# Original Article SIRT1 regulates C2C12 myoblast cell proliferation by activating Wnt signaling pathway

Liang Wang<sup>1</sup>, Ruilin Xue<sup>1</sup>, Chengcao Sun<sup>1</sup>, Cuili Yang<sup>1</sup>, Yongyong Xi<sup>1</sup>, Feng Zhang<sup>1</sup>, Qiqiang He<sup>1</sup>, Suqing Wang<sup>1</sup>, Fang Zhao<sup>2</sup>, Yadong Zhang<sup>3</sup>, Dejia Li<sup>1</sup>

<sup>1</sup>Department of Occupational and Environmental Health, Wuhan University, Wuhan, China; <sup>2</sup>Zhongnan Hospital of Wuhan University, Wuhan, China; <sup>3</sup>The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

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Abstract: Sirtuin type 1 (SIRT1) is a potent NAD<sup>+</sup> dependent deacetylase that deacetylates histone and nonhistone proteins to regulate gene expression and protein activity. Emerging evidences have indicated that SIRT1 plays a significant role in diverse cellular processes including cell growth, differentiation, development, and physiological function in muscle cells; however the signaling mechanisms involved remain to be established. In order to investigate its potential role in muscle biological processes, we administrated C2C12 cells which were isolated from skeletal muscle tissue of dystrophic mice with SIRT1 activator resveratrol (REV), inhibitor nicotinamide (NAM) and Wnt inhibitor FH535. By CCK-8, BrdU assay, real-time PCR and Western blot, we investigated whether the SIRT1 has a function in C2C12 cells by promoting  $\beta$ -catenin accumulation. Our results demonstrate that SIRT1 increases cell proliferation of C2C12 myoblast in a SIRT1-dependent manner. And SIRT1 significantly up-regulates the expression of cyclin D1, C-myc and Dvl2 in vitro as well as stimulates the accumulation of the Wnt/ $\beta$ -catenin. In conclusion, this study indicates that SIRT1 promotes the proliferation of C2C12 myoblast cells, at least partly via Wnt signaling pathway.

Keywords: SIRT1, Wnt, C2C12 myoblast cells, proliferation, muscle regeneration

#### Introduction

SIRT1, a well-known closest homologue of Sir2 (silent mating type information regulation 2) [1], is an NAD<sup>+</sup>-dependent histone/protein deacetylase to regulate longevity mediated caloric restriction in model mammalian organisms [2]. SIRT1 is also an important regulatory factor of cell defense [3] and participates in a very broad and complex array of cellular processes such as cell cycle, inflammation; energy metabolism and DNA repair [4]. REV, which is a naturally occurring polyphenol found in grapes and red wine [5], has been found to significantly activate SIRT1. Interestingly, SIRT1 activation can significantly improve muscular pathology in dystrophic mdx mice [6]. And it is reported that NAM is a potent inhibitor of SIRT1 and it can effectively inhibit SIRT1 activity both in vivo and in vitro [7, 8].

Accumulating evidences suggest that SIRT1 may promote the cell proliferation, and activa-

tion of SIRT1 by induced growth of muscle mass of mdx mice [9]. For example, SIRT1 has been proven to regulate neurite outgrowth and cell regeneration by repression of the mTOR Signaling [10]. Moreover, overexpression of SIRT1 can promote muscle precursor cell proliferation and cell cycle progression [11]. Associated with the SIRT1-mediated proliferation of C2C12 cells were the bidirectional decreases and increases in the expression of the cyclin-dependent kinase inhibitors p21 (Waf1, Cip1) and p27Kip1, respectively [12]. Although SIRT1 can improve the myoblast proliferation, the specific mechanism(s) underlying remains to be clarified.

Intriguingly, Wnt signaling is a similar hot topic in muscle development and can potentially be targeted for therapeutic treatment of musculoskeletal diseases [13]. In embryonic development, Wnt signaling pathway can promote the proliferation and differentiation of embryonic stem cells to produce myoblasts. Meanwhile,

Wnt signaling plays an important role in the development from side portion of mesodermal stem cells to skeletal muscle cells [14, 15]. Therefore, it attaches a lot of significance to reveal the interaction between SIRT1 and Wnt signaling and provides another approach for the therapy of muscular dystrophy. There are three major branches of Wnt signaling pathway: the canonical Wnt signaling pathway (Wnt/βcatenin pathway), Wnt-planar cell polarity pathway (Wnt-PCP pathway) and Wnt-calcium pathway (Wnt-Ca<sup>2</sup> pathway) [16]. The canonical Wnt signaling pathway regulates the amount of β-catenin into the nucleus, thereby directly regulates the presence of putative Lef1/Tcf upon binding of Wnt ligands to specific Fz-LRP5/6 complexes [17]. Wnts also signal through β-catenin-independent pathways that couple to the Frizzled (Fzd) receptor and recruit shared components, including axin, glycogen synthase kinase 3 (GSK3) and disheveled (Dvl) [18]. The recent study on mechanism insights into the specificity of interaction between mammalian Wnt and FZD proteins have presented that different Wnt-FZD pairs showed differential effects on phosphorylation of Dvl2 and Dvl3 [19]. Accumulation of TCF/β-catenin can potentially target certain cell proliferation genes such as c-myc, cyclin D1 [20], and the deletion of Dvl 1/2/3 in HEK293T, MCF7 and HeLa cells leads to lower expression of C-myc, cyclin-D1 and SIRT1 protein [21].

Here, we present for the first time a significant functional link between SIRT1 and Wnt in C2C12 cells which has been previously implicated in osteoblast progenitors and breast cancer cells [22, 23]. We co-incubated the C2C12 cells with SIRT1activator, SIRT1 inhibitor and Wnt inhibitor, and then checked the cell growth and the expression of Wnt. Given that SIRT1 has been shown to regulate the expression of Dvl protein to promote C2C12 cell growth, and that Wnt signaling have a lot of analogous function in muscle development, we hypothesized that Wnt signaling would be involved in SIRT1 mediated regulation of C2C12 myoblast cell proliferation. These results will further elucidate the relationship between SIRT1 and Wnt signaling on the regulation of muscle regeneration, and provide the basis for new methods or targets for the therapy of muscular dystrophy.

### Materials and methods

### Cell culture

The C2C12 myoblast cell lines were obtained from the school of animal science and technology of Huazhong Agricultural University. The cells were cultured in a humidified air of 5%  $CO_2$  at 37°C in Dulbecco's modified Eagle's medium (DMEM, NHyClone) supplemented with 1% penicillin/streptomycin (Gino Biomedical Technology Company) and 10% fetal bovine serum (HyClone). Different concentrations of reagents were added to the cells culture when the C2C12 cells grew to approximate 80% and at least three independent repeated trials were performed throughout the study.

### Reagents and antibodies

NAM was from Beyotime Institute of Biotechnology (Hangzhou, China) and REV was purchased from Sigma-Aldrich (St. Louis, MO, USA). Immediately prior to use, a 0.1 M stock solution of REV stored at -20°C was diluted to the desired concentration with the culture medium and the final concentration of DMSO for all treatments was maintained at 0.2%. FH535 was from Selleck Chemicals (Texas, USA). Cell Counting Kit-8 (CCK-8) was from Dojindo Laboratories (Kumamoto, Japan). The antibodies used were anti-β-catenin rabbit monoclonal (Abcam, USA), anti-SIRT1 rabbit monoclonal (Abcam, USA), anti-β-actin mouse monoclonal (Anbo, E0012, Changzhou, China). The secondary antibodies were purchased from Abbkine (California, USA). Other reagents were from Goodbio technology or HyClone.

### CCK-8 assay

The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Technologies (DOJINDO, Japan). Exponentially growing C2C12 myoblast cells ( $8 \times 10^3$  cells/well, 100 µl) were seeded into 96-well plates 20 to 24 h prior to replacing fresh serum free medium containing the indicated concentrations of drug. We added FH535 at first and then added NAM after 30 min in the group of the FH535 combined with NAM. After incubation for 24 h, culture medium was replaced by drug-free medium (100 µl), the effect of drug was examined by CCK-8 according to the manufacturer's instructions. Briefly, 10 µl of CCK-8 was added to each well, and the

	(-1 )	
Target	Primer Sequence	Accession
Sirt1	F: CCTTGGAGACTGCGATGTTA	NM_019812.2
	R: ATGAAGAGGTGTTGGTGGC	
β-catenin	F: GCCACAGGATTACAAGAAGC	NM_001165902.1
	R: CCACCAGAGTGAAAAGAACG	
β-Actin	F: TGGTGGGAATGGGTCAGAAG	NM_007393.3
	R: GTAGAAGGTGTGGTGCCAGA	
Dvl2	F: GGCTTGTGTCGTCAGATACC	NM_007888.3
	R: TTTCATGGCTGCTGGATAC	
C-myc	F: GAAACCCCGCAGACAGCCA	NM_010849.4
	R: ACGGAGTCGTAGTCGAGGTC	
cyclin D1	F: CTTCCTCTCCAAAATGCCAG	NM_007631.2
	R: TGGAGGGTGGGTTGGAAATG	

 Table 1. Primer sequences used for quantitative polymerase chain reaction (qRT-PCR)

cells were further incubated away from light at 37°C for 1 to 3 h. Absorbance was measured with excitation at 450 nm using a microplate reader (Bio-Rad).

### BrdU assay

The BrdU cell proliferation ELISA kit was obtained from Beyotime Biotechnology (Shanghai, China) and the cells were cultured in growth media for 12 h followed by BrdU (5-Bromo-2deoxyUridine) treatment for 1 h before harvesting. The anti-BrdU antibody (BD, 552598,) was incubated at 4°C for 18 h, and the secondary goat anti-mouse IgG antibody conjugated with Alexa Fluor 594. The incubation time was 2 h at room temperature, protected from light. Nuclei were visualized by DAPI (49, 69 diamidino-2-phenylindole), and images were acquired by Olympus microscope (IX71, Shinjuku-ku, Tokyo, Japan).

# Quantitative reverse transcription-polymerase chain reaction

Total RNA of C2C12 myoblast cells was isolated with the Trizol (Invitrogen, Carlsbad, CA, USA) following the product protocol. First-strand cDNA was synthesized using PrimeScript<sup>TM</sup> RT reagent Kit (TaKaRa, RR037A, Dalian, China). DNA amplification was performed in StepOne (Applied BioSystems, Foster City, CA) using SYBR Green PCR kits (TaKaRa, RR420A, Dalian, China) for  $\beta$ -catenin, SIRT1, Dvl2, C-myc, cyclin D1 and  $\beta$ -actin. The primer sequences are listed in **Table 1**.

### Western blot

C2C12 myoblast cells were harvested in PBS, centrifuged at 5,000 g for 10 min at 4°C and then removed the supernatant. Samples were homogenized in ice-cold RIPA lysing buffer (Beyotime, Hangzhou, China) with 1% protease inhibitor cocktail (Sangon Biotech, Shanghai, China) and 1% phosphatase inhibitors (Goodbio technology, Wuhan, China), and incubated the suspension on ice for 20 to 30 min. Then samples were centrifuged at 10,000 g for 10 min at 4°C. The protein concentration of the supernatant was measured using the BCA Protein Quantification Kit (Best-Bio, Shanghai, China). Supernatant fractions of equal protein concentration were

subjected to SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore, Bedford, MA). Blocking was performed in Tris-buffered saline with 5% nonfat dry milk for 2 h at room temperature. Then the membranes were probed with a primary antibody at 4°C overnight. The membranes were washed (6×5 min) with TBST, and incubated with secondary antibody that corresponds to the primary antibody for 2 h at room temperature. The membranes were washed as above, and HRP activity was detected using Chemiluminescence Reagent (Beyotime, Hangzhou, CHN) and exposure to G: BOX Chemi ×T<sup>4</sup> Gel imaging system instrument (SYNGENE, England). Blots were quantified by densitometric analysis using the ImageJ software.

### Statistical analysis

All values were presented as means  $\bar{\chi}\pm$ SEM of at least three individual experiments. To check whether difference was statistically significant, we carried out the one-way ANOVA, which was statistically informative despite the limited number of samples in each group, followed by the Bonferroni method for multiple comparisons (Prism 5, GraphPad Software, CA). The one-way ANOVA is based on the null hypothesis that all groups have the same mean and Bonferroni method was adopted for potential significant differences. BrdU-positive rate was analyzed by chi square test (SPSS v18). The difference was considered significant if the probability value was no more than 0.05.



**Figure 1.** The role of SIRT1 in proliferation of C2C12 myoblast cells. A. CCK-8 analysis of C2C12 cells treated with different concentration of NAM. C2C12 cells (8×10<sup>3</sup> cells/100  $\mu$ l) were seeded in 96-well plates in triplicate, incubated for 20-24 h, and 30 min later treated with different concentrations of REV or NAM for 24 h, and then subjected to CCK-8 assay to analyze proliferation of C2C12. B. CCK-8 analysis of C2C12 cells treated with REV. C. Cell proliferation changed over time (24 h, 48 h, 72 h) in three treating groups: the control, NAM (50 mM), REV (100  $\mu$ M). Each value represents the mean ± standard error of the mean of triplicate determinations from three independent cell preparations. \*P<0.05, \*\*P<0.01, compared with the value of the control. Statistical analysis was conducted using one-way ANOVA.

### Results

# SIRT1 regulates the proliferation of C2C12 myoblast cells

To elucidate the role of SIRT1 in C2C12 myoblast cells growth control, we treated the cells with REV and NAM. The cell proliferation index of C2C12 myoblast cells were investigated by CCK-8 assay. As shown in Figure 1A, inhibition of SIRT1 by NAM resulted in lower proliferation index than that of the untreated control group (P<0.05). Furthermore, the results demonstrated that the cell proliferation index of C2C12 myoblast cells decreased with the concentration of NAM when it ranged from 0 to 200 mM, which presented in a dose dependent manner (The Pearson correlation coefficient was 0.993). C2C12 myoblast cells number stabilized at increased doses of NAM at 50 mM and the BrdU positive rate reached 26.75%. However, when NAM concentration reached

beyond 50 mM, interestingly, the cell survival rate was rather lower (P<0.01) and the cell condition was steadily deteriorated. Consistent with a role for NAM in regulating cell proliferation in a dose dependent manner, our results indicated that NAM inhibited cell growth in a time dependent manner. As shown in **Figure 1C**, cells were treated with NAM at 50 mM and detected every 24 h. From the first day, C2C12 cells were gradually depleted and the decrease in number was predominantly significant compared with the primary state.

To further confirm the effect of SIRT1 on C2C12 regeneration, we administrated different concentrations of REV on C2C12 myoblast cells and measured the cells growth level after 24 hours. The results showed that after treating with REV in different concentrations, the cell proliferation index was significantly higher than that of the untreated control group (P<0.05) (**Figure 1B**). Meanwhile, the results demon-



**Figure 2.** BrdU assay was used to detect the proliferation level.  $1 \times 10^5$  cells were seeded in a 35 mm tissue culture dish and were incubated for 24 h, then these cells in four groups were treated with NAM (50 mM) and REV (100  $\mu$ M). A. At the next day, the cells were fixed and immunostained for BrdU (red) and with DAPI (blue) then merged the two pictures of the same field. The scale bar is 100  $\mu$ m. B. The total numbers of cells (DAPI-staining cells) per field in three groups. C. BrdU-positive rate (BrdU-staining cells/DAPI-staining cells) of C2C12 myoblasts was analyzed. Each value represents the mean ± standard error of the mean of triplicate determinations from three independent cell preparations. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 vs the value of the control; n=3; error bars ± standard error of means. Statistical analysis was conducted using one-way ANOVA. Original magnification is ×200.

strated that the cell proliferation index of C2C12 myoblast cells increased with REV in a dose dependent manner when the REV concentration ranged from 0 to 100 µM (The Pearson correlation coefficient was 0.983). However, when the concentration of REV reached more than 100 µM, the cell proliferation index decreased and the proliferation of cells was significantly inhibited (Figure 1B). C2C12 myoblast cells proliferation index stabilized at increased doses of REV (Figure 3B, P<0.001) at 100 µM and the BrdU positive rate reached 65.80% (Figure 2C). Similarly, when treating with 100 µM REV, C2C12 cells regenerated in time-dependent manner. These stimulate effects of REV on C2C12 cells was statistically significant.

# Inhibition of Wnt by FH535 promotes the proliferation of C2C12 myoblast cells

It has been demonstrated that FH535 is a potent inhibitor of the Wnt/ $\beta$ -catenin signaling pathway and a significant number of evidence

has proven that FH535 inhibits proliferation of cells including Liver Cancer Stem Cells (LCSC) and HCC cell lines [24]. Consistent with the effect in cancer cells, our results showed that after treatment with the FH535 in different concentrations from 0 to 50 uM, the cell proliferation index was significantly lower than that of the control group (P<0.05) (**Figure 3A**). 24 h after treating with FH535 at 15  $\mu$ M, degeneration in C2C12 was remarkably evident (P<0.05) and became worse as it developed (**Figure 3B**). Total number of cells 201±10.27 (**Figure 4B**) and BrdU positive rate (27.73±1.26%) were both significant compared to the control group (**Figure 4C**).

# FH535 aggravates the inhibition of C2C12 myoblast cells proliferation by regulating SIRT1

In order to investigate the role of Wnt signal pathway in SIRT1 mediated regulation of C2C12 myoblast cell proliferation, we divided C2C12 myoblast cells into four groups: the control group, FH535 (15  $\mu$ M), combination of FH535



**Figure 3.** CCK-8 assay to C2C12 cells were treated with different concentrations of FH535 for 24 h. A. FH535 decreased the proliferation of C2C12 myoblast cells. Each value represents the mean  $\pm$  standard error of the mean of triplicate determinations from three independent cell preparations. \*P<0.05, \*\*P<0.01, compared with the value of the control. B. Cell proliferation changed over time (24 h, 48 h, 72 h) in four treating groups : the control, FH535 (15  $\mu$ M), NAM (50 mM) + FH535 (15  $\mu$ M), REV (100  $\mu$ M) + FH535 (15  $\mu$ M). Each value represents the mean  $\pm$  standard error of the mean of triplicate determinations from three independent cell preparations. \*P<0.05, \*\*P<0.01, compared with the value of the control. Statistical analysis was conducted using one-way ANOVA.



**Figure 4.** BrdU assay was used to detect the proliferation level of cells in four groups: the control group, FH535 (15  $\mu$ M) group, NAM (50 mM) + FH535 (15  $\mu$ M) group, REV (100  $\mu$ M) + FH535 (15  $\mu$ M) group. A. At the next day, the cells were fixed and immunostained for BrdU (red) and with DAPI (blue) then merged the two pictures of the same field. The scale bar is 100  $\mu$ m. B. The total numbers of cells (DAPI-staining cells) per field in the four groups. C. BrdU-positive rate of C2C12 cells was analyzed. Each value represents the mean ± standard error of the mean of triplicate determinations from three independent cell preparations. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 vs the value of the control; n=3; error bars ± standard error of means. Statistical analysis was conducted using one-way ANOVA. Original magnification is ×200.

(15  $\mu M)$  with NAM (50 mM) and REV (100  $\mu M)$  respectively. We observed that FH535 acted as

a negative factor on cells which had been activated by REV (Figures 3, 4). After treatment

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**Figure 5.** Regulation of SIRT1 leading to changes in mRNA expression of Wnt/ $\beta$ -catenin signaling. C2C12 cells were administrated with different drugs for 48 h and the expression of  $\beta$ -catenin, SIRT1, Dvl2, C-myc, cyclin D1 mRNA were detected by quantification real-time PCR method. Each value represents the mean ± standard error of the mean of triplicate determinations. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, compared with the value of the control. Statistical analysis was conducted using one-way ANOVA.

with FH535 and NAM, the cell proliferation index and BrdU positive rate were significantly lower than that of NAM alone (P<0.05) (**Figures 1C, 3B**). Together, these results showed that inhibition of Wnt by FH535 may further intensify the effect of SIRT1 inhibition and aggravates the degeneration of C2C12 myoblast cells proliferation by NAM. This effect may be due to that FH535 have a positive function on Wnt expression, and that SIRT1 may ameliorate C2C12 myoblast cell proliferation by inducing Wnt signaling pathways.

### SIRT1 inhibition decreases expression of Wnt signaling pathway and exacerbates the inhibition by FH535

To examine the mechanisms underlying defective regeneration in NAM treating cells, we measured the expression of SIRT1 and Wnt signaling pathways by RT-PCR and Western Blot, respectively. According to **Figure 5**, NAM significantly reduced the relative level of SIRT1 mRNA. Interestingly, the expression of Wnt/ $\beta$ catenin resulted in an overt decrease at the same time, as well as its target Dvl2, indicating that Wnt/ $\beta$ -catenin may act as a downstream of SIRT1.

This hypothesis was confirmed by the later Western Blot assay, as it shown in **Figure 7**. Down-regulation of SIRT1 induced an apparent loss of Wnt/ $\beta$ -catenin protein level, which is in accord with RT-PCT results. The expression of Wnt/ $\beta$ -catenin and its downstream signals were significantly lower in cells treated with FH535 and NAM than that with NAM alone (P<0.05, **Figure 6**). These data demonstrated that SIRT1 inhibition decreased expression of Wnt signaling pathways.

### SIRT1 activation increases expression of Wnt in vitro and ameliorates the suppression induced by FH535

Upon activation by REV, SIRT1 activators increase bone mass in aged mice by preventing  $\beta$ -catenin sequestration [25]. It was proven that REV in the dose of 100  $\mu$ M effectively inhibited the regeneration and depressed Wnt2, Wnt5a and Notch2 expression of SiHa and HeLa cells [26]. But in C2C12 cells, RSV



**Figure 6.** NAM and REV affected the inhibition of FH535 on Wnt/ $\beta$ -catenin signaling. C2C12 cells were administrated with different drugs for 48 h and the expression of  $\beta$ -catenin, SIRT1, Dvl2 and C-myc mRNA were detected by quantification real-time PCR method. A. qRT-PCR of  $\beta$ -catenin. B. qRT-PCR of Dvl2 mRNA. C. qRT-PCR of C-myc mRNA. D. qRT-PCR of SIRT1 mRNA. Each value represents the mean ± standard error of the mean of triplicate determinations. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, compared with the value of the control. Statistical analysis was conducted using one-way ANOVA.

adjusts cell cycle progression, cell cycle arrest and primary progress of differentiation, by regulating the expression of several key cell cycle regulators, myogenic regulatory factors and muscle-specific structural proteins [27]. Interestingly, the researches on the connection between C2C12 and Wnt shows that WNT/βcatenin signaling regulates myogenesis in several ways, and the depletion of these steps of myogenesis induces muscle growth defects [28]. However, whether this relationship between SIRT1 and Wnt signaling extended to the activation of SIRT1 in C2C12 cells was unknown. To address this, we systematically detected the impact of SIRT1 increase on the change in the expression of Wnt signaling.

As is indicated in **Figure 5**, REV markedly enhanced the relative mRNA level of SIRT1,

cyclin D1, WNT/ $\beta$ -catenin and Dvl2, which is different from the finding in several cell lines. In general, REV inhibits proliferation of most cancer cells and induces apoptosis of epithelial cells [29]. Inversely, REV modulates fibroblast cells to regenerate and produces the recovery of muscle mass following disuse that results in REV-associated improvement in the plantaris muscle of aged rats [30]. Consistent with these reports, we observed that SIRT1 activation ameliorated the deterioration of cell proliferation state and the reduction of WNT/ $\beta$ -catenin, Dvl2 and cyclin D1 induced by inhibition of Wnt signaling.

We next investigated the relative change in proteins and Western Blot detections proved that REV treatment inhibited the expression of  $\beta$ -catenin (Figure 7). Given that the effects of

## SIRT1 and C2C12 proliferation



**Figure 7.** A. Western Blot analysis for relative expression of SIRT1 and  $\beta$ -catenin protein. C2C12 cells were treated with different drugs for 48 h. B. Quantitative analysis of the SIRT1. C. Quantitative analysis of the  $\beta$ -catenin. The histogram shows a quantitative representation of the levels protein obtained from a laser densitometric analysis of three independent experiments. Each value represents the mean ± standard error of the mean of triplicate determinations. \*P<0.05, \*\*P<0.01 vs the value of the control. Statistical analysis was conducted using one-way ANOVA.

NAM and RES on Wnt regulation are SIRT1dependent, we investigated whether these two proteins interact in the specified way. Treating with FH535 slightly reduced SIRT1 protein level, however, the change was not significant. In general, the above results confirmed that the observed effects of REV were indeed mediated through SIRT1.

### Discussion

Both Wnt and SIRT1 have been linked to C2C12 cell regeneration with SIRT1 activation inducing a protective effect while inhibition of Wnt conferring a detrimental effect. SIRT1 plays a critical role in a wide variety of cellular processes including proliferation, differentiation and function, however, the mechanisms responsible for its effects are not fully understood. SIRT1 has been found to promote the proliferation and suppressed the differentiation of myoblast precursors [31] and overexpression of SIRT1 can promote the proliferation of skeletal muscle precursor cell, while reversely it is reported that SIRT1 inhibits cell proliferation in colon cancer [32], which may be due to the different cell type. Our results demonstrate SIRT1 activator REV can promote the proliferation of C2C12 myoblast cells: meanwhile its inhibitor NAM can decrease the proliferation of C2C12 myoblast cells. SIRT1 promotes the proliferation of C2C12 myoblast cells only when REV concentration was no more than 100 µM. When REV concentration was more than 100 µM, the survival rate of C2C12 myoblast cells decreased. This may be due to the toxic effect of the solvent DMSO on cells, on the other hand, the REV can not only activate SIRT1, but also can inhibit lipoxygenase, cyclooxygenase and various protein kinases, therefore, as a result REV may be harmful to cells when its concentration is too high [31, 33].

It is well-established that SIRT1 participates in a very broad and complex array of physiological processes, for example, cellular differentiation, oxidative stress response, metabolism, longevity and various diseases including muscular

dystrophy [34]. Activation of SIRT1 promotes cell proliferation both in the liver [35] and colon [36], and SIRT1 has been proven to correlate with decrease of muscle mass, skeletal muscle strength [37] and oxidative damage in mdx mice [38]. However, overexpression of SIRT1induced proliferation of muscle cells in vivo and in vitro has not been reported yet. In this study, we have shown that SIRT1 regulates skeletal muscle regeneration in C2C12 cells model, and that the stimulating effect is both approximately in dose-dependent manner and time-dependent manner. And in order to study the mechanisms and feature of SIRT1-inducing proliferation in muscle cells, we have test several muscle regeneration-related signaling pathways such as mTOR, myostatin and Wnt signaling pathways. And our results showed that Wnt signaling pathway plays a significant role in SIRT1inducing proliferation in muscle cells, which has not been reported yet elsewhere.

Canonical Wnt signaling pathway is reported to promote myoblast generation in embryonic period [39], and FH535, the inhibitor of Wnt. is highly potent and effective. FH535 can simultaneously inhibit the peroxisome (PPAR) and β-catenin/TCF/LEF combination [40], and it has no marked effect on the quantity of other proteins. We discovered that FH535 not only reduce cell proliferation index, but also morphologically changes cell size and shape. C2-C12 cells became round and spindle-shaped, failed to attach firmly to the culture bottles. To clarify the relationship between SIRT1 and Wnt in C2C12 cells, we firstly co-cultured cells with NAM and FH535, which showed sharp decrease in growth rate compared with control group. Secondly, we detected mRNA expression of Dvl2, which has been reported to decrease significantly and inhibit the degradation of β-catenin in absence of SIRT1 expression in colon and lung cancer cells [41]. Our results showed that DvI2 expression level was much lower in NAM group than that in RSV group. It is reported that C-myc and cyclin D1 are the down-stream of Wnt signaling pathway of regulating cell cycle proteins [42], and we also found that C2C12 myoblasts treated with FH535 could significantly down-regulated mRNA expression levels of C-myc and cyclin D1 compared with the control group. Thirdly, as expected, FH535 and NAM significantly suppressed β-catenin mRNA and protein levels, while RSV promoted them remarkably, thus in conclusion SIRT1 can positively regulate Wnt signaling pathway in proliferation progress in C2C12 myoblasts. Activation of SIRT1 activity with REV increased the  $\beta$ -catenin expression level, while in contrast suppression of SIRT1 activity by NAM decreased them notably.

These findings indicate that SIRT1 regulates Wnt/β-catenin to trigger its concentration in the nucleus and stimulation of genes for proliferation in C2C12 cells. Intriguingly, a previous study noted that oxazepam-induced cellular oncogenesis displayed lower level of DNA methvlation (Crebbp, Dnmt3b) and histone modification (SIRT1), while the expression of the Wnt/ $\beta$ catenin signaling pathway increased [43]. And the suppression of SIRT1 deacetylase by siRNA or NAM in epithelial cells Hepatitis B virus X (HBX) protein could activate  $Wnt/\beta$ -catenin and the presence of HBX might promoted the interaction between β-catenin and SIRT1, leading to protection of  $\beta$ -catenin by inhibitory action of SIRT1 [44]. Why regulation of Wnt signaling by SIRT1 exhibits distinct results in different kinds of cells? We infer that the signaling transfer mechanism could display a contrary trend in C2C12 cells VS human hepatic cells, and that the deacetylation induced by SIRT1 in β-catenin was the root cause that led to nuclear inflow in C2C12 cells but outflow in hepatic cells. This might happen, for instance, if either kind of cells could control an outflow signaling identifying the deacetylated  $\beta$ -catenin, but C2C12 cells particularly occupied an inflow signaling that identifies the redundant and excrescent deacetylated  $\beta$ -catenin in the outflow signaling.

Taken together, our results indicated that Wnt participated in the regulation of C2C12 myoblast cells proliferation via SIRT1. Further studies are warranted to show how SIRT1 affect Wnt signaling, for example, siRNA interference of SIRT1 and Wnt signaling to further study the interaction relationship. Furthermore, it will be promising to detect the effect of Wnt signaling in SIRT1-depleted mdx mice, which will contribute to elucidate the role of SIRT1 in the regulation of muscle regeneration and provide the basis for new methods or targets for the therapy of muscular dystrophy.

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### Disclosure of conflict of interest

None.

Address correspondence to: Dr. Dejia Li, Department of Occupational and Environmental Health, Wuhan University, 115 Donghu Road, Wuhan, China. E-mail: djli@whu.edu.cn

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