Astragaloside IV promotes the proliferation and migration of osteoblast-like cells through the hedgehog signaling pathway

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Abstract. The present study aimed to investigate the effects of astragaloside IV on osteoblast-like cell proliferation and migration, in addition to the underlying signaling pathway. In order to observe the effect on proliferation, a Cell Counting Kit-8 assay and flow cytometry were used. To detect cell migration ability, cell scratch and Transwell cell migration assays were performed. The RNA and protein expression levels of hedgehog signaling molecules, including Sonic hedgehog (SHH) and GLI family zinc finger 1 (GLI1), were examined by reverse transcription-quantitative polymerase chain reaction and western blot analyses. To inhibit the hedgehog signaling pathway, cyclopamine was used. Astragaloside IV, at a dosage of 1x10^{-5} \mu g/ml in MG-63 cells and 1x10^{-4} \mu g/ml in U-2OS cells, resulted in the enhanced proliferation and migration of cells, and the gene expression levels of the SHH and GLI1 were significantly increased. The combination of astragaloside IV and cyclopamine reduced MG-63 and U-2OS cell proliferation and migration, and inhibited the gene expression of SHH and GLI1. Astragaloside IV enhanced the proliferation and migration of human osteoblast-like cells through activating the hedgehog signaling pathway. The results of the present study provide a rational for the mechanism in astragaloside IV promoting the proliferation and migration of osteoblasts via the hedgehog signaling pathway.

Introduction

Dental implants have become the optimal treatment for dentition defects or missing teeth. Osseointegration is a key factor associated with implants and involves three stages: Peri-implant bone formation, reconstruction, and maturation. There are a series of events involved in the formation of bone, including proliferation, migration, and the recruitment of osteoblasts surrounding the implant surface. Previous studies have demonstrated that the migration, recruitment and adsorption efficiency of osteoblasts on the implant surface directly affects the occurrence, development and final effect of osseointegration (1). The process of osseointegration is affected by numerous factors in patients with systemic diseases, including diabetes, osteoporosis and cancer, and the success rates of implants in these patients are markedly reduced. A systematic review reported that, in the first year of loading, there is an increasing trend in the failure of dental implant teeth in patients with diabetes (2). Therefore, how to improve the osseointegration of implants in patients with certain diseases remains a focus of clinical investigations.

Various traditional Chinese medicine materials have been shown to promote osteoblast proliferation and migration and bone formation using in vivo and in vitro experiments (3). Astragal Radix is the root of Astragalus membranaceus (Fisch.) Bge.var. Mongholicus (Bge.) Hsiao or EE Astragalus membranaceus (Fisch.) Bge. Astragal Radix has been shown to exhibit a range of pharmacological effects, including anticancer, anti-inflammatory, antioxidant and anti-osteoporotic properties (4-10). Astragaloside IV (AST-IV, 3-O-b-D-xylopyranosyl-6-O-b-D-glucopyranosyl-cloastragenol) is the principal effective compound isolated from Astragal Radix. A previous study reported that pretreatment with astragaloside IV significantly reverses the loss of neuronal cell viability and prevents MPP+-guided SH-SY5Y cell death, and that astragaloside IV has a neuroprotective effect (11). Astragaloside IV can enhance the proliferation of bone marrow mesenchymal stem cells in vitro, so as to the neural stem cells (12,13) and promote angiogenesis and the reproduction of human umbilical vein endothelial cells (14). The combined application of
astragaloside and tanshinone IIA promotes the migration of mesenchymal stem cells in cardiovascular disease therapy (15). Furthermore, a previous study revealed the effects of astragaloside IV on osteoclasts, demonstrating that astragaloside IV caused the inhibition of osteoclastogenesis and attenuation of osteolysis (16). However, the regulatory action of astragaloside IV on osteoblasts and the associated mechanisms remain, at present, to be fully elucidated.

A previous study demonstrated that the hedgehog signaling pathway regulates the differentiation of skeletal muscle cells in the process of skeletal repair and regeneration (17). In addition, the hedgehog signaling pathway is associated with bone formation, which can promote the proliferation and differentiation of chondrocytes and osteoblasts (18-21).

The hedgehog signaling pathway is conserved in evolution, and is key in embryonic development and homeostasis regulation, growth and cell migration in adult tissues (22). The hedgehog family contains three protein ligands, including sonic hedgehog (Shh), India hedgehog (Ihh) and desert hedgehog (Dhh). These signal via a mechanism involving two transmembrane proteins, namely smoothened (Smo) and patched (Ptc). When a hedgehog protein binds to Ptc, Smo is rendered constitutively active and activates an intracellular signaling cascade, leading to the upregulated transcription of the downstream nuclear transcription factor GLI family zinc finger (Gli)1 and Gli2 (23). Avergent activation of the hedgehog signaling may lead to oncogenesis in various tissues, including basal cell carcinoma, medulloblastoma, pancreatic, colon and gastric cancer, and glioblastoma (24-28). However, the role of the hedgehog signaling pathway in the proliferation and migration of astragaloside IV in osteoblasts remains to be fully elucidated.

In the present study, human MG-63 and U-2OS osteoblast-like cells were treated with astragaloside IV, following which cell functions and the activation of hedgehog signaling were evaluated. Furthermore, the Smo inhibitor, cyclopamine, was used to inhibit the hedgehog signaling pathway, following which the gene expression levels of SHH and GLI1 and cell functions were evaluated.

Materials and methods

Reagents. Fetal bovine serum (FBS), modified Eagle's medium (MEM) and McCoy's 5A medium were obtained from HyClone Laboratories, GE Healthcare Life Sciences (Logan, UT, USA). Penicillin/streptomycin solution, phosphate-buffered saline (PBS), 0.05% Trypsin-EDTA and dimethyl sulfoxide (DMSO) were obtained from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Astragaloside IV and cyclopamine (purity >99%, HPLC) were obtained from Sigma-Aldrich, Merck Millipore (Darmstadt, Germany). The chemical structure and molecular weight of astragaloside IV is shown in Fig. 1. The astragaloside was dissolved in DMSO, and the concentration of the original solution was 25 µg/ml. The stock solution was diluted according to the treatment group when used; the DMSO did not exceed 0.5% (V/V).

Cell culture. The MG-63 and U-2OS human osteosarcoma cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). The MG-63 and U-2OS cells were cultured in MEM and McCoy's 5A medium supplemented with 10% FBS, respectively. The two cell lines were incubated at 37°C in a 5% CO2 humidified atmosphere.

Cell Counting Kit-8 (CCK-8) assay. Cell growth was evaluated using a CCK-8 assay (Dojindo Molecular Technologies, Inc., Shanghai, China). The cells were plated in 96-well plates, 100 µl of medium was added to each well, which contained 10,000 cells. The cells were cultured overnight and were then treated with gradient dilutions of astragaloside IV (1x10^{-4}, 1x10^{-3}, 1x10^{-2}, 1x10^{-1}, 1, and 10 µg/ml concentrations). After 48 h, 100 µl CCK8 reagents were mixed into each well. Following an additional incubation at 37°C for 4 h, the absorbance value of each well at 450 nm was measured using a VERS Amx microplate reader. The number of living cells measured by the absorbance at the wavelength 450 nm was measured with a monochromator microplate. Cell viability=[(As-Ab)/((Ac-Ab)] x100%; where As is the absorbance of the astragaloside IV group, Ac is the absorbance of the control group, and Ab is the absorbance of blank group.

Flow cytometry. The cells were seeded in 6-well plates with 2 ml of complete growth medium/well, containing the appropriate number of cells to yield 60% confluence 12 h following plating. After 12 h, the complete growth medium was replaced with serum-free medium for 24 h. After 24 h, the cells were cultured in complete growth medium and treated with astragaloside IV or astragaloside IV combined with cyclopamine for 48 h. BrdU (1 mM/ml) was then added for 4 h at 37°C, and the cells were harvested. The cells were stained with anti-BrdU and 7-AAD using the BD Pharmingen™ BrdU Flow kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's protocol. The cell cycle distribution was determined by flow cytometry (FACSArina™ II, BD Biosciences).

Wound-healing assay. Cell migration was measured using a wound-healing assay. The cells were seeded in 6-well plates and, when the cells had formed a confluent monolayer, a p200 micro-pipette tip was used to create a scratch in a straight line. Culture medium was used to clean the cells once and they were incubated in 0.1% FBS culture medium. Images of the cells at multiple points along the scratch were captured every 24 h using an inverted microscope. Images were captured at x100 magnification.

Transwell cell migration assay. The cells were harvested following treatment with astragaloside IV for 48 h. The cells were then suspended in serum-free medium and placed in the upper chamber of the Transwell array (8-µm pore size; Merck Millipore). Medium containing 20% FBS was added to the lower chamber, following incubation at 37°C for 48 h, a cotton swab was used to carefully wipe away the cells in the upper chamber. The migrated cells that had adhered to the membrane of the lower chamber, were fixed in 4% formaldehyde solution for 10 min, and stained with 0.1% crystal violet for 15 min. The numbers of migrating cells were then counted with an inverted microscope. The values were averaged and images were captured.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The extraction of total cell RNA was performed using TRIzol reagent
The RNA integrity was analyzed on a 1.0% agarose gel and detection of RNA quantity was performed using a NanoDrop 2000C Spectrophotometer (Thermo Fisher Scientific, Inc.). Subsequently, 1 µg of total RNA was reverse transcribed with a PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) for cDNA synthesis and genomic DNA removal, according to the manufacturer’s protocol. The qPCR procedure was performed on the basis of the instructions of the SYBR premix Ex Taq™ II kit (Tli RNaseH Plus; Takara Biotechnology Co., Ltd.) and performed in triplicate using a Takara real-time PCR system (Takara Biotechnology Co., Ltd.). The thermocycling conditions were as follows: 30 sec at 95˚C, followed by 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec. Gene-specific primers were designed using the online primer design tool Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The lengths of amplifications were between 100 and 250 bp. GAPDH served as an internal control. The specificity of amplification was evaluated by the analysis of the dissociation curve, and the relative abundance of genes was determined using the 2^(-ΔΔCq) method (29). The primer sequences were as follows: SHH forward, 5'-GTG GCC G A G A A G A C C C T A -3' and reverse, 5'-CA A G C G T T C A A C T T T G T C C T T A -3'; GLI1 forward, 5'-CGG G C A C C A T C C A T T T C T A C -3' and reverse, 5'-G G C A C A G T C A G T C T G T C T T C C T T -3'; and GAPDH forward, 5'-GG ACC GTCA AGG C T G -3' and reverse, 5'-TT T G T GAAGAGC CCCAGTCGGA-3'.

Western blot analysis. Total protein was isolated with RIPA lysis buffer (Pulilai Gene Technology Co., Ltd., Beijing, China). The cells were lysed in RIPA buffer on ice for 30 min, and supernatant was then collected through centrifugation at 12,000 x g for 5 min at 4˚C. Protein concentrations were determined with a BCA protein assay kit (Pulilai Gene Technology Co., Ltd.). Total proteins (40 µg protein/lane) were separated by 12% SDS-PAGE and transferred onto a PVDF membrane. The membrane was blocked with 5% (W/V) non-fat milk at room temperature for 1 h, incubated with primary antibodies against Shh (1:1,000; cat. no. 2207), Gli1 (1:1,000; cat. no. 3538) and β-actin (1:1,000; cat. no. 4970) at 4˚C overnight, and then incubated with anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no. 7074) (all from Cell

Figure 1. AST-IV enhances human osteoblast-like cell growth and proliferation. (A) Structure of AST-IV (structural formula, C41H68O14; molecular weight, 784.97). (B) MG-63 and (C) U-2OS cells were treated with DMSO as controls or various concentrations of AST-IV for 48 h. Cell viability was determined with a Cell Counting Kit-8 assay. Following treatment of (D) MG-63 cells with AST-IV (1x10^{-2} µg/ml) and of (E) U-2OS cells with AST-IV (1x10^{-3} µg/ml) or DMSO as a control for 24, 48, 72 and 96 h, cell viability was determined. *P<0.05, AST-IV group compared with control group and #P<0.05, compared with the 24 h group, determined by one-way analysis of variance. AST-IV, astragaloside IV; DMSO, dimethyl sulfoxide.
Signaling Technology, Inc., Danvers, MA, USA) at room temperature for 1 h. The bands were visualized with an ECL reagent (Thermo Fisher Scientific, Inc.). β-actin was used as the loading control.

Statistical analysis. All results are expressed as the mean ± standard deviation of three independent experiments performed in triplicate. Statistical analyses were performed using the SPSS 17.0 software package (SPSS, Inc., Chicago, IL, USA) and one-way analysis of variance with Dunnett’s test was used to compare the means between two groups. \( P<0.05 \) was considered to indicate a statistically significant difference.

Results

Astragaloside IV promotes the proliferation of human osteoblast-like cells. To investigate the effects of astragaloside IV on osteogenesis in human osteoblast-like cells, cell growth and proliferation were analyzed. The structure of astragaloside IV was drawn using Chemdraw 14.0 (Fig. 1A). A CCK-8 assay was performed to detect cell growth. The MG-63 and U-2OS cells were treated with increasing concentrations of astragaloside IV (1x10^-3, 1x10^-4, 1x10^-3, 1x10^-2, 1x10^-1, 1, and 10 µg/ml) for 48 h. The results showed that astragaloside IV at the indicated concentrations (MG-63, 1x10^-2 µg/ml; U-2OS, 1x10^-3 µg/ml) significantly increased the growth of the human osteoblast-like cells (\( P<0.05 \), Fig. 1B and C). As a further demonstration of the association between time and the concentration of astragaloside IV following 24, 48, 72 and 96 h of treatment, the MG-63 and U-2OS cells treated with astragaloside IV for 48 h (MG-63, 1x10^-2 µg/ml; U-2OS, 1x10^-3 µg/ml) had significantly decreased cell proliferation rate was observed (\( P<0.05 \), Fig. 1D and E). Taken together, the results indicated that astragaloside IV increased the growth and proliferation of the human osteoblast-like cells.

Astragaloside IV stimulates the migration of human osteoblast-like cells. The migratory capacity of osteoblasts is key in osteogenesis. To evaluate the functions of astragaloside IV in MG-63 and U-2OS cell migration efficiency, a wound-healing assay and Transwell cell migration assay were performed. Astragaloside IV was observed to markedly induce the wound-healing of MG-63 and U-2OS cells following pre-treatment for 48 h (\( P<0.05 \), Fig. 2A and B). Furthermore, the results of the Transwell cell migration analysis demonstrated that the cell migration efficiencies of the MG-63 and U-2OS cells were enhanced following astragaloside IV treatment for 24 or 48 h, and the differences were significant when compared with the control group (\( P<0.05 \), Fig. 2C and D). These results indicated that astragaloside IV is critical for stimulating the migration of human osteoblast-like cells.

Astragaloside IV enhances the hedgehog signaling pathway. Several studies have suggested that the Shh-induced hedgehog pathway is crucial for regulating the proliferation and migration of tumor cells and promoting the proliferation, differentiation and maturation of osteoblasts. The present study investigated whether astragaloside IV affects the hedgehog signaling pathway. To investigate the role of the hedgehog signaling pathway in astragaloside IV-enhanced cell proliferation and migration, the human osteoblast-like cells were treated with cyclopamine, which is an inhibitor of Smo, to inhibit the hedgehog pathway (30). The MG-63 and U-2OS cells were treated with increasing concentrations of cyclopamine (2.5, 5.0, 7.5, and 10 µmol/l) for 48 h. The cell viability analysis using the CCK-8 assay demonstrated that cyclopamine significantly suppressed the growth of the MG-63 and U-2OS cells in a dose-dependent manner (Fig. 3A and B).

The present study aimed to detect the mechanism of action of astragaloside IV combined with cyclopamine on components of the hedgehog signaling pathway. The results of the RT-qPCR and western blot analyses results demonstrated that the mRNA and protein levels of SHH and GLI1 were significantly upregulated in MG-63 and U-2OS cells treated with astragaloside IV for 48 h (Fig. 3D and E). The mRNA and protein expression levels of GLI1 and SHH were marginally elevated in cells treated with astragaloside IV combined with cyclopamine, however the increase was markedly reduced, compared with that in MG-63 and U-2OS cells treated with astragaloside IV (Fig. 3F and G).

The results of the analysis of gene expression levels of SHH and GLI1 in MG-63 and U-2OS cells following treatment with astragaloside IV suggested that astragaloside IV promoted activation of the hedgehog signaling pathway.

Hedgehog signaling pathway inhibitor eliminates astragaloside IV-induced proliferation and migration of the osteoblast-like cells. The present study then analyzed the effect of astragaloside IV combined with cyclopamine on cell proliferation in human osteoblast-like cells. In the MG-63 cells, the two drugs acted together to inhibit cell proliferation, and the percentage of cells in the S phase was reduced. In the U-2OS cells, there was no effect on cell proliferation compared with the control (Fig. 4A and B). These results indicated that the effect of astragaloside IV on osteoblast-like cell proliferation was reduced by cyclopamine.

Furthermore, the Transwell cell migration assay was used to assess the impact of cyclopamine on cell migration efficiency. The results demonstrated that the cell migration ability of MG-63 and U-2OS cells was promoted following treatment with astragaloside IV combined with cyclopamine for 48 h (\( P<0.05 \), Fig. 4C and D); however, the cell migration efficiency was lower than that resulting from the treatment of osteoblast-like cells with astragaloside IV alone. Taken together, these findings suggested that astragaloside IV-promoted cell proliferation and migration, and that these effects were significantly inhibited by hedgehog signaling pathway inhibition.

Discussion

In the process of bone remodeling, bone is continuously broken down and reformed, which occurs through the balance between osteoblasts and osteoclasts (31). Osseointegration comprises a cascade of complex mechanisms. First, the drilling
of an implant cavity is a traumatic insult to bone and leads to distinct phases of wound healing (32,33). Secondly, new bone is generated from the borders of the drill hole (distance osteogenesis) or from osteogenic cells on the surface of the
implant (contact osteogenesis). In distance osteogenesis, osteoblasts migrate to the surface of the implant cavity, whereas in contact osteogenesis, osteogenic cells migrate directly onto the implant surface and generate new bone (34). Therefore, the proliferative ability and migratory behavior of osteoblasts are key in osseointegration (35).

The optimization of the implant surface assists in promoting osseointegration by promoting the migration, adhesion, proliferation and differentiation of osteoblasts. Sandblasting, etching, and hydrophilicity have been successfully used on the implant surface. To further improve the speed and quality of osseointegration, novel methods of surface modification, including discrete crystal deposition, laser ablation, surface coating and surface treatment with proteins, drugs or growth factors, are being investigated (36). The present study demonstrated that astragaloside IV can promote the proliferation and migration of human osteoblast-like cells.

Figure 3. AST-IV regulates the mRNA and protein levels of hedgehog signaling pathway components in MG-63 and U-2OS cells. (A) MG-63 and (B) U-2OS cells were treated with CP (2.5, 5.0, 7.5, and 10.0 µg/ml) for 48 h, and cell growth was assessed using a Cell Counting Kit-8 array. Data are presented as the mean ± standard deviation of three independent experiments. (C) Components detected in the hedgehog signaling pathway. Expression levels of GLI1 and SHH genes were detected in MG-63 and U-2OS cells following treatment with dimethyl sulfoxide as a control or AST-IV (MG-63, 1x10⁻² µg/ml; U-2OS, 1x10⁻¹ µg/ml) or AST-IV (MG-63, 1x10⁻¹ µg/ml; U-2OS, 1x10⁻² µg/ml) combined with CP (2.5 µmol/l) for 48 h. mRNA levels in the (D) MG-63 and (E) U-2OS cells were analyzed by reverse transcription-quantitative polymerase chain reaction analysis and normalized to GAPDH. Protein expression levels in (F) MG-63 and (G) U-2OS were analyzed by western blot analysis. Actin was used as an internal control. *P<0.05 and **P<0.01. Experiments were performed in triplicate, determined by one-way analysis of variance. AST-IV, astragaloside IV; CP, cyclopamine; GLI1, GLI family zinc finger 1; SHH, sonic hedgehog.
The effects of various doses of astragaloside IV (1x10^{-7}-10 µg/ml) for 48 h on human osteosarcoma cells in vitro were examined in the present study. The results indicated that astragaloside IV promoted MG-63 cell and U-2OS cell proliferation and migration, respectively, at relatively low concentrations (MG-63 cells, 1x10^{-7} µg/ml; U-2OS cells, 1x10^{-5} µg/ml). Several previous studies have reported that a high concentration of astragaloside IV (10-100 µg/ml) can inhibit tumor cell growth, migration and invasion in lung cancer (37), breast cancer (8), hepatoma (38), and glioma (39). In addition, the content of astragaloside IV in Astragali Radix is ~0.04%, and the clinical application dosage of Astragali Radix is ~20 g per day. Therefore, there is ~8 µg of astragaloside IV in 20 g of Astragali Radix. The results of the in vitro experiments in the present study indicated that astragaloside IV promoted MG-63 cell and U-2OS cell proliferation and migration, respectively, at relatively low concentrations (MG-63 cells, 1x10^{-7} µg/ml; U-2OS cells, 1x10^{-5} µg/ml). This result indicated that the
dosage of astragaloside IV required in promoting the proliferation and migration of osteoblast-like cells is lower than the clinical application dosage of Astragali Radix.

Previous studies have reported that numerous cytokines are involved in the proliferation and migration of bone cells, including bone morphogenetic protein, insulin like growth factor, and wingless and int ligands. Furthermore, they have demonstrated that high mobility group box 1 protein (HMGBl) significantly promotes the migration of osteoblasts in vitro and the Toll-like receptor (TLR)2/TLR4-dependent nuclear factor-kB pathway is involved in HMGBl-induced osteoblast migration (40-43). In the present study the hedgehog signaling pathway was found to be involved in the process of astragaloside IV-enhanced cell proliferation and migration in MG-63 and U-2OS cells.

Shh is a 45-kDa signal protein that regulates the proliferation, differentiation and morphology of numerous cell types. Several studies have reported that the hedgehog signaling pathway is important in the proliferation and differentiation of osteoblasts, and is involved in fracture healing and bone repair (44,45). Gli1 and Gli2 proteins are the main transcription factors in hedgehog signaling. Shh can activate Gli1 and Gli2, and high protein expression levels of Gli1 and Gli2 indicate that the hedgehog signaling pathway is activated. The activation of Gli1 and Gli2 can directly promote the expression of a set of genes, including oncogenes and genes involved in cell cycle, for example, Cyclin D, Cyclin E and Myc.

In the present study, the expression of key proteins in the hedgehog signaling pathway in human osteoblast-like cells were detected following treatment with astragaloside IV. The results demonstrated that astragaloside IV caused a marked increase in the mRNA and protein levels of GLI1 and SHH, culminating in the observation that astragaloside IV activated hedgehog signaling.

To further investigate whether astragaloside IV potentiated the osteogenesis of human osteoblast cells via the hedgehog signaling pathway, the cells were treated with cyclopamine. Cyclopamine is an inhibitor of the hedgehog signaling pathway, is naturally produced and belongs to the group of steroidal jerveratrum alkaloids. The results indicated that the effect of astragaloside IV on cell proliferation and migration was markedly reduced by cyclopamine. Following treatment with astragaloside IV combined with cyclopamine in MG-63 and U-2OS cells, the increase in the expression of genes involved in hedgehog signaling was not statistically significant.

In conclusion, the findings of the present study suggested that activation of the hedgehog signaling pathway by astragaloside IV significantly enhanced human osteoblast-like cell proliferation and migration, and that astragaloside IV may serve as a growth factor to promote osseointegration. To the best of our knowledge, the present study is the first to demonstrate the effect of astragaloside IV on osteoblasts and that the hedgehog signaling pathway was the direct target of astragaloside IV. These results identify a therapeutic target for the promotion of bone formation in implants and a rationale for the development of astragaloside IV for use in clinical therapy.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

JL and YH designed the study. JL and YC produced the outline and reviewed the manuscript. YC and LHG analyzed all the results. LHG performed the majority of the experiments and drafted the manuscript. RTZ performed the statistical analysis and figure editing. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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