Abstract. The positive correlation between the number of M2 phenotype TAMs (M2-TAMs) and tumour development suggests a supportive role of M2-TAMs in glioma progression. In the present study, the molecular link between glioma cells and M2-TAMs was investigated and it was demonstrated that transforming growth factor-β1 (TGF-β1) secreted by M2-TAMs is key in facilitating the stemness and migration of glioma cells. Cluster of differentiation (CD)133 and CD44, markers for the M2 phenotype, were assessed by western blotting. A sphere formation assay and trans-well assay were applied to test the stemness and migration abilities of glioma cells following co-cultured with M2-TAMs. Stemness markers CD133 and CD44, epithelial-mesenchymal transition -associated markers and mothers against decapentaplegic homolog (SMAD)2/3 and sex determining region Y -box 4/2 (SOX4/2) levels were also evaluated by western blotting. A xenograft tumor mouse model was used to demonstrate the tumor forming ability of glioma cells. The results showed that the U251 glioma cells co-cultured with M2-TAMs exhibited high level of sphere formation, stemness and migration ability. Recombinant TGF-β1 protein treatment was able to achieve the same effects on U251 cells, whereas a TGF-β pathway inhibitor reversed the stemness and migration abilities of the glioma cells induced by M2-TAMs. It was also demonstrated that TGF-β1 secreted by M2-TAMs upregulated the phosphorylation of SMAD2/3 and the expression of SOX4/2 in glioma cells. In a mouse xenograft model, solid tumours formed by U251 cells co-cultured with M2-TAMs or pre-treated with TGF-β1 were larger in size and had a higher growth rate. Taken together, results of the present study demonstrated that M2-TAMs promoted the stemness and migration abilities of glioma cells by secreting TGF-β1, which activated the SMAD2/3 pathway and induced the expression of SOX4 and SOX2. These results highlight the mechanism by which M2-TAMs and glioma interact and demonstrate potential therapeutic strategies for glioma treatment.

Introduction

Malignant glioma is the most prevalent primary brain tumour in the world, with >350,000 patients diagnosed with glioma worldwide each year (1). Despite the development in glioma therapy, the overall survival rate following the diagnosis of patients remains poor (2,3). In previous years, emerging reports have suggested that the migration and self-renewal abilities of glioma cells contribute substantially to tumour development, including tumour initiation, metastasis, drug resistance and recurrence (4‑8). However, the influence of the tumour microenvironment on stemness and the migration of glioma cells remains to be fully elucidated.

Increasing evidence has indicated that the tumour microenvironment is key in tumour development, including glioma (9,10). The tumour microenvironment comprises tumour cells, tumour stroma, blood vessels, infiltrating inflammatory cells and various associated tissue cells. Tumour-infiltrating inflammatory cells are mobilised and recruited by tumour-derived factors, which contribute to the tumour microenvironment. Macrophages are derived from monocytic precursors and can undergo various differentiation or polarization processes in tissues (11,12). Macrophage polarization is the process of expressing different functional programmes in response to microenvironmental signals (13). There are multiple polarization statuses based on functional states that can acquire specific phenotypes (14). Tumour-associated macrophages (TAMs) can be divided into M1 and M2 subtypes, which have different roles in tumours, based on their polarization status (15). Studies have demonstrated that M1 and M2 subtype macrophages exhibit tumour-suppressive and tumour-promoting functions, respectively (16). M2 phenotype TAMs (M2-TAMs) serve as the primary contributors to tumour-infiltrating leukocytes and can be identified by several surface markers, including CD163, CD206, Fizz1 and Arg1 (17,18). M2-TAMs directly facilitate tumour initiation, progression and metastasis through the secretion of proteolytic molecules to promote extracellular
matrix remodelling (19) or nonproteolytic proteins to stimulate tumour cell proliferation, migration and invasion (20,21). In addition, M2-TAMs interfere with the antitumour functions of other immune cells (22). Consequently, investigating the specific mechanism underlying the tumour-supportive role of M2-TAMs in glioma progression is essential.

Furthermore, the high activity of the transforming growth factor-β (TGF-β) pathway in human glioma is associated with a poor prognosis (23). M2-TAMs have been demonstrated to contribute to the accumulation of TGF-β1 in glioma tissues (24). The role of TGF-β1 in tumour development, including cell proliferation, invasion, immune suppression and microenvironment modification, has been well researched (24-28). In addition, reports have demonstrated that TGF-β1 can promote the self-renewal ability of glioma cells (29). However, how the TGF-β pathway affects the biological properties of glioma cells, including stemness and migration abilities, remains to be fully elucidated.

The TGF-β pathway is induced by binding to pairs of receptor serine/threonine kinases, known as type I and type II intracellular effectors mothers against decapentaplegic homolog (SMAD2/SMAD3, which form a complex with SMAD4 and enter the nucleus for target gene recognition and transcriptional regulation (30,31). Sex-determining region Y-box (SOX) factors are a family of transcriptional regulators that comprise 20 members. SOX2 is important in glioma progression (32). The downregulation of SOX2 via RNA interference in glioma cells impairs their proliferation and tumour formation ability in vivo (33), whereas the ectopic elevation of SOX2 increases cell proliferation and self-renewal activity (33,34). SOX2, mediated by other members of the SOX family, including SOX4, which functions downstream of the TGF-β pathway (26), is one of the crucial factors for the maintenance cancer stemness (29). The inhibition of TGF-β has been demonstrated to suppress the expression of SOX4, leading to a decrease in the level of SOX2 and impairment of glioma tumourigenicity (26). However, the effects of M2-TAMs on the expression of SOX family members to mediate stemness and migration abilities in glioma cells remain to be fully elucidated.

The present study aimed to elucidate the effects and specific mechanisms of M2-TAMs on the stemness and migration of glioma cells. It was demonstrated that M2-TAMs induced the stemness and migration abilities of glioma cells via secreting TGF-β1, leading to activation of the SMAD2/3 pathway and the upregulation of SOX4 and SOX2, whereas the TGF-β pathway inhibitor SB431542 was shown to eliminate their interaction. Furthermore, implanted tumours in a mouse model, formed by glioma cells pre-treated with TGF-β1 protein or co-cultured with M2-TAMs, exhibited an increase in tumour size and growth rate compared with those formed by glioma cells exposed to TGF-β inhibitor or no treatment. Taken together, the results provided novel insights and strategies for the treatment of gliomas.

Materials and methods

Cell culture and reagents. The U251 human glioma cell line and the THP-1 human monocyte cell line were obtained from the American Type Culture Collection (Manassas, VA, USA). The U251 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% foetal bovine serum (FBS; HyClone, GE Healthcare Life Sciences, Logan, UT, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.). The THP-1 cells were cultured in RPMI-1640 (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were cultured at 37°C in a humidified incubator with 5% CO₂. Recombinant human TGF-β1 protein (cat. no. ab50036) was purchased from Abcam (Cambridge, MA, USA), and the TGF-β inhibitor SB431542 (cat. no. HY-10431) was purchased from Medchem Express (Monmouth Junction, NJ, USA). Phorbol myristate acetate (PMA; cat. no. P1585) was purchased from EMD Millipore (Billerica, MA, USA). Interleukin (IL)-4 (cat. no. ab222347) and IL-13 (cat. no. ab221410) were purchased from Abcam.

Preparation of M2 phenotype TAMs and co-culture. The M2-polarised macrophages were generated as previously described (35). Briefly, the THP-1 cells (1x10⁵ cells/ml) were seeded into the upper insert of a six-well Transwell plate (Corning Inc., Corning, MA, USA) and were treated with 320 nM PMA for 6 h at 37°C, followed by incubation with PMA and IL-4 (20 ng/ml) and IL-13 (20 ng/ml) for an additional 18 h at 37°C. The samples were then washed with PBS to remove all PMA, and the M2-TAMs were co-cultured with U251 cells (2x10⁵ cells per well) without direct contact for 48 h at 37°C. The co-cultured U251 cells were then washed and harvested for subsequent experiments.

ELISA. The supernatants of the THP-1 cells and polarised M2-polarised macrophages were centrifuged at 1,000 x g for 5 min under 4°C prior to ELISA. The levels of TGF-β1 (cat. no. DB-100B), epidermal growth factor (EGF; cat. no. DEG00), and IL-10 (cat. no. D1000B) were measured using commercial ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. Each sample was evaluated in triplicate.

Sphere formation assay of U251 cells. The U251 cells with or without co-culture were plated on ultralow attachment plates (Corning Inc.) at a density of 20,000 cells/ml in serum-free DMEM, supplemented with B27 (Invitrogen; Thermo Fisher Scientific, Inc.) and 10 ng/ml EGF (BD Biosciences, San Jose, CA, USA). Images of the spheres were captured using a light microscope (Eclipse; Nikon Corporation, Tokyo, Japan) and the spheres were quantified following 10 days of culture.

Transwell assay. A total of 5x10⁵ U251 cells were suspended in serum-free DMEM and plated into the upper insert of a six-well Transwell plate (Corning Inc.), and serum-containing medium was added to the lower chamber. The cells were incubated at 37°C for 8 h. The non-migratory cells in the upper insert were gently removed using cotton swabs, and the migratory cells were fixed with 4% paraformaldehyde in room temperature for 10 min, followed by staining with crystal violet solution. Images were captured using a light microscope (Eclipse; Nikon Corporation) and quantified by counting cell numbers of five randomly picked fields of view for each well.
Western blot analysis. Western blot analysis was performed as described previously (35). Briefly, the total proteins of U251 cells and M2-TAMs were extracted using RIPA lysis buffer (Abcam) containing the protease inhibitor PMSF (EMD Millipore). The concentration of proteins was determined by Pierce BcA Protein Assay kit according to manufacturer’s protocol (Thermo Fisher Scientific, Inc.). A total of 20 µg of proteins were subjected to 10% SDS-PAGE and were transferred onto polyvinylidene fluoride membranes (EMD Millipore). The membranes were blocked with 5% milk in room temperature for 1 h and then were incubated with rabbit anti-cd133 (cat. no. 86781), rabbit anti-cd44 (cat. no. 37259, 1:2,000), rabbit anti-cd206 (cat. no. 91992, 1:500), rabbit anti-N-cadherin (cat. no. ab98952), rabbit anti-vimentin (cat. no. ab86809), mouse anti-CD163 (cat. no. 93498), rabbit anti-SOX2 (cat. no. 3579), mouse anti-SOX4 (cat. no. 9523), rabbit anti-p-SMAD2 (cat. no. ab86809), mouse anti-CD163 (cat. no. 9525), rabbit anti-p-SMAD2 (cat. no. 18338, 1:500), rabbit anti-p-SMAD3 (cat. no. 9520, 1:500) from Cell Signaling Technology, Inc. (Danvers, MA, USA), and rabbit anti-GAPDH (cat. no. Sc-32233; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) antibodies at a dilution of 1:1,000 unless indicated otherwise overnight at 4°C. The membranes were then washed by PBS with 0.05% Tween-20 and incubated with the appropriate HRP-conjugated secondary antibodies (cat. no. 7074 for rabbit and 7076 for mouse) from Cell Signalling Technology, Inc., at a dilution of 1:5,000 for 2 h at room temperature. The proteins were detected using the enhanced chemiluminescence detection reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). GAPDH was used as a normalised control. Relative protein levels were quantified by the comparison of grey values by ImageJ 1.51 (National Institutes of Health, Bethesda, MD, USA).

Tumour implantation. All experimental procedures involving animals were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Xiangya Hospital, Central South University (Changsha, China). In total 40 female BALB/c nude mice of 7-8 weeks old and 16-18 g body weight were purchased from SJA Laboratory Animal Company (Changsha, China) and maintained under 27°C in a HEPA-filtered environment with cages in a 12-h light/dark cycle room, with food and water sterilized by autoclaving. To generate a subcutaneous xenograph mouse model, the mice were injected subcutaneously with 2x10⁶ U251 cells into the right flanks following the indicated treatment for 48 h (n=8 mice per group). The tumour sizes were measured every 5 days. After 30 days, the mice were sacrificed by cervical dislocation, and solid tumours were harvested.

Statistical analysis. All experiments were performed at least three times. The data are expressed as the mean ± standard deviation. The statistical significance between the specific group and control was analysed using SPSS 13.0 statistical software (IBM Corps., Armonk, NY, USA). Statistical evaluation was performed using Student's t-test (two-tailed) between two groups or one-way analysis of variance followed by Tukey’s post hoc test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

M2-polarised macrophage identification and detection of TGF-β1 secretion. M2-polarised macrophages were obtained from THP-1 human monocyte cell line polarization by PMA, IL-4, and IL-13 treatment. Markers for the M2 phenotype, CD206 and CD163, were assessed by western blot analysis. As presented in Fig. 1A and B, the protein levels of CD206 and CD163 were significantly increased following stimulation compared with the levels in THP-1 cells. The relative growth factors and cytokines secreted from the M2-polarised macrophages were assessed by ELISA. Compared with the original THP-1 cells, M2-polarised macrophages exhibited significant increases in the concentrations of secreted TGF-β1, EGF and IL-10 (Fig. 1C). Due to the crucial role of TGF-β1 in tumour development, TGF-β1 was selected for further investigation.

M2-TAMs enhance the stemness and migration abilities of glioma cells. To demonstrate the influence of M2-TAMs on...
glioma cells, the co-culture system was prepared. Following co-culture for 48 h, the U251 cells were harvested, and tumour formation capacity was assessed by the sphere formation assay. Compared with the U251 cells co-cultured with THP-1 cells, the U251 cells co-cultured with M2-TAMs exhibited a notable increase in sphere formation ability (Fig. 2A and B). Similarly, the expression levels of the stemness markers CD133 and CD44 were significantly enhanced in the U251 cells (Fig. 2C and D), indicating a significant increase in the stemness ability of glioma cells.

The tumour microenvironment is important in facilitating tumour migration. In the Transwell migration assay, co-culture with M2 phenotype macrophages significantly promoted the migration ability of the glioma cells (Fig. 2E and F). Additionally, the protein levels of epithelial-mesenchymal transition (EMT)-associated markers were altered, with increased levels of mesenchymal markers vimentin, N-cadherin, MMP-2 and MMP-9, and decreased levels of the epithelial marker E-cadherin (Fig. 2G and H). In summary, these results indicated that M2-TAMs facilitated the stemness and migration abilities of glioma cells.

TGF-β1 derived from M2-TAMs directly increases the stemness and migration abilities of glioma cells. Due to the upregulation of TGF-β1 levels secreted from M2-TAMs and its importance in tumour development, it was hypothesised that M2-TAMs affecting the stemness and migration abilities of glioma cells was achieved by TGF-β1 function. To determine the contribution of TGF-β1 to glioma cells, the U251 cells were treated with human recombinant protein TGF-β1 (200 nM) or TGF-β pathway inhibitor SB431542 (2 µM). Similar to co-culture with M2-TAMs, TGF-β1 treatment increased U251 cell sphere formation and the expression levels of stemness markers CD133 and CD44, whereas the TGF-β inhibitor significantly reversed its effects (Fig. 3A-d). Similarly, TGF-β1 treatment directly elevated the migration ability of the U251 cells and promoted EMT, whereas its inhibitor led to inhibition of these effects (Fig. 3E-H). These results indicated that TGF-β1 derived from M2-TAMs is important in the regulation of the stemness and migration abilities of glioma cells.

TGF-β1 derived from M2-TAMs induces the stemness and migration abilities of glioma cells via the SMAD2/3 pathway. To determine the mechanism of the increased stemness and migration abilities of glioma cells induced by M2-TAMs, downstream signals of the TGF-β pathway in U251 cells were detected. As shown in Fig. 4A and B, the levels of p-SMAD2 and p-SMAD3 were significantly upregulated following M2 coculture and TGF-β1 treatment, whereas the TGF-β pathway inhibitor eliminated these effects (Fig. 4A and B). As the stemness of glioma cells was increased, the levels of stemness-associated proteins SOX2 and SOX4 were
Figure 3. TGF-β1 derived from M2-TAMs directly influence the stemness and migration of glioma cells. (A) Glioma cell sphere formation ability and (B) results of statistical analysis in the indicated groups. Scale bar=2,500 µm. (C) Representative western blot images and (D) statistical results of stemness markers CD133 and CD44 in the indicated groups. (E) Glioma cell migration ability was measured using a Transwell assay in the indicated groups. Scale bar=2,000 µm. (F) Statistical analysis of migration ability in E. (G) Representative western blot images and (H) results of statistical analysis of EMT-associated proteins of glioma cells in the indicated groups. The results are representative of three independent experiments. The error bars represent the mean ± standard deviation. P-values were determined by one-way analysis of variance followed by Tukey’s post hoc test. *P<0.05, **P<0.01 and ***P<0.001; M2-TAMs, M2 phenotype tumour-associated macrophages; co, co-culture; TGF-β1, transforming growth factor-β1; N-cad, N-cadherin; E-cad, E-cadherin; MMP, matrix metalloproteinase; ns, not significant; Con, control.
determined and demonstrated to be elevated in the U251 cells in the co-culture system and those treated with TGF-β1 (Fig. 4C and D). These results suggested that TGF-β1 secreted from M2-TAMs activated the TGF-β1-SMAD2/3 pathway to induce the expression of SOX4 and SOX2 and promote the stemness and migration abilities of glioma cells.

**M2-TAMs facilitate solid tumour formation in a mouse model.** To investigate whether M2-TAMs can affect glioma cell capacity for solid tumour formation *in vivo*, U251 cells were subcutaneously implanted following different treatments. As shown in Fig. 5A and B, the final tumour sizes formed by cells co-cultured with M2-TAMs or those treated with TGF-β1 were increased compared with those of the control group. Additionally, TGF-β inhibitor treatment decreased the size of the tumours, which were induced by co-culture with M2-TAMs. The growth rate of tumours formed by U251 cells was also accelerated by M2-TAM co-culture or TGF-β1
treatment, and the TGF-β inhibitor reversed these effects. Therefore, these data demonstrated that M2-TAMs facilitated solid tumour formation of glioma cells in vivo through secreting TGF-β1 protein.

Discussion

The tumour-associated microenvironment provides cues to cancer cells that regulate their self-renewal and metastatic potential (36,37). The tumour microenvironment features inflammation, which is the major contributing factor to tumour formation and metastasis (38). Among tumour-infiltrating immune cells, TAMs are the major constituents of the inflammation-associated microenvironment in tumours (39). Previous studies have demonstrated a positive correlation between the number of M2-TAMs and glioma development, particularly glioma stem-like cells (21). However, the present study investigated the function and mechanism of M2-TAMs regarding the stemness and migration abilities of glioma cells and the associated specific pathways involved in this process. The results of the present study demonstrated that M2-TAMs increased the stemness and migration abilities of glioma cells via secreting TGF-β1, leading to activation of the SMAD2/3 pathway and the upregulation of SOX4 and SOX2. These findings provide novel insights, strategies and therapeutic targets for the treatment of glioma progression.

The initial results of the present study showed that M2-TAMs derived from the THP-1 human monocyte cell line upregulated M2 subtype surface markers CD206 and CD163 (Fig. 1A and B). Compared with THP-1 cells, M2-TAMs exhibited notably higher levels of TGF-β1, EGF and IL-10, indicating the successful transition to M2 subtype macrophages. Subsequently, to determine the cross-talk between M2-TAMs and glioma cells, a co-culture system was established, enabling communication through secreting factors from M2-TAMs. M2-TAMs have been demonstrated to promote cell invasion in pancreatic islets cancer (19) and facilitate the stemness of cancer stem cells in breast cancer (40). In the present study, the glioma cells exhibited a higher tumour sphere formation capacity and increased expression of stemness markers following co-cultured with M2-TAMs, compared with those co-cultured with THP-1 cells, indicating enhancement in their stemness and tumour development ability induced by M2-TAMs. The M2-TAMs were also shown to promote the migration ability of glioma cells and facilitate EMT transition.

Previous reports have demonstrated that the level of TGF-β receptor 2 (TGFBR2), as a specific receptor for TGF-β1, is higher in glioma stem-like cells (21). Repression of the expression of TGFBR2 in glioma notably decreased their invasion ability, even when co-cultured with M2-TAMs, indicating that the TGF-β pathway contributed the major function in the interaction between M2-TAMs and glioma stem-like cells. The results demonstrated that direct TGF-β1 protein treatment was able to achieve similar effects to those induced by M2-TAMs in glioma cell performance, including sphere formation capacity, self-renewal and migration abilities. However, the TGF-β inhibitor significantly eliminated the effects that were induced by M2-TAMs.

The TGF-β signalling cascade is initiated by binding and activating its receptors (TGFBR1 and TGFBR2), leading to the phosphorylation of intracellular effectors SMAD2/SMAD3 (30). To demonstrate the TGF-β pathway activity in glioma cells, the protein levels of pSMAD2 and pSMAD3 were detected. The resulting data indicated that co-culture with M2-TAMs or TGF-β1 protein stimulation significantly increased SMAD2/3 phosphorylation activity, whereas the TGF-β pathway inhibitor was shown to repress these effects (Fig. 4A and B). These results suggested that the TGF-β1-SMAD2/3 pathway is key in the cross-talk between M2-TAMs and glioma cells. Although Ye et al (21) also investigated M2-TAMs in glioma, the study focused on the association between M2-TAMs and glioma stem-like cells, and did not examine the specific associated pathways downstream of TGF-β1. The present study demonstrated for the first time, to the best of our knowledge, that M2-TAMs increase the stemness and migration abilities of glioma cells via the TGF-β1-SMAD2/3 pathway.

SOX2 has been shown to be important in the maintenance of stem cell activity, particularly for cancer stem cells (32). SOX2 has been identified as a frequently amplified gene in small cell lung cancer (41). In breast cancer, SOX has been reported to be upregulated in cancer stem cells (42). The downregulation of SOX2 in glioma stem cells impairs their proliferation and tumour formation ability (33). SOX4 acts downstream of the TGF-β pathway and regulates the expression of SOX2 (26). The inhibition of TGF-β signalling is able to suppress the expression of SOX2 through inhibiting SOX4 (26). SOX4 can also regulate the TGF-β-induced EMT process (43). The results of the present study showed that, when co-cultured with M2-TAMs or treated with TGF-β1 protein, the glioma cells exhibited increased expression of SOX4, leading to elevated SOX2 at the same time. Therefore, these results suggested that TGF-β1 activated the SMAD2/3 pathway to induce the expression of SOX4 and SOX2, promoting the stemness and migration abilities of the glioma cells. These results are the first, to the best of our knowledge, to suggest that the SOX4/SOX2 axis is involved in the regulation of M2-TAMs in the stemness and migration abilities of glioma cells.

The in vivo tumour graft assay further supported the above conclusion. The size and growth rate of tumours formed by U251 cells were significantly increased by co-culture with M2-TAMs and TGF-β1 protein treatment. Additionally, TGF-β inhibitor treatment eliminated these effects induced by M2-TAMs. From these results in vivo, it was concluded that M2-TAMs accelerated the growth of solid tumours by the TGF-β1 pathway, which may result from the increased stemness and migration abilities of glioma cells that were observed in the in vitro results.

In conclusion, the present study investigated the contribution of M2-TAMs to the stemness and migration abilities of glioma cells. It was demonstrated that TGF-β1 in the tumour microenvironment secreted from M2-TAMs activated the SMAD2/3 pathway and then increased the expression levels of SOX4 and SOX2. This resulted in elevation of the stemness and migration abilities of the cells in vitro, by altering the gene expression pattern associated with stemness and the EMT process, and increased solid tumour sizes in vivo. The development of therapeutic strategies against the communication among M2-TAMs and glioma cells may be a potential approach to monitor glioma initiation and progression.
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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

ZL guarantees the integrity of the entire study and was responsible for the design of the study and clarified associated intellectual content. ZL also performed experimental studies, data acquisition and data analysis and then edited the whole manuscript and reviewed it. WK performed experimental studies and manuscript editing. QZ performed data analysis and manuscript preparation. YZ performed data acquisition and reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures involving animals were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Xiangya Hospital, Central South University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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