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTCCTTCTCAGACTTTCCGCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MyHC IIa</td>
<td>Forward</td>
<td>TTCCAGAGGGCTAAGGTGGTC</td>
<td>NM_001039545</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCCAGGCCAGTGATGTTGTAAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MyHC IIx</td>
<td>Forward</td>
<td>CAACCCATAGCAGCTACGCCT</td>
<td>NM_030679</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CATCAGAAGTGAAAGCCGAAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MyHC IIb</td>
<td>Forward</td>
<td>CTGTCGGACTCAAGCTGCTGCC</td>
<td>NM_010855</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCGCTCCTTTTCAGACTTCCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sox6</td>
<td>Forward</td>
<td>AAGCCACCTCTCTCATTTCAC</td>
<td>KF933861</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCCAGTCAGCATCTTTGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>AGGGCATTTGGGCTACAC</td>
<td>NM_008084</td>
<td>211</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGGTCAGGTTTCTACCTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward</td>
<td>CCACGAAAACCTACCTTTCACTCC</td>
<td>DQ845171</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTGATCTCCTTTGCATCCTGT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Agarose gel electrophoresis of PCR products of pSox6 gene. Lane M: DNA molecular weight marker; lane 1: PCR products; lane 2: control (-) ddH₂O/template.

Figure 3. Colony PCR confirmation of E. coli clones carrying the pMD19-T-pSox6 plasmid. Lane M: DNA molecular weight marker; lanes 1–3: PCR products.
87.7%, 86.71%, 90.91%, and 89.49% homology with the known Sox6 sequences of human (GenBank accession number: AF309034), mouse (GenBank accession number: U32614), rat (GenBank accession number: NM_001024751), and cattle (GenBank accession number: NM_001191418), respectively. The amino acid sequence of pSox6 (Figure 4) was compared with sequences of mammalian Sox6s available from GenBank. The degree of sequence identity between pSox6 and human (AAK26115), mouse (AAC52263), rat (NP_001019922), and cattle (NP_001178347) was 89.9%, 90.74%, 95.26%, and 90.53%, respectively. There is 100% sequence identity in the functionally critical domains (HMG box, leucine-zipper motif, glutamine-rich Q-box in the N-terminal region, Q-box in the C-terminal region) among those proteins (Figure 4), indicating that pSox6 may have the same function as the reported Sox6 (Connor et al., 1995; Hagiwara et al., 2000; Smits et al., 2001; Hagiwara et al., 2005; Hagiwara et al., 2007; Quiat et al., 2011).

Figure 4. Nucleotide sequence of pSox6 cDNA and its deduced amino acid sequence. Nucleotides and amino acids are numbered on the left. The individual leucines of the leucine-zipper are boxed. The Q-box at the N-terminal region is underlined. The Q-box at the C-terminal region is indicated by white letters in black boxes. The Sry-related HMG box is indicated by white letters in gray boxes. The stop codon is indicated with an asterisk.
3.2. Tissue distribution of pSox6

Expression of pSox6 mRNA in various tissues was assessed by real-time quantitative PCR. As shown in Figure 5, pSox6 transcript was more abundantly expressed in the heart and skeletal muscle than in the kidney, fat, liver, spleen, and lung. The tissue distribution pattern of Sox6 has been previously examined in other mammals, including mice (Connor et al., 1995; Hagiwara et al., 2000; Hagiwara et al., 2005), rats (Narahara et al., 2002), and humans (Cohen-Barak et al., 2001; Ueda et al., 2004). LL and EDL muscles are typical fast muscles that mostly contain the MyHC IIa, MyHC IIx, and MyHC IIb; whereas SOL muscle, a typical slow muscle, mainly contains a large amount of MyHC I (O’Connor et al., 2003; Suryawan et al., 2009; Wang et al., 2012). In this study, we showed that pSox6 mRNA was more abundantly expressed in the LL and EDL muscles than in the SOL muscle (Figure 5), suggesting that differential expression of pSox6 between the fast and slow muscle fibers might be involved in the regulation of expression of fiber-type–specific genes.

3.3. Effect of pSox6 overexpression on MyHC isoform expression in vivo

Sox6 is a transcriptional repressor of the MyHC I gene (Hagiwara et al., 2005; Hagiwara et al., 2007; Quiat et al., 2011). To examine whether pSox6 regulates MyHC isoform expression in vivo, the pSox6 expression plasmid was constructed and injected into mouse TA muscles. As shown in Figure 6, the mRNA level of MyHC I significantly decreased; however, the mRNA levels of MyHC IIa, MyHC IIx, and MyHC IIb significantly increased in TA muscles of pcDNA3.1(+)–pSox6-injected mice, suggesting that overexpression of pSox6 leads to transition from MyHC I toward MyHC IIa, MyHC IIx, and MyHC IIb.

In summary, we have cloned pSox6, which encodes a 801-aa protein with the HMG box, leucine-zipper motif, and glutamine-rich Q-box. Its mRNA level in various porcine tissues was analyzed. Overexpression of pSox6 downregulated MyHC I expression and upregulated MyHC IIa, MyHC IIx, and MyHC IIb expressions. This is the first report of molecular cloning and characterization of Sox6 gene from pigs.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (no. 31472110) and the National Basic Research Program of China (no. 2012CB124701).

References


Figure 5. Relative mRNA expression of pSox6 in different tissues. Total RNA from different tissues of 3 healthy pigs was used for real-time quantitative PCR. Samples were performed in duplicate. The amount of Sox6 mRNA was normalized to the amount of β-actin mRNA. Data were presented as mean ± SE (n = 3), in arbitrary units. LL, longissimus lumborum muscle; EDL, extensor digitorum longus muscle; SOL, soleus muscle.

Figure 6. Effect of pSox6 overexpression on MyHC isoform expression in vivo. RNA was isolated from the TA muscles of BALB/c mice injected intramuscularly with 50 µg of the plasmid pcDNA3.1(+)–pSox6 or the empty vector pcDNA3.1(+). MyHC I, MyHC IIa, MyHC IIx, and MyHC IIb mRNA levels were determined using real-time quantitative PCR 7 days after injection. The amounts of MyHC I, MyHC IIa, MyHC IIx, and MyHC IIb mRNA were normalized to the amount of GAPDH mRNA. Results were presented as mean ± SE (n = 3). **P < 0.01 and ***P < 0.001, as compared with empty vector.


