Mitochondrial-bound hexokinase 1 can affect the glucolipid metabolism and reactive oxygen species production in goose fatty liver

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ABSTRACT
To investigate the functions of hexokinase 1 (HK1) in the formation of goose fatty liver, a total of 40 healthy 63-day-old male Landes geese were selected and randomly assigned to a control group and an overfeeding treatment. In addition, the overexpression or RNA interference assay of HK1, and transcriptome analysis after HK1 overexpression were performed in the goose primary hepatocytes. Data showed that the mRNA expression of hepatic HK1 was upregulated in overfed treatment compared to control on the 19 days of overfeeding. The expression of HK1 was increased in 50 mM glucose treatment in hepatocytes. Moreover, overexpression of HK1 tended to increase the relative lipid accumulation level and had weakened fluorescence intensity of reactive oxygen species (ROS), while knockdown of HK1 resulted in a tendency of relative lipid content decrease and had enhanced fluorescence intensity of ROS in cells in comparison to the control. The verification of transcriptome analysis indicated that the expression of ceruloplasmin (CP), acyl-CoA dehydrogenase medium-chain (ACADM), phosphoglucomutase 2 (PGM2), and phospholipase A2 group IVA (PLA2G4A) was significantly induced by HK1 overexpression, while that of enoyl-CoA hydratase, short chain 1 (ECHS1), cytochrome P450 family 2 subfamily C member 19 (CYP2C19), carnitine palmitoyltransferase 1 A (CPT1A), and oxidative stress-induced growth inhibitor 1 (OSGIN1) was inhibited. In summary, HK1 could promote fat deposition by affecting the process of glucolipid metabolism in the formation of goose fatty liver. Additionally, HK1 might decrease the ROS level in hepatocytes by regulating the expression of redox-related genes.

HIGHLIGHTS
- The HK1 gene may promote the goose fatty liver production.
- The HK1 gene may be used as a research target to decrease the reactive oxygen species level in fatty liver.

Introduction
Non-alcoholic fatty liver disease (NAFLD) in humans and mammals is probably progressed from simple steatosis to non-alcoholic steatohepatitis, cirrhosis, and even hepatic carcinoma, posing a serious threat to human and animal health (Hossain et al. 2016). In contrast, the goose fatty liver is a physiologically fatty liver that can reach eight to ten times heavier than normal liver and no overt injury or pathological symptoms are found during its formation (Geng et al. 2015, 2016). These differences suggest that there is a unique protective mechanism in goose liver. Therefore, the goose fatty liver can be considered as a unique model to clarify the mechanism of NAFLD formation.
reported that a majority of mitochondria-related genes expression was increased in overfed geese compared to normally fed geese (Osman et al. 2016), suggesting the mitochondria of goose may participate in the development of fatty liver in a different way. Hexokinase 1 (HK1) is one of them and it is a rate-limiting enzyme in glycolysis (Li et al. 2020). In addition, the N-terminal of HK1 protein contains a hydrophobic domain that allows it to bind to the voltage-dependent anion channel, thereby forming a mitochondria-bound hexokinase that inhibits mitochondrial cytochrome C release and cell apoptosis (Arzoine et al. 2009). However, the function of HK1 in the development of NAFLD remains unclear. Hence, this study was conducted to study the role of HK1 in goose fatty liver. The research may enhance our understanding of the HK family genes in the development of NAFLD.

**Materials and methods**

**Animals, experimental design, and diets**

A total of 40 healthy 63-day-old male Landes geese with similar body weight were selected and randomly divided into a control treatment and an overfeeding treatment. The geese in the control group were allowed ad libitum feeding, while the geese in the overfeeding treatment were overfed using an electronic machine. The feed consist of cooked maize (maize boiled for 5 min), 1% plant oil, and 1% salt. Geese in the overfeeding group were subjected to 1 week of training to increase their adaption of overfeeding. The daily feed intake was gradually enhanced from 100 g to 300 g this week. Afterwards, the formal overfeeding was conducted at 70 days of age. In brief, the feed intake was 500 g for three meals per day in the first 5 days, followed by 1200 g for 5 meals per day in the following 14 days. The geese were raised in cages individually and had free access to water. At 77, 84, 89 days of age, six geese per treatment were randomly selected and killed with an electrolethaler. The samples of liver, breast muscle, and abdominal fat were separated, harvested, and frozen in liquid nitrogen. All animal research procedures were approved by the Institutional Animal Care and Use Committee of the Yangzhou University Animal Experiments Ethics Committee.

**Preparation of goose primary hepatocytes**

The goose primary hepatocytes were isolated from Landes goose embryos on 23 days of incubation based on the method of our lab (Osman et al. 2016). After the cell separation is completed, the number of cells was counted and the cells were diluted with complete culture medium to $1 \times 10^6$ cells/mL and placed in 12-well dishes. Finally, the dishes were incubated in a 5% CO$_2$ incubator (ThermoForma 3110, Ohio, USA) at 37°C and the medium was renewed after the first 6 h of incubation.

**Treatment of primary hepatocytes with glucose**

After 24 h of incubation, the culture medium was removed, and the cultured goose primary hepatocytes were rinsed with PBS. Hepatocytes were treated with 25 mM or 50 mM glucose (Sigma-Aldrich, St. Louis, MO, USA) for 14 h, and those treated with complete culture media alone was used as the control.

**Vector construction and cell transfection**

HK1 coding sequences (CDS) were cloned into the pcDNA3.1 vector to construct the pcDNA3.1/HK1 expression vector by a commercial company (Suzhou GenePharma Co., Ltd, Suzhou, China). The CDS size of HK1 was 2778 bp. The cells that were transfected with empty vector were considered as the control treatment and with pcDNA3.1/HK1 vector was taken as the overexpression treatment. Vectors were transfected into hepatocytes using Lipofectamine 2000 reagent (Biosharp, Hefei, China) according to the protocol of the manufacturer. Six replicates were used in each treatment. The Opti-MEM medium (ThermoFisher Scientific, Waltham, MA, USA) was changed with complete medium after 6 h of transfection. Cells were cultured for 24 h at 37°C in 5% CO$_2$ incubator.

**RNA interference assay**

The small interfering RNA (siRNA) was designed to target the goose HK1 CDS and purchased from Suzhou GenePharma Co., Ltd (Suzhou, China). The sequences of the sense and antisense strands of siRNAs target the goose HK1 and its negative control siRNA were shown in Supplementary Table 1. The siRNAs were separately transfected into cells using Lipofectamine 2000 (Biosharp, Hefei, China) according to the instructions. The dosage of siRNA in this study was 100 nmol and there are six replicates for each treatment. After 6 h of transfection, the culture medium was replaced from Opti-MEM (ThermoFisher Scientific, Waltham, MA, USA) to complete medium. The primary hepatocytes were harvested after 24 h of culture. The siRNA was evaluated based on their efficiency at suppressing the
HK1 expression. After evaluation, the siRNA with the best efficiency was used in the following study.

**Oil red O staining and relative lipid content quantification**

After transfecting with overexpression vector or siRNA targeting HK1 for 6h, the primary goose hepatocytes were cultured for 24h at 37°C in a 5% CO2 incubator. Then the complete medium was removed and oil red O staining was conducted using the commercial kit (Solarbio Science and Technology Co., Ltd., Beijing, China). The Oil Red O staining solution was discarded, and the dye retained in the cells was eluted into 1mL of isopropanol. Immediately, the absorbance was determined at 510 nm using a microplate reader (Thermo Scientific, Waltham, MA, USA) and isopropanol was used as a blank control (Zheng and Cai 2019). The relative lipid content in overexpression or siRNA treatment was calculated using fold change compared with the control or negative group (set as 1).

**Reactive oxygen species detection**

Intracellular ROS was investigated using a ROS assay kit (Solarbio Science and Technology Co., Ltd., Beijing, China) according to the manufacturer’s instructions. Cells were incubated for 24h after the overexpression vector or siRNA targeting HK1 were transfected for 6h. The cell medium was removed and 1mL of 2,7-dichlorofluorescein-diacetate (DCFH-DA) was added. After 20min incubation at 37°C in an incubator, cells were washed three times with a serum-free medium. The images of intracellular ROS were captured with an Olympus IX-73 inverted fluorescence microscope.

**RNA-sequencing analysis**

The hepatocytes transfected with pcDNA3.1 containing goose HK1 CDS (overexpression treatment) and empty vector (control) were used for RNA-sequencing analysis (RNA-Seq). After 24h of culture, total RNA was extracted from eight samples (four samples from each group) using Trizol (Invitrogen, Carlsbad, CA). Sequencing libraries were generated using a library prep kit for Illumina (NEB, USA) and the library preparations were sequenced on an Illumina hiseq platform and paired-end reads were generated. Differential expression analysis was performed using the DESeq R package (1.18.0). The resulting P-values were adjusted using Benjamini and Hochberg’s approach. The differentially expressed genes (DEGs) were defined as genes with a fold change of overexpression treatment over control > 2 or < 0.5, and P-value < .05. Gene Ontology (GO) enrichment analysis of DEGs was performed using the GOseq Software (Release2.12). In addition, KOBAS software (v2.0) was chosen to test the statistical enrichment of DEGs in Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways.

**Real-time quantitative PCR analysis of gene expression**

Total RNA isolation from samples of tissues and cells was performed by Trizol Reagent (TaKaRa Biotechnology Co. Ltd., Dalian, China) method. The total RNA concentration and quality were evaluated at 260 nm using a spectrophotometer (Nano Drop Technologies, Inc., Wilmington, DE, USA). A Prime Script™ RT Master Mix kit (TaKaRa Biotechnology Co. Ltd., Dalian, China) was then used to reverse-transcribe total RNA into cDNA. Real-time PCR was performed in an ABI 7500 Real-time Quantitative PCR (RT-qPCR) System (Applied Biosystems, Foster City, CA, USA) using SYBR® Premix Ex Taq™ kits (Takara Biotechnology Co. Ltd., Dalian, China). The relative mRNA expression level of target genes was analysed according to the $2^{-\Delta \Delta Ct}$ method after normalisation against the reference gene of glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) (Schmittgen and Livak 2008). Primer sequences of all genes used for RT-qPCR are listed in Table 1.

| Table 1. Primer sequences for real-time quantitative PCR analysis. |
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| Genea | GenBank number | Primer sequence (5’ to 3’) | Product size |
| HK1 | XM_013172930.1 | F:AAAAGGCTTCAGGCTACAGA 182 R:TTGGCAGCTATGTGGGAAAGT | 154 |
| CP | XM_013199280.1 | F:GACGCTGTATCCAGAGGCAGAA 171 R:GAGGATACAGGAAAGCTGGA | 154 |
| ACDM | XM_013201323.1 | F:GCCTCCTAGCCTGCTTAC 190 R:ATGGAGATGCTTGGTCATC | 190 |
| PGM2 | XM_013185934.1 | F:GGATTTGCATTGACTCCCATC 163 R:GCTGTACCCAGGCTCTGAT | 163 |
| PLA2G4A | XM_013177931.1 | F:GCTGGCCCAACTTGGAAGAAGG 240 R:TTGGCTGTACCCAGGCTCTT | 240 |
| ECHS1 | XM_013172470.1 | F:CATGGAGATCGTTGCTACCTG 150 R:GCAAGAGCTGGATGGAGGAC | 150 |
| OSGIN1 | XM_013192414.1 | F:GTCCGTTGAGTCTGTTGACA 150 R:CCTGAGGCTGGATGGAGGAC | 150 |
| CYP2C19 | XM_013178201.1 | F:GCCATCAATGATCCCTTCAT 150 R:GCCGTAGGCTGGATGGAGGAC | 150 |
| CPT1A | XM_013199522.1 | F:TCCAGCATGTGAAAGACAGC 151 R:GCCTGAGGCTCTGAATCATC | 151 |

HK1: hexokinase 1; CP: ceruloplasmin; ACDM: Acyl-CoA dehydrogenase medium-chain; PGM2: phosphoglucomutase 2; PLA2G4A: phospholipase A2 group VA; ECHS1: enoyl-CoA hydratase, short chain 1; CYP2C19: cytochrome P450 family 2 subfamily C member 19; CPT1A: carnitine palmitoyltransferase 1 A; OSGIN1: oxidative stress induced growth inhibitor 1.
**Statistical analysis**

Data analysis was performed using SPSS 22.0 statistical software (SPSS Inc, Chicago, USA). The significance was analysed by Student’s t-test for pairwise comparisons. Data were considered significant at $P < .05$, and $P$ value between .05 and .1 was considered as a trend. Results were presented as means and standard error of the means (SEM).

**Results**

**Effects of tissue type and overfeeding on HK1 mRNA expression**

The mRNA expression of hepatic HK1 was upregulated in the overfed treatment compared to control on the 19 days of overfeeding ($P < .05$, Figure 1A). Data showed that the expression of HK1 in abdominal fat of overfed geese was also upregulated on the 14 days of overfeeding ($P < .05$, Figure 1C). Whereas geese in the overfed group had lower HK1 mRNA expression level in breast muscle than the control treatment on the 14 and 19 days of overfeeding ($P < .05$, Figure 1B).

**Regulation of HK1 expression by glucose**

As shown in Figure 2, the expression of HK1 in goose primary hepatocytes was upregulated by 50 mM glucose compared to expression in the control ($P < .05$).

**Effects of overexpression or interference of HK1 on relative lipid accumulation**

The abundance of the HK1 transcript was dramatically increased in cells transfected with the pcDNA3.1/HK1 vector compared to that in cells transfected with empty vector ($P < .05$, Figure 3A). In addition, the HK1 expression was downregulated in siRNA –1, siRNA-2, and siRNA-3 treatment compared to control ($P < .05$, Figure 3B), especially in siRNA-1 group. Data showed that overexpression of HK1 resulted in a tendency to increase ($P = .062$, Figure 4A) the relative lipid level,
while transfected with siRNA targeting HK1 tended to decrease ($P = .064$, Figure 4B) the relative lipid content of cells in comparison to the control.

**Impacts of overexpression or interference of HK1 on ROS level**

As shown in Figure 4, the fluorescence intensity in HK1 overexpression treatment was weakened compared with that of the control group. Compared with negative control, the fluorescence intensity in siRNA group was enhanced.

**RNA-sequencing analysis**

From RNA-Seq analysis on the transcriptomes of cells transfected with empty vector and HK1 overexpression vector, a total of 1609 genes (933 genes up-regulated and 676 down-regulated) were identified as DEGs (Supplementary Figure 1). The enriched GO and KEGG pathways include hexose metabolic process, monosaccharide metabolic process, arachidonic acid metabolism, linoleic acid metabolism, fatty acid metabolism, fatty acid elongation, and so on (Supplementary Figure 2, 3, 4). To confirm the results from RNA-Seq analysis, 8 DEGs were selected for RT-qPCR verification. Consistently, the expression of ceruloplasmin (CP), acyl-CoA dehydrogenase medium-chain (ACADM), phosphoglucosmutase 2 (PGM2), and phospholipase A2 group IVA (PLA2G4A) was significantly induced by HK1 overexpression, while that of enoyl-CoA hydratase, short chain 1 (ECHS1), cytochrome P450 family 2 subfamily C member 19 (CYP2C19), carnitine palmitoyltransferase 1A (CPT1A), and oxidative stress-induced growth inhibitor 1 (OSGIN1) was inhibited ($P < .05$, Figure 6).

**Figure 2.** Effects of treatment with glucose on mRNA expression of hexokinase 1 in goose primary hepatocytes. Primary hepatocytes were isolated from goose embryos after 23 days of incubation. Hepatocytes were treated with 25 mM or 50 mM glucose in a complete cell culture medium. Meanwhile, the hepatocytes untreated with glucose were used as the control group. HK1: hexokinase 1. **P < .05. All data are presented as means ± SEM (n = 6).**

**Figure 3.** Effects of transfection with overexpression vector (A) or siRNAs (B) on mRNA expression of hexokinase 1 in primary goose hepatocytes. HK1: hexokinase 1. Primary hepatocytes were isolated from goose embryos after 23 days of incubation. Hepatocytes transfected with empty pcDNA3.1 vector were taken as the control treatment, and those transfected with pcDNA3.1 containing the goose HK1 coding sequence were used as the overexpression treatment. In addition, hepatocytes transfected with scrambled siRNA were used as a control. The sense strand sequences of siRNA-1, siRNA-2, siRNA-3 were 5'-GCCUGAAGAUCAUCAUACUTT-3', 5'-GCCAAGAAGGUUGAACAATT-3', and 5'-GCCAGAUUUCAGAGACGUUTT-3', while the anti-sense strand sequences were 5'-AUGUAUGAUCAUCAGCGCTT-3', 5'-UUGUUCAAACCCUCUUGCTT-3', and 5'-AAGGUCUCUGAAACUGGCTT-3'. **P < .05, ***P < .01. All data are presented as means ± SEM (n = 6).
Discussion

Our previous study had shown that a large number of mitochondria-related genes were induced in goose fatty liver, including the HK1 gene that is located in the mitochondrial out membrane (Osman et al. 2016). HK1 is known to be the first rate-limiting enzyme in glycolysis and therefore plays an important role in glucose metabolism and energy homeostasis (Li et al. 2020). The mitochondria-bound HK can preferentially use the ATP produced by mitochondrial oxidative phosphorylation to ensure that the HK phosphorylates the glucose entering the cell and promotes glycolysis (Pedersen 2007). On one hand, glycolysis provides energy to the physiological activity of the body. On the other hand, the products such as dihydroxyacetone phosphate and pyruvate produced during this metabolism can be used to synthesise fatty acids. During overfeeding, geese consume a large amount of high-energy maize feed, which is absorbed as glucose after digestion in the intestinal tract and produces acetyl coenzyme A via the glycolysis pathway. Next, the fatty acids are synthesised in large quantities using acetyl coenzyme A and react with α-phosphoglycerol to produce triglyceride, thereby leading to a large accumulation of fat in the hepatocytes and resulting in the formation of fatty liver in geese (Hermier et al. 1994). In the present study, the expression of HK1 was significantly upregulated in the liver of overfed goose, suggesting that the glycolysis process was indeed enhanced during the late overfeeding stage. Geng et al. (2015) found that overfeeding can induce insulin resistance (IR) and activate lipid metabolism-related pathways. The IR exacerbates hyperglycaemia, and when large amounts of blood glucose enter the liver, it acts as a substrate to increase the expression of hexokinase, including the HK1 gene. The findings of this study indicated that a higher concentration of glucose increased HK1 expression in hepatocytes. The formation of goose fatty liver is accompanied by elevated blood glucose (Geng et al. 2015), therefore, the upregulated HK1 expression in the livers of overfed geese may be attributed to the induction of hyperglycaemia. These findings suggested that the increased mRNA expression of HK1 promoted the use of glucose by the glycolytic pathway and accelerated the synthesis and deposition of fat in the liver of overfed geese. In addition, the results also showed that the HK1 expression in the pectoral muscle of the overfed group was lower than that of the control group at 14 and 19 days of overfeeding. A possible reason is the IR in the pectoral muscle of the geese in the overfed group reduces the uptake of glucose by the myocytes (Malkki 2015), thus weakening the induction of HK1 expression by hyperglycaemia. Adipose tissue mainly stores fat or fatty acids synthesised by the liver or ingested from food. In this study, the HK1 expression was found to be
elevated in abdominal fat at 14 days of overfeeding, which may be also related to the induction of hyperglycaemia.

The results showed that overexpression of HK1 increased the relative lipid accumulation at about 20% compared to the control in goose primary hepatocytes. Conversely, data indicated that the relative lipid content in HK1 knockdown treatment is about 20% lower than in the control group. Similarly, the activity of the HK enzyme was significantly elevated in the liver of obese rats (Huupponen et al. 1989). Ferre et al. (2003) indicated that HK overexpression promoted hepatic accumulation in rodents of the liver. These findings implied that HK1 may affect fat metabolism in goose liver cells. The increased HK1 expression can promote the synthesis of intracellular fatty acids, which are stored as fat droplets in the cells after forming triglycerides with glycerol. The enriched GO and KEGG pathways of DEGs after HK1 overexpression were mainly enriched in biological processes of carbohydrate metabolism and lipid metabolism. As reported in previous studies, the function of PGM2 was related to glycolysis (Allendorf et al. 1983), and ACADM and ECHS1 were involved in regulating fatty acid β-oxidation (Matsubara et al. 1990; Yamada et al. 2015). David et al. (2008) reported that PLA2G4A was associated with arachidonate metabolism, which is part of lipid metabolism. The validation of DEGs through RT-qPCR showed that HK1 can influence the expression levels of PGM2, ACADM, ECHS1, PLA2G4A that associated with carbohydrate and lipid metabolism. Thus, the results of RNA-seq are consistent with the findings of oil red

Figure 5. Effects of transfection with overexpression vector or siRNA targeting hexokinase 1 on the level of reactive oxygen species in primary goose hepatocytes. HK1: hexokinase 1. Primary hepatocytes were isolated from goose embryos after 23 days of incubation. Hepatocytes transfected with empty pcDNA3.1vector were taken as the control treatment (A), and those transfected with pcDNA3.1 containing the goose HK1 coding sequence were used as the overexpression treatment (B). In addition, hepatocytes transfected with scrambled siRNA were used as a negative control and siRNA was used as an experimental treatment (D). The sense strand sequence of siRNA was 5'-GCCUGAAGAUAUAUCAUTT -3', and the antisense strand sequence was 5'-AUGUAUGAUUUCAUGGCTT -3'.

A

B

C

D
staining, suggesting that HK1 affects the glycolipid metabolic process and promotes fat deposition in the goose fatty liver.

Numerous studies have demonstrated that the structural and functional changes in mitochondria are closely related to the formation of NAFLD (Sanyal et al. 2001; Pessayre and Fromenty 2005). In the hepatocytes of NAFLD mice, the ROS production is significantly elevated (Nassir and Ibdah 2014), and excess ROS induces the expression of inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-6 (Su et al. 2011; Reczek and Chandel 2015; Lee et al. 2019), leading to inflammation and promoting the development of NAFLD. The mitochondria-bound hexokinase performed a key role as a preventive antioxidant against oxidative stress and could reduce mitochondrial ROS levels (da-Silva et al. 2004). Mailloux et al. (2011) also found that hexokinase 2 could suppress mitochondrial ROS production and maintain aerobic respiration. Interestingly, overexpression of HK1 also reduced ROS level, while knockdown of HK1 increased ROS level in goose hepatocytes. These findings suggest that HK1 may decrease the ROS production in the formation of goose fatty liver. Osman et al. (2016) concluded that the increased expression of mitochondria-related genes in goose fatty liver was mainly enriched in the GO terms of energy metabolism, generation of precursor metabolites, cellular respiration, and mitochondrial fusion, which is different from the loss of mitochondrial mass in mammalian NAFLD. There is no overt injury or pathological symptoms, and the expression of the pro-inflammatory factor TNF-α is significantly suppressed in goose fatty liver (Liu et al. 2016), suggesting that there is a mechanism to suppress the inflammation during the formation of goose fatty liver. Thus, the increased expression of HK1 may be a protective mechanism in the goose liver.

The CP was also reported to have an antioxidant function because the gene could inhibit the oxidation of lipids and scavenger ROS (Halliwell and Gutteridge 1990; Tapryal et al. 2009). In addition, Luo et al. (2021) indicated that over-expression of CPT1A increased the ROS level in liver of mice. Our results showed that overexpression of HK1 increased the expression of CP but suppressed the expression of CPT1A in cells. In addition, overexpression of HK1 decreased the expression of CYP2C19, one gene that belongs to the CYP2C subfamily of Cytochrome P450 enzymes that involved in the generation of ROS (Flachsbart et al. 2011). These results suggest that HK1 may influence ROS level in mitochondria of goose fatty liver by regulating the expression of redox-related genes.

Conclusions

The HK1 expression was induced in the goose fatty liver during the late overfeeding period in comparison to the normal liver, and this induction may be associated with glucose. In addition, HK1 promoted fat deposition by affecting the glycolipid metabolism process and reduced ROS levels in mitochondria by regulating the expression of redox-related genes in goose fatty liver. These findings will not only help to...
elucidate the unique protective mechanism of goose fatty liver, but also provide a reference for the study of HK family genes in the development of NAFLD.

**Ethical approval**

All animal research procedures were approved by the Institutional Animal Care and Use Committee of the Yangzhou University Animal Experiments Ethics Committee with permission number SYXK(Su) IACUC 2012–0029.

**Disclosure statement**

All authors report no conflicts of interest.

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**Data availability statement**

The data that support the findings of this study are available from the corresponding author, [D. Q. G.], upon reasonable request.

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