Treatment of Hypertensive Heart Disease by Targeting Smad3 Signaling in Mice

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INTRODUCTION

Hypertension remains a major cause of chronic heart disease.1 Hyper- tension cardiac remodeling, characterized by progressive cardiac fibrosis and inflammation associated with high blood pressure, is a major complication of hypertension.2,3 Angiotensin II (Ang II) has been regarded as a key mediator in hypertensive cardiac remodel- ing.4,5 Many studies have reported that Ang II mediates fibrosis directly and indirectly via transforming growth factor β (TGF-β)/Smad3 signaling because Ang II can activate Smad3 directly via the AT1-p38/Extracellular signal-regulated Kinase (ERK) mitogen-activated protein kinase (MAPK)-Smad crosstalk pathway and indirectly by inducing TGF-β.6–9 Thus, activation of TGF-β/Smad signaling may be a central mechanism in the pathogenesis of hypertensive cardiac disease. In the context of fibrosis, Smad3 is pathogenic because Smad3 can bind directly to many collagen matrix promoters, including COL1A2, COL2A1, COL3A1, COL5A1, COL6A1, and COL6A3 genes, to mediate fibrogenesis.10 The functional importance for Smad3 in hypertensive complications is demonstrated by the findings that mice lacking Smad3 are protected against renal and myocardial fibrosis in response to Ang II.10,11,15 In contrast, Smad7, an inhibitor of TGF-β/Smad signaling, is protective and functions to inhibit Smad3-mediated fibrosis via its negative feedback mechanism. This is also supported by the findings that deletion of Smad7 enhances, but overexpression of Smad7 inhibits Ang II-induced hypertensive renal and cardiac complications.9,16–20 It is now well recognized that during fibrogenesis, Smad3 signaling is overactivated, whereas Smad7 is degraded or lost, suggesting that the imbalance between Smad3 and Smad7 signaling may be a key mechanism in progressive fibrosis. This is also supported by the findings that deletion of Smad3 protects against, but deletion of Smad7 promotes fibrosis as seen in hypertensive kidney and cardiovascular diseases.13–16,18,20 Thus, overexpression of Smad7 has been shown as a novel therapeutic strategy to inhibit Smad3-driven cardiac and renal fibrosis.9,17,19,21,22 However, it remains unknown whether inhibition of Smad3 directly with a Smad3 inhibitor has therapeutic potential for hypertensive cardiopathy. This was examined by treating an established hypertensive...
mouse model with a specific Smad3 inhibitor SIS3. The therapeutic effect and mechanisms of SIS3 on hypertensive cardiomyopathy were also investigated.

RESULTS

Treatment with SIS3 Protects against Ang II-Induced Cardiac Dysfunction Independently of Blood Pressure

As shown in Figure 1A, compared with normal saline control, chronic Ang II infusion significantly increased blood pressure in all mice over days 3–28, and treatment with SIS3 from day 14 onward did not alter the levels of systolic blood pressure. Echocardiography also detected that the cardiac functions, including left ventricle ejection fraction (LVEF), LV fractional shortening (LVFS), and LV mass, were significantly impaired in all mice at day 14 after Ang II infusion and became more severe injuries at day 28 after Ang II infusion (Figures 1B–1E). In contrast, hypertensive mice treated with SIS3 from day 14 onward showed protection against the decline in the LVEF and LVFS and an increase in LV mass when compared with the DMSO-control treated mice (Figures 1B–1E). These results suggested that the cardioprotective effect of SIS3 on established hypertensive cardiomyopathy is blood pressure independent.

Treatment with SIS3 Protects against Myocardial Fibrosis in an Established Mouse Model of Ang II-Induced Hypertension

Because mice lacking Smad3 are protected against myocardial fibrosis in response to Ang II and ischemic injury,11–13 we next examined whether blocking Smad3 signaling with SIS3 has any impact on myocardial fibrosis. Immunohistochemistry revealed that compared with the saline-infused normal mice, mice with Ang II infusion for 14 days developed moderate myocardial fibrosis as demonstrated by extensive collagen I and III, fibronectin, and α-SMA+ myofibroblasts accumulation in the LV tissues, particularly in the focal area with severe myocardial damage and in the perivascular area (Figure 2; Figure S1). Further analysis at the protein levels by western blot and at the mRNA levels by real-time PCR confirmed these findings (Figure 3). All of these fibrotic parameters were further significantly increased with massive myocardial fibrosis at day 28 (Figures 2 and 3; Figure S1). In contrast, treatment with a Smad3 inhibitor SIS3...
over the period of days 14–28 halted the progression of myocardial fibrosis to the levels comparable with those at day 14 before SIS3 treatment (Figures 2 and 3; Figure S1).

**Treatment with SIS3 Inhibits Cardiac Inflammation in an Established Mouse Model of Ang II-Induced Hypertension**

We have previously shown that mice lacking Smad3 are protected against renal and cardiac inflammation in response to Ang II.\(^{13,14}\) We thus examined whether inhibition of Smad3 has any impact on cardiac inflammation. Immunohistochemistry revealed that no leukocytic infiltration was evident in saline infusion mice; however, Ang II infusion increased moderate cardiac inflammation, such as CD3+ T cell and F4/80+ macrophage infiltration, at day 14 (Figure 4A). Real-time PCR also showed a marked upregulation of tumor necrosis factor alpha (TNF-α), MCP-1, intercellular cell adhesion molecule-1 (ICAM1), and interleukin (IL)-1β at day 14 after Ang II infusion (Figure 4B). All of these changes became more severe at day 28 in DMSO-treated mice but were blocked by treatment with SIS3 (Figure 4), revealing the anti-inflammatory effect of SIS3 on Ang II-induced hypertensive heart disease.

**SIS3 Treatment Inhibits Ang II-Induced Myocardial Fibrosis by Blocking TGF-β1/Smad3 Signaling**

We then investigated the mechanisms through which inhibition of Smad3 protects against Ang II-induced myocardial fibrosis. Because Ang II-induced TGF-β1 expression leads to renal and myocardial fibrosis via a Smad3-dependent mechanism,\(^{6–9,13–15}\) we thus examined whether the anti-fibrotic effect of SIS3 is associated with inactivation of TGF-β/Smad3 signaling. As shown in Figure 5, immunohistochemistry, western blot, and real-time PCR analyses showed that chronic Ang II infusion activated TGF-β/Smad3 signaling, including upregulation of TGF-β1 and increased Smad3 phosphorylation, over the 14- to 28-day period. Treatment with SIS3, but not DMSO control, virtually blocked Ang II-induced activation of TGF-β/Smad3 signaling in the cardiac tissue, demonstrating the therapeutic effect of SIS3 on blocking TGF-β/Smad3 signaling under the progressive phase of hypertensive myocardopathy.

**Blockade of Smad3 Diminishes Ang II-Induced Cardiac Inflammation by Attenuating NF-κB Signaling via the Smurf2-Dependent Ubiquitin Degradation of Cardiac Smad7**

We have previously shown that deletion of Smad3 protects against Ang II-induced E3-ligase Smurf2, thereby preventing Smad7 from Smurf2-mediated proteasomal ubiquitous degradation in hypertensive nephropathy.\(^{14}\) We have also previously detected that Smad7 is capable of inducing expression of IkBα, an inhibitor of NF-κB signaling, to suppress NF-κB-driven renal inflammation *in vitro* and *in vivo*.\(^{23}\) We thus hypothesized that the anti-inflammatory effect of SIS3 on cardiac inflammation may be attributed to inactivated NF-κB signaling by upregulating cardiac Smad7. As shown in Figures 6 and S2, compared with saline-control mice, Ang II infusion caused degradation of cardiac Smad7 over days 14–28 (Figure 6A), which was tightly associated with upregulation of an E3-ligase Smurf2 in both mRNA and protein levels (Figure 6B). Importantly, inhibition of cardiac Smad7 resulted in a marked degradation of IkBα, therefore increasing NF-κB/p65 phosphorylation and its nuclear translocation (Figure 6C; Figure S2). Conversely, mice treated with SIS3 were protected against Ang II-upregulated Smurf2 and the degradation of cardiac Smad7 (Figures 6A and 6B), resulting in inactivating NF-κB signaling by increasing cardiac IkBα expression (Figure 6C; Figure S2). Thus, treatment with SIS3 inhibited NF-κB-driven cardiac inflammation by preventing cardiac Smad7 from Smurf2-mediated ubiquitous degradation.

**In Vitro Evidence for the Anti-cardiac Fibrotic and Anti-inflammatory Effects of SIS3 on Ang II-Induced Activation of Cardiac Fibroblasts**

To confirm the mechanism and direct therapeutic effect of SIS3 on Ang II-induced cardiac fibrosis and inflammation, we treated primary mouse cardiac fibroblasts with Ang II (1 μM) in the presence or absence of SIS3 (1 μM) or losartan (1 μM). Results shown in Figures 7A and 7B revealed that addition of Ang II could induce a marked phosphorylation of Smad3 as early as 30 min, which was blocked by either SIS3 or losartan. Importantly, like losartan, which blocks the Ang II-AT1 signaling, treatment with SIS3 inactivated Smad3 signaling and suppressed Ang II-induced upregulation of pro-inflammatory cytokines, such as IL-1β and TNF-α, and fibrosis, including collagen I and α-SMA mRNA expression (Figures 7C–7F).

We next examined whether SIS3 treatment has an inhibitory effect on cardiac fibroblast growth in response to Ang II. Results shown in Figure 7G clearly demonstrated that, like losartan treatment again, addition of SIS3 blocked Ang II-induced cardiac fibroblast proliferation as determined by the methyl-thiazoldiphenyl tetrazolium (MTT) assay.

**DISCUSSION**

It is well established that chronic Ang II infusion activates TGF-β/Smad3 signaling to mediate progressive myocardial fibrosis with impaired cardiac function.\(^{13,16,17}\) In the present study, we found that targeting TGF-β/Smad3 signaling directly with a Smad3 inhibitor SIS3 in established hypertensive heart disease protected against progressive cardiac injury by preventing the decline in LVEF and LVFS, an increase in LV mass, and the development of severe cardiac inflammation and fibrosis. These findings provided direct evidence for the treatment of hypertensive heart disease by targeting Smad3 signaling. In addition, results from this study also demonstrated...
that SIS3 may be a novel and effective therapeutic agent for chronic cardiovascular disease.

It is now well established that Smad3 is a common downstream signaling molecule and transcriptional factor leading to tissue fibrosis. Indeed, Smad3 can be activated not only by TGF-β but also by many pathogenic mediators, including Ang II, advanced glycation end products (AGEs), and C-reactive protein (CRP) via the p38/ERK MAPK-Smad crosstalk pathway. It is also known that Smad3 binds many collagen promoters to trigger fibrogenesis. Thus, mice lacking Smad3 are protected against fibrosis in many diseases, including hypertensive renal and cardiovascular diseases. These findings strongly suggest that targeting Smad3 may represent promising research into the new drug development for treating diseases with progressive fibrosis. SIS3 is a small molecule that blocks Smad3 phosphorylation and Smad3 binding to the target DNA. Therefore, treatment with SIS3 inactivated Smad3 signaling and thus blocked Smad3-mediated myocardial fibrosis in a fully established hypertensive cardiac disease as found in this study, in addition to diabetic and obstructive nephropathy and cancer as previously reported. Inhibition of the Smurf2-mediated Smad7 proteasomal ubiquitin degradation pathway may also be a mechanism whereby treatment with SIS3 blocked Smad3-mediated myocardial fibrosis. It is well recognized that Smad7 is an inhibitory Smad that inactivates Smad signaling by recruiting E3 ubiquitin ligases such as Smurf2 to target the TGF-β receptor complex for degradation through the proteasomal-ubiquitin degradation pathway. Smurf2 consists of multiple WW domains that can interact with Smad7 to induce its ubiquitin-dependent degradation. We have previously reported that Ang II induces Smurf2 to cause degradation of Smad7 via a Smad3-dependent mechanism because deletion of Smad3 inhibits Smurf2 while upregulating Smad7, thereby blocking progressive renal fibrosis in hypertensive nephropathy. Once Smad7 is degraded, Ang II-induced activation of Smad3 is further enhanced, thereby promoting severe myocardial fibrosis. Thus, overexpression of Smad7 inhibits, but disruption of Smad7 enhances Smad3-mediated fibrosis in response to Ang II. In the present study, treatment with SIS3 inhibited Ang II-induced activation of Smad3 and Smurf2-dependent Smad7 ubiquitin degradation, which in turn blocked Smad3-mediated myocardial fibrosis via a Smad7-dependent negative feedback mechanism. Inhibition of the Smurf2-dependent Smad7 ubiquitin degradation pathway may also contribute to the inhibitory effect of SIS3 on Ang II-induced, NF-κB-mediated cardiac inflammation. It is well recognized that Ang II activates the NF-κB signaling pathway to mediate inflammation. Recent studies also demonstrated that activation of NF-κB is negatively regulated by Smad7 because Smad7 is capable of...
Figure 4. Treatment with SIS3 in the Established Hypertensive Cardiac Disease from Day 14 to 28 after Ang II Infusion Inhibits Progressive Cardiac Inflammation

(A) Representative immunostaining pictures and quantitative analysis of CD3+ T cells and F4/80+ macrophages infiltrating the cardiac tissues, particularly in the perivascular area. (B) Real-time PCR analysis of cardiac TNF-α, MCP-1, ICAM-1, and IL-1β. Data are mean ± SEM from groups of six mice. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 versus saline control; #p < 0.015, ##p < 0.01, ####p < 0.0001 versus DMSO and Ang II treatment at day 28; &p < 0.05, &&p < 0.01, &&&p < 0.001 versus Ang II infusion prior to SIS3 treatment at day 14. Scale bar: 50 μm.
inducing IκBα, an inhibitor of NF-κB, to inhibit NF-κB-driven inflammatory responses in Ang II-induced hypertensive cardiovascular and kidney diseases.16–22 Thus, overexpression of Smad7 inhibits, but deletion of Smad7 enhances NF-κB-driven inflammation as seen in many diseases.16–22 In the present study, targeting Smad3 impaired Smurf2-mediated Smad7 degradation, thereby preventing Ang II-induced NF-κB signaling. This may be a key mechanism by which SIS3 treatment protected against Ang II-induced cardiac inflammation.

Inhibition of cardiac inflammation by targeting Smad3 also may be associated with suppression of cardiac MCP-1 expression. It is known that MCP-1 is a direct target gene of Smad3, and Smad3 is critical for

Figure 5. Treatment with SIS3 Inhibits Ang II-Induced Activation of TGF-β/Smad3 in the Established Mouse Model of Hypertension

(A and B) Representative immunostaining pictures for phosphorylated Smad3 (phospho-Smad3; dark brown nuclear staining) and TGF-β1 (dark brown staining). (C and D) Quantitative analysis of p-Smad3 and TGF-β1. (E) TGF-β1 expression detected by real-time PCR. (F and G) Western blot analysis of p-Smad3 protein. Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.0001 versus saline control; *p < 0.05, **p < 0.01, ***p < 0.0001 versus DMSO and Ang II treatment at day 28; &p < 0.05, &&p < 0.01 versus Ang II infusion prior to SIS3 treatment at day 14. Scale bar: 50 μm.
Figure 6. Treatment with SIS3 Blocks Ang II-Induced Smurf2-Mediated Degradation of Cardiac Smad7 and Inhibits Ang II-Activated NF-κB Signaling in the Established Mouse Model of Hypertension

(A) Western blot and real-time PCR analysis of cardiac Smad7. (B) Western blot and real-time PCR analysis of cardiac Smurf2. (C) Western blot and real-time PCR analysis of phosphorylated IkBα, IkBα, phosphorylated NF-κB/p65, and p65. Data represent mean ± SEM from groups of six mice. *p < 0.05, **p < 0.01, ***p < 0.001 versus saline control; #p < 0.015, ##p < 0.01 versus DMSO and Ang II treatment for 28 days; &p < 0.05, &&p < 0.01 versus Ang II infusion before SIS3 treatment at day 14.
TGF-β-induced MCP-1 expression during vascular inflammation. Therefore, mice lacking Smad3 are protected against hypertensive renal and cardiac inflammation by inhibiting MCP-1-dependent infiltration and activation in response to Ang II. Consistent with this known mechanism, inhibition of MCP-1-mediated macrophage infiltration and activation may be another mechanism through which treatment with SIS3 blocked cardiac inflammation.

In conclusion, the present study demonstrates that SIS3 is a specific Smad3 inhibitor that can effectively inhibit Ang II-induced, TGF-β1/Smad3-mediated myocardial fibrosis and NF-κB-driven cardiac inflammation. Thus, results from this study suggest that SIS3 may be a novel therapeutic agent for hypertensive myocardialopathy, and targeting Smad3 may represent a new and effective therapy for chronic heart disease clinically.

MATERIALS AND METHODS
A Mouse Model of Ang II-Induced Hypertension and SIS3 Treatment
Hypertension was induced in male C57BL/6J mice (aged 8 weeks) by subcutaneous infusion of Ang II at a dose of 1.46 mg/kg/day for 14 or 28 days via osmotic minipumps (Model 2004; ALZA, Palo Alto, CA, USA) as previously described. SIS3 (S0447; Sigma, St. Louis, MO, USA), a novel specific inhibitor that has been shown to inhibit Smad3 phosphorylation and DNA binding in response to TGF-β1, was diluted in DMSO and injected intraperitoneally (i.p.) daily from day 14 to 28 after Ang II infusion at an optimal dose of 2.5 mg/kg/day. The dose used in this study was based on previous studies in various mouse models, including obstructive and diabetic nephropathy and cancer. Control-treated mice received DMSO only. In addition, a group of normal mice that received saline infusion via osmotic minipumps were used as normal control. Groups of six to eight mice were used in this study, and all mice were euthanized by cardiac blood collection under anesthesia with ketamine (80 mg/kg) and xylazine (15 mg/kg) i.p. daily from day 14 to 28 after Ang II infusion. Systolic blood pressure was measured in conscious mice by the noninvasive tail-cuff method using the CODA blood pressure system (Kent Scientific, Torrington, CT, USA) following
the manufacturer’s instruction. LV tissues were collected for immunochemical, real-time PCR, and western blot analysis. The experimental procedures were approved by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong.

**Echocardiography**

Echocardiography was conducted in both chronic saline and Ang II infusion mice with or without SIS3 treatment at day 14 prior to SIS3 treatment and at day 28 after Ang II infusion with or without SIS3 treatment as previously described. The functions of the LV were assessed by two-dimensional and M-mode echocardiography using a Vevo770 high-resolution ultrasound imaging system (VisualSonics, Toronto, ON, Canada) with a RMV 707B scan head (30 MHz) (VisualSonics) after lightly anesthetizing with ketamine/xylazine (30 MHz) (50 mg/kg) and xylazine (50 mg/kg) i.p. In brief, mice were anesthetized with i.p. administration of ketamine/xylazine (30 MHz) (50 mg/kg) and xylazine (50 mg/kg) i.p. All animals were studied using a 0.25-mm² graticule.

**Immunohistochemistry**

Immunohistochemistry was performed in paraffin sections using a microwave-based antigen retrieval method. The antibodies used in this study were as follows: collagen I (1310-01; Southern Biotech, Birmingham, AL, USA), collagen III (1330-01; Southern Biotech), α-smooth muscle actin (ab230458; Abcam), fibronectin (sc-6953; Santa Cruz Biotechnology, Santa Cruz, CA, USA), TGF-β1 (sc-146; Santa Cruz Biotechnology) and phosphorylated Smad3 (phospho-Smad3; #9520; Cell Signaling Technology), phospho-NF-κB/phospho-p65 (ab86299; Abcam), phospho-IκBα (#2859; Cell Signaling Technology), IκBα (sc-371; Santa Cruz Biotechnology), phospho-Smad3 (sc-9520; Cell Signaling Technology), Smad3 (51-1500; Invitrogen, Waltham, MA, USA), Smad7 (sc-11392; Santa Cruz Biotechnology), and Smurf2 (sc-393848; Santa Cruz Biotechnology). After being washed, the membranes were incubated with LI-COR IRDye 800-conjugated secondary antibodies, anti-mouse (#24849; Rockland Immunochemicals, Limerick, PA, USA) and anti-rabbit (#36595; Rockland Immunochemicals), in the dark for 1 h at room temperature. Signals were scanned and visualized by Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). The ratio of the target protein was subjected to GAPDH and was quantified with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**Western Blot Analysis**

Protein from LV tissues was extracted with Radio-Immunoprecipitation Assay (RIPA) lysis buffer, and western blot analysis was performed as described previously. In brief, after blocking nonspecific binding with 5% BSA, membranes were incubated overnight at 4°C with primary antibodies against collagen I (1310-01; Southern Biotech), collagen III (1330-01; Southern Biotech), α-smooth muscle actin (ab230458; Abcam), fibronectin (sc-6953; Santa Cruz Biotechnology), GAPDH (Chemicon; Merck), phospho-NF-κB/phospho-p65 (ab86299; Abcam), phospho-IκBα (#2859; Cell Signaling Technology), IκBα (sc-371; Santa Cruz Biotechnology), phospho-Smad3 (sc-9520; Cell Signaling Technology), Smad3 (51-1500; Invitrogen, Waltham, MA, USA), Smad7 (sc-11392; Santa Cruz Biotechnology), and Smurf2 (sc-393848; Santa Cruz Biotechnology). After being washed, the membranes were incubated with LI-COR IRDye 800-conjugated secondary antibodies, anti-mouse (#24849; Rockland Immunochemicals, Limerick, PA, USA) and anti-rabbit (#36595; Rockland Immunochemicals), in the dark for 1 h at room temperature. Signals were scanned and visualized by Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). The ratio of the target protein was subjected to GAPDH and was quantified with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**Primary Culture of Cardiac Fibroblasts**

Cardiac fibroblasts were isolated from the LV of C57/BL6 mice and were characterized by positive for vimentin but negative for desmin (a smooth muscle marker) and factor VIII (an endothelial cell marker) as described previously. Cardiac fibroblasts at passage 3 were stimulated with Ang II (1 µM) for 0, 0.5, 1, 3, 6, and 12 h for measuring phospho-Smad3 by western blotting and for collagen I, α-SMA, TNF-α, IL-1β, and mRNA expression by real-time PCR.

**MTT Assay**

Cardiac fibroblasts were seeded on a 96-well plate at the density of 1 × 10⁵/well and treated with Ang II (1 µM) in the presence or absence of

**Real-Time PCR**

LV mRNA expression was quantitatively analyzed by real-time PCR with primers against mouse mRNA as previously described. In brief, total RNA was isolated from LV tissues using RNeasy Isolation Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer’s instructions. Real-time PCR was performed using Bio-Rad iQ SYBR Green supermix with the Opticon2 (Bio-Rad, Hercules, CA, USA). The primers used for real-time PCR included collagen I, collagen III, α-SMA, Fibronectin, TGF-β1, IL-1β, TNF-α, MCP-1, ICAM-1, Smad7, Smurf2, and GAPDH as described previously. Primers for Smurf2 were: forward 5′-GTGCTGTTGTGGATGAGAAT-3′ and reverse 5′-CCTGCTGCGTTGTCCTTTGT-3′. Reaction specificity was confirmed by melting curve analysis. The ratio for the mRNA was normalized with GAPDH and expressed as mean ± SEM.
SIS3 (1 μM) and losartan (1 μM) for 24 h. MTT (5 mg/mL; Invitrogen) was added to each well in a final concentration of 0.5 mg/mL and incubated for 4 h at 37°C. After supernatant was removed and 100 μL DMSO was added to each well, then absorbance at 570 nm was measured using a plate-reading spectrophotometer. All data were calculated as a ratio against control.

Statistical Analysis
Data obtained from this study were expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA, followed by Newman-Keuls posttest using GraphPad Prism 6.0 (Graph Pad Software, San Diego, CA, USA).

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.omtmd.2020.08.003.

AUTHOR CONTRIBUTIONS
J.M., Y.Q., and J.C. performed experiments, analyzed the data, and wrote the manuscript. X.-r.H. and L.W. performed animal model, data collection, and analysis. X.Y. and H.-y.L. designed and supervised the study and revised the manuscript.

CONFLICTS OF INTEREST
The authors declare no competing interests.

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