Original Article

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Myoblast differentiation of C2C12 cell may related with oxidative stress

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SUMMARY Muscle is a contractile tissue responsible for maintaining posture and the movement of all parts of the body. Prolonged oxidizative stress can lead to the damage of cells, tissues, and organs. In this study, we investigated the possibility of oxidative stress in the process of myoblast differentiation of C2C12 cells. First, the myoblast differentiation model of C2C12 cells was constructed and verified by Giemsa staining. The expression of hypoxia inducible factor1-alpha (HIF1-α), hypoxia inducible factor1-beta (HIF1-β), Von Hippel-Lindau (VHL), lysyl oxidase (Lox), EGL-9 family hypoxia-inducible factor 1 (EGLN1), proline 4-hydroxylase alpha 1 (P4HA1) and heme oxygenase-1 (HOMX1) in the process of myoblast differentiation was verified by *in vitro* experiments and Gene Expression Omnibus (GEO) bioinformatic analysis. We found that with the increased expression of myogenic factor 5 (MYF5), myogenic differentiation 1 (MYOD1), and Desmin, myotube fusion became more obvious during the process of C2C12 cell differentiation. Both experimental and GEO analysis indicated that the expression of HIF1-α, HIF1-β, VHL, LOX, EGLN1 and P4HA1 increased, and the expression of HOMX1 decreased during myogenic differentiation. Therefore, we suggest that the myoblast differentiation of C2C12 cells may be related to oxidative stress. Their possible relationship was proposed, though further studies are needed.

Keywords C2C12 cells, myoblast differentiation, oxidative stress

1. Introduction

Skeletal muscle differentiates through clonal proliferation of myoblasts, directed differentiation, and mutual fusion into multinucleated myotubules to finally become mature muscle fibers (*1*). Myogenic differentiation is a process regulated by myogenic regulation-transcription factors (MRFs) including MYOD1, myogenin and MYF5 (*2-4*). The early stages of development are dominated by induction of MYF5 and MYOD1 (*5*). MYF5 leads to rapid proliferation of myoblasts (*6*), while the up-regulation of MYOD1 results in stagnation of the cell cycle and transition from proliferation to differentiation.

Oxidative stress (OS) is an imbalance between production and accumulation of oxygen reactive species (ROS) in cells and tissues and the ability of the body to detoxify these reactive products (*7*), and is highly related to the process of homeostasis and the function of skeletal muscle. Active oxygen can not only damage the structure of cells and thus their function, but also affect cell's growth, proliferation, and differentiation (*8*). Redox signal is an important regulator of skeletal muscle protein synthesis and proteolytic cell signaling pathways (*9*). Previous studies have shown that oxidative stress also plays a vital role in the pathogenesis of sarcopenia (*10*). High ROS levels can modify the structure and function of cell proteins and lipids, leading to cell dysfunction, including impaired energy metabolism, altered cell signaling and cell cycle control (*7*). The production of ROS in the sarcoplasmic reticulum physiologically enhances muscle contractility (*11*) and regeneration of skeletal muscle (*12*). Recent studies have shown that ROS is produced by mitochondrial electron transport chain complex I and is an indispensable mediator of muscle differentiation (*13*).

The oxygen concentration in mature skeletal muscle cells is about 1-10%, and physiological hypoxia is the optimal condition for myoblast differentiation. Therefore,

oxidative stress promotes myoblast differentiation, which is very important for the repair of muscle injury. However, how oxidative stress relates to myogenic differentiation and the potential mechanism has never been investigated extensively. It has been reported that HIF1-α, HIF1-β, VHL, Lox, EGLN1, P4HA1 and HOMX1 are associated with tissue myoblast differentiation and oxidative stress. The following experiments were conducted to explore the molecular mechanisms involved.

2. Materials and Methods

2.1. Cell culture and differentiation

C2C12 cells were cultured in high dulbecco's modified eagle medium (Gibco) containing 10% fetal bovine serum (Gibco), 100 IU/mL penicillin and 100 IU/mL streptomycin (Beyotime). Subsequently, the cells were switched to differentiation medium (DM) containing 2% horse serum (Gibco), 100 UI/mL penicillin and 100 μg/mL streptomycin in DMEM for 0, 3, 5 and 7 days of differentiation.

2.2. Giemsa dyeing

The cells were gently washed 3 times with phosphate buffered saline (PBS) before addition of anhydrous methanol solution to fix them (cover the cells) for 15 min. The methanol solution was aspirated and the monolayer of cells were rinsed twice with fresh anhydrous methanol. Before staining, anhydrous ethanol was added to absorb water, and then diluted 10% Giemsa working dye was added. The cells were incubated at 37℃ for 15 min, and washed with PBS.

2.3. Real-time Quantitative PCR

The cells were cultured in a six-well plate. Total RNA was extracted from three replicates per group using Trizol. RNA purity and integrity were evaluated using a NanoDrop-2000 spectrophotometer.

Complementary DNA (cDNA) was generated using a TAKARA kit. The first step is to remove genomic DNA: Random Primer (6 mer) 1μL, dNTP Mix (10 nm) 1μL, template RNA 2000 ng, RNase-free ddH_2O supplemented 10 μL, 65℃ for 5 min, 4℃ for 2 min; Step 2 Reverse transcriptional reaction: 4 μL 5X Primer Script Buffer, 0.5 μL RNase Inhibitor, 1 μL Primer Script Reverse Transcriptase and 4.5 μL RNase-free ddH_2O were added into the reaction products of the first step. The cDNA was synthesized using the following reaction conditions: 30℃ for 10 min; 42 ℃ 60 min; 70 ℃ for 15 min. The product was stored at -80℃.

QPCR was performed using 2X SYBR Green qPCR Mix (SparkJade, Bio, China) on a Lightcycler 480 to confirm the relative levels of expression of genes in the C2C12 cells. The total volume of the PCR reaction was 10 μL, containing 0.5 μL of each primer (10 μM), 1 μL cDNA, 5 μL 2X SYBR Green qPCR Mix, 3 μL RNase-free ddH_2O. PCR cycling conditions were as follows: initial 5 min denaturation at 95℃, followed by 45 cycles of amplification at 95℃ for 10 sec, 60℃ for 10 sec and 72℃ for 15 sec. To quantify the expression of each candidate gene, the mRNA expression levels were normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Relative gene expression was analyzed using a comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$). RT-qPCR was performed in triplicate for each sample and was repeated three times for each assay. Sequences of the forward and reverse primers used are shown in Table 1.

2.4. Western blotting

Protein concentration was determined using the BCA protein concentration assay kit (Biosharp, China) after lysing cells in RIPA Buffer (CWBIO, China) supplemented with 1% PMSF (CWBIO, China). The cells were washed with ice-cold PBS and exposed to RIPA Buffer supplemented with 1% PMSF cocktail solution for 1 h on ice. Insoluble material was removed by centrifugation at 16600g for 25 min at 4˚C. Proteins (50-100 μg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (0.45 μm, Biosharp, China). The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 2 h at room temperature. The blots were then incubated with primary antibody overnight at 4˚C. Antibodies used for western blot analysis were rabbit

Table 1. Q-PCR primer sequences

Primer Name	Primer sequence (5′-3′)
Mus-HIF1-α	F: CATGATGGCTCCCTTTTTCA
	R: GTCACCTGGTTGCTGCAATA
Mus-HIF1-β	F: TGCCTCATCTGGTACTGCTG
	R: TGTCCTGTGGTCTGTCCAGT
Mus-VHL	F: CTGCGTCTGCCCTTTGTAG
	R: TCACCAGGAAGCAAAACTGA
Mus-Lox	F: CAGGCTGCACAATTTCACC
	R: CAAACACCAGGTACGGCTTT
Mus-P4HA1	F: CGTGGGGAGGGTATCAAAAT
	R: ATGGTAGCGGCAGAACAGTC
Mus-EGLN1	F: CGTCTCTCAGTGATTCCAACC
	R: ACTGTTAGGTCGGTCGAAGC
Mus-Desmin	F: GTGAAGATGGCCTTGGATGT
	R: AAGGTCTGGATCGGAAGGTT
Mus-MYOD1	F: AGCACTACAGTGGCGACTCA
	R: GGCCGCTGTAATCCATCAT
Mus-MYF5	F: CTGCTCTGAGCCCACCAG
	R: GACAGGGCTGTTACATTCAGG
Mus-HOMX1	F: AGGGTCAGGTGTCCAGAGAA
	R: GTTCTGCTTGTTGCGCTCTA
Mus-GAPDH	F: CATCCCAGAGCTGAACG
	R: CTGGTCCTCAGTGTAGCC

anti-Desmin (ab32362), rabbit anti-MYOD1 (ab203383), rabbit anti-MYF5 (ab125301), and rabbit anti-GAPDH. The blots were washed three times for 5 min with TBST and then incubated with horseradish peroxidase-labeled secondary antibody for 1 h at 37°C. Goat-anti-rabbit lgG (1:25000; Proteintech, USA) was used as the secondary antibody. After additional washes, the signal was detected using an ECL Chemiluminescence Substrate Kit (Biosharp, China). The protein signals were visualized by exposing the membranes in a Chemiluminescence Gel Imaging System (18200880; Alliance, UK). The level of expression of each protein was normalized to that of GAPDH. The results were quantified using ImageJ-win64 software (Rawak Software Inc., Stuttgart, Germany).

2.5. Gene expression from GEO database

To analyze the expression of oxidative stress related molecules in myogenic differentiation models, *HIF1-α*, *HIF1-β*, *VHL*, *LOX*, *EGLN1*, *P4HA1* and *HOMX1* were screened with GEO profiles database.

2.6. Statistical analysis

The results are presented as mean ± standard error of the mean (SEM). Statistical comparisons were made with a one-way ANOVA and the Tukey multiple comparison test using GraphPad Prism software, version 7.0 (GraphPad Software Inc., San Diego, CA, USA) to identify significant differences. *P*-values < 0.05 were considered statistically significant (*represents $P < 0.05$, **represents $P < 0.01$, ***represents $P < 0.001$, and ****represents $P < 0.0001$). All experiments were performed at least three times.

3. Results

3.1. Construction of myoblast differentiation model of C2C12 cells

We used Giemsa dyeing and expression of myoblast related genes to investigate whether the model of myoblast differentiation was successfully constructed. In the process of differentiation, the number of myotubes increased in the field of vision (Figure 1). As shown by western blotting and qPCR, the expression of MYOD1, MYF5 and Desmin gradually increased over seven days during the process of C2C12 myogenic differentiation (Figure 2). These results indicate that the myoblast differentiation model of C2C12 cells was established successfully.

3.2. Expression of oxidative stress related molecules in myoblast differentiation

The mRNA expression of oxidative stress related molecules at different time periods of myogenic differentiation of C2C12 cells are shown in Figure 3. With myogenic differentiation, the relative expression of *HIF1-α*, *HIF1-β*, *VHL, Lox*, *P4HA1*, and *EGLN1* genes increased. The expression of *HOMX1*, however, showed the opposite trend, with the highest expression seen prior to induction, and the lowest expression on day seven.

Through online analysis in GEO repository, GSE5447 and GSE55034 were selected for data mining analysis. The GSE5447 dataset included 6 samples of gene expression analysis during the 0 h, 6 h, and 24 h process of differentiation and after the addition of deltaNP73α which is an inhibitor in C2C12 cells. The GSE55034 dataset contained gene expression analysis

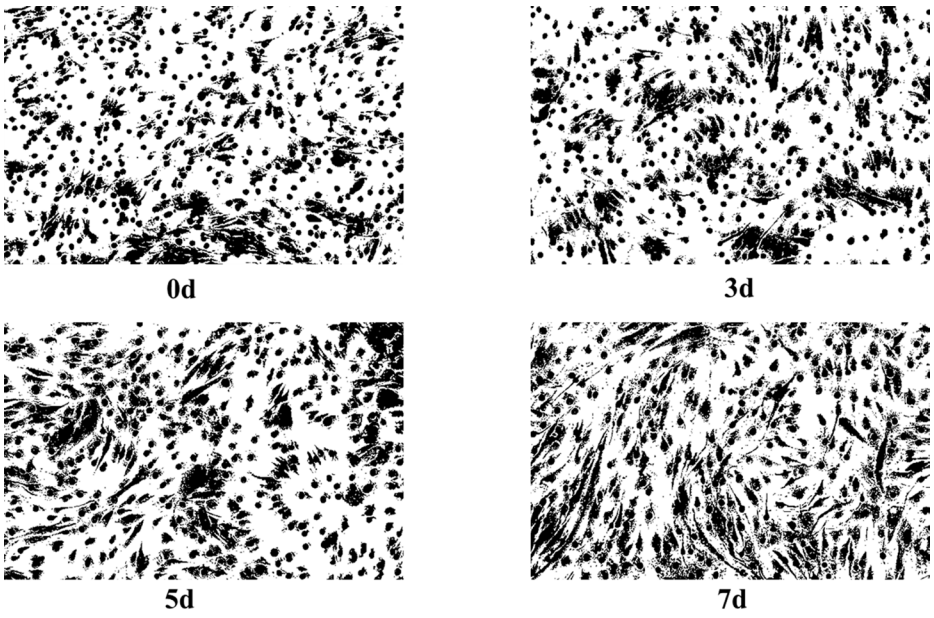

Figure 1. Giemsa staining of C2C12 during myogenic differentiation under the induction of 2% horse serum. (A) before induction. **(B)** induction for 3 days. **(C)** inudction for 5 days. **(D)** induction for 7 days.

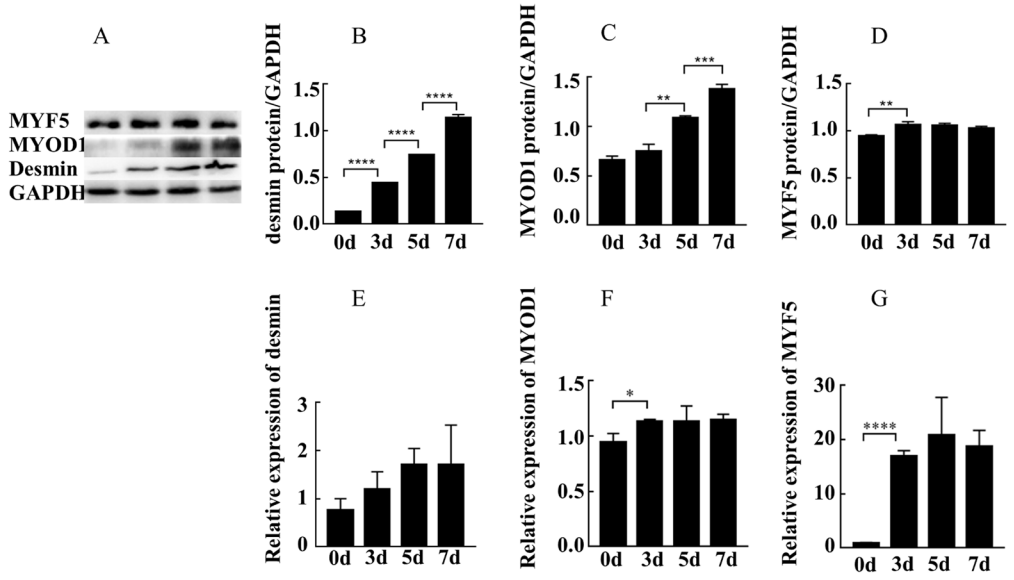

Figure 2. Expression of myogenic molecular markers. (A) The expression of MYF5, MYOD1 and Desmin protein during myogenic differentiation. (B-D) Gray scale analysis of Western Blot results. (E-G) The expression of *MYF5*, *MYOD1*, and *Desmin* mRNA during myogenic differentiation.

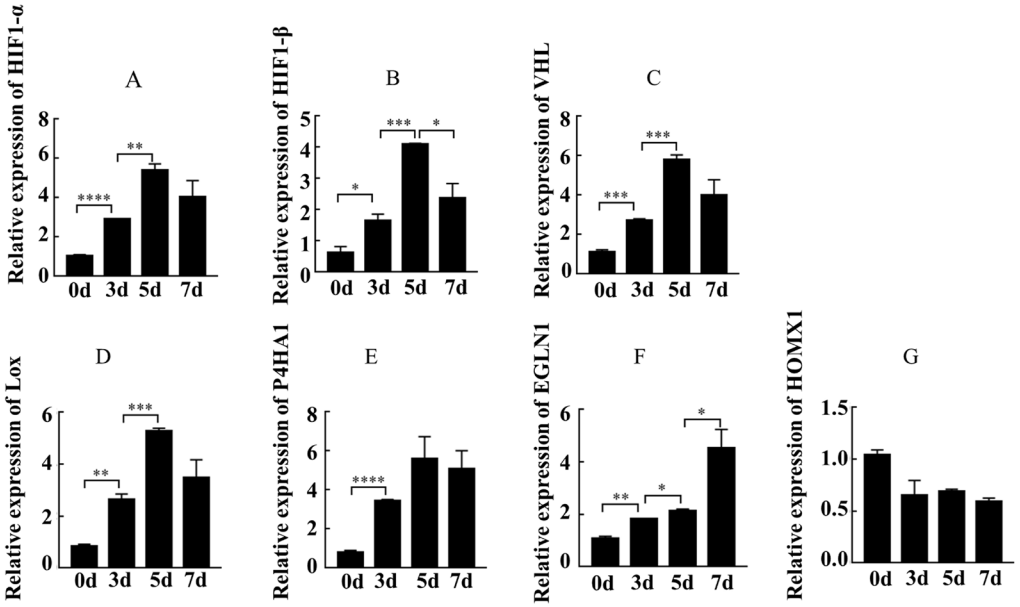

Figure 3. Expression of oxidative stress molecules at the mRNA level during myogenic differentiation. (A) *HIF1-α*, **(B)** *HIF1-β*, **(C)** *VHL*, **(D)** *Lox*, **(E)** *P4HA1*, **(F)** *EGLN1*, **(G)** *HOMX1*.

during myogenic differentiation in human cells with or without myogenic stimuli. *HIF1-α*, *HIF1-β*, *VHL*, *EGLN1*, *Lox*, and *P4HA1* were highly expressed during myogenic differentiation (Figure 4A-4F), and the expression of *HOMX1* decreased with differentiation (Figure 4G), which was consistent with the experimental results.

4. Discussion

Both GEO online analysis and experimental data showed that the expression of *HIF1-α*, *HIF1-β*, *VHL*, *LOX*, *P4HA1*, *EGLN1* genes were up-regulated at the mRNA level, while the expression of *HOMX1* was down-regulated during myogenic differentiation. Based on the above results, we highlight the possible relationship between ROS and myogenic differentiation as follows (Figure 5).

The mammalian *EGLN* family encodes proline hydroxylases, which is involved in the regulation of growth, differentiation and apoptosis of muscle cells, and the expression of EGLN1 is up-regulated in

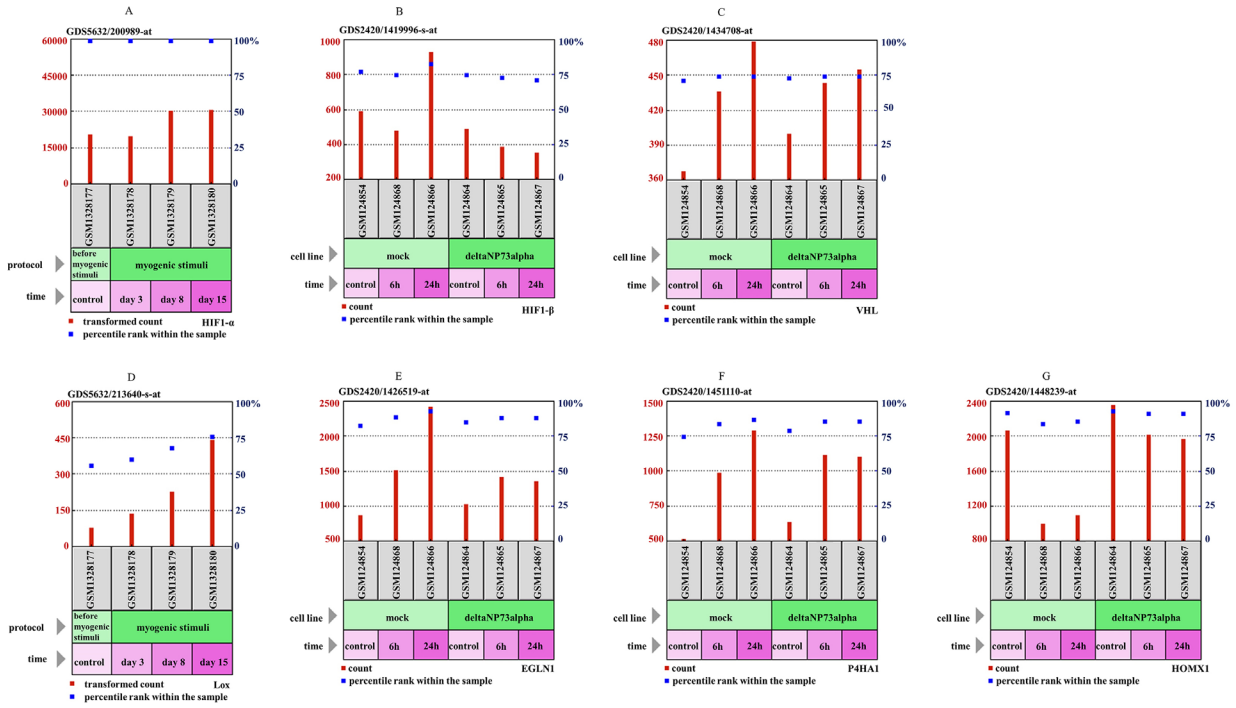

Figure 4. Expression of oxidative stress molecules during myogenic differentiation through bioinformatics prediction. (A) *HIF1-α*, **(B)** *HIF1-β*, **(C)** *VHL*, **(D)** *Lox*, **(E)** *P4HA1*, **(F)** *EGLN1*, **(G)** *HOMX1*.

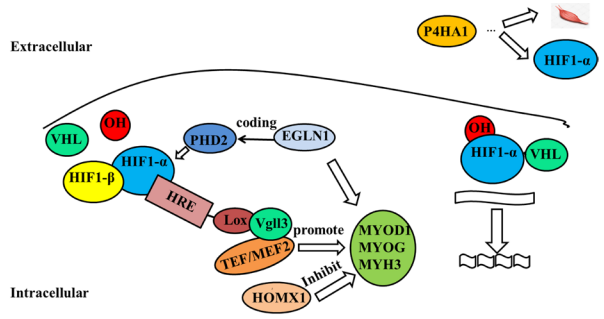

Figure 5. Schematic diagram showing the possible interacting mechanisms between myogenic differentiation and oxidative stress. Under hypoxia, HIF1-PHD2 axis promotes myogenic differentiation through HRE, leading to the expression of MYOD1, MYOG and MYH3. HOMX1 Inhibits myogenic differentiation.

induced vascular smooth muscle cells. The *EGLN1* gene mutation is associated with erythrocytosis and high-altitude hypoxia adaptation (*14,15*). Under hypoxia, the prolyl-4-hydroxylase2 (PHD2) protein encoded by the *EGLN1* gene inhibits hydroxylation of the proline of HIF1-α, HIF-α and β subunits preventing formation of a complete HIF dimer to initiate transcription, thereby increasing downstream target gene expression (*16*). Then HIF1-α binds to the hypoxia response element (HRE) of Lox, promoting the transcription and expression of Lox. Lox combines with vestigial-like family member 3 (VGLL3) co-activator and this conjugate binds with transcriptional enhancer factor/myocyte enhancer factor-2 (TEF/MEF2) to promote the subsequent expression of MYOD1, myogenin (MYOG), myosin heavy chain 3 (MYH3) genes, thereby stimulating differentiation (*17*). HMOX1, a cell-protective enzyme, is induced in response to oxidative stress, during which it protects tissues and mitigates damage (*18*). Studies have found that the rate-limiting enzyme HMOX1 in the heme degradation pathway effectively inhibits the differentiation of myoblasts by targeting Myomirs and the inhibition of c/EBPδ (*19*), through inhibiting the expression of the primary regulator MYOD1 (*5*). As a tumor suppressor, VHL hydrolyzes proteins through the ubiquitin-proteasome pathway in mammals. VHL can interacts with myogenin (*20*) or HIF1-α (*21*) to degrade it, but VHL down-regulates the expression of myogenin protein in a concentration-dependent manner (*22*). Additionally, P4HA1 is necessary for collagen synthesis and deposition (*23*). Mutation of the *P4HA1* gene causes a congenital connective tissue disease associated with tendon and muscle damage (*24*). In addition, biopsy of a 2-year-old patient with a P4HA1 gene mutation showed muscle fiber atrophy and decreased collagen immune response in the muscle basement membrane (*24*). Through unbiased gene co-expression analysis, the HIF-1 pathway was identified as a potential downstream target of *P4HA1* (*25*). So P4HA1 affects myoblast differentiation and oxidative stress, but the specific molecular mechanism is still unclear.

Therefore, we concluded that the myoblast differentiation of C2C12 cells may be related to oxidative stress and more studies are required to better understand the specific molecular mechanisms between them.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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