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RESEARCH ARTICLE

The microRNA, miR-29c, participates in muscle development through targeting the *YY1* gene and is associated with postmortem muscle pH in pigs

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Abstract Previous studies indicated that miR-29c is important for muscle development in mice and human, but its role in pigs is unknown. In this study, we detected the expression of miR-29c in Meishan longissimus lumborum (LL) muscle. The results showed that miR-29c was gradually upregulated during development of skeletal muscle in pig. Moreover, the expression of *YY1* and *Akt3* genes, which were confirmed to be targeted by miR-29c in mice, was decreased along with muscle development. Furthermore, the expression level of miR-29c was significantly higher in adult Meishan pigs than Large White pigs, while the expression of *YY1* and *Akt3* genes was significantly lower in Meishan pigs. These results indicated that the expression pattern of miR-29c was opposite to that of *YY1* and *Akt3* genes in pigs. Also, the luciferase assay indicated that miR-29s can target the *YY1* gene in pigs. In addition, we identified a T to C mutation in the primary transcript of miR-29c, which was associated with the postmortem muscle pH in pigs. Based on these results, we concluded that miR-29c is also important in skeletal muscle development of pigs.

Keywords pig, miR-29c, skeletal muscle, expression, SNP

1 Introduction

MicroRNAs are a class of small non-coding RNAs[1,2] can inhibit target genes through binding with their 3′ untranslated regions (3′ UTR)[3]. Previous studies showed that microRNAs can participate in cell proliferation, differentiation and apoptosis[4–6]. The miR-29 family contains three members, miR-29a, miR-29b and miR-29c[7], and they have similar expression patterns and biofunctions. MiR-29 can participate in many physiological and chemical processes. It has been reported that miR-29a can inhibit apoptosis and protect the mitochondrial functions during forebrain ischemia through targeting the pro-apoptosis *PUMA* gene in astrocytes[8]. Also, many studies have shown that miR-29s can inhibit tissue fibrosis through downregulating collagen genes and inhibition of IGF-1 and PDGC growth factors[5,9,10]. Furthermore, miR-29s can restrain the activities of DNA methyltransferases and demethylases[11], promote murine osteoclastogenesis[12] and inhibit tumorigenesis[13].

MiR-29s are also important in skeletal muscle development. A recent study reported that miR-29 was downregulated in the dystrophic muscle and restoration of the expression of miR-29 in muscle tissue can improve dystrophy pathology by promoting regeneration and inhibiting of fibrogenesis[2]. Also, miR-29s can repress proliferation and promote differentiation of myoblasts in skeletal muscle development by targeting the *Akt3* gene[6]. Furthermore, they can regulate myogenesis via the NF-KB-*YY1*-miR-29 signaling pathway in mice[14]. Although some functions of miR-29s in muscle development have been reported in mice and human, this has not been studied in pigs. In this study, we focused on the role of miR-29c in muscle development in pigs.

2 Materials and methods

2.1 Tissues and animals

The longissimus lumborum (LL) muscle samples of

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Meishan pigs at different stages, 50-day-old fetuses (E50d), 95-day-old fetuses (E95d) and adult stage (12 month), and Large White pigs at adult stage (12 month), were collected. The samples were stored at −80°C until assayed by qPCR. A Large White population of 233 animals was selected for trait association analysis. All the pigs were slaughtered at about 90 kg and meat quality traits of intramuscular fat content (IMF) by Soxhlet extraction and 24 h postmortem muscle pH (pHu) determined by pH meter. In addition, muscle drip loss (DLS) and loin eye area were determined by methods described in previous studies[1,15].

2.2 Cells and transfection

PK-15 cells (a porcine kidney epithelial cell line) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT, USA) with 12% (v/v) fetal bovine serum. Cells were transferred to 24 well plates with growth medium, 24 h before transfection. Cells were transfected with miRNA mimics (GenePharma, Shanghai, China) and plasmid using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). Opti-MEM I Reduced Serum Medium (Gibco, Grand Island, NY, USA) was used to dilute Lipofectamine 2000 and nucleic acids. Transfection procedure was performed follow the manufacturer's instructions.

2.3 Cloning for dual-luciferase assay

The psiCHECK-2 dual-luciferase reporter vector (Promega, Madison, WI, USA) housing the 3′ UTR of *YY1* gene was used to examine the effect of miR-29 on *Renilla* luciferase production. *YY1* 3′ UTR was amplified with the use of forward primer 5′ CCGCTCGAGCTCTATC TTGCTCTGTAATCTCG 3′ and reverse primer 5′ ATAA-GAATGCGGCCGCTCCAATTTCTGGGAGGCTCA3′. A 2-base substitution in the seed sequence of miR-29 was introduced to create mutant forms of miR-29 when synthesized. The miRNA mimics and 3′ UTR dual-luciferase vector were co-transfected into cells using Lipofectamine 2000 (Invitrogen). Cells were assayed with the Dual-Luciferase Reporter Assay System (Promega) 24 h after transfection.

2.4 Quantitative PCR

Total RNA (including miRNA) was extracted from tissues with TRIzol reagent (Invitrogen). Concentration and quality of RNA were assessed by the NanoDrop 2000 (Thermo, Waltham, MA, USA) and denatured gel electrophoresis. Reverse transcription was performed using Prime Script™ RT Reagent Kit with gDNA Eraser (TAKARA BIO INC, Otsu, Shiga, Japan) with miRNA specific primers added to initiate cDNA synthesis. The quantitative PCR (qPCR) 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) (Appendix A, Table S1).

2.5 Genotyping

One SNP under study was selected in the ssc-miR-29b-2/c cluster. The SNP was genotyped by a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The PCR product (752 bp) of the SNP was digested with *Nco*I restriction enzyme (Fermentas, Life Sciences, USA) at 37°C overnight. This restriction enzyme recognizes the sequence T-C. The T-allele carrying the PCR product was cleaved once by the enzyme generating two fragments (631 and 121 bp). All digestion products were separated by agarose gel electrophoresis and the association analysis was performed by SAS program (Appendix A, Table S1).

3 Results

3.1 MiR-29c can target *YY1* gene

Previous studies have confirmed that *AKT3* and *YY1* are the target genes of miR-29 in mouse[6,16]. To test whether the *YY1* gene was also targeted by miR-29c in pigs, the conservation of target site of the miR-29c in the *YY1* gene was analyzed. We found that it was completely conserved among human, mice and pig (Fig. 1a). Then we performed the luciferase activation assays. The fragment of the 3′UTR of the pig *YY1* gene, which contains the binding site of miR-29s, was cloned into the luciferase vector. Subsequently, the interaction between miR-29 and 3′UTR of the *YY1* gene was evaluated through luciferase activity analysis. The results showed that miR-29a, miR-29b and miR-29c could significantly inhibit the luciferase activity when the 3′ UTR was inserted (Fig. 1b). Moreover, the mutant miR-29 with two nucleotides substituted in the seed sequence did not inhibit the luciferase activity (Fig. 1c). These results indicated that the *YY1* gene was also targeted by miR-29s in pigs.

3.2 Differential expression analysis of miR-29c, *YY1* and *Akt3*

The expression of miR-29c at E95d was 1.8 fold higher than that of E50d and it was sharply increased approximately 170 fold at the adult stage (Fig. 2a). In addition, the expression patterns of *YY1* and *Akt3* genes were also measured. The results showed that expression of both was decreased during muscle development in Meishan pigs (Fig. 2b, Fig. 2c). Moreover, expression of miR-29c was significantly higher in Meishan than that in Large White at

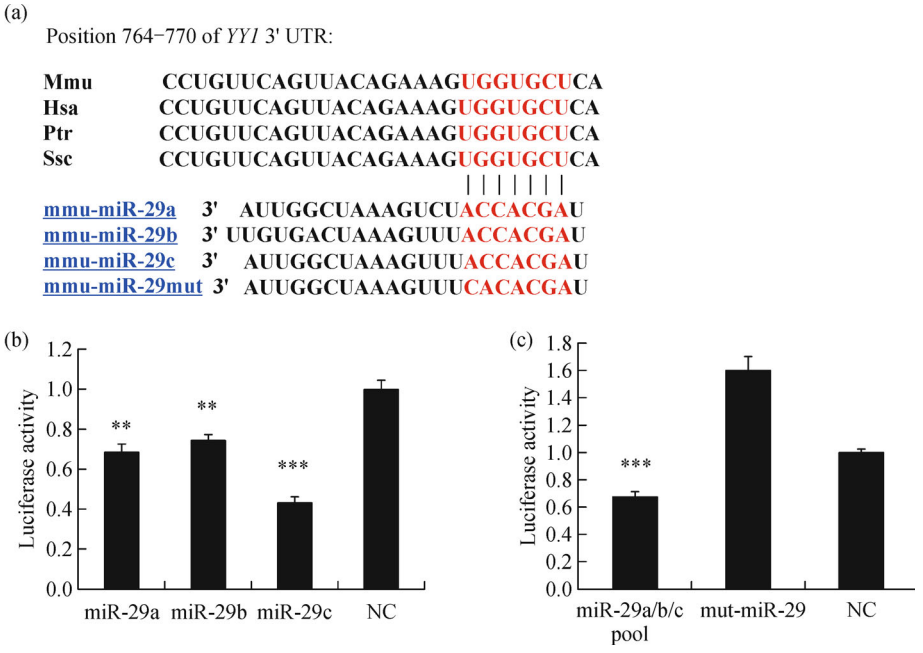

Fig. 1 Identification of the target genes of miR-29c. (a) The potential binding site of ssc-miR-29 in *YY1* 3′ UTR, and the investigation of the conservation of this binding site in different species (http://www.targetscan.org); (b) identification of the target of *YY1* gene using dual luciferase reporter system. The 756 bp 3′ UTR of *YY1* gene was first inserted into the psiCHECK-2 luciferase vector, and then co-transfected into PK-15 cells together with miR-29a, miR-29b or miR-29c, and finally the luciferase activity was detected at 24 h post-transfection; (c) luciferase activity was detected when the mutant miR-29 (2 nt substitution was introduced into the seed sequence of miR-29), pooled miR-29 or NC (negative control) were co-transfected with the luciferase vector inserted with the 765bp 3′ UTR of *YY1* gene. Results are shown as mean±S.E.M, means are of measurements from three independent individuals. ** Indicates a significant difference ($P < 0.01$); *** indicates a significant difference ($P < 0.001$).

the adult stage (Fig. 3a). In contrast, the expression of *Akt3* and *YY1* was significantly lower in Meishan than in Large White (Fig. 3b, Fig. 3c). The expression patterns of *YY1* and *Akt3* genes were opposite to that of miR-29c. These results also indicated that *YY1* and *Akt3* genes are targeted by miR-29c in pigs.

3.3 The SNP in the ssc-miR-29b-2/c cluster influences pHu

We detected a C/T SNP in the ssc-miR-29b-2/c cluster at 443 nt downstream of pre-miR-29b-2 and 33 nt upstream of pre-miR-29c, which caused a *Nco*I polymorphism (Fig. 4a, Fig. 4b). For genotyping, the 752 bp DNA fragment was amplified and the genotype was identified using PCR-RFLP analysis. As indicated, the CC genotype has one 752 bp band; the TT genotype has 631 and 121 bp bands, and the CT genotype has three bands (Fig. 4c). Furthermore, we found that the expression of miR-29c in the CC genotype was significant higher than in TT genotype (Fig. 4d).

The trait association of the Large White population showed that the polymorphism of this locus was significantly associated with the pHu trait. However, there was no significant association between this SNP and other muscle quality traits. According to our results, the mean value of pHu of CC individuals was significantly higher than TT individuals (5.67±0.03 vs 5.52±0.03, $P < 0.01$) (Table 1). This result showed that this polymorphism in miR-29c is important in determining the pHu trait of pig skeletal muscle.

4 Discussion

Previous studies indicated that miR-29s is important in myogenesis processes and it can target *YY1* and *Akt3* genes in mice[6,16]. In this study, we found that in pigs the expression of miR-29c was upregulated, while *YY1* and *Akt3* were downregulated during muscle development. Also, through luciferase analysis we found that the porcine *YY1* gene can be targeted by miR-29c. Therefore in pigs, miR-29c may also participate in muscle development through targeting *YY1* and *Akt3* genes. It has been reported that *YY1* and *Akt3* are important in myogenesis and muscle growth. In mice, our previous study found that *Akt3* can promote the proliferation of myoblasts[6]. *YY1* is a transcription factor containing a zinc finger DNA binding domain[17]. It has been confirmed that *YY1* had negative

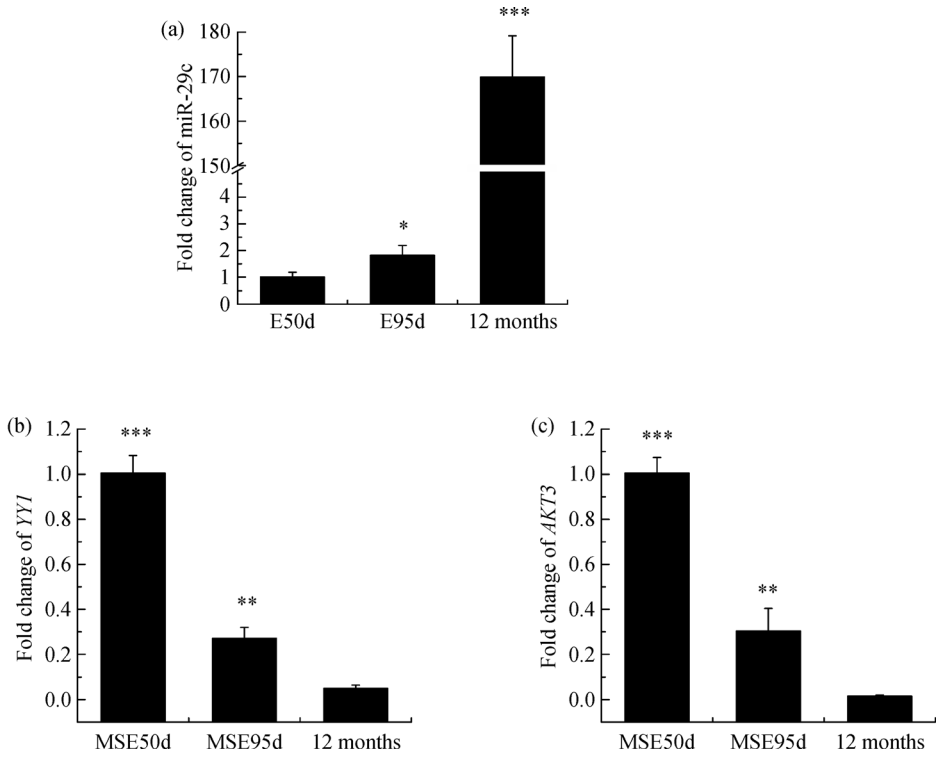

Fig. 2 Expression profiles of miR-29c, *YY1* and *Akt3* during muscle development. (a) The expression of miR-29c in LL muscle of Meishan pigs at E50d, E95d, and adult stage (12 months) was detected by qPCR; (b) the expression *YY1* genes in LL muscle of Meishan at three stages was detected by qPCR; (c) the expression *Akt3* genes in LL muscle of Meishan at three stages was detected by qPCR. The expression level of E50d was set as 1. Expression of miR-29c was normalized to *U6*. Expression of *Akt3* and *YY1* genes were normalized to *β-Tubulin*. Results are shown as mean±S.E.M., means are of three independent individuals. * Indicates a significant difference ($P < 0.05$); ** indicates a significant difference ($P < 0.01$); *** indicates a significant difference ($P < 0.001$).

effects on myogenesis of muscle cells through inhibiting the expression of myogenic differentiation marker genes including α-actin, Tnnc, Tnni2 and MyHC[18]. Moreover, *YY1* can inhibit myogenesis through transcriptional regulation of non-coding RNAs including miR-1, miR-29 and lncRNA Yam-1[14,19]. Based on these results, we conclude that miR-29s can regulate skeletal muscle development through targeting *YY1* and *Akt3* genes in pigs.

In this study, we also found that miR-29 was associated with the pH value trait of pigs. Previous studies have shown that the pH value was associated with glycometabolism, and the concentration of glycogen in muscle tissue was positively correlated with its pHu value[20,21]. Also, previous studies indicated that both *Akt3* and *YY1* played positive roles in glucose and energy metabolism of the muscle tissue. In mice, *YY1* knock out lead to hyperactivation of the *insulin/IGF* signaling, which can suppress the diabetic-like symptoms arising when treated with rapamycin. The YY1 protein can bind to the promoter regions of insulin/IGF and inhibit their transcription, which reduced the metabolism efficiency of blood glucose. Furthermore, *YY1* can form a complex with mTOR and PGC-1α to regulate mitochondrial genes expression and energy metabolism[22]. A previous study indicated that *Akt3* also participated into glucose transport, and impaired *Akt3* expression was related to insulin resistance of muscle tissue in humans[1]. In addition, glucose homeostasis was impaired in *Akt2* and *Akt3* genes double knockout mice and they displayed glucose and insulin intolerance[22–24].

These studies indicated that *YY1* and *AKT3* are important in glucose and energy metabolism. Thus, miR-29c may affect the muscle pHu value through targeting *Akt3* and *YY1* genes.

5 Conclusions

In conclusion, miR-29c was upregulated, while *Akt3* and *YY1* genes were downregulated, during muscle development of pigs. The miR-29c can participate in muscle development through targeting *Akt3* and *YY1* genes. A T to C mutation was detected in the miR-29c genomic sequence, which was association with pHu trait in pigs. Therefore, we conclude that miR-29 is important for the skeletal muscle development in pigs.

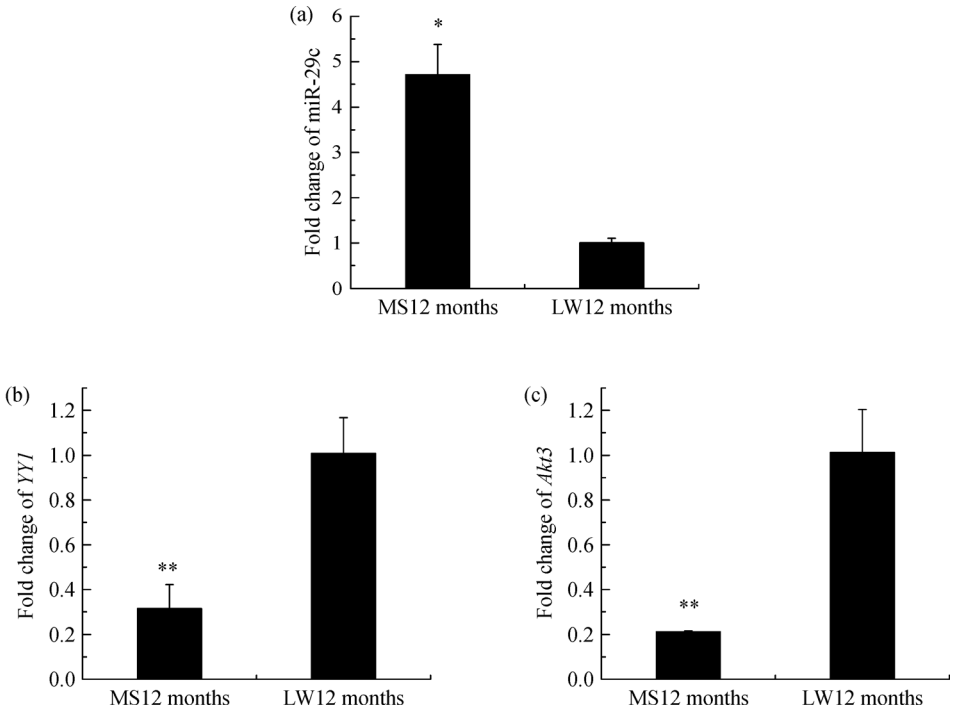

Fig. 3 Expression profiles of miR-29c, *YY1* and *Akt3* genes in different breeds. (a) The expression of miR-29c in LL muscle of Meishan and Large White at the adult stage (12 months); (b) the expression of *YY1* genes in LL muscle of Meishan and Large White at the adult stage (12 months); (c) the expression of *Akt3* genes in LL muscle of Meishan and Large White at the adult stage (12 months).The expression level of Large White was set as 1. Expression of miR-29c was normalized to *U6*. Expression of *YY1* and *Akt3* genes was normalized to *β-Tubulin*. Results are shown as mean±S.E.M., means are of three independent individuals. * Indicates a significant difference ($P < 0.05$); ** indicates a significant difference ($P < 0.01$); *** indicates a significant difference ($P < 0.001$).

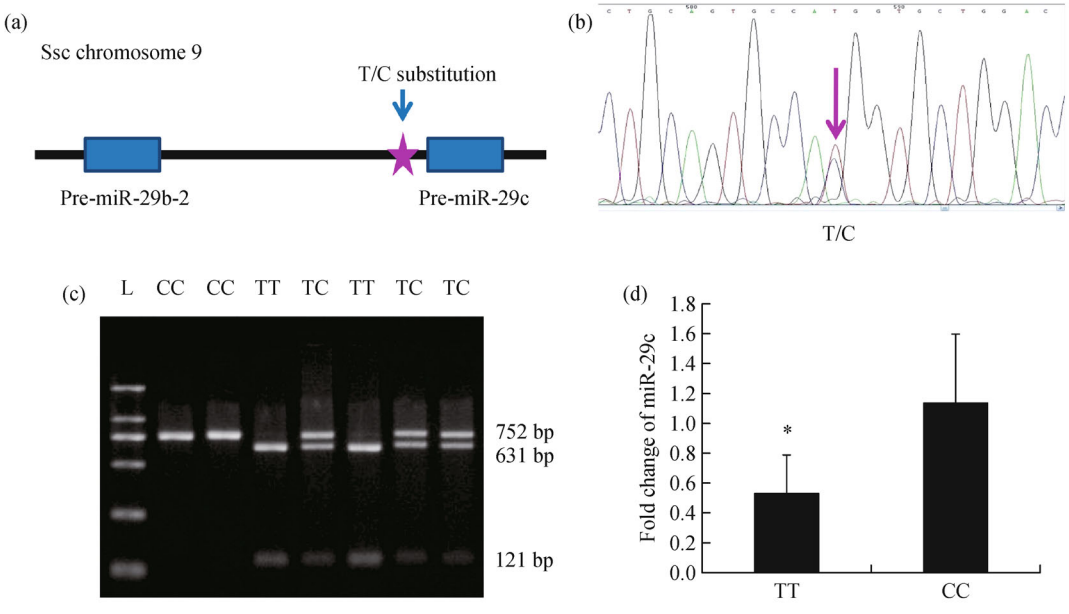

Fig. 4 SNP identification in the pre-miR-29b-2/c cluster. (a) The scheme of the SNP site in the pre-miR-29b-2/c cluster on SSC9; (b) the sequence of the T/C SNP. The SNP is labeled by an arrow; (c) the gel photo of the genotype digested using restriction enzyme *Nco*I. The CC homozygote had one band of 752 bp; the TT homozygote had two bands of 631 and 121 bp; TC heterozygote had bands of 752, 631 and 121 bp. L indicates the DNA ladder; (d) the expression level of miR-29c in TT and CC homozygote. The expression level of miR-29c in CC homozygote was set as 1.0, expression of miR-29c was normalized to *U6*.

Table 1 Association study of the T/C SNP in the miR-29b-2/c cluster and meat quality traits

Genotype	No.	Muscle pHu value	Muscle drip loss/%	Intramuscular fat content/%	Loin eye area /cm^2
CC	46	5.67±0.03^a	1.60±0.21	2.27±0.08	46.17±0.91
TC	121	5.57±0.02^b	1.69±0.13	2.23±0.05	45.73±0.56
TT	65	5.53±0.03^b	1.97±0.17	2.20±0.07	45.71±0.76
P Value					
CC-TC		0.0098**	0.7329	0.6749	0.6827
CC-TT		0.0011**	0.1743	0.4975	0.7009
TC-TT		0.2332	0.1873	0.7055	0.9845

Note: Least square mean values with different letters are significantly different.

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Supplementary material The online version of this article at http://dx.doi.org/10.15302/J-FASE-2015075 contains supplementary material (Appendix A).

Compliance with ethics guidelines Weiya Zhang, Wei Wei, Yuanyuan Zhao, Shuhong Zhao, and Xinyun Li declare that they have no conflict of interest or financial conflicts to disclose.

All applicable institutional and national guidelines for the care and use of animals were followed.

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