Tanshinone-IIA attenuates the deleterious effects of oxidative stress in osteoporosis through the NF-κB signaling pathway

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Received August 10, 2016; Accepted May 16, 2017

DOI: 10.3892/mmr.2018.8741

Abstract. Osteoclasts are responsible for bone resorption caused by bone microstructural damage and bonerelated disorders. Evidence shows that tanshinone IIA (Tan-IIA), a traditional Chinese medicine, is used clinically as a drug for the treatment of cardiovascular and cerebrovascular diseases. However, the efficacy and mechanism underlying the effect of Tan-IIA on the viability of osteoclasts remain to be fully elucidated. The present study investigated the therapeutic effects of Tan-IIA on osteoblast differentiation and oxidative stress in vitro and in vivo. Cell viability was analyzed and oxidative stress was examined in the osteoblasts. Wnt1−/− mice were used to investigate the therapeutic effects of Tan-IIA on spontaneous tibia fractures and severe osteopenia. The bone strength, collagen and mineral were examined in the tibia. Osteoblast activity was also analyzed in the experimental mice. The Tan-IIA-induced differentiation of osteoclasts and the mechanism of action were investigated in osteocytes. The data showed that Tan-IIA treatment improved cell viability. The data also demonstrated that Tan-IIA decreased the levels of H2O2, accumulation of reactive oxygen species and apoptosis of osteoblasts. Tan-IIA inhibited the deleterious outcomes triggered by oxidative stress. In addition, Tan-IIA inhibited the activation of nuclear factor (NF)-κB and its target genes, tumor necrosis factor (TNF)-α, inducible nitric oxide synthase and cyclooxygenase 2, and increased the levels of TNF receptor-associated factor 1 and inhibitor of apoptosis protein-1/2 in the osteocytes. Furthermore, it was shown that Tan-IIA reduced the propensity to fractures and severe osteopenia in mice with osteoporosis. Tan-IIA also exhibited improved bone strength, mineral and collagen in the bone matrix of the experimental mice. It was found that the Tan-IIA-mediated benefits on osteoblast activity and function were through the NF-κB signaling pathway. Taken together, the data obtained in the present study suggested that Tan-IIA had protective effects against oxidative stress in osteoblastic differentiation in mice with osteoporosis by regulating the NF-κB signaling pathway.

Introduction

Osteoporosis is a set of bone diseases caused by a variety of factors featuring normal calcification, normal proportions of calcium salt and substrate, decreased bone quantity per unit volume, and metabolic bone lesions (1). Reports have shown that osteoporosis affects a higher proportion of individuals in the elderly population (2). Statistics indicate that osteoporosis is becoming a serious health problem in the aging population due to weakened bone structure and fractures in elderly individuals (3,4). The results of pathological investigations indicate that osteoclast activity and subtypes are crucial for the function of physiology and pathological implications for future treatment of osteoporosis (5). Osteoporosis readily leads to hip fractures, which are associated with functional impairment, maintenance of bone damage, loss of independence and increased mortality rates (6,7). Furthermore, clinical practice guidelines recommend that all patients with osteoporosis receive chronic oral glucocorticoid treatment. However, the therapeutic efficacy is limited in patients with osteoporosis who receive osteoporosis pharmacotherapy in terms of bone mineral density testing and bone strength. Therefore, examining more efficient molecular agents for the clinical treatment of osteoporosis is urgently required.

Tanshinone IIA (Tan-IIA) is a traditional Chinese medicine extracted from Danshen, which has been used clinically for the treatment of various human diseases (8,9). A previous study indicated that Tan-IIA decreased the protein expression of epidermal growth factor receptor and insulin-like growth factor I receptor, inhibiting the phosphoinositide 3-kinase/Akt/mammalian target of rapamycin pathway in gastric carcinoma in vitro and in vivo (10). In addition, Tan-IIA was shown to exert inhibitory effects on the hypoxia-induced enhancement of store-operated calcium entry in pulmonary arterial smooth muscle cells through the protein kinase G-proliferator-activated receptor-γ signaling pathway (11). The pharmacological and therapeutic
properties of Tan-IIA in the cardiovascular system have attracted interest (12). A previous study showed that Tan-IIA treatment alleviated rat gingival connective tissue overgrowth induced by cyclosporine A (13). Emerging experimental investigations and clinical trials have demonstrated that Tan-IIA prevents cardiac injury, hypertrophy and atherogenesis through estrogen receptor-α-induced oxidative stress (14). Although the efficacy of Tan-IIA has been observed in different diseases (15,16), the efficacy and molecular mechanism underlying the effect of Tan-IIA have not been reported previously. In the present study, the effects and molecular mechanisms underlying the effects of Tan-IIA on osteocytes were evaluated in vitro and in vivo. The present study also examined the efficacy of Tan-IIA on anti-apoptotic effects in the treatment of osteoporosis. Current evidence suggests that Tan-IIA is an efficient anti-apoptotic agent in osteoporosis.

Oxidative and reductive stress are essential for the dynamic phases experienced by cells undergoing adaptation towards an endogenous or exogenous noxious stimulus (17). Mitochondrial malfunction is the common denominator arising from the aberrant functioning of the rheostat, which maintains homeostasis between oxidative and reductive stress in osteoblasts (18,19). Previous evidence suggests that the mechanism underlying osteoporosis is important for osteoclast activation in the treatment of osteoporosis. Maladaptation during oxidative stress may be pivotal in the pathophysiology of osteoporosis (20,21). A previous study provided evidence that targeting antioxidant drug therapy is an efficient clinical regimen for reducing the deterioration of osteoporosis in the aforementioned oxidative stress signaling pathway (22,23). Evidence has also revealed that endoplasmic reticulum stress is a key risk factor in the pathogenic progression of osteoporosis. Therefore, the present study investigated Tan-IIA-mediated oxidative stress in osteoblasts from mice with osteoporosis. The data showed that Tan-IIA may be an efficient anti-osteoporotic agent in vitro and in vivo. The present study also investigated whether Tan-IIA has an anti-apoptotic and protective role in the treatment of osteoporosis in a mouse model. The data suggested that Tan-IIA ameliorated osteoblast activity and functioned through the nuclear factor (NF)-κB signaling pathway. Taken together, the data suggested that Tan-IIA exerted protective effects against oxidative stress in osteoblastic differentiation of mice with osteoporosis via regulation of the NF-κB signaling pathway and endoplasmic reticulum stress-dependent pathways, which may contribute to the treatment of osteoporosis.

Materials and methods

Ethics statement. The present study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Taihe Hospital Affiliated to HuBei University of Medicine (HuBei, China). All experimental protocols on animals were performed in accordance with the National Institutes of Health (24) and approved by the Committee on the Ethics of Animal Experiments Defence Research of Tianjin Medical University General Hospital (Tianjin, China). All surgery and euthanasia were made to minimize suffering.

Animal experiments. A total of 64 female Wnt1<sup>sw</sup>/sw mice (10-week-old, 32-40 g) with osteoporosis were purchased from the Jackson Laboratory (Ben Harbor, ME, USA) on a mixed C57BL/6 background. All animals were fed under pathogen-free conditions. All animals were housed under controlled temperatures in a 12 h light/dark cycle with free access to food and water. The Wnt1<sup>sw</sup> mice with osteoporosis were randomly divided into three groups (n=32 in each experimental group) and received treatment with Tan-IIA (10 mg/kg), Alendronate (ADN) or PBS. Treatments were performed via an intraperitoneal injection manner (200 μl) once a day. The detailed procedures were according to those of a previous report (25). The therapeutic efficacies of Tan-IIA were analyzed according to a previous study (26).

Reverse transcription-quantitative-polymerase chain reaction (RT-qPCR) analysis. Osteoblasts were isolated from experimental mice following treatment with Tan-IIA, ADN or PBS as described previously (27). Total RNA from the osteoblasts was extracted using an RNAeasy Mini kit (Qiagen Sciences, Inc., Gaithersburg, MD, USA) according to the manufacturer’s protocols. The expression levels of B-cell lymphoma (Bcl)-2, alkaline phosphatase (ALP), p53, NF-κB, inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)2, tumor necrosis factor (TNF) receptor-associated factor 1 (TRAF-1), TNF-α, Caspase-3, Apaf-1, and inhibitor of apoptosis protein (IAP) in the osteoblasts were determined using RT-qPCR analysis (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). All forward and reverse primers were synthesized by Invitrogen; Thermo Fisher Scientific, Inc. (Table I). RT-qPCR was performed using a TaqPath™ 1-Step RT-qPCR Master Mix RT-qPCR kit (cat. no. A15300; Thermo Fisher Scientific, Inc.). Thrombocytopenic conditions included 45 amplification cycles, denaturation at 95°C for 120 sec, primer annealing at 62.5°C for 30 sec with touchdown to 56.5°C for 45 sec and applicant extension at 72°C for 60 sec). Changes in relative mRNA expression levels were calculated using the 2<sup>-ΔΔCq</sup> method (28). The results are expressed as the n-fold change, compared with the control (β-actin).

Western blot analysis. The osteoblasts from the experimental mice with osteoporosis treated with Tan-IIA, ADN and PBS were homogenized in lysate buffer containing protease-inhibitor and were centrifuged at 8,000 x g at 4°C for 10 min. The supernatant of the mixture was used for analysis of proteins of interest. For detection of proteins, the transmembrane proteins were extracted using a transmembrane protein extraction kit (Qiagen Sciences, Inc.) according to the manufacturer’s protocols. Protein concentrations were determined using a Bicinchoninic Acid protein assay (Pierce; Thermo Fisher Scientific, Inc.) and protein samples (40 μg) was separated by 12.5% polyacrylamide gel electrophoresis. Proteins were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The SDS assays were performed as previously described (29). For the western blot analysis, primary antibodies: p65 (1:1,000, ab16502; Abcam, Cambridge, UK), IKK-β (1:1,000, ab178870; Abcam), IκBα (1:1,000, ab109300; Abcam) and β-actin (1:1,000, ab8227; Abcam) were added following blocking (5% skimmed milk) for 1 h at 37°C, followed by the incubation with hors eradish peroxidase-conjugated immunoglobulin G (cat. no. STAR206P; Bio-Rad Laboratories, Inc.) was used at a
Scientific, Inc.) with Tan-IIA, ADN or PBS (1.5 mg/ml) in osteogenic medium (Invitrogen; Thermo Fisher Scientific, San Jose, CA, USA). The cells were cultured for 12 h at 37˚C. At 85% confluence, the cells were cultured with CellQuest Pro software (version 3.2; BD Biosciences, Tokyo, Japan). The examination of intracellular oxidative stress was performed using flow cytometry and DCFH-DA. The osteoblasts were harvested from the experimental mice on day 60, and trypsinized and labeled with DCFH-DA. The osteoblasts were harvested from the experimental mice after treatment as described previously (30). Immunohistochemical staining was performed using an avidin-biotin-peroxidase technique. Paraffin-embedded tissue sections (4 µm) were prepared and epitope retrieval was performed for further analysis. The paraffinized sections were subjected to H2O2 (3%) for 10-15 min, and were subsequently blocked in regular blocking solution for 10-15 min at 37˚C. Finally, the sections were incubated in anti-cluster of differentiation 31 (1:1,200, ab28364; Abcam), and anti-Ki67 (1:1,200, ab15580; Abcam), respectively, at 4˚C for 12 h following blocking. All sections were washed three times and incubated with horseradish peroxidase-conjugated Immunoglobulin G (1:2,000, product code: ab97057; Abcam) for 1 h at 37˚C and were observed in six randomly selected views under a fluorescence microscope at 488 nm (Olympus BX41; Olympus Corporation, Tokyo, Japan).

Oxidative stress assay. The examination of intracellular oxidative stress was performed using flow cytometry and DCFH-DA. The osteoblasts were harvested from the experimental mice on day 60, and trypsinized and labeled with DCFH-DA for 45 min at 37˚C. Subsequently, the osteoblasts were treated with H2O2 in the presence Tan-IIA, ADN or PBS for 30 min at room temperature. The data were further analyzed with CellQuest Pro software (version 3.2; BD Biosciences, San Jose, CA, USA).

Phenotypic characterization of osteoblast differentiation. The osteoblasts were seeded at a density of 1x10^5 cells/cm^2 for 12 h at 37˚C. At 85% confluence, the cells were cultured in osteogenic medium (Invitrogen; Thermo Fisher Scientific, Inc.) containing 5% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) with Tan-IIA, ADN or PBS (1.5 mg/ml) in the presence of the indicated reagents. The procedures used for characterizing osteoblast differentiation were as described in a previous study (31).

Statistical analysis. All data are presented as the mean ± standard error of the mean. Unpaired data were analyzed using Student's t-test. Comparisons of data between multiple groups were analyzed using one-way analysis of variance followed by a Bonferroni post hoc test. Statistical analyses were performed using SPSS 19.0 (IBM SPSS, Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Tan-IIA ameliorates the decreased viability of osteoblasts and inhibits osteoclast differentiation. To investigate the effects of Tan-IIA on osteocyte growth, osteoblasts and osteoclasts were treated with Tan-IIA for 24 h. As shown in Fig. 1A and B, the results showed that Tan-IIA treatment increased the viability of the osteoblasts in a dose-dependent manner, compared with ADN and PBS. The viability of osteoblasts was also improved by Tan-IIA following treatment with increasing concentrations of H2O2 between 0.025 and 1.2 mM for 24 h (Fig. 1C). The present study also investigated the effects of Tan-IIA on osteoclastogenesis. The results (Fig. 1D) showed that Tan-IIA treatment inhibited osteoclast differentiation. The morphology of osteoclasts also confirmed the efficacy of Tan-IIA on osteoclast differentiation (Fig. 1E). Collectively, these results suggested that Tan-IIA not only ameliorated the decreased viability of osteoblasts, but effectively inhibited osteoclast differentiation.

Tan-IIA treatment inhibits the bone-resorbing activity and apoptosis of osteoclasts in vitro. The present study also investigated whether Tan-IIA affects the function of osteoblasts and osteoclasts. It was found that Tan-IIA treatment inhibited the apoptosis of osteoblasts induced by receptor activator of γc (αTcR) and osteoclasts. It was found that Tan-IIA treatment inhibited the bone-resorbing activity and apoptosis of osteoclasts in vitro.

| Gene name | Reverse | Forward |
|-----------|---------|---------|
| P53 | 5'-TTAAGCTTTTGGGCTGGGC-3' | 5'-ATGGTGCGCATGAACCTGG-3' |
| Bcl-2 | 5'-CGTCAATACGAAAGACAC-3' | 5'-TTCTACCTCCGATCCACT-3' |
| Caspase-3 | 5'-ATGGAGAAACAAACAACCTCAG-3' | 5'-TTGCTCCATGTATGGCTTTAC-3' |
| Apaf-1 | 5'-AGCAAGTTGGTGCATCCCTCCGAT-3r | 5'-ATAGCAACAAAGCTTCCGGTA-3r |
| ALP | 5'-CCAATACGTCGCTGTAAC-3G | 5'-CATACGTCATCCGAC-3- |
| TNF-α | 5'-AATCCCTTTATTACC-3' | 3'-GTCTTCTCAAGGGCTGC-3' |
| iNOS | 5'-CTGCAGGTCTTTCAGCTCGG-3' | 5'-GTGGAACACAGGGGTATG-3' |
| COX2 | 5'-TGAACACGAGCTTGGTCACTTGG-3' | 5'-AGGCCCTTGCCACTTGTGA-3' |
| TRAF-1 | 5'-AGAACCAGGAAGATGGCGA-3' | 5'-TGAAGAGACAGCCGACACC-3' |
| IAP | 5'-GGCATACGTCGTAAC-3G | 5'-GTGGAACACAGGGGTATG-3' |
| β-actin | 5'-ACGGTCAGTCACTATACG-3' | 5'-ACGGTCAGTCACTATACG-3' |

Apaf-1, apoptotic protease-activating factor 1; ALP, alkaline phosphatase; Bcl-2, B-cell lymphoma-2; COX2, cyclooxygenase 2; IAP, inhibitor of apoptosis protein; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor-α; TRAF-1, TNF receptor-associated factor 1.
NF-κB ligand (RANKL), whereas the apoptosis of osteoclasts was promoted by treatment with Tan-IIA (Fig. 2A). In addition, the expression levels of caspase-3 and apoptotic protease-activating factor 1 were significantly upregulated in osteoclasts, but downregulated in osteoblasts (Fig. 2B and C). It was also observed that the expression levels of the anti-apoptotic genes Bcl-2 and p53 were increased and decreased in osteoblasts, respectively (Fig. 2D and E). Furthermore, Tan-IIA treatment significantly increased the bone-resorbing activity for osteoblasts, however, no effects were observed in osteoclasts in vitro (Fig. 2F). Taken together, these data suggested that Tan-IIA treatment was beneficial in bone-resorbing activity through regulation of the apoptosis of osteoblasts and osteoclasts.

**Tan-III treatment shows beneficial effects in mice with osteoporosis.** The biomechanical properties of Wnt1<sup>−/−</sup> bones were examined to assess the in vivo effects of Tan-III on
Osteoporosis. First, bone strength was assessed in experimental mice treated with Tan-IIA, ADN and PBS. The data showed that ADN increased bone strength, compared with that in the PBS group, however, Tan-IIA increased the bone strength of mice with osteoporosis, compared with that in the ADN and PBS groups (Fig. 3A-C). In addition, the present study analyzed the bone mineral and matrix composition of the experimental mice treated with Tan-IIA, ADN or PBS. The results (Fig. 3D) showed that Tan-IIA treatment led to an increasing ratio of proline to amide I. The relative mineral content was calculated by the ratio of phosphate to amide I, which was also increased in the Tan-IIA-treated Wnt1<sup>sw/sw</sup> mice (Fig. 3E). The evidence showed that Tan-IIA treatment increased the ratio of phosphate to proline in the Wnt1<sup>sw/sw</sup> mice (Fig. 3F). Overall, these data suggested that Tan-IIA was beneficial for the treatment of mice with osteoporosis by decreasing the mineral and collagen composition of the bone matrix.

**Tan-IIA improves osteoporosis by regulating oxidative stress in osteoblasts from experimental mice.** In order to analyze the efficacy of Tan-IIA in osteoblasts and osteoclasts from experimental mice treated with Tan-IIA, ADN and PBS, the present study analyzed oxidative stress in the mice with osteoporosis. The mRNA expression of ALP, a biomarker of osteoblastogenesis, was downregulated by Tan-IIA treatment for 24 h (Fig. 4A). The levels of H<sub>2</sub>O<sub>2</sub> and accumulation of ROS were also decreased in the osteoblasts from the experimental mice (Fig. 4B and C). In addition, the results revealed that the expression levels of superoxide dismutase (SOD) and thiobarbituric acid reactive substances (TBARS) were decreased...
in the osteoblasts from the experimental mice treated with Tan-IIA, compared with those in mice treated with ADN or PBS (Fig. 4D and E). The results also demonstrated that the levels of reactive nitrogen species (RNS) were decreased in Tan-IIA-mediated oxidative stress in osteoblasts from the experimental mice (Fig. 4F). These data suggested that Tan-IIA inhibited the deleterious effects on the osteoblasts of experimental mice triggered by oxidative stress.

**Tan-IIA improves osteoporosis via regulation of the NF-κB signaling pathway.** To understand the mechanism underlying the Tan-IIA-induced suppression of oxidative stress in osteoblasts, the present study first analyzed the activity of NF-κB in osteocytes. The results (Fig. 5A) showed that Tan-IIA treatment inhibited the activity of NF-κB in osteocytes. Tan-IIA treatment also suppressed the activation of NF-κB phosphorylation in the osteoblasts (Fig. 5B). The levels of NF-κB target genes (TNF-α, iNOS, and COX2) were decreased (Fig. 5C-E), and the levels of TRAF-1 and IAP were increased (Fig. 5F and G) in osteoblasts. The results of the western blot analysis revealed that the levels of p65, inhibitor of NF-κB (IkBα) and IkB kinase (IKK-β) were downregulated following treatment with Tan-IIA (Fig. 5H). Collectively, these results indicated that Tan-IIA improved osteoporosis by regulating the NF-κB signaling pathway.

**Discussion**

Osteoporosis is a comprehensive disease occurring in skeletal tissues, which is increasing in the aging population (32). The clinical consequences of osteoporosis include fractures of the upper extremities, hip and even spine, resulting in loss of function and independence, impaired quality of life, and increasing morbidity and mortality rates (33). Therefore, understanding the molecular mechanism of osteoporosis and identifying more efficient drugs for the treatment of osteoporosis are important for the clinical treatment of osteoporosis. In the present study, the efficacy and mechanism of Tan-IIA on osteocytes were investigated using a mice model of osteoporosis. In addition, osteocyte viability and osteoclast differentiation were examined to identify the therapeutic effects of Tan-IIA on osteoporosis. The effects of Tan-IIA on oxidative stress were investigated in vitro and in vivo. Notably, the mechanism underlying the Tan-IIA-mediated signaling pathway was analyzed. The data obtained suggested that Tan-IIA exhibited protective effects against oxidative stress in osteoblast differentiation in mice with osteoporosis via regulation of the NF-κB signaling pathway. The results also indicated that oxidative stress was important in the progression of osteoporosis and may be an efficient target, consistent with previous reports (34,35).

Previous reports have shown that the excessive accumulation of ROS and the subsequent activation of oxidative stress act as contributory functions in the development and progression of osteoporosis in the skeletal system (36,37). The involvement of the production of ROS in age-related osteoporosis and glucocorticoid-induced osteoporosis have been well documented (23). In addition, evidence has indicated that oxidative stress can damage various cellular components of osteoblasts, and contribute to the aggravation of osteoporosis (22,38). Oxidative stress is also considered an important pathogenic factor on bone mineral density and bone loss (20). Increasing evidence has shown that cell autophagy is important in the response to oxidative stress (21), and oxidative damage to osteoblasts can be considered a target for alleviating the condition of osteoporosis through the endoplasmic reticulum stress pathway (39). In the present study, the efficacy of Tan-IIA on the improvement of osteoblasts was confirmed, and it was also shown that Tan-IIA promoted osteoblastic differentiation.
through oxidative stress, which may be associated with changes in bone matrix formation and bone mineralization in the progression bone rarefaction.

Previous investigations have been performed to understand the Tan-IIA-associated therapeutic regimen via targeting specific molecules and disrupting the dopaminergic system, leading to various symptoms of cardiovascular diseases (40,41). However, the molecular mechanism of Tan-IIA in mediating the treatment of osteoporosis has not been investigated previously. Lee et al (42) suggested that the activation of NF-κB was associated with osteoclast differentiation, and their investigations indicated that the downregulation of RANKL-induced osteoclast differentiation assisted in the recovery of osteoporosis through inhibiting IκB degradation. In the present study, the findings indicated that Tan-IIA treatment may be a potential drug candidate for the treatment of osteoporosis. The results suggested that Tan-IIA inhibited the deleterious outcomes triggered by oxidative stress. In addition, Tan-IIA inhibited the activation of NF-κB and its target genes, TNF-α, iNOS and COX2, and increased the expression of TRAF-1 and IAP1/IAP2 in osteocytes.

In conclusion, the present study aimed to elucidate the underlying mechanisms responsible for Tan-IIA-mediated anti-apoptotic effects, and its effects on oxidative stress and signaling pathways. The investigation was extended to understand the Tan-IIA-mediated improvement of osteoporosis in a mouse model. The results confirmed the protective effects of Tan-IIA against oxidative stress and its beneficial effect on osteoblast differentiation. Extensive evidence has indicated oxidative stress as a novel mechanism, which contributes to the development and progression of osteoporosis, with its increase leading to degenerative disease in osteoporosis (43,44). The results of the present study confirmed those of previous reports, demonstrating that Tan-IIA ameliorated the apoptosis of osteoblasts and improved osteoblast function through the NF-κB signaling pathway. Future investigations of Tan-IIA may clarify the most promising outcomes to be investigated as a potential agent for osteoporosis.

Acknowledgements

Not applicable.

Funding

This study was supported by grant from the Natural Science Foundation of Tianjin (grant no. 2011THJH104PU).

Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors’ contributions

SZ is the executor for this paper and performed most of the experiments. WW designed the experiments. ZL, YY and HJ prepared the figures and conducted the data statistics.

Ethics approval and consent to participate

All experimental protocols on animals were performed in accordance with the National Institutes of Health (44) and approved by the Committee on the Ethics of Animal Experiments Defence Research of Tianjin Medical University General Hospital (Tianjin, China). All surgery and euthanasia were made to minimize suffering.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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