AMPK-dependent regulation of GLP1 expression in L-like cells

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Abstract

This study examined whether AMPK, an evolutionarily conserved sensor of cellular energy status, determines the production of glucagon-like peptide-1 (GLP1). A negative relation existed between phosphorylation of AMPKα and the expression and secretion of GLP1 during changes in energy status in STC-1 cells, an L-like cell line. High concentration of glucose (25 mmol/L) decreased AMPKα phosphorylation, whereas it stimulated the expression and secretion of GLP1 relative to 5.6 mmol/L glucose. Serum starvation upregulated AMPKα phosphorylation, whereas it reduced GLP1 production significantly. Stimulation of AMPK phosphorylation by AICAR and overexpression of wild-type AMPKα1, constitutively active AMPKα1 plasmids, or AMPKα1 lentivirus particles suppressed proglucagon mRNA and protein contents in STC-1 cells. Inactivation of AMPK by Compound C, AMPKα1 siRNA or kinase-inactive AMPKα1 mutant increased the expression and secretion of GLP1. Our results suggest that AMPKα1 may link energy supply with the production of GLP1 in L-like cells.

Introduction

The proglucagon gene (GCG) encodes both glucagon and glucagon-like peptides (GLPs) in pancreatic α-cells and intestinal enteroendocrine L cells as well as neurons, respectively (Baggio & Drucker 2007). Glucagon is predominantly generated in pancreatic α-cells by prohormone convertase (PC)-2, whereas in the gut and brain, GCG expression leads to GLP1 synthesis after cleavage by PC-1/3 (Mojsov et al. 1986, Drucker & Asa 1988). The biologically active forms of GLP1 are GLP1(7–37) and GLP1(7–36)NH2 (Orskov et al. 1993). The half-life of bioactive GLP1 in the circulation is less than 2 min owing to rapid inactivation by the ubiquitous proteolytic enzyme dipeptidyl peptidase-4 (DPP4) (Deacon et al. 1995). DPP4 rapidly converts GLP1(7–37) and GLP1(7–36) NH2 to their inactive metabolites GLP1(9–37) or GLP1(9–36)NH2 (Hansen et al. 1999). GLP1 secretion occurs primarily in response to nutrient ingestion, through a complex array of direct and indirect mechanisms (Baggio & Drucker 2007). Postprandial GLP1 levels peak within 15–30 min of nutrient consumption. GLP1, a potent antihyperglycemic hormone, induces glucose-dependent stimulation of insulin secretion, whereas suppresses glucagon secretion (Herrmann et al. 1995). Such glucose-dependent action is attractive particularly because when the plasma glucose concentration is in the normal fasting range, GLP1 no longer stimulates insulin release, thus avoiding
hypoglycemia (Elahi et al. 1994). Insulinotropic activity of GLP1 in type 2 diabetes mellitus, therefore, offers great potential for treatment of hyperglycemia without causing hypoglycemia (Elahi et al. 1994). Current GLP1-based therapies include long-acting GLP1 receptor (GLP1R) agonists (Buse et al. 2004) and inhibitors of GLP1 degradation (Aschner et al. 2006). With the introduction of GLP1-based treatments into the clinical practice, interest in factors that regulate the release of endogenous GLP1 has been heightened.

AMPK, which consists of a catalytic α-subunit and noncatalytic β- and γ-subunits, is a fuel sensor and regulator that plays a vital role in cellular energy homeostasis (Kim & Lee 2005). Enzyme activity is regulated both allosterically by AMP and by reversible phosphorylation at Thr-172 of the α-subunit by an upstream kinase (AMPK kinase or AMPKK) (Hardie et al. 1999). AMPK controls whole-body glucose homeostasis by regulating metabolism in multiple peripheral tissues such as skeletal muscle, liver, adipose tissues and pancreatic β-cells (De Morentin et al. 2011). By responding to diverse nutritional and hormonal signals, AMPK serves as an integrator of intertissue signals among peripheral tissues and the hypothalamus to control energy intake and whole-body energy balance (Minokoshi et al. 2004, De Morentin et al. 2011). The effects of AMPK on GLP1 synthesis in L cells remain largely uncharacterized.

Our previous studies demonstrate that mTORC1 enhances GLP1 synthesis (Xu et al. 2015a,b). As AMPK has been characterized to suppress mTOR signaling (Inoki et al. 2006), we hypothesize that AMPK contributes to the regulation of GLP1 production. Here, we present evidence that AMPKα1 regulates the production of GLP1 in STC-1 cells. Our data identify AMPK as a critical mechanism regulating proglucagon gene expression and GLP1 secretion, thus expanding its interest as a potential target for the treatment of type 2 diabetes mellitus.

Materials and methods

Materials

Deprotin was from Sigma Chemical. Control lentiviral activation particles, AMPKα1 lentiviral activation particles, Polybrene, control siRNA, AMPKα1 siRNA and mouse anti-AMPKα1 antibody were purchased from Santa Cruz Inc (Santa Cruz, USA). AICAR, mouse anti-β-actin, rabbit anti-AMPKα (Thr172) and rabbit anti-AMPKα antibodies were obtained from Cell Signaling Technology. Rabbit anti-AMPKα2 antibody and mouse anti-GLP1 were from Abcam. IRDye-conjugated affinity-purified anti-rabbit, anti-mouse IgGs were purchased from Rockland (Gilbertsville, PA, USA). TRizol reagent and the reverse transcription (RT) system were from Promega. Lipofectamine was purchased from Invitrogen. Compound C and Glucagon-Like Peptide-1 Active enzyme immunoassay kit were purchased from Millipore.

Cell culture and transfection

The intestinal secretin tumor cell line (STC-1) was purchased from ATCC. All experiments were performed using passages 20–25. STC-1 cells were maintained in DMEM medium supplemented with 2.5% fetal bovine serum and 10% horse serum at 37°C in an atmosphere of 5% CO₂ air. For transient transfection, cells were plated at optimal densities and grown for 24 h. Cells were then transfected with the following constructs: GFP, wild-type AMPKα1 (WT), constitutively active form of AMPKα1 (AF), or the dominant negative form of AMPKα1 (DN) plasmids (Corradetti et al. 2004, Inoki et al. 2006) using Lipofectamine reagent according to the manufacturer’s instruction.

Infection of AMPK α1 lentivirus

Lentiviral infection was performed according to the manufacturer’s instruction. The infected STC-1 cells were seeded in a 6-well tissue culture plate and grown to about 80% confluency. A mixture of complete medium with Polybrene at a final concentration of 5μg/mL was prepared, and 3 mL of the mixture were added to each well for the treatment of type 2 diabetes mellitus.

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well of cultured cells. Lentiviral activation particles were thawed at room temperature and mixed gently before use. AMPKα1 lentiviral activation particles or control lentiviral activation particles were administrated to cultured STC-1 cells. Culture plates were swirled gently to mix the lentivirus. Then, the cells were incubated for 48 h.

Recruitment of human subjects and collection of colon mucosa

Three obese male participants with type 2 diabetes and 3 age-matched lean normal glycemic male participants were enrolled in the study. Anthropometric data are provided in Table 1 (ESM). Participation in this study was voluntary, and written informed consent was obtained from each participant. The guidelines of the Declaration of Helsinki (2000) of the World Medical Association were followed. All protocols were approved by the Research Ethics Committee of The First Affiliated Hospital of Jinan University.

All participants were fasted for 8 h. An enteroscopy was performed in sedated participants using a colonoscope (CF-HQ290i; Olympus). Mucosal biopsies were taken from colon. Tissue samples were extracted for protein and RNA using lysis buffer and RNA TRIzol, respectively.

Western blot analysis

Colon mucosa and culture cells were quickly harvested, rinsed thoroughly with PBS and then homogenized on ice in the protein lysis buffer (50 mmol/L Tris–HCl; 15 mmol/L EGTA; 100 mmol/L NaCl; 0.1% Triton X-100 supplemented with protease inhibitor cocktail, pH 7.5). After centrifugation for 10 min at 4°C, the supernatant was used for Western blot analysis. Protein concentration was measured by Bradford’s method. Proteins were loaded onto SDS-PAGE gels and then transferred to nitrocellulose membranes. The membranes were incubated for 1 h at room temperature with 5% fat-free milk in Tris-buffered saline containing Tween 20, followed by incubation overnight at 4°C with the primary antibodies. Specific reaction was detected using IRDye-conjugated second antibody and visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

RNA extraction and quantitative real-time PCR analysis

For gene expression analysis, RNA was isolated from colon mucosa and STC-1 cells using TRIzol (Invitrogen) and reverse transcribed into cDNAs using the First-Strand Synthesis System for RT-PCR kit (Invitrogen). SYBR Green–based quantitative RT-PCR was performed using the Mx3000 multiplex quantitative PCR system (Stratagene). Triplicate samples were collected for each experimental condition to determine relative expression levels. Sequences for the primer pairs used in this study are as follows: mouse proglucagon (accession no. NM_008100.3), sense 5′-ATT GCC AAA CGT CAT GAT GA-3′ and antisense 5′-GGC GAC TTC TTC TGG GAA GT-3′; mouse β-actin (accession no. NM_007393.3), sense 5′-ATC TGG CAC CAC ACC TTC-3′ and antisense 5′-AGC CAG GTC CAG ACG CA-3′; human proglucagon (accession no. NM_002054.4), sense 5′-GCA CAT TCA CCA GTG ACT ACA GCA-3′ and antisense 5′-TGG CAG CTG GCC CGT CCA AAT A-3′; human TFIIB (accession no. NM_001514.5), sense 5′-ACC AGC GTG TTG GAT GCT C-3′ and antisense 5′-CCC ACA TCA ATA ACC CGG TC-3′.

Measurements of GLP1

Measurements of GLP1 secretion were performed as described previously (Xu et al., 2015b). Samples were collected in the presence of aprotonin (2 μg/mL), EDTA (1 mg/mL) and Diprotin (0.1 mmol/L). Cell culture medium was harvested and stored at ~80°C before use. Glucagon-like peptide-1 (active forms) was assayed using the enzyme immunoassay kit according to the manufacturer’s instruction, which is highly specific for the immunologic measurement of active GLP1(7–36 amide) and GLP1(7–37) in plasma and will not detect other forms of GLP1 (e.g., 1–36 amide, 1–37, 9–36 amide or 9–37).

Statistical analysis

All data were expressed as mean ± S.E.M. Statistical significance was analyzed with an unpaired Student’s t-test. Data were considered significant when P < 0.05.

Results

Effects of glucose and serum deprivation on the phosphorylation of AMPKα and expression of GLP1 in STC-1 cells

As shown in Fig. 1A, phosphorylation of endogenous AMPKα on Thr172 in STC-1 cells was decreased by high glucose relative to low glucose. This change was associated with an increase in GLP1 synthesis and secretion. mRNA (Fig. 1B) and protein levels (Fig. 1A) of proglucagon and GLP1 secretion
(Fig. 1C) were significantly higher when STC-1 cells were cultured at 25 mmol/L glucose than at 5.6 mmol/L glucose.

The effects of serum deprivation on AMPKα phosphorylation and GLP1 production were next examined. Serum starvation significantly inhibited the proglucagon mRNA and protein, as well as GLP1 secretion, but stimulated AMPKα phosphorylation (Fig. 1D–F).

These in vitro experiments indicate that negative energy balance upregulates AMPKα phosphorylation, which subsequently inhibits GLP1 production in L-like cells. We then examined the effects of long-term changes in nutritional status on AMPKα phosphorylation and GLP1 production in colon mucosa of human subjects. As shown in Fig. 2A, levels of phosphorylated AMPKα in colon mucosa were significantly decreased in obese patients with $41.2 \pm 7.8 \text{ kg/m}^2$ body mass index (BMI) relative to lean human subjects (BMI $23.3 \pm 2.2 \text{ kg/m}^2$). In contrast to the changes in AMPKα, proglucagon mRNA (Fig. 2B) and protein (Fig. 2A) demonstrated a significant increase in obese subjects compared with lean people.

Figure 1
Effects of glucose and serum deprivation on the phosphorylation of AMPKα and expression of GLP1 in STC-1 cells. Cultured STC-1 cells were incubated for 24h in the presence of 5.6 mmol/L (low) or 25 mmol/L (high) glucose supplemented with 2.5% fetal bovine serum and 10% horse serum (A–C). STC-1 cells were maintained in high glucose DMEM medium supplemented with indicated serum or serum deprivation for 24h (D–F). Shown are representative Western blots for phospho-AMPKα (pAMPKα, Thr172) and proglucagon. AMPKα and β-actin were used as loading controls (A and D). Proglucagon mRNA (B and E) was analyzed by quantitative PCR, normalized to β-actin and expressed as fold change from control. Medium GLP1 (C and F) was determined by enzyme immunoassay. Results are expressed as mean ± s.e.m. Experiments were repeated for three times. *Denotes $P < 0.05$ vs control.
Inhibition of GLP1 synthesis and secretion by AMPKα1 in STC-1 cells

The direct effects of AICAR, an activator of AMPK, on GLP1 synthesis and secretion were next examined in cultured STC-1 cells. Exposure of STC-1 cells to AICAR at the doses ranging from 31.25 to 250 μmol/L for 24 h caused a concentration-dependent increase in AMPKα1 phosphorylation (Fig. 3A) and a decrease in proglucagon mRNA (Fig. 3B) and protein (Fig. 3A) and medium content of GLP1 (Fig. 3C).

Consistent with the pharmacological AMPK, overexpression of wild-type AMPKα1 (WT) or constitutively active AMPKα1 (AF) plasmids significantly inhibited the proglucagon mRNA and protein, as well as GLP1 secretion in cultured STC-1 cells (Fig. 4A–C). On the other hand, there is no significant change in GLP1 production in STC-1 cells overexpressing AMPKα2 plasmids (Supplementary Fig. 1, see section on supplementary data given at the end of this article). Overexpression of constitutively active AMPKα1 upregulated the expression and phosphorylation of AMPKα1 (Fig. 4A). Further, overexpression of AMPKα1 lentivirus in cultured STC-1 significantly enhanced the phosphorylation and expression of AMPKα1 (Fig. 4D). The increment of AMPKα1 was associated with a decrease of proglucagon mRNA and protein levels, as well as GLP1 secretion (Fig. 4F).

Stimulation of GLP1 synthesis and secretion by inhibition of AMPKα1 in STC-1 cells

The direct effects of selective and reversible inhibition of AMPK on GLP1 synthesis and secretion were next examined by treating cultured STC-1 cells with Compound C. As shown in Fig. 5A–C, Compound C, at a dose ranging from 1.25 to 20 μmol/L, induced a concentration-dependent increase in GLP1 synthesis and secretion. Compound C caused a concentration-dependent inhibition in AMPKα1 phosphorylation, which was associated with an increment in proglucagon mRNA and protein levels, as well as GLP1 release (Fig. 5A–C). Further, Compound C pretreatment abolished the AICAR-induced reduction in GLP1 synthesis and secretion, and enhancement of AMPKα1 phosphorylation (Fig. 5D–F).

Transfection of a kinase-inactive AMPK mutant (AMPKα1-DN) plasmid caused a marked the GLP1 synthesis and secretion (Fig. 6A). Levels of phospho-AMPKα1 were significantly decreased in cells transfected with AMPKα1-DN.

On the other hand, siRNA knockdown of endogenous AMPKα1 significantly increased proglucagon mRNA and protein levels (Fig. 6 A and B), as well as GLP1 secretion (Fig. 6C) in cultured STC-1 cells. Knockdown of AMPKα1 markedly inhibited the phosphorylation and expression of AMPKα1 (Fig. 6A).

Discussion

The major finding of this study is that AMPK in STC-1 cells functions to alter the expression and secretion of GLP1. This conclusion is supported by the following distinct
observed: (1) there exists a negative relation between AMPKα phosphorylation and the production of GLP1 in response to negative energy supply such as glucose deficiency and serum deprivation in cultured STC-1 cells or positive energy balance in obese human subjects; (2) stimulation of AMPKα1 by either pharmacological or genetic approaches suppresses proglucagon mRNA and protein content in STC-1 cells; and (3) conversely, inhibition of AMPKα1 signaling by Compound C, AMPKα1 siRNA or a dominant negative form of AMPKα1 increases the expression and secretion of GLP1.

Enteroendocrine L cells are mainly expressed in the distal ileum and colon. In response to nutrients such as glucose and monounsaturated fatty acids, these...
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Figure 5
Stimulation of GLP1 synthesis and secretion by Compound C in STC-1 cells. (A–C) Cultured STC-1 cells were treated with varying concentrations of Compound C for 24 h. Phospho-AMPKα, AMPKα and proglucagon protein were analyzed by Western blotting (A). Results of quantitative PCR analysis of proglucagon mRNA are expressed as fold increase from vehicle control using β-actin as loading control (B). Medium GLP1 (C) was determined by enzyme immunoassay. Results are expressed as mean ± s.e.m. Experiments were repeated for three times. *P<0.05 vs control. (D–E) Cultured STC-1 cells were treated with DMSO, AICAR (125 μM), Compound C (10 μM) or AICAR plus Compound C for 24 h. Phospho-AMPKα, AMPKα and proglucagon protein (D) were analyzed by Western blotting. Proglucagon mRNA (E) was analyzed by RT-PCR. Medium GLP1 (F) was determined by enzyme immunoassay. Results are expressed as mean ± s.e.m. Experiments were repeated for 3 times. *P<0.05 vs control.

Figure 6
Effects of AMPKα1 gene manipulation on GLP1 production. STC-1 cells were transfected with control siRNA or AMPKα1 siRNA for 48 h. (A) Representative Western blots of phospho-AMPKα, proglucagon, AMPKα1 and β-actin. (B) Proglucagon mRNA. (C) Medium GLP1 (Active) concentration. Results were expressed as mean ± s.e.m. n=3; *P<0.05 vs control.

cells secrete GLP1 (Mansour et al. 2013). The molecular mechanism by which L cells sense nutrients to and then alter the secretion of GLP1 remains largely unknown. Adenosine 5′-monophosphate (AMP)–activated protein kinase (AMPK), mammalian target of rapamycin (mTOR) and peroxisome proliferator–activated receptors (PPARs) have been reported to serve as crucial cellular energy sensors to regulate cell metabolism (Jiang et al. 2015).
We thus speculate that GLP1 biosynthesis and secretion can be modulated by these nutrient-sensing molecules. Our previous report has demonstrated that mTORC1 may function to integrate nutritional and hormonal signals such as ghrelin with the synthesis and secretion of GLP1 within L cells (Xu et al. 2015a,b). This study extends the nutrient-sensing mechanism in L cells to AMPK signaling. Our finding demonstrates that AMPKα1 is altered by cellular energy status in STC-1 cells, an L-like cell line, and in colon mucosa of human subjects. Phosphorylation of AMPKα1 increases during negative energy balance such as glucose deficiency or serum deprivation, whereas its level decreases during long-term energy surplus in obese human subjects. These observations are consistent with other studies demonstrating that AMPK is critical for nutrient and hormone sensing in a variety of cells ranging from central nervous system to peripheral tissues (Kim & Lee 2005). In hypothalamic neurons, AMPK integrates nutritional signals with feeding behavior (Minokoshi et al. 2004, De Morentin et al. 2011, Stark et al. 2013). In muscle and liver, AMPK improves insulin sensitivity (Friedrichsen et al. 2013, Hasenour et al. 2013). In pancreatic islets, AMPK links the extracellular glucose with transcription of proproinsulin and secretion of insulin (Da Silva Xavier et al. 2000, 2003). Using pharmacological and genetic intervention for gain or loss of function of AMPKα1, we demonstrate that AMPKα1 negatively regulates production and secretion of GLP1 in L cells. By simultaneously inducing glucose-dependent stimulation of insulin secretion and suppressing glucagon secretion, GLP1 functions as a potent antihyperglycemic hormone (Baggio & Drucker 2007). Modulation of GLP1 synthesis and secretion by AMPK in L cells may thus coordinate the level of glucose in an organism with secretion of insulin, leading to the fine control of glucose homeostasis.

AMPK is a serine/threonine protein kinase that acts as a sensor of cellular energy homeostasis. It is expressed as a heterotrimer consisting of one catalytic α-subunit and 2 regulatory subunits (β and γ) (Stapleton et al. 1997). The α-subunit of AMPK is encoded either by the PRKAA1 or PRKAA2 gene, whereas the β- and γ-subunits are encoded by the PRKAB1 or PRKAB2 and the PRKAG1, PRKAG2 or PRKAG3 genes, respectively, yielding 12 possible heterotrimeric complexes, which provide a molecular basis for the multiple roles of the highly conserved AMPK signaling system in nutrient regulation and utilization in distinct mammalian cells (Stapleton et al. 1997, Gao et al. 1996). The different complexes of AMPK confer tissue specificity. Isoform-selective activators or inhibitors of AMPK may thus provide new therapeutic tools for the treatment of type 2 diabetes mellitus. Intestinal AMPK also fulfills key functions in metabolic processes. The prevailing catalytic subunit of AMPK complex in small intestine is α1 and its preferential associated regulatory partners are β2 and γ1 (Harmel et al. 2014). Because each isof orm varies from its sibling forms, the complement of isoforms present in a complex can influence the role and response of AMPK within the cell. In muscle, AMPKα2 is the predominant subunit representing two-thirds of the total ααRNA (Zong et al. 2002). Exercise increases nuclear translocation of AMPKα2 (Goodyear & Kahn 1998, Birk & Wojtaszewski 2006), while in AMPKα2 by overexpressing its domain-negative mutant decreased glucose uptake in skeletal muscle in response to contraction (Mu et al. 2001, Steinberg et al. 2006). In pancreatic β-cells, the α2-subunit displays substantial nuclear localization and is thus implicated in the control of gene expression. On the other hand, α1-containing complex whose total activity exceeds that of α2-containing complex by 5- to 10-fold is mainly cytosolic and is proposed to regulate plasma membrane ion channels and secretion of insulin (da Silva Xavier et al. 2000, 2003). Previous studies have showed that AICAR, an AMPK activator, increases GLP1 secretion in rodents but not in fetal rat intestinal cells, murine GLUTag or human NCI-H716 L cells (Maida et al. 2011, Mulherin et al. 2011). Our findings suggest that AMPKα1 alters both the transcription and secretion of GLP1 in STC-1 cells. AMPKα1-containing complex suppresses proglucagon mRNA and protein and the secretion of GLP1 by overexpressing this subunit. Inhibition of AMPKα1 signaling by AMPKα1 siRNA or a dominant negative form of AMPKα1 increases the expression and secretion of GLP1. Our experiments thus provide evidence that AMPKα1 modulates GLP1 synthesis and release in the STC-1 cell line, a remarkable intestinal L cell model. These findings are consistent with our previous observations demonstrating that mTOR activity stimulates GLP1 production in L-like cells (Xu et al. 2015a,b). AMPK has been well characterized to suppress mTOR signaling through tuberous sclerosis complex 1 and 2 (TSC1/2) (Inoki et al. 2006). AMPK may thus inhibit GLP1 through mTOR in L cells.

As an insulinotropic and glucagonostatic hormone that inhibits appetite and reduces body weight, GLP1 is a promising therapy for type 2 diabetes (Baggio & Drucker 2007). GLP1 receptor agonists (Buse et al. 2004...
and dipeptidyl peptidase-4 inhibitors (Aschner et al. 2006) have been widely used as treatment targeting β-cell dysfunction in patients with type 2 diabetes. However, the use of these drugs may be associated with certain adverse effects on pancreatic and thyroid tissues. Both animal studies and analyses of drug databases have indicated an association of GLP1 receptor agonists with pancreatitis, pancreatic cancer and thyroid cancer (Bjerre et al. 2010, Parks & Rosebraugh 2010). Our study provides an alternative strategy for manipulation of GLP1 by altering AMPK activity in colon mucosa. If the relationship of AMPK signaling with GLP1 production exists in normal and diabetic humans, it might be feasible to target AMPK in colon mucosa for the therapy of type 2 diabetes. In summary, the data presented here demonstrate that AMPKα1 may link energy supply with the production of GLP1 in L-like cells.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-16-0099.

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
Xu G and Zhang W designed the research; Jiang S, Zhai H, Li D, Huang J, Zhang H and Li Z performed the research; Xu G and Jiang S analyzed the data; Xu G and Zhang W wrote and edited the paper. All authors contributed to the discussion and revised the article and all of them approved the final versions of the manuscript. Xu G and Zhang W are responsible for the integrity of the work as a whole.

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