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Single-dose local intraosseous injection of simvastatin suppresses breast cancer with tumor vascular normalization

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ABSTRACT

Tumor vessels play important roles in cancer development and angiogenesis has been characterized as an essential process for tumor cell tumor growth. Our previous studies found that a single-dose local intraosseous simvastatin injection rapidly and long-termly mobilized bone marrow-derived endothelial progenitor cells to peripheral blood, promoting angiogenesis and ameliorating ischemia injury. However, whether intraosseous injection of simvastatin participates in cancer progression and the role of angiogenesis enhancement in this process remain unknown. In this study, we found that intraosseous injection of simvastatin improves tumor vascular structure, along with increasing the percentage of pericyte coverage on tumor vessels, and reducing vascular permeability, tumor hypoxia and tumor necrosis. Further, we demonstrate that a single-dose local intraosseous simvastatin injection suppresses tumor growth, facilitates sensitivity of chemotherapy and prolongs survival in breast cancer-bearing mice. In addition, oral application, intravenous, subcutaneous and intraperitoneal injection of simvastatin do not show these effects. Taken together, these results demonstrate that intraosseous injection of simvastatin suppresses breast cancer with tumor vascular normalization, which might be a promising strategy for cancer treatment.

Introduction

Cancer is still the leading cause of death and the burden is increasing worldwide [1,2]. However, it is difficult to achieve satisfactory therapeutic effects for cancer at present [3,4]. Angiogenesis is essential for tumorigenesis and tumor progression [5]. Anti-angiogenic therapy, such as bevacizumab, a tyrosine kinase inhibitor, designs to inhibit angiogenesis by suppressing VEGF receptor signaling [6], disrupts the vascular supply, starves tumor of nutrients and oxygen and should be of benefit to patients. While, current evidence suggests that cancer cells are able to circumvent antiangiogenic therapy and develop resistance to targeted drugs, and the excessive nutritional demands of the expanding tumor lead to the generation of more abnormal tumor vessels [7,8]. Moreover, additional antiangiogenic therapy with standard-of-care treatments provided minimal clinical benefits and not all cancer patients were sensitive to the targeted drugs [9]. Therefore, it is extremely urgent to find a new strategy to treat cancer.

Simvastatin, an inhibitor of 3-hydroxy-3-methyl glutaryl-coenzyme reductase, is widely used to limit cholesterol synthesis and prevent cardiovascular disease [10]. It is effective, safe, and has been universally acknowledged for more than 30 years in clinical treatment. Besides, the administration route of simvastatin has high potential for clinical translation. Traditionally, simvastatin is administered orally (p.o.). However, the bioavailability is low due to its extensive first-pass metabolism, during which less than 5% of orally administered simvastatin can reach the circulatory system [11]. Intraosseous (i.o.) administration in bone marrow through a technique proposed in the 1920s [12], provides a safe, simple, and fast method for gaining access to the circulation [13] and bone marrow puncture is widely performed for clinical therapy. Our previous research showed that single-dose local intraosseous injection of simvastatin significantly promoted angiogenesis and bone formation [14,15]. Recent studies showed that p.o. administered simvastatin plays a potential role in suppressing cancer [16–19]. Tumors are inseparable from angiogenesis. However, the effects of i.o. injection of simvastatin on the progression of cancer remain unknown.

The tumor microenvironment (TME) may reduce the effectiveness of virtually all types of anticancer therapies [20]. The rise of the "tumor vascular normalization" strategy by Jain provides a new idea for cancer

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treatments in 2001 [21]. This strategy suppresses cancer development by improving tumor vascular structure, increasing pericyte coverage on vessels, reducing vascular permeability, tumor hypoxia, and tumor necrosis, and facilitating sensitivity to chemotherapy [22]. In recent years, increasing research has found that bone plays an important endocrine role in affecting or being influenced the whole-organism physiology [23]. Bone and bone marrow are tightly associated and can be considered as one unique functional unit that regulates disease development [24]. The bone marrow microenvironment is a niche that releases signals to maintain homeostasis. For example, bone marrow-derived pericytes play crucial roles in trophic support and stabilization of vessels [25], to promoting tumor vascular normalization. Thus, it is necessary to find a new way to suppress cancer via local i.o. treatment.

In this study, we compared the roles of simvastatin by p.o. application, intravenous (i.v.) injection, subcutaneous (s.c.) injection, intraperitoneal (i.p.) injection and local i.o. injection, and found that only i.o. injection of simvastatin inhibited breast cancer development with tumor vascular normalization. However, the other administration routes of simvastatin did not show these effects. Thus, this study might provide a promising strategy for the treatment of breast cancer.

Materials and methods

Cell lines

A breast cancer cell line (4T1-luc) stably expressing luciferase was provided by Dr. Xiaoqing Ren from Peking University Third Hospital. These cells were cultured routinely in RPMI 1640 (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (Gibco, USA) supplemented with 1% penicillin/streptomycin, and maintained in humidified incubators at 37 °C and 5% CO_2.

Breast cancer mouse model

Five-to six-week-old female BALB/c mice (Charles River. Beijing, China) were bred and maintained under specific pathogen free conditions, provided with sterilized food and water and housed in a barrier facility with a 12 h light/dark cycle. The mice were inoculated with 4T1-luc cells (1×10^6) into the fourth mammary fat pads. Tumors were measured along two orthogonal axes (a = length, b = width) and tumor volume was calculated by the formula: volume = $a \times b^2/2$ [26]. Simvastatin was administered after tumors were established (tumor volume 50–80 mm^3, approximately 8–10 days post inoculation). Mice were randomly divided into ten groups, receiving one of the following ten treatments: p.o. control solution, p.o. simvastatin (National Institutes for Food and Drug Control, Beijing, China), i.v. control solution, i.v. simvastatin, s.c. control solution, s.c. simvastatin, i.p. control solution, s.c. simvastatin, i.p. simvastatin, i.o. control solution and i.o. simvastatin. The body weight of the mice and the tumor volume were measured every two days. Two weeks later, D-luciferin (PerkinElmer, USA) was injected into the abdominal cavity of the mice and tumor growth was monitored in vivo by the Spectrum in vivo imaging system (IVIS) (Perkin Elmer, USA) 10 min after injection. Subsequently, mice were sacrificed, and the tumors were excised, weighed, and cut into blocks, fixed in 10% formalin, and embedded in paraffin for hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) or snap-frozen in liquid nitrogen for Western blot analysis.

For survival rate, mice were observed every day. The number of dead mice was recorded, and the survival rate was analyzed by the Kaplan-Meier method.

Preparation and application of simvastatin

For oral application, simvastatin was dissolved in 1% carboxyl methylcellulose, and mice were administered 1% carboxyl methylcellulose as a control or simvastatin (10 mg/kg/day, 50 μL/mouse) for two weeks [27]. For i.v. injection, simvastatin was dissolved in 2% DMSO diluted in saline and mice were administered 2% DMSO as a control or simvastatin (5 mg/kg, 100 μL/mouse, twice a week) for two weeks [28]. For s.c. injection, simvastatin was mixed in Poloxamer 407 (BASF, Ludwigshafen, Germany; 25% w/w), which was dissolved in phosphate-buffered saline (pH 7.4, 4 °C) by gentle mixing overnight [29]. Mice were s.c. administered Poloxamer 407 gels as a control or simvastatin (11 mg/kg, 100 μL/mouse, every other day) for two weeks [30]. For i.p. injection, simvastatin was dissolved in 1% carboxyl methylcellulose, and mice were administered 1% carboxyl methylcellulose as a control or simvastatin (5 mg/kg, 50 μL/mouse, every other day) for two weeks [31]. For the local i.o. injection, simvastatin was mixed in Poloxamer 407 and mice were local i.o. administered Poloxamer 407 gels as a control or simvastatin (2.5 mg/kg, 20 μL/mouse) for once.

For i.o. injection, mice were anesthetized by an intraperitoneal injection of 10% chloral hydrate (3.3 mL/kg) and fixed in the supine position. A hole was made by inserting the needle of a 1 mL syringe carefully into the bone marrow of the right tibia along the medial patellar ligament. Then, the control or simvastatin gels were injected into the bone marrow.

This study was approved by the Ethics Committee of Biomedical Science of Peking University.

Western blot

Western blot was performed as previously described [29]. Signals were detected by a LAS500 Imaging System (GE, NY, USA). For hypoxia inducible factor (HIF)-1α (Cell Signaling Technology, Danvers, MA, USA) protein detection, 40 μg of total protein from each sample was loaded.

H&E and IHC

H&E and IHC were performed on formalin-fixed, paraffin-embedded tissues from mice as previously described [29]. H&E staining was evaluated by a pathologist to confirm the necrotic area in tumor tissues. An antibody against HIF-1α (Cell Signaling Technology, Danvers, MA, USA) and NG2 (bs-23788R, Bioss, Beijing, China) were used as the primary antibody for IHC.

Immunofluorescence

Immunofluorescent staining of pericytes in tumors was detected with frozen tumor sections that were prepared as mentioned above. The tumor tissues were co-immunostained with rabbit anti-CD31 (1:50) and Cy3-conjugated mouse anti-α-smooth muscle actin (α-SMA) (1:300) antibodies at 4 °C overnight, followed by a DyLight 488-conjugated goat anti-rabbit secondary antibody (1:200) for 1 h at room temperature. Images were captured with a TCS-SP laser scanning confocal microscope (Leica Microsystems, Mannheim, Germany). The pericyte coverage index is presented as the percentage of CD31/α-SMA.

Hypoxyprobe staining

We assessed hypoxic changes in the tumors after treatment with either simvastatin or controls by using the Hypoxyprobe™-1 Plus Kit (EMD Millipore, Billerica, MA). Briefly, mice were injected with 60 mg/kg Hypoxyprobe™-1 intravenously. One hour following injection, mice were sacrificed and tumors were dissected and fixed with 4% paraffin. Then, tumors were stained with the anti-Hypoxyprobe-1 antibody (1:50) according to the manufacturer's instructions [32].

Reactive oxygen species (ROS) detection

The production of ROS was measured by dihydroethidium (DHE, Sigma-Aldrich, St. Louis, MO, USA) staining. Briefly, frozen tumor sections (embedded in optimal cutting temperature compound) were prepared for incubation with freshly prepared DHE (30 μM) for 30 min in dark at 37 °C. After the sections were washed with PBS, they were stained with DAPI

(Invitrogen, MA, USA) for 10 min and monitored by confocal microscopy and the fluorescent intensity of DHE (indicating levels of ROS) was quantified using Image-Pro Plus 6.0 software (NIH).

Statistical analysis

Data were presented as the mean ± s.d. Two-tailed Student's *t*-test was applied to compare two groups in Prism 5.0 (GraphPad Software, San Diego, CA, USA). For survival data, Kaplan-Meier survival curves were prepared, and significant differences were analyzed using the log-rank test. Grayscale values that were obtained by Western blot and gelatin zymography were analyzed by ImageJ software. $p < 0.05$ was considered statistically significant.

Results

Local intraosseous injection of simvastatin improved tumor vascular structure

Our previous studies indicated that i.o. injection of simvastatin in bone marrow promoted bone formation and angiogenesis [14,15]. Thus, we established a mouse breast cancer model to clarify whether simvastatin i.o injection plays a role in tumor development via tumor vessels.

To investigate the role of simvastatin in the vascular structure, we performed a confocal assay to co-immunostained CD31 (all vessels, red) and FITC-α-SMA (mature pericytes, green) in mouse tumor tissues and surprisingly found that instead of reducing the pericyte coverage, simvastatin i.o. injection remarkably increased the percentage of pericyte-coated vessels, as exhibited in Fig. 1A and B. Similarly, simvastatin i.o. injection upregulated the expression of NG2, another marker of pericytes (Fig. 1C). Moreover, the vessel permeability of tumors was significantly decreased in the simvastatin group, as determined by comparing the extravasation of Evans blue dye into the interstitium of tumors (Fig. 1D). These results indicated that locally i.o.-injected simvastatin improved tumor vascular structure and may participate in tumor vascular normalization.

Local intraosseous injection of simvastatin decreased tumor hypoxia via decreasing ROS level

To confirm the role of simvastatin in tumor vascular normalization, we further analyzed necrosis and hypoxia in TME and found that local i.o. injection of simvastatin significantly reduced the tumor necrotic area in tumor tissues by H&E staining (Fig. 2A, B). Moreover, local i.o.-injected simvastatin revealed an antioxidant effect. Fig. 2C showed that i.o.-injected simvastatin decreased the number of Hypoxyprobe-stained hypoxic cells. Compared with the control group, HIF-1α expression was downregulated significantly in the simvastatin group by Western blot (Fig. 2D). The IHC assay further confirmed that the number of HIF-1α-positive cells was markedly reduced at the protein level in the i.o.-injected simvastatin group (Fig. 2E). These results indicated that i.o.-injected simvastatin decreased tumor hypoxia. ROS was found to increase HIF-1α in cancer cells to regulate angiogenesis and tumor growth [33]. Thus, we analyzed ROS levels in the tumors and found that ROS generation was decreased in the simvastatin group (Fig. 2F). Taken together, injection of simvastatin i.o. might affect tumor vascular normalization to improve the TME via inhibiting ROS production.

Local intraosseous injection of simvastatin suppressed breast cancer in vivo

The tumor vascular normalization strategy was proposed as an alternative approach to treat cancers. Thus, we explored the role of simvastatin in breast cancer development and compared the therapeutic effects of simvastatin among p.o., i.v. s.c., i.p. and i.o. applications.

A mouse breast cancer model was established with 4T1-luc cells, and simvastatin was applied p.o., i.v., s.c., i.p. or i.o. We measured the body weight of the mice and the tumor volume every two days for two weeks.

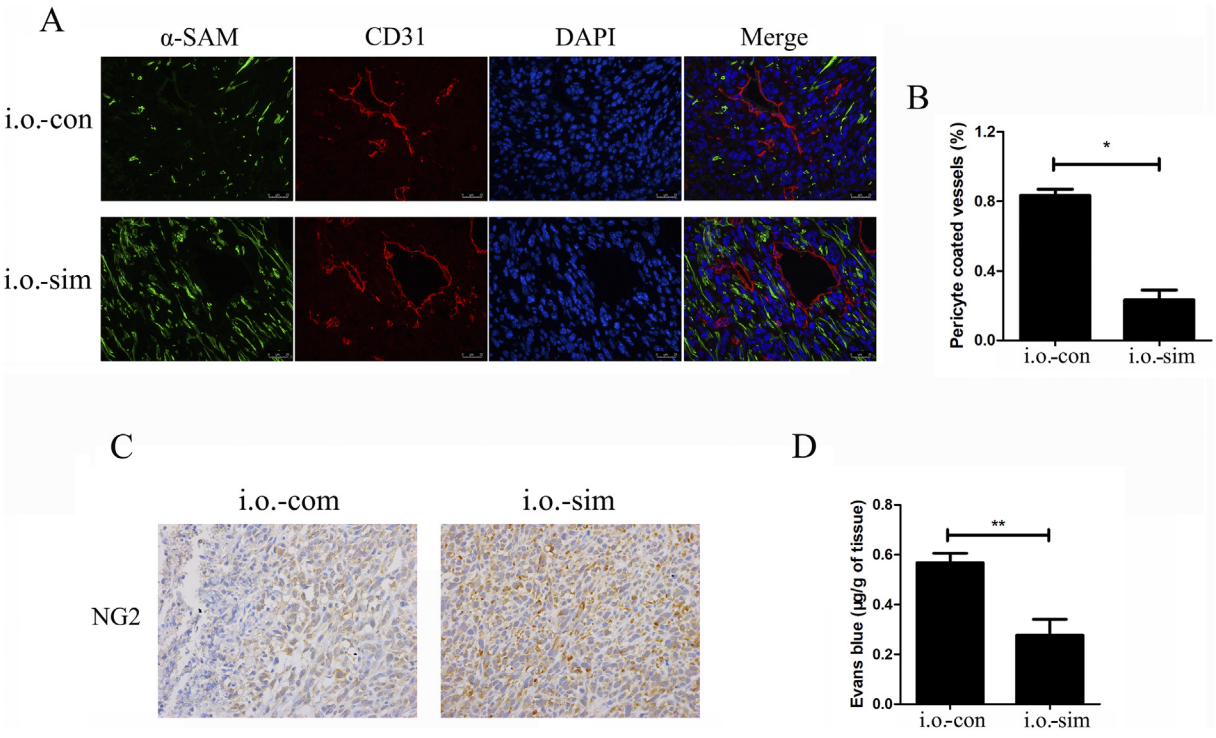

Fig. 1. Local intraosseous injection of simvastatin improved tumor vascular structure. A. Double staining for CD31 (green) and α-SMA (red) of i.o.-injected tumor tissues by immunofluorescence. B. The pericyte coverage index ($\alpha\text{-SMA}^+/\text{CD31}^+$) was calculated. C. IHC staining for NG2 in i.o.-con and i.o.-sim tumors. D. Tumor vessels permeability was analyzed by Evans blue dye. Data were presented as the mean ± s.d ($^*P < 0.05$ $^{**}P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

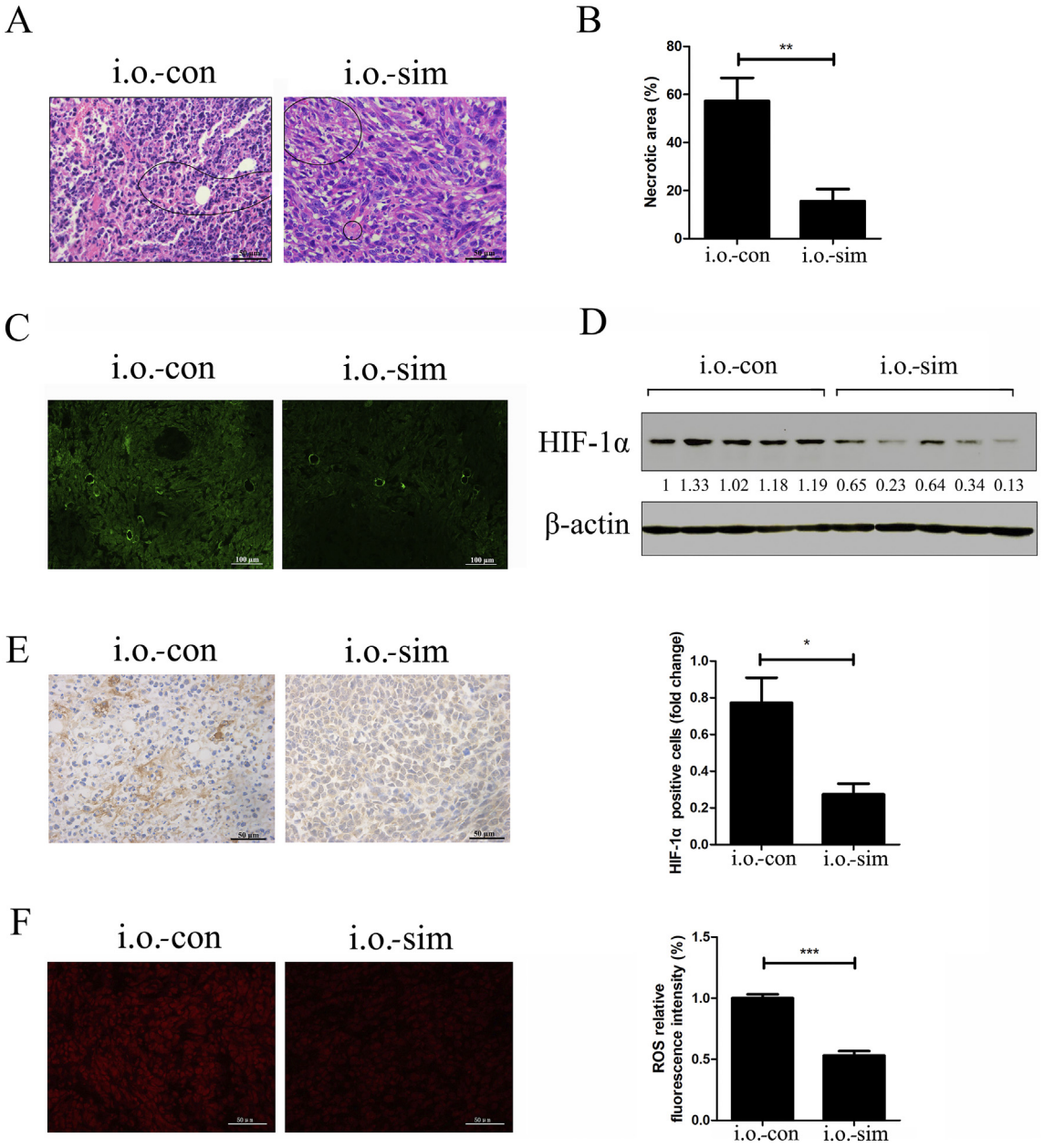

Fig. 2. Local intraosseous injection of simvastatin decreased tumor hypoxia by decreasing ROS. A. H&E staining was analyzed in the tumors of the locally i.o-injected mice (200 × magnification). B. The percentage of necrotic area was calculated. C. Hypoxyprobe-staining assay was performed by immunofluorescence analysis of tumor tissues. D. The expression of HIF-1α was analyzed by Western blot of tumor tissues. E. Representative immunohistochemistry staining for HIF-1α in tumor tissues (200 × magnification). F. Representative images of DHE (red) using a fluorescent ROS probe with locally injected tumor tissues. Data were presented as the mean ± s.d (*P < 0.05 **P < 0.01, ***$P < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

As shown in Fig. 3A–E, mouse body weight gain was obviously suppressed in all groups, except for i.o.-simvastatin group mice, which maintained a good state (Fig. 3E). In addition, only i.o. injection of simvastatin (Fig. 4J) suppressed tumor growth exhibited in tumor volume changes in Fig. 4F–J. Two weeks after simvastatin application, i.o. injection of simvastatin suppressed tumor growth in vivo as shown in the IVIS Spectrum Imaging System images (Fig. 4A) and excised tumor images (Fig. 4B–F). The tumor weights were also reduced only in the i.o.-simvastatin mice in comparison with the i.o.-control mice. However, other administration routes of simvastatin did not reduce the tumor weighs (Fig. 4G), which were consistent with the tumor images. Besides, we compared the survival of breast tumor-bearing between traditional p.o. application and i.o. application. Injection of simvastatin i.o. significantly prolonged the survival of breast tumor-bearing mice compared with i.o. control mice, but p.o. applied simvastatin did not (Fig. 4H, I). Taken together, only i.o. applied simvastatin exert these suppressive effects in breast cancer mouse model.

Locally intraosseous injection with simvastatin improved the sensitivity to chemotherapeutic drugs in breast cancer

Tumor vessel normalization increases tumor perfusion and decreases tumor hypoxia, which may improve the general response to anticancer chemotherapy [34]. To evaluate the role of simvastatin in chemotherapy drug delivery, we administered the chemotherapeutic agent doxorubicin (DOX) combined with i.o control (DOX + i.o.-con) or DOX combined with i.o. simvastatin (DOX + i.o.-sim) and determined the effect on breast cancer-bearing mice. As shown in Fig. 5A, the antitumor effect was improved by DOX + i.o.-sim, which significantly prolonged the mouse survival

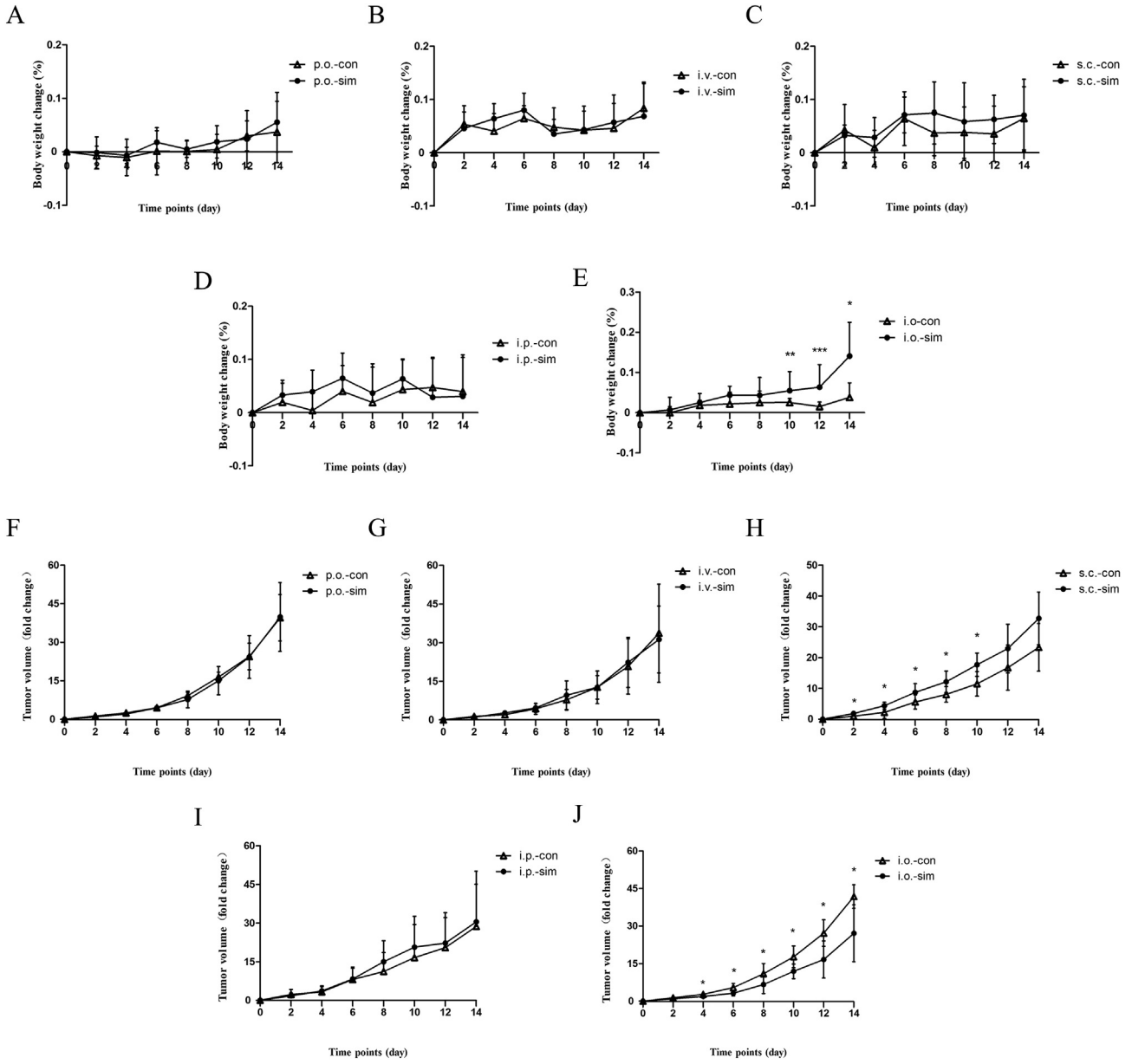

Fig. 3. Local intraosseous injection of simvastatin maintained the mice states and suppressed the tumor volume of breast cancer-bearing mice. Forty 5 to 6-week-old female Balb/c mice were inoculated with 1×10^6 4T1-luc cells into the fourth mammary fat pads and randomly divided into ten groups ($n = 10$). Ten days after inoculation, mice were respectively administered simvastatin or control via p.o., i.v., s.c., i.p. or i.o.. A-E. The change in body weight was analyzed after simvastatin application via p.o., i.v., s.c., i.p. or i.o., respectively. F-J. The tumor volume was calculated by the formula: volume = a × $b^2/2$ and analyzed after simvastatin application by p.o., i.v., s.c., i.p. or i.o., respectively.

compared with the DOX + i.o.-con group mice. H&E staining was performed and we observed that DOX + i.o. injection of simvastatin significantly reduced the tumor necrotic area in tumor tissues than the DOX + i.o.-con group (Fig. 5B). These findings were consistent with the hypothesis that local i.o. injection of simvastatin increased the efficacy of chemotherapeutic drugs in breast cancer.

Discussion

Simvastatin is widely used and its efficacy and safety have been universally acknowledged for several decades. In addition, it received over-the-counter status in numerous countries. Our previous results showed that local i.o.-injected simvastatin increased the mobilization of EPCs and osteogenic bone marrow-derived mesenchymal stem cells to peripheral blood [14,35], promoted peripheral nerve regeneration [36], and enhanced bone formation and angiogenesis [15,29]. In this study, we demonstrated that local i.o.-injected simvastatin suppressed the growth of breast cancer, while none of p.o., i.v., s.c. or i.p. application of simvastatin showed these antitumor effects. Local i.o. injection of simvastatin prolonged breast cancer-bearing mouse survival and inhibited tumor growth with tumor vessel normalization by improving tumor vascular structure, reducing tumor section necrosis, tumor hypoxia, and ROS and increasing chemotherapeutic drug sensibility. This could be a potential clinically translatable strategy for cancer therapy.

Angiogenesis is the process of new vessel formation, through which the vascular system expands during embryonic and postnatal development [37]. However, angiogenesis may be involved in tumor progression [38]. To eliminate the suspicion that i.o.-injected simvastatin may promote tumor development due to our earlier results that locally i.o.-injected simvastatin promoted angiogenesis [14,15], we established a breast cancer

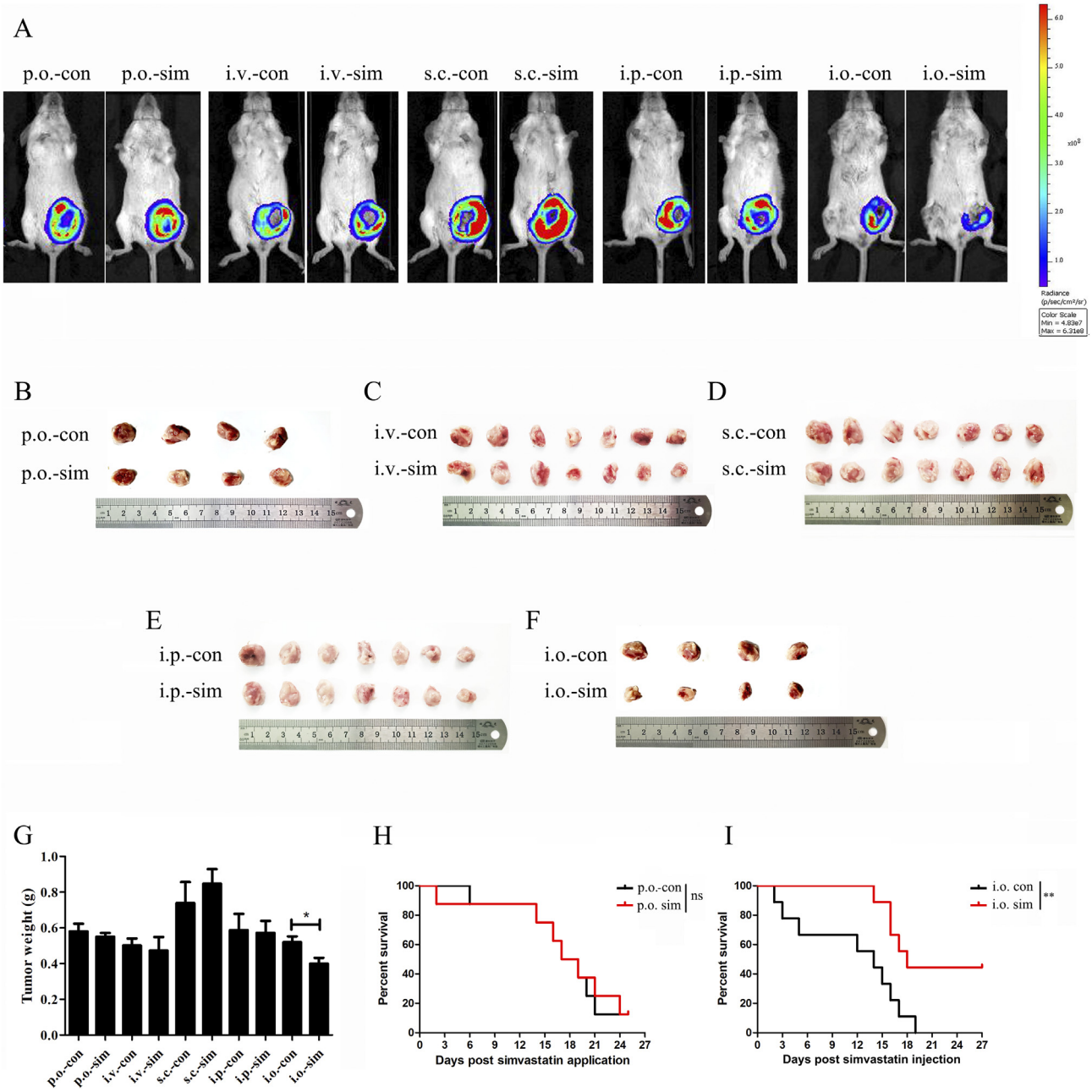

Fig. 4. Local intraosseous injection of simvastatin suppressed breast cancer. Breast tumor-bearing mice were sacrificed two weeks after simvastatin application. A. Mice were monitored in vivo by the IVIS Spectrum Imaging System. B–F. The orthotopic tumors were dissected and photographed. G. The tumor weights were analyzed. H. Kaplan-Meier analysis of mouse survival in p.o. groups. I. Kaplan-Meier analysis of mouse survival in i.o. groups ($n = 9$). Data were presented as the mean ± s.d (*P < 0.05 **P < 0.01).

model and found that i.o. injection of simvastatin suppressed tumor growth and prolonged survival with tumor vessel normalization. These results suggest that i.o. injection of simvastatin increases the number of normal vessels in tumors, which facilitates the delivery of chemotherapeutic drugs and the infiltration of immune cells to kill cancer cells.

Recently, simvastatin was reported to exhibit potential anticancer activities, such as inhibiting breast cancer, lung cancer and gastric cancer [16–19]. In this study, we found that current administrated strategies of simvastatin did not exert satisfactory tumor suppressive roles in breast cancer. It has been reported that traditionally administered simvastatin may display excellent synergistic functions combined with other therapies, such as application after surgery [39] or combination with anti-PD-1 antibody as an adjuvant [40]. In addition, some studies have reported that a lack of beneficial effects were obtained from statins treatment for cancer [41]. These may result from the low bioavailability of simvastatin. Here, we propose the novel strategy of a single i.o. injection of low-dose simvastatin to address this problem. We speculate that cells in bone marrow may be more sensitive to simvastatin, leading to enhancing the bioavailability of simvastatin to the utmost.

As a unit, the bone and bone marrow show a tight functional correlation [24]. Cancer cells cause destruction of bone, and cytokines secreted by bone marrow cells may also take part in cancer progression, during which, crosstalk is established between bone and cancer [42]. Bone marrow compartment is composed of multiple cells, such as hematopoietic

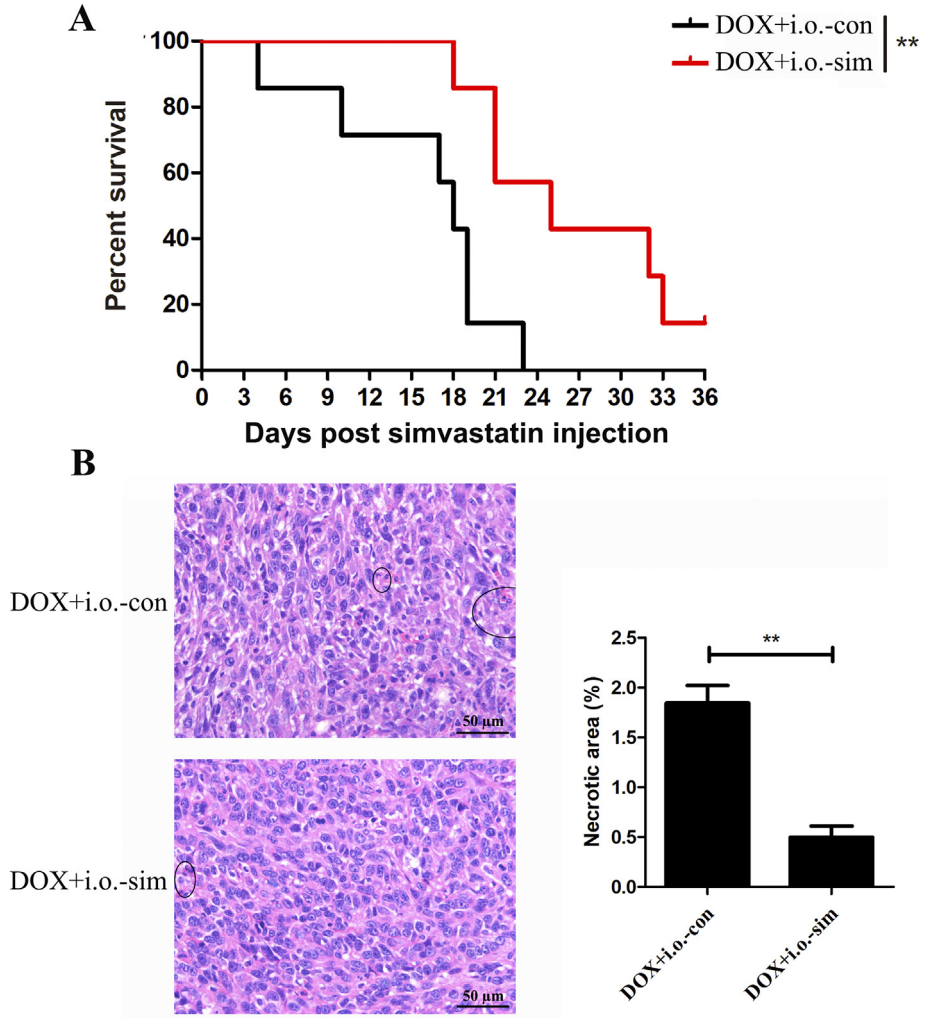

Fig. 5. Fourteen 5 to 6-week-old female Balb/c mice were inoculated with 1×10^6 4T1 cells into the fourth mammary fat pads. Ten days after inoculation, mice were administered DOX by intravenous injection and randomly divided into two groups ($n = 7$): i.o. control and i.o. simvastatin. A. Kaplan-Meier analysis of mouse survival is presented. B. H&E staining was analyzed in the tumors of the DOX + i.o.-con and DOX + i.o.-sim mice (200 × magnification) and the percentage of necrotic area was calculated (**P < 0.01).

stem/progenitor cells, mesenchymal stem cells, immune cells, osteoclasts and osteoblasts [43], and is a favorable metastatic site for cancer cells [44,45]. Signals from the bone marrow microenvironment may regulate the dormant and proliferative states of cancer cells. Hematopoietic stem/progenitor cells are recruited from the bone marrow and differentiated into tumor-supporting cells [46,47] and mesenchymal stem cells produce cytokines to protect cancer cells from immunosurveillance [44]. Therefore, targeting bone marrow activity seems to be an ideal treatment strategy for cancers. Local i.o. injection of simvastatin may regulate bone marrow-derived cells and cancer development.

Taken together, our findings suggest that local i.o. injection of simvastatin inhibits breast cancer with tumor vascular normalization, including improving tumor vascular structure, decreasing tumor hypoxia and ROS and enhancing the sensitivity of chemotherapeutic drugs. This study combines bone and vessels and provides a novel therapeutic strategy for the treatment of breast cancer.

Conclusions

In conclusion, our findings suggested that i.o. injection of simvastatin prolonged the survival of breast tumor-bearing mice, suppressed tumor growth with tumor vessel normalization, and showed clinical benefit when simvastatin was used in combination with chemotherapeutics in breast cancer. Therefore, i.o. injection of simvastatin might be a novel and promising strategy for cancer treatment.

Ethics approval and consent to participate

All animal experiments in this research were performed in accordance with currently prescribed guidelines and followed a standard protocol approved by the Biomedical Ethics Committee of Peking University (reference number: LA2017246).

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CRediT authorship contribution statement

Wanqiong Yuan: Methodology, Data curation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Bao Hai:** Methodology, Data curation. **Xiaoqing Ren:** Methodology, Investigation, Data curation. **Junxiong Zhu:** Methodology, Investigation. **Chenggui Zhang:**

Methodology, Investigation. **Zhiyuan Guan:** Methodology, Investigation. **Jialin Jia:** Methodology, Investigation. **Hong Wang:** Methodology, Investigation. **Baoshan Cao:** Conceptualization. **Chunli Song:** Conceptualization, Methodology, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2020.100867.

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