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Characterization of the promoter region of bovine *SIX4*: Roles of E-box and MyoD in the regulation of basal transcription

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ABSTRACT

The sine oculis homeobox 4 (*SIX4*) gene belongs to the Six gene family and encodes an evolutionarily conserved transcription factor. Previous studies have demonstrated that *SIX4* plays an essential role in proper muscle regeneration. However, the mechanisms regulating *SIX4* transcription remain elusive. In the present study, we determined that bovine *SIX4* was highly expressed in the *longissimus thoracis* and in undifferentiated Qinchuan cattle muscle cells (QCMCs) and that its protein localizes to both the cytoplasm and the nucleus. To elucidate the bovine the molecular mechanisms of *SIX4* regulation, 1.3 kb of the 5′-regulatory region was obtained. MyoD and Ebox recognition sites were identified in the core promoter region at −522/-193 of the bovine *SIX4* using a series of 5′ deletion promoter plasmids in luciferase reporter assays. An electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay in combination with site-directed mutation and siRNA interference demonstrated that MyoD binding occurs at MyoD and Ebox recognition sites through direct and indirect mechanisms and play important roles in the transcriptional regulation of the bovine *SIX4* promoter. Taken together, these interactions provide insight into the regulatory mechanisms of *SIX4* transcription in mediating skeletal muscle growth in cattle.

1. Introduction

The initiation of the skeletal myogenesis that occurs during embryogenesis is a complex and successive process that involves a multitude of genes, as well as signalling and sequence-specific transcription factors (TFs) [1]. It is widely accepted that myogenic regulatory factors (MRFs), including myoblast determining factors (MyoD), Myf5, myogenin (MyoG) and MRF4 play central roles in myogenesis during the extensive proliferation of muscle progenitor cells into myoblasts [2,3]. MyoD and Myf5 act as myogenic determination factors, whereas MRF4 and MyoG induce the final differentiation programme [2,3]. In vertebrates, MRFs are involved in activating the transcription of additional myogenic TFs, such as binding paired box families [4] and enhancer factor 2 (MEF2) [5]. In addition, MyoD expression is strongly induced during adult muscle regeneration as satellite cells become activated in part through the action of *SIX4* [6,7].

The sine oculis homeobox 4 (*SIX4*) gene belongs to the Six gene family [8] and is readily detectable in muscle precursors of the developing limb buds and are critical for embryonic and adult myogenesis [9,10]. Previous genetic experiments have demonstrated that *SIX1* and *SIX4* can promote the regeneration of adult skeletal muscle through enhancing the expression of MyoD [11]. In addition, *SIX4*-null mice that are normal in their muscle developmental phenotype, which has been attributed to compensation by other Six family members, have discouraged further assessment of the role of *SIX4* during adult muscle regeneration [9,10]. Interestingly, *SIX1* and *SIX4* harmonize in driving the transformation of slow-twitch fibres to the fast-twitch phenotype in synergy with the cofactor Eya1 during myogenesis development [12].

Abbreviations: TF, transcription factors; QCMCs, Qinchuan cattle muscle cells; MRFs, myogenic regulatory factors; MEF2, enhancer factor 2; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; ORF, open reading frame; TSS, translational start site.

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Although much is known about *SIX4* involved in regulating the formation of muscles, the mechanisms regulating the transcription of bovine *SIX4* during myogenesis is far from complete. In this study, we analysed the molecular mechanisms of *SIX4* gene regulation via the 5′-regulatory region. In addition, the relative mRNA expression pattern of bovine *SIX4* in tissue and its subcellular localization was determined. Our results provide a solid basis for further insight into the transcriptional regulatory mechanisms for *SIX4* in mediating skeletal muscle growth in cattle.

2. Materials and methods

2.1. Quantitative PCR analysis of gene expression patterns

Ten tissue samples (heart, liver, spleen, lung, kidney, abomasum, small intestine, abdominal fat, *longissimus thoracis* muscle and testicular tissue) were obtained from three Qinchuan foetal bovines. QCMCs were isolated from the Qinchuan foetal bovine samples. For inducing QCMC differentiation, cells at 70% confluence were switched to differentiation medium containing DMEM/F-12 and 2% horse serum (Gibco, Invitrogen, USA). Total RNA was extracted using RNAiso (TaKaRa, Dalian, China) and reverse transcribed using a PrimeScriptTM RT Reagent Kit (Takara) following the manufacturer's instructions. QRT-PCR was performed in triplicate using a SYBR® Premix Ex TaqTM II Kit (Takara) on an ABI 7500 Real-Time PCR system. Gene expression levels were normalized to that of GAPDH, and fold change was determined using the $2^{-\Delta\Delta Ct}$ method [13].

2.2. Western blotting

For Western blot, cell proteins were extracted using T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Protein was separated on 10% SDS-PAGE gels and incubated with the SIX4 antibody (sc-390779, Santa Cruz, USA) and GAPDH antibody (sc-293335, Santa Cruz). Signals were enhanced by ECL Plus (Thermo Fisher Scientific) and visualized by exposure of X-ray films to chemical luminescence using the ChemiDoc™ XRS + System (Bio-Rad, Hercules, CA, USA).

2.3. QCMCs culture and immunofluorescence

QCMCs were cultured in 6-well culture plates, and fixed with 4% paraformaldehyde for 15 min at room temperature. The following primary anti-SIX4 antibody (1:1000 dilution sc-390779) and fluorescent-labelled secondary anti-rabbit IgG H&L antibody (Alexa Fluor® 555) (1:500 dilution, Abcam) were used in immunofluorescence. The cells were incubated with the primary antibody overnight at 4 °C, and then incubated with secondary antibody protected from light at 37 °C for 1 h. Then, 4′6-Diamidino-2-phenylindole (DAPI, Sigma) was used at 50 ng/ml to stain the nuclei. The immunofluorescence images were captured with an Olympus IX71 microscope and electronically assigned to the red or blue channels (Olympus Optical, Tokyo, Japan).

2.4. 5′-Rapid amplification of cDNA ends (5′-RACE)

To identify the TSS site of the bovine *SIX4* gene, 5′-RACE was performed on total RNA from the *longissimus thoracis* muscle using a BD SMARTTM RACE cDNA amplification kit (Clontech Inc., CA, USA) according to the manufacturer's protocol.

2.5. Luciferase reporter constructs

An ~1.3 kb promoter region of the bovine *SIX4* gene (NCBI accession NO.: AC_000167.1, region from 73124222 to 73125547), including the translational start site, was amplified using gene-specific primers (*SIX4*-F/R, Supl. 1). PCR amplifications were performed using genomic DNA from Qinchuan cattle blood as a template. The fragment primers *SIX4*-P1/R, *SIX4*-P2/R, *SIX4*-P3/R and *SIX4*-P4/R (Supl. 1) were designed to contain unidirectional deletions of the bovine *SIX4* promoter and the *SIX4*-F/R products as a template. Object fragments were ligated into the pGL3-basic vector luciferase reporter construct and were named pGL3-P1, pGL3-P2, pGL3-P3 and pGL3-P4.

2.6. Cell transfection

C2C12 cells were cultured in DMEM containing 25 mM glucose supplemented with 10% new-born calf serum (Invitrogen) at 37 °C and 5% CO_2. Briefly, cells were grown in triplicate in 24-well plates until reaching a confluency of 70%, after which 800 ng of the expression construct (pGL3-P1, pGL3-P2, pGL3-P3 or pGL3-P4) was co-transfected with 10 ng of pRL-TK normalizing vector by using Lip3000 (Invitrogen) according to the manufacturer's instructions. The pGL3-basic vector served as a negative control. At 6 h after transfection, the media was replaced with DMEM containing 2% horse serum (HS) (Gibco) to induce differentiation of the C2C12 myoblasts into myotubes. Cell lysates were collected 48 h post-transfection and prepared for luciferase activity analysis using the Dual-luciferase® Reporter Assay System (Promega, USA) following the manufacturer's instructions.

2.7. Site-directed mutagenesis

The potential TF-binding sites for MyoD and Ebox motif were analysed using the Genomatix suite (http://www.genomatix.de/) and mutated with the corresponding primers (Supl. 1) using Fast Directed Mutagenesis Kit (Takara) according to the manufacturer's protocol.

2.8. MyoD knockdown and overexpression

The siRNAs against MyoD was designed as previously described [14], containing the control and the negative control (Supl. 1). C2C12 cells cultured in 24-well plates were transiently co-transfected with 50 nM siRNA and 800 ng each of the corresponding plasmids pGL3-522/-193, pGL3-522/-193^{Mut1}, pGL3-522/-193^{Mut2} or pGL3-522/-$193^{Mut1\ \&\ Mut2}$. The pcDNA3.1-MyoD expression plasmid was constructed by RT-PCR from Qinchuan cattle mRNA (NCBI: AB110599.1). For MyoD overexpression, C2C12 cells cultured in 24-well plates were transiently co-transfected with 400 ng each of pcDNA3.1-MyoD and the corresponding plasmids pGL3-522/-193, pGL3-522/-193^{Mut1}, pGL3-522/-193^{Mut2} or pGL3-522/-$193^{Mut1\&Mut2}$. The pcDNA3.1 plasmid served as a negative control.

2.9. Electrophoretic mobility shift assays (EMSAs)

Nuclear extracts were obtained from QCMCs according to the manufacturer's protocol and used for EMSAs by using a LightShift Chemiluminescent EMSA Kit (Thermo Fisher Corp., Waltham, MA, USA) with modifications. Briefly, 200 fmol of 5′-biotin labelled MyoD probe (Supl. 1) was incubated with 10 μg of nuclear extracts, 2 μL of 10 × binding buffer, 1 μL of poly dI.dC and 1 μL of 50% glycerol in a volume of 20 μL. For the competition assay, unlabelled or mutated probes were added to the reaction mixture for 10 min before adding the labelled MyoD probe. Finally, 10 μg of MyoD (sc-31940, Santa Cruz) antibody was added to the reaction mixture for the super-shift assay. The DNA-protein complexes were separated

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