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Role of hepatic neuregulin 4 in the regulation of gluconeogenesis in mice

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ABSTRACT

Aims: Enhanced hepatic gluconeogenesis is an important cause of hyperglycemia in type 2 diabetes. However, the regulatory mechanisms underlying disordered hepatic gluconeogenesis remains largely unclear. In the present study, we investigated the potential role of hepatic neuregulin 4 (Nrg4) in the regulation of gluconeogenesis in mice.

Main methods: Microarray analysis was performed in primary mouse hepatocytes treated with or without 8-Br-cAMP. Primary mouse hepatocytes transfected with Nrg4 overexpressing or shRNA adenovirus were used to detect the expressions of the key gluconeogenic genes and glucose output. Hepatic Nrg4 expression levels were measured in fasted C57/BL6 mice, obese *ob/ob* mice, diabetic *db/db* mice and Goto-Kakisaki (GK) rats. Pyruvate tolerance test was performed and gluconeogenic gene expressions were detected 7 days after Nrg4 shRNA adenovirus was injected into male C57BL/6 and *db/db* mice.

Key findings: Microarray analysis revealed that Nrg4 expression was significantly induced by 8-Br-cAMP in primary mouse hepatocytes, along with the upregulation of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). Adenovirus-mediated overexpression or knockdown of Nrg4 in primary mouse hepatocytes increased or decreased PEPCK and G6Pase expressions as well as hepatic glucose production. Hepatic Nrg4 expression was induced by fasting in normal C57/BL6 mice, and markedly upregulated in obese *ob/ob* mice, diabetic *db/db* mice and GK rats. Hepatic Nrg4 knockdown in C57BL/6 and *db/db* mice improved pyruvate tolerance, with the downregulation of PEPCK, G6Pase, and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α).

Significance: Hepatic Nrg4 plays a crucial role in the regulation of gluconeogenesis and may be a therapeutic target of type 2 diabetes.

1. Introduction

Type 2 diabetes is a serious chronic disease characterized by hyperglycemia due to insufficient insulin secretion or ineffective insulin action [1], which has become an important public health problem around the world. Liver plays a crucial role in the maintenance of normal glucose homeostasis by controlling the balance of hepatic glucose storage and production [2]. In humans, gluconeogenesis contributes to the vast majority of total hepatic glucose production under fasting condition [3]. Enhanced hepatic gluconeogenesis is a major cause of fasting hyperglycemia in type 2 diabetic patients [4]. However, the mechanism of disordered hepatic gluconeogenesis remains largely unknown.

Neuregulin 4 (Nrg4) is a kind of epidermal growth factor-like molecule belonged to the Neuregulins family, acting as an extracellular ligand of ErbB3 and ErbB4 tyrosine kinase receptors to trigger downstream signaling pathways [5,6]. It has been demonstrated that Nrg4 plays an essential role for tissue development, epithelial cell survival, synapse growth and elaboration [5,7–9]. In recent years, accumulating evidence has shown that Nrg4 is involved in metabolic regulation in mice, especially in lipid metabolism. Nrg4 is abundantly expressed in liver and adipose tissues of mice [6]. Nrg4 expression in adipose tissue

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Table 1
Primer sequences used for real-time quantitative PCR.

Gene	Species	Forward sequence (5′ to 3′)	Reverse sequence (5′ to 3′)
β-Actin	Mouse	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
Nrg4	Mouse	CCTACTATCCCCAGCCCATTCT	TGCCGACAGATTACTTTCGCT
PEPCK	Mouse	GTGCTGGAGTGGATGTTCGG	CTGGCTGATTCTCTGTTTCAGG
G6Pase	Mouse	ACTGTGGGCATCAATCTCCTC	CGGGACAGACAGACGTTCAGC
PGC-1α	Mouse	ATACCGCAAAGAGCACGAGAAG	CTCAAGAGCAGCGAAACGTCACAG
18S	Rat	CACGGGTGACGGGGAATCAG	CGGGTCGGGAGTGGGTAATTTG
Nrg4	Rat	GGTCCTCGTCACTCTTGCCA	GGTATTGCCTGTCTCCACCAG

is related to obesity. Nrg4 secreted from the brown adipose tissue (BAT) protects against diet-induced insulin resistance and hepatic steatosis through attenuating hepatic lipogenic signaling and promoting hepatic fatty acid oxidation [6,10]. Therefore, Nrg4 is regarded as a beneficial endocrine factor for obesity-associated metabolic disorders.

Insulin and glucagon are two antagonistic hormones for the regulation of hepatic gluconeogenesis. Glucagon promotes hepatic gluconeogenesis *via* activating cAMP/PKA/CREB signaling pathway and stimulating the expressions of key gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) [11,12]. In the present study, we aimed to identify differentially expressed genes involved in cAMP-stimulated gluconeogenesis in primary mouse hepatocytes. Interestingly, our gene chip result revealed that Nrg4 expression was significantly induced by 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), along with the upregulation of PEPCK and G6Pase. We further investigated the involvement of Nrg4 in hepatic gluconeogenesis *in vitro* and *in vivo* as well as its mechanism.

2. Materials and methods

2.1. Materials

Male C57BL/6 mice aged 6–8 weeks were purchased from Shanghai Slack Experimental Center (Shanghai, China). GK rats, *ob/ob*, *db/db* mice and corresponding control animals were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Hank's balanced salt solution (HBSS), collagenase Type IV, phosphate buffer solution (PBS), Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Hepatocyte medium was obtained from ScienCell Research Laboratories, Inc. (Carlsbad, CA, USA). Bovine serum albumin (BSA), dexamethasone, 8-Br-cAMP, sodium pyruvate and sodium lactate were purchased from Sigma (St. Louis, MO, USA). All the primers mentioned in the article were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). Anti-PEPCK and anti-G6Pase were from Abcam (Cambridge, UK), anti-tubulin was from Cell Signaling Technology (Danvers, MA, USA), and anti-Hsp90 was from Millipore (Billerica, MA, USA).

2.2. Primary mouse hepatocyte isolation, culture, and treatment

Primary hepatocytes were isolated by the two-step perfusion method. Mouse liver was perfused with 10 ml calcium-free 1 × HBSS through hepatic portal vein, and then 20 ml HBSS containing calcium and 0.05% collagenase IV was perfused in a recirculating manner until sufficient digestion. Hepatocytes suspension was filtered through a 70 μm nylon mesh, centrifuged at 1000 rpm for 5 min at 4 °C, and washed by 1× PBS for three times. The cell pellet was resuspended in hepatocyte medium and plated onto 6-well or 12-well plates at about a 3×10^5/ml density for culture.

Primary mouse hepatocytes were cultured at 37 °C in a humidified 5% CO_2 incubator. After 24 h, the hepatocyte medium was replaced by DMEM containing 5 mM glucose, 0.25% BSA, 10 U penicillin, 10 μg streptomycin and 100 nM dexamethasone for 12–16 h pretreatment. And then cells were incubated with glucose/phenol red-free DMEM containing gluconeogenic substrates (10 mM sodium lactate and 1 mM sodium pyruvate) in the presence or absence of 100 μM 8-Br-cAMP for 8 h.

2.3. Microarray analysis

Total RNA was extracted from primary mouse hepatocytes incubated with or without 8-Br-cAMP by using Trizol (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA). Sample labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology, Santa Clara, CA, USA). Agilent DNA Microarray Scanner (part number G2505C) was used to scan the hybridized arrays. Agilent Feature Extraction software (version11.0.1.1) was used to analyze acquired array images. Microarray analysis was performed by KangChen Bio-tech (Shanghai, China).

2.4. RNA extraction and real-time quantitative PCR

Total RNA was extracted from primary mice hepatocytes or mice livers using Trizol. RNA quality was evaluated with NanoDrop-2000 spectrophotometer by the A260/A280 and A260/A230 ratio. cDNA was synthesized by GoScript reverse transcription kit (Promega Corporation, Madison, WI, USA). Real-time quantitative PCR (RT-qPCR) was performed with Applied Biosystems7300 Real-Time PCR machine (Applied Biosystems, Foster City, CA, USA) using a SYBR Green Premix Ex Taq (Takara, Shiga, Japan). Primer sequences are described in Table 1. The expression abundance of genes was normalized to 18S rRNA or β-actin mRNA levels in each sample.

2.5. Glucose output measurement

Glucose concentration of the medium was measured by the Glucose Oxidase Method Measurement Kit (Applygen Technologies Inc., Beijing, China). Briefly, after treatment, 200 μl medium per well was transferred into a new vial and centrifuged at 2000 ×*g* for 10 min at 4 °C. Then 5 μl supernatant was removed into 195 μl working solution and incubated in 37 °C water bath for 30 min in a 96-well plate. The glucose concentration was qualified with an OD reading at 550 nm.

2.6. Adenovirus infection

For interference, Nrg4 specific targeted short hairpin RNA (sh-Nrg4: 5′-CAGCATCCCAAGCGAAAGTAA-3′) adenovirus and control adenovirus expressing scrambled negative shRNA (sh-Ctrl:5′-TTCTCCGAAC GTGTCACGT-3′) were constructed by GeneChem Co., Ltd. (Shanghai, China). For overexpression, adenovirus encoding a full-length mouse Nrg4 and control adenovirus encoding green fluorescent protein (GFP) were constructed by GeneChemCo., Ltd. (Shanghai, China). Primary mouse hepatocytes were transfected with 4×10^7 pfu shRNA adenovirus or 1×10^7 pfu overexpression adenovirus according to the

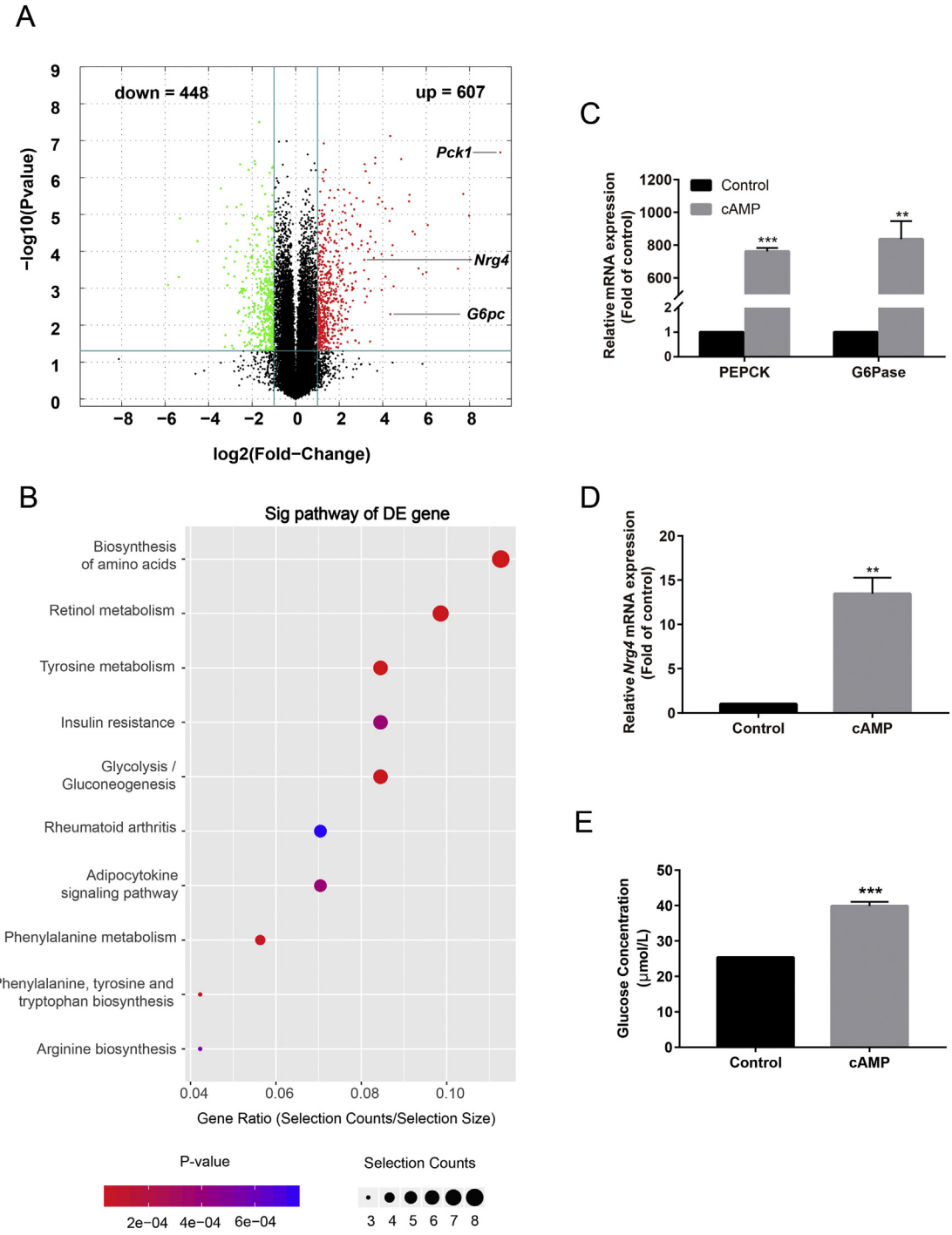

Fig. 1. Microarray expression analysis reveals the correlation between Nrg4 and hepatic gluconeogenesis. (A) Primary mouse hepatocytes treated with or without 100 μM 8-Br-cAMP for 8 h was extracted for microarray analysis (n = 3) and Volcano plot of the differentially expressed genes (Fold change > 2.0, $P < 0.05$) was performed. (B) KEGG pathway analysis of 84 upregulated genes (Fold change > 5.0, $P < 0.05$). (C and D) RT-qPCR analysis of PEPCK, G6Pase, and Nrg4 mRNA expressions in the primary hepatocytes. (E) Hepatic glucose output of the primary hepatocytes was measured. Data were given as mean ± SEM for three separate experiments. $^{**}P < 0.01$, and $^{***}P < 0.001$ *vs* control group.

manufacturer instructions for 24 h, and then cells were performed for further experiments.

2.7. Western blot

Primary mouse hepatocytes were treated with 1× RIPA lysis buffer for total protein extraction. Protein concentration was evaluated by the BCA protein determination method. Degenerated samples were added into 10% SDS-PAGE gel for electrophoresis. After transfer, the PVDF membrane was blocked with 5% BSA and incubated with antibodies at a 1:1000 dilution. Images were taken by a LAS-4000 Super CCD Remote Control Science Imaging System (Fuji, Japan).

2.8. Animal treatment

All the animals were housed under 12/12 h light/dark cycles with free access to food and water. 8-week-old male C57BL/6 mice or 4-week-old male *db/db* mice were injected with 200 μl saline solution

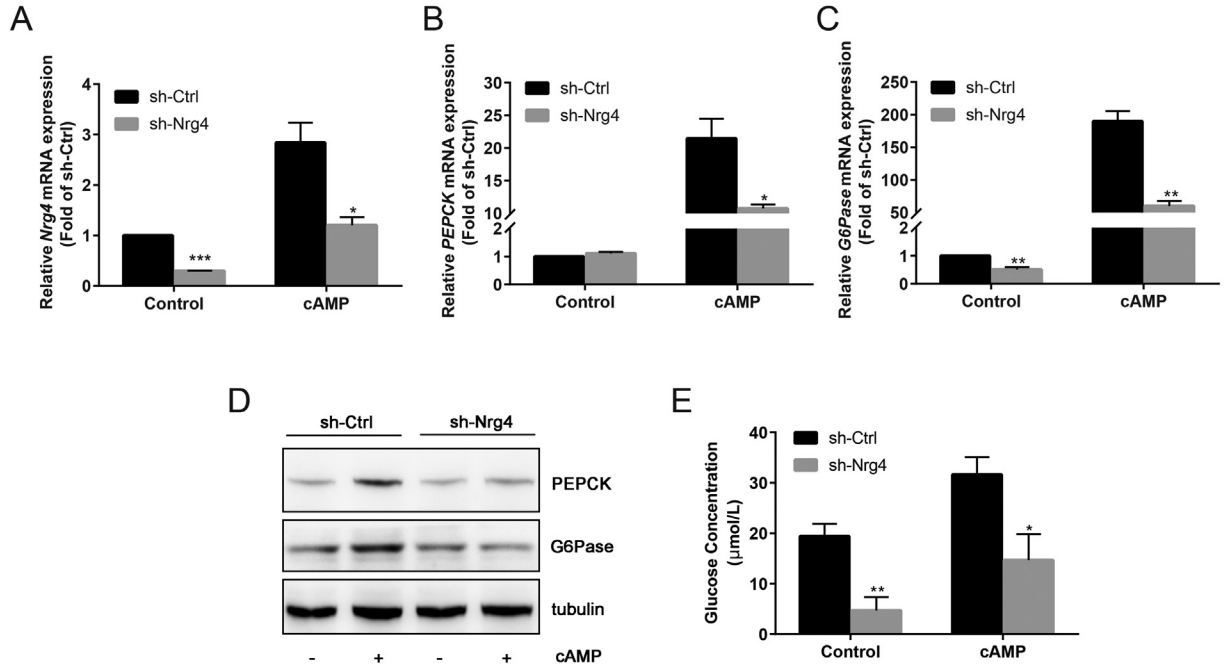

Fig. 2. Nrg4 knockdown inhibits gluconeogenesis in primary mouse hepatocytes. After primary mouse hepatocytes were transfected with control adenovirus (sh-Ctrl) and Nrg4 interference adenovirus (sh-Nrg4) for 24 h and incubated with 100 μM 8-Br-cAMP for 8 h, (A–C) Nrg4, PEPCK, and G6Pase mRNA expressions were detected by RT-qPCR, (D) PEPCK and G6Pase protein expressions were detected by Western blot, and (E) glucose production was measured. Data were given as mean ± SEM for at least three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *vs* control group.

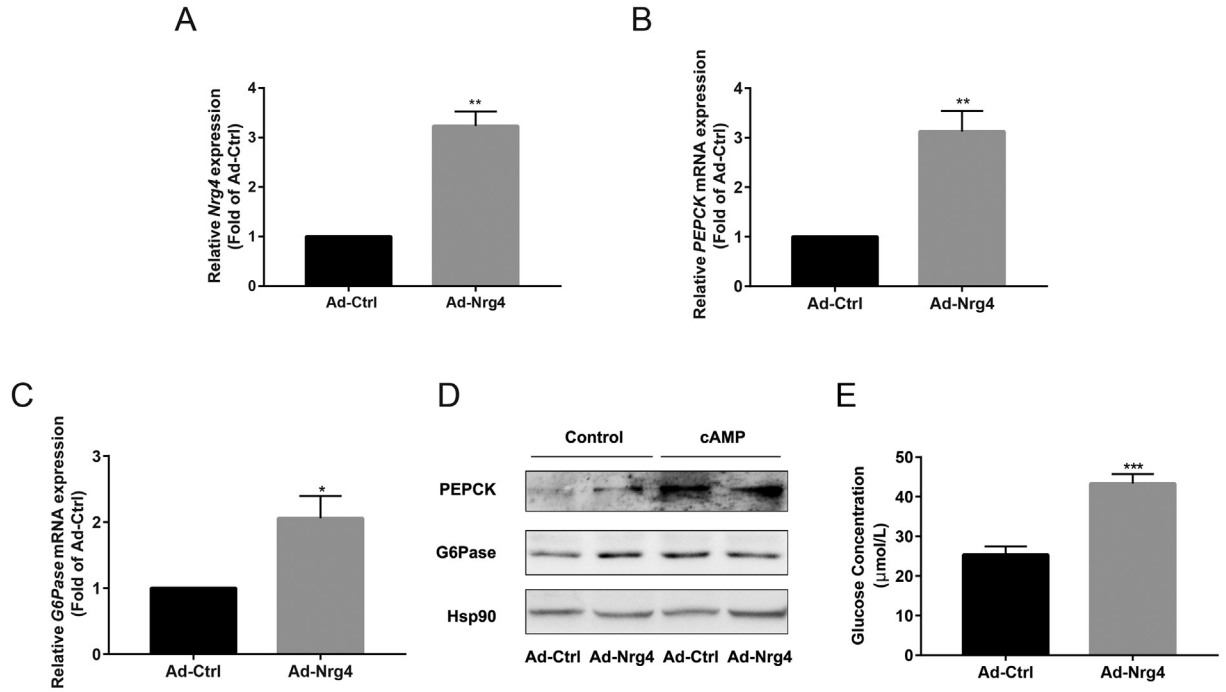

Fig. 3. Nrg4 overexpression increases gluconeogenesis in primary mouse hepatocytes. (A–C) Nrg4, PEPCK and G6Pase mRNA expressions in primary mouse hepatocytes transfected with control adenovirus (Ad-Ctrl) or Nrg4 overexpression adenovirus (Ad-Nrg4) in the absence of 8-Br-cAMP. (D) PEPCK and G6Pase protein expressions in primary mouse hepatocytes transfected with Ad-Ctrl or Nrg4 Ad-Nrg4 in the presence or absence of 8-Br-cAMP. (E) Glucose output in primary mouse hepatocytes transfected with Ad-Ctrl or Ad-Nrg4 in the absence of 8-Br-cAMP. Data were given as mean ± SEM for three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *vs* control group.

containing 2.4×10^9 pfu control or Nrg4 shRNA adenovirus through the tail vein.

The intraperitoneal pyruvate tolerance test was performed in 16 h fasted C57/BL6 and *db/db* mice 7 days after adenovirus injection. Sodium pyruvate was delivered *via* intraperitoneal injection at a dose of 2 g/kg body weight. The tail vein blood glucose was determined using an Accu-Chek Performa glucometer (Roche, Basel, Switzerland). All animal protocols were reviewed and approved by the Animal Care Committee of Ruijin Hospital, Shanghai Jiaotong University School of Medicine.

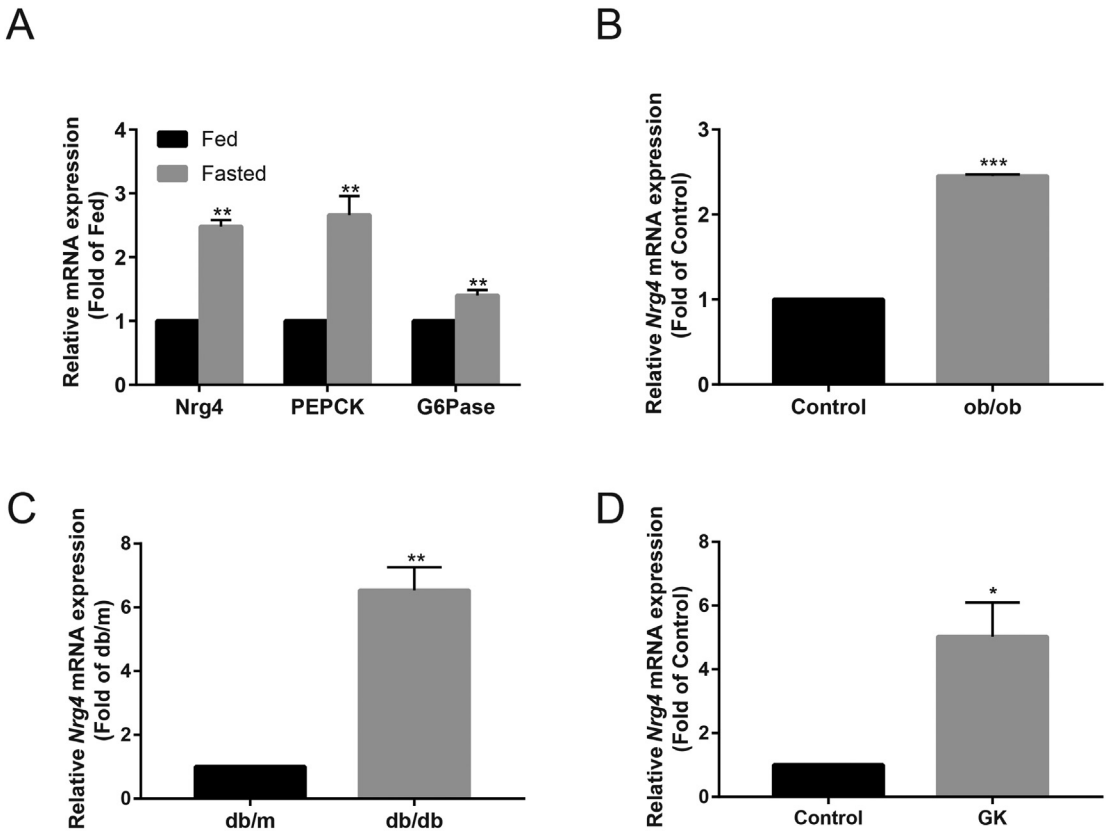

Fig. 4. Increased hepatic Nrg4 expression in diabetic or obese animals. (A) Hepatic Nrg4 mRNA expression in male C57BL/6 mice under fed or fasted condition for 16 h ($n = 4$ per group). (B) Hepatic Nrg4 gene expression in male control mice or obese *ob/ob* mice ($n = 3$ per group). (C) Hepatic Nrg4 mRNA expression in male *db/m* control mice or diabetic *db/db* mice ($n = 3$ per group). (D) Hepatic Nrg4 gene expression in male control Wistar rats or diabetic GK rats ($n = 3$ per group). Data were given as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *vs* control group.

2.9. Statistics

Data are performed as means ± SEM. Comparisons were performed by using the Student's *t*-test for 2 groups. Significance was established at $P < 0.05$.

3. Results

3.1. Microarray expression analysis reveals the correlation between Nrg4 and hepatic gluconeogenesis

To identify the differentially expressed genes involved in hepatic gluconeogenesis, the global gene expression patterns were analyzed for primary mouse hepatocytes incubated with and without 100 μM 8-Br-cAMP for 8 h. Microarray analysis revealed 1055 differentially expressed genes between control group and 8-Br-cAMP treatment group (fold change > 2.0, $P < 0.05$), including 607 upregulated genes and 448 downregulated genes. According to the volcano plot shown in Fig. 1A, the expressions of two key gluconeogenic enzymes PEPCK (encoded by *Pck1*) and G6Pase (encoded by *G6Pc*) were significantly upregulated in 8-Br-cAMP treatment group, demonstrating the effective stimulation of hepatic gluconeogenesis. Among these differentially expressed genes, we further analyzed 124 genes with fold change > 5.0 as candidate genes for the regulation of hepatic gluconeogenesis. According to KEGG pathway analysis of 84 significantly upregulated genes, glycolysis/gluconeogenesis was one of the top ten enriched pathways (Fig. 1B). Nrg4 exhibited an abundant gene expression level in control group and was raised by 8.92 folds after 8-Br-cAMP treatment in primary mouse hepatocytes (Fig. 1A). Interestingly, in recent years several studies have shown that circulating Nrg4 level was elevated in patients with type 2 diabetes [13,14]. Therefore, it is reasonable to suppose that Nrg4 is involved in the regulation of hepatic gluconeogenesis and pathogenesis of type 2 diabetes. RT-qPCR revealed that PEPCK, G6Pase, and Nrg4 expressions were strongly elicited by 8-Br-cAMP in primary mouse hepatocytes (Fig. 1C and D). As expected, hepatic glucose output was also markedly increased in 8-Br-cAMP treatment group (Fig. 1E). These results validate the reliability of microarray analysis.

3.2. Nrg4 regulates gluconeogenesis in primary mouse hepatocytes

To determine the role of Nrg4 in hepatic gluconeogenesis, primary mouse hepatocytes were transfected with control adenovirus or sh-Nrg4 adenovirus for 24 h, followed by treatment with 100 μM 8-Br-cAMP for 8 h. As shown in Fig. 2A, Nrg4 expression was decreased over 50% in primary mouse hepatocytes transferred with sh-Nrg4 in both control and 8-Br-cAMP treatment groups. The knockdown of Nrg4 expression significantly suppressed PEPCK and G6Pase mRNA (Fig. 2B and C) and protein (Fig. 2D) expressions. In consistent with the downregulations of the two key gluconeogenic enzymes, hepatic glucose production was also reduced with the silence of Nrg4 expression in primary mouse hepatocytes (Fig. 2E). Conversely, overexpression of Nrg4 (Fig. 3A) in primary mouse hepatocytes led to increased mRNA expressions of PEPCK and G6Pase (Fig. 3B and C). Forced Nrg4 expression induced a similar trend for PEPCK and G6Pase protein expressions in the absence of 8-Br-cAMP, but did not further enhance 8-Br-cAMP-stimulated expressions of the two enzymes (Fig. 3D). Correspondingly, hepatic glucose output was markedly elevated by Nrg4 overexpression in the absence of 8-Br-cAMP (Fig. 3E). These results indicate that Nrg4 regulates gluconeogenesis *via* stimulating PEPCK and G6Pase expressions.

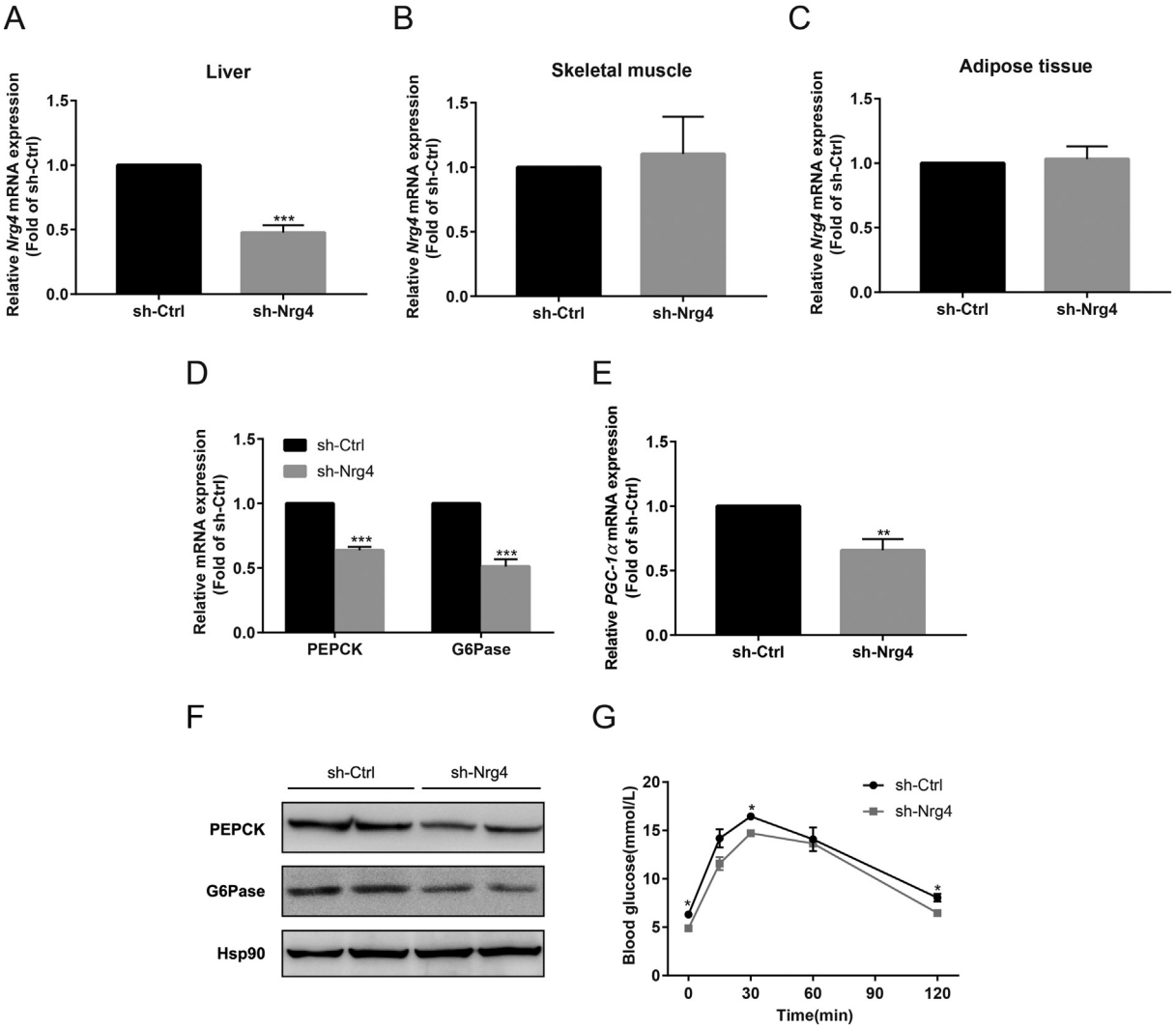

Fig. 5. Knockdown of Nrg4 suppressed hepatic gluconeogenesis in C57BL/6 mice. (A–C) Nrg4 mRNA expressions in liver, skeletal muscle, and adipose tissue of male C57BL/6 mice injected with sh-Ctrl adenovirus or sh-Nrg4 adenovirus *via* tail vein. (D and E) Hepatic PEPCK, G6Pase, and PGC-1α mRNA expressions in C57BL/6 mice injected with sh-Ctrl adenovirus or sh-Nrg4 adenovirus. (F) Hepatic PEPCK and G6Pase protein expressions in C57BL/6 mice injected with sh-Ctrl adenovirus or sh-Nrg4 adenovirus. (G) Pyruvate tolerance test was performed 7d after adenovirus injection. Data were given as mean ± SEM from 6 mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* control group.

3.3. Increased hepatic Nrg4 expression in the liver of diabetic animals

To explore whether Nrg4 is involved in the regulation of in hepatic gluconeogenesis *in vivo*, we firstly detected hepatic Nrg4 expression in response to different nutritional signals. As shown in Fig. 4A, hepatic Nrg4 expression was increased in C57BL/6 mice after 16 h fasting, along with elevated PEPCK and G6Pase expressions. Next, Nrg4 expression levels in the liver were investigated in the several animal models of diabetes and obesity. Compared with corresponding controls, hepatic Nrg4 expressions were markedly increased in obese *ob/ob* mice, diabetic *db/db* mice, and diabetic GK rats (Fig. 4B–D). All these results further indicate that hepatic Nrg4 expression is tightly correlated with glucose metabolism.

3.4. Knockdown of Nrg4 decreases hepatic gluconeogenesis in C57BL/6 and db/db mice

To investigate the *in vivo* effect of Nrg4 on hepatic gluconeogenesis, sh-control adenovirus or sh-Nrg4 adenovirus was injected into male C57BL/6 mice through tail vein. Nrg4 expression in liver was remarkably reduced in C57/BL6 mice injected with sh-Nrg4 (Fig. 5A), without significant alternation in skeletal muscle and epididymal white adipose tissue between the two groups (Fig. 5B and C). Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), a crucial transcriptional coactivator, stimulates PEPCK and G6Pase expression under the fasting state [15]. Nrg4 knockdown in liver resulted in a reduction of PEPCK, G6Pase, and PGC-1α mRNA expressions (Fig. 5D and E). PEPCK and G6Pase protein expressions showed a similar result (Fig. 5F). To determine whether Nrg4 downregulation led to a decrease in hepatic gluconeogenesis, intraperitoneal pyruvate tolerance test was performed 7 days after sh-Nrg4 adenovirus injection. As expected, blood glucose levels at 0, 30, 120 min after pyruvate challenge were remarkably decreased in sh-Nrg4-treated C57/BL6 mice compared with control mice (Fig. 5G).

We further injected sh-control or sh-Nrg4 adenovirus into male *db/db* mice to examine whether Nrg4 knockdown in liver could rescue the elevated gluconeogenesis. Like in C57BL/6 mice, injection of sh-Nrg4 also decreased Nrg4 mRNA expression in liver of *db/db* mice (Fig. 6A), not in skeletal muscle and epididymal adipose tissues (Fig. 6B and C). Correspondingly, PEPCK, G6Pase, and PGC-1α expressions were significantly inhibited in sh-Nrg4-injected *db/db* mice (Fig. 6D–F). No significant differences in body weight and food intake were found

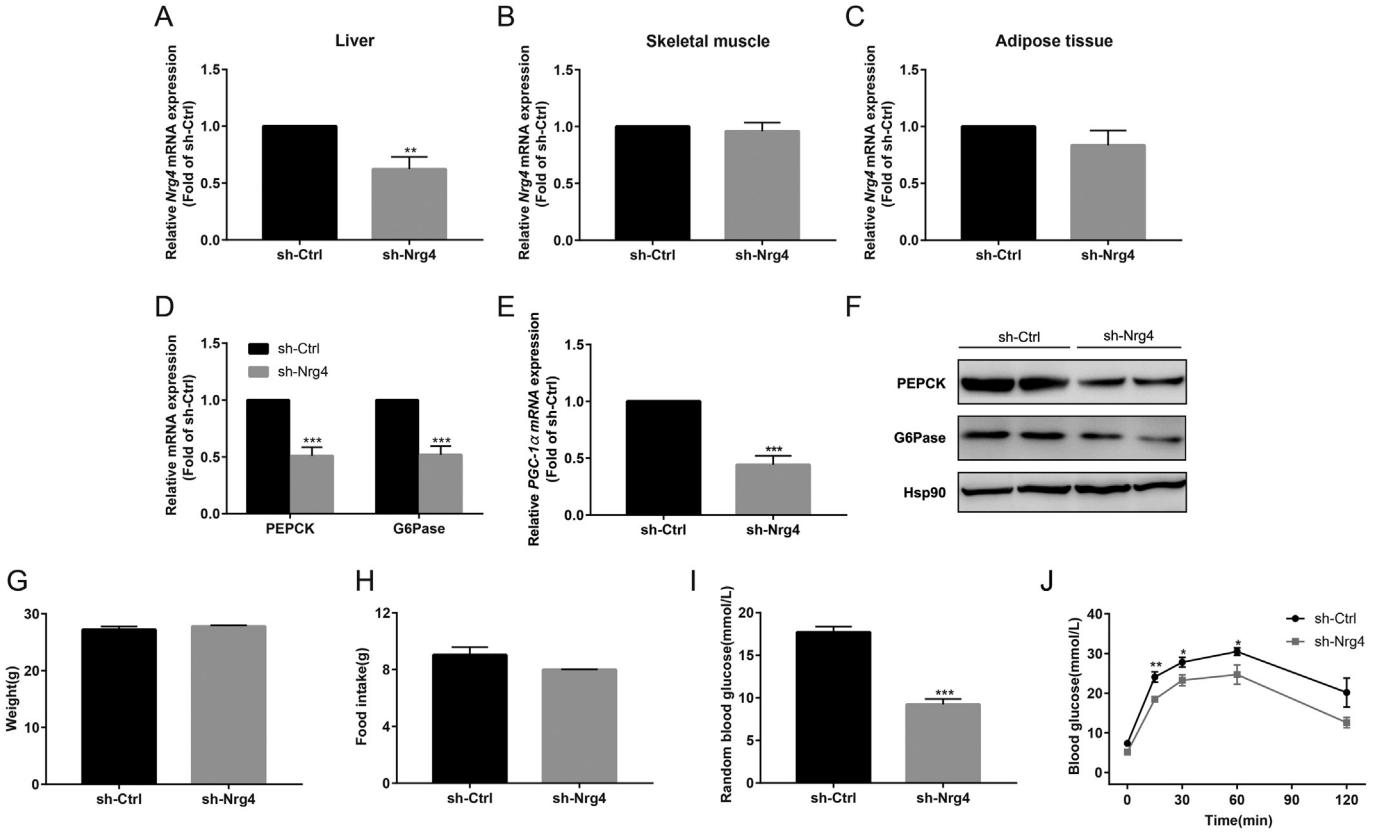

Fig. 6. Knockdown of Nrg4 attenuated abnormal hepatic gluconeogenesis in *db/db* mice. (A–C) Nrg4 mRNA expressions in liver, skeletal muscle, and adipose tissues of *db/db* mice injected with sh-Ctrl or sh-Nrg4 adenovirus were detected by RT-qPCR ($n = 6$ per group). (D and E) PEPCK, G6Pase, and PGC-1α mRNA expressions in the liver. (F) PEPCK and G6Pase protein expressions in the liver. (G) Body weight 6d after adenovirus injection and (H) food intake 7 days after virus injection. (I) Random blood glucose 6 days after adenovirus injection. (J) Pyruvate tolerance test (2 g/kg) was performed 7 days after adenovirus injection. Data were given as mean ± SEM from 6 mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* control group.

between two groups (Fig. 6G and H). However, random blood glucose level was markedly decreased after sh-Nrg4 injection (Fig. 6I). Under the pyruvate challenge, *db/db* mice injected with sh-control displayed a significant elevation of blood glucose, while sh-Nrg4-treated *db/db* mice showed a less response to pyruvate (Fig. 6J). These results suggest that Nrg4 knockdown in the liver may improve global glucose metabolism *via* suppressing hepatic gluconeogenesis.

4. Discussion

Elevated hepatic gluconeogenesis is a crucial contributor of hyperglycemia in diabetes. In this current study, we used 8-Br-cAMP-treated primary mouse hepatocytes, which mimic the fasting action of glucagon, to identify differentially expressed genes involved in gluconeogenesis by microarray analysis. The result revealed that Nrg4 expression in primary mouse hepatocytes was markedly induced by 8-Br-cAMP treatment, along with increased PEPCK and G6Pase expressions. Adenovirus-mediated overexpression or knockdown of Nrg4 in primary mouse hepatocytes stimulated or inhibited PEPCK and G6Pase expressions as well as hepatic glucose output. Knockdown of Nrg4 in the liver of C57/BL6 and *db/db* mice suppressed hepatic gluconeogenesis, suggesting that Nrg4 is an important regulator of gluconeogenesis.

As a growth factor, Nrg4 is related to breast cancer, prostate cancer, gastrointestinal malignant lymphoma, gastric cancer and oral leukoplakia in human [16–23]. Recently, Nrg4 was identified as a BAT-enriched endocrine factor to alleviate diet-induced insulin resistance and hepatic steatosis *via* blocking hepatic lipogenesis and chronic inflammation as well as promoting hepatic fatty acid oxidation and energy expenditure [6,10]. Subsequent clinical studies also implied a link of Nrg4 to energy metabolism. Circulating Nrg4 levels are inversely associated with the risks of metabolic syndrome (MS) and subclinical cardiovascular disease in obese adults [24,25]. The patients with non-alcoholic fatty liver disease (NAFLD) exhibited a significant decrease in serum Nrg4 concentration compared to control subjects without NAFLD [26], suggesting that Nrg4 is a protective factor in the development of metabolic disorders. It has been demonstrated that Nrg4 mRNA expressions are significantly decreased in adipose tissues in murine and human obesity [6]. However, our study revealed a significant elevation of Nrg4 mRNA levels in the liver of obese or diabetic rodents, indicating that Nrg4 has a tissue-specific expression pattern under different pathological conditions. At present, all metabolic benefits of Nrg4 were attributed to its endocrine action as an adipokine [7,11,22]. Nothing is known about the effect of Nrg4 in other tissues on the local or systemic metabolism. The autocirne/paracrine effect of Nrg4 is greatly neglected.

There are several studies demonstrating contradictory results about the correlation of Nrg4 with diabetes. Two independent studies showed that circulating Nrg4 levels were significantly elevated in the pre-diabetic and diabetic patients compared with controls without diabetes, suggesting that Nrg4 is an independent risk factor of diabetes [13,14]. Interestingly, another study demonstrated a decrease in serum Nrg4 levels of patients with type 2 diabetes mellitus [27]. In addition, women with gestational diabetes mellitus had lower circulating levels of Nrg4 compared with non-GDM pregnant controls [28]. Therefore, the peripheral effect of Nrg4 on glucose homeostasis need to be further investigated. Hepatic gluconeogenesis is essential for the maintenance of normal blood glucose level [3]. Our study showed that Nrg4 expression in mouse hepatocytes was strongly induced by 8-Br-cAMP or starvation,

suggesting an involvement of Nrg4 in the regulation of gluconeogenesis. Knockdown of Nrg4 in mouse hepatocytes reduced hepatic glucose production *in vitro* and *in vivo*, along with the decreased expressions of two key gluconeogenic enzymes PEPCK and G6Pase. Apparently, Nrg4 expression level in the liver is positively associated with the risk of diabetes.

During fasting, increased circulating glucagon promotes hepatic gluconeogenesis *via* cAMP/PKA signal pathway. The activation of PKA promotes CREB Ser133 phosphorylation and cAMP-regulated transcriptional co-activator 2 (CRTC2) dephosphorylation, stimulating the transcript of PGC-1α gene and its downstream target genes including PEPCK and G6Pase [11]. In the present study, knockdown of Nrg4 in the liver of C57BL/6 mice and *db/db* mice inhibited PGC-1α expression. Thus, it is possible that PGC-1α mediates Nrg4-regulated PEPCK and G6Pase expressions.

5. Conclusion

In summary, our study demonstrates for the first time that hepatic Nrg4 plays an important role in the regulation gluconeogenesis *in vitro* and *in vivo*. Nrg4 knockdown in liver attenuates enhanced hepatic gluconeogenesis in *db/db* mice *via* suppressing PEPCK, G6Pase, and PGC-1α expressions. Therefore, Nrg4 specific-silencing in liver will provide a potential therapeutic strategy for type 2 diabetes.

CRediT authorship contribution statement

Linlin Zhang: Conceptualization, Data curation, Investigation, Methodology, Software, Writing - original draft. **Mengyao Bai:** Conceptualization, Data curation, Investigation, Methodology, Software, Writing - original draft. **Hongju Tang:** Data curation, Investigation, Methodology. **Feiye Zhou:** Investigation, Methodology, Software. **Qin Zhu:** Investigation, Data curation. **Shushu Wang:** Investigation, Software. **Kecheng Zhu:** Investigation, Data curation. **Qianqian Liu:** Investigation, Methodology. **Yun Liu:** Investigation, Methodology. **Xiao Wang:** Data curation, Formal analysis. **Yabin Ma:** Conceptualization, Data curation, Methodology, Software, Project administration, Writing - review & editing. **Libin Zhou:** Conceptualization, Data curation, Methodology, Software, Project administration, Writing - review & editing.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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