Anti-tumor efficacy of ultrasonic cavitation is potentiated by concurrent delivery of anti-angiogenic drug in colon cancer

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ABSTRACT

This study investigated the efficacy of concurrent delivery of an anti-angiogenic drug and ultrasonic cavitation therapy in a mouse model of human colon cancer. A biotinylated form of the anti-angiogenic drug Endostar was conjugated to a streptavidin-coated microbubble (MB). Mice bearing subcutaneous tumors (HT29) were divided into 4 groups. Group 1 served as an untreated control. Group 2 served as a cavitation control and received naked microbubbles and sham ultrasonic cavitation (MB + sham cavitation). Group 3 received naked microbubbles and ultrasonic cavitation (MB + cavitation). Group 4 received Endostar-loaded microbubbles and ultrasonic cavitation (Endostar-MB + cavitation). Ultrasonic cavitation was performed using a high-power custom-built sonicator. Contrast-enhanced ultrasound imaging (CEUS) was used to measure tumor blood flow before and after ultrasonic cavitation. In vivo fluorescence imaging was performed to monitor changes in tumor volume. Immunohistochemistry was performed to assess CD31, VEGFR-2 and alpha-v beta-3 integrin expression within the tumor. Apoptosis of the tumor cells was determined by TUNEL assay, and ultrastructural changes within the tumor were examined by electron microscopy. Ultrasonic cavitation with Endostar-MB demonstrated a significantly greater inhibition of tumor blood flow on day 7 and tumor growth on day 16 compared with naked MB and control groups. The Endostar-MB treated mice showed significantly decreased expression VEGFR-2 and alpha-v beta-3 integrin, and increased apoptosis of tumor cells and degradation of the tumor ultrastructure. Our findings indicated that the anti-vascular and anti-tumor effects of ultrasonic cavitation could be potentiated by simultaneously delivering an anti-angiogenic drug in colon cancer.

1. Introduction

Although it has traditionally been perceived as a tool for diagnostic imaging, ultrasound is emerging as a highly effective, low-cost and truly noninvasive tool for therapy [1–3]. Of particular note is high-intensity focused ultrasound (HIFU), which is now used for noninvasive thermal ablation of deep-seated solid tumors [4–6]. More recently, the introduction of microbubble contrast agents into the therapeutic ultrasound process has received increasing attention [7–12] due to their potential for localized drug delivery and potentiation of cavitation effects. Ultrasound contrast agents are highly echogenic microbubbles with many unique properties with relevance to ultrasound-mediated therapy. At high acoustic pressures (typically beyond those used for imaging), ultrasound causes acoustic cavitation and microbubble destruction, and can produce extremely high mechanical stresses [13–15] over a very localized region. Some preclinical studies have demonstrated that cavitation therapy with microbubbles can induce a reduction or complete abolishment of blood flow within the tumor [16,17]. Although the precise mechanism(s) at work have not been elucidated, it is possible that anti-angiogenic effects caused by cavitation-induced ischemia are responsible for the observed anti-tumor effect.

The use of targeted microbubbles may enable concentration of the treatment at the desired location upon activation with ultrasound [18–20], and various targeting ligands have been conjugated to the surface of microbubbles to achieve site-specific...
accumulation [21–23]. We hypothesized that local delivery of an anti-angiogenic drug may potentiate the anti-vascular effect of cavitation therapy, and lead to a sustained anti-tumor effect.

Endostar, an anti-angiogenic drug, is a modified version of recombinant human Endostatin and has been approved by the SFDA (State Food and Drug Administration) for the treatment of non-small cell lung cancer in China [24–26]. In the present study we describe an ultrasonic cavitation strategy with Endostar-loaded microbubble able to target tumor angiogenesis and release its payload upon focal ultrasound treatment for anti-tumor efficacy. The efficacy of this strategy was evaluated in a colon cancer model using real-time quantitative CEUS and in vivo fluorescence imaging.

2. Materials and methods

2.1. Cell culture

A human colon cancer cell line HT-29 transfected with green fluorescence protein gene (HT-29-GFP) was obtained from AntiCancer, Inc., (San Diego, CA). Cells were cultured in RPMI 1640 (GIBCO Life Technologies, New York, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone, Logan, UT) at 37 °C in 5% CO2 saturated humidity. All media was supplemented with penicillin/streptomycin (Gibco-BRL, Grand Island, NV).

2.2. Animal care

48 BALB/C male nude mice, aged 4–6 weeks and weighing 20–25 g, were purchased from the Beijing Kelihua laboratory animal center (Beijing, P.R. China). All mice were maintained in a HEPA-filtered environment at 24–25 °C and humidity was maintained at 50–60%. All animals were fed with autoclaved laboratory rodent diet. Animal experiments were approved by the Animal Committee of Nanjing Origin Biosciences, China.

2.3. Xenograft mouse tumor model

A mouse model of human colon cancer (cell line HT29-GFP) was used to assess the efficacy of drug loaded ultrasound contrast agents with ultrasound treatment for inhibition of tumor growth. Stocks of HT-29-GFP tumors were established by subcutaneously injecting 5 × 106 HT-29-GFP cells in the flank of nude mice. Colon tumors, grown s.c. in nude mice, were harvested at the exponential growth phase and resected under aseptic conditions. Strong GFP expression of the HT-29-GFP tumor tissue was confirmed by fluorescence microscopy. Necrotic tissues were removed and viable tissues were cut with scissors and minced into 1-mm3 pieces. Animals were anesthetized by injection of 0.02 ml of solution of 50% ketamine, 38% xylazine, and 12% acepromazine maleate. Two pieces of tumor fragment were transplanted to the flank of nude mice with 8-0 surgical sutures. All surgical procedures and animal manipulations were conducted under HEPA-filtered laminar-flow hoods with a ×8 surgical microscope (Shanghai Precision Instruments, Y2Z005S, Shanghai, China).

2.4. Preparation of drug loaded ultrasound contrast agent

Targetstar-SA (MB, Targetson Inc., San Diego, CA; distributed in China by Origin Biosciences) was used as the microbubbles in this study. Targetstar-SA is an ultrasound contrast agent comprised of a perflurocarbon gas core encapsulated by a lipid shell. The outer shell is derivatized with streptavidin, which binds biotinylated ligands at a density of 80–220 molecules per microbubble. The agents are suspended in aqueous saline at a concentration of approximately 1 × 109 molecules per ml, and have a mean diameter of approximately 2.0 μm. Endostar (Sincere Pharmaceutical, Nanjing, P.R. China) was used as the therapeutic payload. Endostar is a small molecule inhibitor of angiogenesis based on Endostatin. Endostar was biotinylated by Signalway Antibody (Nanjing, P.R. China). Microbubbles were incubated with biotinylated Endostar at room temperature for 20 min at a ratio of 0.7 nmole of biotinylated Endostar per 106 microbubbles. The unreacted Endostar was removed from the microbubbles by centrifugal washing, per the manufacturers recommended protocol.

The presence of microbubble-bound Endostar was assessed using fluorescence microscopy and flow cytometry. Endostar was conjugated to the MBs and fluorescently labeled using a rabbit anti-Endostatin antibody and a FITC conjugated anti-rabbit IgG secondary antibody (Abcam). The conjugated microbubble concentration was quantified with a hemocytometer. The payload of Endostar was determined by quantitation of microbubble-bound Endostar by BCA Protein Assay (Pierce, Rockford, IL, USA). Naked Targetstar-SA microbubbles were used directly from the vial without the addition of Endostar.

2.5. Administration of ultrasound contrast agents

48 Tumor-bearing mice were randomly divided into 4 groups of 12 mice once the average tumor size had reached 120 mm3. Mice were anesthetized with ketamine, acepromazine and xylazine and then placed in a supine position on a heated stage. A dose of 1 × 107 microbubbles in 70 μl per mouse was administered by retro-orbital injection with a 27 gauge needle. Group 1 served as an untreated control. Group 2 received naked microbubbles and a sham ultrasound cavitation (MB + sham cavitation). Group 3 received naked microbubbles and ultrasonic cavitation (MB + cavitation). Group 4 received Endostar loaded microbubbles and ultrasonic radiation (Endostar-MB + cavitation).

2.6. Cavitation therapy and contrast-enhanced ultrasound imaging

The animals in group 3 and 4 received 3 consecutive daily cavitation therapies, using the same acoustic conditions. For cavitation therapy, the transducer was coupled to the skin by covering the tumor with acoustic coupling gel. Imaging was performed in CnTI mode at a mechanical index (MI) of 0.04 and transmission frequency of 3.5 MHz. Imaging gain settings were optimized and held constant during the experiment. Right after injection of microbubbles, ultrasound images were captured to obtain the contrast signal from the tumor tissue as well as from adherent and freely circulating microbubbles. Digital raw data were stored as cine loops up to 2 min for analysis. One board-certified abdominal radiologists (CZ with 14 years of CEUS experience) reviewed and analyzed the data offline using the perfusions software QONTRAST (Bracco, Italy). A region of interest (ROI) was drawn freehand around the peripheral margin of the tumor using an electric-cursor, being careful to avoid the surrounding non-tumor tissue. A time-intensity curve (TIC) for the selected tumor tissue was derived automatically by the software, and the following parameters were generated: peak intensity (PI), which is maximum signal intensity reached during the transit of the microbubble bolus; regional blood volume (RBV), which is proportional to the area under the time-intensity curve; mean transit time (MTT); and regional blood flow (RBF), which is the ratio of the RBV to MTT.

2.7. In vivo fluorescence imaging

Tumor bearing mice were monitored by real-time whole-body fluorescence imaging for tumor growth. The imaging was performed before the cavitation and on day 3, 7, 11 and 16 after cavitation therapy. Tumor size was measured and volume was calculated using the formula \( V = \frac{4}{3} \pi r^3 \), where \( W \) and \( L \) represent the perpendicular minor dimension and major dimension, respectively. A fluorescence stereo microscope (MZ650; Nanjing Optic Instrument Inc., China) equipped with a D510 long-pass and HQ600/50 band-pass emission filters (Chroma Technology, Brattleboro, VT) and a cooled color charge-coupled device camera (Qimaging, BC, Canada) was used to image intact tumors in live mice. Selective excitation of GFP was produced through an illuminator equipped with HQ470/40 and HQ540/40 excitation band-pass filters (Chroma Technology, Brattleboro, VT). Images were processed and analyzed with the use of IMAGE PRO PLUS 6.0 software (Media Cybernetics, Silver Spring, MD).

2.8. Immunohistochemistry

At the end of the study, all mice were sacrificed on day 16 after the cavitation therapy and the tumors and the tumors were removed and weighed. The parts of tumor sample were fixed in 10% buffered formalin and paraffin-embedded. For immunohistochemistry, sections were incubated with primary antibodies against CD31, VEGFR-2 and alpha-v beta-3 integrin (all from BD Biosciences, San Diego, CA) overnight at 4 °C after permeabilization with a solution of 0.1% sodium citrate and 0.1% Triton X-100 and blocking with 10% normal horse serum and 0.5% Triton X-100 in PBS. The slides were incubated with horse radish peroxidase-labeled secondary antibody (1:200, Maxxin Bio-Tech Co., Ltd., Fuzhou, China) for 30 min at room temperature. After color development using diaminobenzidine (Maxxin Bio-Tech Co., Ltd.), the slices were counterstained with hematoxylin and mounted with a neutral resin medium. The whole slide was first viewed at 100-times magnification in order to identify a “hot spot” representing the area of the highest vessel density. The field was then switched to ×400 magnification for analysis. For each slide, the microvessel
density (MVD) was calculated as the average number of CD31+ vessels in 4 fields of view. The same method was used to calculate the average optical density (AOD) of VEGFR-2 and alpha-v beta-3 integrin expression.

2.9. TUNEL analysis for detection of apoptosis

Apoptosis of the tumor cells following cavitation therapy was determined by TUNEL staining, using a commercially available kit (In Situ Cell Death Detection Kit, POD; Roche, Germany). The parts of tumor sample were deparaffinized and dehydrated according to standard protocols. Tissue sections were incubated with Proteinase K working solution for 30 min at 21–37 °C. The slides were then washed with PBS (pH 7.2–7.6) twice. The positive control was incubated with DNase I for 10 min at 15–25 °C. The negative control was incubated with label solution (without terminal transferase) instead of the TUNEL reaction mixture. The slides were then washed with PBS (pH 7.2–7.6) three times. Converter-POD was added on slides and incubated in a humidified chamber for 30 min at 37 °C. The slides were then washed with PBS (pH 7.2–7.6) three times. The DAB substrate was added to slides and incubated for 10 min at 15–25 °C. The slides were then washed with PBS (pH 7.2–7.6) three times. The slides were mounted and analyzed microscopically (Olympus, Melville, NY, USA). The apoptotic index (AI) was calculated by counting the number of TUNEL+ nuclei visible on high-power-field at least 3 fields/sample and expressing the results as percentage of the total number of cells in the same fields. Apoptotic cells were recognized by the appearance of brown or tan stained nuclei.

2.10. Electron microscopy analysis

Tumor samples for electron microscopy were fixed in 10% glutaraldehyde and then with 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated in graded solutions of ethanol, and embedded in LX – 112 (Fullium). Ultramicrotome sections, contrasted with uranyl acetate and lead citrate were examined and photographed by transmission electron microscope (JEM-3100F TEM, JEOL Ltd., Japan). The sections were screened for tumor cell morphology.

2.11. Statistical analysis

Statistical analysis was performed with SPSS 16.0 software. Data were presented as mean ± SD. The Shapiro-Wilk test was applied to test for normality. One-way analysis of variance (ANOVA) was used when the data showed a normal distribution. The Mann–Whitney U-nonparametric test was used when the data had a non-normal distribution. P values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Cavitation therapy reduces tumor vascular perfusion

Fluorescence microscopy and flow cytometry revealed Endostar bound on microbubbles (Fig. 1). Quantitation of microbubble-bound Endostar showed an Endostar payload of 1.2 µg per 1E8 microbubbles dose.

The effect of drug loaded ultrasonic cavitation therapy on tumor vasculature was assessed in intact animals by contrast-enhanced ultrasound perfusion imaging immediately before and after cavitation treatment (Fig. 2 and supplement file). Cavitation treatment led to significant decrease of blood flow into the tumor (Fig. 2A). All three vascular perfusion parameters (PI, RBV, and RBF) were significantly reduced in the MB + Cavitation group [PI (22.41 ± 9.51)%, RBV (222.75 ± 88.21) mL, RBF (23.38 ± 8.87) mL/s] and Endostar-MB + Cavitation group [PI (27.2 ± 9.94)%, RBV (249.88 ± 64.99) mL, RBF (29.52 ± 5.3) mL/s] compared to MB + Sham Cavitation group [PI (40.2 ± 15.5)%], RBV (433.94 ± 217.5) mL, RBF (39.5 ± 13.06) mL/s] on day 1 after the third cavitation (P < 0.01), indicating a rapid effect. However, vascular perfusion in the MB + Cavitation group was partially recovered by 7 days after the cavitation [PI (36.76 ± 9.31)%, RBV (499.6 ± 117.4) mL, RBF (41.44 ± 19.49) mL/s], while perfusion in the Endostar-MB + Cavitation group remained low [PI (25.01 ± 15.15)%], RBV (153.28 ± 72.35) mL, RBF (23.03 ± 8.87) mL/s] on day 1 after the third cavitation (P < 0.01) (Fig. 2B). This suggests synergistic inhibitory effects between ultrasonic cavitation and locally-delivered Endostar.

3.2. Cavitation therapy reduces tumor volume

The effect of cavitation treatment on tumor growth was evaluated by whole-body fluorescence imaging. Tumor size was measured on day 0, 3, 7, 11 and 16 following cavitation treatments. Representative fluorescence images and mean tumor volumes are shown in Fig. 3A and B, respectively. The tumor sizes were significantly reduced in MB + Cavitation [(143 ± 51.14) mm³] and
Endostar-MB + Cavitation group \((134.2 \pm 38) \text{ mm}^3\) compared to untreated control \((260.22 \pm 61.19) \text{ mm}^3\) and MB + Sham cavitation group \((255.6 \pm 61.78) \text{ mm}^3\) from day 7 after cavitation treatment \((P < 0.01)\). On day 16 after cavitation treatment, tumor inhibition effect was still observed in MB + Cavitation \((226.97 \pm 86) \text{ mm}^3\) and Endostar-MB + Cavitation group \((106.1 \pm 53.38) \text{ mm}^3\) as compared to untreated control \((361.6 \pm 67.89 \text{ mm}^3)\) and MB + Sham cavitation group \((349.37 \pm 59.33) \text{ mm}^3\) \((P < 0.01)\). However, the more significant reduction of tumor size in Endostar-MB + Cavitation group \((106.1 \pm 53.38) \text{ mm}^3\) was found as compared with MB + Cavitation group \((226.97 \pm 86) \text{ mm}^3\) \((P < 0.01)\), indicating more sustained tumor inhibition by the cavitation with Endostar loaded microbubbles than the naked microbubbles.

3.3. Cavitation therapy with local endostar is anti-vascular and anti-angiogenic

Immunohistochemical staining for CD31, VEGFR-2 and alpha-v beta-3 integrin was performed to assess the effect of drug loaded ultrasonic cavitation on tumor angiogenesis. Positive expression of CD31, VEGFR-2 and alpha-v beta-3 integrin was present in untreated tumors (Fig. 4). Decreased CD31 expression was found in MB + Cavitation and Endostar-MB + Cavitation groups, suggesting an anti-vascular effect (Fig. 3B). Tumors in the Endostar-MB + Cavitation group showed significantly decreased expression levels of VEGFR-2 and alpha-v beta-3 integrin compared with control and sham treated. Interestingly, no change in these angiogenesis markers was observed in the MB + Cavitation group \((P > 0.05)\) (Fig. 5). This suggests that ultrasonic cavitation alone exhibits an anti-vascular effect, while the additional delivery of Endostar is required to exert an anti-angiogenic effect.

3.4. Cavitation therapy induces tumor apoptosis

The effect of cavitation treatment on tumor apoptosis was analyzed by TUNEL staining. TUNEL-positive cells were counted only in regions of intact tumor, being careful to avoid the central necrosis typically observed in xenografts. Representative fields of view from each group are shown in Fig. 6A, and demonstrate the higher rate of apoptosis in mice treated with MB + Cavitation or Endostar-MB + Cavitation as compared to MB + Sham Cavitation and untreated groups. The number of apoptotic cells in 3 random fields from 3 different tumors in each group was counted, and the apoptotic index is shown in Fig. 6B.

3.5. Cavitation therapy induces ultrastructural rearrangement

A high magnification electron micrograph of the endothelial cells of tumor blood vessels in the control group showed intact nuclear membranes and normal chromatin (Fig. 7A). However,
Fig. 3. Inhibitory effect of drug loaded ultrasonic cavitation on tumor growth. Whole body fluorescence imaging was performed to measure tumor size at different time points during the study as described in the MATERIALS AND METHODS. (A) Sequential in vivo whole body fluorescence imaging of tumor progression after ultrasonic cavitation. (B) Tumor growth curves after treatments.

Fig. 4. Effect of drug loaded ultrasonic cavitation on expression of tumor angiogenesis makers. Representative immunohistochemical images for the expression of CD31, VEGFR-2 and alpha-v beta-3 integrin (400× magnification). Immunohistochemical staining of the tumor tissue were performed as described in the MATERIALS AND METHODS.
electron microscopic picture from MB + Cavitation and Endostar-MB + Cavitation groups revealed absent or broken nuclei and nuclear membranes of the endothelial cells, with fragmented DNA present in the cytoplasm (Fig. 7B and C). In some pictures from Endostar-MB + Cavitation group, various mitochondrial vacuoles of different sizes were observed in the cytoplasm (Fig. 7D). The predominance of fragmented DNA is attributable to the high percentage of cells undergoing apoptosis.

Fig. 5. Effect of drug loaded ultrasonic cavitation on expression of tumor angiogenesis makers. Quantitation of the expression of CD31, VEGFR-2 and alpha-v beta-3 integrin by average optical density (AOD) and microvessel density (MVD). *P < 0.01, when compared with untreated group.

Fig. 6. Effect of drug loaded ultrasonic cavitation on tumor apoptosis. The tumor sections were stained with TUNEL to measure apoptosis of tumor cells as described in the MATERIALS AND METHODS. (A) Representative TUNEL images for cell apoptosis (400x magnification). (B) Quantitation of cell apoptosis by apoptotic index (AI). *P < 0.01, when compared with untreated group.
4. Discussion

The unique physiological barriers presented by the tumor environment and associated vasculature impose a limit to the therapeutic efficacy of systemically delivered anticancer agents [27]. Several strategies for specifically targeting the tumor have previously been applied in an effort to develop efficient and noninvasive ways of delivering anticancer agents and optimizing therapeutic protocols [28]. Therapeutic ultrasound using microbubble-mediated ultrasonic cavitation has demonstrated great potential as a tool for targeted disruption of tumor vasculature. This technique is being explored for local gene transfection, targeted drug delivery and release, blood–brain barrier disruption and thrombolysis [29–31].

Several studies have investigated the efficacy of microbubble mediated ultrasonic cavitation in tumor xenograft models. However, to our knowledge the current study is the first to explore drug loaded microbubbles at multiple time points [16,17]. This study utilized a new type of microbubble conjugated with an anti-angiogenesis drug (Endostar) and investigated dynamic change of tumor vasculature at multiple time points before and after cavitation therapy. Real-time imaging of vascular perfusion revealed a decrease in blood flow and volume following treatment. The presence of extensive endothelial apoptosis found in TUNEL staining and electron microscopy suggests that destruction of the vasculature is responsible for the diminished blood flow to the tumor. Interestingly, the anti-vascular effect in tumors treated with cavitation therapy and naked microbubbles persisted for only 7 days, after which time blood flow resumed to normal. In animals treated with Endostar-loaded microbubbles and cavitation therapy, no such resumption in blood flow was observed. This suggests a noteworthy synergy between the acoustic and anti-angiogenic drug treatment.

Several mechanisms for this synergy are possible. High-energy microstreaming and liquid jets arising from the oscillation and collapse of microbubbles can produce injury to vessel wall, thereby triggering blood coagulation and the formation of thromboses. Additionally, deposition of such high energy may result in transient holes (sonoporation) in endothelial cells, thereby allowing for the direct passage of Endostar to the tumor cells. Although an anti-vascular response was found in our study, our study has not quantified the dose of Endostar delivered. Derivation of a dose-response curve and assessment of multiple dosing are critical studies that should be performed in the future.

In a previous study by our group, we demonstrated an anti-tumor effect due to ultrasonic cavitation with naked microbubbles [17]. In the current study, a head-to-head comparison demonstrated that the inhibitory effect on tumor growth for Endostar-loaded microbubbles was greater than that for naked microbubbles. This suggests an enhanced anti-tumor effect due to concurrent delivery of an anti-angiogenic drug and ultrasonic cavitation therapy.

CD31, VEGFR2 and αVβ3 integrin play important roles in regulating endogenous angiogenesis [32,33]. Tumor angiogenesis was also selected as a therapeutic target due to the accumulating evidence documenting the complex behavior of anti-angiogenic drugs in clinical practice [27,28]. Endostar exerts its antiangiogenic effects by blocking the VEGF-induced tyrosine phosphorylation of KDR/Flk-1 to inhibit formation of new blood vessels in tumor [24–26]. We analyzed the expression of these surface markers in the tumor samples in order to elucidate the anti-angiogenic mechanism underpinning the effect of Endostar-loaded microbubbles. The Endostar-MB + Cavitation treated mice showed significantly decreased expression levels of VEGFR-2 and alpha-v beta-3 integrin and microvessel density as compared with other three groups, indicating Endostar may be released from...
microbubbles by ultrasound cavitation and play an important role for inhibition of tumor angiogenesis.

To further clarify the anti-cancer mechanism of Endostar-loaded microbubble ultrasound cavitation, we stained tumor sections with TUNEL to measure apoptosis of tumor cells. We found that ultrasound cavitation with naked or Endostar-loaded microbubbles caused significant apoptosis of tumor cells, indicating tumor cell apoptosis is associated with microbubble-mediated ultrasound cavitation regardless of whether a drug-loaded microbubble is used. Previous studies have observed similar morphological changes after cavitation therapy, including cell shrinkage, vacuole formation, chromatin condensation, karyorrhexis and the formation of apoptotic bodies [13,16,17]. Our electron microscopy examination revealed an absence of nuclear membranes of treated endothelial cells, with fragmented DNA and the presence of abundant mito-

Author contributions

Pintong Huang designed the study and wrote the manuscript. Chao Zhang and Yu Sun conducted in vivo experiments and analyzed the data. Jian Chen and Ying Zhang performed the ex vivo experiments and analyzed data. Weihui Shentu and Zhijian Yang analyzed data, supervised the overall study and assisted with manuscript preparation.

Conflict of Interest

On behalf of all authors of this paper, I declare that this study will not lead to any financial or other kinds of conflicts of interest.

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Appendix A. Supplementary material

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