Infantile haemangioma (IH) is a tumour commonly found in children and has a high morbidity rate, particularly in Caucasians (~10%)\(^1,2\). IHs are composed of disorganised blood vessels and immature cells, and are characterised by rapid development in their early stages\(^1,3,4\). Although typically harmless, some IHs are disfiguring, destructive and even life-threatening. Additionally, approximately 40%–80% of cases result in permanent cutaneous residua even after regression\(^5\). To overcome the disease, several drugs have been utilised. Corticosteroids, the traditional first line therapy, often cause adverse effects\(^6\). Propranolol, the preferred systemic therapy for IHs, also has intolerable side effects such as bronchial hyperreactivity\(^7\). Moreover, it has been reported that over 10% of IHs did not respond to propranolol therapy, and approximately 16% regrew after administration of propranolol\(^8\). Alternative ther-

**Objective:** To explore the potential therapies for infantile haemangiomas by targeting survivin, a member of the inhibitor of apoptosis protein family, using its specific small molecule inhibitor YM155.

**Methods:** The expression of survivin in human haemangioma tissue was explored using immunohistochemistry and immunohistofluorescence. Cell cycle analysis and EdU assays were used to measure cell proliferation. Hoechst33342 and Annexin V/PI double staining were performed to measure cell apoptosis. The capacity for self-renewal and multilineage differentiation potential of haemangioma stem cells (HemSCs) were measured by clone formation assays and multiple differentiation assays. Murine haemangioma models were established to explore the therapeutic efficacy of YM155 in vivo.

**Results:** Strong staining of survivin in stromal cells was observed in the proliferative haemangioma tissue. In vitro studies demonstrated that YM155 induced cell cycle arrest and proliferation suppression of HemSCs, and also caused cell apoptosis at a higher concentration. YM155 impaired the self-renewal capacities and damaged multiple differentiation potentials of HemSCs. Importantly, YM155 suppressed blood vessel formation and cell proliferation, and induced cell apoptosis in murine haemangioma models.

**Conclusion:** The present study demonstrated that targeting survivin using its specific suppressant, YM155, prevented the progression of infantile haemangioma by suppressing cell proliferation, inducing cell apoptosis and disrupting the differentiation potential of HemSCs. These results indicate a novel and promising therapeutic approach for the treatment of infantile haemangioma.

**Key words:** haemangioma stem cells, infantile haemangioma, survivin, YM155


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Infantile haemangioma (IH) is a tumour commonly found in children and has a high morbidity rate, particularly in Caucasians (~10%)\(^1,2\). IHs are composed of disorganised blood vessels and immature cells, and are characterised by rapid development in their early stages\(^1,3,4\). Although typically harmless, some IHs are disfiguring, destructive and even life-threatening. Additionally, approximately 40%–80% of cases result in permanent cutaneous residua even after regression\(^5\). To overcome the disease, several drugs have been utilised. Corticosteroids, the traditional first line therapy, often cause adverse effects\(^6\). Propranolol, the preferred systemic therapy for IHs, also has intolerable side effects such as bronchial hyperreactivity\(^7\). Moreover, it has been reported that over 10% of IHs did not respond to propranolol therapy, and approximately 16% regrew after administration of propranolol\(^8\). Alternative ther-

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1 The State Key Laboratory Breeding Base of Basic Science of Stomatology & Key Laboratory of Oral Biomedicine Ministry of Education, School & Hospital of Stomatology, Wuhan University, Wuhan, P.R. China.
2 Department of Orthodontics, School and Hospital of Stomatology, Wuhan University, Wuhan, P.R. China.
3 Department of Oral and Maxillofacial Surgery, School & Hospital of Stomatology, Wuhan University, Wuhan, P.R. China.

**Corresponding author:** Dr Wei ZHANG, The State Key Laboratory Breeding Base of Basic Science of Stomatology & Key Laboratory of Oral Biomedicine Ministry of Education, School & Hospital of Stomatology, Wuhan University, Wuhan 430079, P.R. China. Tel: 86-27-87686158; Fax: 86-27-87873260. Email: wzhang88@whu.edu.cn

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apies with high specificity and efficiency are therefore still needed to treat IHs.

Survivin (also known as BIRC5), a member of the inhibitors of apoptosis (IAP) protein family, inhibits apoptosis-related proteins, regulates cell division, and is related to stress response\(^9\). Survivin regulates progression of the cell cycle by interacting with the Aurora B kinase/inner centromere protein (INCENP)/Borealin/Dasra B complex to form a chromosomal passenger\(^9\). By interacting with mitochondrial caspase-9, survivin also significantly prevents programmed cell death in an intrinsic apoptotic approach\(^10,11\). Survivin is one of the most frequently elevated proteins in virtually solid tumours and is largely undetectable or expressed at very low levels in normal tissue, suggesting that it is a promising target for cancer therapy\(^9,12,13\). High expression levels of survivin were also found in IHs\(^14\). Sepantronium bromide (YM155; 1-(2-methoxyethyl)-2-methyl-4,9-dioxo-3-(pyrazin-2-ylmethyl)-4,9-dihydro-1H-naphtho[2,3-d]imidazolium bromide) is a small imidazolium-based compound that selectively suppresses survivin expression and displays potent anti-tumour activities in various cancers\(^15-17\). Herein, we speculate that inhibition of survivin using YM155 in human IHs.

**Materials and methods**

**Clinical samples, immunohistochemistry and immunofluorescence**

Thirteen pathologically confirmed human IH samples (Table 1) with no treatment history and six normal skin samples resected during cleft lip operations were collected at the Hospital of Stomatology, Wuhan University. The study was approved by the review board of the ethics committee of the Hospital of Stomatology. Written informed consent for use of IH specimens was obtained from all parents or guardians. The procedures for obtaining human tissue were performed according to the National Institutes of Health guidelines. Immunohistochemistry and immunofluorescence were performed as previously reported\(^20,21\).

**Cell isolation and culture**

Human HemSCs were isolated from proliferating haemangioma tissues as previously described\(^19\). Tissues were cut into pieces and digested with collagenase and dispase for 1 hour. Single-cell suspension was selected using anti-CD133–coated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and the selected cells were cultured on fibronectin (1 μg/mm\(^2\))-coated plates. Human bone marrow stem cells (BMSCs) were purchased from ScienCell (San Diego, CA, USA). Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously described\(^21,22\). Sepantronium bromide (YM155) was purchased from Selleck (Houston, TX, USA)

**Cell cycle analysis and 5-Ethynyl-2’-deoxyuridine (EdU) incorporation assay**

For cell cycle analysis, HemSCs were trypsinised, before being washed and fixed in 70% ethanol at 4°C for 30 minutes. HemSCs were incubated with RNase (100 μg/ml; Roche Applied Science, Penzberg, Germany) and propidium iodide (PI) (10 μg/ml; Sigma, St Louis, MO, USA) for 15 minutes at room temperature.
Cell cycle distribution was analysed on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) using CellQuest software (Becton Dickinson). The proliferation of HemSCs was analysed using the Cell-Light EdU Apollo488 In Vitro Imaging Kit (RiboBio, Guangzhou, China) according to the manufacturer’s instructions.

**Determination of apoptosis**

Apoptosis induced by YM155 in HemSCs was determined by way of morphological evaluation by Hoechst staining, double staining of Annexin V-FITC/PI, quantification of cytoplasmic histone-associated DNA fragments with a Cell Death Detection ELISA PLUS kit (Sigma-Aldrich, St Louis, MO, USA), and Western blot analysis for Bax–Bcl-2 ratio and caspase-3 cleavage.

**Clone formation assays**

100 μl HUVECs (10 cells/ml) were added into each well (96-well plate). After culturing for 14 days, the cells were fixed and stained with crystal violet staining solution (5 mg/ml). The positive rate for clone formation was calculated by the number of positive staining wells/total wells.

**Tube formation assays**

HemSCs were seeded at $3 \times 10^5$ cells/well on a 48-well plate coated with Matrigel (BD Biosciences, San Jose, CA, USA) and were treated with either endothelial basal medium (as a control) or endothelial basal medium containing 20 nm YM155. After 18 hours, capillary-like structures were photographed under a light microscope (Leica, Wetzlar, Germany) and analysed using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA).

**Adipogenic differentiation and oil red O staining**

As previously described, HemSCs were seeded in a 24-well plate with adipogenic media (ScienCell) containing YM155 for 3 days of treatment. After cells had been further cultured in adipogenic media for 11 days, they were stained with oil red O solution. Staining intensities were quantified through cell lysis in pure isopropanol and measured at 490 nm using a 96-well microplate reader (BioTek, Winooski, VT, USA).

**Murine model for human IHs**

Male BALB/c nude mice (18 to 20 g, 6 to 8 weeks old) were purchased from the Wuhan University Center for Animal in pressurised ventilated cages according to institutional regulations. $2 \times 10^6$ HemSCs were trypsinised and resuspended in 200 μl Matrigel. The mixture was subcutaneously injected into the flanks of the nude mice. The mice were divided into two groups randomly, and received YM155 (5 mg/kg, intraperitoneally injected twice per week; n = 4) or normal saline (vehicle, 100 μl, intraperitoneally injected twice per week; n = 4) every other day, three times. The animals were euthanised and Matrigel plugs were harvested after 14 days. The samples were authenticated as previously reported.

**Ethics statement**

Experiments involving human specimens were approved by the review board of the Ethics Committee of the Hospital of Stomatology, Wuhan University. Written informed consent was obtained from all parents or primary caregivers. The procedures for obtaining human tissue were performed according to the National Institutes of Health guidelines. Animal studies were approved and overseen by the Institutional Animal Care and Use Committee, Center for Animal Experiment, Wuhan University.

**Statistical analysis**

Student $t$ tests were used to analyse the difference between the two groups. A two-way analysis of variance (ANOVA) was used to analyse the grouped results. $P < 0.05$ was considered statistically significant.

**Results**

**Aberrant expression of survivin in human IH tissues**

The expression of survivin in the proliferating (n = 6) and involuting (n = 7) haemangioma tissues was explored using immunohistochemistry and immunohistofluorescence. Normal skin tissue was used as a control (n = 6). The results showed that survivin was intensely expressed in both proliferating and involuting haemangioma tissues (Figs 1a and 1b). High expression levels of survivin were found in CD31-positive endothelial cells in both proliferating and involuting haemangiomas. However, nuclear staining of survivin was only observed in the stromal cells of the proliferating haemangioma tissue. Moreover, mRNA expression of survivin in hae-
mangiomas measured by real-time quantitative polymerase chain reaction (PCR) showed higher expression of survivin in proliferating haemangiomas compared to that of involuting lesions (Fig 1c). In addition, the results of the immunofluorescence confirmed the expression of survivin in both endothelial and stromal cells in proliferating haemangiomas, but only in endothelial cells in involuting lesions (Fig 1d). These data suggest that there is an aberrant expression of survivin in proliferating IHs.

**YM155 selectively inhibits growth of HemSCs**

It has been reported that HemSCs located around blood vessels in proliferating hemangiomas contribute to the rapid development of IHs. We speculated that HemSCs might be survivin positive. Thus, we analysed expression levels of survivin in HemSCs and control cells, including BMSCs and HUVECs. Expression levels of survivin were higher in HemSCs compared to BMSCs and HUVECs, as seen in both protein (HemSCs vs BMSCs, $P < 0.01$; HemSCs vs HUVECs, $P < 0.01$) and mRNA levels (HemSCs vs BMSCs, $P < 0.01$; HemSCs vs HUVECs, $P < 0.01$; Figs 2a and b). YM155, a specific and widely used survivin suppressant, was used to examine the antiproliferative effects of survivin on HemSCs by targeting survivin. As shown through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays in Fig 2c, YM155 suppressed the growth of HemSCs in a concentration dependent manner. YM155 decreased cell growth by almost 90% at a concentration of 80 nM after 24 hours of treatment. Moreover, HemSCs were more sensitive to YM155 treatment than BMSCs and HUVECs. In addition, the viability of these cells was measured using a Vi-CELL Cell Viability Analyzer (Beckman Coulter, Brea, CA, USA). As shown in Fig 2d, the viability of HemSCs started to decline at a concentration of 20 nM of YM155 ($P < 0.05$), whereas a significant decrease in the viability of BMSCs and HUVECs was found at 40 nM ($P < 0.01$). This suggests that HemSCs are more sensitive to suppression of survivin than the other two cell types. After YM155 treatment at concentrations of 5 and

![Fig 1 Expression of survivin in IH and normal skin tissues.](image)
10 nM, cell growth of HemSCs was decreased without a decline in cell viability, indicating that growth inhibition of HemSCs following YM155 treatment at 10 nM was not caused by cell death. In summary, YM155 exhibited higher efficacy in suppressing cell growth of HemSCs compared to BMSCs and HUVECs.

**YM155 halts cell cycle and suppresses cell proliferation in HemSCs**

Due to the pivotal role of survivin in cell cycle regulation and the ability of YM155 to remarkably reduce cell growth of HemSCs at low concentrations, cell proliferation and cell cycle were then analysed using EdU incorporation assays and flow cytometry, respectively. As shown in Figs 3a and c, HemSCs with EdU positive staining were significantly reduced at 5 nM ($P < .001$) and at higher concentrations (10 nM and 20 nM) ($P < 0.001$). Additionally, results from flow cytometry demonstrated that the cell cycle of HemSCs almost halted after YM155 treatment (Figs 3b and d), consistent with the extinction of EdU positive cells. As expected, YM155 treatment suppressed the protein expression of survivin in a dose-dependent manner (Fig 3e). Moreover, the results from Western blots revealed that cell cycle–related proteins, including cyclin D1 and phospho-Rb, were dramatically downregulated in HemSCs after YM155 treatment in a dose-dependent manner. In summary, the survivin suppressant YM155 halted cell cycle progression and suppressed cell proliferation of HemSCs at a low concentration.

**YM155 induces cell apoptosis of HemSCs**

YM155 also induced cell apoptosis, as previously reported. Thus, we explored whether YM155 treatment could induce cell apoptosis of HemSCs using Hoechst33342 staining. As shown in Fig 4a, the segregation of chromosomes in cell division was readily observed in the HemSCs without YM155 treatment. However, chromatin condensation emerged in the stem cells after treatment with 20 nM YM155. Furthermore, Annexin V/PI double staining showed that the Annexin V/PI double positive cell population (late apoptotic cells) was slightly increased in HemSCs treated with YM155 at 5 nM and 10 nM, and significantly elevated at a concentration of 20 nM (Fig 4b). As quantified in Fig 4c, YM155 caused an apoptotic cell population of over 30% at 20 nM. The apoptotic cell population of HemSCs was then measured by DNA fragmentation ELISA assays. Apoptotic cells were increased significantly by ~8 fold compared to that of the control group after YM155 (20 nM) treatment (Fig 4d). The expression of apoptosis-related proteins was analysed by Western blots (Fig 4e). The expression of cleaved-Caspase-3 (Cl-caspase-3) and Bax in HemSCs was significantly increased after YM155 treatment with 10 nM and 20 nM concentrations, while Bcl-2 was downregulated in a dose-dependent manner. Overall, the above results suggest that YM155 induced cell apoptosis.
Fig 3 YM155 induces cell cycle arrest of HemSCs. (a) Cell proliferation was detected by EdU incorporation assays and (b) cell cycle was analysed by measuring DNA content after propidium iodide (PI) staining. Quantification analyses of (c) cell proliferation and (d) cell cycle were performed. (e) The expression of survivin, phospho-Rb at Ser870 and cyclin D1 were measured by Western blots. β-Actin was used as a loading control. Data are expressed as mean ± SEM. *P < 0.05, **P < 0.001 vs vehicle group.

Fig 4 YM155 induces cell apoptosis of HemSCs. (a) Detection of apoptosis in HemSCs by Hoechst 33258 staining; the arrows indicate the apoptotic cells and (b) Annexin V-FITC/PI double staining. (c) Quantitation analysis of apoptotic cell population (Annexin V+/PI−, Annexin V+/PI+, and Annexin V−/PI+ population). (d) Quantitation analysis of DNA fragmentation using the Cell Death Detection ELISAPLUS. (e) The expression of cleaved-caspase-3 (Cl-caspase-3), Bax and Bcl-2 was measured by Western blots. β-Actin was used as a loading control. Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01 vs vehicle group.
of HemSCs in a dose-dependent manner, especially at 20 nM.

**YM155 impairs self-renewal and multilineage differentiation potential of HemSCs**

More recent research indicates the essential role of survivin in the maintenance of pluripotency in stem cells. We explored whether targeting survivin in HemSCs impaired their stemness. The self-renewal capacity of HemSCs was examined using clone formation assays. As shown in Fig 5a, almost 75% of HemSCs formed clones without treatment; however, only about 10% of HemSCs formed clones after YM155 treatment. We also analysed the multilineage differentiation of HemSCs after YM155 treatment. As previously reported, HemSCs were able to differentiate into endothelial cells and form capillary-like structures. Here, the tube formation assays revealed that YM155 (20 nM) impeded the network formation of the stem cells (Fig 5b), indicating that YM155 disrupted differentiation of stem cells into endothelial cells. Moreover, YM155 decreased the mRNA expression of vascular endothelial growth factor A (VEGF-A) in HemSCs in a dose-dependent manner (Fig 5c), potentially contributing to the damaged tube formation. On the other hand, YM155 enhanced actin polymerization of stem cells, as the staining of phalloidin suggested the formation of F-actin (Fig 5d). Moreover, increased expression of alpha smooth muscle actin (α-SMA) was observed in HemSCs after YM155 treatment (Fig 5e). These results suggest that YM155 induced differentiation of HemSCs towards a mesenchymal phenotype. YM155 also prevented HemSC differentiation into adipocytes. As shown in Figs 5f to h, the oil red O staining demonstrated that after YM155 treatment for the first 3 days, HemSCs failed to differentiate into adipocytes. The real-time PCR results further revealed decreased mRNA expression of LPL (Fig 5g).
and C/EBPα (Fig 5h). PPAR-γ, the core transcription factor regulating adipogenesis, was also remarkably downregulated in a dose-dependent manner in HemSCs after YM155 treatment (Fig 5i). In summary, YM155 impaired the self-renewal capacity and multilineage differentiation potential of HemSCs.

**YM155 suppresses the progression of haemangioma in the murine model**

To explore the potential of YM155 in clinical applications, a haemangioma murine model was established. One week after the establishment of the model, YM155 was intraperitoneally injected every other day, at 5 mg/kg, three times (Fig 6a). After 2 weeks, the implants in the control group were red and full of blood vessels, while the Matrigel implants in the YM155-treated group were pale and showed a lack of blood vessels (Fig 6b). Decreased microvessel density (MVD) was confirmed by counting the micro blood vessels containing red blood cells in haematoxylin-eosin (H&E) staining slides. This confirmed that YM155 significantly suppressed blood vessel formation in the murine haemangioma models ($P < 0.001$, Fig 6c). YM155 at the indicated concentration did not cause significant toxicity as the increase in mouse weight was not affected (Fig 6d). The decrease in expression of human CD31 in the YM155-treated implants proved the ability of haemangiomas to impair blood vessel formation. Additionally, the decline in expression of cyclin D1 indicated a significant decrease in the proliferation of haemangioma cells in the models ($P < 0.001$, Fig 6e and quantified in Fig 6f). Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assays revealed increased apoptosis of haemangioma cells after YM155 treatment (Fig 6d). Finally, real-time PCR assays showed that YM155 treatment decreased the expression of survivin and VEGF-A in the haemangioma implants (Fig 6g). Overall, the results demonstrated that YM155 exhibited pronounced therapeutic effects in the haemangioma murine model by decreasing cell proliferation and suppressing blood vessel formation, as well as triggering cell apoptosis.
Discussion

A previous study reported abundant expression of survivin in specimens of human IH tissues. More recently, a study discovered that a hydrogel-endothelial cell implant could mimic haemangioma and that survivin and the Hippo pathway modulated this development. Additionally, it was reported that YM155 inhibits the embryonic angiogenesis via PAK1-survivin/VEGF signalling pathway. These studies suggest the importance of survivin in angiogenesis and the development of haemangioma. In the present study, we showed that survivin was highly expressed in stem cells derived from HemSCs. The specific suppressant of survivin, YM155, not only induced cell cycle arrest and apoptosis of HemSCs in vitro, but also halted the rapid development of the haemangioma in the murine models. Thus, targeting survivin using the suppressant YM155 is a promising alternative therapeutic strategy for IH treatment.

More studies suggest that survivin plays an essential role in stem/progenitor cells, including haematopoietic progenitor cells, keratinocyte stem cells, neural stem cells and embryonic stem cells (ESCs). A recent study uncovered the structural basis of YM155 for selective induction of human pluripotent stem cells. IH is a disease believed to originate from immature progenitor cells or stem cells. Thus, we decided to explore the expression of survivin in human IH samples. In line with a previous study, survivin was abundantly expressed in both proliferating and involuting tissues. However, we noticed that there was strong nuclear staining of survivin in the perivascular cells of the proliferating haemangiomas, which was rarely observed in those of the involuting lesions. As a result, we speculated that the survivin-positive staining might be located in the stem cells. As expected, expression levels of survivin in isolated HemSCs were higher compared to those in BMSCs and endothelial cells. The relatively high expression of survivin also conferred the susceptibility of haemangiomas to survivin targeted therapy.

Therefore, we analysed the effects of the survivin-specific inhibitor, YM155, on cell growth and apoptosis of HemSCs. YM155 significantly induced cell apoptosis at a nanomolar concentration (20 nM). It was also reported that survivin could activate the Cdk2/Cyclin E complex and phosphorylate Rb, thus resisting G1 arrest and promoting G1/S transition. Through these means, survivin maintained rapid stem cell division. HemSCs isolated from proliferating IHs were immature progenitor cells with robust proliferation, in which survivin could potentially be involved. Indeed, YM155 dramatically halted the cell cycle and suppressed cell proliferation. These findings supported the notion that targeting survivin using YM155 could be a promising approach for IH treatment.

Recent studies suggest that survivin also contributes to the maintenance of pluripotency in various stem cells. Survivin, which is downstream of the Wnt/β-catenin/Tcf4 signalling pathway, is essential for retaining a less differentiated phenotype and high proliferative status in corneal epithelial progenitor cells. Moreover, Kapinas et al. demonstrated that survivin functions as a transcription factor/cofactor that regulates the transcription profile of pluripotency markers Oct4 and Nanog, resulting in a loss of pluripotency in human ESCs due to survivin inhibition. In the present study, suppression of survivin in HemSCs reduced their self-renewal capacities and disrupted their multi-lineage differentiation potential. We found that YM155 destroyed the endothelial and adipogenic differentiation of stem cells but increased the expression of α-SMA, suggesting that suppression of survivin accelerated the maturation of HemSCs. This finding was similar to the effects of rapamycin on these specific stem cells reported by Greenberger et al. The results also indicated that survivin might be a crucial downstream molecule of the mTOR signaling pathway in IHs. Moreover, based on the considerable expression of survivin in endothelial cells and the evidence that survivin is essential in promoting proliferation of endothelial progenitor cells, the aggressive vasculogenesis and angiogenesis found in IHs were both affected by targeting survivin.

YM155, currently in phase I/II clinical trials for patients with several cancer types, was the most important antagonist against survivin due to its ability to suppress survivin promoter activity. A recent study proved that YM155 was efficient in inhibiting pluripotent stem cell–derived teratoma formation by only eliminating undifferentiated cells without affecting the survival and function of differentiated cells.

Conclusion

We proved that YM155 might be a promising approach for IH therapy, as it significantly reduced microvessel formation, suppressed the proliferation of haemangioma cells and induced cell apoptosis both in vitro and in vivo.

Conflicts of interest

The authors declare no conflicts of interest related to this study.
Author contribution

Drs Bei Ke WANG and Wei ZHANG conceived the project, designed the experiments and drafted the manuscript; Dr Wei ZHANG purified the haemangioma stem cells; Drs Bei Ke WANG, Hui Min LI and Jie Gang YANG performed the cell culture, YM155 treatment, Western blotting, real-time PCR and other in vitro experiments; Drs Jie Gang YANG, Jian Gang REN and Yu CAI performed immunohistochemistry and immunofluorescence of human samples; Drs Bei Ke WANG, Hui Min LI and Wei ZHANG performed the animal study; Drs Ji Hong ZHA, Yi Fang ZHAO and Jun JIA provided human samples; Drs Yi Fang ZHAO and Jun JIA revised the paper. All authors read and approved the final manuscript.

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