

# miR-29 targets *Akt3* to reduce proliferation and facilitate differentiation of myoblasts in skeletal muscle development

W Wei<sup>1</sup>, H-B He<sup>1</sup>, W-Y Zhang<sup>1</sup>, H-X Zhang<sup>1</sup>, J-B Bai<sup>1</sup>, H-Z Liu<sup>1</sup>, J-H Cao<sup>1</sup>, K-C Chang<sup>2</sup>, X-Y Li<sup>\*1</sup> and S-H Zhao<sup>1</sup>

MicroRNAs (miRNAs) are a type of endogenous noncoding small RNAs involved in the regulation of multiple biological processes. Recently, miR-29 was found to participate in myogenesis. However, the underlying mechanisms by which miR-29 promotes myogenesis have not been identified. We found here that miR-29 was significantly upregulated with age in postnatal mouse skeletal muscle and during muscle differentiation. Overexpression of miR-29 inhibited mouse C2C12 myoblast proliferation and promoted myotube formation. miR-29 specifically targeted *Akt3*, a member of the serine/threonine protein kinase family responsive to growth factor cell signaling, to result in its post-transcriptional downregulation. Furthermore, knockdown of *Akt3* by siRNA significantly inhibited the proliferation of C2C12 cells, and conversely, overexpression of *Akt3* suppressed their differentiation. Collectively and given the inverse endogenous expression pattern of rising miR-29 levels and decreasing *Akt3* protein levels with age in mouse skeletal muscle, we propose a novel mechanism in which miR-29 modulates growth and promotes differentiation of skeletal muscle through the post-transcriptional downregulation of *Akt3*.

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MicroRNAs (miRNAs) are endogenous noncoding small RNAs comprising 21–23 base pairs. The miRNA sequence is complementary to the 3' untranslated region (UTR) of its target mRNA, and the resulting complementary binding inhibits gene expression at the post-transcriptional level by mediating mRNA breakdown or translation inhibition.<sup>1,2</sup> miRNA has been confirmed to be involved in multiple physiological and biochemical processes, including tumorigenesis, hematopoiesis, neurogenesis, immunity, cell proliferation, and apoptosis.<sup>3–6</sup> miRNA has also been shown to participate in muscle development or repair in mice, rats, pigs, and humans.<sup>7–11</sup> Previous studies have identified numerous muscle-associated miRNAs, including miR-1, miR-29, miR-133, miR-206, and miR-214, which have important roles in regulating myogenesis and differentiation of skeletal or cardiac muscle.<sup>12–15</sup>

The miR-29 family comprises three members, miR-29a, miR-29b, and miR-29c. Their seed sequences are identical, and they have similar expression patterns and functions. Functional studies have shown that miR-29 participates in different physiological processes. It represses Tc1 expression, thereby inhibiting B cell chronic lymphocytic leukemia in mice.<sup>16,17</sup> Overexpression of miR-29a/b/c causes insulin resistance, indicating its possible role in type 2 diabetes mellitus.<sup>18</sup> miR-29a/b has also been shown to promote osteogenesis by participating in the Wnt signaling circuit or targeting inhibitors of osteoblast differentiation.<sup>19,20</sup> However, most studies have focused on the role of miR-29 as a

tumorigenic inhibitor. For example, miR-29 restores normal patterns of DNA methylation by targeting DNA methyltransferase 3A and 3B, thus inhibiting tumorigenesis in lung cancer.<sup>21</sup> miR-29 directly targets p85 alpha and CDC42 in HeLa cells, suppresses Mcl-1 and Bcl-2 in hepatocellular carcinoma, and induces apoptosis in a p53-dependent manner or through a mitochondrial pathway.<sup>22,23</sup> A recent study shows that miR-29 acts as a tumor suppressor in rhabdomyosarcoma by regulating the expression of CCND2 and E2F7.<sup>24</sup>

Importantly, the miR-29 family has been found to be involved in muscle development. Knockdown of miR-29b *in vivo* induced cardiac fibrosis in mice.<sup>15</sup> Repression of miR-29a/b upregulated the expression of ADAMTS-7, and subsequently induced vascular smooth muscle cell (VSMC) calcification.<sup>25</sup> These results indicate that miR-29 is important in the function of cardiac muscle and VSMCs. We previously found that miR-29a, miR-29b, and miR-29c were significantly upregulated in *longissimus* muscle of adult pigs, compared with 33- and 65-day old fetuses.<sup>26</sup> In myotonic dystrophy type 1, miR-29b and miR-29c were found to be downregulated.<sup>27</sup> Furthermore, miR-29 is downregulated in Duchenne muscular dystrophy, and restoration of its expression could improve dystrophy by promoting myogenic differentiation and inhibiting fibrogenic differentiation.<sup>28,29</sup> These results provide evidence for the role of miR-29 as a critical regulator in skeletal muscle, but its effector mechanism is not clear. Current evidence indicates

<sup>1</sup>Key Laboratory of Animal Genetics, Breeding and Reproduction of Ministry of Education, College of Animal Science and Technology, Huazhong Agricultural University, Wuhan, PRC and <sup>2</sup>School of Veterinary Medicine and Science, Sutton Bonington Campus, University of Nottingham, Nottingham, UK

\*Corresponding author: X-Y Li, Key Laboratory of Animal Genetics, Breeding and Reproduction of Ministry of Education, College of Animal Science and Technology, Huazhong Agricultural University, Wuhan 430070, PRC. Tel: +86 27 87387480; Fax: +86 27 87280408; E-mail: xyli@mail.hzau.edu.cn

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**Abbreviations:** miRNAs, microRNAs; UTR, untranslated region; VSMC, vascular smooth muscle cell; YY1, Yin Yang1; Rybp, Ring1 and YY1-binding protein; Mfap5, microfilament-associated protein 5; Q-PCR, quantitative polymerase chain reaction; EdU, 5-Ethynyl-2'-deoxyuridine

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that in undifferentiated myoblasts, TGF- $\beta$ -Smad3 signaling enhances the recruitment of a complex, which contains Yin Yang1 (YY1), Rybp (Ring1 and YY1-binding protein), and Polycomb protein (recruited by YY1), thereby negatively regulating miR-29 expression.<sup>30,31</sup> On the other hand, under normal myogenic differentiation conditions, miRNA-29 promotes myogenic differentiation of C2C12 cells by inhibiting Smad3, which in turn inhibits TGF- $\beta$ -mediated regulation of HDAC4, or YY1, Rybp, Polycomb-repressive complex, Ezh2, collagens, Lims1, and Mfap5 (microfibrillar-associated protein 5), thereby preventing the transdifferentiation of C2C12 cells into myofibroblasts.<sup>28,30,32,33</sup> These studies show that miR-29 indirectly promotes myogenesis through inhibiting the conversion of myoblasts to myofibroblasts; however, it is unclear if miR-29 could perform a more direct myogenic role by targeting effector genes that mediate skeletal muscle cell proliferation or differentiation.

Akt kinase family comprises three members: Akt1, Akt2, and Akt3. Akt3 is mainly known for its role in tumorigenesis. Raised Akt3 is found to associate with breast and prostate cancers,<sup>34</sup> and ovarian cancer cells.<sup>35</sup> The probable underlying mechanism is that AKT3 promotes cell proliferation by accelerating G2–M phase transition.<sup>36</sup> High expression of Akt3 is also associated with malignant melanomas. Knock-down or inhibition of Akt3, but not Akt1 or Akt2, induced apoptosis in melanoma cells; therefore, the Akt3 signaling cascade is considered a therapeutic target in melanoma treatment.<sup>37–39</sup> Downregulation of AKT3 by miRNAs, such as miR-15a and miR-16-1 or miR-93, resulted in the inhibition of proliferation in multiple myeloma and breast cancer stem cells.<sup>40,41</sup> Recent studies found that Akt3 has a potential role in promoting the proliferation of human and rat aortic VSMCs.<sup>42,43</sup> Moreover, marked cardiac hypertrophy was found in cardiac-specific Akt3 transgenic mice, suggesting a role for Akt3 in cardiac muscle development.<sup>44</sup> However, there is no clear evidence of a functional link between the Akt3 isoform and skeletal muscle or myoblasts.

The aim of this study is to elucidate the underlying mechanism by which miR-29 modulates the proliferation and differentiation of myoblasts, and its relationship with Akt3. We demonstrated that in aging mouse skeletal muscles, miR-29 was upregulated, but Akt3 expression was downregulated. Akt3 promoted myoblast proliferation and delayed muscle differentiation, and was downregulated by miR-29 at the post-transcriptional level.

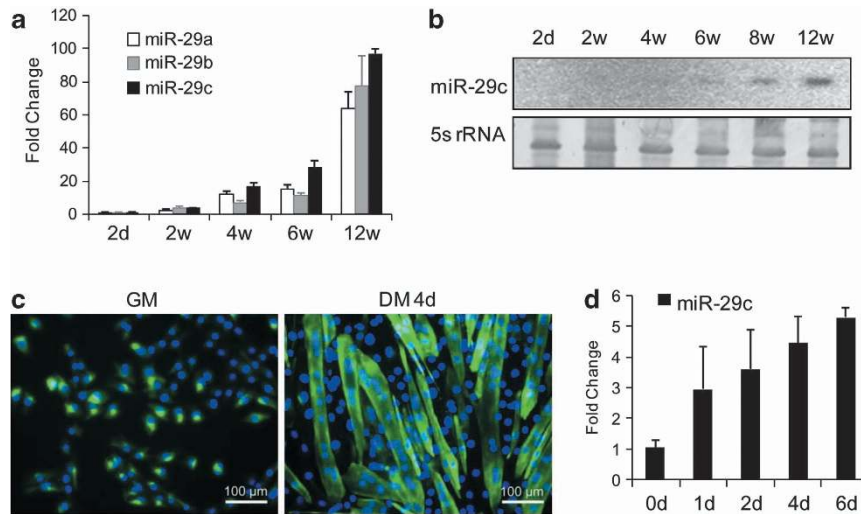
## Results

**miR-29 was significantly upregulated during postnatal skeletal muscle growth in mice.** Our previous study showed that expression of miR-29 family members was significantly higher in the *longissimus* muscle of adult pigs than those from 33- and 65-day fetuses,<sup>26</sup> indicating its potential role in skeletal muscle development. In order to confirm the expression profile of miR-29, we collected hindleg muscles from postnatal Balb/c mice at 2 days, 2 weeks, 4 weeks, 6 weeks, and 12 weeks, and analyzed the expression of miR-29 in these samples by Q-PCR. As anticipated, we found that the expression of miR-29a, miR-29b, and miR-29c was upregulated in an age-dependent

manner. Expression of miR-29 in the skeletal muscle of 12-week-old mice was over 60-fold higher than in 2-day-old mice (Figure 1a). The three miR-29 members showed similar rising expression patterns with age. Furthermore, northern blotting confirmed the upregulation of miR-29c during postnatal muscle growth (Figure 1b). Next, we investigated the expression profile of miR-29c *in vitro* in murine C2C12 myoblasts and myotubes (Figure 1c). miR-29c was upregulated during C2C12 differentiation (Figure 1d). These results indicated that miR-29 expression was upregulated during postnatal skeletal muscle growth and muscle differentiation.

**miR-29 repressed proliferation of C2C12 cells.** In order to detect the effects of miR-29 on muscle proliferation, C2C12 myoblasts transfected with synthetic pooled miR-29 mimics (miR-29a/b/c mimics mixed equally) or scrambled negative control (NC) miRNA were grown to near confluence and subjected to propidium iodide-cell cycle flow cytometry (Supplementary Figure 1). There was a significantly higher number of cells in the G0/G1 and G2 stages in the miR-29-transfected group than in the NC group (Figure 2a). This suggests that miR-29 could arrest C2C12 cells in the G0/G1 stage. The proliferation index was lower in miR-29-transfected cells than in NC-transfected cells (Figure 2b). xCELLigence system-based real-time monitoring of C2C12 cell proliferation found that miR-29-transfected cells displayed a reduced growth rate compared with corresponding NC miRNA-transfected cells from 24 h post transfection (Figure 2c). To perform overexpression of miR-29b and miR-29c, a 1007-bp DNA fragment, housing the primary transcript of mmu-miR-29b-2 and the mmu-miR-29c cluster, was cloned into the pEGFP-C1 vector (miR-29bc-C1). Twenty-four hours post transfection with the recombinant GFP-miR vector, Q-PCR showed that miR-29b and miR-29c were successfully overexpressed in C2C12 cells (Figure 2d). miR-29bc-C1 and pEGFP-C1 (control) transfected cells were nuclear stained with 5-Ethynyl-2'-deoxyuridine (EdU) (a dye only inserts in the replicating DNA molecules) and subjected to flow cytometry to identify cell populations that were dual positive for EdU and GFP. miR-29bc-C1-transfected cells showed significantly less mitotic activity, as evident by a significant reduction in dual EdU- and GFP-labeled cells (Figure 2e). Collectively, these results indicated that miR-29 overexpression inhibited C2C12 cell proliferation.

**miR-29 promoted C2C12 myoblast differentiation.** To establish the involvement of miR-29 in muscle cell differentiation, C2C12 myoblasts were transfected with synthetic pooled miR-29 mimics or NC miRNA. Twenty-four hours later, growth medium was replaced with differentiation medium to induce cell differentiation. Immunofluorescence detection of fast skeletal myosin showed that more cells underwent differentiation and myotube formation in the pooled miR-29 group than in the NC group at 72 h (Figure 3a). Several myoblast differentiation marker genes were also detected at 48 h of differentiation by Q-PCR. Along with the successful overexpression of miR-29, expression of the *myogenin*, *MyHC IIId*, and *MCK* genes was significantly upregulated in cells transfected with the pooled miR-29 mimics than in the cells transfected with NC miRNA



**Figure 1** Upregulation of miR-29 in mouse skeletal muscle with age. (a) Expression of miR-29 in the hindleg muscles of postnatal Balb/c mice was determined by Q-PCR. The ages of the mice were 2 days, 2 weeks, 4 weeks, 6 weeks, and 12 weeks. Fold change was in relation to expression in 2-day-old mice. (b) Northern blotting analysis of miR-29 in thigh muscles from postnatal Balb/c mice. 5s rRNA was used as the reference gene. (c) Immunofluorescence detection of fast skeletal myosin (Alexa Fluor 488 dye, green) in C2C12 myoblasts (GM) and myotubes at 4 days of differentiation (DM 4d). Cell nuclei were positive for DAPI (blue). Scale bar = 100  $\mu$ m. (d) miR-29c expression in C2C12 cells differentiated for 0, 1, 2, 4, and 6 days. Fold change in relation to expression on 0 day. Expression of miR-29 was normalized to U6 and is presented as mean  $\pm$  S.E.M. (from three independent individuals or replicates per time point)

(Figure 3b). Together, these results showed that miR-29 promoted myoblast differentiation.

**miR-29 directly targeted 3' UTR of *Akt3*.** In order to uncover the mechanisms by which miR-29 suppresses proliferation in myoblasts, we examined potential targets of miR-29 involved in the cell cycle. *Akt3* was identified by TargetScan analysis as a prime target, with a highly conserved complementary miR-29-binding site in its 3' UTR across vertebrates from chickens to humans (Figures 4a and b).

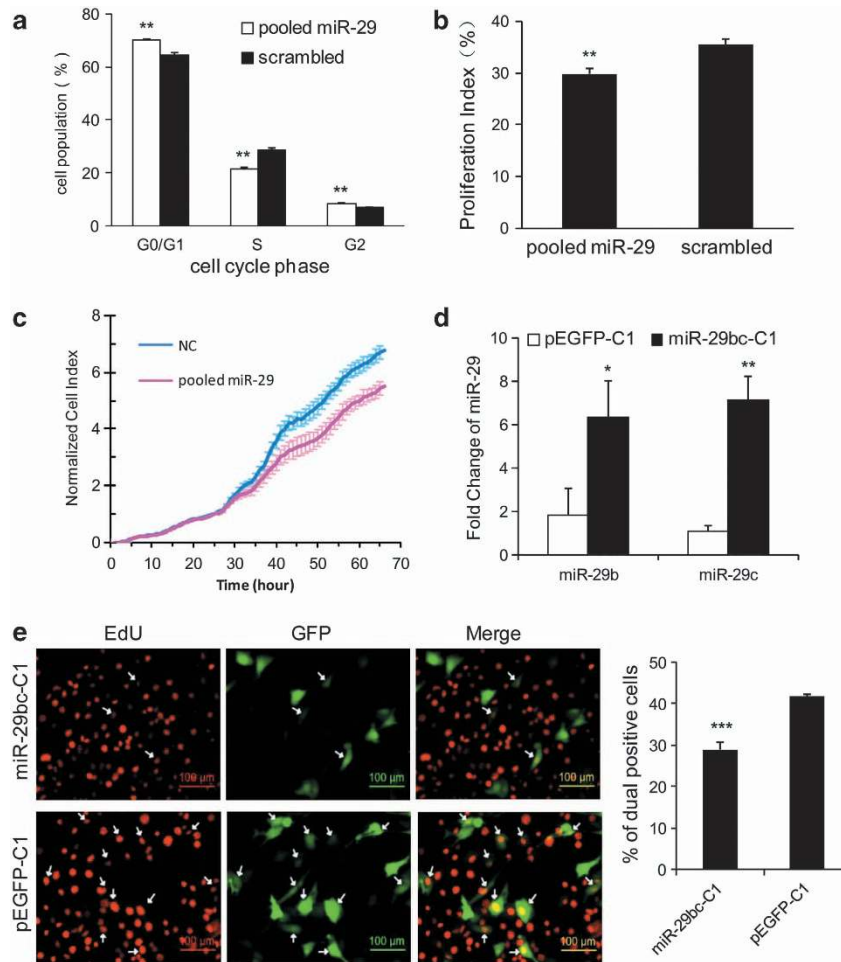
To establish miR-29 function at the *Akt3* 3' UTR, a fragment of *Akt3* 3' UTR containing the binding site of miR-29 was spliced to the 3'-end of the *Renilla* luciferase reporter gene in the dual-luciferase assay cassette (Figure 4c). *Renilla* luciferase expression was significantly reduced in BHK-21 cells when the construct was co-transfected with miR-29a, miR-29b, or miR-29c (Figure 4d). However, miR-29 had no appreciable inhibitory effect on a mutated *Akt3* 3' UTR dual-luciferase construct (containing 2-base substitutions in the binding site of miR-29) (Figure 4d). Likewise, a 2-base mutation introduced into the seed sequence of miR-29 (29mut) abrogated any inhibition of the *Akt3* 3' UTR construct, although the significant suppression effect was still observed in the cells transfected with the pooled miR-29 mimics (Figure 4e). These results demonstrated the specific inhibition of *Akt3* expression by miR-29.

The inhibitory effect of miR-29a/b/c on endogenous *Akt3* protein expression was evident in C2C12 myoblasts, even though no significant inhibition was detected at the mRNA level (Figure 4f). The impact of miR-29 on the protein expression of other members of the Akt family, and p85a, a regulatory subunit of phosphatidylinositol 3-kinase (PI3K), was evaluated by western blotting. *Akt1* and *Akt2* were not inhibited in C2C12 myoblasts transfected with pooled miR-29 mimics (Figure 4h). Protein p85 $\alpha$ , previously shown to be a

target of miR-29 in HeLa cells,<sup>22</sup> like *Akt3* protein, was downregulated by miR-29 (Figure 4h). Therefore *Akt3* protein expression was directly inhibited by miR-29.

**Inverse expression relationship between miR-29 and *Akt3* protein *in vivo*.** The functional relationship between the miR-29 and *Akt3* expression in Balb/c mouse skeletal muscle was examined. *Akt3* mRNA expression in murine muscle showed a rising trend with age, from 2-day to 12-week-old animals (Figure 5a). However, *Akt3* protein expression was significantly downregulated with age, as determined by western blotting (Figure 5b). Immunohistochemical detection of *Akt3* in murine muscle sections corroborated the finding of reduced *Akt3* protein expression with age across the same age range (Figure 5c). The post-transcriptional (protein) regulation of *Akt3* by miR-29 therefore showed an inverse relationship of expression, such that the increase of miR-29 in skeletal muscle with age (Figures 1a and b) coincided with the corresponding decrease in *Akt3* protein expression (Figure 5d).

***Akt3* inhibited myogenic differentiation and promoted proliferation of C2C12 cells.** Transfection of siAkt3 into C2C12 myoblasts substantially knocked down the expression of *Akt3* mRNA and protein (Figure 6a), which resulted in increased cell arrest in the G0/G1 stage and decreased proliferation rate (Figures 6b and c, and Supplementary Figure 2), an outcome similar to miR-29 overexpression (Figures 2a and b). Overexpression of *Akt3* by transfection of *Akt3*-3.1, as evidenced by raised expression of *Akt3* mRNA and protein (Figure 6d), resulted in no apparent change in cell cycle (Figure 6e). However, there was a significant reduction in the number of myotubes inferred by the immunofluorescence detection of fast skeletal myosin expression when *Akt3* was overexpressed (Figure 6f).



**Figure 2** miR-29 repressed proliferation of C2C12 cells. C2C12 cells were transiently transfected with pooled miR-29a/b/c mimics or scrambled NC, and the cell cycle phase (a) and proliferation index (b) were analyzed by propidium iodide flow cytometry. (c) C2C12 cells were transfected with pooled miR-29 mimics or NC upon the cell index reaching 1.0, and cell growth dynamics were continuously monitored using the xCELLigence system. (d) miR-29bc-C1 or pEGFP-C1 plasmids were transfected into C2C12 myoblasts and 24 h later, the cells were processed for Q-PCR detection of miR-29 b/c. Expression of miR-29c was normalized by U6. (e) After transfection with miR-29bc-C1 or pEGFP-C1, C2C12 cells were stained with EdU. Cells dual positive for EdU and GFP are indicated by white arrows (left graph), and the percentage of dual positive cells was determined by flow cytometry (right graph). The scale bar stands for 100  $\mu$ m. The results are presented as mean  $\pm$  S.E.M. (three independent replicates per group). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$

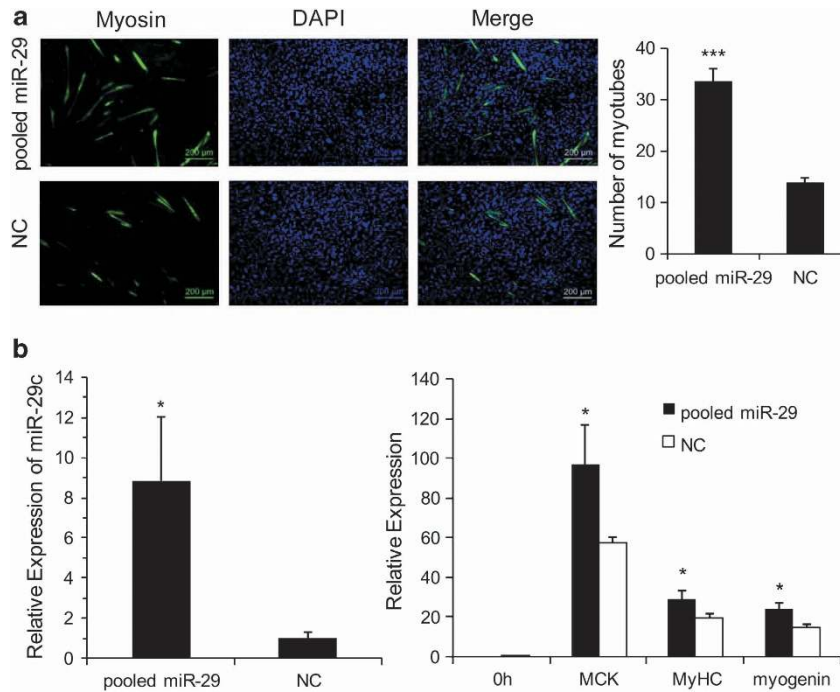
Myoblast differentiation marker genes were also detected at 48 h of differentiation by Q-PCR. Expressions of the *MyHC IIId* and *MCK* genes were significantly downregulated in cells transfected with Akt3-3.1, relative to cells transfected with pcDNA-3.1 empty plasmid (Figure 6g). Together, these results show that Akt3 promoted myoblast proliferation at the expense of muscle differentiation.

**Akt3 could restore cell cycle and block myoblasts' differentiation in miR-29 overexpression.** Since Akt3 was shown to be a target of miR-29 during cell cycle and differentiation, its principal role was further evaluated by its overexpression in C2C12 myoblasts in the presence of transfected pooled miR-29, which resulted in increased number of cells that progressed from G0/G1 stage to S and M stages (Figures 7a and b and Supplementary Figure 3). As expected, the presence of pooled miR-29 with control empty plasmid (pCDNA-3.1) in C2C12 myoblasts led to increased cell arrest at the G0/G1 stage. Furthermore, the

increase in muscle gene expression (*MCK*, *MyHC IIId*, and *myogenin*) as a consequence of miR-29 transfection was attenuated by the coexpression of Akt3 (Figure 7c). Therefore, the overexpression of Akt3 could restore cell cycle or proliferation index, over-riding the differentiation effect of miR-29.

## Discussion

miRNAs were recently discovered as a class of regulators in skeletal muscle development in mice, rats, pigs, and human.<sup>7-11</sup> The most notable muscle-specific miRNAs are miR-1, -133, and -206. miR-1 is highly expressed in adult pig muscle. miR-133 is moderately expressed in adult muscle, but barely detectable in fetal and neonate muscles. miR-206 is abundant in porcine skeletal muscle of different ages, but is lowly expressed in proliferating satellite cells.<sup>8</sup> The principal effects of miRNAs are on the modulation of proliferation and differentiation of muscle cells. For instance, miR-206



**Figure 3** Promotion of C2C12 cell differentiation by miR-29. (a) C2C12 myoblasts transfected with pooled miR-29 mimics or NC were differentiated for 3 days. Immunofluorescence detection with fast myosin antibody was used to identify myotubes (green). The nuclei were stained blue with DAPI. The scale bar stands for 200  $\mu$ m. The number of myotubes is presented as mean  $\pm$  S.E.M. (14 random fields were captured for each treatment group). (b) C2C12 myoblasts transfected with pooled miR-29 mimics or NC were induced to undergo differentiation for 48 h. Subsequent detection of miR-29c, *myogenin*, *MyHC IId*, and *MCK* was determined by Q-PCR. The results are presented as mean  $\pm$  S.E.M. (three independent replicates per group). \* $P < 0.05$ ; \*\*\* $P < 0.001$

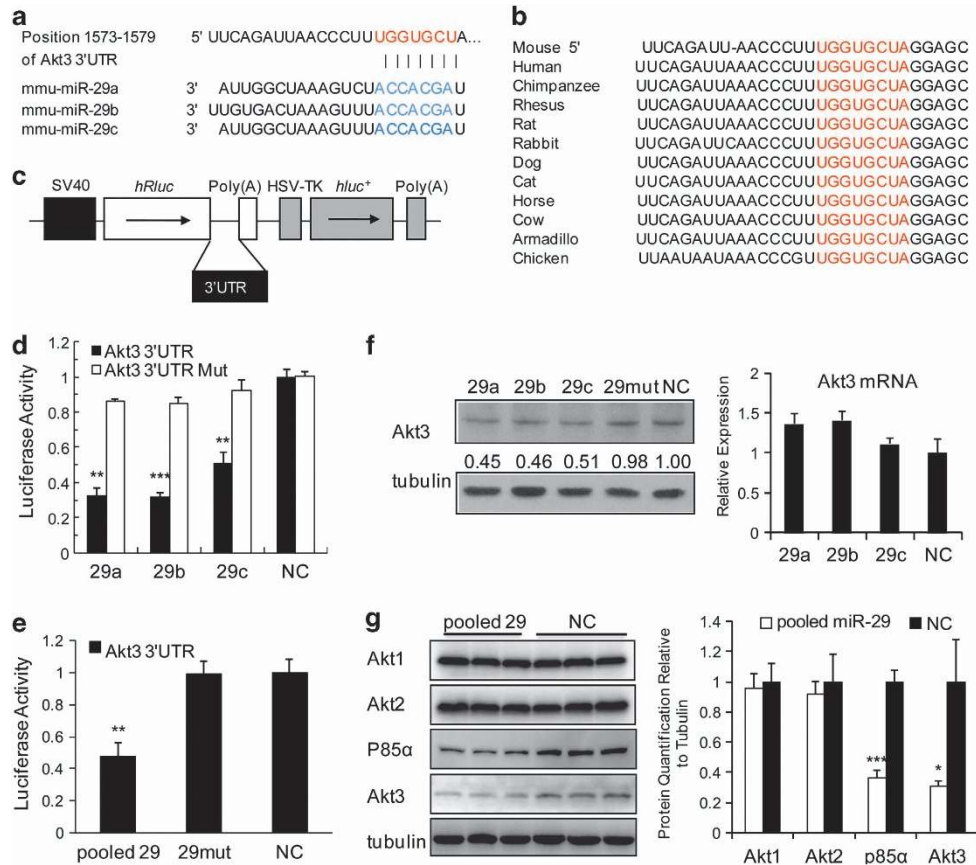
promotes differentiation by inhibiting *Id1-3* and *MyoR*, both of which are inhibitors of myogenic transcription factors.<sup>13</sup> miR-1 promotes myogenesis by repressing *HDAC4*, whereas miR-133 enhances myoblast proliferation by inhibiting serum response factor.<sup>14</sup> Besides, miR-100, -192, -335, -214, and -221/222 were also proved to be participating in muscle proliferation and differentiation.<sup>11,12,45</sup> Based on these, miRNAs could have therapeutic application.<sup>3</sup>

We previously showed that miR-29 expression is developmentally upregulated in porcine skeletal muscle from fetal to adult growth.<sup>26</sup> Similar developmental expression of miR-29 was observed in mice and humans.<sup>27,32,33</sup> These results suggest that miR-29 is transcriptionally regulated by similar mechanisms in the skeletal muscle among mammals. Activated NF- $\kappa$ B has been shown to stimulate the expression of YY1 transcription factor that in turn binds to a consensus site upstream of the miR-29b-2/c cluster, thereby inhibiting its transcription.<sup>33,46</sup> Additionally, others have reported direct transcriptional suppression of the miR-29b-1/miR-29a cluster promoter by NF- $\kappa$ B.<sup>47</sup> Activation of NF- $\kappa$ B was shown to decrease during mouse skeletal muscle differentiation.<sup>48</sup> Indeed, NF- $\kappa$ B activation signaled by a range of inflammatory cytokines (including tumor necrosis factor-like weak inducer of apoptosis) could inhibit myogenic differentiation.<sup>49,50</sup> Conversely, inhibition of NF- $\kappa$ B stimulates skeletal muscle differentiation and regeneration, making NF- $\kappa$ B a potential therapeutic target for Duchenne muscular dystrophy and other muscle-wasting disorders.<sup>51–53</sup> Additionally, putative binding sites of *MyoD*, *myogenin*, and *Mef2* are present upstream of the miR-29b-2/c cluster,<sup>36</sup>

indicating their possible roles in myogenic differentiation via miR-29 regulation.

miR-29 is widely recognized as an activator of tumor suppression and apoptosis. In cancer biology, *DNMT3A*, *DNMT3B*, *p85 alpha*, *CDC42*, *Bcl-2*, *Mcl-1*, *CCND2*, and *E2F7* are established targets of miR-29.<sup>21–24,54</sup> So far, the effector targets of miR-29 that mediate skeletal muscle differentiation are still needed to be identified. In the present study, miR-29 was shown to inhibit myoblast proliferation by targeting *Akt3*. All members of the Akt family are known to be intimately involved in numerous biological programs, whereas *Akt3* isoform is specifically involved in some cancers such as melanomas.<sup>34–38,40</sup> There is evidence for *Akt3* in the regulation of cell growth in VSMCs and heart.<sup>42–44</sup> Presently, we demonstrated that *Akt3* can promote myoblast proliferation and is a direct target of miR-29. Knocking down *Akt3* expression mimicked the overexpression of miR-29 in prohibiting the proliferation of C2C12 cells (Figures 6b and c). In addition, *p85 $\alpha$* , another target of miR-29 in tumor suppression, was inhibited in the miR-29-overexpressed myoblasts<sup>22</sup> (Figure 4g). Collectively, miR-29 inhibits myoblast proliferation by targeting the proliferation-associated genes *Akt3* and *p85 $\alpha$* .

Previous work has shown that miR-29 targets collagen, *Mfap5*, *Lims1*, polycomb group members, and other key factors such as *YY1*, *HDAC4*, and *Rybp*, to inhibit myoblast conversion to myofibroblasts, thereby promoting myogenesis in an indirect manner.<sup>28,30–33</sup> Conversely, decrease in miR-29 could suppress myogenesis in chronic kidney-disease mice.<sup>10</sup> We showed here that miR-29-mediated silencing of *Akt3* in



**Figure 4** Akt3 3' UTR as a target of miR-29. The predicted binding site of miR-29 in the 3' UTR of Akt3 (a) is highly conserved among vertebrates (b). The Akt3 3' UTR was inserted into the dual-luciferase reporter vector psi-CHECK2 at the 3' end of the *Renilla* luciferase gene (*hRluc*). The constitutive firefly luciferase (*hLuc+*) expression was used as an internal normalization control (c). The Akt3 3' UTR or Akt3 3' UTR Mut (containing a 2-base substitution in the binding site for miR-29) construct was co-transfected with miR-29a, miR-29b, miR-29c, or NC, as indicated, into BHK-21 cells, and normalized *Renilla* luciferase activity was determined (d). Pooled miR-29 mimics, miR-29 mut (housing a 2-base mutation), or NC was co-transfected with the Akt3 3' UTR dual-luciferase vector, as indicated, into BHK-21 cells, and normalized *Renilla* luciferase activity was assayed (e). Akt3 protein expression in C2C12 myoblasts was detected at 48 h post transfection with miR-29a/b/c, miR-29 mut, or NC. Comparable tubulin levels served as loading controls in all western blotting assays (f). (g) Protein detection of members of the Akt3 family and p85α in C2C12 myoblasts transfected with pooled miR-29a/b/c and NC. Results are presented as mean ± S.E.M. (three independent replicates per group). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$

myoblasts could delay cell proliferation, and that *Akt3* overexpression suppressed myoblast differentiation. In addition, we found that the cell cycle and differentiation effects of miR-29 could be significantly reduced in the presence of *Akt3* overexpression (Figure 7). This is a more direct effect of miR-29-induced differentiation, compared with the indirect effect of inhibition of the above-mentioned fibrogenic route.

In conclusion, our study has provided new insights into the fundamental role of miR-29 in the promotion of muscle differentiation, at least in part, by inhibiting *Akt3* at the post-transcriptional level to mediate its myogenic effects. miR-29 and its target genes may therefore be potential candidates for intervention aimed at improving muscle growth in farm animals, as well as in skeletal muscle-wasting conditions.

#### Materials and Methods

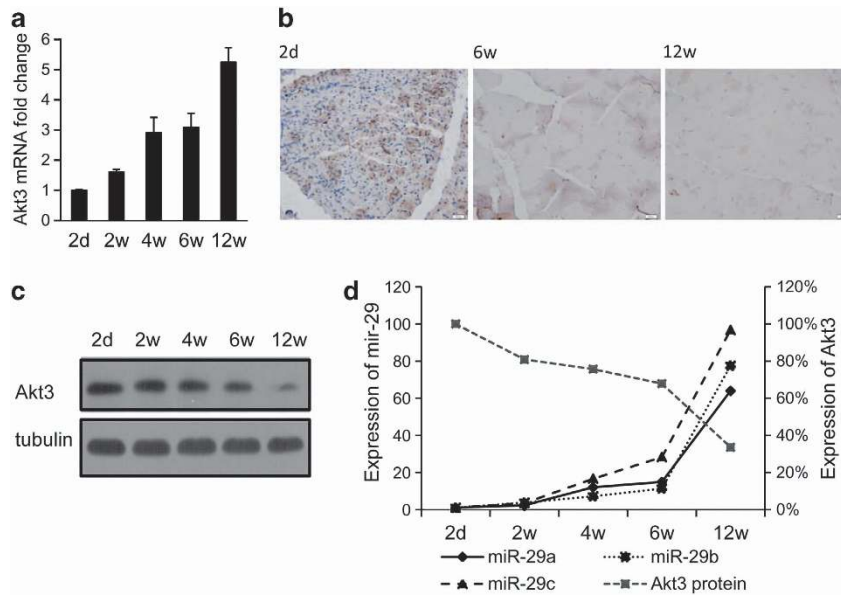
**Animals and cells.** Male Balb/c mice were obtained from Hubei Centre for Disease Control and Prevention. Five groups of mice, three per group, at different ages were used: 2 days, 2 weeks, 4 weeks, 6 weeks, and 12 weeks of age. Three muscle samples from the hindleg of each mouse were collected. Two muscle samples from each mouse were frozen in liquid nitrogen, stored at  $-80^{\circ}\text{C}$  for use in real-time Q-PCR and western blotting. The third sample was stored in 4%

paraformaldehyde for immunohistochemistry. The experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (1996), and protocols approved by The Hubei Province for Biological Studies Animal Care and Use Committee.

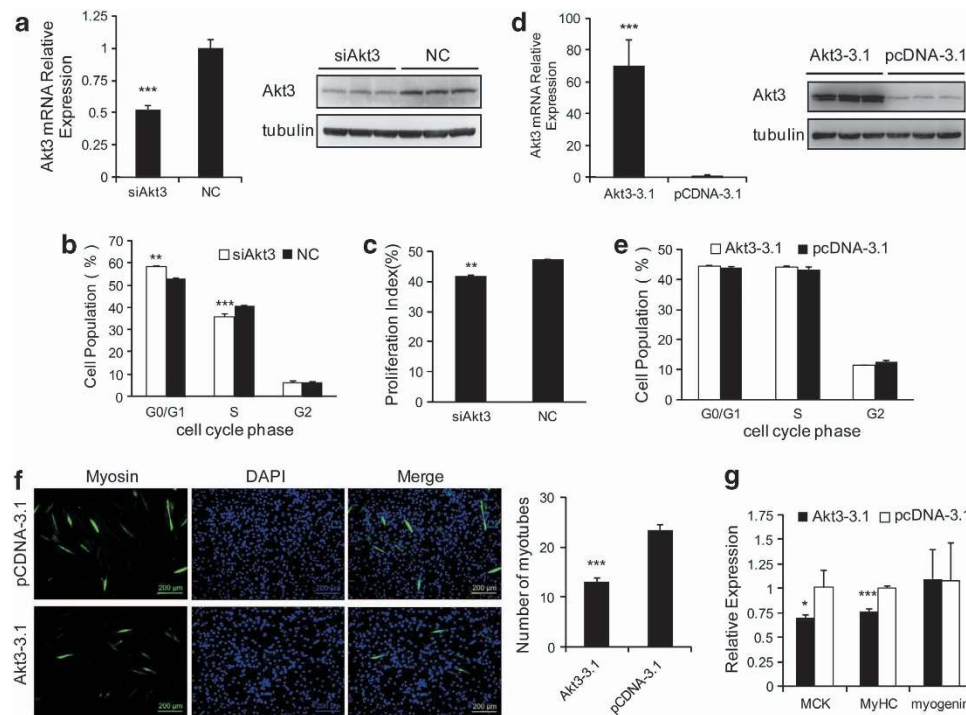
Mouse C2C12 myoblasts were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT, USA) with 10% (v/v) fetal bovine serum. To induce differentiation, culture medium was switched to DMEM with 2% horse serum.

**Quantitative (Q)-PCR.** Total RNA (including miRNA) was extracted from tissues or cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Fermentas, Ottawa, ON, Canada). The concentration and quality of RNA were assessed by the NanoDrop 2000 (Thermo, Waltham, MA, USA) and denatured gel electrophoresis. Reverse transcription was performed using M-MLV reverse transcriptase (Invitrogen). Random primers, oligo (dT)<sub>12-18</sub> or miRNA-specific primers were added to initiate cDNA synthesis. The Q-PCR reaction was carried out in the LightCycler 480 II (Roche, Basel, Switzerland) system, and the reaction mixture used LightCycler 480 SYBR Green I Master (Roche). The sequence of miRNA-specific primers and Q-PCR detection primers can be found in Supplementary Table 1.

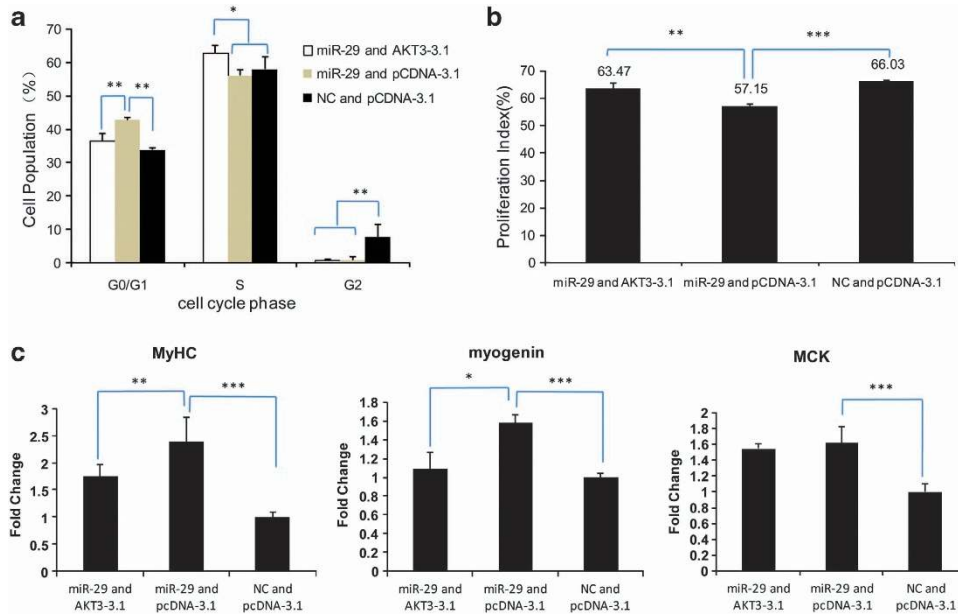
**Immunofluorescence.** After transfection and differentiation, C2C12 cells cultured in six-well plate were washed with phosphate-buffered saline (PBS) twice, and fixed in 4% paraformaldehyde for 15 min. They were then washed twice with



**Figure 5** Inverse *in vivo* expression relationship between miR-29 and Akt3. Rising mRNA expression of Akt3 in thigh muscles of postnatal Balb/c mice of ages 2 days, 2 weeks, 4 weeks, 6 weeks, and 12 weeks (a). Akt3 expression was normalized to tubulin expression. Reduction in Akt3 protein expression in thigh muscles of Balb/c mice with increasing age. Immunohistochemical detection of Akt3 protein in Balb/c thigh muscles of different ages (b). Scale bar = 20  $\mu$ m. Inverse relationship with age between endogenous miR-29a/b/c and Akt3 protein expression in postnatal mouse muscles (c). Relative expression of Akt3 was normalized to tubulin levels. Expression of miR-29 was normalized to U6 levels (d). The results are presented as mean  $\pm$  S.E.M. (three independent individuals per group)



**Figure 6** Akt3 inhibited differentiation and promoted proliferation of C2C12 cells. Transfection of siAkt3 into C2C12 myoblasts knocked down the expression of Akt3 mRNA (left) and protein (right), detected by Q-PCR and western blotting (a), which resulted in raised cell population in the G0/G1 phase and in reduced cell population in the S phase (b), and overall reduction in cell proliferation (c) as determined by flow cytometry. Overexpression of Akt3 via transfection of Akt3-3.1 expression vector into C2C12 myoblasts was detected as raised Akt3 mRNA (left) and protein (right) levels (d). Overexpression of Akt3 in C2C12 myoblasts had no appreciable effect on cell interphase cycle (G0/G1, S, and G2), as determined by flow cytometry (e). Immunofluorescence detection of fast skeletal myosin (green myotubes) in Akt3 expression plasmid-transfected C2C12 cells after 3 days of differentiation (f). Nuclei were stained blue with DAPI. Scale bar = 200  $\mu$ m. Number of myotubes graphically presented as mean  $\pm$  S.E.M. (right). C2C12 myoblasts transfected with Akt3-3.1 or pcDNA-3.1 were induced to undergo differentiation for 48 h. Subsequent RNA detection of *myogenin*, *MyHC IId*, and *MCK* was determined by Q-PCR, normalized to *tubulin* expression (g). All the Q-PCR and flow cytometry results are presented as mean  $\pm$  S.E.M. (three independent replicates per group). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$



**Figure 7** Akt3 overexpression could overcome the cell cycle and differentiation effects of miR-29 in C2C12 cells. C2C12 myoblasts were transiently co-transfected with pooled miR-29 and Akt3 expression vector without the 3' UTR miR-29 recognition site (Akt3-3.1), miR-29 and pCDNA-3.1, or NC and pCDNA-3.1 as control, followed by 3 days of differentiation. The cell cycle phase (a) and proliferation index (b) were analyzed by propidium iodide flow cytometry. RNA expression of *myogenin*, *MyHC*, and *MCK* from corresponding transfections was quantified by Q-PCR (c). Fold change calculation was with reference to control transfection (NC and pCDNA-3.1). The results are presented as mean  $\pm$  S.E.M. (three independent replicates per group). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$

PBS, incubated in ice-cold 0.25% Triton X-100 at room temperature for 10 min, and further washed thrice. The cells were incubated in blocking solution (3% bovine serum albumin, 0.3% Triton X-100, 10% fetal bovine serum in PBS) at 4 °C overnight. The next day, after three washes with PBS, the cells were incubated in primary monoclonal anti-myosin (skeletal, fast) antibody (Sigma Life Science, St Louis, MO, USA) at room temperature for 1 h. The cells were washed three times, and incubated with secondary Alexa Fluor 488 IgG, IgM (H + L) antibody (Invitrogen). After 1 h, the secondary antibody was removed, and the cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) in the dark. After washing three times, images were captured with a Nikon ECLIPSE TE2000-S (Nikon, Tokyo, Japan).

**Northern blotting.** Total RNA was separated on 15% urea-PAGE gel and transferred to positively charged nylon membrane (PerkinElmer, Waltham, MA, USA) using Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA, USA). Oligonucleotide probes used for hybridization with miRNA were labeled with [<sup>32</sup>P] at their 5'-end. 5s rRNA was used as the loading control. The sequences of the probes were: miR-29c: 5'-TAACCGATTCAAATGGTGCTA-3'; 5s rRNA: 5'-CGGTATCCAGGCGGTCT-3'.

**Cloning for dual-luciferase assay.** The psiCHECK-2 dual-luciferase reporter vector (Promega, Madison, WI, USA) housing the 3' UTR of Akt3, which was *XhoI* and *NotI* cloned to the 3'-end of the *Renilla* gene was used to examine the effect of miR-29 on *Renilla* production. Akt3 3' UTR was amplified with the use of forward primer 5'-CGCTGAGACTGATTCCTG-3' and reverse primer 5'-CTT TACACCCAGCAGTC-3'. Site-directed mutation was used to introduce a 2-base substitution into the miR-29-binding site of Akt3 3' UTR (Akt3 3' UTR Mut) by mutagen primers 5'-AGCTCCTAGCCACAAGGGTAAATC-3' and 5'-ACCCCTTG TGGCTAGGAGCTGACAC-3'. A 2-base substitution in the seed sequence of miR-29 was introduced to create mutant forms of miR-29 (miR-29 Mut) when synthesized. The miRNA mimics and 3' UTR dual-luciferase vector were co-transfected into cells using Lipofectamine 2000 (Invitrogen). Medium was replaced with fresh growth medium 6 h after transfection. Cells were incubated for 24 h, and assayed with the Dual-Luciferase Reporter Assay System (Promega).

**Transfections.** One day before transfection, cells were plated in growth medium without antibiotics. Cells were transfected with miRNA mimics or NC-nonspecific miRNA (GenePharma, Shanghai, China) using the Lipofectamine

2000 transfection reagent (Invitrogen). Opti-MEM I Reduced Serum Medium (Gibco, Grand Island, NY, USA) was used to dilute Lipofectamine 2000 and nucleic acids. Transfection procedure was performed according to the manufacturer's instructions. The miRNA mimics were synthesized as duplexes. The miRNA sequences were: mmu-miR-29a: 5'-UAGCACCAUCUGAAAUCGG UUA-3', 3'-ACCGAUUUCAGAUGGUGCUAAU-5'; mmu-miR-29b: 5'-UAGCACCA UUUUAAAUCAGUGUU-3', 3'-CACUGAUUUCAAUGGUGCUAAU-5'; mmu-miR-29c: 5'-UAGCACCAUUUGAAAUCGGUUA-3', 3'-ACCGAUUUCAAAUGGU GCUAUU-5'; mut-miR-29: 5'-UAGACCAUUUGAAAUCGGUUA-3', 3'-UAACCG AUUUCAAAUGGUCUA-5'. The NC was similar to miRNA in composition, but not in specificity: 5'-UUCUCCGACGUGUCACGUTT-3', 3'-TTAAGAGGCUUGCA CAGUGCA-5'. The sequence of siAkt3 was 5'-AACCAGGATCATGAGAACTT-3'.

### Cell proliferation analysis

**Cell cycle flow cytometry.** Thirty six hours after transfection, trypsinised cells were fixed in 70% (v/v) ethanol overnight at -20 °C overnight. Following incubation in 50  $\mu$ g/ml propidium iodide solution (which contains 100  $\mu$ g/ml RNase A and 0.2% (v/v) Triton X-100) for 30 min at 4 °C, the cells were analyzed using a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) and the ModFit software (Verity Software House, Topsham, ME, USA). The proliferative index (PI) stands for the proportion of mitotic cells from a total of 20 000 cells examined.

**EdU assay.** Six hours after transfection with pEGFP-C1 or miR-29bc-C, C2C12 cells were cultured with fresh growth medium containing EdU (final concentration, 10  $\mu$ M) for 24 h. Cells were trypsinized, fixed with 4% paraformaldehyde, permeabilized, and analyzed by flow cytometry to detect the population of GFP-positive cells and EdU-stained cells.

**xCELLigence real-time cell analyzer.** C2C12 cells were seeded on a 16-well E-Plate and allowed to grow for 24 h. When the cell index reached 1.0 ~ 1.5, they were transfected with miRNA mimics using the FuGENE HD transfection reagent (Roche). Cell growth and proliferation were monitored by an xCELLigence RTCA DP instrument (Roche).

**Western blotting.** Protein lysates were generated with the use of M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, followed by protein transfer to polyvinylidene fluoride membranes (Millipore,



Billerica, MA, USA) using Mini Trans-Blot Cell (Bio-Rad). Primary antibodies specific for Akt1, Akt2,  $\beta$ -tubulin (Cell Signaling Technology, Boston, MA, USA; 1 : 1000 dilution), Akt3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1 : 1000 dilution), p85 $\alpha$  (Abcam, Hong Kong, China; 1 : 1000 dilution), and HRP-labeled anti-rabbit/mouse/goat IgG secondary antibody (Beyotime, Jiangsu, China; 1 : 2000 dilution) were used to detect protein expression.

**Immunohistochemistry.** After fixation with 4% paraformaldehyde, the skeletal muscle samples were embedded in paraffin, and 6- $\mu$ m thick serial sections were made. The streptavidin-biotin-peroxidase complex method was used to determine the expression of Akt3. The primary antibody was rabbit anti-Akt3 (Cell Signaling Technology, 1 : 200 dilution), and the secondary antibody was biotinylated sheep anti-rabbit antibody. Diaminobenzidine and Harris' hematoxylin were used to counterstain the sections. Images were captured using Olympus BH-2 System (Olympus, Shinjuku, Tokyo, Japan).

**pcDNA-3.1-Akt3 expression vector without the 3' UTR miR-29 recognition site (Akt3-3.1).** Akt3-coding sequence was amplified by the forward primer 5'-GGATCACAGATGCAGCTACC-3' and reverse primer 5'-GTAGAAAGGCAACCTCCACAC-3', and inserted into pcDNA-3.1. The primary transcript of the mmu-miR-29b-2 and mmu-miR-29c cluster was amplified by the forward primer 5'-CTACCGACACTATGCATCTTG-3' and reverse primer 5'-AGTGATCTCTAACCTTGGCAG-3', and cloned into the pEGFP-C1 vector. A stop codon was added between the EGFP gene and multiple cloning sites to stop the translation of EGFP.

**Statistical analysis.** All results are presented as mean  $\pm$  S.E.M. based on at least three replicates for each treatment. Unpaired Student's *t*-test was used for *P*-value calculations.

**Ethics standards.** The experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (1996), and protocols approved by the Hubei Province for Biological Studies Animal Care and Use Committee.

### Conflict of Interest

The authors declare no conflict of interest.

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