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Leucine promotes porcine myofibre type transformation from fast-twitch to slow-twitch through the protein kinase B (Akt)/forkhead box 1 signalling pathway and microRNA-27a

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Abstract

Muscle fibre types can transform from slow-twitch (slow myosin heavy chain (MyHC)) to fast-twitch (fast MyHC) or vice versa. Leucine plays a vital effect in the development of skeletal muscle. However, the role of leucine in porcine myofibre type transformation and its mechanism are still unclear. In this study, effects of leucine and microRNA-27a (miR-27a) on the transformation of porcine myofibre type were investigated *in vitro*. We found that leucine increased slow MyHC protein level and decreased fast MyHC protein level, increased the levels of phospho-protein kinase B (Akt)/Akt and phospho-forkhead box 1 (FoxO1)/FoxO1 and decreased the FoxO1 protein level. However, blocking the Akt/FoxO1 signalling pathway by wortmannin attenuated the role of leucine in porcine myofibre type transformation. Over-expression of miR-27a decreased slow MyHC protein level and increased fast MyHC protein level, whereas inhibition of miR-27a had an opposite effect. We also found that expression of miR-27a was down-regulated following leucine treatment. Moreover, over-expression of miR-27a repressed transformation from fast MyHC to slow MyHC caused by leucine, suggesting that miR-27a is interdicted by leucine and then contributes to porcine muscle fibre type transformation. Our finding provided the first evidence that leucine promotes porcine myofibre type transformation from fast MyHC to slow MyHC via the Akt/FoxO1 signalling pathway and miR-27a.

Key words: Leucine: Porcine myofibre type transformation: Protein kinase B/forkhead box 1 signalling pathway: MicroRNA-27a

Muscle fibre, as the basic unit of muscle composition, is one of the main factors affecting the meat quality of livestock. It has been reported that the amount of muscle fibres is invariable after livestock birth; however, the muscle fibre types transform continuously under the influence of the external environment[1]. According to the polymorphism of myosin heavy chain (MyHC), muscle fibre is divided into four different types including type I with MyHC I (slow MyHC), type IIa with MyHC IIa, type IIx with MyHC IIx and type IIb with MyHC IIb[2,3]. MyHC IIa, MyHC IIx and MyHC IIb are collectively called fast MyHC. Under the influence of the external environment, muscle fibre types can transform from slow-twitch (slow MyHC) to fast-twitch (fast MyHC) or vice versa[4]. The transformation of muscle fibres can be regulated by myogenic factors, such as myogenin and myocyte enhancer factor-2 (MEF2)[5,6] and also by a variety of signalling pathways. For example, it has been reported that serine phosphorylation and protein expression of protein kinase B (Akt), a serine/threonine protein kinase, are greater in oxidative muscle compared with glycolytic muscle[7,8]. Forkhead box 1 (FoxO1) is one of the downstream transcription factors of the phosphoinositide 3-kinase (PI3K)/Akt signalling pathway[9]. Kamei *et al.*[10] reported that over-expression of FoxO1 decreased the type I fibre-related gene expression and the number of type I fibres in mouse skeletal muscle.

Leucine is a branched-chain amino acid that mainly metabolises in skeletal muscle because the key enzymes of leucine metabolism are mainly distributed in skeletal muscle[11]. The balance of protein synthesis and degradation is crucial to the protein deposition of skeletal muscle. It is well known that leucine can stimulate muscle protein synthesis and suppress protein degradation[12,13]. Besides the regulation of protein turnover, leucine also plays a vital role in the process of myogenesis[14,15]. During the process of myogenesis, the formation of muscle fibre is an ultimate integral part, which is regulated by nutritive or non-nutritive factors. It has been reported that

Abbreviations: Akt, protein kinase B; FoxO1, forkhead box 1; miR-27a, microRNA-27a; miRNA, microRNA; MyHC, myosin heavy chain; PI3K, phosphoinositide 3-kinase.

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leucine increased the expression of oxidative muscle fibre marker genes, including sirtuin 1 (SIRT1), AMP-activated kinase (AMPK) and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), in C2C12 myotubes and mice skeletal muscle[16–18]. Vaughan *et al.*[19] also found that treatment with leucine promoted oxidative preference and capacity of skeletal muscle cells. Although the role of leucine in oxidative fibre formation is well established[16–19], the effect of leucine on skeletal muscle fibre type transition is still unclear.

MicroRNA (miRNA) are highly conserved, non-coding RNA that have been regarded as important regulators in a variety of biological processes. Regulation of miRNA expression by nutrients has been recognised gradually[20–22]. Our previous studies have provided evidence that microRNA-27a (miR-27a) promotes proliferation and differentiation of C2C12 cells[23,24]. However, the role of miR-27a in skeletal muscle fibre type transition and whether miR-27a contributes to leucine's function in skeletal muscle fibre type transition are still unknown.

In this study, we investigated the role of leucine in porcine skeletal muscle fibre type transition *in vitro*. Its underlying mechanism was also investigated.

Methods

Ethics statement

All animal procedures were performed according to protocols approved by the Animal Care Advisory Committee of Sichuan Agricultural University.

Culture and treatment of porcine myoblasts

Porcine myoblasts were isolated form 3-d-old male Duroc × Landrace × Yorkshire pigs. The cells were isolated as described previously[25]. When the density reached about 80 % confluence, the cells were shifted to differentiation medium containing Dulbecco's modified Eagle's medium (DMEM)/F12 with 2 % horse serum (Invitrogen). After 72 h of differentiation, porcine myotubes cultured in differentiation medium were treated with different concentrations of leucine (0, 0·5, 2 and 4 mM) for 4 d.

Transfection of microRNA mimics and inhibitor

When porcine myoblasts reached about 80 % confluence, the cells were induced to differentiate in differentiation medium, which was replaced every day. In all, 3 d later, the cells were transfected with 100 nM miR-27a mimics, 100 nM miRNA mimics negative control, 200 nM miR-27a inhibitor or 200 nM miRNA inhibitor negative control (GenePharma). The process of transfection was conducted using the Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. In all, 24 h after transfection, the lipofectamine complex was removed, and the cells were continued to culture for 3 d.

Real-time quantitative PCR

Total RNA was extracted from cultured porcine myotubes using RNAiso Plus reagent (TaKaRa). RNA concentrations were determined by Nano-Drop ND 2000c Spectrophotometer

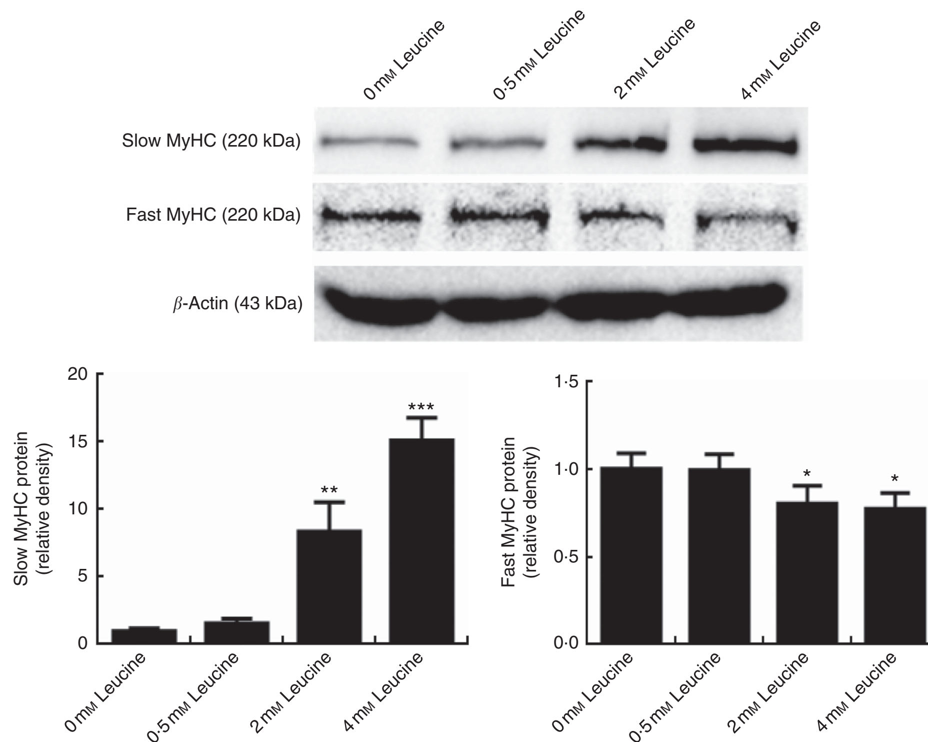

Fig. 1. Leucine promotes porcine myofibre type transformation from fast myosin heavy chain (MyHC) to slow MyHC. After 72 h of differentiation, porcine myotubes cultured in differentiation medium were treated with different concentrations of leucine (0, 0·5, 2 and 4 mM) for 4 d. Slow MyHC and fast MyHC protein levels were determined by Western blot analysis. Equal loading was monitored with anti-β-actin antibody. Values are means of the densitometry results from three independent experiments, with their standard errors represented by vertical bars. * $P<0·05$, ** $P<0·01$ and *** $P<0·001$ as compared with control.

(Thermo Scientific). In all, 10 ng of total RNA from each sample was reverse-transcribed using TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems). The expression of miRNA was determined using the TaqMan MicroRNA Assay kit (Applied Biosystems) in CFX96 Real-Time PCR Detection System (Bio-Rad). Relative expression of miRNA was quantified using $2^{-\Delta\Delta C_t}$ method and was normalised by U6 small nuclear RNA.

Western blot

Protein samples were extracted from adhered porcine myotubes using radioimmunoprecipitation assay (RIPA) buffer (Pierce). Briefly, cells were washed once with PBS and then lysed in lysis buffer for 30 min at 4°C. The cell lysates were centrifuged at 14 000 ***g*** for 15 min at 4°C, and liquid supernatant was collected. Bicinchoninic acid (BCA) protein assay kit (Pierce) was used to determine the protein concentration by Nano-Drop ND 2000c Spectrophotometer (Thermo Scientific). In all, 20 µg of protein extractions were separated by 12 % SDS–polyacrylamide gel and then transferred to polyvinyldifluoride (PVDF) membrane (Millipore) using wet Trans-Blot System (Bio-Rad). After blocking with TRIS-buffered saline Tween 20 (TBS/T) containing 5 % bovine serum albumin for 2 h at room temperature, the membranes were incubated with primary antibodies at 4°C overnight against slow MyHC (1:400; Sigma, catalogue no. M8421), fast MyHC (1:400; Sigma, catalogue no. M4276), Akt (1:1000; Cell

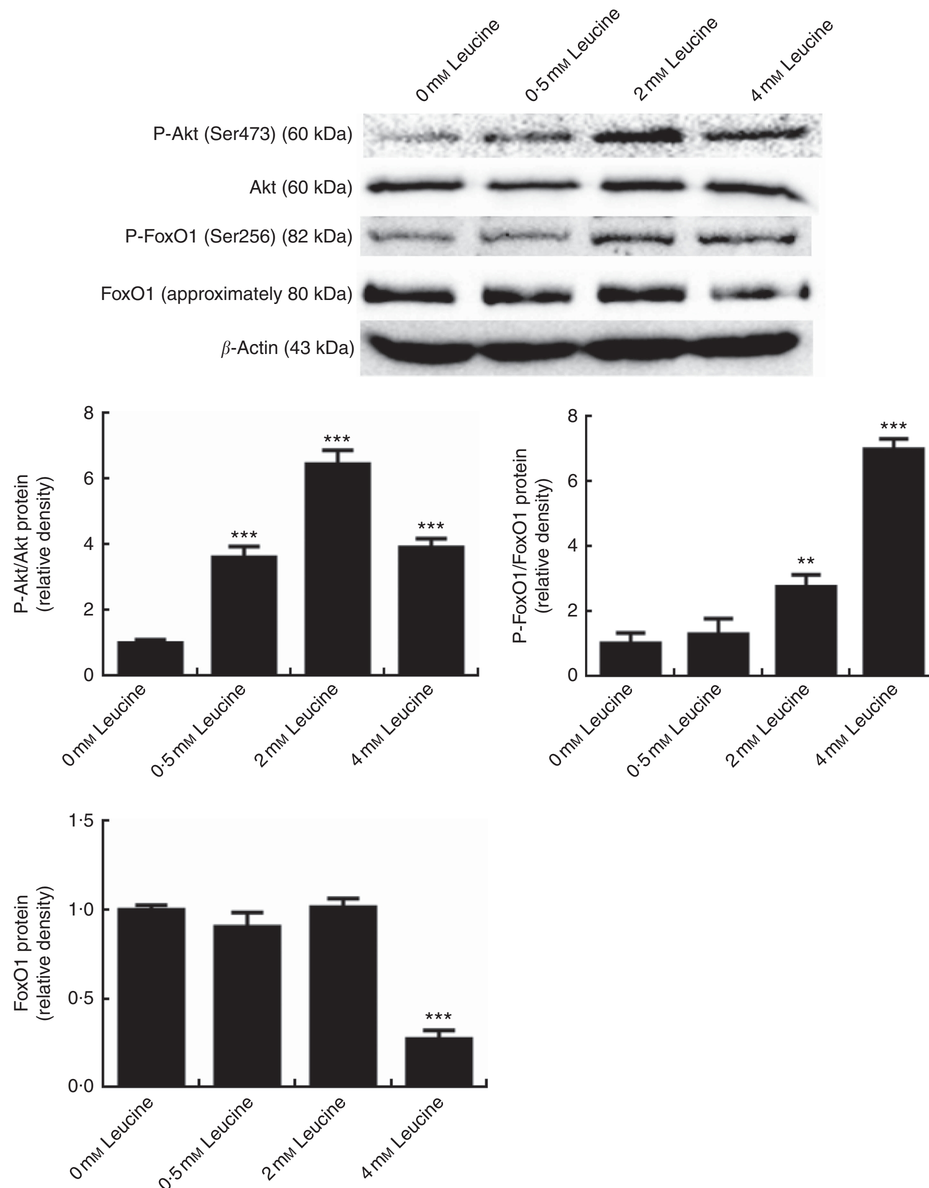

Fig. 2. Effect of leucine on the protein kinase B (Akt)/forkhead box 1 (FoxO1) signalling pathway. Samples were prepared as described in Fig. 1. Akt, phospho-Akt (P-Akt), phospho-forkhead box 1 (P-FoxO1) and FoxO1 protein levels were determined by Western blot analysis. Equal loading was monitored with anti-*β*-actin antibody. Values are means of the densitometry results from three independent experiments, with their standard errors represented by vertical bars. ** $P<0·01$ and *** $P<0·001$ as compared with control.

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Signaling, catalogue no. 9272), phospho-Akt (P-Akt, 1:1000; Cell Signaling, catalogue no. 9271), FoxO1 (1:1000, Cell Signaling, catalogue no. 2880), phospho-forkhead box 1 (P-FoxO1, 1:1000, Cell Signaling, catalogue no. 9461) or β-actin (1:3000; Santa Cruz, catalogue no. sc-1616). The PVDF membranes were washed with TBS/T three times for 10 min each, incubated with second antibodies for 1 h at room temperature and then washed with TBS/T three times. Clarity Western enhanced chemiluminescence (ECL) Substrate (Bio-Rad) was used to visualise signals. The Gel-Pro Analyzer (Media Cybernetics) was used to quantify protein expression, and the ratio of target proteins expression was normalised to β-actin.

Statistical analysis

Data expressed as means with their standard errors were analysed by one-way ANOVA or Tukey's tests using statistical software SPSS, version 11.5 (SPSS Inc.). In all analyses, $P < 0{\cdot}05$ was considered statistically significant.

Results

Effect of leucine on porcine myofibre type transformation

To determine whether leucine plays a role in porcine myofibre type transformation, we probed the protein levels of slow MyHC and fast MyHC in samples from cells treated with leucine. As shown in Fig. 1, leucine increased slow MyHC protein level and decreased fast MyHC protein level.

Effect of leucine on the protein kinase B/forkhead box 1 signalling pathway

To determine whether the Akt/FoxO1 signalling pathway is activated in response to leucine treatment, we probed for P-Akt and P-FoxO1 levels after leucine treatment. As shown in Fig. 2, leucine increased the level of P-Akt/Akt and P-FoxO1/FoxO1, indicating that leucine activates the Akt/FoxO1 signalling pathway. Fig. 2 also showed that leucine decreased the protein level of FoxO1.

Leucine regulates porcine myofibre type transformation through the protein kinase B/forkhead box 1 signalling pathway

To explore whether leucine regulates porcine myofibre type transformation through the Akt/FoxO1 signalling pathway, leucine and wortmannin (a specific PI3K/Akt inhibitor) were added to the differentiation medium simultaneously. As shown in Fig. 3, leucine increased slow MyHC protein level and decreased fast MyHC protein level, whereas wortmannin attenuated this effect, indicating that leucine regulates porcine skeletal myofibre type transformation via the Akt/FoxO1 signalling pathway.

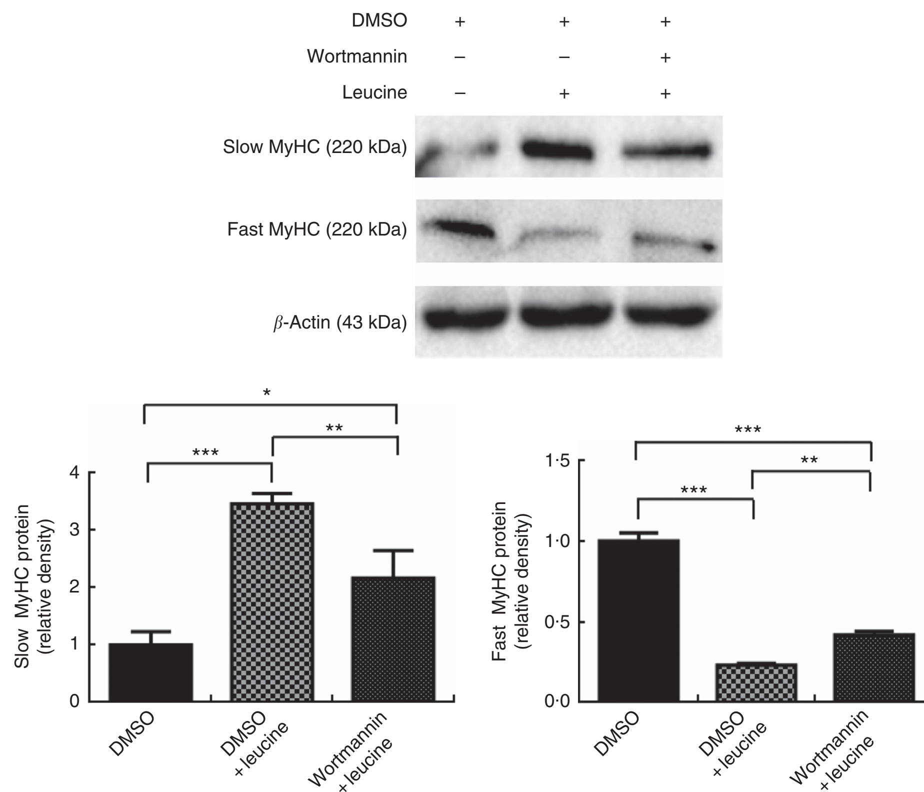

Fig. 3. Leucine regulates porcine myofibre type transformation through the protein kinase B/forkhead box 1 signalling pathway. After 72 h of differentiation, porcine myotubes cultured in differentiation medium were treated with 4 mM leucine and 1 μM wortmannin for 4 d. Dimethyl sulfoxide (DMSO) was used to dissolve wortmannin. Slow myosin heavy chain (MyHC) and fast MyHC protein levels were determined by Western blot analysis. Equal loading was monitored with anti-β-actin antibody. Values are means of the densitometry results from three independent experiments, with their standard errors represented by vertical bars. * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$ and *** $P < 0{\cdot}001$ as compared with control.

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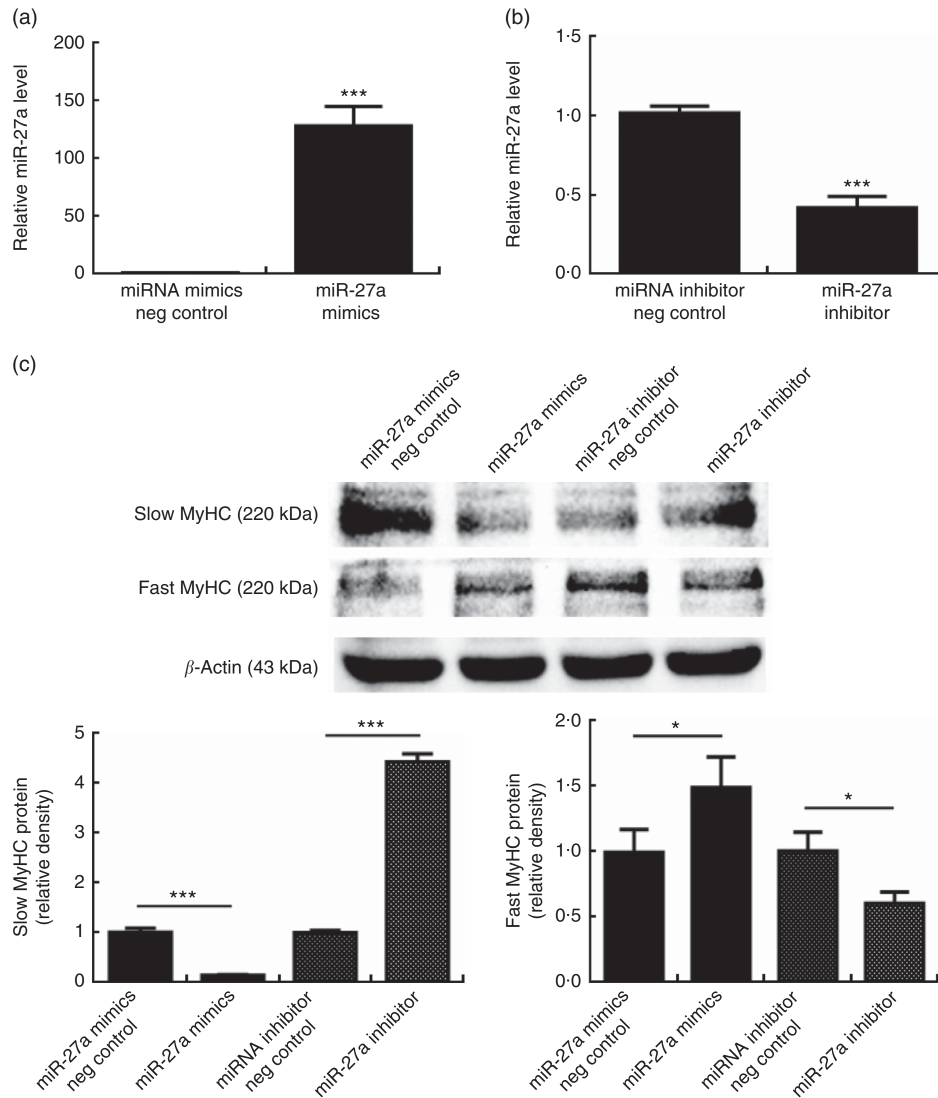

Fig. 4. Effect of microRNA-27a (miR-27a) on porcine myofibre type transformation. After 72 h of differentiation, porcine myotubes cultured in differentiation medium were transfected with 100 nM microRNA (miRNA) mimics negative (neg) control, 100 nM miR-27a mimics, 200 nM miRNA inhibitor negative control or 200 nM miR-27a inhibitor for 3 d. MiR-27a expression (a, b) was determined by real-time quantitative PCR normalised to the amount of U6 small nuclear RNA. Slow myosin heavy chain (MyHC) and fast MyHC protein levels (c) were determined by Western blot analysis. Equal loading was monitored with anti-β-actin antibody. Values are means of the densitometry results from three independent experiments, with their standard errors represented by vertical bars. * $P<0.05$ and *** $P<0.001$ as compared with negative control.

Effect of microRNA-27a on porcine myofibre type transformation

To determine whether miR-27a plays a role in porcine myofibre type transformation, we transfected miR-27a mimics, miRNA mimics negative control, miR-27a inhibitor or miRNA inhibitor negative control into porcine myotubes. As shown in Fig. 4(a) and (b), the level of miR-27a was significantly increased in cells transfected with miR-27a mimics, compared with miRNA mimics negative control, whereas miR-27a inhibitor decreased the level of miR-27a, indicating that the transfection was efficient. Under these conditions, over-expression of miR-27a decreased slow MyHC protein level and increased fast MyHC protein level. In contrast, inhibition of miRNA-27a increased slow MyHC protein level and decreased fast MyHC protein level (Fig. 4(c)).

Effect of leucine on microRNA-27a expression in porcine myotubes

Expression of miR-27a in leucine-treated porcine myotubes was determined by real-time quantitative PCR. As shown in Fig. 5, expression of miR-27a in porcine myotubes was decreased following leucine treatment.

MicroRNA-27a contributes to leucine-induced porcine myofibre type transformation

Next, we examined whether the inhibition of miR-27a could be relevant for the myofibre type transformation effect of leucine on porcine myotubes. To accomplish this aim, porcine myotubes were transfected with miR-27a mimics followed by

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leucine treatment. As shown in Fig. 6, leucine increased slow MyHC protein level and decreased fast MyHC protein level, whereas miR-27a mimics attenuated the effect of leucine on slow MyHC and fast MyHC expressions.

Discussion

Leucine, a well-known anabolic factor that regulates skeletal muscle protein turnover, plays a vital role in muscle protein synthesis and degradation[12,13]. Leucine also plays an important role in the formation of skeletal muscle fibres[16–19]. In this study, we showed that leucine promoted slow MyHC expression and inhibited fast MyHC expression, indicating that leucine promotes porcine myofibre type transformation from fast-twitch to slow-twitch.

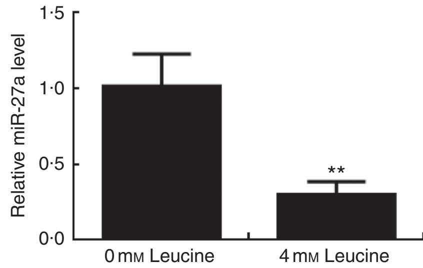
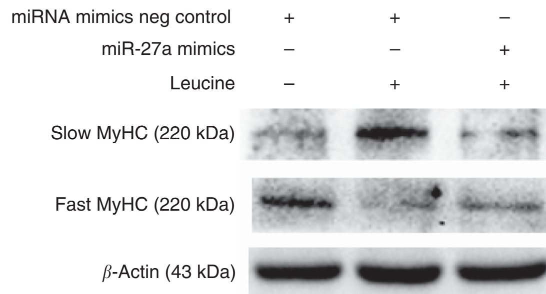


Fig. 5. Effect of leucine on microRNA-27a (miR-27a) expression. After 72 h of differentiation, porcine myotubes cultured in differentiation medium were treated with 4 mM leucine for 4 d. miR-27a level was determined using real-time quantitative PCR normalised to the amount of U6 small nuclear RNA. Values are means from three independent experiments, with their standard errors represented by vertical bars. ** $P<0·01$ as compared with control.

It has been reported that the expression of Akt was higher in oxidative muscle[7,8]. FoxO1, a downstream transcription factor of the PI3K/Akt pathway, has a closer relation with muscle myofibre type distribution under controlled conditions; moreover, the expression level of FoxO1 in fast-twitch muscle is higher than that in slow-twitch muscle[26–29]. Swimming endurance training promoted fast- to slow-twitch transformation in mouse muscle, accompanied by reduction in FoxO1 expression; in addition, over-expression of FoxO1 increased the formation of fast-twitch fibres in C2C12 cells[29]. Wortmannin or LY294002, the specific blocker of the PI3K/Akt pathway, can suppress the activity of Akt, increase the expression of FoxO1 and then result in the accumulation of oxidative fibre[30–32]. In the present study, we showed that leucine could affect the Akt/FoxO1 signalling pathway, and wortmannin attenuated the effects of leucine on porcine fast MyHC and slow MyHC expressions, indicating that the Akt/FoxO1 signalling pathway contributes to leucine-induced porcine skeletal myofibre type transformation.

miRNA are a class of endogenous non-coding RNA that play critical roles in a variety of biological processes[33,34]. Studies



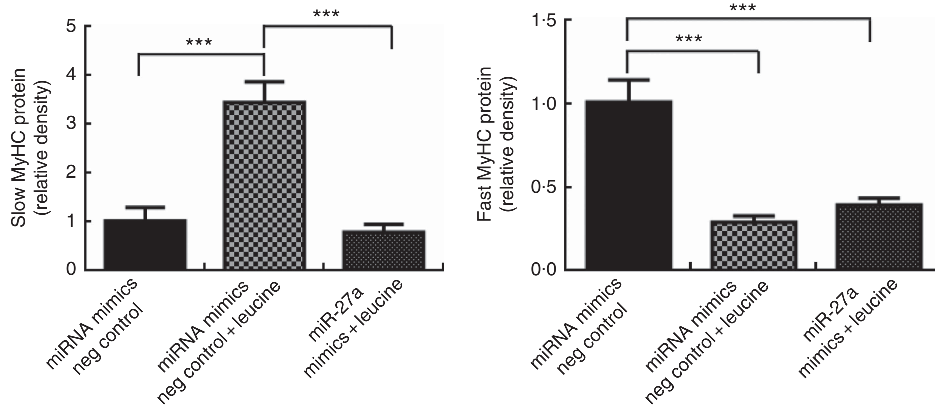


Fig. 6. MicroRNA-27a (miR-27a) contributes to leucine-induced porcine myofibre type transformation. After 72 h of differentiation, porcine myotubes cultured in differentiation medium were transfected with 100 nM microRNA (miRNA) mimics negative (neg) control or 100 nM miR-27a mimics. After 24 h of transfection, 4 mM leucine was added to differentiation medium simultaneously for another 72 h. Slow myosin heavy chain (MyHC) and fast MyHC protein levels were determined by Western blot analysis. Equal loading was monitored with anti-β-actin antibody. Values are means of the densitometry results from three independent experiments, with their standard errors represented by vertical bars. *** $P<0·001$ as compared to control.

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have shown that the expression of majority of miRNA was different in slow- and fast-twitch muscles, indicating that miRNA might play a crucial role in muscle fibre type transition[35,36]. Allen & Loh[37] reported that the expression of miR-27a and b was abundant in the adult mouse muscle, and expression of miR-27a and b in slow-twitch muscle was higher than that in fast-twitch muscle. It has been reported that over-expression of miR-27b might decrease slow muscle fibre composition in C2C12 cells[38]. In the present study, we found that transfection of miR-27a mimics in porcine myotubes decreased slow MyHC protein expression and increased fast MyHC protein expression, whereas transfection of miR-27a inhibitor gave an opposite result, indicating that miR-27a promotes porcine myofibre type transformation from slow-twitch to fast-twitch. Recently, growing studies have reported that nutrients could regulate the expression of miRNA[20–22]. In this study, we observed that treatment with leucine decreased the expression of miR-27a in porcine myotubes. We also observed that over-expression of miR-27a repressed transformation from fast MyHC to slow MyHC caused by leucine. These results indicated that leucine promotes porcine skeletal myofibre type transformation from fast-twitch to slow-twitch by inhibiting the expression and function of miR-27a. miRNA negatively regulate gene expression at the post-transcriptional level via binding to the 3′-untranslated region of target mRNA. Although human FoxO1 and mouse FoxO1 are targeted by miR-27a[39,40], there is no evidence that porcine FoxO1 is a target of miR-27a, as leucine (4 mM) down-regulated both the expression of miR-27a and the protein expression of FoxO1 in this study.

In conclusion, we provided the first evidence that leucine promotes porcine myofibre type transformation from fast MyHC to slow MyHC through the Akt/FoxO1 signalling pathway and miR-27a.

Acknowledgements

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X. C. and Z. H. conceived the study and designed the experiments. S. Z. carried out the experiments, analysed the data and wrote the manuscript. X. C., Z. H., D. C., B. Y., H. C., J. H., J. L., P. Z., J. Y. and Y. L. contributed reagents/materials/analysis tools. Z. H. revised the manuscript. All authors read and approved the final manuscript.

The authors declare that there are no conflicts of interest.

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