Fatty acid-binding protein 4 silencing protects against lipopolysaccharide-induced cardiomyocyte hypertrophy and apoptosis by inhibiting the Toll-like receptor 4–nuclear factor-κB pathway

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Abstract
Objective: To explore the effects and potential mechanisms of fatty acid-binding protein 4 (FABP4) in a lipopolysaccharide (LPS)-induced in vitro septic cardiomyopathy model.

Methods: Rat cardiomyocyte H9c2 cells were transfected with small interfering RNA (siRNA) against FABP4 (siFABP4), then induced with LPS. The following parameters were measured: cell viability, lactate dehydrogenase release, cardiac hypertrophy and related marker expression, apoptosis, inflammatory cytokine release and expression, and the activation of Toll-like receptor 4 (TLR4) and nuclear factor-κB (NF-κB) pathways.

Results: LPS increased the mRNA and protein expression of FABP4 in H9c2 cells. FABP4 silencing by siFABP4 significantly inhibited LPS-induced cardiac hypertrophy and reduced the mRNA expression of the myocardial hypertrophy markers atrial natriuretic peptide and brain natriuretic peptide. siFABP4 also attenuated LPS-induced increase in TUNEL-positive apoptotic cells, caspase-3 and caspase-9 activities, and the release and expression of proinflammatory cytokines. Mechanistically, we found that FABP4 silencing inhibited the mRNA and protein expression of TLR4 and suppressed the NF-κB signaling pathway, as evidenced by reduced nuclear NF-κB p65 and increased cytoplasmic I-κBα expression in LPS-stimulated H9c2 cells.

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Conclusion: FABP4 silencing reduces LPS-induced cardiomyocyte hypertrophy and apoptosis by down-regulating the TLR4/NF-κB axis.

Keywords
Cardiomyocytes, lipopolysaccharide, fatty acid binding protein 4, hypertrophy, apoptosis, inflammation, Toll-like receptor 4

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Introduction
The bacterial endotoxin lipopolysaccharide (LPS) is thought to be the leading cause of multiple organ failure in the systemic inflammatory disorder sepsis.1,2 One essential organ liable to injury in sepsis is the heart.3 Indeed, sepsis-induced myocardial dysfunction is one of the most severe complications of sepsis, and a major cause of mortality.4 LPS has been shown to be involved in the complex pathophysiological process of septic myocardial injury through several mechanisms, including increased inflammatory mediator expression and reactive oxygen species production, and the abnormal regulation of some genes.5,6 However, the exact mechanism by which LPS causes cardiac injury is still unclear.

Fatty acid-binding protein 4 (FABP4) is a cytosolic lipid-binding protein that regulates glucose and lipid metabolism and the inflammatory process.7 FABP4 plays a vital role in various metabolic and cardiovascular diseases such as type 2 diabetes,8 insulin resistance, and atherosclerosis.9 Previous studies also reported the expression of FABP4 in capillary endothelial cells and epicardial fat tissue of the heart.11,12 Recently, FABP4 overexpression was reported to aggravate cardiomyocyte injury and hypertrophy,13 while cardiomyocyte FABP4 expression was induced by hypoxia–reoxygenation injury, and FABP4 silencing ameliorated apoptosis through activating the phosphoinositide 3-kinase/Akt pathway.14 FABP4 might also be involved in the pathological process of sepsis because septic patients were reported to have elevated serum FABP4 concentrations compared with healthy controls.15 FABP4 was induced in hepatic tissue by cecal ligation and puncture and LPS treatment, and FABP4 overexpression enhanced inflammatory response and reduced survival.16 Moreover, the FABP4 inhibitor BMS309403 prevented sepsis-induced acute lung injury.17 Considering the broad proinflammatory role of FABP4 in cardiomyocytes and sepsis, the elevated expression of cardiac FABP4 may be common in sepsis-induced myocardial dysfunction. Therefore, it is essential to explore the role of FABP4 in LPS-induced cardiomyocyte injury.

In the present study, FABP4 expression levels were silenced using small interference RNA (siRNA) in rat cardiomyocytes, and the impacts of siFABP4 on LPS-induced viability, apoptosis, hypertrophy, and the inflammatory response were investigated. The regulatory implications of siFABP4 on the expression of Toll-like receptor (TLR)4 and nuclear factor (NF)-κB were also examined.

Materials and methods

Cell culture
The rat H9c2 cardiomyocyte cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum at 37°C in a humidified incubator with 5%
CO₂. The culture medium was changed daily, and cells were passaged when they reached 80% to 90% confluence. Cells were treated with LPS by incubating them in DMEM containing LPS (10 µg/ml) for 6, 12, 24, and 48 hours.

**Small interfering RNA (siRNA)**

H9c2 cells were seeded in six-well plates, grown to about 80% confluence, and then incubated with serum-free medium overnight. siRNAs targeting the *FABP4* gene (siFABP4) and nonsense siRNA (negative control, siNC) were designed and synthesized by GenePharma (Shanghai, China). The siFABP4 sequence was: sense 5'-AC TAATTAGCAAGCAATCACT-3', antisense: 5'-AGTGATTGCTTGCTAATTAGTG-3'; and the siNC sequence was: sense 5'- CGATACCGTTAACTATACAAA-3', antisense 5'-TGGGGATATTTAACGTTATCGT-3'. siFABP4 or siNC (final concentration 100 nM) were mixed with the Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA), then added to cells and incubated at 37°C with 5% CO₂. Real-time quantitative (RT-q)PCR and western blot analyses were used to confirm the interference efficiency after 48 hours (see below).

**Cell viability assay**

The cell counting kit (CCK)-8 assay (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) was used to assess cytotoxicity by measuring cell viability. H9c2 cells were seeded in 96-well plates at a density of 1 x 10⁴ cells/well and treated with 10 µg/mL LPS for 6, 12, 24, and 48 hours, or cells were transfected with siFABP4 as described above then treated with 10 µg/mL LPS for 24 hours. Medium was replaced with fresh medium, then CCK-8 reagents (10%) were added and incubated at 37°C for 1 hour. The absorbance at 450 nm was measured by a microplate reader.

**Lactate dehydrogenase (LDH) assay**

Cell injury was evaluated by measuring the amount of LDH in extracellular fluid from damaged cardiomyocytes. Briefly, after LPS and/or siRNA treatments, the medium of H9c2 cells was collected and centrifuged at 3,000 x g for 10 minutes at room temperature to obtain the supernatant. LDH release into the surrounding medium was then measured by spectrophotometry with an LDH assay kit (Nanjing Jiancheng Bioengineering Institute). All data are expressed as U/dL.

**Cell size measurement**

H9c2 cells were seeded in six-well plates for various treatments, then fixed with 4% paraformaldehyde for 30 minutes. After washing three times in cold phosphate-buffered saline (PBS), cells were permeabilized with 1% Triton X-100 and blocked with 5% bovine serum albumin. They were then incubated with an anti-α-actinin antibody (dilution 1:100, Abcam, Cambridge, UK) overnight at 4°C, and subsequently with a secondary antibody conjugated with Alexa Fluor® 488 (dilution 1:100, Abcam) for 1 hour at room temperature. Cells were counterstained with 4',6-diamidino-2-phenylindol (DAPI; BBI Life Sciences, Shanghai, China) and observed and imaged using an IX-73 inverted fluorescence microscope (Olympus, Tokyo, Japan). Cell surface areas were measured from five random fields of view per well (a total of 100 cells) and analyzed by ImageJ software (National Institute of Health, Bethesda, MD, USA).
Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

H9c2 cardiomyocytes were cultured in six-well plates, then fixed with 4% paraformaldehyde and permeabilized with 1% Triton X-100 as described above. They were incubated with 50 μL TUNEL reaction fluid (Beyotime Institute of Biotechnology, Jiangsu, China) at 37°C for 1 hour to detect cells undergoing apoptosis. Cells were washed twice with PBS, stained with DAPI to label nuclei, then observed under a fluorescence microscope (Olympus BX41). Five photographs (magnification ×200) were taken randomly for each sample to calculate the percentage of TUNEL-positive cells.

Capase-3 and capase-9 activity assays

After various treatments, cells were lysed with 100 mL of lysis buffer for 15 minutes on ice, then centrifuged at 10,000 × g for 15 minutes. A total of 10 μL supernatants were mixed with 90 μL AC-DEVD-pNA or AC-LEHD-pNA (both 2 mM, Beyotime Institute of Biotechnology) for 2 hours at 37°C to measure capase-3 and capase-9 activities, respectively. The absorbance at 405 nm was measured using a microplate reader.

Enzyme-linked immunosorbent assay (ELISA)

Cells were transfected with siFABP4 and treated with 10 μg/mL LPS for 24 hours. The culture medium was then collected, and commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to measure interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α expression. Experiments were performed at least three times.

Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted by TRIzol agent (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer’s instructions, and 1 μg RNA was used to synthesize cDNA with the PrimeScript RT Reagent kit (Takara Bio Inc., Dalian, China). RT-qPCR was performed using a SYBR Green RT-qPCR kit (Takara Bio Inc.) on an ABI Prism 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. The qPCR system (20 μl) contained 10 μl SYBR EX Taq–Mix, 1 μl forward and reverse primers, 1 μl cDNA, and 8 μl ddH2O. PCR primers are shown in Table 1, and PCR conditions were: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 s and 60°C for 1 minute. The relative expression of mRNA was determined with the 2−ΔΔCt method.

Table 1. Sequences of primers used in this study.

| Gene symbol | Forward primer (5’–3’) | Reverse primer (5’–3’) |
|-------------|------------------------|-----------------------|
| ANP         | ACCAAAGGCTTCTTCTCCTCT  | TTCTACCCGGCATTCTTCTCC |
| BNP         | AGAACAATCCACGAGTGCAAG  | AAACAACCTCAGCCCGTCACA |
| FABP4       | ACATGAAAGAAGTGGGAGTTGC | AAGTACTCTGACGGGATGAGC |
| IL-1β       | CACCTCTCAAGCAGAGCACAG  | GGGTTCCATGGTAGAATCAC |
| IL-6        | TACATTTGCCAGCGATTCAGG  | CACAGCCAGTCTCCTCATT  |
| TNF-α       | AAATGGCTCCCTCCTCATGACT  | TCTGTCTGTGGTTTGGTACGAC |
| TLR4        | AAGTTATTTGCTGGTGCTGCTAG| GAGGTAGGGTTTTGTGCTAAG |
| GAPDH       | AACCTTGGGCTATTGGAAGG   | ACACATTGGGGGTAGAACA  |
Western blot

Radioimmunoprecipitation assay lysis buffer was used to extract total protein, and cytoplasmic and nuclear proteins were extracted using related Extraction Reagents (Pierce Biotechnology, Inc., Rockford, IL, USA). The BCA protein assay kit (Beyotime Institute of Biotechnology) was used to measure protein concentrations. Total protein (50 μg) was run on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% low-fat milk and incubated with primary antibodies against FABP4 (dilution 1:200, Abcam), nuclear factor (NF)-κB p65 (dilution 1:500, Abcam), and I-κBa (dilution 1:500, Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4°C. After washing with PBS, the membrane was incubated with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (dilution 1:250, Santa Cruz Biotechnology) for 1 hour at room temperature. Anti-β-actin (dilution 1:2000, Abcam) and anti-lamin B (dilution 1:1000, Abcam) primary antibodies were used as internal controls for cytoplasmic and nuclear fractions, respectively. Bands were visualized using a chemiluminescent detection system (Thermo Fisher Scientific), and the optical densities were analyzed using ImageJ software.

Statistical analysis

Data are expressed as means ± standard deviation (SD) and were analyzed by SPSS 20.0 statistical software (Armonk, NY: IBM Corp.). Differences between three or more groups were compared by one-way analysis of variance, followed by post hoc testing using Bonferroni’s correction. P < 0.05 was regarded as statistically significant.

Results

Expression of FABP4 in rat cardiomyocytes treated with LPS

To explore the role of FABP4 in septic cardiomyocyte injury, we investigated whether LPS treatment affects FABP4 expression in cardiomyocytes. H9c2 cells were incubated with 10 μg/mL LPS for 6, 12, 24, and 48 hours. The CCK-8 assay showed that LPS treatment significantly reduced cell viability, while significantly more LDH was detected in the culture media of LPS-treated cells compared with control cells at 12, 24, and 48 hours (P < 0.05, Figure 1a, 1b). RT-qPCR revealed that LPS significantly increased mRNA expression of the hypertrophy markers atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) (P < 0.001, Figure 1c, 1d). FABP4 mRNA and protein expression was also significantly increased by LPS treatment in a time-dependent manner (P < 0.001 by 24 hours, Figure 1e, 1f). These results indicate that FABP4 is involved in the pathological process of septic cardiomyocyte injury.

We then used siRNA to knockdown FABP4 expression in cardiomyocytes, as evidenced by significantly reduced mRNA and protein expression after 48 hours (P < 0.001, Figure 2a, 2b). Knockdown of FABP4 significantly increased cell viability and decreased supernatant LDH levels after LPS treatment (P < 0.01 and P < 0.001, respectively; Figure 2c, 2d). These results suggest that FABP4 has an aggravating effect on LPS-induced injury of cardiomyocytes.

siFABP4 suppressed cardiac hypertrophy in cardiomyocytes treated with LPS

To determine the role of FABP4 in cardiac hypertrophy, we performed immunofluorescence using an α-actinin antibody. LPS treatment was shown to induce a hypertrophic response in H9c2 cells, which was
markedly attenuated by siFABP4, resulting in a significantly reduced cardiomyocyte size (cell surface area) (P < 0.01, Figure 3a, 3b). Moreover, siFABP4 significantly attenuated the LPS-induced upregulation of cardiomyocyte hypertrophy markers ANP and BNP (P < 0.001, Figure 3c, 3d).

**siFABP4 protected H9c2 cells from LPS-induced apoptosis**

We next investigated whether FABP4 influences the LPS-induced apoptosis of cardiomyocytes. H9c2 cells treated with siFABP4 showed a significant decrease in TUNEL-positive staining compared with controls (P < 0.01, Figure 4a, 4b). Consistently, siFABP4 significantly attenuated the LPS-induced increase in apoptosis biomarkers caspase-3 and caspase-9 (P < 0.001, Figure 4c, 4d). These results indicate that FABP4 promotes LPS-induced H9c2 cell apoptosis.

**siFABP4 inhibited the inflammatory response induced by LPS in cardiomyocytes**

The concentrations of IL-1β, IL-6, and TNF-α in the culture medium were next analyzed by ELISA. LPS treatment significantly increased IL-1β, IL-6, and TNF-α levels in the culture medium of H9c2 cells compared with controls (P < 0.001), while siFABP4 knockdown significantly reduced this (P < 0.01) (Figure 5a–c). In line with
this, RT-qPCR revealed that siFABP4 significantly reduced the LPS-induced upregulation in IL-1β, IL-6, and TNF-α mRNA expression (P < 0.01, Figure 5d–f). These data indicate that siFABP4 decreases LPS-induced proinflammatory cytokine expression in cardiomyocytes.

**siFABP4 inhibited the expression of LPS-induced TLR4 and NF-κB translocation in cardiomyocytes**

RT-qPCR showed that LPS treatment significantly increased TLR4 mRNA expression (P < 0.001), and that this was significantly reduced in cells exposed to siFABP4 (P < 0.001, Figure 6a). In support of this, western blotting revealed that siFABP4 significantly decreased the expression of TLR4 protein in LPS-treated H9c2 cells (P < 0.001, Figure 6b–d). Moreover, siFABP4 significantly reversed the LPS-induced reduction in cytoplasmic I-κBα protein (P < 0.01) and significantly increased the nuclear translocation of NF-κB p65 in H9c2 cells (P < 0.001) (Figure 6e–i). These data indicate that FABP4 silencing inhibits LPS-induced...
TLR4 expression and the NF-κB signaling pathway.

Discussion

In the present study, we investigated the expression and function of FABP4 in H9c2 cells treated with LPS. FABP4 expression in H9c2 cells was shown to be increased by LPS treatment, while FABP4 silencing by siRNA markedly decreased cardiac hypertrophy, apoptosis, and the production of proinflammatory cytokines. FABP4 silencing also suppressed the expression of TLR4 and blocked NF-κB translocation from the cytoplasm to the nucleus in LPS-treated H9c2 cells.

FABP4 plays a crucial role in the cardiovascular system. Serum FABP4 concentrations were previously positively correlated with an increased risk of heart failure. However, the role of FABP4 in septic cardiomyopathy has never been investigated. Our study showed that FABP4 plays a promotive role in cardiomyocyte hypertrophy, with enhanced FABP4 expression detected in cardiomyocytes following LPS treatment. Considering that high serum FABP4 levels

Figure 3. siFABP4 inhibited LPS-induced cardiomyocyte hypertrophy. Cultured H9c2 cardiomyocytes were transfected with siFABP4 or siNC and then treated with LPS (10 μg/mL) for 24 hours. (a) Cardiomyocytes were stained with α-actinin (green) and DAPI (blue). Representative photographs are shown (scale bar: 5 μm). (b) Quantitative analysis of cardiomyocyte surface area from a total of 100 cardiomyocytes per group. ANP (c) and BNP (d) expression was evaluated by RT-qPCR using GAPDH as the internal control. ***P<0.001 vs. cardiomyocytes in the control group; ##P<0.01, ###P<0.001 vs. cardiomyocytes with LPS. FABP4, fatty acid-binding protein 4; LPS, lipopolysaccharide; siFABP4, FABP4-specific small interfering RNA; siNC, negative control siRNA; DAPI, 4',6-diamidino-2-phenylindole; ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; RT-qPCR, real-time quantitative PCR; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
have been reported in patients with sepsis.\textsuperscript{15} FABP4 appears to be not just a marker of cardiac dysfunction in sepsis, but to also have possible essential roles in sepsis-induced cardiomyocyte injury. Therefore, it is of value to investigate the functions of FABP4 in cardiomyocytes, as in sepsis-induced acute lung injury.\textsuperscript{17} LPS is a common mediator of sepsis-induced myocarditis and is used to induce myocardial inflammatory lesions.\textsuperscript{20} In this study, we showed that 10 μg/mL LPS treatment significantly reduced cardiomyocyte viability while increasing the LDH content and the percentage of apoptotic cells, thus resulting in cardiomyocyte injury. Moreover, LPS-induced injury was attenuated by siFABP4, as evidenced by increased cell viability and reduced apoptosis. The pro-survival effect of FABP4 silencing was also confirmed in another study that reported the protective effects of FABP4 siRNA in H9c2 cells after hypoxia reoxygenation.\textsuperscript{14} Cardiomyocyte hypertrophy is an important adaptive response of the heart, and is characterized by an enlarged cardiomyocyte size.\textsuperscript{21} This myocardium enlargement is irreversible and ultimately results in cardiac failure.\textsuperscript{22} Previous studies showed that LPS-induced cardiac dysfunction through cardiomyocyte hypertrophy, and stimulated caspase expression in cardiomyocytes, which

Figure 4. siFABP4 inhibited LPS-induced cardiomyocyte apoptosis. (a) Cardiomyocytes were stained with TUNEL (green) and DAPI (blue). Representative photomicrographs are shown (scale bar: 5 μm). (b) Quantification of TUNEL-positive cells. Cell apoptosis was determined by measuring caspase-3 (c) and caspase-9 (d) activities. ***P<0.001 vs. cardiomyocytes in the control group; ###P<0.01, ####P<0.001 vs. cardiomyocytes with LPS. FABP4, fatty acid-binding protein 4; siFABP4, FABP4-specific small interfering RNA; LPS, lipopolysaccharide; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; DAPI, 4′,6-diamidino-2-phenylindole.
induced end-stage nuclear apoptosis, cardiac myofilament protein degradation, and sarcomere disorganization.\textsuperscript{23,24} In the present study, we found that FABP4 promoted hypertrophy in LPS-treated cardiomyocytes, which is in line with the observation that FABP4 overexpression enhanced cardiac hypertrophy induced by transverse aorta constriction.\textsuperscript{13} While siFABP4 clearly inhibited LPS-induced cardiomyocyte injury, apoptosis, and hypertrophy in our study, the mechanisms involved in this should be investigated further.

Increased inflammatory mediator production, including that of IL-1\(\beta\), IL-6, and TNF-\(\alpha\), is regarded as one of the main mechanisms underlying LPS-induced myocardial injury. These inflammatory mediators influence downstream molecular signaling pathways and play essential roles in LPS-induced myocardial injury, cardiomyocyte apoptosis, and heart failure.\textsuperscript{25,26} FABP4 has been shown to adjust LPS-induced proinflammatory activity and to modulate lipid trafficking and responses in cells.\textsuperscript{27} Moreover, high levels of circulating FABP4 were associated with various diseases involving a systematic inflammatory response, including obesity, insulin resistance, type 2 diabetes, asthma, and atherosclerosis.\textsuperscript{28} Recent studies have also shown that FABP4 can alter allergic airway inflammation.\textsuperscript{29} FABP4 has increasingly been thought of as a promoter of inflammatory responses, but few studies have investigated its role in the septic cardiomyocyte model. We discovered that FABP4 silencing in H9c2 cells significantly attenuated the increased concentrations of IL-1\(\beta\), IL-6, and TNF-\(\alpha\) induced by LPS. This supports previous findings of a promotive role for FABP4 in the inflammatory response, as shown by FABP4 inhibition decreasing IL-1\(\beta\), IL-6, and TNF-\(\alpha\) expression in LPS-induced alveolar epithelial cells and renal tubular epithelial cells.\textsuperscript{17,30}
To understand the mechanisms involved in protecting against cardiac injury, we examined TLR4 and NF-κB signaling pathways and found that they were strongly induced by LPS in H9c2 cells, and markedly reversed by FABP4 silencing. TLR4 is a key receptor of the innate immune system and is involved in the LPS-induced inflammatory response. TLR4 in cardiomyocytes is expressed, activated, and translocated to the nucleus in response to LPS, causing an inflammatory response and cell apoptosis. We showed that FABP4 silencing inhibited TLR4 expression and the translocation of NF-κB p65 from the cytoplasm to the nucleus in response to LPS. This suggests an alleviating effect of FABP4 silencing on LPS-induced inflammatory responses. The modulation of TLR4 by FABP4 is also supported by another study which showed that a novel monoclonal antibody against FABP4 attenuated the inflammatory responses induced in mice receiving a high-fat diet through inhibiting the TLR4 signaling pathway.
Conclusions
This study showed that FABP4 mRNA and protein expression was induced by LPS in cardiomyocytes. Moreover, silencing FABP4 protected cardiomyocytes from LPS-induced injury by inhibiting apoptosis, hypertrophy, and the inflammatory response. We identified FABP4–TLR4–NF-κB as a signaling axis activated by LPS in cardiomyocytes that might mediate sepsis-induced myocardial dysfunction. Future in vivo studies are required to confirm the protective role and mechanisms of FABP4 in myocardial injury.

Availability of data and materials
The raw data used and analyzed for the current study are available from the corresponding author on reasonable request.

Declaration of conflicting interest
The authors declare that there is no conflict of interest.

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Author contributions
Fangyuan Sun performed the experiments and wrote the manuscript; Gang Chen performed the experiments and collected data; Ming Lei designed and supervised the study, and revised the manuscript. All authors read and approved the manuscript.

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