Uncoupling protein 2 negatively regulates glucose-induced glucagon-like peptide 1 secretion

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Abstract

It is known that endogenous levels of the incretin hormone glucagon-like peptide 1 (GLP1) can be enhanced by various secretagogues, but the mechanism underlying GLP1 secretion is still not fully understood. We assessed the possible effect of uncoupling protein 2 (UCP2) on GLP1 secretion in mouse intestinal tract and NCI-H716 cells, a well-characterized human enteroendocrine L cell model. Localization of UCP2 and GLP1 in the gastrointestinal tract was assessed by immunofluorescence staining. Ucp2 mRNA levels in gut were analyzed by quantitative RT-PCR. Human NCI-H716 cells were transiently transfected with siRNAs targeting UCP2. The plasma and ileum tissue levels of GLP1 (7–36) amide were measured using an ELISA kit. UCP2 was primarily expressed in the mucosal layer and colocalized with GLP1 in gastrointestinal mucosa. L cells secreting GLP1 also expressed UCP2. After glucose administration, UCP2-deficient mice showed increased glucose-induced GLP1 secretion compared with wild-type littermates. GLP1 secretion increased after NCI-H716 cells were transfected with siRNAs targeting UCP2. UCP2 was markedly upregulated in ileum tissue from ob/ob mice, and GLP1 secretion decreased compared with normal mice. Furthermore, GLP1 secretion increased after administration of genipin by oral gavage. Taken together, these results reveal an inhibitory role of UCP2 in glucose-induced GLP1 secretion.

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Introduction

It is widely recognized that oral ingestion of glucose triggers more insulin release than intravenous delivery of glucose, which results in a similar plasma glucose profile – a phenomenon known as the ‘incretin effect’ (Elrick et al. 1964, McIntyre et al. 1965, Perley & Kipnis 1967). This is mainly attributed to two hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP1) (McIntyre et al. 1964, Thorens 1995). GLP1 is a product of post-translational cleavage of proglucagon (proG) peptide; the majority of it is produced in L cells of the ileum and colon. Although cleavage of proG results in several different forms of GLP1, GLP1 (7–36) amide has been reported to be the most abundant form secreted from L cells (Goke et al. 1991, Fehmann et al. 1995).

Carbohydrates in the gut lumen elicit release of GLP1 from L cells and GIP from K cells (Hirasawa et al. 2005). It appears that glucose within the lumen of the gut acts on the luminal surface to stimulate GLP1 secretion (Meier & Nauck 2005). A variety of signaling mechanisms have been proposed to explain how L cells might sense glucose, including ATP-sensitive potassium (KATP) channel closure, sodium glucose cotransporter activity, and activation of sweet taste receptors (Herrmann et al. 1995, Ritzel et al. 1997, Rorsman 1997, Gribble et al. 2003, Reimann et al. 2006, Margolskee et al. 2007, Rozengurt & Sternini 2007). However, the underlying mechanism of GLP1 release in vivo is still debated both in terms of relative contributions of direct vs indirect glucose-sensing pathways and identity of the signaling pathways within L cells. Thus, we sought to determine which glucose-sensing mechanism in the gut lumen might underlie this L-cell response.

It is known that one mechanism for sensing glucose in pancreatic endocrine β-cells is uncoupling protein 2 (UCP2)-mediated insulin secretion. UCP2 is a member of the inner mitochondrial membrane anion carrier superfamily (Ricquier & Bouillaud 2000, Rousset et al. 2007). Proton leak activity of UCP2 has an important role in negative regulation of insulin secretion via its inhibition of ATP synthesis in β-cells (Ježek 2002). Pancreatic endocrine β-cells are glucose-sensing cells equipped with a KATP channel consisting of two subunits, SUR1 and Kir6.2 (Donley et al. 2005). Intestinal endocrine L cells secreting GLP1 are also glucose-sensing cells and coexpress the KATP channel subunits in vivo (Nielsen et al. 2007). Ucp2 mRNA is widely expressed, including in the gastrointestinal tract (Nedergaard & Cannon 2003, Mattiasson & Sullivan 2007).
However, the physiological function of Ucp2 in the gastrointestinal tract remains unknown. Combining these pieces of evidence raises the question of whether there is any relationship between UCP2 and GLP1 secretion in the gastrointestinal tract.

In this study, we investigated whether L cells secreting GLP1 also expressed UCP2, and whether UCP2 may regulate GLP1 secretion in L cells in a similar manner to its regulation of insulin secretion in β-cells. We measured plasma GLP1 levels, ileum tissue GLP1 levels, and plasma glucose levels before and after oral administration of glucose in UCP2-deficient mice, wild-type littermates, normal C57BL/6J mice, and ob/ob mice. We also used NCI-H716 cells, a well-characterized human L-cell model, to further verify the effect of UCP2 on secretion of GLP1.

Materials and methods

Animals and sample preparation

Animals and treatment

UCP2-deficient mice on a C57BL/6J background and their wild-type littermates, ob/ob mice on a C57BL/6J background, and C57BL/6J mice were obtained from the animal center at Nanjing University. Mice were maintained and the experiments were performed according to the National Institutes of Health Guide for Care and Use of Laboratory Animals. All mice (males, aged 8–10 weeks) were maintained at 23 °C with a regular 12 h light:12 h darkness cycle. Following overnight fasting with free access to water, UCP2-deficient mice (n = 20) and wild-type littermates (n = 20) were given glucose (2 g/kg) by oral gavage and killed 15, 30, or 60 min thereafter. We used ob/ob mice (n = 5) and C57BL/6J mice (n = 5) to investigate the expression of UCP2 in the gut, and plasma levels of GLP1, in the state of pathological high blood glucose. Ob/ob mice and C57BL/6J mice were fasted for 2 h, and basal plasma glucose and GLP1 levels in ileum tissue were then measured. We also administered genipin to ob/ob mice via oral gavage, collected samples 1 h after genipin administration, and detected GLP1 levels in ileum tissue and glucose in the blood.

Tissue samples

Samples from ileum tissue were frozen in liquid nitrogen and kept for RNA preparation, fixed in 4% paraformaldehyde for immunohistochemistry, or immediately taken for preparation of fresh mitochondrial protein. Dipeptidyl peptidase IV inhibitor (Linco Research, St Charles, MO, USA) was added to the blood samples collected for the detection of GLP1.

RNA isolation and quantitative RT-PCR

The reagents used for RNA isolation and quantitative RT-PCR included TRIzol (Invitrogen), oligo(dT)18 primers and AMV reverse transcriptase (Invitrogen), and primers and TaqMan probes (Shanghai Shenggene Molecular Biotechnology Co., Ltd., Shanghai, China).

Ileum tissue samples were dissected and immediately frozen in liquid nitrogen. Total cellular RNA was extracted using TRIzol reagent. The purity and concentration of RNA were determined by measuring the absorbance at 260 and 280 nm, where A260/280 > 1.7 was considered as sufficient purity. One microgram of RNA was reverse transcribed into cDNA using oligo(dT)18 primers and AMV reverse transcriptase at 42 °C for 1 h. Quantitative RT-PCR was performed with an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) using TaqMan probes (Shanghai Shenggene Molecular Biotechnology); threshold cycle numbers were obtained by ABI Prism 7000 SDS software version 1.0. The primers and probe sequences used in this study were as follows: Ucp2 gene (sense primer, 5'-CCATGTTGCCCCAGTATG-3'; antisense primer, 5'-TGAGTTGCGTTTCAGGAG-3'; and probe, 5'-FAM-CTGTGGCCCCAGTATG-3'-ROX); β-actin (sense primer, 5'-GGACACCGAATTTCTACAATG-3'; antisense primer, 5'-GGGGGTGTTGAAGTTCTCAAAC-3'; and probe, 5'-FAM-CTGTGGCTCAAAC-3'-ROX). Amplification conditions were one cycle at 95 °C for 5 min followed by 40 cycles at 95 °C for 30 s and 60 °C for 1 min, and one final cycle at 72 °C for 4 min.

Western blot

Primary antibodies used for western blot analysis were polyclonal antibody-recognizing UCP2 (C-20, sc6525, Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:700 and a polyclonal anti-cytochrome c antibody (6H2, sc13561, Santa Cruz Biotechnology), diluted 1:1000.

Mitochondrial protein from the NIC-H716 cells was isolated using 1 ml extraction buffer (250 mM sucrose, 1 mM EDTA, and 10 mM Tris, pH 7.4) supplemented with protease inhibitors (1 mmol/l phenylmethylsulfonyl fluoride, 2 μmol/l leupeptin, and 1× aprotinin). The mixture was centrifuged at 800 g for 10 min. The resulting supernatant was centrifuged at 10 000 g for 10 min and the mitochondrial pellet was suspended in 25 μl TES buffer. Mitochondrial protein concentration was determined colorimetrically using a BCA Protein Assay (Pierce, Rockford, IL, USA). Mitochondrial proteins (15 μg) were mixed with 3× sample buffer (0.5 M phosphate buffer, pH 7.0, 30% [w/v] glycerol, 7.5% [w/v] SDS, and 0.75 mM bromophenol blue), boiled for 5 min, and separated by electrophoresis on a 12.5% SDS–PAGE gel. After separation,
proteins were transferred to Immobilon PVDF membranes (Millipore Corporation, Bedford, MA, USA). UCP2 proteins were detected with a polyclonal antibody-recognizing UCP2 (Santa Cruz Biotechnology) at a dilution of 1:700 followed by incubation with HRP-conjugated monkey anti-goat IgG secondary antibody (Santa Cruz) at a dilution of 1:2000 and detected with enhanced chemiluminescence (ECL detection system; NEN, Boston, MA, USA). To validate equal protein loading among various lanes, PVDF membranes were stripped and reprobed with a polyclonal anti-cytochrome c antibody (Santa Cruz Biotechnology) at a dilution of 1:1000.

Localization of UCP2 and GLP1 in mouse ileum tissue and NCI-H716 cells

Localization of UCP2 and GLP1 in mouse ileum

The antibodies used for tissue immunostaining were polyclonal rabbit anti-GLP1 (bs-0038R; Beijing Biosynthesis Biotechnology Co., Beijing, China) and polyclonal goat anti-UCP2 antibody (C-20, sc6525, Santa Cruz Biotechnology). For double-labeling experiments involving a combination of GLP1 and UCP2 antibodies, tissue sections were incubated with the primary antibodies (anti-UCP2, 1:50) at 4 °C overnight and then washed with PBS. For the secondary antibody, FITC (green) goat anti-rabbit (1:200) was added for 30 min at RT in the dark and then washed with PBS. Slides were fixed in 4% paraformaldehyde in PBS for 15 min and then washed with PBS. Anti-GLP1 (1:100) was added and incubated at 4 °C overnight, followed by a further wash with PBS, application of the secondary antibody (Cy3 (Red) donkey anti-goat (1:200), for 30 min at RT in the dark), and a final wash with PBS. Slow Fade equilibration buffer (Molecular Probes cat #S2828) was applied, and the slides were mounted with 10 µl Slow Fade (Molecular Probes cat #S2828), covered, and sections viewed with a fluorescence microscope.

Localization of UCP2 and GLP1 in NCI-H716 cells

Antibodies used for immunofluorescence staining in NCI-H716 cells were anti-GLP1 (C-17; Santa Cruz) and anti-UCP2 (LS-B1911; LifeSpan BioSciences). Cells were grown on coverslips, fixed in 4% paraformaldehyde in PBS for 10 min, washed with PBS, and cooled with 100% methanol at −20 °C for 20 min. Thereafter, cells were washed with PBS and permeabilized with 0.1% Triton X-100 for 10 min. After blocking with Dako blocking solution, primary antibody (anti-GLP1, 1:100) was added and incubated at 4 °C overnight. For the secondary antibody, Cy3 (Red) donkey anti-goat (1:200) was applied for 30 min at RT in the dark followed by a PBS wash. Anti-UCP2 (1:50) was added and incubated at 4 °C overnight. For the secondary antibody, FITC (green) goat anti-rabbit (1:200) was added for 30 min at RT in the dark. Hoechst was used as a nuclear counterstain for 45 min. Following a final wash with PBS and addition of Slow Fade equilibration buffer (Molecular Probes cat #S2828), slides were mounted with 10 µl Slow Fade (Molecular Probes cat #S2828).

Culture of NCI-H716 cells and secretion studies

Human enteroeordocrine NCI-H716 cells were maintained in suspension culture as described by the American Type Culture Collection (Manassas, VA, USA). Two days before the experiments, cells were seeded into 24-well culture plates precoated with Matrigel as described previously (Reimer et al. 2001). On the day of the experiments, supernatants were replaced with PBS containing 1 mM CaCl₂ and dipeptidyl peptidase IV inhibitor. The solutions were adjusted to pH 7.2. Cells were incubated at 37 °C for 1 h either without glucose or with a range of glucose concentrations and RNA interference targeting UCP2 (final concentration 300 nM, as described in the next section). GLP1 was measured by ELISA and normalized to protein content.

siRNA preparation and NCI-H716 cell transfection

The siRNA sequences targeting UCP2 and a nonspecific siRNA control were purchased from Shanghai Shenggine Molecular Biotechnology. Subconfluent differentiated NCI-H716 cells were transiently transfected with siRNAs using Lipofectamine 2000 according to the manufacturer’s protocol (Life Technologies). The entire mixture was then added to the cells in one dish, resulting in a final siRNA concentration of 300 nM. Cells were usually examined 48 h after transfection.

GLP1 measurement

The level of serum GLP1 (7–36) amide was measured using an ELISA kit (Linco Research). This assay relies on a monoclonal antibody fixed in a coated micro-well plate binding to the N-terminal region of active GLP1. The concentration of active GLP1 is proportional to the fluorescence generated by umbelliferone, which is produced by alkaline phosphatase-catalyzed hydrolysis of methyl umbelliferyl phosphate (conjugated with anti-GLP1 monoclonal antibodies). Samples (100 µl of each sample) were added to each assay well. ELISA has a working range of 2–100 pM. Extraction of GLP1 (7–36) amide from ileum was carried out with ethanol/acid (5:1 v/v) solution (5 ml/g tissue). Samples were homogenized at 24 000 r.p.m. and kept for 24 h at 4 °C. Homogenates were centrifuged (2000 g) and the supernatant was decanted and diluted in saline. Concentrations of GLP1 (7–36) amide in tissue extracts were measured using an ELISA method (GLP1 ELISA kit; Linco Research).
Statistical analyses

Data are expressed as mean ± s.e.m. Statistical significance was calculated by one-way ANOVA or unpaired two-tailed t-test. P values <0·05 were regarded as significant differences.

Results

Localization of UCP2 and GLP1 in mouse ileum tissue and NCI-H716 cells

As shown in Fig. 1A, immunofluorescence staining using anti-GLP1 and anti-UCP2 antibodies demonstrated localization of UCP2 and GLP1 in certain cells in mouse ileum (400× magnification). (B) Immunofluorescence staining using anti-UCP2 (green) antibody demonstrated localization of UCP2 in certain cells in UCP2-deficient mouse and wild-type mouse ileum (400× magnification). (C) Immunofluorescence staining with anti-GLP1 and anti-UCP2 antibodies in NCI-H716 cells, an L-cell model. This showed anti-UCP2 antibody-positive staining (green) and anti-GLP1 antibody-positive staining (red) (400× magnification).

Figure 1 Localization of UCP2 and GLP1 in mouse ileum and NCI-H716 cells. (A) Immunofluorescence staining using anti-GLP1 (red) and anti-UCP2 (green) antibodies demonstrated localization of UCP2 and GLP1 in certain cells in mouse ileum (400× magnification). (B) Immunofluorescence staining using anti-UCP2 (green) antibody demonstrated localization of UCP2 in certain cells in UCP2-deficient mouse and wild-type mouse ileum (400× magnification). (C) Immunofluorescence staining with anti-GLP1 and anti-UCP2 antibodies in NCI-H716 cells, an L-cell model. This showed anti-UCP2 antibody-positive staining (green) and anti-GLP1 antibody-positive staining (red) (400× magnification).

UCP2-deficient mice have higher plasma GLP1 levels after glucose loading

As shown in Fig. 2, the initial blood glucose level (basal plasma level of glucose) in wild-type littermates was slightly higher compared with UCP2-deficient mice, but there were no significant differences (see left panel, UCP2-deficient mice vs wild-type littermates: 5·3±0·2 vs 6·4±0·4, P>0·05). There was no significant difference in basal plasma levels of GLP1 between UCP2-deficient mice and wild-type littermates (see right panel, UCP2-deficient mice vs wild-type littermates: 3·7±0·1 vs 3·5±0·2, P=0·066). Following administration of glucose (2·0 g/kg) via oral gavage, blood glucose increased in both UCP2-deficient mice and wild-type littermates and peaked at 15 min after administration. The blood glucose levels in wild-type littermates were much higher than those in UCP2-deficient mice after glucose loading (Fig. 2, left panel). However, plasma GLP1 levels in UCP2-deficient mice were significantly higher than those in wild-type littermates at 15, 30, and 60 min after oral glucose loading (15 min: 9·4±1·0 vs 6·9±0·3 mmol/l, P<0·001; 30 min: 7·9±0·3 vs 5·6±0·4 mmol/l, P<0·001; 60 min: 4·9±0·1 vs 3·38±0·10 mmol/l, P<0·01; Fig. 2, right panel).

Figure 2 UCP2-deficient mice have higher serum GLP1 and lower plasma glucose levels. Blood glucose (left panel) and plasma GLP1 (right panel) levels were assessed at time 0 and 15, 30, and 60 min after an oral glucose load (2·0 g/kg). UCP2-deficient mice (total number: 20, each time point n=5) and wild-type mice (total number: 20, each time point n=5) **P<0·01, ***P<0·001. Full colour version of this figure available via http://dx.doi.org/10.1530/JME-11-0114.
Downregulated UCP2 increased secretion of GLP1 in L cells after glucose stimulation

Time course experiments revealed that release of GLP1 from NCI-H716 cells into the supernatant: NCI-H716 cells were incubated with 30 mM glucose and the supernatants were collected at 0 (control), 5, 15, 30, 60, and 120 min to measure GLP1 levels. In the dose-dependent experiment, NCI-H716 cells were incubated with 0 (control), 5, 10, 20, 30, and 60 mM glucose for 1 h, and the levels of GLP1 in the supernatants were determined. *P < 0.05 vs control. (B) The knockdown efficiency of si-UCP2 in NCI-H716 cells. NCI-H716 cells were left untransfected (blank), mock transfected (no siRNA), or transfected with si-UCP2. After 48 h, the mRNA levels of Ucp2 were determined by quantitative qRT-PCR with normalization to β-actin, and the expressions of UCP2 protein were detected by western blotting. Cytochrome c was used as a loading control. **P < 0.01 vs untransfected (blank). (C) NCI-H716 cells treated with si-UCP2 increased glucose-mediated GLP1 secretion. NCI-H716 cells were incubated with growth medium only as a normal control (blank), or 300 nM si-UCP2 for 48 h plus 30 mM glucose for 1 h. GLP1 concentrations in the supernatant after 1 h culture were determined using a GLP1 assay kit. *P < 0.05 vs untransfected (blank). Full colour version of this figure available via http://dx.doi.org/10.1530/JME-11-0114.

Acute inhibition of UCP2 by genipin can improve GLP1 secretion in ob/ob mice

In ob/ob mice following administration of genipin (an aglycone known to block UCP2 function) by oral gavage, GLP1 in ileum tissue increased compared with animals administrated saline (Fig. 5B). However, there was no effect on blood glucose in ob/ob mice as a result of genipin treatment (Fig. 5A), suggesting that acute inhibition of UCP2 by genipin can improve GLP1 secretion in ob/ob mice.
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Discussion

The ‘incretin effect’ of GLP1 has been known for many years, and its role in the overall regulation of insulin release in vivo is well established. However, regulation of GLP1 secretion itself is not well understood. The presence of a UCP2 response to glucose in enteroendocrine L cells may identify a new mechanism by which intestinal hormone secretion is regulated. Our data indicate that glucose-induced secretion from L cells is partly mediated via UCP2.

Compared with wild-type littermates, UCP2-deficient mice had higher plasma levels of GLP1 after administration of glucose; however, there was no significant difference in the basal level of GLP1 (UCP2-deficient mice vs wild-type littermates: 3.7 ± 0.1 vs 3.5 ± 0.2, \( P=0.063 \)). This result indicates that UCP2 had no effect on baseline GLP1 secretion but impacted on postprandial secretion of GLP1. In vitro, decreased UCP2 expression in NCI-H716 cells by siRNA interference resulted in increased glucose-mediated GLP1 secretion. These results indicate that UCP2 is intimately involved in glucose-stimulated secretion of GLP1 from L cells.

In our study, we found that blood glucose levels in wild-type littermates were much higher than those in UCP2-deficient mice after glucose loading (Fig. 2, left panel). This may be due to UCP2-deficient mice producing more insulin or releasing more GLP1 after glucose loading.

The finding that there are many more L cells in the distal (ileum and colon) vs proximal (duodenum and proximal jejunum) gut led to the suggestion that the early rapid rise in plasma GLP1 after glucose load was not due to direct stimulation of L cells. ‘Proximal-to-distal’ models proposed that indirect neurally mediated signaling, initiated by glucose-sensing K cells or other non-L cells of the proximal gut, leads to release of GLP1 from L cells of the distal gut (Knudsen et al. 1975, Holst 2007). However, there is considerable evidence that luminal glucose directly leads to GLP1 secretion from the proximal gut. First, although in humans there are more L cells in the gut distally than proximally, there are still many L cells in the duodenum and jejunum (Theodorakis et al. 2006). Secondly, the time course (onset, peak, and duration) of glucose-elicited release of GLP1 in humans is consistent with that of glucose reaching the proximal intestine (Schirra et al. 1996, Balks et al. 1997, Theodorakis et al. 2004, Kim et al. 2005). Thirdly, applying small amounts of glucose solution by catheter into the duodenum in humans leads to increased plasma GLP1 levels (Vilsbøll & Holst 2004). Finally, many animal studies have shown that glucose directly stimulates GLP1 secretion, for example, in an isolated perfused porcine ileum experiment (Hansen 2004).

To investigate the potential pathophysiological relevance of UCP2 to GLP1, we conducted experiments with ob/ob mice. Ob/ob mice have blood glucose levels in the diabetic range, indicating a defect in their β-cell response, as well as significantly increased levels of Ucp2 mRNA and protein in their pancreatic islets. UCP2 plays an important role in the pathogenesis of type 2 diabetes by inhibiting β-cell insulin secretion.
(Lameloise et al. 2001, Zhang et al. 2001), but it is not clear whether UCP2 levels are also increased in L cells in the small intestine and colon in obesity-induced type 2 diabetes. We compared Ucp2 mRNA levels in ileum tissue from ob/ob mice and C57BL/6 mice and found that Ucp2 mRNA levels in ileum from ob/ob mice were increased in the fasting state, whereas GLP1 levels in ileum tissues from ob/ob mice were decreased. This result suggests that chronic high glucose induces high levels of Ucp2 mRNA in ob/ob mouse intestine and impaired the GLP1 secretion.

Genipin can rapidly inhibit UCP2-mediated proton leak. In pancreatic islet β-cells, genipin increases mitochondrial membrane potential and ATP levels while closing K_ATP channels in a UCP2-dependent manner, stimulating insulin secretion. Importantly, acute addition of genipin to isolated islets reverses high glucose- and obesity-induced β-cell dysfunction (Zhang et al. 2006). Our results indicate that acute inhibition of UCP2 by genipin can improve GLP1 secretion in ob/ob mice, suggesting that UCP2 negatively regulates GLP1 secretion in chronic high-glucose state.

It is well known that tissue-specific post-translational processing of proG results in the production of a diversity of peptides in the intestine and pancreas. Glicentin, oxyntomodulin, GLP1, and GLP2 are the proG-derived peptides produced in the L cells of the intestine, whereas glucagon is the major product of processing in the A cells of the pancreas (Dhanvantari & Brubaker 1998). Whether UCP2 affects the proG-derived peptides produced by influencing post-translational processing of proG in the L cell of the intestine is unclear and will be explored further in future studies.

In summary, our in vivo and in vitro results suggest that UCP2 inhibits GLP1 secretion. Our findings provide evidence that UCP2 may serve as a negative regulator of GLP1 secretion in the gastrointestinal tract.

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 Z, J L, X L, and Y L carried out the field collection and/or preparation of the samples. H Z and J L performed cell culture and RNAi. X L performed animal experiments. Y L participated in the statistical analysis and interpretation of the data. H Z prepared the first draft of the manuscript. C-Y Z designed the study. K Z participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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