FHL2 Regulates Proliferation Differentiation and Autophagy of Bovine Skeletal Muscle Satellite Cells Through Wnt/ β -catenin Signaling Pathway

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Abstract

The mechanism of physiological regulation of bovine skeletal muscle development is a complex process, and FHL2 has been studied in association with β -catenin activity and has previously been reported to play a role in skeletal muscle. However the mechanism of FHL2 action in regulating skeletal muscle development in bovine skeletal myosatellite is unclear. Here, we report that FHL2 can both promote the proliferation and differentiation of bovine myosatellite cells through the wnt signaling pathway and bovine skeletal muscle satellite cells through cellular autophagy. The results of western blotting, rt-qPCR, cell transfection assay showed that FHL2 gene expression was enhanced during the proliferation of skeletal muscle satellite cells, and FHL2 knockdown inhibited the proliferation and differentiation of bovine satellite cells and promoted the atrophy of myotubes. Furthermore, immunoprecipitation assays yielded that FHL2 knockdown decreased β -catenin activity in BMSCs and activated β -catenin-mediated wnt signaling pathway in combination with DVL-2, and that FHL2 knockdown induced autophagy in bovine satellite cells. Therefore, the FHL2 gene is a key gene in the regulation of bovine satellite cells.

Introduction

Skeletal muscle growth is mainly due to the differentiation of myosatellite cells, a class of myogenic stem cells with the ability to differentiate. Skeletal muscle cells differentiate directionally into myogenic cells, which proliferate and differentiate and fuse to form myotubes and eventually form mature muscle fibers (Glass 2003; Vinciguerra et al. 2010; Massie et al. 1996). The process of skeletal muscle growth is influenced by myogenic regulatory factors (MRFs) (MyoD, Myf5, MyHC), myocyte enhancer factor 2 (MEF2), Wnt signaling pathway, Shh, BMP and Notch pathway, as well as growth factors TGF- β , FGFs and IGFs, etc (Moncaut et al. 2013).

The wnt signaling pathway was involved in various biological processes, including the proliferation and differentiation of skeletal muscle satellite cells (Joiner et al. 2013). In the classical wnt signaling pathway, wnt ligands can form receptor complexes. When the receptor complexes were absent, β -catenin protein levels were reduced for ubiquitinated protein hydrolysis due to the GSK3-dependent phosphorylation of residues near the N-terminus (Rudnicki and Williams 2015). GSK3 has many putative substrates and its activity is regulated by multiprotein complexes, including APC and Axin, Axin interacts with DVL, which received upstream signals in the cytoplasm and stabilizes β -catenin by inhibiting the formation of Axin and GSK3 β . Wnt was involved in the expression of MRFs, and Wnt1 activates Myf5 through the wnt classical signaling pathway and affects skeletal muscle development (Maltzahn et al. 2012; Brunelli et al. 2007; Chen et al. 2005; Ikeya and Takada 1999). The Wnt signaling pathway mainly regulated the differentiation of skeletal muscle satellite cells, and previous studies have demonstrated demonstrate the importance of the Wnt signaling pathway in the proliferation and differentiation of skeletal muscles.

FHL proteins, which structurally comprise four LIM structural domains and an n-short half-LIM domain, can interact with a variety of proteins to regulate cell proliferation and differentiation, The FHL protein family regulated cell proliferation and tumorigenesis and was preferentially found in cardiac and skeletal

muscles and was expressed in the gonads, prostate, adrenal glands and intestines (Fimia et al. 2000; Labalette et al. 2008). FHL2 has been detected in skeletal muscles, and via interactions with the β -catenin protein, it enhance muscle formation by mediating the Wnt signaling pathway (Hamidouche et al. 2008). This suggests that FHL2 plays a role in the regulation of skeletal muscle development and that FHL2 may potentially to play a role in the Wnt/ β -catenin-mediated skeletal muscle cell development.

Methods

Animal procedures

This animals experiment was approved by the Animal Ethical and Welfare Committee (AEWC), NO. IACUC-NXU1013. Ningxia university –school of agriculture. And all methods were carried out in accordance with Ningxia university experiments guidelines and regulations. The study was carried out in compliance with ARRIVE guidelines, all methods were allowed by Ningxia university-school of agriculture.

Sample collected

The tissue samples were collected from Ningxia Xuanheyuan Agriculture and Animal Husbandry Co. Ltd, the muscle tissue was collected from the hind leg muscle (2-3cm) of cows slaughtered after electric shock, stored immediately in PBS 30ml supplemented with 10% diclonal antibody and 5% diphenycin, and transported back to the laboratory at 37 °C.

Cell culture

BMSCs were maintained in medium containing of DMEM (Sigma, St. Louis, MO, USA), 10% Fetal Bovine Serum (FBS) (Sigma), and 1% Antibiotic-Antimycotic (ABAM) (Sigma) under the condition of 37°C, 5% CO₂. Then, we changed growth medium to differentiation medium when the cells reached to 90% confluence with DMEM, 2% horse serum (Gibco) and 1% ABAM contained.

FHL2 knockdown

In order to silence FHL2, we structure pcDNA3.1 vector (containing a FHL2 short hairpin RNA (shRNA) cassette) first, then we verified the transfection efficiency of pcDNA3.1 vector, when the confluence of inoculated cells reached 70%-90%. referring to the lipofectamine 3000 (Invitrogen) transfection kit method. The negative control was transfected with sh, and the experimental group was transfected with sh-FHL2.

RNA isolation and real-time quantitative PCR (qRT-PCR)

Total RNA was isolated by TRIzol reagent (Invitrogen, USA), and used approximately 2 μ g RNA was reverse transcribed to cDNA by the Takara PrimeScript RT reagent kit (Dalian, Liaoning, China). Amplification was performed according to the SYBR® Premix Ex Taq (Takara, Japan) protocol using a CFX96™ Real-Time PCR Detection System (Bio-Rad). The qRT-PCR data were normalized relative to the

expression of β -actin (endogenous control) and the $2^{-\Delta\Delta C_t}$ method was applied to quantify mRNA expression level. Details of primers are summarized in Table 1.

Western blotting (WB)

Following medium removal, BMSCs were washed with phosphate-buffered saline then lysed with RIPA buffer (Solarbio, Beijing, China). Thirty micrograms of protein per lane was separated by 5% SDS-PAGE and then transferred to polyvinylidene fluoride. Subsequent to blocking with 5% non fat milk for 1 h at room temperature, the membranes were incubated by primary antibodies overnight on a rocker at 4 °C. After washing with TBST for three times, secondary antibody was diluted 3000 times with TBST and incubated at room temperature for 30min for development and fixing. Then the optical density of the target band was analyzed with Alpha software processing system. Western blotting was performed using ECL Prime detection reagent.

All antibodies are as follows: mouse anti-MyHC (Sigma, 1:2000), mouse anti-MyoG (Santa Cruz Biotechnology, CA, USA, 1:2000), rabbit anti-FHL2 (Santa Cruz, 1:1000), rabbit anti-Active- β -catenin (Cell Signaling Technology, Danvers, MA, USA, 1:1500), rabbit anti-Dvl-2 (Abcam, Cambridge, UK, 1:1000), rabbit anti-Axin-1 (Cell Signaling Technology, 1:1000), rabbit anti-GSK3 β (Abcam, 1:1000), rabbit anti-Atrogin-1 (Novus Biological, Abingdon, UK), rabbit anti-Histone H3 (Cell Signaling Technology, 1:2000), rabbit anti- β -actin (Sigma, 1:5000) and mouse anti- β -actin (Sigma, 1:5000).

Immunoprecipitation (IP)

For IP analysis, BMSCs were harvested with IP lysis buffer and total cell lysate containing proteins and were immunoprecipitated with DVL-2 or FHL2 antibodies. The immune complex was washed with IP lysis buffer (Beyotime, Shanghai, China) at least three times. The sample was boiled for 5 minutes, whose supernatant was electrophoresis, and transferred to a polyvinylidene fluoride membrane for WB analysis.

Cell Counting Kit-8 (CCK-8) assay

We quantified living cells at 0, 12, 24 and 36 hours after culturing with a non-radioactive CellTiter 96 assay kit (BestBio, Shanghai, China). Absorbance at 450 nm reflected the amount of living cells. Cells were seeded at a density of 5×10^3 cells/well in a 96-well plate, and incubated with 10 μ l CCK-8 for additional 3h at 37 °C; the absorbance at 450 nm was measured using a Microplate Reader. The samples from each treatment at each point had ten replicates. The cell proliferation rate is expressed as the slope connecting absorbance at various time periods of culture.

5-Ethynyl-20-deoxyuridine (EdU) assay

Cell proliferation of transfected BMSCs was also assessed with an EdU assay kit (RiboBio, Guangzhou, China). After the 48-hour shRNA transfection, BMSCs were grown in 96-well plates, exposed to 5nM EdU for 2 hours, and then fixed with 4% paraformaldehyde; the proliferating BMSCs were stained with Apollo

staining solution, and nucleic acids were stained with Hoechst. Five fields were randomly selected and EdU-stained cells were observed using a Leica DMI8 fluorescence microscope.

Immunofluorescent staining

Myoblasts were cultured in 12-well plates and fixed in 4% formaldehyde for 30 min after transfection for 48 h. Cells were washed with PBS three times and permeabilized with 0.5% Triton X-100 (Sigma) for 10 min. Next, we used 1% Bovine Serum Albumin (BSA) in PBST (PBS + 0.05% tween-20) to block cells and incubated with mouse anti-myhc (Sigma, 1:2000) 1:20, at 4°C overnight. The cells were then incubated with fluorescent secondary antibody in the dark for 90 min. The cell nuclei were visualized by DAPI staining (Sigma). Image-pro plus® Media Cybernetics. Inc., Rockville, MD, USA was used for immunofluorescence cumulative optical density (IOD) analysis.

Statistical analysis

All experiments were repeated 3-5X. Statistically differences were calculated with an unpaired two-tailed Student's t test. P value of < 0.05 was considered significant. Data are expressed as means ± standard error of the mean (SEM).

Results

The expression of FHL2 increased during BMSCs development

To determine the function of FHL2 in BMSCs, firstly, we studied the expression pattern of FHL2 in the proliferation and differentiation of BMSCs. With the time of BMSCs proliferation and differentiation, the expression level of FHL2 gene (Fig 1a) and protein expression level of FHL2 (Fig 1b) gradually increased. At the same time, the protein expression level of myosin heavy chain (MyHC) was also detected during BMSCs differentiation, The level of FHL2 and MyHC protein expression increased in the same trend (Fig. 1b). The results indicate that FHL2 may play an important role in the proliferation and differentiation of bovine satellite cells.

FHL2 knockdown inhibited BMSCs proliferation

In order to explore the effect of FHL2 during proliferation of BMSCs, shRNA was used to knockdown the expression of FHL2 in BMSCs. The expression levels of FHL2 mRNA (Fig 2a) and protein (Fig 2b) were significantly decreased after sh-FHL2 transfection. Next, we analyzed the expression of proliferation-related genes (CyclinD1, CDK2 and PCNA) to determine the proliferation of BMSCs after transfection sh-FHL2. The mRNA expression of proliferation-related genes CyclinD1, CDK2 and PCNA were significantly decreased after sh-FHL2 transfection (Fig 2d). Finally, The CCK8 induced sh-FHL2 significantly inhibited the proliferation of BMSCs significantly at 24h and 36h after transfection (Fig 2c, 2e). These results suggest that FHL2 knockdown inhibit the proliferation of BMSCs.

FHL2 knockdown inhibited BMSCs differentiation

We quantified myogenic markers in FHL2 knockdown cells to reveal the effect of FHL2 on the differentiation of bovine muscle satellite cells into myotubes. As indicated in Fig. 3a, differentiated cells incorporated with sh-FHL2 showed significantly decreased expression of encoding myogenin (MyoG), myosin heavy chain 3 (Myh3), myosin heavy chain 7 (Myh7) and myoglobin (Mb). Both direct microscopy and immunofluorescence showed that myotube formation was significantly reduced in BMSCs of FHL2 knockdown (Fig 3b, Fig. 3c). In addition, BMSCs transfected with sh-FHL2 had lower protein expression of MyoG and MyHC than control-group (Fig. 3d), further supporting the conclusion that FHL2 knockdown inhibits myotube formation.

FHL2 knockdown increases atrophy of BMSCs

Dexamethasone can cause myotubes atrophy, and Muscle Atrophy F-box (Atrogin-1) gene promotes atrophy of myotubes. We verified the expression of FHL2 after muscle atrophy was induced by Dexamethasone for 24h using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and WB. The results demonstrated that FHL2 mRNA and protein were expressed at significantly higher levels after treatment with dexamethasone (Fig 4a and 4b). In addition, sh-FHL2 in myotubes promoted expression levels of Atrogin-1 and Cathepsin L, and protein levels of Atrogin-1 (Fig 4c and 4d). Furthermore, WB analysis showed that atrogin-1 was increased after sh-FHL2 and dexamethasone treatment (Fig 4d). The results indicate that FHL2 knockdown may contribute to muscular atrophy.

FHL2 knockdown disrupted the DVL-2-mediated Wnt signaling pathway in BMSCs

To further investigate the mechanism of action of FHL2 in regulating cell proliferation, differentiation and autophagy, we performed transcriptome sequencing after interfering with FHL2 expression. Transcriptome sequencing identified a number of genes associated with muscle development (Fig 5a). KEGG was enriched in 15 pathways, most significantly in Glycine serine and Threonine metabolic pathway (Fig 5b). The Wnt/ β -catenin signaling pathway is involved in regulating cell proliferation and differentiation. The qRT-PCR result illustrated that mRNA expression levels of c-Myc, wnt5a, wnt10b and *lef1*—Wnt signaling pathway related factors—decreased dramatically in BMSCs (Fig 5c). The expression of FHL2 shRNA was lower than control shRNA through TOPflash/FOPflash ratio (Fig 5d). The results showed that FHL2 may regulate the proliferation and differentiation of BMSCs by Wnt signaling pathway. We found that the protein expression levels of DVL-2 and β -catenin decreased significantly in the sh-FHL2 group (Fig 5e and 5f). Axin1 is a negative regulator of the Wnt signaling pathway, Axin1 was degraded when treated with Wnt3a (Wnt pathway activator), whereas sh-FHL2 almost eliminated the degradation of Axin1 (Fig 5g). Furthermore, Wnt3a treatment reduced the ability of β -catenin to enter the nucleus by sh-FHL2 (Fig 5h). We know that the inhibitor 1-azakenpaullone (1-AKP) can mediate the signaling pathway activated by DVL-2 (Kunick et al. 2004). This study revealed that MyHC⁺ has been activated by 1-AKP in sh-FHL2 group and control group (Fig 5i). Added 1-AKP significantly inhibited the expression of Axin1 and GSK3 β (Fig 5j). We confirmed that 1-AKP activated Wnt signaling pathway. Then we treated BMSCs with 1-AKP, the protein expression of Axin1 and GSK3 β were significantly reduced in BMSCs after sh-FHL2 transfection (Fig 5k). In order to further explore the relationship between FHL2 and DVL-2. The

immunoprecipitate sample precipitated with the Dvl2 antibody in BMSCs contained FHL2 (Fig 5l). Our results demonstrated that FHL2 can combine DVL-2 to enhanced β -catenin expression and possibly exerted its efficacy via Wnt/ β -catenin signaling pathway.

FHL2 knockdown induces autophagy in BMSCs

Previous studies have shown that autophagy can negatively control Wnt signalling pathway. Therefore, we speculate that the combination of FHL2 and DVL2 to regulate the wnt signaling pathway may be involved in the regulation of cell autophagy. Therefore, this study examined the mRNA expression levels (Beclin1, Beclin2 and p62) of autophagy-related. Western blotting analysis showed that beclin1 and beclin2 levels gradually increased after FHL2 silencing, and the expression level of P62 also increased significantly (Figure 6a). Western blotting results also confirmed that sh-FHL2 promoted the expression of Beclin1 protein and reduced the expression of P62 protein. The changes of LC3 protein levels to detect autophagy induction revealed an increase in LC3 expression in FHL2 knockdown BMCSs compared to controls (Figure 6b). We transfected BMSCs with tandem mRFP-GFP-LC3 plasmid as reporter of autophagic flux(Klionsky and Al 2016). Autophagosomes that have not fused with lysosomes appear as yellow (mRFP and GFP) puncta, whereas autolysosomes appeared as red (mRFP) puncta, Compared with control cells, there was a significant increase in the number of autolysosomes in FHL2 knockdown(Figure 6c and 6d). FHL2 knockdown induced the autophagic flux in BMSCs.

Table 1. Primer information for quantitative real-time PCR analysis

Genes	Forward Primer (5'-3')	Reverse Primer (5'-3')
<i>FHL2</i>	CTCTGCGCTTCTCAGCGATA	GGCAGGAAGTTACACCGGAA
<i>MyH3</i>	CTGGAGGAAATGAGGGATGA	CACTCTTGAGAAGGGGCTTG
<i>MyoG</i>	TGGGCGTGTAAGGTGTGTAA	TATGGGAGCTGCATTCACTG
<i>MyH7</i>	CTTCAACCACCACATGTTCG	GCTTCTGGAAGTTGCTGGAC
<i>Cathepsin L</i>	TGCTTCGATTCTCCATCCG	CGTGGCACTAAAAGCCCAAC
<i>Atrogin-1</i>	CTCCAGGAAACAAAGACCCGA	GGTCTGCCCGGGGGTTTTAT
<i>Wnt5a</i>	CAACTGGCAGGACTTTCTCAA	CATCTCCGATGCCGGAAC
<i>Wnt10b</i>	CTCTGCCACAGCCAACTCT	ATCGAACTTGCCTGGCTTGA
<i>c-Myc</i>	GTAATTCCAGCGAGAGGCAGA	CTAGGCTAGCTCGGCTCTTC
<i>Lef1</i>	CCCTGTGTTGTTCCGGCCTC	ATTGGAAGGATGCGTCAGGG
<i>CyclinD1</i>	ATGAAGGAGACCATCCCCCT	CGCCAGGTTCCACTTGAGTT
<i>CDK2</i>	AGGGAACGTACGGAGTTGTG	GACATCCAGCAGCTTGACAAT
<i>PCNA</i>	TCCAGAACAAGAGTATAGC	TACAACAGCATCTCCAAT
<i>β-actin</i>	CATCCTGACCCTCAAGTA	CTCGTTGTAGAAGGTGTG

Discussion

The role of the FHL2 gene in the regulation of ovarian function has been extensively studied, in skeletal muscle which was a class of myogenic progenitor cells (also called satellite cells) with significant regenerative capacity. Moreover, the mechanisms of FHL2 have been incompletely reported. The main function of FHL2 is to regulate cell proliferation and development. However, the mechanism by which FHL2 regulates the proliferation and differentiation of bovine skeletal muscle satellite cells remained unknown. There is evidence that FHL2, which was highly expressed in skeletal and cardiac muscle, is a muscle-specific inhibitor of LEF/TCF target genes and it played a different regulatory role in myogenesis by binding to β -catenin to stimulate myogenic cell differentiation (Martin and B. 2002). In addition, FHL2 can induce IL-6 activation in skeletal muscle cells and promote myogenesis (Wong et al. 2012). We therefore propose that FHL2 can regulate the proliferation and differentiation of skeletal muscle cells.

FHL2 was localized in the nucleus and cytoplasm, and it regulated myoblast proliferation and differentiation (Mourabit et al. 2004). In the nucleus, FHL2 promoted myogenic differentiation (Wei et al. 2005). To clarify the role of FHL2 in BMSCs, our study showed that FHL2 knockdown not only resulted in inhibition of proliferation of BMSCs, but also it inhibited myotube formation, and these data suggesting that FHL2 regulates proliferation, differentiation and myotube atrophy in BMSCs. The regulatory effects of FHL2 on skeletal muscle satellite cells were similar to those found in previous studies.

Previous studies have shown that FHL2 interacts with cytoplasmic and transcription factors such as, Runx2, β -catenin, Foxo, Hand1, etc.([Johannessen et al. 2006](#)). These interactions enabled FHL2 to regulate intracellular signaling pathways, such as Wnt signaling pathway, NF- κ B pathway, as a transcriptional co-activator or repressor regulatory protein.([Tran et al. 2016](#)). We propose that FHL2 interacts with DVL-2 as a mediator protein to regulate the proliferation and differentiation of bovine skeletal muscle cells through the Wnt signaling pathway. In our study, we detected an interaction between FHL2 and DVL-2. FHL2 silencing repressed the Wnt/ β -catenin pathway, including the Wnt pathway target genes, namely, c-Myc, Wnt5a, Wnt10b, and LEF1, and reduced the transcriptional activity of DVL-2 and β -catenin. These results suggested that FHL2 might affect proliferation and differentiation of bovine skeletal muscle through the Wnt/ β -catenin signaling pathway. FHL2 interacted with DVL-2 in the Wnt signaling pathway to regulate skeletal muscle development ([T. et al. 2011](#)), and the GSK3 β inhibitor 1-AKP affects the Wnt signaling pathway through DVL-2 ([Kunick et al. 2004](#)), and we found that the expression of Axin1 and GSK3 β was significantly decreased when FHL2 knockdown cells were treated with 1-AKP. This suggested that the DVL-2 played a role in the regulation of the FHL2-mediated Wnt signaling pathway. Our findings further support the hypothesis that FHL2 regulates skeletal muscle development by binding to the key mediators of the Wnt/ β -catenin pathway. Overall these studies improved our understanding of the FHL2 protein.

Cellular autophagy, which plays a role in various physiological events such as cell proliferation, cell differentiation, proliferation, death and growth, is also involved in the repair of damaged skeletal muscle and the reversal of muscle atrophy([Call and Nichenko 2020](#); [Rienzo et al. 2019](#)). Meanwhile, autophagy may play an important role in the maintenance and integrity of muscle mass. Previous studies have used western blotting to examine the extent of cellular autophagy by detecting the accumulation of LC3 protein during muscle cell development([Sandri 2010](#); [Fortini et al. 2016](#)). To investigate the relationship between FHL2 and autophagy, in our study, the expression of essential autophagy genes such as the regulatory proteins Beclin1, Beclin2 and p62([Zhu et al. 2018](#)), was measured after FHL2 knockdown. FHL2 knockdown promoted the expression of LC3 and Beclin1/2 and suppressed the expression of p62, showing that FHL2 might be involved in autophagy induction and plays an integral role in the system. In addition, by constructing the mRFP-GFP-LC3 dual fluorescent autophagy indicator system for labeling and tracking LC3 as well as changes in autophagic flow, this study showed that FHL2 knockdown increased the number of autophagosomes, thus indicating that the knockdown of FHL2 promotes cellular autophagy, FHL2 knockdown maintain cellular homeostasis and play a role in myocytogenesis and myotube formation, thus promoting cellular autophagy and activating cellular autophagy([Zihao Liu et al. 2019](#)). Thus, our study suggests that FHL2 can regulate cellular autophagy.

Therefore, our study found that FHL2 was involved in the proliferation and differentiation of skeletal muscle satellite cells and myotube formation. Additionally, FHL2 interacted with DVL-2 to enhance β -catenin expression and regulated the Wnt/ β -catenin signaling pathway. Moreover, FHL2 knockdown induced an autophagic flux in BMSCs, thus regulating cellular autophagy. Thus, FHL2 was a potential key gene in the regulation of bovine satellite cells.

One of the limitations of our study is that we only assessed the role of FHL2 in the proliferation and differentiation of bovine skeletal muscle satellite cells and in myotube formation. However, environment and a combination of other genes may also influence the development of skeletal muscle tissue, which we were not investigated in our study. Secondly, due to the complexity of the FHL2-associated pathways, we did not further validate other pathways and membrane-signaling factors in the Wnt signaling pathway. Lastly, we only relied on bovine cell cultures to validate this gene, in our study. To further confirm the role of FHL2 in skeletal muscles, we would need to knockout the FHL2 gene in various animal models in future research.

Conclusions

In conclusion, our study shows that FHL2 is essential to bovine satellite cell proliferation and differentiation through its interaction with DVL-2 protein. Our study has shown that FHL2 modulates a variety of pathologies of cardiac and skeletal muscle, therefore understanding the molecular regulatory mechanisms of FHL2 is of great significance for molecular breeding and the treatment of muscle diseases

Declarations

Author Contributions:Yun Zhu and Peng Li conceived and designed the study. Yun Zhu generated the experimental data. Yun Zhu analysed the data with assistance from Peng Li. Yun Zhu wrote the paper with input from Xiaolong Kang.Xingang Dan.Yun Ma and Yuangang Shi.

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Conflicts of Interest: The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

References

Brunelli S, Relaix F, Baesso S, Buckingham M, Cossu G (2007) Beta catenin-independent activation of MyoD in presomitic mesoderm requires PKC and depends on Pax3 transcriptional activity. *Developmental Biology* 304 (2):604-614

Call JA, Nichenko AS (2020) Autophagy: an essential but limited cellular process for timely skeletal muscle recovery from injury. *Autophagy*

- Chen AE, Ginty DD, Fan CM (2005) Protein kinase A signalling via CREB controls myogenesis induced by Wnt proteins. *Nature* 433 (7023):317-322
- Fimia GM, Cesare DD, Sassone-Corsi P (2000) A Family of LIM-Only Transcriptional Coactivators: Tissue-Specific Expression and Selective Activation of CREB and CREM. *Molecular and Cellular Biology* 20 (22)
- Fortini P, Ferretti C, Iorio E, Cagnin M, Garribba L, Pietraforte D, Falchi M, Pascucci B, Baccarini S, Morani F (2016) The fine tuning of metabolism, autophagy and differentiation during in vitro myogenesis. *Cell Death & Disease* 7 (3):e2168
- Glass DJ (2003) Molecular mechanisms modulating muscle mass. *Trends in Molecular Medicine* 9 (8):0-350
- Hamidouche Z, Ha E, Vaudin P, Charbord P, Fromigué O (2008) FHL2 mediates dexamethasone-induced mesenchymal cell differentiation into osteoblasts by activating Wnt/beta-catenin signaling-dependent Runx2 expression. *Faseb Journal Official Publication of the Federation of American Societies for Experimental Biology* 22 (11):3813
- Ikeya M, Takada S (1999) Wnt signaling from the dorsal neural tube is required for the formation of the medial dermomyotome. *Development* 125 (24):4969-4976
- Johannessen M, Møller S, Hansen T, Moens U, Ghelue MV (2006) The multifunctional roles of the four-and-a-half-LIM only protein FHL2. *Cellular and Molecular Life Sciences CMLS* 63 (3):268-284
- Joiner DM, Ke J, Zhong Z, Xu HE, Williams BO (2013) LRP5 and LRP6 in development and disease. *Trends in Endocrinology & Metabolism* 24 (1):31-39
- Klionsky DJ, Al ZME (2016) Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *AUTOPHAGY* 1 (12)
- Kunick C, Lauenroth K, Leost M, Meijer L, Lemcke T (2004) 1-Azakenpauillone Is a Selective Inhibitor of Glycogen Synthase Kinase-3?? *ChemInform* 14 (2):413-416
- Labalette C, Nouet Y, Sobczak-Thépot J, Armengol C, Levillayer F, Gendron MC, Renard CA, Regnault B, Chen J, Buendia MA (2008) The LIM-only protein FHL2 regulates cyclin D1 expression and cell proliferation. *Journal of Biological Chemistry* 283
- Maltzahn JV, Chang NC, Bentzinger CF, Rudnicki MA (2012) Wnt signaling in myogenesis. *Trends in Cell Biology* 22 (11)
- Martin, B. (2002) The LIM-only protein FHL2 interacts with β -catenin and promotes differentiation of mouse myoblasts. *The Journal of Biophysical and Biochemical Cytology* 159 (1)

- Massie BM, Simonini A, Sahgal P, Wells L, Dudley GA (1996) Relation of systemic and local muscle exercise capacity to skeletal muscle characteristics in men with congestive heart failure. *Journal of the American College of Cardiology* 27 (1):140
- Moncaut N, Rigby P, Carvajal JJ (2013) Dial M(RF) for myogenesis. *Febs Journal* 280 (17)
- Mourabit HE, Müller S, Tunggal L, Paulsson M, Aumailley M (2004) Analysis of the adaptor function of the LIM domain-containing protein FHL2 using an affinity chromatography approach. *Journal of Cellular Biochemistry* 92 (3):612-625
- Rienzo MD, Antonioli M, Fusco C, Liu Y, Fimia GM (2019) Autophagy induction in atrophic muscle cells requires ULK1 activation by TRIM32 through unanchored K63-linked polyubiquitin chains. *Science Advances* 5 (5):eaau8857
- Rudnicki MA, Williams BO (2015) Wnt Signaling in Bone and Muscle. *Bone* 80:60-66
- Sandri M (2010) Autophagy in skeletal muscle. *FEBS letters* 584 (7):1411-1416
- T., Pulvirenti, M., Van, Der, Heijden, L., A., Droms, J. (2011) Dishevelled 2 Signaling Promotes Self-Renewal and Tumorigenicity in Human Gliomas. *Cancer Research*
- Tran MK, Kurakula K, Koenis DS, Vries CD (2016) Protein–protein interactions of the LIM-only protein FHL2 and functional implication of the interactions relevant in cardiovascular disease. *BBA - Molecular Cell Research* 1863 (2):219-228
- Vinciguerra M, Musaro A, Rosenthal N (2010) Regulation of Muscle Atrophy in Aging and Disease. *Oxygen Transport to Tissue XXXIII* 694:211-233
- Wei Y, Labalette C, Renard CA, Neuveut C, Buendia MA (2005) Study of the role of FHL2 in beta-catenin-associated transcription regulation and oncogenesis. *Cancer Research* 65
- Wong CH, Mak WY, Li MS, Tsui KW (2012) The LIM-only protein FHL2 regulates interleukin-6 expression through p38 MAPK mediated NF-κB pathway in muscle cells. *Cytokine* 59 (2)
- Zhu X, Zhang C, Hospital HC (2018) The Characteristics and Correlation of the Content of Autophagy Labelled Protein Beclin-1 and P62 in Pancreatic Cancer Patients. *The Practical Journal of Cancer*
- Zihao Liu SH, Yan Wang, Can Cui, Qing Zhu, Xiaosong Jiang, Chaowu Yang, Huarui , Du CY, Qingyun Li, Haorong He, Xiaoxu Shen, Yuqi Chen, Yao Zhang, Lin Ye, Zhichao , Zhang DL, Xiaoling Zhao and Huadong Yin (2019) The LIM-Only Protein FHL2 is involved in Autophagy to Regulate the Development of Skeletal Muscle Cell. *International journal of biological sciences*

Figures

Figure 1

The expression of FHL2 during proliferation and differentiation in bovine satellite cells (BMSCs). (a) Quantitative real-time PCR of FHL2 mRNA in bovine satellite cells cultured in GM (growth medium) for 1 and 3 days (G1, G3) or DM (differentiation medium) for 1, 3 and 5 days (D1, D3, D5). (b) Western blotting analysis of FHL2 and MyHC levels in G1, G3, D1, D3 and D5. Data indicates the mean $\pm$ SEM from at least three separate experiments. * $P < 0.05$; ** $P < 0.01$.

Figure 2

Consequence of FHL2 silencing on bovine satellite cell (BMSC) proliferation. Cells were incorporated with FHL2 shRNA or pcDNA3.1 for 48 hours and examined as follows: relative expression levels of FHL2 mRNA (a) and protein (b) as shown by qRT-PCR and western blotting, respectively; (c) CCK-8 assay of the proliferation rate; (d) relative mRNA expression levels of CyclinD1, CDK2 and PCNA; and (e) Representative images of cell staining with Hoechst (left panel), EdU (center panel), merged images of both stains (right panel), and a graph representing the consequence of FHL2 silencing on the proliferation rate of pairs of BMSCs. Data indicates the mean $\pm$ SEM from at least three separate experiments. * $P < 0.05$; ** $P < 0.01$.

Figure 3

Consequence of FHL2 silencing on differentiation of bovine satellite cells (BMSCs). (a) BMSCs were grown in differentiation induction medium for 72 hours and transfected with FHL2 shRNA and pcDNA3.1 for 48 hours, then examined as follows: (a) relative mRNA expression levels of MyoG, MyH3, MyH7 and Mb; (b) representative microscopic images (the red arrow point myotubes); (c) immunofluorescence of MyHC merged image after transfection of FHL2 shRNA and control, and calculated percentage of myotube area; (d) western blotting analysis of MyHC and MyoG expression. Data indicates the mean $\pm$ SEM from at least three separate experiments. * $P < 0.05$; ** $P < 0.01$).

Figure 4

Effect of FHL2 on bovine myotube atrophy. Relative expression of FHL2 mRNA (a) and protein (b) FHL2 levels after 24 hours of treatment with DMSO or dexamethasone in bovine myotubes as shown by qRT-PCR and western blotting. (c) Relative expression levels of Atrogin-1 and Cathepsin L mRNA after transfection with FHL2 shRNA or pcDNA3.1. (d) Western blotting analysis showing Atrogin-1 expression levels with or without 24 hours of dexamethasone treatment following transfection with FHL2 shRNA or pcDNA3.1. Data indicate the mean $\pm$ SEM from at least three separate experiments. * $P < 0.05$; ** $P < 0.01$.

Figure 5

FHL2 knockdown disrupted the DVL-2-mediated Wnt/β-catenin signaling axis. BMSCs were incorporated with FHL2 sh-RNA or pcDNA3.1 and examined as follows: (a) RNA-seq was used to analyze the heat map of changes in the expression levels of selected differential genes in bovine satellite cells of after transfected sh-control and sh-FHL2 plasmids for 3 days. (b) KEGG enriched 16 pathways in sh-FHL2 cells relative to sh-control cells. (c) relative expression levels of c-Myc, wnt5a, wnt10b and lef1, as shown by

qRT-PCR; (d) luciferase activity of the TOP/FOP reporters; western blotting analysis of (e) active nuclear β -catenin protein levels, (f) DVL-2 protein levels, (g) Axin1 protein levels in 24 hours Wnt3a- or saline-treated BMSCs, (h) Levels of active nuclear β -catenin 24 h after treatment with Wnt3A or normal saline, (i) Immunofluorescence staining for MyHC in control and sh-FHL2 cells exposed to 1-AKP or DMSO after 2 d in differentiation medium. (j) Axin1 and GSK3 β protein levels in control cells treated with 1-AKP or DMSO. (k) Axin1 and GSK3 β protein levels in FHL2-silenced cells treated with 1-AKP or DMSO. (l) reciprocal co-immunoprecipitation (IP) analysis of FHL2 and DVL-2. Data indicates the mean $\pm$ SEM from at least three separate experiments. *P < 0.05; **P < 0.01.

Figure 6

FHL2 knockdown activated autophagy in bovine satellite cells. (a) qRT-PCR analysis the mRNA levels of Beclin1, Beclin2 and P62 in sh-control and sh-FHL2 cells. (b) western blotting analysis the protein levels of Belcin1 and P62 levels in sh-control and sh-FHL2 cells. (c) The autophagosomes of FHL2 knockdown and control cells were observed under a confocal microscope. (d) The histogram shows the number of autophagosomes. Data indicates the mean $\pm$ SEM from at least three separate experiments. *P < 0.05; **P < 0.01.