Role of BMI1 in epithelial ovarian cancer: investigated via the CRISPR/Cas9 system and RNA sequencing

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Abstract

Background: B-cell-specific Moloney murine leukemia virus integration site 1 (BMI1) might be an appropriate biomarker in the management of epithelial ovarian cancer (EOC). However, the biological role of BMI1 and its relevant molecular mechanism needs further elaboration. Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system is an excellent genome-editing tool and is scarcely used in EOC studies.

Methods: We first applied CRISPR/Cas9 technique to silence BMI1 in EOC cells; thereafter we accomplished various in vivo and in vitro experiments to detect biological behaviors of ovarian cancer cells, including MTT, flow cytometry, Transwell, real-time polymerase chain reaction and western blotting assays, etc.; eventually, we used RNA sequencing to reveal the underlying molecular traits driven by BMI1 in EOC.

Results: We successfully shut off the expression of BMI1 in EOC cells using CRISPR/Cas9 system, providing an ideal cellular model for investigations of target gene. Silencing BMI1 could reduce cell growth and metastasis, promote cell apoptosis, and enhance the platinum sensitivity of EOC cells. BMI1 might alter extracellular matrix structure and angiogenesis of tumor cells through regulating Focal adhesion and PI3K/AKT pathways.

Conclusion: BMI1 is a potential biomarker in EOC management, especially for tumor progression and chemo-resistance. Molecular traits, including BMI1 and core genes in Focal adhesion and PI3K/AKT pathways, might be alternatives as therapeutic targets for EOC.

Keywords: B-cell-specific Moloney murine leukemia virus integration site 1 (BMI1), Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9, Epithelial ovarian cancer (EOC), Focal adhesion pathway, PI3K/AKT pathway

Background

Epithelial ovarian cancer (EOC) is the leading cause of mortality among gynecologic cancers, and the majority of patients are diagnosed in advanced stages [1, 2]. Approximately, 70% of the patients would relapse even if they have received optimal cytoreductive surgery (CRS) combined with standard platinum-based chemotherapy. The 5-year survival rate for advanced-stage patients is very low [3]. It has been a major research focus and challenge to search for appropriate tumor markers and effective therapeutic targets because the etiology of EOC is largely unknown and the tumors present heterogeneity in multiple dimensions.

As a core member of Polycomb group proteins (PcG), B-cell-specific Moloney murine leukemia virus integration site 1 (BMI1) plays an important role in epigenetics, participates in important cellular events and is identified as aberrantly expressed in various human cancers [4–11]. In our previous study, we also found significant over-expression of BMI1 in metastatic lymph nodes and recurrent tumors compared to primary ovarian carcinomas. In addition, intensive expression of BMI1 in metastatic and recurrent tumors was an independent prognostic factor for survival and relapse, respectively [12]. However, the biological functions and related mechanism of BMI1 in EOC lack elaboration.

Among a variety of genome-editing techniques, the clustered regularly interspaced short palindromic repeats,
(CRISPR)/Cas9 system has enormous prospects in tumor genesis and progression researches. The technique is highly effective at thoroughly shutting off gene expression with ~ 20 bp guide RNAs (gRNAs) [13, 14], by which can establish ideal cellular models for functional investigation. To date, studies involving the CRISPR/Cas9 technique in ovarian cancer studies are still scarce.

Therefore, we 1) explored the feasibility of the CRISPR/Cas9 system as a genome editing tool to construct BMI1 knock-out EOC cell models, 2) investigated the changes in biological behaviors after silencing BMI1 using in vitro and in vivo experiments, and 3) identified the differences in mRNA profiles between wild-type and BMI1 knock-out EOC cells using transcriptome sequencing technique in order to reveal the potential molecular mechanism. 4) revealed the potential of BMI1 serving as a biomarker in EOC management with basic research evidences.

**Methods**

**Knocking out BMI1 in EOC cell lines using CRISPR/Cas9 technique**

**Cell culture**

The ovarian cancer cell line SKOV3 was obtained from Institute of Basic Medical Sciences Chinese Academy of Medical Sciences & School of Basic Medicine, Peking Union Medical College. The SKOV3 cells were cultured in McCoy5A medium (Macgene; Beijing, CN), supplemented with 10% fetal bovine serum (FBS, HyClone; Logan, UT, US), 100 U/ml penicillin (Solarbio; Beijing, CN), and 100 μg/ml streptomycin (Solarbio; Beijing, CN).

**CRISPR/Cas9 vector construction and transfection**

Specific gRNAs (Table 1) were designed using http://crispr.mit.edu/, and they were subsequently connected to linear pX330-BbsI vectors. To enhance editing efficiency, two gRNAs were synthesized targeting distinct domains of BMI1. Successively, two expression vectors (pX330-Cas9-gRNA1 and pX330-Cas9-gRNA2) were established, and co-transfected SKOV3 simultaneously with pLLexp-puro plasmid. Then, we used puromycin (1 μg/ml) to screen out those cells which have not been successfully transfected. Eventually, we cultured the viable cells surviving drug-sifting in 96-well plates using limited dilution method and amplified all the single-cell derived sub-clones.

**Validation of BMI1 knock-out**

Genomic DNA was extracted from each sub-clone for polymerase chain reaction (PCR) and Sanger sequencing.

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**Table 1** Sequences of gRNAs and primers

| Name               | Sequences (5′>3′) | PAM/ Length |
|--------------------|------------------|-------------|
| **CRISPR gRNAs for BMI1** |                  |             |
| gRNA1              | CACCGAAGGTGTATTTGTCGTT | AAACGGTAAAGCAATAACACGTTC ACC | |
| gRNA2              | CACCGTGGTCTGTCTGTGAAC | AAACAGTTCAACAAGGCAACAACAC | TGG | |
| **Primers for PCR** |                  |             |
| BMI1\(^a\)         | TTGATGCCCAACACCATATAAGAAT | AATTACAACAGGAATTTCACAACA | 494 bp 194 bp |
|                   | CTAAACCAATGATTTATGCAAAT |                      | |
| **Primers for real-time PCR** |              |             |
| Caspase3           | GAAATTGTGGAATGTCGTGA | CTACAACGATCTCCCTCTGGAAA 166 bp |
| Bcl-2              | CTAAGGGTATGAAAGCCTGTA | CTCGTGAAATCTAAAGGTCTGT 111 bp |
| COL1A1             | GTGCGTATGCTGATCTGTA | CGGTGGTTCTTGGTGCTGT 119 bp |
| COL4A1             | GCAATACCTCTGAACAATAAACAGG | GCCCAATCTACCTGAGCACA 240 bp |
| TNC                | TCCAGCTCTGGTGCTGATCT | TGATGGTGGTGTGGTGAAGACA 131 bp |
| LAMA3              | TGTCATACTACCTCCTCTGC | TCCAGTCTCTTGGCGGGTGT 181 bp |
| ITGA7              | CAGCGAGGATGGACCATCAC | CCAAAGGGGAGGTAGTGGCTATC 203 bp |
| ITGB4              | CTCAACCCAGATGATCGCCTC | CGGGATTACCTGTGTCCTGTA 133 bp |
| AKT3               | AATGGACAAGAAGCTACACGG | TGTGTTGGTATGAGGGCATTCC 130 bp |
| CREB5              | AAAGACTCGCACAATAACACG | AACCTCGGCGACGACTAGC 88 bp |
| PK3CA              | GAAACAAGACGGCTTTGACCT | CCTACGAGTGGTCCTCTCTGTA 76 bp |
| PK3CD              | AGCCGGAGAAGCTACACGCT | GTGTCAGGTTAGGAGGTTCAAC 122 bp |
| BIRC3              | TTTCCGGTGGCTTATACAAACT | GCACAGTGGTAAGAATCTCTCAT 96 bp |
| GAPDH              | ACAAATGGTATGGGAGAAGG | GCCATCAGCGCCAGCTTTTC 101 bp |

\(^a\)Longer fragments obtained by the first BMI1 primer is more suitable for subsequent Sanger sequencing, whereas the second primer is better for revealing fragments shortening after silencing BMI1 on Agarose gel.
of the targeted fragments. PCR primers are listed in Table 1. Protein was subsequently extracted from subclones with shortened nucleic acid sequences for western blotting. All validated BMI1 knock-out clones were then amplified and stored.

Comparison of biological behaviors using in vitro and in vivo experiments

**MTT assays**

Cell viability was detected using MTT assays. Twenty microliters of MTT reagent (5 mg/ml) was added to each well (96-well plate) and incubated for 4 h and then terminated by adding 100 µl dimethylsulfoxide (DMSO) and incubating at 37 °C in the dark for 10 min. Cell proliferation was assessed by measuring the absorbance at 570 nm and 630 nm wavelength (Optical density, OD 570-630nm). Growth curves were drawn with doubling time calculated for each group by consecutive MTT assays for seven days.

**Flow cytometry (FCM)**

The cell pattern of each sub-clone was detected using C6 FCM (BD Biosciences; New Jersey, US). Briefly, cell pretreatment comprised cold-ethanol fixation for 2 h and propidium iodide (PI, Keygene; Wageningen, the Netherlands) staining for 30 min in the dark. Modfit software (Verity; Maine, US) was used for analysis. Similarly, apoptosis could be compared by FCM after Annexin V-fluorescein isothiocyanate (FITC) and PI staining.

**Transwell migration and invasion assays**

The cell migration assay was performed using a Transwell chamber (BD Falcon™, San Jose, CA). These chambers were inserted into 24-well culture plates. Wild-type SKOV3 cells and BMI1 knock-out clones in 200 µl serum-free culture solution were added to the upper chambers. Ten percent FBS-containing McCoy’s 5A medium was added into the lower chambers to serve as the chemo-attractant. For invasion assays upper chambers were pre-coated with Matrigel (BD BioCoat™, BD Biosciences, San Jose, CA). After incubation for 24 h, the medium, the gel and uncrossed cells in the upper chambers were removed, while the migrated/invaded cells at the lower side of the membranes were fixed with paraformaldehyde (4%) and stained with crystal violet. Pictures were taken at 200X magnification, and cell numbers from five random microscopic fields were counted for statistical comparison.

**Real-time PCR and western blotting**

Expression levels of marker proteins in apoptosis were compared between wild-type and BMI1 knock-out cells by real-time PCR and western blotting. Primers and antibodies are listed in Tables 1 and 2, respectively.

| Name      | Corporation     | Dilution ratio |
|-----------|-----------------|----------------|
| BMI1      | Cell signaling  | 1:1000         |
| Caspase3  | Abcam           | 1:1000         |
| Bcl-2     | Abcam           | 1:500          |
| COL1A1    | Abcam           | 1:1000         |
| COL4A1    | Bioworld        | 1:500          |
| TNC       | Abcam           | 1:2000         |
| LAMA3     | Abcam           | 1:2000         |
| ITGA7     | Bioworld        | 1:500          |
| ITGB4     | Abcam           | 1:2000         |
| AKT3      | Proteintech     | 1:500          |
| CREBS     | Proteintech     | 1:500          |
| PIK3CA    | Abcam           | 1:1000         |
| PIK3CD    | Abcam           | 1:500          |
| BIRC3     | Abcam           | 1:1000         |
| β-Tubulin | Bioworld        | 1:3000         |
| β-Actin   | Cell signaling  | 1:1000         |

Total RNA was isolated using Trizol reagent (Invitrogen; California, US). For quantitative analysis, mRNA levels of target sequences were compared: RNA was first retro-transcribed with random primers using TransScript First-Strand cDNA Synthesis Kit (TransGen; Beijing, CN), and then real-time PCR was carried out using TransStart Tip Green qPCR SuperMix (TransGen; Beijing, CN) with specific primers. The comparative Ct method was used to calculate the relative abundance of mRNA compared to GAPDH expression.

Harvested EOC cells were washed in phosphate buffer (PBS) and lysed in ice-cold cell lysis buffer with freshly added 0.01% protease inhibitor and then incubated on ice for 15 min. Cell debris was discarded after ultrasonic breaking and centrifugation at 14000 rpm for 10 min at 4 °C. Afterwards, the supernatant was run on a sodium-dodecyl sulphate (SDS)-PAGE gel, transferred to a polyvinylidene fluoride (PVDF) membrane, hybridized with specific antibodies, and developed using electrochemiluminescence (ECL) methodology.

**Chemotherapeutic response**

MTT assays were also used to estimate the cells’ sensitivity to cisplatin, carboplatin and paclitaxel (National Institutes for Food and Drug Control; CN). Each antineoplastic medicine was diluted at gradient concentrations and added to 96-well plates (6 wells per group per concentration). After incubation at 37 °C for 48 h, MTT assays were performed as previously described. With OD_{570nm} and OD_{630nm} detected, the growth inhibition ratio was calculated under each concentration for each group (Formula 1). Afterwards, the drug concentration
at which 50% of the cells were prevented from proliferating was obtained using GraphPad Prism 5.0 software (California, US).

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\text{Growth inhibition rate} = \left( \frac{1 - \text{Test well OD}_{70/630nm} - \text{Blank well OD}_{70/630nm}}{\text{Lowest Concentration well OD}_{70/630nm} - \text{Blank well OD}_{70/630nm}} \right) \times 100\%
\]

Xenografted tumor experiments
Female nude mice (BALB/c) were purchased from Beijing Vital River Laboratory Animal Technology Corporation (Beijing, CN). All mice were housed and fed under specific pathogen-free conditions in an institution approved by the Beijing Laboratory Animal Research Center. All studies were approved and supervised by Peking Union Medical College Hospital. All mice used were 5 weeks old when the experiments initiated. Six mice were assigned per group. Cells cultivated from different groups (2 × 10⁷ cells) were injected subcutaneously. To compare the ability of tumor formation in vivo, the transplanted tumors were checked and dimensioned every 2 days. Feeding was stopped at the same time for each group after 10 weeks. Tumor formation rate and average tumor size (Length×Width² × 0.5) were calculated and compared.

Investigation of underlying molecular mechanism using RNA sequencing
Total RNA was extracted from wild-type SKOV3 and BMI1 knock-out clones, and transcriptome libraries were subsequently constructed using a KAPA stranded mRNA-Seq Kit (KAPA Biosystems; Massachusetts, US). High-throughput sequencing was accomplished on a Hiseq2000 platform (Illumina; California, US). Bioinformatics analyses included estimation of gene-expression amount, clustering, GO (Gene ontology) and KEGG (Kyoto Encyclopedia of Genes and Genome). Differential gene-expression profiles were validated by real-time quantitative PCR and western blotting; corresponding primers and antibodies are listed in Tables 1 and 2. Eventually, potential pathways involving BMI1 in tumor genesis and progression were proposed.

Statistical analysis
Biological behaviors and transcriptome profiles were compared between wild-type and BMI1 knock-out EOC cells. Each experiment was performed in triplicate assays and repeated at least three times. All values were expressed as the means ± SD (standard deviation). Statistical significance was determined using two-sided Student's t-test or Fish's exact test, and a value of \( P < 0.05 \) was considered significant. Statistical analyses were performed using Statistical Package for the Social Sciences 20.0 (IBM; New York, US).

Results
Knock-out BMI1 in EOC cells using CRISPR/Cas9 system
Specific gRNAs were inserted into pX330-Cas9 vectors as shown in Fig. 1 with a success rate of 100%. After transfection and drug sifting, we obtained 23 single-cell derived BMI1 knock-out clones after limited-dilution culture. Figure 1 also depicts the validation results of target-gene editing: Agarose electrophoresis results revealed PCR products from the treated group had shortened nucleic acid sequences (9/23, Fig. 1b); target fragments of these sub-clones were confirmed deleted by Sanger sequencing (9/9); and western blotting experiments further verified that all nine sub-clones completely lack expression of BMI1 protein (Fig. 1c).

Silencing BMI1 suppressed cell proliferation, migration and invasion of EOC cells
Growth curves showed that BMI1 knocking out reduced cell proliferation straight from the second through the seventh day, compared to the wild-type SKOV3 (Fig. 2a). The doubling times for BMI1 knock-out and wild-type clones were 2.10 ± 0.7 d and 5.62 ± 1.57 d, respectively (\( P < 0.05 \)). BMI1 silencing also led to cell pattern alteration, as the proportion of EOC cells in S phase significantly decreased (23.7% vs. 12.2%, Fig. 2b). Cell invasiveness assessed using Matrigel-coated Transwell chambers presented a significant inhibition (Fig. 2c). Figure 2d shows reduced migration of SKOV3 cells transfected with CRISPR/Cas9-gRNAs. The numbers of migrated/invaded cells in either group are listed in Table 3. In addition, in vivo experiments (Fig. 2e) revealed that 1) tumor formation rate of subcutaneous inoculation reduced from 100% to 50% after BMI1 silencing and 2) the average xenografted tumor size was dramatically smaller in the BMI1 knock-out group (5.3 mm³) than in the wild-type group (1986.7 mm³).

BMI1 knock-out promoted apoptosis of EOC cells
We next examined the effect of BMI1 knocking out on cell apoptosis. First, FCM detected a higher apoptosis rate in BMI1 knock-out EOC cells than in the untreated group (3.3 ± 0.2% vs. 1.9 ± 0.2%, \( P < 0.05 \)). Established markers of apoptosis include caspasas. Therefore, we determined the expression of caspase 3 cleavages in control and BMI1-gRNAs-transfected EOC cells. An increase of active caspase 3 was observed in BMI1 knock-out cells (Fig. 2f). Bcl-2 suppresses apoptosis in a variety of cell systems, including functioning in a feedback loop system with caspasas. We detected a remarkable reduction of Bcl-2 expression after knocking-out BMI1, indicating an ongoing apoptotic process (Fig. 2f, Additional file 1: Figure S1).

Silencing BMI1 enhanced platinum sensitivity of EOC cells
We tested whether BMI1 silencing would affect chemotherapeutic responses of EOC cells. After drawing cell
Fig. 1  

a. BMI1 gRNAs successfully inserted into pX330-BbsI vectors;  
b. Sub-clones with shortened nucleic acid sequences on Agarose gel after PCR;  
c. Sub-clones with no expression of BMI1 protein entirely detected by western blotting

Fig. 2  

Changes of Biological behaviors after silencing BMI1:  
a. cell growth curve;  
b. cell cycle pattern;  
c. Transwell invasion;  
d. Transwell migration;  
e. in vivo experiments;  
f. apoptosis markers;  
g. chemotherapeutic responses
growth inhibition curves under gradient drug concentrations (Fig. 2g). IC50s of either sub-clone to cisplatin, carboplatin and paclitaxel were calculated (Table 3). The data demonstrated that BMI1 knock-out sensitized EOC cells to platinum medications (P < 0.05), including both cisplatin and carboplatin. However, similar reactions to paclitaxel were observed in BMI1-silenced and untreated cells (P > 0.05).

**BMI1 modulated focal adhesion and PI3K/AKT pathways in EOC cells**

Total RNA extracted from either group was qualified for RNA sequencing, revealing appropriate OD260nm/280nm values (1.8–2.0) and abundances (≥5.0 μg per group). Raw reads were filtered and aligned to reference sequences. With GO analyses, we demonstrated that the majority of differentially expressed genes after BMI1 silencing participated in extracellular matrix (ECM) construction and blood vessel development. KEGG analyses revealed that BMI1 might alter the biological behaviors of EOC cells by modulating the focal adhesion and PI3K/AKT pathways. Expressions levels of related markers at both mRNA and protein levels were validated (Fig. 3): shutting-off BMI1 down-regulated transcription of COL1A1, COL4A1, TNC, ITGA7, ITGB4 and Bcl-2, (Fig. 3): shutting-off BMI1 down-regulated transcription of COL1A1, COL4A1, TNC, ITGA7, ITGB4 and Bcl-2, while it up-regulated transcription of AKT3, LAMA3, CREB5 and BIRC3. Western blotting experiments on paraffin sections were random DNA sequences (~30 bp), which were demonstrated later to be complementary to phage sequences [17, 18]. Recently, this technique has been generating excitement for its ability to modify genetic information rapidly and thoroughly. Compared to traditional techniques (e.g. RNA interfering, transcription activator-like effector nuclease), CRISPR/Cas9 system possesses several advantages: 1) the gRNAs are easy to design and stable in double-chain form; 2) target sequences are directly cut on DNA level, inducing an entire shut-off of gene expression; 3) multiple genes could be edited simultaneously, and DNA sequences could be knock-in as well as knock-out [13, 14]. We have successfully applied the CRISPR/Cas9 system and established a stable EOC cell model with BMI1 silenced entirely for further investigations.

BMI1 protein is one component of the polycomb repressive complex 1 (PRC1), which catalyzes lysine 119 mono-ubiquitination of histone H2A (H2AK119Ub1). H2AK119Ub1 is thought to be complementary to phage sequences [17, 18]. Recently, this technique has been generating excitement for its ability to modify genetic information rapidly and thoroughly. Compared to traditional techniques (e.g. RNA interfering, transcription activator-like effector nuclease), CRISPR/Cas9 system possesses several advantages: 1) the gRNAs are easy to design and stable in double-chain form; 2) target sequences are directly cut on DNA level, inducing an entire shut-off of gene expression; 3) multiple genes could be edited simultaneously, and DNA sequences could be knock-in as well as knock-out [13, 14]. We have successfully applied the CRISPR/Cas9 system and established a stable EOC cell model with BMI1 silenced entirely for further investigations.

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platinum sensitivity of EOC cells. Similarly, Wang et al. revealed that down-regulating BMI1 increased the amount of reactive oxygen species, stimulated the DNA damage repair pathway, and eventually promoted the cisplatin-induced apoptosis [28]. Altogether, these evidences might shed light upon the most vexing conundrum in EOC management: chemo-resistance.

The CRISPR/Cas9 technique has provided an excellent cell model for analysis of one single variable: BMI1. We further investigated the most relevant genes using RNA sequencing. Most genes differentially expressed after silencing BMI1 were engaged in tissue development, ECM organization and blood vessel development, which might alter the microenvironment and angiogenesis of tumors, and then lead to transformation of malignant phenotypes: decrease in COL1A1 and COL4A1 might redress the reconstruction disorder of ECM [29, 30]; regulation of TNC and LAMA could increase tissue tension, reduce invasion and prevent migration of ovarian cancer cells [31, 32]. However, the clinical significance of these biomarkers in EOC management still needs validation because of controversial results from different studies. Furthermore, KEGG analyses revealed that the focal adhesion and PI3K/AKT pathways were involved after silencing BMI1. Aberrant expression of focal adhesion kinase (FAK) was related to the uncontrolled proliferation, suppressed apoptosis,
invasion, angiogenesis and immune-depression of tumor cells [33]. One meta-analysis demonstrated that up-regulation of FAK indicated shorter overall survival in a variety of human cancers with a pooled hazards ratio of 1.815 and site specificity [34]. A growing number of studies have proved the activation of the PI3K/akt/mTOR pathway in EOC [35–38], although the Cancer Genome Atlas research detected a low mutational rate (<5%) of PI3KCA, AKT and PTEN in high-grade serous ovarian carcinomas (HsOCs) [39]. Other research found that PI3K genes were frequently mutated in non-HsOCs, which were relatively insensitive to platinum [38]. Another promising thing is that diverse anti-neoplastic drugs targeting the PI3K/akt/mTOR pathway are under clinical investigations [39, 40].

Conclusions
It is feasible to use the CRISPR/Cas9 system to construct ideal EOC cell models with target gene expression thoroughly shut off. Silencing BMI1 can change various biological behaviors of EOC cells, including reducing cell proliferation, migration, invasion, and promoting cell death; BMI1 might be a promising predictor for platinum sensitivity. BMI1 may accelerate tumor genesis and metastasis through dysregulating tumor ECM and blood vessel assemblies. Molecular traits, including BMI1 and crucial genes in the focal adhesion and PI3K/AKT pathways, might be potential biomarkers and novel therapeutic targets for EOC.

Additional file

Additional file 1: Figure S1. PARP expression detected by western blotting. (PNG 29 kb)

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Availability of data and materials
Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Authors’ contributions
Conceptualization: ZQ, GT, SK; Formal analysis: ZQ, QQ, GT; Funding acquisition: GT, SK; Investigation: ZQ, QQ, GT; Methodology: ZQ, QQ, GT; Project administration: GT, SK; Resources: ZQ, QQ, CD, YJ, GT, SK; Software: ZQ, QQ; Supervision: CD, YJ, SK; Validation: ZQ, CD, YJ, GT; Writing - original draft: ZQ, GT; Writing - review & editing: GT, CD, YJ, SK. All authors read and approved the final manuscript.

Ethics approval and consent to participate
All mice were housed and fed under specific pathogen-free conditions in an institution approved by the Beijing Laboratory Animal Research Center. All studies were approved and supervised by Peking Union Medical College Hospital.

Competing interests
The authors declare that they have no competing interests.

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