

New Phenomenon

Sirt2 induces C2C12 myoblasts proliferation by activation of the ERK1/2 pathway

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Sirtuins are Class III histone deacetylases (HDACs) that have emerged as important regulators of diverse biological processes, and comprised of seven members (Sirt1 to Sirt7) [1]. Sirt2 predominantly resides in the cytoplasm, deacetylates lysine40 on α -tubulin, and co-localizes with microtubules and HDAC6 [2]. Sirt2 plays an important role in the control of mitotic exit in cell cycle, and the increased Sirt2 activity severely delays cell cycle progression through mitosis [3–5]. Sirt2 is hyper-phosphorylated and accumulated in the M phase, which regulates mitotic progression [3]. In glioblastoma cancer stem cells, sphere-forming capacity and cell proliferation are significantly decreased by Sirt2 inhibitors [6]. The effect of Sirt2 on cell proliferation seems to vary in a tissue-specific and disease-specific manner; thus, the role of Sirt2 in myoblast proliferation is to be elucidated. The present study aimed to elucidate the effect of Sirt2 on C2C12 cell proliferation and cell cycle progression by infecting C2C12 cells with a recombinant adenovirus encoding *Sirt2* gene.

C2C12 cells were infected with Ad-Sirt2, and Sirt2 mRNA and protein expression levels were tested by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot assays. Primers used are shown in **Supplementary Table S1**. It was shown that Sirt2 mRNA and protein expressions significantly increased in the Ad-Sirt2 group by 51- and 2.5 fold ($P < 0.01$), respectively (**Fig. 1A,B**), indicating that Sirt2 was highly expressed in Ad-Sirt2-infected C2C12 cells. Sirt2 is a primary deacetylase, so we detected the deacetylase activity of Sirt2 by checking Ac-Lys protein expression. **Figure 1C** shows that Ac-Lys40 protein expression in the Ad-Sirt2 group was less than that in the Ad-GFP group. In conclusion, the mRNA and protein expressions and the deacetylase activity of Sirt2 were increased in the Ad-Sirt2 group. **Figure 2** shows that Sirt2 promoted C2C12 cell growth. The percentage of C2C12 cells displayed no difference among the three groups after 24 h of adenovirus treatment, whereas it increased by 1.5 fold in the Ad-Sirt2 treatment group 48 h post infection (**Fig. 2A**; $P < 0.01$). Ki-67 (Santa Cruz, USA) was used as a marker to evaluate cell proliferation.

Thirty-six hours after adenovirus treatment, the percentages of Ki-67-positive cells in the Ad-GFP and the Ad-Sirt2 treatment groups were 12.33% and 18.01%, respectively, and the Ki-67 expression was increased by Sirt2 overexpression (**Fig. 2B**; $P < 0.01$).

We then assessed the cell cycle promotion caused by Sirt2 and analyzed the cell cycle distribution by using flow cytometry. The percentage of cells in G_0/G_1 phase decreased in the Ad-Sirt2 treatment group (47.19 ± 2.0), when compared with the Ad-GFP group (64.70 ± 1.1). The percentages of S and G_2/M phase cells increased from 15.79 ± 0.9 and 19.51 ± 1.4 to 29.72 ± 1.6 and 23.09 ± 0.8 in the Ad-GFP and the Ad-Sirt2 groups, respectively (**Table 1** and **Supplementary Fig. S1**). We determined the mRNA levels of cyclin D1, cyclin E1, and cdk2 by using a RT-PCR. Compared with the Ad-GFP group, cyclin D1 and cdk2 levels in the Ad-Sirt2 group were significantly increased by 1.75- and 2.24 folds, respectively, whereas the cyclin E1 level had no significant change and the p21 level was significantly decreased by 89.43% (**Fig. 3**).

ERK1/2 activation is typically indispensable for cell proliferation [7–9]. ERK1/2 interacts with Sirt2 exogenously and endogenously, and the deacetylase activity of Sirt2 is upregulated by ERK1/2 signaling [10]. To confirm whether Sirt2 affects this signaling pathway in C2C12 cells, we detected ERK1/2 and PI3K protein expression and phosphorylation level by using western blot. Compared with the Ad-GFP group, Sirt2 promoted the phosphorylation of ERK1/2 in C2C12 myoblasts, but the phosphorylation status of PI3K did not show any significant alteration (**Fig. 4A**). In brief, Sirt2 overexpression activated the ERK1/2 signaling pathway in C2C12 cells.

To further investigate the correlation between the effect of Sirt2 on C2C12 proliferation and the ERK 1/2 signaling, a specific inhibitor of ERK1/2, 5 $\mu\text{mol/l}$ U0126 (Sigma, St Louis, USA) was used to block ERK1/2 signaling [11]. We found that U0126 suppressed the ERK1/2 pathway and inhibited cell proliferation. Sirt2 overexpression could rescue the blocked C2C12 proliferation, which was caused

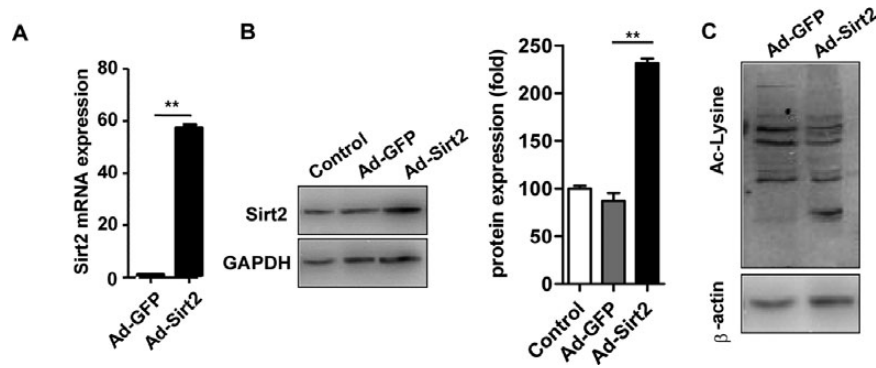


Figure 1. Sirt2 expression in C2C12 proliferation (A) qRT-PCR was used to analyze the Sirt2 mRNA expression level. (B) Sirt2 protein expression was detected by western blot analysis using an anti-Sirt2 antibody (Cell Signaling Technology, Beverly, USA). GAPDH (Santa Cruz) was used as an internal control. (C) Sirt2 deacetylase activity was assayed by using an Ac-lysine antibody (Cell Signaling Technology), β -actin (Santa Cruz) was used as an internal control. * $P < 0.05$, ** $P < 0.01$.

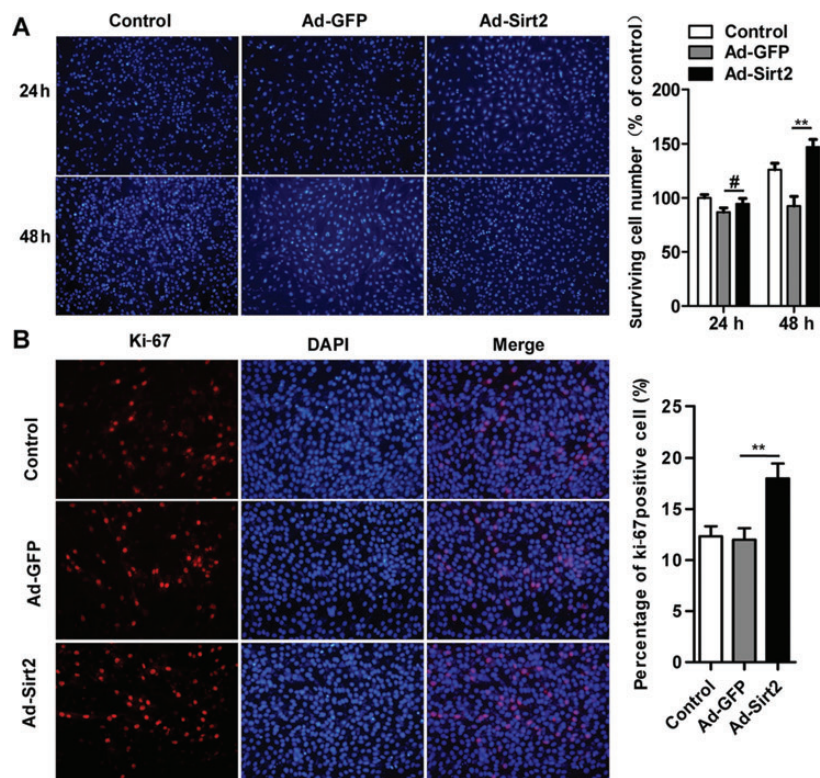


Figure 2. Sirt2 promoted C2C12 cell proliferation C2C12 cells were or were not infected with Ad-GFP or Ad-Sirt2 for 24, 36, or 48 h. Ad-GFP cells were served as controls. (A) Cell nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI) (blue) and the cells were quantified from ten images at 24 and 48 h time point. (B) Cells were stained for Ki-67 (red) and nuclei were stained with DAPI (blue), Ki-67-positive cells were quantified from 10 images at 36 h timepoint. Magnification, $\times 10$. Data were expressed as mean \pm SE of three independent determinations. * $P < 0.05$, ** $P < 0.01$.

Table 1. Sirt2 influences the cell cycle in C2C12 cells

Group	Cell cycle (%)		
	G_0/G_1	S	G_2/M
Control	59.87 \pm 1.7	20.01 \pm 1.2	20.12 \pm 1.5
Ad-GFP	64.70 \pm 1.1**	15.79 \pm 0.9**	19.51 \pm 1.4
Ad-Sirt2	47.19 \pm 2.0**	29.72 \pm 1.6**	23.09 \pm 0.8*

G_0 , diploid population in the G_0 phase of the cell cycle; S, cell population in the S phase; M, tetraploid population in the M phase. Statistics from three separate experiments, values represent mean \pm SE of cells in G_0/G_1 , S and G_2/M of the cell cycle. * $P < 0.05$, ** $P < 0.01$.

by U0126 (Fig. 4B). Moreover, Sirt2 differentially weakened the U0126-induced alterations in cyclin D1, cdk2, and p21 mRNA expression (Fig. 4C).

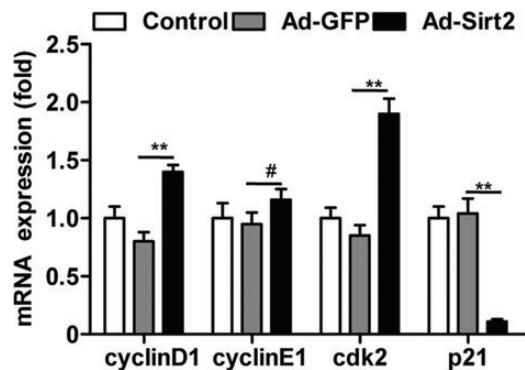


Figure 3. Effect of Sirt2 on C2C12 cell cycle-related gene C2C12 cells were or were not infected with Ad-GFP or Ad-Sirt2 for 48 h, real-time qPCR was used to detect mRNA expression. The expression of each gene was normalized to *GAPDH* expression. Data were expressed as mean \pm SE of three independent determinations. * P < 0.05, ** P < 0.01, # P > 0.05.

Taken together, these data indicated that Sirt2 could enhance myoblast proliferation, shorten the G₁ phase in murine C2C12 myoblasts by inducing cell cycle regulatory proteins cyclin D1 and cdk2 kinase, and suppressing p21. Moreover, Sirt2 induced cell proliferation by activating ERK1/2 in C2C12 myoblasts. This study provided an updated understanding of the effects of Sirt2 on skeletal muscle cells, and future research should address two questions on the basis of our current observations:

- (1) Sirt2 overexpression promotes C2C12 cell proliferation, but it should be explored whether the Sirt2 inhibitor or downregulated Sirt2 can lead to opposite results.
- (2) Given that Sirt2 plays an active role in cell proliferation, does Sirt2 also participate in muscle regeneration?

Supplementary Data

Supplementary data are available at *ABBS* online.

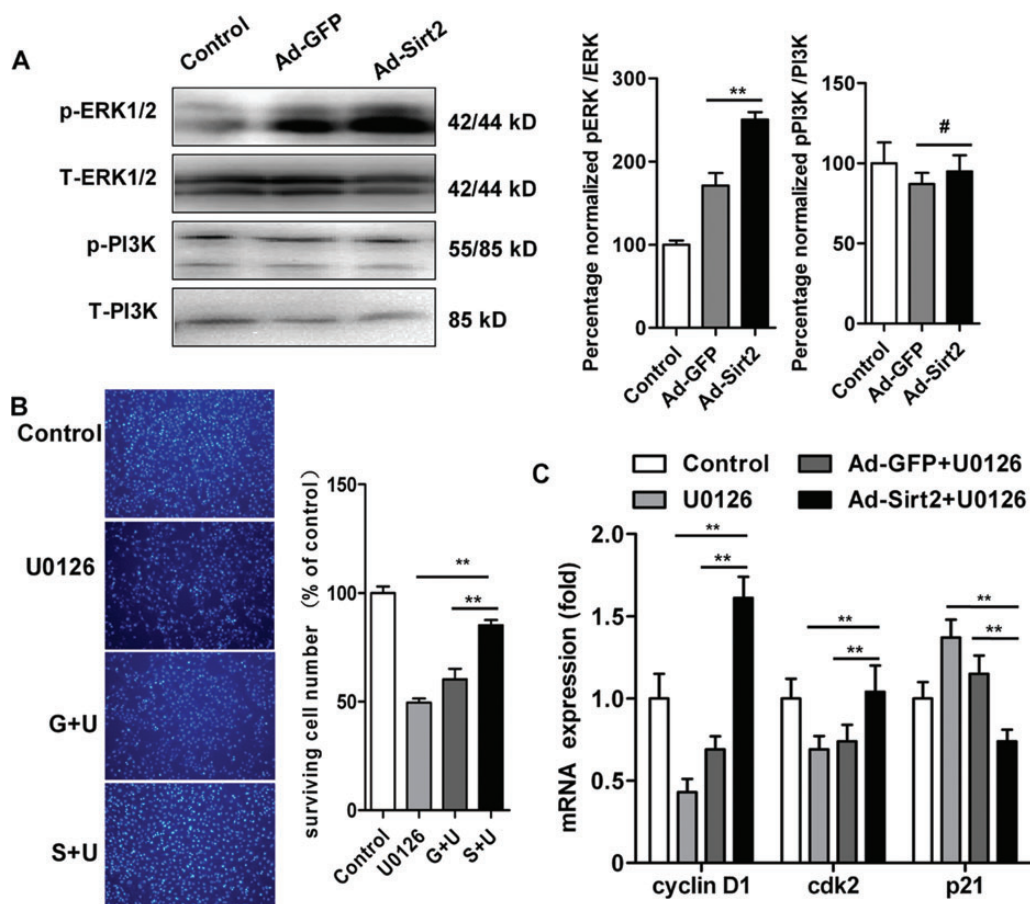


Figure 4. Sirt2 promoted C2C12 cell proliferation through ERK1/2 (A) Western blot analysis was used to assess the activity of signaling pathway in excess/absence of Sirt2. The activations of ERK1/2 and PI3K were measured by using anti-antibodies or anti-phospho antibodies (Cell Signaling Technology), respectively, and quantitative analyses were performed by densitometry. (B) Quantification of the myoblast number of four treatments from 10 images at 48 h timepoint. Magnification $\times 10$. (C) Sirt2 recovered U0126-induced inhibition in C2C12 cell proliferation. Results are expressed as mean \pm SE of three independent determinations. ** P < 0.01, # P > 0.05.

Funding

This work was supported by grants from the National Natural Science Foundation of China (31272410), the Scientific and Technological Innovation Project for Youths of Northwest A&F University (ZD2012007), the Program for Innovative Research Team in the Northwest A&F University, and the National Swine Industry Technology System (CARS-36-04B).

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