

Original Research

# Danshensu Attenuated Epithelial-Mesenchymal Transformation and Chemoresistance of Colon Cancer Cells Induced by Platelets

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## Abstract

**Background:** The interactions between platelets and tumor cells are well-known to play important roles in the progression of malignant tumors. Danshensu, a main water-soluble component of *Salvia miltiorrhiza*, can resist platelet aggregation and exert significant anti-tumor effects on various types of tumors. However, whether Danshensu could inhibit the progression of malignant tumors by suppressing the activities of platelets had not been reported. **Methods:** The effects of Danshensu on the platelet activity and epithelial-mesenchymal transformation (EMT)-like invasive phenotype of SW620 colon cancer cells were assessed by stimulating with the supernatants from co-cultured platelets and SW620 cells with direct contact (SCP). The expression and secretion of proteins were determined by western blot and enzyme-linked immunosorbent assay (ELISA), respectively. Hematoxylin and eosin (H&E) staining was performed to analyze the histopathology of tumor tissues and immunohistochemical staining was conducted to examine the protein expression in tumors. **Results:** Co-incubation of SW620 cells with platelets directly or SCP both generated long spindle-shaped invasive phenotype. Pretreatment of platelets with Danshensu (25  $\mu$ M) inhibited the morphological changes of SW620 cells induced by SCP, which was associated with the inhibitory effects of Danshensu on platelet secretion. Danshensu diminished the secretion of a list of biological factors in SCP, including interleukin (IL)-6, tumor necrosis factor alpha (TNF- $\alpha$ ), IL-1 $\beta$  and vascular endothelial growth factor (VEGF) that are all involved in tumor cell EMT and chemoresistance. Moreover, Danshensu up-regulated the expression of E-cadherin but down-regulated the levels of N-cadherin and Vimentin, resulting in the repression of SW620 cell migration. It was also shown that Danshensu enhanced the sensitivity of SW620 cells to oxaliplatin by suppressing the expression of MDR1. Furthermore, Danshensu could not only reduce the growth of subcutaneous tumors and liver metastasis that induced by SCP, but also down-regulated the expression of MDR1 *in vivo*. Mechanistic studies revealed that Danshensu suppressed the activation of the TGF- $\beta$ /Smad signaling pathway. **Conclusions:** Danshensu attenuated EMT-like characteristics and chemoresistance by inhibiting secretion capability of platelets and activation of the TGF- $\beta$ /Smad signaling pathway, suggesting that it may be optimized to be a therapeutic agent for fighting against colon cancer.

**Keywords:** platelet; colon cancer; Danshensu; epithelial mesenchymal transformation; chemoresistance

## 1. Introduction

Platelets have been associated with the progression of numerous types of solid tumors and poor prognosis in clinical practice [1]. Interestingly, there is a pathogenic feedback loop between platelets and tumor cells. In other words, tumor cells can activate platelets that play crucial roles in promoting the progression of cancer, including tumor growth, angiogenesis and metastasis [2]. Besides, there is a significant correlation between metastasis occurrence and the presence of superabundant platelets, which has often been found in colon cancer [3]. Tumor cells can activate platelets via a series of specific pathways involving integrin  $\alpha$ V $\beta$ 3 [4], Fc $\gamma$ RIIa [5], ADP, CLEC-2 and podoplanin [6]. Moreover, aspirin exerts therapeutic and prophylactic

effects on colon cancer metastasis potentially by inhibiting platelet activity [7]. Overall, suppressing platelet activation provides an effective and efficient route for the treatment of tumors [5,8].

*Salvia miltiorrhiza* has been employed to treat cardiovascular and malignant diseases for a long time [9]. Danshensu is one of the main active ingredients from *Salvia miltiorrhiza* that is traditional Chinese medicine widely used for antiplatelet aggregation and anti-tumor therapy [10]. As suggested by the screening of antiplatelet components from the aqueous extract of *S. miltiorrhiza*, Danshensu could significantly reduce platelet aggregation induced by multiple factors [11]. A large number of clinical and preclinical studies demonstrated that platelets could promote the develop-



ment of tumor cells, and classic antiplatelet drugs including aspirin was able to prohibit tumorigenesis and metastasis [12]. In addition, we previously reported that Danshensu played an important role in inhibiting metastasis in spontaneous and experimental melanoma metastasis model as well as in non-small cell lung cancer models [13,14]. Moreover, Danshensu suppressed the progression of non-small cell lung cancer via inhibiting COX-2 activity and further attenuated metastasis *in vivo*. Of note, Danshensu could directly exert its inhibitory effect on tumor cell growth only at high concentration (100  $\mu$ M Danshensu had a significant inhibitory effect on A549 cell proliferation after 48 h treatment) [14]. Although Danshensu was capable of inhibiting metastasis, whether the anti-metastatic activity was mediated by platelets remained unclear. In the present study, we aimed to clarify the role of Danshensu in tumor progression and to further elucidate whether it was associated with the inhibition of platelet activity.

## 2. Materials and Methods

### 2.1 Chemicals and Bioreagents

Danshensu (PubChem CID: 439435) was purchased from Helin Biological Engineering Co., Ltd. (Xi'an, China). Oxaliplatin (OXA) were purchased from Aladdin Biological Technology Co., Ltd. (Shanghai, China).

### 2.2 Cell Culture

SW620 human colon cancer cell line was purchased from Jiangyin Cambridge Biotechnology Co., Ltd. (Nanjing, China), and maintained in Dulbecco's modified eagle medium (DMEM) (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA).

### 2.3 Cell Viability Assay

Cell viability was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assays according to the manufacturer's protocol ( $n = 6$ ). In short, SW620 cells ( $1 \times 10^4$  cells/well) were seeded in a 96-well plate. Different concentrations of OXA were added to treat the SW620 cells for 24 h. The absorbance value was measured by a microplate reader (BioTek Instruments, Inc., Winooski, USA).

### 2.4 Preparation of Platelets and Supernatant

Platelet-rich plasma for research use only was provided by Jiangsu Province Blood Center (Nanjing, China). Platelets were prepared as described previously [15]. The platelets ( $2 \times 10^8$ /mL) were then exposed to colon cancer cells ( $1 \times 10^6$ /mL) or thrombin (0.5 U/mL) for 15 min following treatment with Danshensu or solvent (basal medium) for 30 min at 37 °C. The supernatant was filtered using a sterile 0.22-micron filter. Before incubation with the SW620 cancer cells, 10% FBS was added into the supernatants. The supernatants from the co-cultured platelets

and colon cancer cells with direct contact (SCP) and the supernatant of platelets activated by thrombin (STP) were collected according to the protocol as described previously and stored at  $-80$  °C freezer (**Supplementary Fig. 1**). This study was approved by the Institutional Ethics Committee of Nanjing University of Chinese Medicine and conducted in accordance with the principles of the Helsinki Declaration. All patients have provided written informed consent before sample collection and subsequent analysis.

### 2.5 Cell Player™ Cell Morphology Monitoring

SW620 cells were incubated with platelets, STP or SCP collected by centrifugation and filtration. The cells were maintained in the supernatants and observed every 1 h by IncuCyte ZOOM™ Live Content Imaging System (Essen BioScience Inc. (Essen) IncuCyte™, Michigan City, Indiana, USA).

### 2.6 Detection of Multiple Inflammatory Factors in Supernatants

The levels of VEGF, IL-6, IL-1 $\beta$  and TNF- $\alpha$  in the supernatants were measured by ELISA kits (Peprotech, Rocky Hill, NJ, USA) according to the manufacturer's instructions ( $n = 3$ ).

### 2.7 ATP Measurement

ATP levels in the supernatants were assessed using an ATP assay kit (Beyotime Biotechnology, China) ( $n = 3$ ). In brief, the platelets ( $2 \times 10^8$ /mL) were exposed to colon cancer cells ( $1 \times 10^6$ /mL) or thrombin (0.5 U/mL) for 15 min after treatment with Danshensu or solvent (basal medium) for 30 min at 37 °C. The supernatants were filtered using a sterile 0.22-micron filter after centrifugation. ATP detection working solution was added to a black 96-well culture plate and was incubated for 3 minutes at room temperature. Then, the supernatants were added to the wells, and the luminescence was measured immediately. The concentration of ATP in the sample was calculated based on the standard curve.

### 2.8 Cell Migration Assay

24-well Transwell migration chambers with 8  $\mu$ m pore size were purchased from Corning incorporated (Kennebunk, ME, USA). Briefly, the treated cells were re-suspended in 200  $\mu$ L serum-free culture medium ( $1 \times 10^6$ /mL). Then, 800  $\mu$ L of complete medium containing 10% FBS was supplemented to the lower chamber. After 24 h, the non-migrated and non-invasion cells were removed by a wet cotton swab. The migration and invasion of the SW620 cells were assessed using 0.1% crystal violet. The SW620 cells were counted in five randomly selected fields, and images were obtained under a microscope (200 $\times$ ) (Leica Microsystems, Wetzlar, Germany). The number of cells was calculated through the membrane, and then the migration scores of SW620 cells were examined ( $n = 3$ ).

## 2.9 Colony Formation Assay

The SW620 cells were cultured in DMEM with 10% FBS in the 6-well plates, and 500 SW620 cells were seeded into each well of the plates ( $n = 3$ ). The plates were incubated at 37 °C for 14 days, and the colony formation of the cells was assessed using 0.1% crystal violet, after which the formed colonies were photographed (Leica Microsystems, Watzlar, Germany).

## 2.10 Western Blot Analysis

Total protein was extracted as previously reported [16]. Briefly, the total protein was extracted using RIPA buffer containing 1 mM PMSF and 1:100 dilution of protease inhibitor cocktail (BestBio, Shanghai, China). The total protein was transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, USA) that was then incubated with mouse anti-human E-cadherin, N-cadherin, vimentin, TGF- $\beta$ , Smad2, phospho-Smad2, Smad3, phospho-Smad3, multidrug resistance 1 (MDR1) and GAPDH polyclonal antibodies (Cell Signaling Technology, 1:1000), followed by incubated with rabbit or mouse secondary monoclonal antibody (Cell Signaling Technology, 1:1000) ( $n = 3$ ). Finally, the protein bands were visualized with Bio-Rad Gel Doc XR+ gel imaging system (Bio-Rad, USA).

## 2.11 Histopathological and Immunohistochemical Staining

All tissue specimens were embedded in paraffin according to standard histological procedures, sectioned (4  $\mu$ m), and stained with hematoxylin and eosin (H&E) or MDR1 antibody (Cell Signaling Technology, 1:500) as described previously [16]. Then, the sections were examined and quantitatively analyzed using PerkinElmer MANTRA microscope (Mantra, PerkinElmer, USA). The mean DAB staining intensity was quantified by Mantra quantitative pathology workstation (Mantra, PerkinElmer, USA) ( $n = 4$ ).

## 2.12 Detection of ALT and AST in Serum

The whole blood sample was placed at room temperature for 2 h, centrifuged at 2500 R/min for 10 min under the condition of 4 °C, and the upper serum was separated. The full-automatic biochemical analyzer (Hitachi 7020) was used. After debugging, according to the glutamic oxaloacetic transaminase determination kit and alanine aminotransferase assay kit (Wako Pure Chemical Industries, Ltd, R1: el157; R2: el158; R1: ej715; R2: eh029) ( $n = 4$ ).

## 2.13 Animal Study

All procedures were approved by the Institutional Animal Care and Use Committee of Nanjing University of Chinese Medicine, and performed in accordance with the use of laboratory animal guidelines of Nanjing University of Chinese Medicine (Nanjing, China, Approval No.

ACU170905). The SW620 cells were pre-incubated in the SCP or the supernatants collected from co-cultured tumor cells and platelets that treated with 25  $\mu$ M Danshensu for 24 h, after which the residues were removed by centrifugation. Six-week-old male BALB/c nu/nu mice were supplied by Model Animal Research Center of Nanjing University (Nanjing, China) and housed under specific pathogen-free (SPF) conditions. Subsequently,  $2 \times 10^6$  SW620 cells suspended in a mixture of 100  $\mu$ L PBS and 100  $\mu$ L Matrigel (BD Bioscience, USA) were subcutaneously injected into each nude mouse. Control animals were subcutaneously injected with untreated SW620 cells. One week later. The mice were intraperitoneally administered with 3 mg/kg OXA on a weekly basis for 4 weeks. The length and width of tumors were measured using a digital caliper every 4 days for 28 days following tumor cell injection. The mice were sacrificed on day 28 after subcutaneous injection of SW620 cells, and the tumor and liver tissues were fixed in formalin and prepared for histopathological examination.

## 2.14 Statistical Analysis

The data were represented as mean  $\pm$  standard deviation (SD) and statistically analyzed using one-way ANOVA or Student's *T*-test. All results were analyzed by SPSS 22.0 software (SPSS Inc., Chicago, USA).  $p < 0.05$  was considered statistically significant.

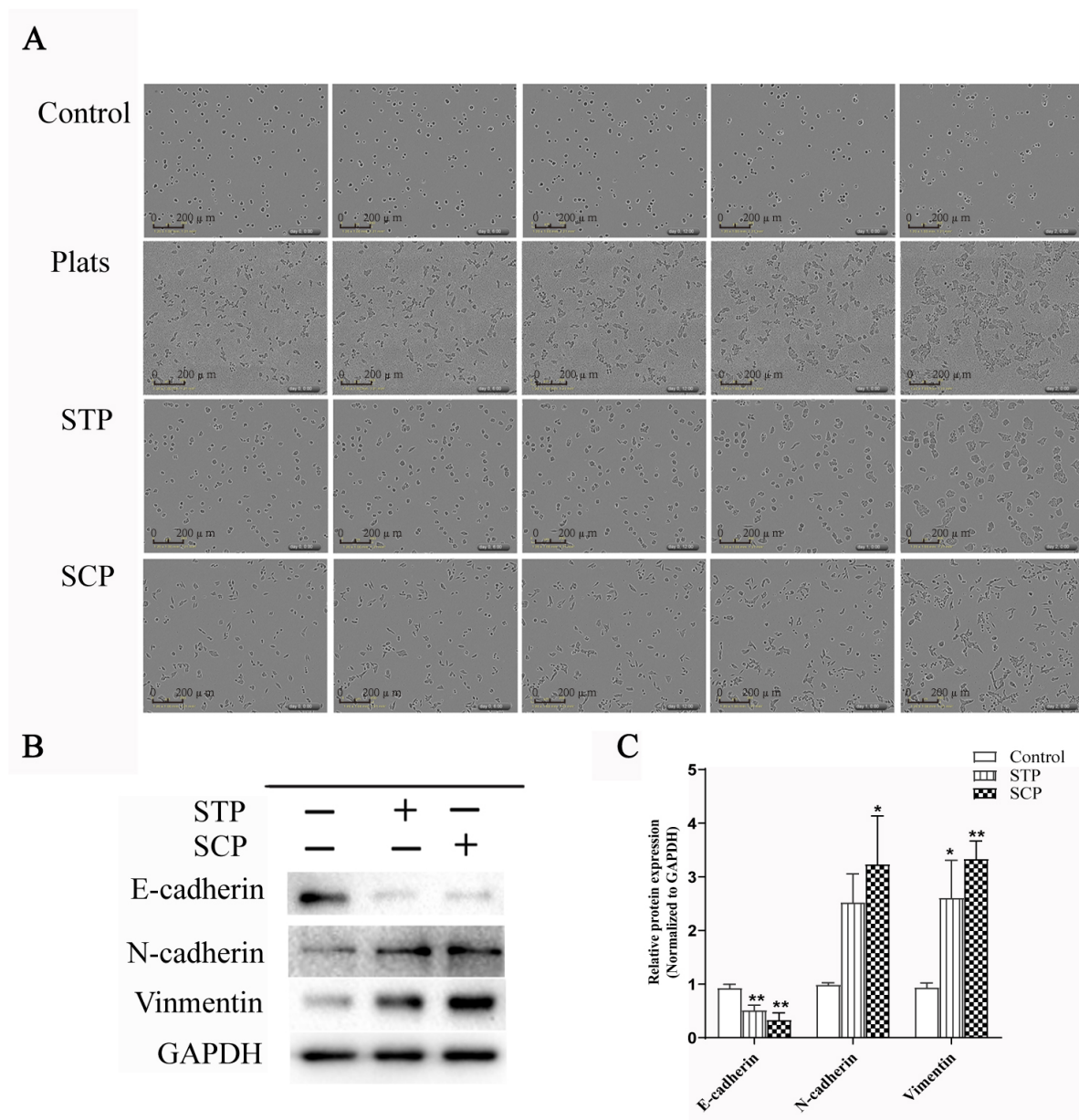
# 3. Results

## 3.1 Platelet-Colon Cancer Cell Interactions Played a Pivotal Role in EMT-Like Invasive Phenotype

It has been well accepted that platelets can induce EMT-like transition and promote metastasis *in vitro* [17]. We observed the morphological changes of SW620 cells by IncuCyte Zoom, a real-time dynamic imaging system. SW620 cells underwent EMT-like morphological changes following the treatment of SCP, STP or platelets for 24 h (Fig. 1A). SW620 cells incubated with SCP had pronounced EMT-like phenotypic changes compared with those incubated with STP. Further, the expression of E-cadherin was down-regulated while the levels of N-cadherin and Vimentin in the SW620 cells were significantly up-regulated following the treatment of SCP for 24 h compare with control (Fig. 1B,C). These data indicated that SW620 exerted significant effects on activating secretion ability of platelets and platelet secretion stimulated the formation of an EMT-like invasive phenotype.

## 3.2 Danshensu Inhibited Platelet Activation Mediated by Colon Cancer Cells and Prevented EMT-Like Invasive Phenotype

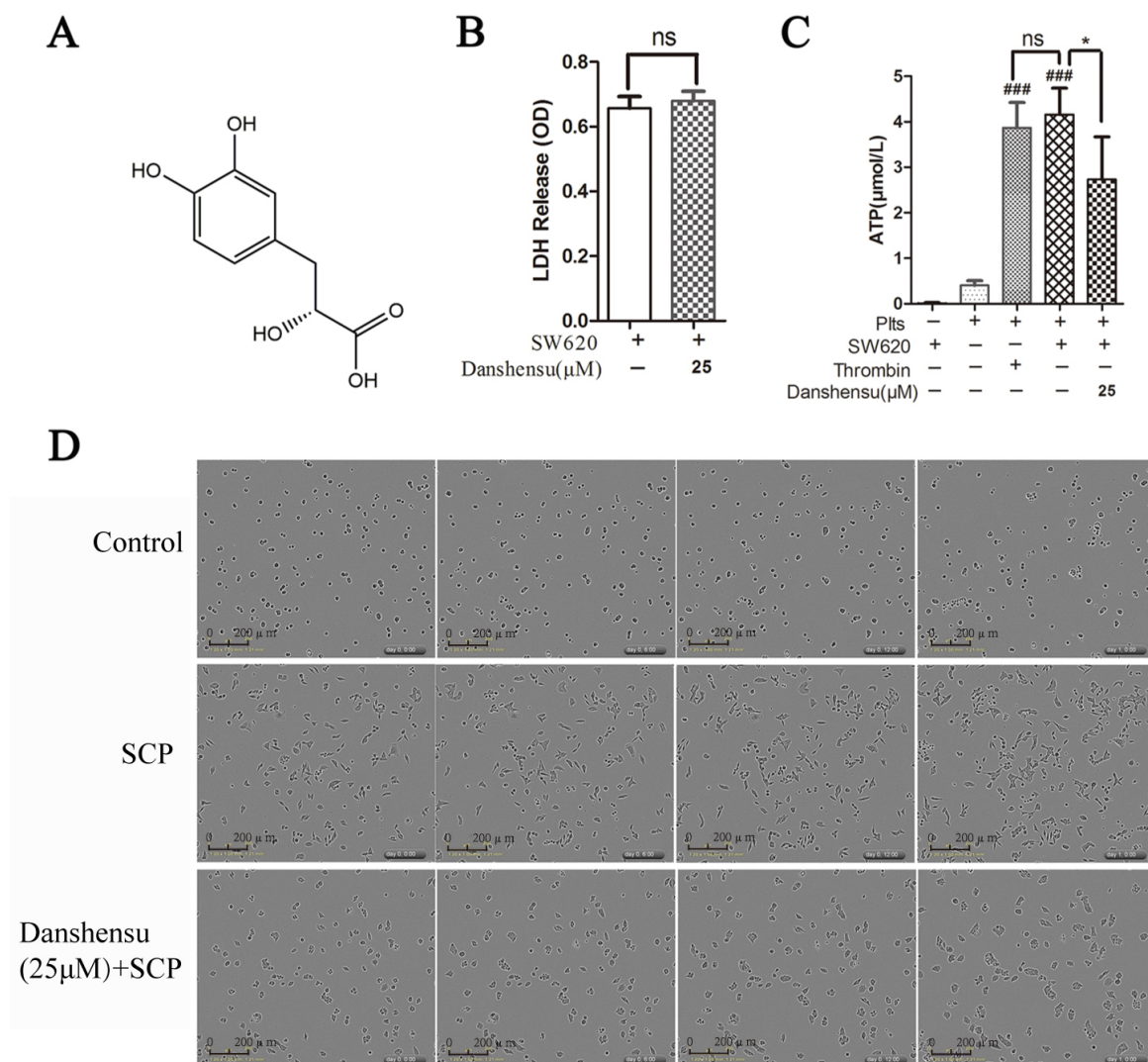
Danshensu was reported to suppress platelet adhesion and aggregation by inhibiting cyclooxygenase-2 (COX-2) and thromboxane B2 [10] (Fig. 2A). We first explored the effects of Danshensu on the proliferation and membrane permeability of SW620 cancer cells by MTT and LDH re-



**Fig. 1. SW620 cancer cells treated with SCP induced an EMT-Like invasive phenotype.** (A) Morphological changes of SW620 cancer cells incubated with platelets, STP or SCP for 48 h. Scale bar indicates 200  $\mu$ m. (B,C) Western blot bands of E-cadherin, N-cadherin and Vimentin in the SW620 cells treated with platelet secretions for 24 h ( $n = 3$ ). The relative expression of E-cadherin, N-cadherin and Vimentin was normalized to GAPDH. Data were represented as mean  $\pm$  SD. \*  $p < 0.05$ , \*\*  $p < 0.01$  versus control.

lease assays (**Supplementary Fig. 2** and Fig. 2B). Strikingly, 25  $\mu$ M Danshensu attenuated the ATP secretion from platelets induced by SW620 colon cancer cells, whereas 25  $\mu$ M Danshensu did not have obvious impacts on the cytotoxicity of SW620 cells (Fig. 2B,C, **Supplementary Fig. 2**). Subsequently, we observed the morphological changes of SW620 cells after incubation with SCP and Danshensu using IncuCyte Zoom, and detected the expression levels of EMT-related proteins by Western blot assay (Fig. 2D). After SW620 cells were stimulated with SCP, the invasive ability of the cells, as well as the expression levels of N-cadherin

and Vimentin were all increased (Fig. 3A,B). However, the levels of E-cadherin were up-regulated while the expression of N-cadherin and vimentin were significantly down-regulated in Danshensu treated group compared with those in the SCP group (Fig. 3C,D). Taken together, the EMT phenotype was reversed in the presence of Danshensu. We next explored the effect of Danshensu on cell migration using Transwell assay in the SW620 cells. The results showed that the platelets treated with Danshensu was able to attenuate the migration of SW620 cells induced by SCP ( $p < 0.001$ , Fig. 3A,B).



**Fig. 2. Danshensu interfered with the interactions between platelets and colon cancer cells.** (A) The structure of Danshensu. (B) LDH release assay showing the effects of Danshensu on the cytotoxicity of SW620 cells ( $n = 3$ ). (C) ATP levels in the supernatants were assessed using an ATP assay kit ( $n = 3$ ). (D) Microscopic images of the SW620 cells in the absence or presence of SCP. Data were represented as mean  $\pm$  SD from three independent experiments. ###  $p < 0.001$  versus control, \*  $p < 0.05$  versus SCP group.

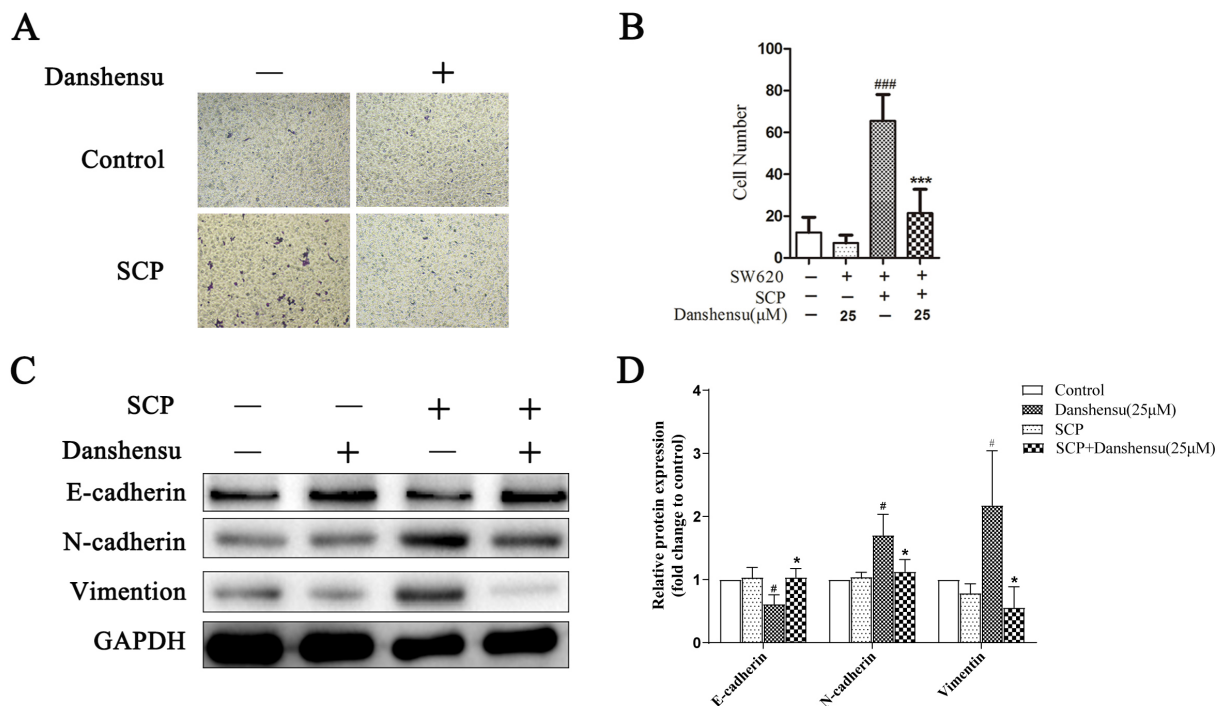
### 3.3 Danshensu Diminished SCP-Induced Clonogenic Survival and Chemoresistance

Chemoresistance is a common problem in cancer treatment. Fischer *et al.* [18] reported that EMT phenotype increased tumor cell chemoresistance, eventually leading to tumor development and difficulty in cancer treatment. OXA is used for colorectal cancer treatment, but the development of chemoresistance is inevitable in clinical practice [19]. Drug efflux protein MDR1 is a main marker for the drug chemoresistance in the colorectal cancer cells [20]. Therefore, we examined the role of SCP in the chemoresistance of SW620 cells. Compared with the control group, SCP group significantly increased the clonogenic survival of SW620 cancer cells. However, prophylactic adminis-

tration of Danshensu onto platelets significantly decreased the clonogenic survival ( $p < 0.001$ , Fig. 4A,B). MTT assay showed that the resistance of SW620 cells to OXA was increased following the stimulation of SCP (Fig. 4C). Nevertheless, intervening with platelets with Danshensu alleviated SCP-induced chemoresistance and MDR1 protein expression (Fig. 4C–E).

### 3.4 Danshensu Antagonized EMT and Chemoresistance Through Limiting Tumor Cell-Induced Platelet Secretion and Repressing the Activation of TGF- $\beta$ Signaling Pathway

It has been known that platelets contain numerous growth factors and cytokines, and the secretions of co-cultured tumor cells and platelets play a crucial role in

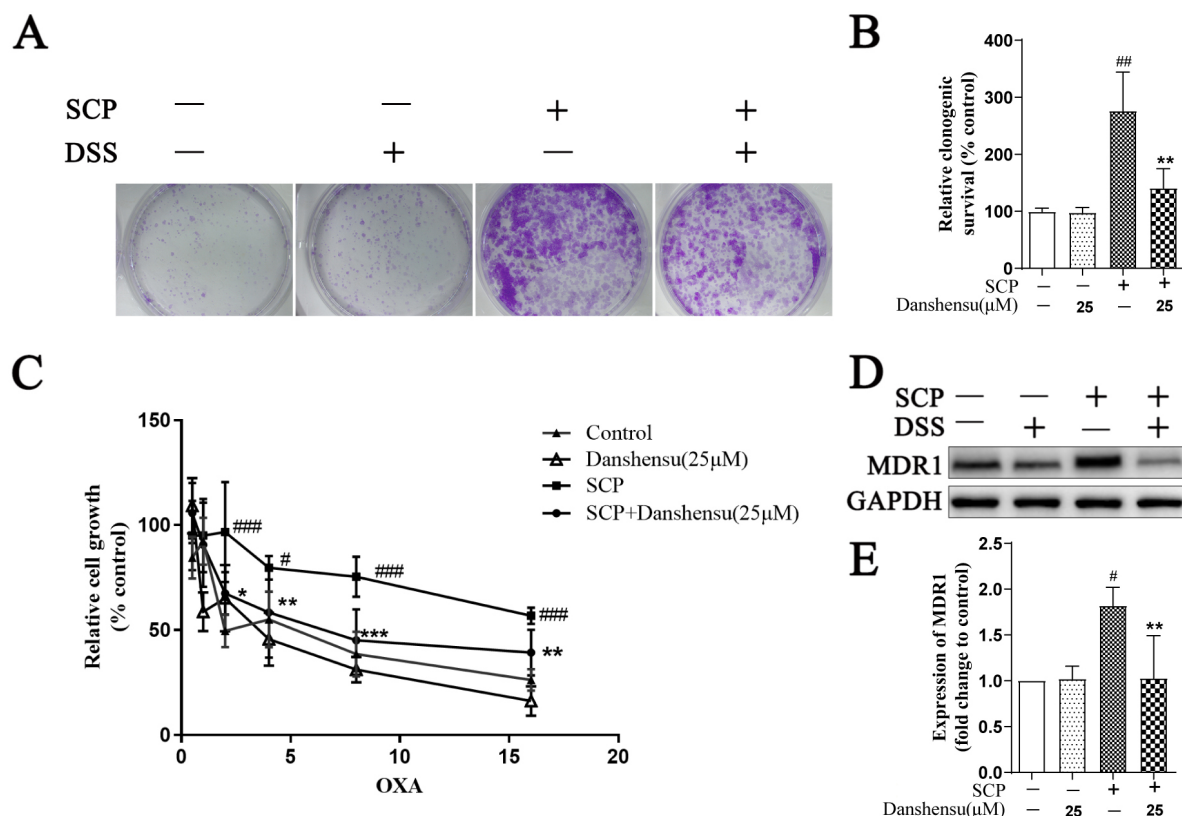


**Fig. 3. Danshensu attenuated the migration of SW620 cells mediated by platelets.** (A,B) The effect of Danshensu on migration of SW620 cells using Transwell. The results were represented as mean  $\pm$  SD from three independent experiments ( $n = 3$ ). (C,D) Western blotting analysis of E-cadherin, N-cadherin and Vimentin in the SW620 cells treated with SCP or Danshensu for 24 h ( $n = 3$ ). Data were represented as mean  $\pm$  SD from three independent experiments. #  $p < 0.05$ , ###  $p < 0.001$  versus control group. \*  $p < 0.05$ , \*\*\*  $p < 0.001$  versus SCP group.

the process of EMT. Notably, we previously reported that a variety of secreted factors from platelets were involved in the malignant progression of tumor cells [15]. In the present study, we examined the levels of various cytokines in the supernatants by ELISA assay. Our results demonstrated that the levels of IL-6, TNF- $\alpha$ , IL-1 $\beta$  and VEGF were increased following the co-culture of tumor cells and platelets. We uncovered that Danshensu administration inhibited the production of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  in the co-cultured tumor cells and platelets, which was associated with the inhibition of the TGF- $\beta$ /Smad signaling pathway (Fig. 5). Additionally, inactivation of the TGF- $\beta$ /Smad signaling pathway suppressed EMT and chemoresistance. To reveal the mechanisms underlying Danshensu limited EMT and chemoresistance, we further investigated whether the TGF- $\beta$ /Smad signaling pathway was regulated by SCP. Compared with those in the control group, the levels of TGF- $\beta$ , p-Smad2 and p-Smad3 in the SW620 cells were significantly increased in the SCP group (Fig. 5E,F). More interestingly, prophylactic administration of Danshensu onto platelets decreased the levels of TGF- $\beta$ , p-Smad2, and p-Smad3 in the SW620 cells (Fig. 5E,F).

### 3.5 Danshensu Retarded the Progression of SCP-Stimulated SW620 Tumors *in vivo*

To demonstrate the role of Danshensu in the progression of SW620 tumors *in vivo*, SCP-stimulated SW620 cancer cells were subcutaneously injected into mice, and the growth of tumors after intraperitoneal administration of oxaliplatin (OXA) was monitored accordingly (Fig. 6A). It was observed that SCP-stimulated SW620 cancer cells were insensitive to OXA and the volume of SCP-stimulated tumors were significantly increased compared to that of the control group (Fig. 6B). However, prophylactic administration of Danshensu boosted the sensitivity of SW620 cancer cells to OXA, and the tumor volume was strikingly decreased following the intervention of Danshensu compared with SCP alone ( $p < 0.01$ , Fig. 6B). Moreover, all mice in the SCP group displayed remarkable liver metastasis and the percentage of liver metastasis was significantly up-regulated compared with that of control group ( $p < 0.001$ , Fig. 6C,D). Surprisingly, prophylactic administration of Danshensu onto platelets prevented the liver metastasis of SCP-stimulated SW620 cancer cells ( $p < 0.001$ , Fig. 6C,D). Additionally, MDR1 levels in the primary tumors were detected by immunohistochemistry. It was revealed that the expression of MDR1 was profoundly impaired following the treatment of Danshensu, in comparison to SCP alone ( $p < 0.001$ , Fig. 6E,F). In addition, liver metastasis can



**Fig. 4. Danshensu diminished SCP-induced clonogenic survival and chemoresistance.** (A,B) The effect of Danshensu on SW620 cell survival using colony formation assay (n = 3). (C) MTT assay showing the chemoresistances of SW620 cells to OXA following the stimulation of SCP (n = 6). (D,E) Western blotting analysis of MDR1 expression level in the SW620 cells, GAPDH was used as a loading control (n = 3). Data were represented as mean  $\pm$  SD from three independent experiments. <sup>#</sup> $p < 0.05$ , <sup>##</sup> $p < 0.01$ , <sup>###</sup> $p < 0.001$  versus control group. <sup>\*</sup> $p < 0.05$ , <sup>\*\*</sup> $p < 0.01$ , <sup>\*\*\*</sup> $p < 0.001$  versus SCP group.

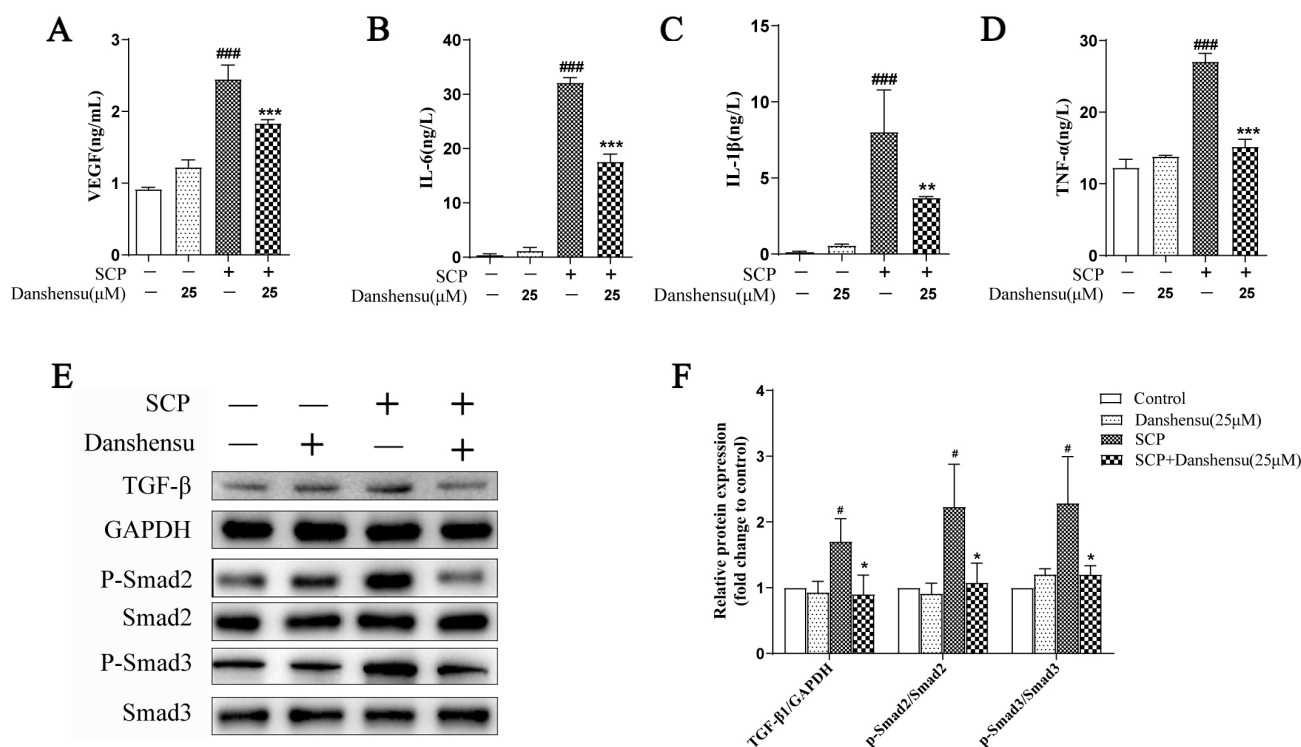
cause the increase of ALT and AST (Fig. 6G,H). Therefore, SW620 cells incubated with SCP can induced chemoresistance and facilitated metastasis *in vivo*, which could be reversed in the presence of Danshensu.

## 4. Discussion

Platelets are small, enucleated cells which are abundant in blood, participating in various physiological and pathological processes of organisms, such as hemostasis, wound healing, inflammatory response, thrombosis, organ transplant rejection and tumor development [21]. Platelet granules, including  $\alpha$ -granules and dense granules, store hundreds of mediators that essentially regulate platelet functions and secrete them after platelet activation to be involved in disease development [22]. In tumor microenvironment, platelets and their granular contents influence cancer progression, and result in abnormal secretion of platelets [23]. Labelle *et al.* [17] reported that direct platelet-tumor cell contact synergistically promoted tumor cell transition to an invasive mesenchymal-like phenotype. However, whether such EMT phenotype is induced by direct contact between platelets and tumor cells or secretions from the

co-cultured platelets and tumor cells remains elusive. Our study compared the results of direct incubation of tumor cells with platelets to those of incubation with SCP based on real-time dynamic monitoring of tumor cell morphologies. Interestingly, SW620 cancer cells underwent obvious EMT-like morphological changes after the stimulation of SCP compared with the control. In addition, the impacts of platelet secretion on tumor cells was evaluated using thrombin as a platelet activator. Given that thrombin-activated platelet secretion also promoted tumor cell EMT, inhibition of platelet secretion might play a pivotal role in suppressing EMT process.

As an aromatic carboxylic acid, Danshensu is one of the most abundant active phenolic acids in the dried root of *S. miltiorrhiza* Bunge (Lamiaceae), which is a widely used traditional Chinese medicine. The effects of Danshensu on platelet aggregation and thrombosis have been well documented [10], and it exerts potential antitumor and anti-angiogenic effects by inhibiting platelet adhesion and promoting tumor microcirculation [13,24]. Our group also demonstrated the inhibitory effects of multiple components from *S. miltiorrhiza* on tumor progression [25].



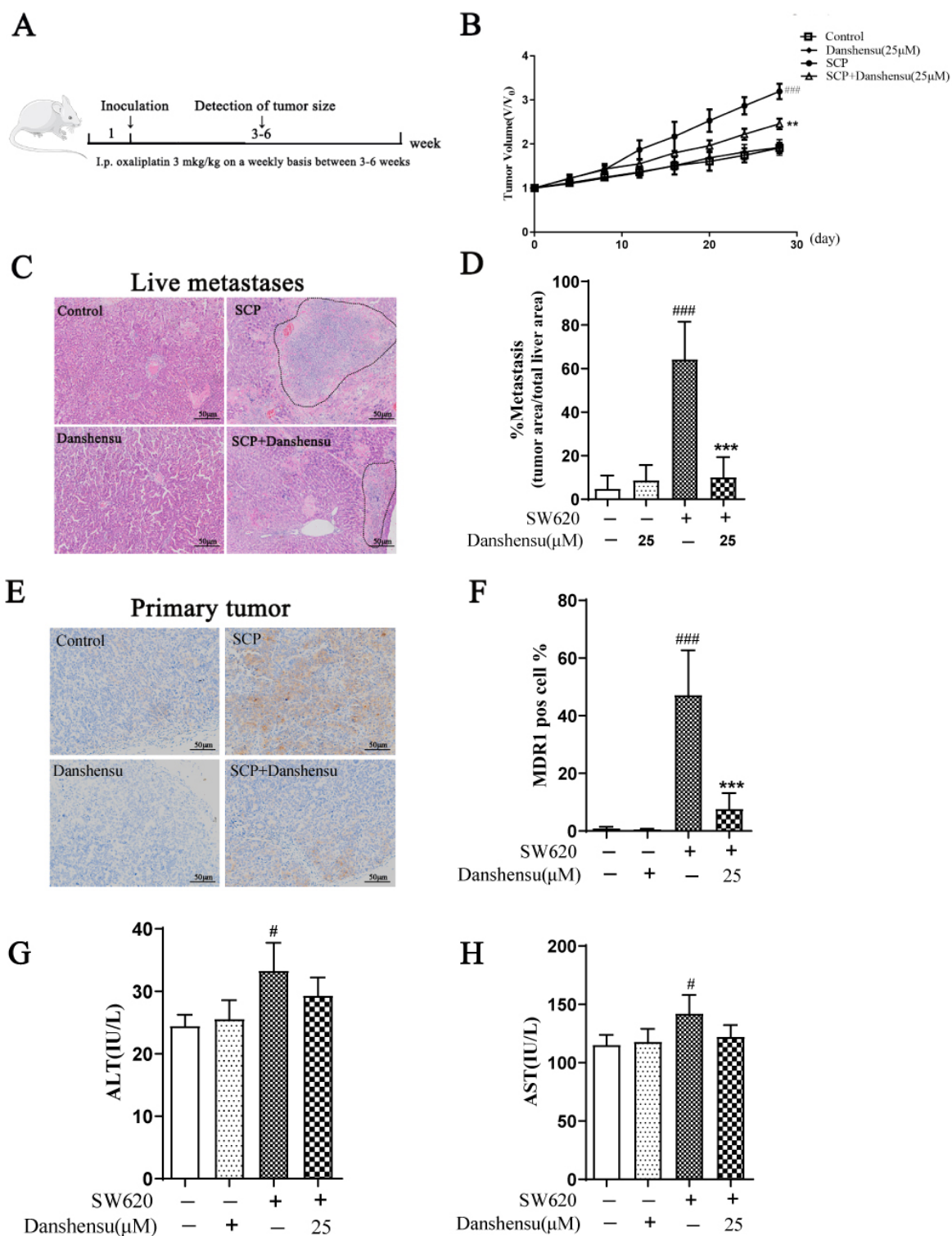
**Fig. 5. Danshensu modulated the cytokine secretion from platelets induced by SW620 cancer cells.** (A–D) The levels of VEGF (A), IL-6 (B), IL-1 $\beta$  (C) and TNF- $\alpha$  (D) in the secretions of platelets interacting with tumor cells by ELISA (n = 3). (E,F) Western blotting analysis of TGF- $\beta$ , p-Smad2, Smad2, p-Smad3 and Smad3 expression levels in the SW620 cancer cells. GAPDH was used as a loading control (n = 3). Data were represented as mean  $\pm$  SD from three independent experiments. # $p$  < 0.05, ### $p$  < 0.001 versus control group. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 versus SCP group.

More specifically, Danshensu significantly prevented tumor metastasis *in vivo* [14]. Although Danshensu presented remarkable antiplatelet and antitumor effects, whether it was involved in platelet-mediated development of malignant tumors still required in-depth studies.

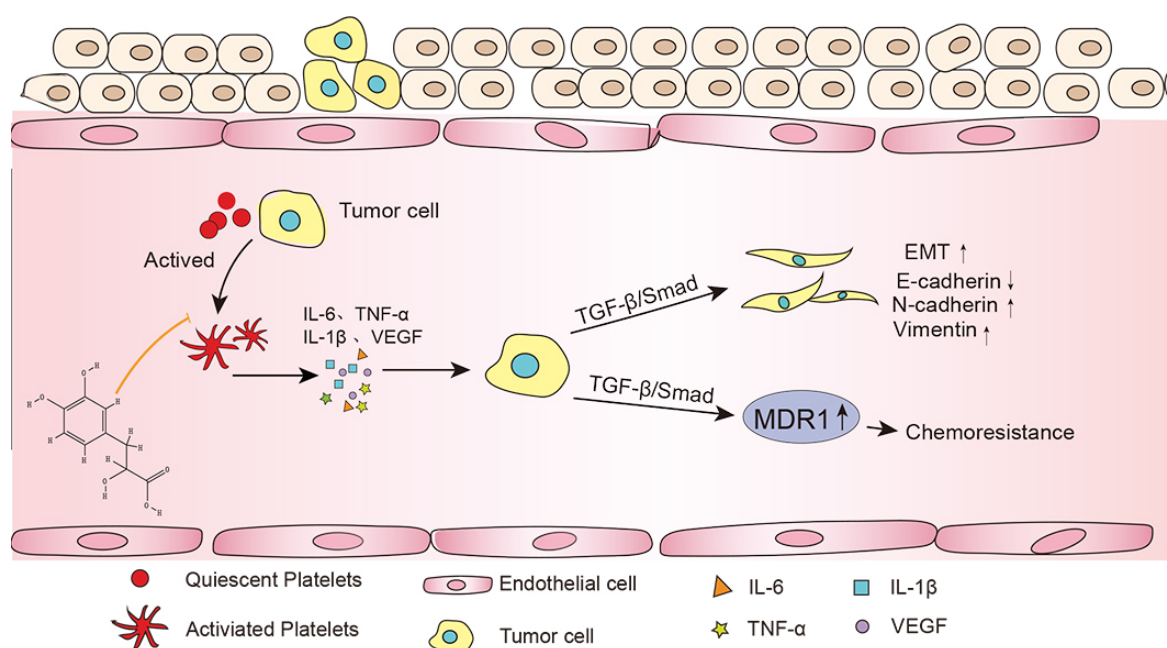
In the present study, we found that Danshensu inhibited the effects of SCP on the EMT and migration of tumor cells by intervening with platelets (Fig. 3), and the secretions of VEGF, IL-6, IL- $\beta$  and TNF- $\alpha$  in the co-cultured platelets and SW620 were reduced after Danshensu intervention (Fig. 5). Additionally, Danshensu prohibited SCP-induced tumor cell chemoresistance, and the expression of MDR1 was decreased after the treatment of Danshensu compared with the SCP group (Fig. 4).

It was reported that EMT enhanced the chemoresistance of tumor cells [18]. The level of TGF- $\beta$  was shown to be closely correlated with EMT, chemoresistance and poor survival outcomes of colon cancer patients [26]. In our study, TGF- $\beta$  was decreased after Danshensu treatment compared with SCP group. Of note, the expression of TGF- $\beta$  was required for the development of cancer, and the decreased level of TGF- $\beta$  was related to Danshensu inhibiting platelet-mediated EMT and chemoresistance in the SW620 cancer cells. The TGF- $\beta$ /Smad signaling pathway participated in the regulation of cell proliferation, survival, angiogenesis and chemoresistance to antitumor therapy [27]. In addition, the Smad2/3 phosphorylation also dramatically contributed to the induction of EMT phenotype of cancer cells [28]. We herein found that the phosphorylation of Smad2/3 was significantly attenuated after following the treatment of Danshensu compared with SCP alone (Fig. 5). We also uncovered that the co-culture supernatant-stimulated tumor cells showed conspicuous chemoresistance both *in vitro* and *in vivo* (Figs. 4,6). Besides, the expression of MDR1 was significantly increased in the primary tumors compared with control group. However, the intervening effects of Danshensu on platelets reduced the chemoresistance of SW620 cancer cells mediated by SCP, which was consistent with what we observed *in vitro* (Fig. 6). Notably, the supernatant-stimulated tumor cells were more prone to metastasize to livers *in vivo*. Nevertheless, in the presence of Danshensu, the metastatic foci in the liver were significantly reduced compared with control (Fig. 6). Furthermore, Danshensu contributed to profound decrease in the growth of tumors and the expression of MDR1 in tumors following the treatment of OXA.

Aspirin is currently used in clinical tumor prevention and incorporated into clinical oncology guidelines due to its striking antiplatelet activity. Moreover, it was also documented to inhibit platelet-induced EMT of circulating tu-



**Fig. 6. Danshensu retarded the progression of SCP-stimulated SW620 tumors *in vivo*.** (A) Schematic diagram of animal experimental procedure. (B) Growth curve of SW620 tumors following different treatments ( $n = 4$ ). (C) H&E staining of the livers. (D) Quantification of liver metastasis ( $n = 4$ ). (E) Immunohistochemical staining of MDR1 expression in the primary tumors ( $n = 4$ ). Scale bar indicates  $50 \mu\text{m}$ . (F) Quantification of MDR1 expression in tumors ( $n = 4$ ). Scale bar indicates  $50 \mu\text{m}$ . (G,H) Detection of ALT and AST levels in serum ( $n = 4$ ). Data were represented as mean  $\pm$  SD from four independent experiments. ###  $p < 0.001$  versus control group. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  versus SCP group.



**Fig. 7. Tanshinol reduced the interaction between platelets and tumor cells and inhibits tumor metastasis.** The interaction between tumor cells and platelets can secrete a large number of cytokines (IL-6, IL-1 $\beta$ , TNF- $\alpha$ , VEGF, etc.). On the one hand, these cytokines can activate the expression of MDR1 and promote chemoresistance of tumor cells. Moreover, they can promote the activation of TGF- $\alpha$ /SMAD pathway and the metastasis of tumor cells. In our study, Danshensu can inhibit the interaction between tumor cells and platelets, and reduced the cytokines such as IL-6, IL-1 $\beta$ , TNF- $\alpha$  and VEGF in SCP. Danshensu also can inhibit the expression of MDR1 and TGF- $\alpha$ /Smad pathway, then affecting the chemoresistance and metastasis of tumor cells.

mor cells [29]. Since aspirin could significantly inhibit the malignant development of colon cancer in the case of long-term and low-dose administration, antiplatelet activity was thought to be extremely important for the adjuvant treatment of malignant tumor development [30]. Targeted inhibition of platelet activity has certain inhibitory effects on the EMT and chemoresistance of tumor cells [31,32].

In our study, although 25  $\mu$ M of Danshensu could not directly inhibit the invasion of SW620 cancer cells, it was able to interfere with the activation of platelets by SW620 cancer cells and achieved the effects of reducing platelet-induced SW620 cancer cell migration and chemoresistance. The pretreatment of 25  $\mu$ M Danshensu attenuated the release of IL-1 $\beta$ , IL-6, VEGF and TNF- $\alpha$  in the co-cultured platelet-tumor cell system, which reduced the effect of platelets on EMT and drug resistance of tumor cells. The underlying mechanism that Danshensu interfered with the interactions between platelets and tumor cells may be associated with the decreased activation of TGF- $\beta$ /Smad signaling pathway. Therefore, Danshensu may emerge as an effective compound that potentially exerts anti-tumor activity through interfering with platelets.

## 5. Conclusions

In conclusion, our study showed that Danshensu resulted in the reduced production of a series of pro-inflammatory cytokines (e.g., IL-1 $\beta$ , IL-6, TNF- $\alpha$  and

VEGF) stimulated by tumor cells. These pro-inflammatory factors could promote the EMT process of cancer cells, leading to the progression of cancer cells. Taken together, Danshensu suppressed tumor metastasis by inhibiting the interactions between platelets and tumor cells, as well as blocking the TGF- $\beta$ /Smad signaling pathway induced by SCP (Fig. 7).

## Author Contributions

AW and YZ conceived, designed and led the project. YC, KL, YX and YW performed the experiments. YC and AW analyzed the data. AW contributed reagents, materials, and analysis tools. YZ and YC wrote the manuscript with input from all authors. All authors have read and approved the final manuscript.

## Ethics Approval and Consent to Participate

All procedures were approved by the Institutional Animal Care and Use Committee of Nanjing University of Chinese Medicine, and performed in accordance with the use of laboratory animal guidelines of Nanjing University of Chinese Medicine.

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## Conflict of Interest

The authors declare no conflict of interest.

## Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/j.fbl2705160>.

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