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Ubiquitin C-Terminal Hydrolase L1 regulates myoblast proliferation and differentiation

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Abstract

Skeletal muscles are dynamic tissues that possess regenerative abilities, which require multiple processes and regulatory factors. Ubiquitin C-Terminal Hydrolase L1 (UCHL1), which is primarily expressed in neuronal tissues, was upregulated in skeletal muscles in disease conditions but its functional role in skeletal muscles is unknown. Using mouse myoblast cells C2C12 as an *in vitro* model, this study reported that UCHL1 elicits different regulation in myoblast cell proliferation and differentiation. We first observed that UCHL1 protein level was continuously declined during cell differentiation. Gene knockdown of UCHL1 by siRNA resulted in a significant decrease in cell proliferation but marked acceleration of cell differentiation and myotube formation. Meanwhile, UCHL1 gene knockdown upregulated myogenic factors myoD and Myogenin (MyoG). In mice, UCHL1 was significantly upregulated in denervated skeletal muscle. Overall, these novel data suggest that UCHL1 may play a role in myogenesis by promoting myoblast proliferation and inhibiting differentiation.

Keywords

Ubiquitin C-terminal Hydrolase L1; myoblast; proliferation; differentiation; myogenin

Introduction

Skeletal muscles are complex and dynamic tissues that have strong renewal and regenerative abilities during normal growth and in response to injury. In skeletal muscle regeneration processes, myoblast cells are derived from activated satellite cells and undergo sequential processes of proliferation, differentiation, fusion, and finally maturation to become myofibers[1–4]. These processes are regulated by multiple myogenic factors, including MyoD, Myf5, and myogenin (MyoG)[5]. The orchestration of these events are critical in normal muscle regeneration [1–3, 6]. Our understanding in the area has been rapidly expanded in the past decades, yet many details of the processes of myogenesis and related myogenic factors remain to be fully addressed.

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Ubiquitin carboxyl-terminal hydrolase L1 (UCHL1), also known as “PGP9.5”, was originally found as a neuronal specific protein that is highly expressed in both central and peripheral nervous systems [7, 8]. Altered UCHL1 has been linked to neurodegenerative diseases. In the brain of patients with Parkinson’s and Alzheimer’s disease (AD), UCHL1 protein level were reduced[9]. In an animal experiment, UCHL1 exhibited a protective effect against beta-amyloid-induced decreases in synaptic function and contextual memory in AD mouse model[10].

It is evident that UCHL1 affects skeletal muscle developments and function. Mice with a spontaneous deletion of exons 7 and 8 in the UCHL1 gene, also known as gracile axonal dystrophy (*gad*) mice, develop abnormal shuffling movement, hind-limb paralysis, and early death[11]. A recent study shows that this is due to the impaired structure and function of neuromuscular junction[12], indicating that UCHL1 affects skeletal muscle function via the neuro-control of muscles rather than the direct effect on the skeletal muscles. However, recent studies suggest that UCHL1 is expressed in human skeletal muscles[13] and mouse myoblast cells C2C12 [14]. UCHL1 expression was increased in skeletal muscles in spinal muscular atrophy diseases [15, 16]. To date, however, the functional role of UCHL1 in muscles is still elusive. In this study, we are presenting novel data suggesting the potential functional role of UCHL1 in the regulation of myoblast proliferation and differentiation.

Methods

Cell culture

C2C12 myoblast cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in growing medium (GM) that was Dulbecco’s Modified Eagle’s Medium (DMEM, ATCC) supplemented with 10% fetal bovine serum (FBS, GE HyClone, Logan, UT) and 1% penicillin-streptomycin. Media were changed every two days. For differentiation, cells were grown in GM to full confluence and then switched into differentiating medium (DM) that was DMEM supplemented with 2 % horse serum (HyClone) and 1% penicillin-streptomycin. Media were changed every two days.

Manipulations of UCHL1 gene expression

UCHL1 gene expression was manipulated using UCHL1 gene knockdown or overexpression. UCHL1 siRNA gene knockdown was performed as described previously[17] with some modifications. Briefly, C2C12 cells were grown in GM to desired confluence. Media were changed with fresh GM or DM, depending on the purpose of experiments. Control or UCHL1 siRNA (Integrated DNA Technologies, IDT, Coralville, Iowa) were premixed with Lipofectamine RNAiMAX transfection Reagent (Invitrogen, ThermoFisher Scientific) at a ratio of 1 ul reagent : 1 ul of 10 uM siRNA in 100 ul of DMEM without FBS, and set in room temperature for 10 minutes. The mixture was then added into cell media with the final siRNA concentration being 10 nM. Cells were incubated in GM or DM with the siRNA for 24 hours. Thereafter, media were changed with fresh GM or DM every two days for a desired period.

For UCHL1 overexpression, C2C12 cells were grown in GM to desired confluence. Media were changed with fresh GM. Control adenovirus or adenovirus expressing full length mouse UCHL1 (Vector BioLabs Malvern, PA) were added into the media. After 24 hours of incubation at 37 C, media were changed with fresh GM.

EdU cell proliferation assay

Cell proliferation was measured using Click-iT EdU microplate assay kit (Invitrogen, C10214) by following the manufactory's instruction. Briefly, C2C12 were grown in a 96-well plate with GM to about 40% confluence, treated with control or UCHL1 siRNA for gene knockdown, or control adenovirus or UCHL1 expressing adenovirus for oveexpression, as described above, for 2 days. Changing with fresh GM, EdU reagent was added in each well with final concentration 10 uM and incubated for 4, 20, or 28 hours. After removing media containing EdU reagent, cells were fixed and incubated with reaction cocktail. Following washes and blocking, cells were incubated with an antibody for EdU conjugated with horse radish peroxidase (HRP). Finally, after washes, the fluorescent probe, AmplexRed mixed with H₂O₂, were added. The fluorescent intensity, which is proportional to EdU incorporation into synthesized DNA during cell proliferation, was detected at excitation 570 nm/emission 585 nm using a microplate reader (Infinite 200M, Tecan). The fluorescent intensity of 6 repeat wells at each incubation time point was averaged.

Western blot

Cells or muscle tissues were homogenized in RIPA buffer containing a protease inhibitor cocktail (Santa Cruz, Dallas, TX) and phosphatase inhibitor (Research Product International, Mount Prospect, IL). Protein concentration of samples were determined by a standard BCA assay and subjected to standard Western blot protocol as described previously [18, 19]. The following primary antibodies were used: UCHL1, MyoD, MyoG, GAPDH (Santa Cruz), myosin heavy chain (MF20), PAX7 (Developmental Studies Hybridoma Bank, DSHB, Iowa City, IA), UCHL3, insulin receptor substrate 1 (IRS1) and phosphorylated IRS1 (S613), Akt and phosphorylated Akt (s473) (Cell Signaling Technologies, Danvers, MA). The appropriate secondary antibodies conjugated with Alex-700 or Alex-800 were purchased from Invitrogen. The fluorescent signals of the blots were detected using a LI-COR scanner (LI-COR Biosciences, Lincoln, NE) and quantified using LI-COR Image Studio or NIH ImageJ software.

Cell fluorescent staining

C2C12 cells were cultured on autoclaved coverslips (Fisher) in a 12-well plate in GM or DM, and treated with siRNA, as described above. At the end of treatments, cells were washed with PBS and fixed with a pre-chilled 50% Ethanol - 50% Methanol mixture for 10 minutes. For proliferation confluence, cells were incubated with Wheat Germ Agglutinin (WGA) conjugated with Texas Red (Invitrogen) for 10 minutes, rinsed with PBS. For immunofluorescent staining, cells were blocked with 5% bovine serum albumin (BSA) and 5% donkey serum in PBST for 1 hour at room temperature. Then cells were incubated with appropriate primary antibodies overnight at 4 C. The primary antibodies against UCHL1 (from rabbit, EPR4118, GeneTex, Irvine, CA, or Abcam Cambridge, MA), or myosin heavy chain (MyHC from mouse, DSHB) were diluted in the blocking solution. After washes, cells

were incubated with the desired secondary antibodies conjugated with Alex-488 with Hoechst for staining nucleus. To show myotubes, cells were incubated with WGA conjugated with Texas Red (Invitrogen) for 10 minutes after secondary antibody incubation. After washes, coverslips with cells were mounted on a glass slide with Fluoromount-G (SouthernBiotech, Birmingham, AL). The images were taken using a confocal laser scanning microscope (Olympus) or a fluorescent Microscope (Olympus).

Data analysis

Data calculation, graphing, and descriptive statistics were performed using Microsoft Excel Data Analysis package. Data were presented as Mean $\pm$ Standard Deviation (SD). Statistical significance between treatment groups and control groups were compared by one-factor analysis of variance (ANOVA) followed by student t-test. Statistical significances were defined as p value less than 0.05.

Results

1, UCHL1 protein level was altered in proliferating and differentiating myoblast C2C12 cells

In mouse myoblast C2C12 cells, we first observed that UCHL1 protein level was relatively high in proliferating cells in growing media (GM) containing 10% FBS. As cells were switched into differentiating media (DM) that contains 2% horse serum, the UCHL1 protein level began to decline, which was continuous throughout the course of differentiation (Figure 1). Meanwhile, during the differentiation, the protein levels of MyoG and myosin heavy chain (MyHC) were continuously increased, while PAX7 and MyoD level declined, which are wellknown characteristics of myoblast differentiation. The changes of UCHL1 protein level during myoblast proliferation and differentiation suggests that UCHL1 may be functionally associated with these processes.

2, UCHL1 is a positive factor for myoblast C2C12 proliferation

The high level of UCHL1 in C2C12 cells in GM suggests a potentially functional significance of UCHL1 for myoblast proliferation. To test this, we used siRNA effectively knocking down UCHL1 in C2C12 cells in GM. Cell staining with WGA conjugated with Texas Red showed that the confluence of cells treated with UCHL1 siRNA was lower as compared with cells with control siRNA (Figure 2A). EdU assay, which detects DNA synthesis as an index of cell proliferation, showed that UCHL1 knockdown significantly reduced C2C12 myoblast proliferation as compared with control knockdown (figure 2B). The inhibitory effect of UCHL1 gene knockdown on C2C12 proliferation was also confirmed using MTT assay (supplemental data Figure S1A). These results suggest that UCHL1 positively regulates myoblast proliferation. However, the overexpression of UCHL1 using adenovirus did not significantly enhance C2C12 proliferation (figure 2C and figure S1B). We reason it as the relatively high level of endogenous UCHL1 that may elicit a maximal effect on cell proliferation. Alternatively, it is possible that UCHL1 has a supporting effect but not stimulating effect on cell proliferation.

3, UCHL1 knockdown accelerated C2C12 differentiation

Because UCHL1 protein level continuously declined during cell differentiation, we then further examined the effect of UCHL1 reduction on C2C12 myoblast differentiation. Cells were grown in GM to full confluence, treated with UCHL1 siRNA or control siRNA, and then switched into DM for differentiation for 2 to 4 days. C2C12 cells with UCHL1 siRNA knockdown showed remarkably faster cell differentiation indicated by more elongated myotubes as compared with the cells with control knockdown at the same time point in DM (Figure 3A). The differentiation was quantified by the ratio of the nuclei that were co-localized with MyHC staining versus total nuclei, which was significantly increased in cells with UCHL1 knockdown as compared with the control after 2 days of differentiation (Figure 3B). These data suggest that UCHL1 may have an inhibitory effect on myoblast differentiation and downregulation of UCHL1 facilitates myoblast differentiation.

4, UCHL1 knockdown increased myogenic factors

Given this dramatic acceleration of myoblast differentiation following the UCHL1 knockdown, we further determined the effect of UCHL1 knockdown on some key myogenic molecules including PAX7, MyoD, and MyoG. Western blot showed that MyoD and MyoG, two key factors that stimulate myoblast differentiation, were significantly increased in cells treated with UCHL1 siRNA compared with cells treated with control siRNA (figure 4B). There was no difference in PAX7 level between the control and UCHL1 knockdown groups. Overall, these results indicate that the acceleration of C2C12 differentiation by UCHL1 gene knockdown is at least partially due to the upregulation of myogenic factors MyoD and MyoG.

IGF-Akt signaling pathway plays an important role in regulation of myogenic factors and muscle growth[20]. However, our results showed that UCHL1 knockdown did not alter the activation of this pathway, indicated by the unchanged phosphorylation of insulin receptor substrate 1 and Akt (figure S2).

5, UCHL1 was upregulated in denervated skeletal muscles

If UCHL1 is involved in myoblast proliferation, it may be upregulated during myogenic response. We tested this in denervated muscles because denervation can cause muscle injury and atrophy, at same time trigger myogenic activity as a compensatory response[21]. In mice with unilateral sciatic denervation, we observed that UCHL1 protein level was significantly increased in denervated muscles as compared with the control muscles. As an evidence of myogenesis, levels of PAX7 and MyoG were also significantly increased in denervated muscles (Figure S3A). Immunofluorescent staining showed that in denervated muscle, increased UCHL1 was co-localized with PAX7 positive staining (Figure 13B), suggesting that UCHL1 is associated with activated satellite cells or proliferating myoblast cells during the regeneration response. These *in vivo* data support the notion that UCHL1 plays a role in regulation of myoblast activation and proliferation during the muscle regeneration processes.

Discussion

This study presents novel evidence showing for the first time that UCHL1 is involved in regulation of myoblast proliferation and differentiation. Our data show that UCHL1 protein level undergoes dynamic changes from proliferation to differentiation of C2C12 myoblast cells. Functionally, the data suggest that UCHL1 is important for myoblast proliferation because knockdown of UCHL1 significantly reduces myoblast proliferation. In contrast, reduction of UCHL1 by gene knockdown dramatically accelerates myoblast differentiation into myotubes. Overall, these novel data suggest that UCHL1 has important impact on early processes of myogenesis through promoting myoblast proliferation and inhibiting differentiation.

In adult myogenesis, such as in muscle regeneration in response to injury, the efficient and sufficient proliferation of satellite cell-derived myoblasts is fundamental and critical for the subsequent steps of myoblast differentiation, myotube fusion, and myofiber maturation[1, 22, 23]. Our data suggest that, with its positive effect, upregulation of UCHL1 is essential for adequate myoblast proliferation, which is important for the later steps of differentiation and fusion. Our *in vivo* data showed that UCHL1 protein level began to increase in denervated muscles after denervation injury. The immunofluorescent staining indicates that the UCHL1 was co-localized with PAX 7 positive staining in skeletal muscles. In denervated muscle, protein levels of UCHL1 and PAX7 were increased. Together with our *in vitro* data, we propose that UCHL1 can promote the proliferation of myoblasts that are newly derived from activated satellite cells to allow sufficient myoblast proliferation at early stage of muscle regeneration.

A recent study reported the upregulation of UCHL1 in skeletal muscles in spinal dystrophy model. In that study, the authors observed that the treatment with UCHL1 inhibitor LDN57444 had adverse effect on the muscle injury, and concluded that the upregulated UCHL1 in that model was compensatory rather than detrimental response[15]. Our data support their conclusion and provide further details to understand and explain their observations. According to our data, the upregulated UCHL1 promotes myoblast proliferation and thus is beneficial for muscle regeneration and repair. Therefore, inhibition of the upregulated UCHL1 in this muscle injury model would have a negative effect on myoblast proliferation and consequently on muscle regeneration.

Our data revealed that knockdown of UCHL1 markedly promoting myoblast differentiation and myotube formation, suggesting that UCHL1 inhibits myoblast differentiation. This inhibitory effect on differentiation of UCHL1 may be important for sufficient proliferation by preventing premature differentiation. On the other hand, downregulation of UCHL1 perhaps would be essential and important for promotion of myoblast differentiation and myotube fusion. Indeed, we observed that in C2C12 cells UCHL1 protein level started to decline as soon as the cells were switched into DM and started differentiation. C2C12 cells with prolonged differentiation showed very low UCHL1 protein level, which is consistent with the fact that in adult muscle fibers, UCHL1 protein level is usually low or even undetectable.

As for the mechanism by which downregulation of UCHL1 boosts myoblast differentiation, our data suggest that it is at least partially due to the increased MyoD and MyoG. MyoD is critical in conversion of myoblast and fibroblast into myotube and muscle fiber[24, 25]. MyoG as a downstream gene of MyoD is critical for muscle development[26]. As suggested by our data, gene knockdown of UCHL1 resulted in a significant increase in MyoD and MyoG level in first two days, suggesting these two myogenic factors may contribute to the accelerated myoblast differentiation and myotube formation.

As a member of UCH deubiquitinase (DUB) family [27, 28]. UCHL1 cleaves ubiquitin at C-terminal from small substrates [29], binds to and stabilize mono-ubiquitin [30], and thus is critical for maintaining free ubiquitin pool. After decades of discovery, the substrates and functions of UCHL1 as a DUB remains to be defined. It would be interesting and important to verify whether the functional role of UCHL1 in regulation of myoblast proliferation and differentiation is dependent or independent of its DUB activity and identify its substrates.

The role of UCHL1 in regulation of myogenesis described in this study is novel, interesting, yet remains to be further characterized. For example, although knockdown of UCHL1 decreased myoblast proliferation, overexpression of UCHL1 did not increase the proliferation. Our interpretation was that it may be because high level of endogenous UCHL1 already has maximal effect on cell proliferation. However, it is also possible that UCHL1 is essential for myoblast proliferation but does not function as a stimulating factor. Secondly, our data show that UCHL1 is downregulated in myoblast differentiation and this downregulation facilitates the differentiation. However, UCHL1 is upregulated during the injury. It is not known how long the UCHL1 upregulation last and how this upregulation is controlled during muscle regeneration. Thirdly, it is important to address how UCHL1 regulates myoD and MyoG expression during myogenesis. Nevertheless, this study reveals a novel function of UCHL1 regulating myoblast cell proliferation and differentiation. This information helps to better understand the role of UCHL1 in skeletal muscle regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

UCHL1	ubiquitin C-terminal hydrolase L1
DUB	deubiquitinating enzyme, or deubiquitinase
PAX7	Paired box protein 7

FBS	fetal bovine serum
DMEM	Dulbecco's Modified Eagle's Medium
GM	growing medium
DM	differentiation medium
BSA	bovine serum albumin
EdU	5-ethynyl-2'-deoxyuridine
WGA	wheat germ agglutinin
MyHC	myosin heavy chain
MyoG	myogenin

Biographies

Hongbo Gao is a PhD graduate student at USD Sanford School of Medicine. She participated in experimental design, carried out major experiments, including cell culture and treatments, animal experiments and tissues preparation, Western blot, data analysis, and manuscript preparation.

Sigurd Harnett was a MD/PhD student at the time when this work was conducting and recently graduated with MD/PhD degree from USD Sanford School of Medicine. He carried out some cell culture, siRNA validation and treatments, and contributed to manuscript preparation.

Yifan Li is an associate professor at USD Sanford School of Medicine. He was responsible for overall experimental design, carried out cell staining, animal surgery and tissue collection, and wrote and revised the manuscript.

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Highlights

- UCHL1 is upregulated in denervated skeletal muscles
- Elicits different effects on myoblast cells proliferation and differentiation.
- Involved in regulation of myogenesis and muscle regeneration

Figure 1.

Altered UCHL1 protein level in C2C12 cells at proliferation and differentiation. The left panels: Representative images of Western blots for UCHL1, UCHL3, MyoG, MyHC, PAX7, MyoD, and GAPDH from C2C12 cells in GM (day 0) or DM for 1 through 4 days. The right panels: Quantifications of the Western blots for UCHL1 and UCHL3 (top), MyoG and MyHC (middle), and PAX7 and MyoD (bottom) from cells in CM (day 0) or DM for 1 through 4 days. “*” indicates $p < 0.05$ as compared with day 0. $n = 4$

Figure 2.

Effect of manipulations of UCHL1 expression on C2C12 cell proliferation. A: Cell staining with WGA-Texas Red showing the cell confluences of proliferating C2C12 following treated with control or UCHL1 siRNA for 2 days. B: the top panel are representative images of Western blot for UCHL1 and GAPDH protein levels of cells in GM treated with control siRNA or UCHL1 siRNA for 2 or 3 days. The bottom bar graph presents the EdU assay of cells proliferation treated with control siRNA or UCHL1 siRNA for 2 through 3 days. “*” indicates $p < 0.01$, $n = 6$ for each group. C: The top are representative images of Western blot for UCHL1 and GAPDH protein levels of cells in GM treated with control adenovirus or adenovirus expressing UCHL1 for 3 or 4 days. The bottom bar graph shows the EdU assay of cells proliferation treated with control adenovirus or adenovirus expressing UCHL1 for 3 through 4 days (OE: overexpression).

Figure 3.

Effect of reduction of UCHL1 on C2C12 differentiation. A: Fluorescent staining of differentiated C2C12 myotubes. Cells treated with control siRNA (top row) or UCHL1 siRNA (bottom row) were immunofluorescently stained with a primary antibody against UCHL1 followed by a secondary antibody conjugated with Alex-488 (green), WGA conjugated with Texas Red staining cell membrane (red), and Hoechst staining nucleus (blue). B: Differentiated C2C12 myotubes were immunofluorescently stained with a primary antibody for myosin heavy chain (MyHC, green) and Hoechst for nuclei (blue). The ratio of the nuclei co-localized with MyHC staining versus total nuclei were present in the bar graph on the right. Black bar is the group with control siRNA and the gray bar is UCHL1 siRNA group. “*” indicates $p < 0.05$. $n = 4$ for each group.

Figure 4.

Effect of UCLH1 gene knockdown on the levels of myogenic factors. A: representative images of Western blot (left) and quantifications (right) for UCLH1 and UCLH3 in C2C12 cells in DM treated with control siRNA or UCLH1 siRNA for 1, 2, or 3 days. B: representative images of Western blot (left) and quantifications (right) for PAX7, MyoD, and MyoG from the same batches of cells above. Black bars are control siRNA groups and gray bars are UCLH1 siRNA groups. “*” indicates $P < 0.05$ as compared with control siRNA groups at the same time point. $n = 4$ for each group.