Lrrc75b is a novel negative regulator of C2C12 myogenic differentiation

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Abstract. Many transcription factors and signaling molecules involved in the guidance of myogenic differentiation have been investigated in previous studies. However, the precise molecular mechanisms of myogenic differentiation remain largely unknown. In the present study, by performing a meta-analysis of C2C12 myogenic differentiation microarray data, we found that leucine-rich repeat-containing 75B (Lrrc75b), also known as AI646023, a molecule of unknown biological function, was downregulated during C2C12 myogenic differentiation. The knockdown of Lrrc75b using specific siRNA in C2C12 myoblasts markedly enhanced the expression of muscle-specific myogenin and increased myoblast fusion and the myotube diameter. By contrast, the adenovirus-mediated overexpression of Lrrc75b in C2C12 cells markedly inhibited myoblast differentiation accompanied by a decrease in myogenin expression. In addition, the phosphorylation of extracellular signal-regulated kinase 1/2 (Erk1/2) was suppressed in the cells in which Lrrc75b was silenced. Taken together, our results demonstrate that Lrrc75b is a novel suppressor of C2C12 myogenic differentiation by modulating myogenin and Erk1/2 signaling.

Introduction

Skeletal muscle differentiation is a highly complex and coordinated biological process which involves a broad spectrum of signaling molecules. It firstly begins with the commitment of satellite cells (muscle stem cells) to myogenic precursor cells known as myoblasts. Subsequently, myoblasts gradually become terminally differentiated myocytes coordinated by a series of regulatory factors. Finally, mononucleated myocytes specifically fuse to form multinucleated myotubes (1,2). To date, many efforts have been devoted to exploring and elaborating the precise regulation of myogenic differentiation. A number of transcription factors and muscle-specific genes, such as paired box (Pax)3/Pax7 (3-5), myogenic differentiation (MyoD) (6), Myogenic factor 5 (MYF5) (7), myogenin (8,9) and myosin heavy chain (MyHC) (10-12) have been confirmed as muscle determination factors. Myogenin is a member of the MyoD family, which is suggested to function in myogenesis. Previous studies have found that myogenin is expressed during myoblast differentiation, and its expression directly affects the progression of myoblasts into skeletal muscle (13,14). Recent studies have demonstrated that several regulators, such as miR-186 (9), multiple EGF like domains 10 (MEGF10) (15) and p53 (16) are involved in myoblast differentiation through the regulation of myogenin. These results provide evidence for a key role of myogenin as a critical regulator of myoblast differentiation. During myogenesis, myogenic regulatory factors (MRFs) are activated and regulate the transcription of genes, such as MyHC (17). In adult skeletal muscle, MyHC mRNA isoforms are expressed in a distinct patterns, including MyHC-I, MyHC-IIa, MyHC-IIx, MyHC-IIb, embryonic (emb) and neonatal (neo) (10,18). It has been confirmed that MyHC is expressed in late and terminal differentiation, and that it is the most suitable marker of muscle fibre (1). A series of signaling molecules, including p38(19), Wnt(20,21), extracellular signal-regulated kinase 1/2 (Erk1/2) (22,23), c-Jun N-terminal kinase (JNK) (24) and mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4) (25), have been shown to be involved in myogenesis. However, the precise molecular
mechanisms of myogenic differentiation remain largely unknown, and a number of novel genes involved in this process remain to be identified.

Microarray technology provides us with a unique opportunity to examine gene expression patterns in a whole genome. However, the heterogeneity of gene expression data could exist across different laboratories, different ChIP platforms or different experimental operations, which can be partly circumvented by meta-analysis so as to yield a more robust result.

In this study, we found that the leucine-rich repeat-containing 75B (Lrrc75b), also known as Alf646023, was downregulated during myogenesis by performing a meta-analysis of C2C12 myogenic differentiation microarray data in the GEO database. It has been demonstrated that many proteins containing leucine-rich repeat (LRR) domains participate in important biological processes, such as signal transduction, cell adhesion, cell development and DNA repair (26). Importantly, studies have revealed the involvement of LRR proteins in cell differentiation. LRR8 (also known as FAD158) is expressed in differentiating 3T3-L1 cells, and the knockdown of LRR8 has been shown to significantly inhibit 3T3-L1 adipocyte differentiation (27). Another LRR protein, LRR17, functions as an inhibitor of RANKL-induced osteoclast differentiation (28).

The aim of this study was to elucidate the potential function of Lrrc75b in myogenesis. Using knockdown and overexpression techniques, we found that Lrrc75b significantly regulated the activity of muscle marker genes and the phosphorylation of Erk. Our results demonstrated that Lrrc75b is a novel negative regulator of myogenesis.

Materials and methods

Meta-analysis of C2C12 myogenic differentiation microarray data. To obtain the differentially expressed genes in C2C12 myogenic differentiation, the GEO database was used (29). Three datasets (listed in Table I) were used and we also used the Affymetrix mouse expression array (including 430 2.0 array, 430A and B array). To the best of our knowledge, these arrays contain more abundant gene probesets. The raw data from each experiment were normalized using ChIP analysis tools and the following thresholds were then used to obtain sets of differentially expressed genes: i) E/B >1.5 or B/E >1.5, use lower 90% confidence bound of fold; ii) E-B >50 or B-E >50 and iii) P-value of 0.05. The upregulated or downregulated gene probesets were converted to official gene symbols using DAVID [National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), USA], a functional annotation tool (30). The differentially expressed genes in at least 2 experiments in all the above 3 experiments were designated as potential myogenesis upregulated or downregulated genes.

Cell culture. C2C12 myoblasts (CRL-1772; ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; both from HyClone Laboratories, Inc., Logan, UT, USA) at 37°C with 5% CO₂. To induce myogenic differentiation, the cells were plated on tissue culture plates and grown to 95% confluence before switching to differentiation medium (DM) (DMEM and 2% horse serum; HyClone Laboratories, Inc.). The cells were replenished with fresh DM every other day until day 5.

Transfection with Lrrc75b siRNA. Stealth RNAi™ pre-designed siRNAs specific for mouse Lrrc75b (MSS208271; Gibco Life Technologies, Rockville, MD, USA) were synthesized by Gibco Life Technologies. Stealth RNAi medium GC control was used as a negative control. siRNA duplexes were transfected into the cells using 2.5 µl/ml Lipofectamine RNAiMAX (Gibco Life Technologies) according to the manufacturer's instructions. Briefly, the C2C12 cells at 40-60% confluence were transfected with 0.2 µM siRNA.

Adenoviral infection. The C2C12 cells were plated in culture dishes at a density of 1.3x10⁵ cells/cm². Adenoviral shuttle vector expressing either pDC316-mCMV-EGFP-CMV-Lrrc75b (C-terminal Myc-tagged) or the empty pDC316-mCMV-EGFP and adenovirus packaging were completed by a professional company (Biowit Technologies, Shenzhen, China). Briefly, mouse Myc-tagged Lrrc75b was synthesized and inserted into vector pDC316-mCMV-EGFP using the restriction enzymes Nhel and HindIII. The adenoviral shuttle vector and virus backbone plasmid pHGlodeltaE13Cre were then co-transfected into 293 cells by polyfetamine. Adenoviruses were generated following the instructions of AdMax™ Adenoviral Vector Creation System and the recombinant adenoviruses were collected and amplified in 293 cells. When the cells grew to 50-70% confluency, they were infected with Ad-Lrrc75b or Ad-GFP at an MOI of 200 for 12 h in growth medium and this was then changed to fresh growth medium. At 48 h post-infection, the cells were harvested for western blot analysis or, the medium was changed to to DM and the cells were cultured for the indicated periods of time (0, 1, 3, 5 days) before harvesting.

Reverse transcription-quantitative (real-time) PCR (RT-qPCR). Total RNA was extracted from the C2C12 cells with TRIzol Reagent (Gibco Life Technologies) following the manufacturer's instructions. Each sample was reverse transcribed into cDNA using the PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time; Takara Bio Inc., Otsu, Japan). This was followed by quantitative PCR (qPCR) using an Applied Biosystems 7500 Real-time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA) with the One-Step SYBR PrimeScript RT-PCR kit II (Takara Bio Inc.) according to the manufacturer's instructions. The housekeeping gene, GAPDH, was used as an internal normalization control to obtain the relative fold changes using the comparative CT method. The sequences of the primers used were as follows: mouse Lrrc75b forward, 5'-ggaccatgagctctggaagt-3' and reverse, 5'-atccagctctccctacca-3'; and mouse GAPDH forward, 5'-cggtgtctaccccaaatg-3' and reverse, 5'-gettacacccttctgatg-3'.

Western blot analysis. The whole-cell lysates were harvested for 30 min on ice in RIPA lysis buffer containing 100 mM PMSF (Beyotime Institute of Biotechnology, Haimen, China) and then centrifuged at 12,000 rpm for 15 min at 4°C. Total protein concentrations were measured by BCA protein assay and equal amounts of proteins were then separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a poly-
vinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk for 1.5 h at room temperature, and then incubated overnight at 4˚C with the following primary antibodies: anti-myogenin (SC-12732, 1:200; F5D; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-MyHC (m4276, 1:1,000; Sigma-Aldrich, St. Louis, MO, USA), anti-phospho-p44/42 MAPK (4377, 1:2,000; p-Erk1/2) (Thr202/Tyr204), anti-p44/42 MAPK (4695, 1:2,000; Erk1/2) and anti-Myc-tagged (2278, 1:300) (all from Cell Signaling Technology, Inc., Beverly, MA, USA) and anti-tubulin (AT819, 1:2,000; Beyotime Institute of Biotechnology). After being washed with TBST 3 times, the membranes were incubated with HRP-labeled goat anti-mouse IgG (A0216, 1:1,000; Beyotime Institute of Biotechnology) or HRP-labeled goat anti-rabbit IgG (AB6721, 1:5,000; Abcam, Cambridge, MA, USA) for 1 h at 37˚C. Bands were visualized using the Luminata Crescendo Western HRP substrate (Millipore, Billerica, MA, USA). The quantification of the band intensities was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**Immunofluorescence microscopy and myotube analysis.** The cells were fixed with 4% formaldehyde for 20 min and washed 3 times in PBS, and then incubated with 0.3% Triton X-100 in PBS for 20 min and blocked in 10% donkey serum at 37˚C for 30 min. Subsequently, the cells were incubated overnight at 4˚C with anti-myosin primary antibody against MyHC (M4276, 1:150; Sigma-Aldrich). The cells were then incubated with Alexa Fluor 594-conjugated secondary antibody (A-21203, 1:200, Gibco Life Technologies) for 1 h at room temperature. The nuclei of the cells were visualized using 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) staining for 10 min. Images of samples were also captured using a fluorescence microscope (DP73; Olympus Corp., Tokyo, Japan). Finally, 5 random fields with representative images per sample were used to calculate the myotube area (area occupied by myotubes relative to the total area) and the fusion index (the ratio of nuclei in MyHC-positive myotubes with ≥2 nuclei to the total number of nuclei in the field).

**Statistical analysis.** The experiments were repeated 3 times, and statistical analyses were performed using the Student's t-test or ANOVA. Statistical comparisons were considered significant at P<0.05.

**Results**

**Meta-analysis of myogenic differentiation microarray data indicating the downregulation of Lrrc75b.** To overcome the weakness of conventional microarray-based data analysis, we designed a meta-analysis strategy for microarray datasets used in this research to build up lists of meta-genes in C2C12 myogenesis. The differentially expressed genes in at least 2 datasets in all the above-mentioned 3 experiments were designated as potential myogenesis upregulated or downregulated genes. The Venn diagram in Fig. 1 shows the distribution of differentially expressed genes in at least 2 datasets in all the above-mentioned 3 experiments were designated as potential myogenesis upregulated or downregulated genes. The Venn diagram in Fig. 1 shows the distribution of differentially expressed (upregulated or downregulated) genes among the GSE5447, GSE4694 and GSE5305 datasets. (B) Intersection of differentially downregulated genes among the GSE5447, GSE4694 and GSE5305 datasets.

**Lrrc75b expression is inhibited during myogenic differentiation.** To investigate the roles of Lrrc75b in myogenic
Differentiation, we first established a C2C12 myoblast differentiation model. C2C12 cells are derived from satellite cells of C3H mice, which is the classical muscle differentiation model (8,31). The results presented in Fig. 2A show that the exposure of C2C12 cells to DM resulted in the formation of myotubes within 3 days and further fusion, forming multinucleated myotubes within 5 days. Moreover, as shown in Fig. 2B, the expression of muscle-specific genes, such as myogenin and MyHC increased upon the induction of differentiation. These results are consistent with those of a previous study (1). To confirm that Lrrc75b is indeed required for myoblast differentiation, RT-qPCR was performed. As shown in Fig. 2C, the mRNA expression of Lrrc75b was markedly decreased during differentiation; similar results were obtained by the analysis of microarray data. This result suggests that Lrrc75b plays a role in the differentiation of C2C12 myoblasts into myotubes.

Knockdown of Lrrc75b promotes the myoblast differentiation of C2C12. To clarify the possible role of Lrrc75b in myogenesis, we established C2C12 cells in which Lrrc75b expression was depleted by using stealth siRNA that target the specific region of the mouse Lrrc75b gene and then examined the cells for their myoblast differentiation ability. As shown in Fig. 3A, the mRNA expression of Lrrc75b significantly decreased by ~70% in the cells transfected with siRNA targeting Lrrc75b (si-Lrrc75b) compared with the cells transfected with the control siRNA. Subsequently, immunofluorescence staining using anti-MyHC antibody was used to detect the formation of myotubes. As shown in Fig. 3B, the knockdown of Lrrc75b significantly promoted myogenic differentiation and resulted in a higher myogenic index, such as a greater myotube area and fusion index (Fig. 3C and D). In addition, as shown in Fig. 3E-G, the results of western blot analysis confirmed that the knockdown of Lrrc75b markedly increased the expression of myogenin and MyHC.

Overexpression of Lrrc75b inhibits myogenic differentiation. Having clarified that the knockdown of Lrrc75b promotes myogenic differentiation, we then wished to determine whether the upregulation of Lrrc75b in C2C12 cells would affect myogenesis. To examine this hypothesis, we induced the overexpression of Lrrc75b using Myc-tagged Lrrc75b adenovirus. As compared to the C2C12 cells infected with GFP adenovirus, the expression of Myc-tagged Lrrc75b adenovirus emerged in the cells infected with the Lrrc75b adenovirus (Fig. 4A). Moreover, as shown in Fig. 4B-D, in the cells infected with Lrrc75b adenovirus, the number of myotubes and myogenic index were markedly lower than those observed in the cells infected with the GFP adenovirus. Furthermore, the results of western blot analysis revealed that the overexpression of Lrrc75b significantly decreased the expression of myogenin (Fig. 4E and F).
As regards the expression of MyHC, infection of the cells with Lrrc75b adenovirus did not exert a significant effect on its expression, neither on day 3 nor day 5. We hypothesized that the reason for this may be that the expression of muscle-specific genes is essential but not sufficient for terminally differentiated myoblast fuse into multinuclear myotubes (32). Lrrc75b affects the phosphorylation of Erk1/2. To further elucidate the mechanisms involved in the regulation of myogenic differentiation by Lrrc75b, we investigated the target effector of the MAPK pathway, p-Erk1/2. At 24 h post-infection with control or si-Lrrc75b, the C2C12 cells underwent myogenic differentiation. The phosphorylation of Erk1/2 was analyzed in the 2 groups of cells at 0, 1, 2 and 3 days. As shown in Fig. 5A-C, the phosphorylation level of Erk1/2 was gradually decreased in both groups of cells at the later stage of differentiation. However, the phosphorylation of Erk1/2 was much weaker in the si-Lrrc75b-transfected cells on days 2 and 3. On the contrary, the C2C12 cells infected with GFP or Lrrc75b adenovirus exhibited an inhibition of myogenic differentiation. As shown in Fig. 5D-F, the phosphorylation of Erk1/2 was increased in the Lrrc75b adenovirus-infected cells on day 5.
Whole genome microarray analyses, viewed as a non-biased, genome wide molecular taxonomy, is helpful for the understanding of the molecular mechanisms of myogenesis. As previously reported (33,34), probably due to platform-to-platform or laboratory-to-laboratory variability, there was a great heterogeneity in the differentially expressed genes of...
C2C12 myogenesis in the 3 datasets. For example, among a total of 3,921 upregulated genes, only 932 genes were upregulated in more than 2 different experiments. Thus, meta-analysis provides a list of more pertinent genes for further study.

To date, many efforts have been devoted to exploring and elaborating the precise regulation of myogenic differentiation. However, the precise molecular mechanisms of myogenic differentiation remain largely unknown and many novel genes involved in this process remain to be identified. In this study, we found that Lrrc75b, a completely unknown function gene, was downregulated during myoblast differentiation. The knockdown of Lrrc75b using siRNA markedly enhanced C2C12 myoblast differentiation, as evidenced by an increase in the myotube area and fusion index. By contrast, the overexpression of Lrrc75b attenuated the differentiation of C2C12 myoblasts into myotubes, decreasing the myotube area and fusion index. Therefore, these findings indicate that Lrrc75b plays an essential role in C2C12 myoblast differentiation.

Skeletal muscle development is a complex and orderly progress wherein mesodermal cells commit to myoblasts and subsequently fuse into multinuclear myotubes (35). This progress is mainly controlled by a family of MRFs, such as myogenin (25). It has been reported that activated myogenin in early differentiation can drive terminal differentiation, the formation of myotubes and muscle fiber maturation (25,36). Our observations that the knockdown of Lrrc75b using siRNA resulted in an increase in myogenin expression, whereas the opposite was observed with the overexpression of Lrrc75b by injection with adenovirus, indicate that Lrrc75b is involved in signaling upstream of myogenin during C2C12 myoblast differentiation into myotubes.

Given that the Erk1/2 signaling pathway plays an important role during myoblast differentiation (37-42), we examined the changes in Erk1/2 phosphorylation in response to myogenic differentiation and found that the phosphorylation of Erk1/2 was decreased. This result is consistent with the findings of a previous study (23). Our results also demonstrated that the phosphorylation of Erk1/2 was decreased following the knockdown of Lrrc75b using siRNA, whereas it was increased in late-stage differentiation with the overexpression of Lrrc75b by infection with adenovirus. Taken together, these data suggest that Lrrc75b plays a negative role in myogenic differentiation and this role is mediated at least partly via Erk1/2.

In conclusion, in this study, we obtained a more pertinent gene set for C2C12 myogenic differentiation through meta-analysis of three microarray datasets from different laboratories. Our results also demonstrate that Lrrc75b is a novel suppressor of C2C12 myogenic differentiation by modulating myogenin and Erk1/2 signaling.

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