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Research Article

LIM homeobox transcription factor Lhx2 inhibits skeletal muscle differentiation in part via transcriptional activation of Msx1 and Msx2

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ABSTRACT

LIM homeobox transcription factor Lhx2 is known to be an important regulator of neuronal development, homeostasis of hair follicle stem cells, and self-renewal of hematopoietic stem cells; however, its function in skeletal muscle development is poorly understood. In this study, we found that overexpression of *Lhx2* completely inhibits the myotube-forming capacity of C2C12 cells and primary myoblasts. The muscle dedifferentiation factors *Msx1* and *Msx2* were strongly induced by the *Lhx2* overexpression. Short interfering RNA-mediated knockdown of *Lhx2* in the developing limb buds of mouse embryos resulted in a reduction in *Msx1* and *Msx2* mRNA levels, suggesting that they are downstream target genes of Lhx2. We found two Lhx2 consensus-binding sites in the −2097 to −1189 genomic region of *Msx1* and two additional sites in the −536 to +73 genomic region of *Msx2*. These sequences were shown by luciferase reporter assay to be essential for Lhx2-mediated transcriptional activation. Moreover, electrophoretic mobility shift assays and chromatin immunoprecipitation assays showed that Lhx2 is present in chromatin DNA complexes bound to the enhancer regions of the *Msx1* and *Msx2* genes. These data demonstrate that *Msx1* and *Msx2* are direct transcriptional targets of Lhx2. In addition, overexpression of Lhx2 significantly enhanced the mRNA levels of bone morphogenetic protein 4 and transforming growth factor beta family genes. We propose that Lhx2 is involved in the early stage of skeletal muscle development by inducing multiple differentiation inhibitory factors.

Abbreviations: ES cells, embryonic stem cells; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PS, penicillin-streptomycin; FGF-2, fibroblast growth factor-2; RT-PCR, reverse transcription PCR; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; PBS, phosphate-buffered saline; MyHC, myosin heavy chain; siRNA, short interfering RNA; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; bps, base pairs; Bmp4, bone morphogenetic protein 4; Tgfβ, transforming growth factor beta

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Introduction

Lhx2 is a LIM homeobox transcription factor essential for eye and brain development as well as erythropoiesis [1]. In addition to its multiple roles in neural induction and morphogenesis [2,3], previous studies revealed that Lhx2 regulates the function of hair follicle stem cells [4,5] and promotes self-renewal of hematopoietic stem cells [6]. We recently discovered that lateral mesoderm-specific overexpression of Lhx2 in differentiating mouse embryonic stem (ES) cells leads to robust amplification of long-term repopulating hematopoietic stem cells [7,8]. It remains to be determined whether Lhx2 affects the development and homeostasis of other types of tissue stem cells, such as mesenchymal stem cells and skeletal muscle satellite cells.

Lhx2 is known to be a key transcription factor for the regulation of cardiac and pharyngeal muscle development [9]. In *Drosophila*, *apterous*, a functional homolog of mouse *Lhx2* [10], has been shown to be essential for muscle development [11]. In mice, *Lhx2* mRNA is abundantly expressed in developing limb buds of mouse embryos [12], and the outgrowth of limb buds is accompanied by proliferation and differentiation of myoblasts in the paraxial mesoderm. Although *Lhx2*-deficient mice exhibit normal limb development, Tzchori et al. showed that *Lhx2*/*Lhx9* double knockout mice display severe limb defects leading to shortened digits [13], which is consistent with the finding that *cLhx2* is required for limb outgrowth in the chick [14]. These studies collectively indicate that Lhx2/Lhx9 play essential roles in the outgrowth of limb buds; however, downstream effectors of Lhx2/Lhx9 in cells in the mesenchymal or muscle lineage have not yet been identified.

The homeodomain of Lhx2 is known to bind a TAATTA sequence in the enhancer/promoter regions of target genes [5]. Representative Lhx2-regulated genes include *glycoprotein hormone α-subunit* and *thyroid-stimulating hormone β-subunit* in the pituitary gland [15,16], and *Pax6* and *Cerberus 1* in neural precursor cells [17]. Most Lhx2 target genes are expressed in a tissue-specific manner. To obtain insight into the function of Lhx2 in skeletal muscle development, we investigated the effects of *Lhx2* overexpression in myoblastic cells. In this study, we present data showing that in mouse limb buds Lhx2 transcriptionally activates the expression of *Msx1* and *Msx2*, thereby inhibiting skeletal muscle differentiation.

Materials and methods

Cell culture and gene transfer

The C2C12 mouse myoblast cell line and 293T human embryonic kidney-derived cell line were purchased from the American Type Culture Collection (Manassas, VA) and maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and 1% penicillin-streptomycin (PS; Sigma). Skeletal muscle satellite cells were isolated from 7–8-week-old female mice and grown in DMEM containing 20% FBS, 1% PS and 10 ng/ml fibroblast growth factor-2 (FGF-2; Peprotech, Rocky Hill, NJ), as described previously [18]. For the induction of muscle differentiation, C2C12 cells and satellite-derived primary myoblasts were cultured in DMEM supplemented with 2% horse serum (Invitrogen) and 1% PS.

Retrovirus vector-mediated gene transduction experiments were carried out as described previously [7] using the retroviral vector pMY-Lhx2 ires-EGFP (or pMY-ires-EGFP) in combination with the PLAT-E virus-producer cell line. Mouse *Msx1* and *Msx2* cDNAs were isolated by reverse transcription (RT)–PCR by utilizing *Lhx2*-transduced C2C12 cells as source materials and inserted into the pMY-ires-EGFP vector.

Mice

Adult mice and pregnant C57BL/6 female mice were purchased from Nihon SLC (Hamamatsu, Japan) and used for the isolation of muscle satellite cells and dissection of limb buds, respectively. All mice were maintained under a 12 h light/dark cycle in a pathogen-free animal facility. All experimental procedures were pre-approved by the ethical committee of Tokyo Metropolitan Institute of Medical Science.

RT-PCR

Total RNAs were prepared using Trizol reagent (Invitrogen). RNA (500 ng) from each sample was reverse-transcribed using PrimeScript RT master mix for first strand cDNA synthesis with random primers (Takara, Otsu, Japan). Part of the cDNA mixture (1/20) was used in a PCR reaction with an annealing temperature of 56 °C, recombinant Taq DNA polymerase (Takara), and the primer sets listed in Table S1. PCR reactions for *glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*) were used as template controls. Real-time RT-PCR was performed with SYBR premix ExTaq II (Takara) using a LightCycler480 system (Roche Applied Science, Indianapolis, IN). Expression levels of mRNA were quantified using Relative Quantification Software with *Gapdh* as an internal control.

Immunofluorescent staining

Cells cultured on Lab-TekII chamber slides (Nunc, Roskilde, Denmark) were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 15 min, and incubated with 2% bovine serum albumin (Sigma) for 1 h at room temperature. The samples were incubated with goat anti-Lhx2 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-myogenin (1:1, F5D; Developmental Studies Hybridoma Bank), or mouse anti-sarcomeric myosin heavy chain (MyHC) (1:1, MF20; Developmental Studies Hybridoma Bank), followed by a corresponding AlexaFluor 546-conjugated secondary antibody (1:500; Molecular Probes, Eugene, OR). Cell nuclei were visualized by staining with Hoechst 33258 (Sigma).

Short interfering (si) RNA-mediated knockdown

A retroviral siRNA expression vector carrying *EGFP* (designated pReGS), a modified version of the pRePS vector [19], was constructed. For knockdown of *Lhx2* mRNA, limb buds were dissected from mouse embryos at 11.5 days post-coitum. They were soaked in 1 ml of *Lhx2*-siRNA retroviral solution in the presence of polybrene (8 μg/ml; Sigma) and centrifuged at 2800 × *g* for 90 min at 25 °C. Infected limb buds were placed on 1.5% agarose (Dojindo, Kumamoto, Japan) in DMEM containing 10% FBS and