

Original Article

Scutellarin suppresses human colorectal cancer metastasis and angiogenesis by targeting ephrinb2

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Abstract: Tumor induced angiogenesis is an attractive target for anti-cancer drug treatment. Scutellarin, which is a native compound derived from *scutellaria altissima* leaves, has already been proved to possess anti-tumor activities. Nevertheless, their effects in colorectal cancer metastasis and angiogenesis have not been evaluated. In order to reveal the anti-angiogenic and anti-metastasis capacity of scutellarin, wound healing and Transwell chamber inserts invasion were done in colorectal cancer cells, and cell proliferation as wells colony formation were conducted to identify the proliferation inhibition of colorectal cancer in vitro. The growth inhibition of scutellarin was further definite by a mouse colorectal xenograft model in vivo. Herein, we demonstrated scutellarin suppressed colorectal cancer cell viability and colony formation in vitro, and remarkably reduced tumor growth in vivo mouse xenografts. Additionally, scutellarin restrained colorectal cancer cells-induced angiogenesis, inhibited human umbilical vascular endothelial cells (HUVECs) migration, tube formation of HUVECs, and micro-vessel formation in chick embryo chorioallantoic membrane (CAM) assay. Altogether, our results exhibited the evidence that scutellarin inhibit colorectal cancer angiogenesis and metastasis via targeting ephrinb2 signaling, with the potential of an anti-tumor agent for cancer treatment.

Keywords: Colorectal cancer, scutellarin, metastasis, angiogenesis, ephrinb2

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related mortality worldwide [1]. The 5 year survival rate approximate 90% in patients with early stage CRC, whereas the rate less than 20% for those patients with metastatic CRC. Hence, colorectal cancer metastasis is the main determinant of survival of patients [2]. Currently, there are no effective therapeutic strategies that selectively inhibit colorectal cancer cells metastasis. Therefore, the exploitation and optimization of a safe and effective agent that be able to directly inhibit CRC metastasis remains a definite goal. It is well known that that there is a wide variation in cancer morbidity among different countries [3]. On account of historically consumed a traditional

diet high in soy; the incidence of clinical CRC in Asians is low. However, the incidences of CRC in Asians who immigrate to the United States and adopt a Western diet increased. These findings suggest that diet habits influence the CRC morbidity, and soy consumption may be in part responsible for reducing the morbidity of CRC [4]. Further, a great deal of epidemiologic evidence implies that soy-rich diet is relevant to an overall low mortality of patients with CRC.

Across series of experimental models, scutellarin has been certified possess anti-cancer activity, typically relative to suppression of cell growth and/or induction of tumor cells apoptosis [5]. In CRC, scutellarin inhibits cell proliferation, and triggers G2/M phase cell cycle arrest and induces apoptosis [6]. In addition, scutel-

larin epigenetic modulate DNA, including DNA promoter methylation and histone modification, bringing about altered expressions pattern of miRNA [7]. However, the effects of scutellarin are in a concentration dependent manner and the most of these effects are observed in conjunction with the high concentrations of scutellarin [8]. At lower concentrations, scutellarin has been proven to inhibit prostate cancer cell motility and metastasis. Nevertheless, the detail role of scutellarin in other cancers remains to be amplified [9]. Therefore, we conducted the current study to reveal whether scutellarin inhibit human colorectal cancer growth and metastasis. Furthermore, owing to the complexity of cancer cells metastasis and our goal to successfully target it in cancer treatment, we attempted to analyze the basic molecular mechanisms of scutellarin in anti-cancer.

Cancer cell metastasis is a complex process, which consists of a cascade of sequential steps involving cellular mobility, invasion adhesion, movement through the circulatory system, and re-implantation within a separate organ. In the metastasize process, cancer cells need conquer three major obstacles. First: cancer cells attachment to the extracellular matrix, such as the basement membrane. Second: cells secrete proteases that induce local degradation of the extracellular matrix. Third: cancer cells migrate and invasion through matrix. Increased cell migration and invasion are essential characteristic of the metastatic phenotype, and are necessary for successfully initiate the metastatic cascade. Based on above, the ability to inhibit cancer cells migration and invasion would remarkably prevent the metastasis. In the present study, we demonstrated the suppressive activities of scutellarin on colorectal cancer cells growth, angiogenesis and metastasis, and revealed the effects of scutellarin on those processes was partly through inhibiting ephrinb2 signaling.

Methods

Cells culture and cell proliferation assay

Colorectal cancer cell lines (SW620, HCT116, LOVO and HT29) were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM or 1640 medium sup-

plemented with 10% FBS. MTT assay was conducted to assess cells viability after scutellarin treatment. Cells (1000 cells/well) were seeded into 96-well plate. After 24 h, fresh medium containing various concentrations of scutellarin was added and cultured for another 24 h. Cells absorbance were assessed using a Varioskan™ Flash Multimode Reader (Thermo Fisher Scientific, MA, USA) at 490 nm. A constitutively active ephrinb2 plasmid was provided by Dr. Michael Tsang. Transfection was performed with lipofectamine 2000 (Thermo Fisher Scientific, MA, USA).

Colony formation assay

Cells were pretreated with scutellarin for 24 h. Then cells (1000 cells/well) were seeded into 6-well plate. After two weeks, the cell colonies were stained with 0.1% crystal violet and the visible cell colonies were counted [10].

Transwell invasion assay

Cells were pre-treated with 0.5, 1 and 2 μ M scutellarin for 24 h. Then 3,000 cells were seeded into the upper chamber of Transwell, which was pre-coated with Matrigel. Media with 10% FBS was placed into the lower chamber of Transwell. After culture for 24 h, non-invaded cells on the upper surface of the membrane were removed and cells on the lower surface of the membrane were stained with 0.1% crystal violet. Photographs of 5 random fields from each group were recorded, and the number of the invaded cells was counted [11].

Wound healing assay

Cells were pre-treated with 0.5, 1 and 2 μ M scutellarin for 24 h. Then, the confluent monolayer of cells was wounded by tip. Fresh media, containing either scutellarin or DMSO, were then added. For each well, 3 pictures were taken with a microscope at two time points (0 and 24 h) after scratching. The wound closure was represented by the percentage of the non-covered wound area base on the wound area at 0 h [12].

Tube formation assay

1×10^4 /well HUVECs were seeded into 96-well plates that pre-coated with Matrigel. HUVECs were seeded in wells that containing different

culture media from control HCT116, or cells treated with various concentrations of scutellarin. The HUVECs were incubated for 6 h, and tube formation ability was quantified by counting the total number of complete tubes per well [13].

Angiogenesis assay on chicken chorioallantoic membrane (CAM)

Fertilized chicken eggs were obtained from chicken house of Nanjing Qinglongshan (Nanjing, Jiangsu, China). On day 8, an air sac was created and a small window in the shell was cut. HCT116 cells culture medium were re-suspended in sterile filter paper. Then, papers were placed onto the CAM. The implanted paper was photographed 5 days after implantation, and the branching of blood vessels was counted [14].

Murine model of colorectal cancer cells metastasis

All the experiments involving animals were reviewed and approved by Animal Care and Use Committee of Nanjing University of Chinese Medicine. Balb/c nude mice were purchased from Vital River Laboratories (Beijing, China). 3×10^6 HCT116 cells were subcutaneously injected on each flank and mice were separated into four groups including, vehicle control, 5, 10 and 20 mg/kg/d scutellarin. The tumor volume was calculated as $0.5 \times (\text{width})^2 \times (\text{length})$ and the weight of orthotopic tumor was measured. Formalin-fixed paraffin-embedded tumor tissues were subjected to immunohistochemical staining with Ki67 and CD34. In vivo metastasis analysis, HCT116 cells were injected into Balb/c mice via the tail veins. Mice were randomly divided into 2 groups and administrated with vehicle or 10 mg/kg scutellarin. After daily administration for 21 days, mice were sacrificed and lungs were fixed and conducted to H&E staining for counting metastasis loci [15].

PCR array

Human tumor metastasis PCR array was provided by Kangchen Bio-tech Inc (Shanghai, China). RNA was extracted using TRIZOL® reagent (Invitrogen, Carlsbad, CA, USA), and followed by synthesis into cDNA using SuperScript. The mixture of cDNA and a 2 × Super array PCR master mix was added into the the PCR array

plate containing the gene-specific primer sets. The ΔCt value of each metastasis-associated gene in each group was calculated and the differential expression of each gene was measured according to $\Delta\Delta\text{Ct}$. The fold-change in difference between the scutellarin treated cells and DMSO treated control cells were compared.

Western blotting

Protein samples were prepared using RIPA lysis buffer. Proteins were separated by 10% SDA-PAGE and were transferred to PVDF membrane. After blocking with 5% non-fat milk at RT, the PVDF was incubated with anti-GAPDH (CST, Danvers, MA, USA) or anti-ephrinb2 (Santa Cruz, Delaware, CA, USA) at 4°C overnight. Goat anti-rabbit secondary antibody (Boster, Wuhan, Hubei, China) was used to incubate the PVDF for 1 h and enhanced chemiluminescence was used to visualize target proteins in BIO-RAD ChemiDoc XRS Imaging system.

Statistical analysis

Results were presented as the mean \pm SD and analyzed by two-sided Student's t test. *P*-values less than 0.05 considered statistical significant.

Results

Scutellarin inhibits CRC cell growth and metastasis in vitro

To evaluate the anti-cancer effect of scutellarin in CRC, four colon cancer cell lines (HCT116, LOVO, SW480 and HT29) were used in cells proliferation assays. As shown in **Figure 1A**, we found that scutellarin inhibited CRC cells proliferation in a dose responsive manner. The IC₅₀ values from each CRC cell line were calculated, showing that scutellarin exerted 50% inhibition in four CRC cell lines under 10 μM . We further corroborated its anti-cancer effect by performing colony formation analysis in vitro. After 24 h pre-treatment with various concentrations of scutellarin, the cells were cultured for 14 days. As depicted in **Figure 1B**, scutellarin markedly suppressed HCT116 and LOVO cells colonies formation in a concentration dependent manner. In order to determine the effect of scutellarin on both HCT116 and LOVO cell migration and invasion in vitro, HCT116 and LOVO cells

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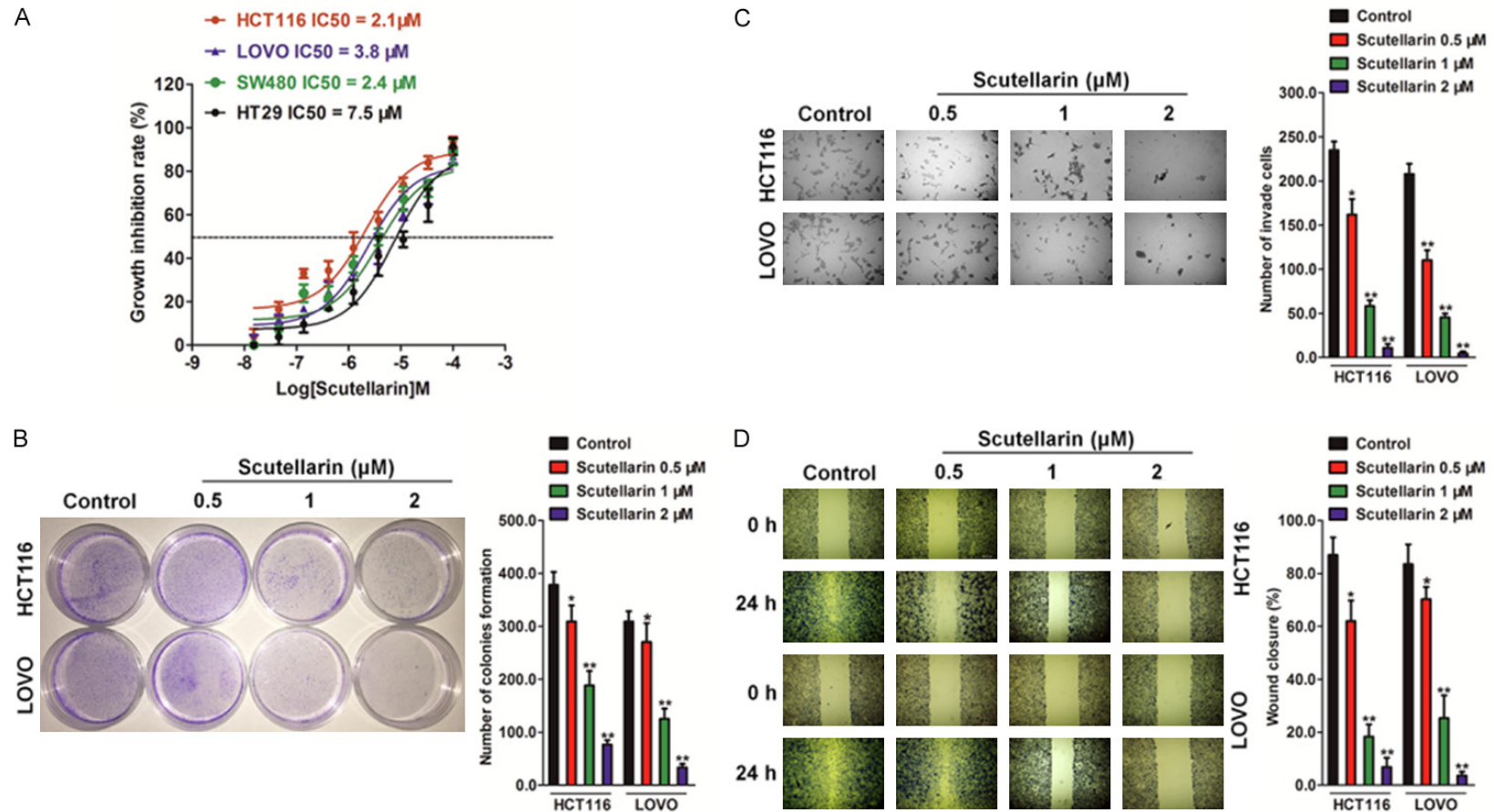


Figure 1. Scutellarin inhibits CRC cell growth and mobility. A. HT29, HCT116, LOVO and SW480 cells were exposed with scutellarin, and cell viability were measured. Data are the mean \pm SD. B. Scutellarin inhibited colony formation of CRC cells. One day after pre-treatment various concentrations of scutellarin, cells were plated into 6-well plate cultured for 14 days and cells colonies were counted. Data represented the mean \pm SD. $*p < 0.05$, $**p < 0.01$ compared to control. C. Transwell analysis was conducted to determine the invasion of CRC cells. Cells invaded the Transwell membrane were counted. $*p < 0.05$, $**p < 0.01$ compared to control. D. Cells were pre-treated with scutellarin for 24 h, the wound were created and percentage of wound closure over 24 h was measured. $*p < 0.05$, $**p < 0.01$ compared to control.

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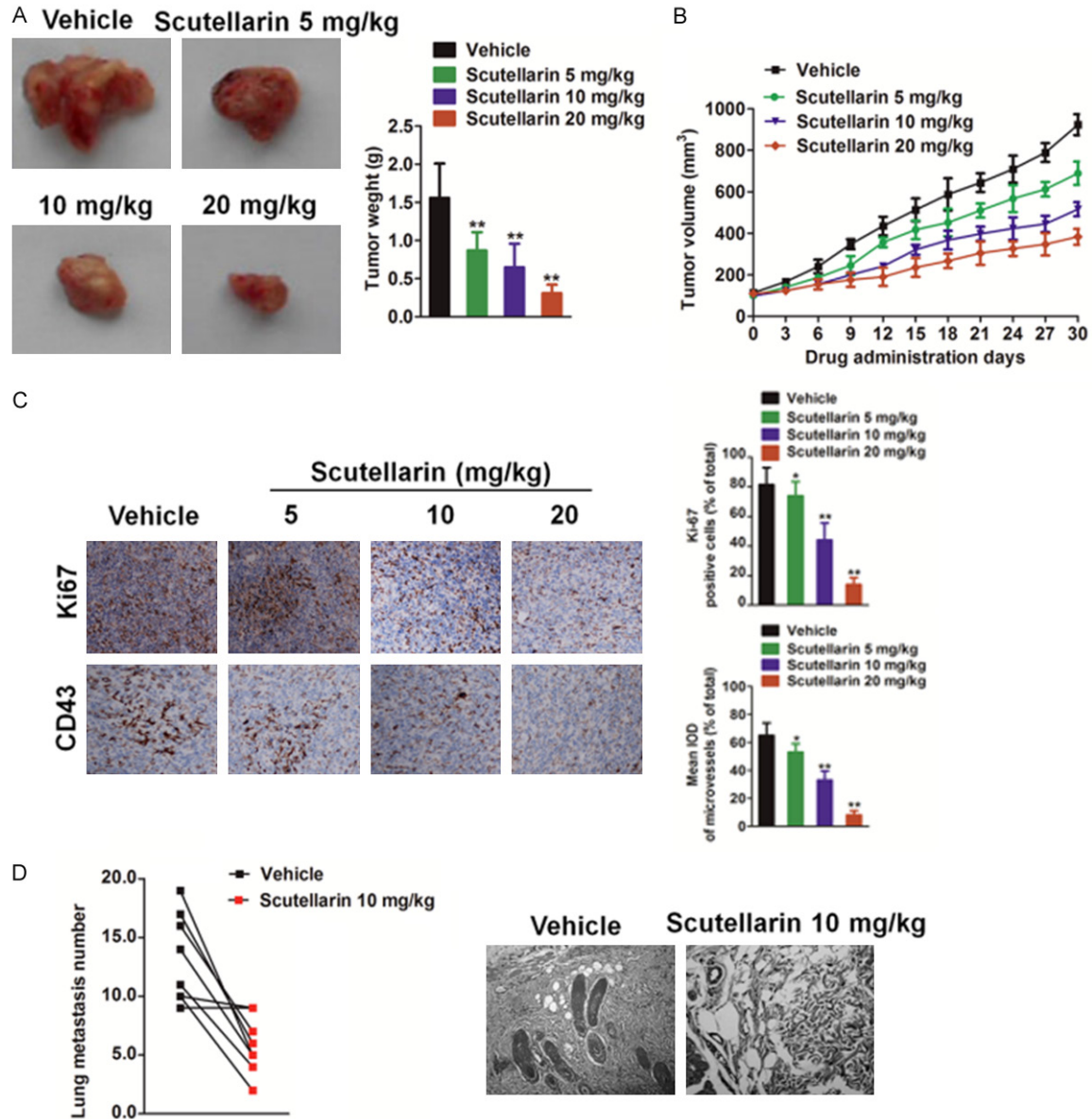


Figure 2. Effect of scutellarin on tumor growth and angiogenesis in vivo. A. Treatment with scutellarin was initiated when tumor volumes reached 100 mm³. Tumor weights were measured at the end of the experiment. B. 1×10^6 HCT116 cells were s.c. injected and nude mice were oral administrated with the scutellarin. Xenograft tumor volumes were measured three times a week by a caliper. C. Immunohistochemical analysis showed that scutellarin inhibited Ki67 positive cells in colorectal cancer xenografts (upper panel). Immunohistochemical analysis showed that scutellarin inhibited CD34-positive blood vessels in colorectal cancer xenografts (lower panel). * $p < 0.05$, ** $p < 0.01$ vs. vehicle. D. Representative picture of the lungs from mice injected intravenously with HCT116 cells after treatment with vehicle or 10 mg/kg scutellarin. * $p < 0.05$, compared to vehicle.

were treated with scutellarin. As shown in **Figure 1C**, scutellarin remarkably inhibited both HCT116 and LOVO cells invasion in vitro. Furthermore, we confirmed the effects in the wound closure analysis. As shown in **Figure 1D**, the wound assay result suggested that scutellarin significantly inhibits wound closure. Altogether, these results demonstrated that scutel-

larin inhibited CRC cells proliferation, mobility and invasion in vitro.

Scutellarin suppresses CRC cells growth and metastasis

Next, human HCT116 CRC nude mouse xenograft model was subjected to validate the results in vitro. As shown in **Figure 2A** and **2B**,

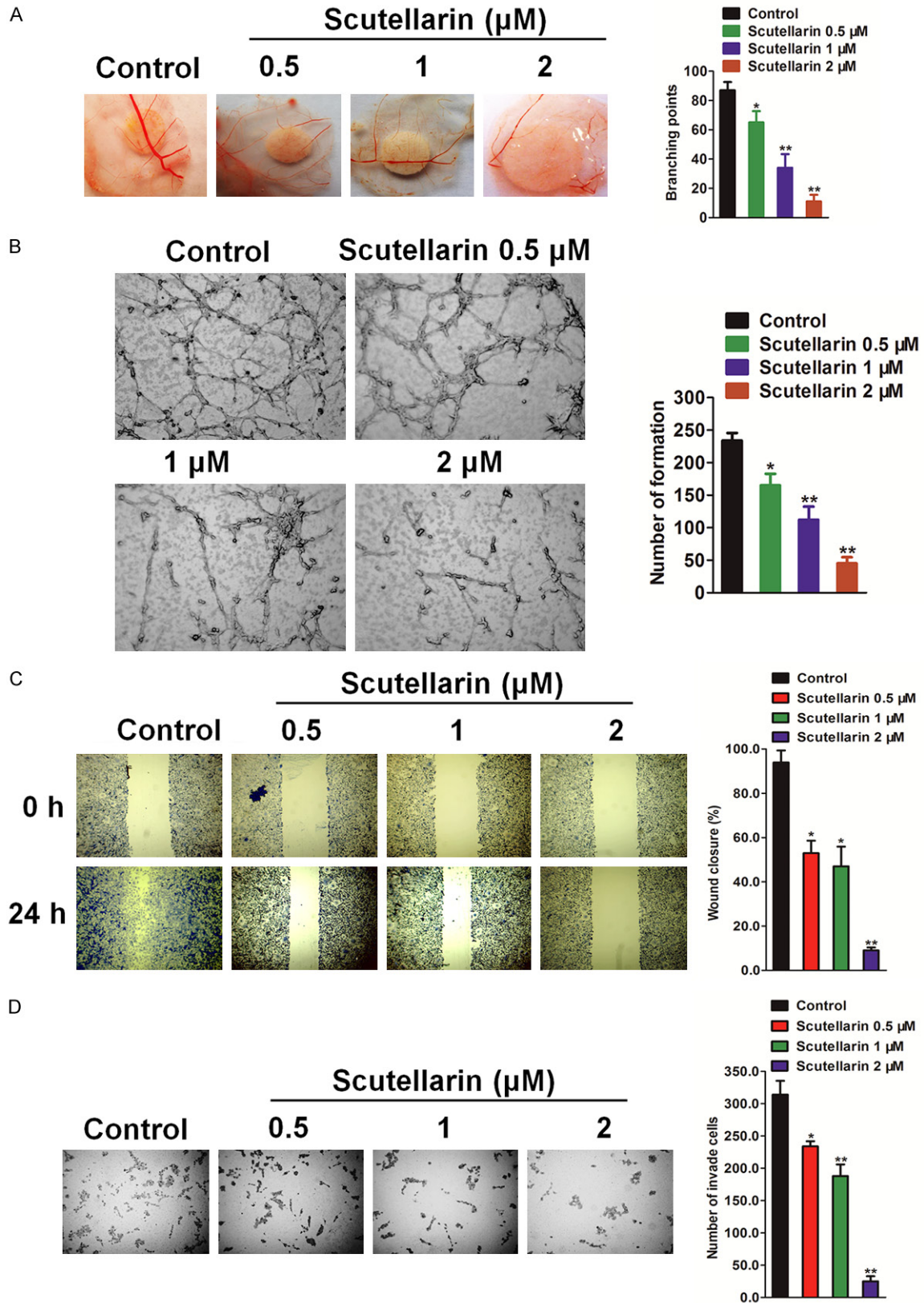


Figure 3. Effect of scutellarin on angiogenesis and tube formation. A. Effect of scutellarin on the angiogenesis in CAM. In the control group, the blood vessels grew normally. Treated with various concentrations of scutellarin, CAM

Scutellarin suppresses colorectal cancer metastasis and angiogenesis

blood vessels were significantly inhibited. B. Effect of scutellarin on the tube formation. In the control group, HUVECs cells formed the tube structure on the Matrigel. In the scutellarin treated group, tube formation was inhibited in dose-dependent manner. Data are expressed as means \pm SD. * $p < 0.05$, ** $p < 0.01$, compared to the control group. C. HUVECs were plated to six-well plate. A scratch was created and cells were treated with CM of HCT116 cells treated or un-treated with scutellarin. The percentage of wound closure was quantified. D. Scutellarin inhibited the invasion of HUVECs induced by the CM of HCT116 cells treated or un-treated with scutellarin. The results shown were representative of three independent experiments. * $p < 0.05$; ** $p < 0.01$, compare to control.

HCT116 tumor-bearing mice treatment with scutellarin exhibited tumor growth inhibition. We further determined the cell proliferation marker (Ki67) and the tumor angiogenesis biomarker (CD34) in orthotopic tumor tissues of by immunohistochemical. Our results suggested that the percentage of Ki67 positive cells was significantly decreased after scutellarin treatments (**Figure 2C**). Meanwhile, scutellarin remarkably reduce microvessel density in tumor tissues, as reflected by decreased CD34 staining (**Figure 2C**). The anti-metastasis effect of scutellarin was evaluated with artificial metastasis model in vivo. After cells injection, mice were treated with vehicle and 10 mg/kg scutellarin every day and continued for 21 days. At the end of this experiment, the lungs were excised and the hematoxylin-eosin staining assay was employed. Quantification analysis of the number of pulmonary metastasis loci revealed that numbers of metastatic nodules in mice treated with scutellarin were decreased as compared to control mice (**Figure 2D**).

Scutellarin inhibited tumor induced angiogenesis

Chorioallantoic membrane (CAM) angiogenesis analysis and tube formation assay were performed to identify the effect of scutellarin on angiogenesis. As compared to the control, scutellarin (0.5-2 μ M) inhibited the CAM angiogenesis (**Figure 3A**). In the control group, the blood vessels grew normally whereas in the scutellarin-treated group, no new capillary vessels were generated. Furthermore, HUVEC cells were treated with cultural medium (CM) from CRC cells that pre-treated with DMSO or scutellarin. As shown in **Figure 3B**, scutellarin markedly decreased the number of the tube structure at the concentrations of 0.5-2 μ M respectively. Chemotactic migration of endothelial cells is a core step in the angiogenic process. To assess the roles of scutellarin on endothelial cell migration stimulated by CM of colorectal cancer cells, we scraped the cell monolayers of HUVECs and performed the wound closure

assay. As shown in **Figure 3C**, stimulation by the CM from control HCT116 cells increased HUVECs migration whereas CM from scutellarin treated HCT116 cells inhibited the migration of HUVECs in a dose-dependently manner. As expected, Transwell invasion results demonstrated that CM of colorectal cancer cells significantly induced HUVECs invasion in vitro, and this effect was significantly impaired by CM from scutellarin treated HCT116 cells (**Figure 3D**).

Identification of angiogenesis-related genes affected by scutellarin

The anti-cancer effects mediated by the scutellarin on CRC cells were further investigated by analyzing the expression of a panel of genes which participate into the tumor metastasis. As shown in **Figure 4A**, the levels of 26 different gene transcripts were analyzed by applying a quantitative RT-PCR array on HCT116 cells treated or un-treated with scutellarin. Among the 26 genes, 13 genes exhibited a down-regulation as compared to the untreated control cells and the most significantly down-regulation gene was ephrinb2. Subsequently, the gene ephrinb2 was further determined at protein level by immunoblotting (**Figure 4B**). To ascertain the potential function of ephrinb2 in scutellarin-regulated CRC cells metastasis and angiogenesis, constitutively active form of ephrinb2 was transfected into HCT116 cells and the level of ephrinb2 was assessed by immunoblotting with anti-ephrinb2 antibody (**Figure 4C**). As shown in **Figure 4D**, active ephrinb2 largely rescued the impaired migration in HCT116 cells treated with scutellarin. In addition, HUVECs cell migration and tube formation analysis were conducted to identify the role of ephrinb2 in the effect of scutellarin on angiogenesis. As a result, scutellarin-inhibited migration (**Figure 4E**) and the tube formation of HUVECs (**Figure 4F**) were abolished by over-expression ephrinb2. Altogether, these results indicated that ephrinb2 was involved in scutellarin suppressed the metastasis of CRC cells and tumor angiogenesis.

Scutellarin suppresses colorectal cancer metastasis and angiogenesis

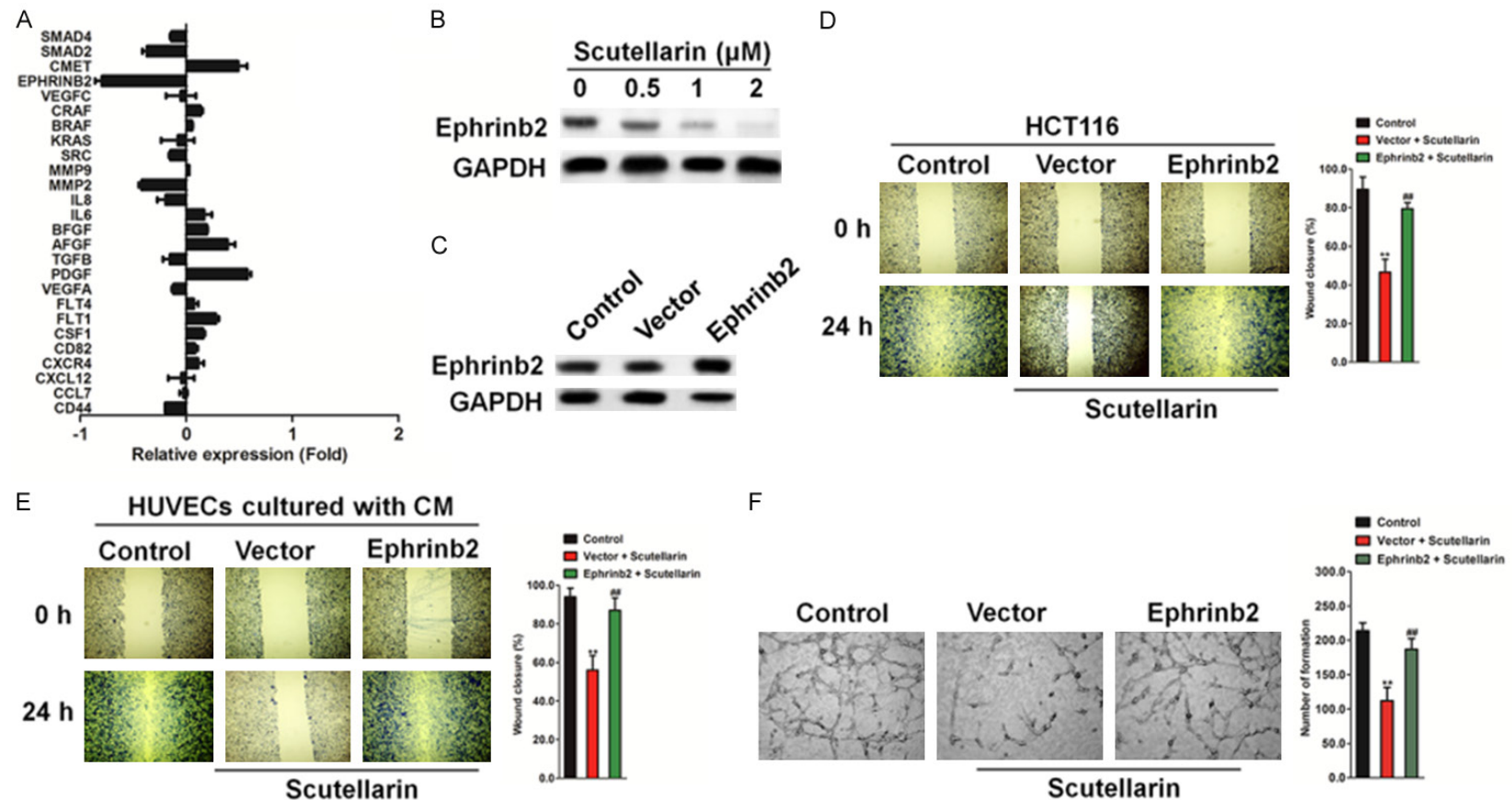


Figure 4. Effects of scutellarin on the expression of cells metastasis related genes. A. 26 genes involved in the migration and invasion processes were screened by PCR array. HCT116 cells were treated or un-treated with scutellarin for 48 h. RNA extraction and retrotranscription, the resulting cDNA was applied in the PCR array and amplified. Data are reported as fold compared to untreated HCT116 cells. B. Immunoblotting assay of cell lysates from HCT116 cells treated or untreated with scutellarin. C. HCT116 cells were transfected with constitutively active form of ephrinb2 and the expression of ephrinb2 was determined by western blot. D. In the presence of scutellarin, wound healing assay was subjected to assess the cell motility after ephrinb2 over-expression. Data are expressed as means \pm SD. $^{**}p < 0.01$ compared to control cells, $^{##}p < 0.01$ compared to cells transfected with vector. E. HUVECs cells were performed to wound closure analysis in the absence of CM from HCT116 cells treated or un-treated with scutellarin. F. Tube formation was performed in the absence of CM from HCT116 cells treated or un-treated with scutellarin. Columns are data collected from three independent experiments and are average \pm SD. values. $^{**}p < 0.01$ compared to control cells, $^{##}p < 0.01$ compared to cells transfected with vector.

Discussion

Compounds extraction separations from traditional Chinese herbs have already been confirmed to be valuable resources for the exploitation of novel anti-cancer agents. One of the expectant compounds is a flavone glycoside named as scutellarin, which is a principal active component of Chinese herb *Erigeron breviscapus* [16]. Substantial studies have demonstrated that scutellarin possess various beneficial biological roles in plenty of mammalian systems, including cell-apoptosis inhibitor, free-radical scavenger, anti-inflammatory and anti-tumor [17]. Nevertheless, no studies about its inhibition on colorectal cancer (CRC) metastasis and angiogenesis have been reported. The results in the current study suggested that scutellarin decreased CRC cell growth, metastasis and accompanied with diminished tumor angiogenesis. We demonstrated that scutellarin inhibited CRC cells migration and invasion in vitro and suppressed cells metastasis in vivo. In vivo murine xenograft model, we revealed the effectiveness of scutellarin in suppressing tumor progression. Furthermore, scutellarin significantly inhibit tumor growth in vivo as assessed by Ki67 expression.

Tumor angiogenesis is an attractive target during cancer treatment in clinic owing to its core role in tumor growth. Inhibition of tumor cells induced angiogenesis brings about a good opportunity of preventing cancer evolved into malignant [18]. A great number of studies demonstrate that the vascular endothelial growth factor (VEGF)/vascular endothelial growth factor receptor (VEGFR) system is involved in tumor angiogenesis. Inhibitors or monoclonal antibody targeting VEGF or VEGFR are currently being explored and development in basic and clinical studies. In our study, the tube formation model and CAM angiogenesis assay showed that scutellarin significantly inhibit angiogenesis in CAM and tube-structure formation of endothelial cells. Then, we examined the molecular mechanisms underlying the anti-angiogenic and anti-metastasis of scutellarin. Investigation to the molecular mechanisms of the pharmaceutical effects of scutellarin revealed that it inhibited the expression of ephrinb2.

Ephrins facilitate angiogenesis both in physiological and pathological conditions, including

cancer angiogenesis [19]. Notably, ephrinb2 and ephb4 control the fate of endothelial cells angiogenesis in arterial and venous through regulation the VEGF signaling pathway. Ephrinb2 determines VEGFR (such as VEGFR3) expression in forward and reverse signaling pathway dependent manner [20]. Previous study identify that ephrinb2 extends to lymphangiogenesis and leads the internalization of VEGFR3 in the cultured lymphatic endothelial cells. Although the roles of ephrins in the developmental angiogenesis have been well elucidated, its function in tumor angiogenesis remains further studied [21]. Currently, several types of ephrins and their receptors have been discovered be over-expression in human cancers including breast cancer, colon cancer and hepatocellular carcinoma [22]. Surprisingly, the down-regulation of other kinds of ephrins and receptors also participate into tumorigenesis; namely, ephB6 in melanoma and ephA1 in colorectal carcinoma [23].

Our results elaborated that scutellarin effectively inhibited CRC metastasis and angiogenesis has significant implications for the therapeutic use of scutellarin in cancer treatment. In this study, we demonstrated that scutellarin was able to inhibit human CRC cell growth, metastasis and angiogenesis via targeting ephrinb2. Our findings provide a strong rationale for pursuing the application of scutellarin to suppress colorectal cancer metastasis and angiogenesis.

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Disclosure of conflict of interest

None.

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Scutellarin suppresses colorectal cancer metastasis and angiogenesis

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Scutellarin suppresses colorectal cancer metastasis and angiogenesis

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