Gene 555 (2015) 223–230

Contents lists available at ScienceDirect

Gene

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

The molecular characterization and temporal–spatial expression of myocyte enhancer factor 2 genes in the goat and their association with myofiber traits

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ARTICLE INFO

Article history:
Received 4 August 2014
Received in revised form 16 October 2014
Accepted 5 November 2014
Available online 13 November 2014

Keywords:
MEF2
Cloning
Temporal–spatial expression
Association analysis
Goat

ABSTRACT

The myocyte enhancer factor-2 (*MEF2*) gene family in vertebrates includes *MEF2A*, *MEF2B*, *MEF2C*, and *MEF2D*, which have important functions in the regulation of muscular growth and development. To investigate their temporal–spatial expression and functions in the goat, these genes were cloned (accession nos. JN967621–24) and their expression patterns characterized at five postnatal stages (3, 30, 60, 90, and 120 days). Association analysis was then applied regarding *MEF2* expression levels and myofiber diameter and density. *MEF2B* was shown to be weakly homologous with other species, the distant branches with other members and the lowest expression levels, suggesting that it is distinct from other family members. Expression of the other three *MEF2* genes was widely distributed, but this was largely accumulated in the skeletal muscle and myocardium compared with the viscera at all developmental stages. *MEF2A* and *MEF2D* expression levels were higher overall than *MEF2B* and *MEF2C* in six tissues, and were significantly positively correlated with the myofiber diameter of the longissimus dorsi. These findings suggest that goat *MEF2* genes mainly function in the skeletal muscle and myocardium, and that *MEF2A* and *MEF2D* are likely to effectively promote muscular growth and development during postnatal stages. *MEF2A* expression was highest in the myocardium, where *MEF2C* expression increased with age, implying that both gene products are related to the growth and development of postnatal myocardium.
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1. Introduction

The myocyte enhancer factor-2 (MEF2) family of proteins includes four members in vertebrates: MEF2A, MEF2B, MEF2C, and MEF2D (Morisaki et al., 1997). These proteins contain an N-terminus, with a conserved MADS-box binding domain and adjacent MEF2 domain (Molkentin et al., 1996), and a less conserved C-terminus responsible for regulation activity and function (Black and Olson, 1998).

MEF2 proteins bind to A/T-rich DNA sequences that present in many promoters and enhancers of muscle-specific skeletal muscle cells (Gossett et al., 1989), and cooperate with myogenic basic helix–loop–helix (bHLH) factors to control morphogenesis and myogenesis (Molkentin et al., 1995; Ornatsky et al., 1997). Of the *MEF2* gene family, *MEF2A* controls the normal development and functional integrity of cardiac muscle (Naya et al., 2002), and is thought to be involved in human heart failure (Bhagavatula et al., 2004; Wang et al., 2003). Moreover, *MEF2A* polymorphisms are associated with growth traits of skeletal muscle in cattle (Juszczuk-Kubiak et al., 2012b) and chickens (Zhou et al., 2010). The binding ability and distribution of *MEF2B* differ between mice and humans, and is distinct from other *MEF2* family members (Morisaki et al., 1997). While *MEF2C* maintains the sarcomere integrity and postnatal maturation of the skeletal muscle (Potthoff et al., 2007). Finally, *MEF2D* has been shown to affect the muscular growth and maturation during pig myogenesis (Zhao et al., 2011). Although the *MEF2* gene family has been extensively studied in humans, mice, pigs, cattle, and chickens, it has not been well characterized in the goat.

Previous investigations into whether the distribution of *MEF2* is limited to the muscle, spleen, and brain (Janson et al., 2001; Leifer et al., 1994; Martin et al., 1994; Yu et al., 1992), or is widespread among various tissues (Arnold et al., 2007; Edmondson et al., 1994) are inconclusive and mainly focused on antenatal stages of development, rarely involving the postnatal period. Additionally, few studies have determined if *MEF2* associates with muscular growth, especially in postnatal stages. Therefore, the present paper aimed to investigate the expression profile of *MEF2* genes in different tissues and to analyze the association between *MEF2* expression levels and muscular growth and development in the postnatal goat.

Abbreviations: MEF2, myocyte-specific enhancer factor 2; MEF2 genes, MEF2A, MEF2B, MEF2C and MEF2D; MC, myocardium; LD, longissimus dorsi; TB, triceps brachii; SM, semimembranosus; Top2β, DNA topoisomerase 2 beta; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase; MYOD, myoblast determination protein; MYOG, myogenin; SHF, second heart field.

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http://dx.doi.org/10.1016/j.gene.2014.11.007
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2. Materials and methods

2.1. Animals and tissues

All procedures in this study were approved by the ethics committee of Sichuan Agricultural University, according to the Instructive Notions with Respect to Caring for Laboratory Animals (China, 2006).

To accurately determine *MEF2* gene expression levels, and correlate these with the muscular growth and developmental in the postnatal goat, various samples were collected from 30 Nanjiang Yellow goats at five different postnatal stages (3, 30, 60, 90, and 120 days; three males and three females per stage) and immediately frozen in liquid nitrogen then stored at −80 °C until required. Samples were collected of viscera (liver and kidney), the myocardium (MC), and skeletal muscle (longissimus dorsi, LD; triceps brachii, TB; and semimembranosus, SM).

2.2. Total RNA extraction and cDNA synthesis

Total RNA was isolated by Trizol reagent (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase I (Takara Bio Inc., Shiga, Japan) to remove genomic DNA. The nucleic acid concentration and purity were examined by NanoDrop (Bio-Rad, Benicia, CA), and its integrity was determined using 1% agarose gel electrophoresis. The cDNA was synthesized using the PrimeScript® 1st Strand cDNA Synthesis Kit (Takara Bio Inc.) according to the manufacturer's protocol.

2.3. Primer design for cloning and quantitative RT-PCR (qRT-PCR)

Primer pairs were designed to amplify caprine *MEF2* genes according to regions of conservation with other species using Primer Premier 5.0 software (Tables 1 and S1), and were synthesized by the Sangon Biotechnology Company (Shanghai, China). The dynamic range and PCR amplification efficiency were determined using a series of cDNA template dilutions.

2.4. Cloning and sequencing of MEF2 genes

Conventional PCR was performed in a 25.0 μL reaction including 12.5 μL 2 × Master Mix Taq (TIANGEN Biotech, Beijing, China), 2 μL cDNA template, 1 μL of each primer, and 8.5 μL double distilled (dd) H_2O. PCR was carried out at 95 °C for 4 min; followed by 35 cycles at 94 °C for 45 s, the primer-specific annealing temperature (Table 1) for 35 s, and 72 °C for 90 s; with a final extension at 72 °C for 10 min. PCR products were separated by electrophoresis (1.5% agarose gel), and target fragments were excised and purified, then ligated into the pMD19-T vector (Takara Bio Inc.). Positive clones were selected using blue–white selection, then sequenced.

2.5. Selection of reference genes and qRT-PCR

To identify suitable reference genes to normalize qRT-PCR data, the expression levels of multiple reference genes were detected in different tissues and at different developmental stages of the postnatal goat, and their expression stability was analyzed by geNorm software (Chen et al., 2014). Finally, *β-actin*, DNA topoisomerase 2 beta (*Top2β*), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase (*YWHAZ*) were selected as reference genes.

Real-time fluorescence qRT-PCR was used to quantify *MEF2* gene expression in six tissues and five stages of development based on SYBR Green II chemistry. The reactions were conducted in 96-well optical reaction plates (Bio-Rad) as previously described (Li et al., 2013). Discrete samples were reacted in triplicate, and a blank control of ddH_2O was set up to monitor contamination. Amplifications were performed using SYBR® Premix Ex TaqTM II (Takara Bio Inc.) according to instructions

Table 1
Primer pairs of cloning and quantitative assay in the present study.

Genes	Accession no.	Primer sequence (5′–3′) [§]	Product size	Tm (°C)
Cloning				
MEF2A	NM_001083638.2	F1: GCAGTGTACCATGCATTAGG	846	56.8
		R1: CTTCGTAGGCATAACTTTGC		
		F2: CAGTGCCAAATGGAGCTGGA	881	58.8
		R2: GCAGTTCACTGCAGTAACAC		
MEF2B	NM_001145793.1	F: AGCAGACAAAGGGCACTCCA	1242	57.7
		R: ATCGCTGCGTCTTCTCCAAG		
MEF2C	NM_001046113.1	F: GGGACGAGAGAGAGAAGAAAGAC	1415	63.6
		R: GGTATAGCACACACACACACTGC		
MEF2D	NM_001205178.1	F1: GTTCATGAGCACAGTGTAAG	918	64.3
		R1: TCCTCCCTGGGAAGTGATGA		
		F2: CAGCCTAAACAAGGTCATCC	937	55.7
		R2: GGAGCACAAGAAAGGAAGTG		
qRT-PCR				
MEF2A	JN967621	F: TGAAAGGAATCGACAGGTCAC	143	59.7
		R: CAGTGCTGGCATACTGAAACA		
MEF2B	JN967622	F: AGAGGAATCGGCAGGTAACAT	130	59.2
		R: TACTGGAAGAGGCGGATGG		
MEF2C	JN967623	F: ATCCTGATGCAGACGATTCAG	115	59.7
		R: GGTGGAACAGCACACAATCTT		
MEF2D	JN967624	F: GGTCTCCCAGTCTACCCACTC	160	63.2
		R: TGAACTGAAGGCTGGTAAGGA		
β-actin	NM_001009784	F: CCTGCGGCATTCACGAAACTAC	87	59.7
		R: ACAGCACCGTGTTGGCGTAGAG		
Top2β	XM_005698949.1	F: GTGTGGAGCCTGAGTGGTATA	137	59
		R: AAGCATTCGCCTGACATTGTT		
YWHAZ	XM_005689196.1	F: ACTACTATCGCTACTTGGCTGAG	84	59
		R: CTTCTTGGTATGCTTGCTGTGA		

[§] F: forward primer sequences, R: reverse primer sequences.

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