Characterization of miR-218/322-Stxbp1 pathway in the process of insulin secretion

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Abstract

MicroRNAs (miRNAs) have been implicated in a variety of physiological processes, however, the function of miRNAs in insulin secretion and type 2 diabetes is still unclear. Stxbp1 plays an essential role in exocytosis, and is crucial for insulin secretion. In this study, we focused on the molecular mechanism of Stxbp1 in insulin secretion by identifying its upstream regulators: miR-218 and miR-322. The expression of Stxbp1 was significantly increased in isolated mouse islets exposed to high levels of glucose within 1 h; while two of its predicted upstream miRNAs were found to be downregulated. Further study found that miR-218 and miR-322 directly interact with Stxbp1 by targeting the 3’UTR of its mRNA. MIN6 cells overexpressing the two miRNAs showed a sharp decline in insulin secretion and a decreased sensitivity to glucose; while the inhibition of the two miRNAs promoted insulin secretion. However, islets treated with prolonged high levels of glucose, which is known as glucolipotoxicity, displayed relatively high expression of miR-218 and miR-322, and a reduced level of expression of Stxbp1 accompanied by the blocking of insulin secretion. In summary, this study identified a pathway consisting of miR-218/322 and Stxbp1 in insulin secretion, contributing to a network of β-cell function involving miRNA.

Key Words

miR-218
miR-322
Stxbp1
insulin secretion
glucolipotoxicity
β-cell function
type 2 diabetes

Introduction

MicroRNAs (miRNAs) are a novel family of small non-coding RNAs, typically 22 nucleotides in length. They negatively regulate gene expression by base pairing to complementary sites on the 3’UTR of target mRNAs, leading to translation repression or mRNA degradation. So far, miRNAs have been found to be involved in a wide range of biological and pathological processes, including cell apoptosis, migration, metabolism, immune response, aging, and organic development (Pelaez & Carthew 2012, Zhang et al. 2015).

Type 2 diabetes (T2D) is characterized by reduced insulin secretion from pancreatic β-cells and insulin resistance at the target cells, which result in increased blood glucose levels (Kahn 2001). It has been reported that impaired insulin secretion has already occurred before the onset of T2D, indicating that the insulin-secretion processes are crucial during the development of the disease (Hosker et al. 1989, Del Prato & Tiengo 2001).

Syntaxin-binding protein 1 (Stxbp1 or Munc18-1) modulates the folded conformation of syntaxin 1A, which acts as an important regulator in intracellular vesicle trafficking (Dong et al. 2007). Although the interaction of Stxbp1 and syntaxin 1A plays a key role
in the process of insulin secretion, the upstream regulators of \textit{Stxbp1} remain undetermined.

\textit{miR-218} has been reported to be implicated in cancer cell proliferation and migration (Xiao et al. 2014), while \textit{miR-322} regulates cell cycle quiescence and cell differentiation in muscle cells (Sarkar et al. 2010). However, few studies have been focused on the role of \textit{miR-218} and \textit{miR-322} in \textbeta-cell function.

In this study, we first assessed the expression of \textit{Stxbp1} and its predicted pairing miRNAs in isolated islets treated with high levels of glucose for a short period. \textit{Stxbp1} was found to be significantly upregulated, while two of the screened miRNAs: \textit{miR-218} and \textit{miR-322} showed the opposite tendency. The subsequent luciferase assays confirmed that \textit{miR-218} and \textit{miR-322} directly interact with 3'UTR of \textit{Stxbp1}; the overexpression of the two miRNAs in islet cell lines leads to a sharp decrease in expression of \textit{Stxbp1} and insulin secretion. The mechanism by which miRNAs regulate expression of \textit{Stxbp1} in islets exposed for long periods to high levels of glucose, known as glucolipotoxic, was also investigated in this study. To conclude, we identified \textit{miR-218}/322–\textit{Stxbp1}–insulin as an important pathway in \textbeta-cell function.

\textbf{Material and methods}

\textbf{Animal preparation}

Male C57BL/6j mice (\textit{Mus musculus}) were housed in a pathogen-free animal facility with access to water and food and allowed to eat and drink \textit{ad libitum}. All of the animals were subject to controlled temperature (22 ± 1 °C) and lighting (lights on 0600–1800 h). All of the experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

\textbf{Isolation of islets from mouse pancreata}

Islets were isolated as described previously (Neuman et al. 2014). Mice were anesthetized by i.p. injection of sodium pentobarbital. Pancreatic islets were then isolated by injection of 500 U/ml of collagenase solution into the pancreatic duct followed by digestion at 37 °C for 28 min with mild shaking. The islets were washed several times with D-Hank’s solution (136 mM NaCl, 0.53 mM KCl, 4.2 mM NaHCO\textsubscript{3}, 0.44 mM KH\textsubscript{2}PO\textsubscript{4}, 0.385 mM Na\textsubscript{2}HPO\textsubscript{4}), separated from acinar cells on a discontinuous Ficoll 400 gradient, viewed under a dissecting microscope, and hand-selected. Finally, the islets were collected and transferred into RPMI1640 medium containing 10% fetal bovine serum (FBS).

The islets were cultured at 37 °C in a humidified atmosphere containing 5% CO\textsubscript{2}/95% air for 12 h (primary culture) for removal of exocrine and other tissues. Subsequently, islets were moved to DMEM containing 5/25 mM glucose and 10% insulin-free FBS for insulin secretion studies (Zhang et al. 2001).

\textbf{PCR analysis}

Assays to quantify mature miRNAs were conducted as described previously (Chen et al. 2005). Total RNA was extracted from the cultured cells using TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. PCR products of \textit{Stxbp1} mRNA were analyzed by agarose electrophoresis, and mRNA levels were further verified by RT-qPCR using subGreen.

In this study, miRNA expression in cells and islets was normalized to U6 snRNA (Schmittgen et al. 2004), and mRNA expression was normalized to \textit{Gapdh}. The relative amount of each gene to internal control was calculated by using the equation $2^{-\Delta Cq}$, in which $\Delta Cq = Cq_{\text{gene}} - Cq_{\text{control}}$. Primers for \textit{Stxbp1} and \textit{Gapdh} were as follows: 5’-CATGAGAGCCATTGTCCCCA-3’ (\textit{Stxbp1}, sense); 5’-AGTGCTTTGTATCCAGCTTGTC-3’ (\textit{Stxbp1}, antisense); 5’-AGAAGGCTGGGGCTCATTTG-3’ (\textit{Gapdh}, sense); 5’-AGGGCCATCCACGTCTTC-3’ (\textit{Gapdh}, antisense).

\textbf{Cell culture}

MIN6 cells were routinely maintained in 1640 medium supplemented with 20% FBS, 1% glutamine, 1% β-mercaptoethanol, and 10 mM HEPES. The cells were cultured at 37 °C with 5% CO\textsubscript{2}.

\textbf{The miRNA target prediction}

The miRNA target prediction and analysis were performed with the algorithms from TargetScan (http://www.targetscan.org/) PicTar (http://pictar.mdc-berlin.de/) and miRanda (http://www.microrna.org/).

\textbf{MIN6 insulin secretion assay}

To determine the insulin secretion of MIN6 cells, cells were cultured in 96-well plates (40 000 cells/well) for 48 h. Then the medium was removed and cells were washed twice with glucose-free Krebs (NaCl 119 mM, KCl 4.74 mM, CaCl\textsubscript{2} 2.54 mM, MgCl\textsubscript{2} 1.19 mM, KH\textsubscript{2}PO\textsubscript{4} 1.18 mM, NaH\textsubscript{2}PO\textsubscript{4} 0.194 mM) and incubated in 5 mM glucose at 37 °C for 28 min, followed by incubation of 100 mg/ml of insulin solution for 1 min. The culture medium was removed, and the cells were washed twice with glucose-free Krebs. The supernatant was stored for further analysis.
1.19 mM, NaHCO₃ 25 mM, and HEPES (pH 7.4) 10 mM) containing 0.05% insulin-free BSA. An aliquot of 100 μl of the above Krebs solution containing the desired amount of glucose was added to each well and cells were further cultured for 1 h.

**Determination of insulin concentration**

Insulin concentrations in the medium were assessed, using mouse insulin as a standard, with a Rat/Mouse Insulin ELISA Kit (EMD Millipore (Billerica, MA, USA), catalog number: EZRMI-13K). In brief, samples were added into a 96-well plate with the rat/mouse insulin antibody at the bottom. To each sample, 20 μl assay buffer, 10 μl rat insulin standards/medium, and 80 μl detection antibody was added. Subsequently, 100 μl enzyme buffer, followed by 100 μl substrate solution buffer, was added to each well. After a final stop solution was added, the insulin content was determined by reading the absorbance at 450 nm.

**Cell transfections**

MIN6 cells were seeded on six-well plates and were transfected by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. For each well, equal doses (100 nmol) of miRNA mimics, inhibitors, siRNAs, or scrambled negative control RNA were used. The cells were harvested at 24 h after transfection for real-time PCR analysis and western blotting.

**siRNA interference assay**

Three sequences from the coding region of mouse Stxbp1 were designed and synthesized by Invitrogen. A scrambled siRNA (Stealth RNAi Negative Control Kit, Invitrogen) was used as a negative control. Sequences of the Stxbp1 siRNAs were as follows: 5’-UAUCCUCCACAAUUUGUAGCC-CUC-3’ (sense) and 5’-GAGGGCAUCACAAUUGGAG-GUAU-3’ (antisense).

**Plasmid construction and luciferase assay**

The entire 3’UTR of Stxbp1 was synthesized and inserted into a p-MIR-report plasmid (Ambion, GenePharm, Shanhai, China). A plasmid with a mutated Stxbp1 3’UTR was also constructed using the same method. For luciferase reporter assays, 2 μg of firefly luciferase reporter plasmid, 2 μg of β-galactosidase expression vector (Ambion), and equal amounts (200 pmol) of mimics, inhibitors, or scrambled control RNA were transfected into cells in six-well plates. The β-galactosidase vector was used as a transfection control. At 24 h after transfection, cells were assayed using luciferase assay kits (Promega).

**Immunofluorescence**

MIN6 cells were briefly washed twice with cold PBS, and then fixed in 4% paraformaldehyde for 10 min at RT. After fixation, the cells were washed with PBS (3×5 min, RT), and then permeabilized and blocked using 2% BSA (Sigma–Aldrich) and 0.05% Triton X-100 in PBS for 1 h at RT. Next, the cells were incubated with primary antibody (STXBP1, Santa Cruz, 1:500) in 2% BSA/PBS in a humidified chamber overnight at 4 °C, and then rinsed in PBS (3×5 min, RT). The cells were then incubated in secondary fluorescent antibodies (Invitrogen) in 2% BSA/PBS in a light-proof container for 1 h at RT. Finally, the cells were stained with DAPI and visualized using a fluorescence microscope.

**Western blotting analysis**

The STXBP1 expression was assessed by western blotting analysis and samples were normalized to GAPDH. Protein extraction was blocked with PBS-5% fat-free dried milk at room temperature for 1 h and incubated at 4 °C for overnight with anti-STXBP1 (1:1000, Santa Cruz), anti-syntaxin (1:1000, Santa Cruz), and anti-GAPDH (1:2000, Santa Cruz) antibodies respectively.

**Statistical analyses**

All data were representative of at least three independent experiments. Data were expressed as mean ± s.d. of three separate experiments. Statistical significance was considered at P<0.05 using the Student’s t-test. In this study, ‘*’ indicates ‘P<0.05’, and ‘**’ indicates ‘P<0.01’.

**Results**

**Glucose-stimulated expression of Stxbp1 and insulin secretion**

High levels of glucose are well known to stimulate insulin synthesis and secretion, and the expression of the related proteins in this process usually shows a significant variation (Sun et al. 2011, Shantikumar et al. 2012). In this study, we first checked the expression of Stxbp1 and insulin secretion in isolated mouse islets cultured in
media containing 5 or 25 mM glucose (high glucose). High levels of glucose enhanced expression of Stxbp1 gradually within 1 h (Fig. 1A), while the Stxbp1 mRNA showed only a slight increase (Fig. 1C and Supplementary Figure S1, see section on supplementary data given at the end of this article). Meanwhile, insulin secretion was used to evaluate the function of β-cells. As expected, insulin secretion was increased under the influence of high levels of glucose (Fig. 1B).

The result confirmed that Stxbp1 is an important positive regulator in the insulin secretion process, and also implied that Stxbp1 expression may be regulated by miRNAs.

### The expression of Stxbp1-related miRNAs in islets

Rapid insulin secretion induced by glucose is partly regulated by autocrine factors, and miRNAs may play an important role in this process (Fred et al. 2010). A list of potential miRNAs that regulate expression of Stxbp1 are shown in Fig. 2A. To clarify whether the promotion of the expression of Stxbp1 was associated with miRNAs, we screened all the predicted miRNAs by qPCR, and found that miR-218 and miR-322 were clearly decreased (Table 1). The observation indicated that miR-218 and miR-322 were involved in rapid glucose-stimulated insulin secretion (GSIS) though regulation of the expression of Stxbp1.

### Stxbp1 is a direct target of miR-218 and miR-322

The expression levels and the computational prediction alone cannot provide direct evidence of the interaction between miRNAs and the target. Thus a luciferase assay was performed to evaluate the association. As is shown in Fig. 3A, the target region in the 3′UTR of Stxbp1 was conserved among humans, mice and rats. Co-transfection of miR-218 mimics with the reporter plasmid containing WT Stxbp1 3′UTR sequence into 293T HEK cells resulted in a 50% reduction in the luciferase signal, and miR-322 mimics decreased the relative luciferase activity to 40% (Fig. 3B). However, the interaction was lost when the plasmid with a mutated sequence was used instead (Fig. 3C).

To verify the specific interaction between miR-218/322 and Stxbp1 mRNA, another predicted miRNA was selected as a negative control. Figure 3D showed that the overexpression of miR-195 in HEK293 cells did not change the relative luciferase activity in both the WT group and the mutant group. A similar result was observed when miR-195 was inhibited in cells.

These results indicate that miR-218 and miR-195 directly target the 3′UTR of Stxbp1 mRNA.

### Downregulation of Stxbp1 by miR-218 and miR-322

To study the biological role of miR-218 and miR-322 in β-cell function, MIN6 cells were transfected with mimics...
and inhibitors of the two miRNAs. The levels of miRNA were determined by real-time quantitative PCR analysis. The relative levels of the two miRNAs were significantly increased after transfection with mimics (Fig. 4A), while they declined when cells were transfected with inhibitors (Fig. 4C). We assessed the expression of Stxbp1 by western blotting assays at 24 h post-transfection. The expression levels of the STXBP1 protein were significantly reduced by the introduction of miR-218 and miR-322, whereas cells transfected with mimic control maintained a considerable amount of STXBP1 protein (Fig. 4B); in contrast, the inhibitors increased the relative expression levels of STXBP1 protein in MIN6 cells (Fig. 4D). These results indicate that miR-218 and miR-322 negatively regulate the expression of STXBP1 protein.

Roles of miR-218, miR-322, and Stxbp1 in rapid insulin secretion

To investigate the effects on insulin secretion of miR-218 and miR-322 targeting Stxbp1, MIN6 cells were transfected with miRNA mimics, inhibitors, or siRNA against Stxbp1 and analyzed for changes in insulin secretion though ELISA assays. The cells transfected with ncRNA or control siRNA served as controls. Efficient interference with Stxbp1 expression is shown in Fig. 4B and D. As shown in Fig. 4E, the insulin secretion rate of MIN6 cells transfected with miRNA mimics was clearly decreased, and cells seemed insensitive to the glucose gradients. While MIN6 cells with blocking of miR-218 and miR-322 displayed a higher rate of insulin secretion compared with the controls (Fig. 4F). The knockdown of Stxbp1 by its siRNA also leads to a complete inhibition of insulin secretion (Fig. 4E).

Table 1 The screening of Stxbp1-related miRNAs in islets treated for a short period with high levels of glucose

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Glucose</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mM</td>
<td>25 mM</td>
</tr>
<tr>
<td>miR-9</td>
<td>1</td>
<td>0.95</td>
</tr>
<tr>
<td>miR-15</td>
<td>1</td>
<td>0.94</td>
</tr>
<tr>
<td>miR-16</td>
<td>1</td>
<td>0.89</td>
</tr>
<tr>
<td>miR-195</td>
<td>1</td>
<td>1.06</td>
</tr>
<tr>
<td>miR-322</td>
<td>1</td>
<td>0.21</td>
</tr>
<tr>
<td>miR-424</td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td>miR-497</td>
<td>1</td>
<td>1.07</td>
</tr>
<tr>
<td>miR-30</td>
<td>1</td>
<td>1.03</td>
</tr>
<tr>
<td>miR-384-5p</td>
<td>1</td>
<td>0.87</td>
</tr>
<tr>
<td>miR-218</td>
<td>1</td>
<td>0.18</td>
</tr>
<tr>
<td>miR-143</td>
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<td>0.92</td>
</tr>
</tbody>
</table>

Taken together, these results indicated that miR-218 and miR-322 negatively regulate insulin secretion from β-cells through silencing Stxbp1 expression.

Role of miR-218, miR-322, and Stxbp1 in glucolipotoxicity

Long-term exposure to high levels of glucose leads to the functional breakdown of islets, which is known as glucolipotoxicity, study of the mechanism of glucolipotoxicity in β-cells may provide a better understanding of
development of T2D (Melloul et al. 1993). In this study, we proved that STXBP1 protein was decreased by nearly 80% in islets exposed to high levels of glucose for 72 h (Fig. 5A), while expression of Stxbp1 was constant in islets exposed to 5 mM glucose (Fig. 5B). To determine whether miRNAs were implicated in this process, we also checked the mRNA levels. The Stxbp1 mRNA showed only a slight decrease in islets cultured with high levels of glucose for a long period (Fig. 5B and Supplementary Figure S2, see section on supplementary data given at the end of this article). Meanwhile, levels of miR-218 and miR-322 were raised to 400% in islets exposed to 25 mM glucose compared with islets cultured with low levels of glucose. We also determined the expression of syntaxin, which is

Figure 4
The role of miR-218 and miR-322 co-targeting Stxbp1 in the process of rapid insulin secretion. (A) Relative levels of miR-218 and miR-322 in MIN6 cells transfected with corresponding mimics. MIN6 cells were transfected with mimics of miR-218 or miR-322, or a mix of mimics of the two miRNAs, and cells were harvested after 24 h of incubation. The miRNA expression was assessed using quantitative PCR. Values are mean ± S.E.M. (n = 3), **P < 0.01. (B) Western blotting analysis of expression of Stxbp1 in MIN6 cells under the influence of siRNA and miRNA mimics. (C) Relative levels of miR-218 and miR-322 in MIN6 cells transfected with miRNA inhibitors. Values are mean ± S.E.M. (n = 3), **P < 0.01. (D) The expression of STXBP1 protein in MIN6 cells with knockdown of miR-218 and miR-322. (E) Immunofluorescence of Stxbp1 in MIN6 cells with overexpression of miR-218 mimics or miR-322 mimics. Scale bars = 50 μm. (F) The effects of siRNA and the overexpression of miR-218 and miR-322 on insulin secretion (n = 3, **P < 0.01). (G) The knockdown of miR-218 and miR-322 promote insulin secretion of MIN6 cells (n = 3, **P < 0.01).
The levels of glucose ([D] The levels of insulin secretion from islets treated with prolonged high levels of glucose on the expression of miR-218 and miR-322 in mouse islets. [C] Effects of long-term high exposure to high levels of glucose. [A] Western blotting analysis of Stxbp1 and syntaxin in islets treated with high levels of glucose for 72 h. Islets were cultured in medium containing 25 mM glucose, the 5 mM glucose was used as a negative control. (n=3, **P<0.01, *P<0.05). (B) PCR analysis of Stxbp1 mRNA in islets incubated with prolonged exposure to high levels of glucose. (C) Effects of long-term high levels of glucose on the expression of miR-218 and miR-322 in mouse islets. (D) The levels of insulin secretion from islets treated with prolonged high levels of glucose (n=3, **P<0.01).

modulated by Stxbp1 and also involved in insulin secretion, and found that the expression of syntaxin was decreased by 25% in β cells cultured with 25 mM glucose. The levels of insulin secretion of these islets were also determined to evaluate the influence of pro-longed high levels of glucose on β cells. As expected, the secreted insulin was decreased from 1.5 ng/islet per h to around 0.5 ng/islet per h.

This implied that miR-218 and miR-322 play an important role in the process of β-cell damage caused by long-term exposure to high levels of glucose.

**Discussion**

Although the number of pathways involving miRNAs is continuously increasing, the role of miRNAs in β-cell function and T2D remains unclear. Recently, miRNAs have been reported to be involved in insulin synthesis, the mechanism still needs further study (Fred et al. 2010, Lu et al. 2010, Wang et al. 2010, Zhao et al. 2010). Although the number of known miRNAs is continuously increasing, information regarding their precise cellular function remains limited. One of the main challenges in understanding the functions of miRNAs is to identify the genuine target genes of miRNAs. In this study, we aimed to identify the novel pathways involving miRNAs and Stxbp1 in the physiological process of insulin secretion.

Although Stxbp1 is well known to participate in the fusion of insulin secretory granules in the plasma membrane of β cells (Gomi et al. 2005, Dong et al. 2007, Yu et al. 2014), the expression of Stxbp1 in the process of rapid insulin secretion has not been reported yet. In this study, we determined the expression of both Stxbp1 mRNA and STXBP1 protein during the physiological process of GSIS for the first time, to our knowledge.

Not surprisingly, the protein level of STXBP1 was obviously upregulated in β cells, accompanied by increased insulin secretion. However, Stxbp1 mRNA showed little change within 1-h of stimulation, thus it is believed that Stxbp1 expression is regulated at the transcriptional level.

The biological roles of miR-218 and miR-322 have been extensively explored, however, studies focused on their function in β cells and T2D were still absent.

By using bioinformatics programs, we selected a group of genuine pairing miRNAs of Stxbp1. We used RT-qPCR to check the expression of these miRNAs in isolated islets cultured in medium containing high levels of glucose, and miR-218 and miR-322 were identified as the candidate regulators of Stxbp1. The two miRNAs were proved to be significantly decreased during the process of rapid GSIS, and the relatively low level of miR-218 and miR-322 may promote the expression of Stxbp1 and insulin secretion.

The subsequent luciferase assays further confirmed that miR-218 and miR-322 directly target the 3′UTR of Stxbp1. The overexpression of the two miRNAs in MIN6
cells leads to the inhibition of Stxbp1 expression and insulin secretion; while the knockdown of the two miRNAs relatively promoted the Stxbp1–insulin pathway.

Glucose has long been recognized as the main stimulator of insulin synthesis and secretion, however, prolonged high levels of glucose, or glucolipotoxicity, decrease insulin secretion, and impair β cell function (Eizirik et al. 1992, Conget et al. 1994, Fred et al. 2010). Studies on the molecular mechanism of glucolipotoxicity provide better understanding of T2D. Reduced insulin release has been commonly linked to defective exocytosis. Stxbp1 positively regulates insulin secretion, and it has been reported to be reduced in the islets of both T2D patients and GK rats (Zhang et al. 2002, Lam et al. 2005, Andersson et al. 2012). Our results indicated that the expression levels of miR-218 and miR-322 were raised in mouse islets exposed to high levels of glucose for a long period (72 h), which is followed by the sharp decrease in expression of Stxbp1 and the blocking of insulin secretion.

In this study, miR-218 and miR-322 were screened out as the upstream regulators of Stxbp1, and played an important role in the process of GSIS. The pathway consisting of miR-218/322 and Stxbp1 was also proved to be involved in β cell dysfunction caused by glucolipotoxicity. Therefore, our results contribute to the network consisting of miRNAs, target proteins, and insulin, providing a potential target for studying the molecular mechanisms of β-cell disorders and diabetes.

Conclusions

In this study, the pathway consisting of miR-218/322 and Stxbp1 was proved to play an important role in the process of glucose-induced rapid insulin secretion, and was also involved in the β-cell dysfunction caused by prolonged exposure to high levels of glucose. Stxbp1 significantly promotes insulin secretion, while its expression was regulated by miR-218 and miR-322 in β cells.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-14-0305.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
H L performed most of the experiments, analyzed data, and wrote the manuscript. Z A and Z Y reviewed and edited the manuscript. Y W, W G, and J X performed some experiments. Z A and Z Y contributed to the discussion and edited the manuscript. X J designed the experiments and wrote and edited the manuscript. X J is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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