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Semaphoring 4D is required for the induction of antioxidant stress and anti-inflammatory effects of dihydromyricetin in colon cancer

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ABSTRACT

Semaphorin 4D (Sema4D) has been involved in cancer progression, the expression of which is associated with the poor clinical outcomes of some cancer patients. Dihydromyricetin (DMY) has antitumor potentials for different types of human cancer cells. However, the pharmacological effects of DMY on colon cancer (CC) or the regulatory effects of Sema4D on this process remain largely unknown. In the present study, we aimed to evaluate the effects of DMY on CC, and to elucidate the role of Sema4D in DMY-induced antitumor effects. DMY inhibited the proliferation and growth of Colo-205 colon cancer cells significantly *in vivo* and *in vitro*. DMY inhibited reactive oxygen species (ROS) and malondialdehyde (MDA) levels, but increased glutathione (GSH) level. Moreover, the activities of antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and heme oxygenase 1 (HO-1) were enhanced by DMY treatment *in vitro*, showing strong anti-oxidative stress effect. In addition, DMY inhibited the secretion of interleukin 1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor (TNF-α) in the supernatant of Colo-205 culture medium. Besides, the expressions of cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS) were suppressed by DMY in dose-dependent manners *in vivo*, showing potent anti-inflammatory effect. Further investigations showed that DMY suppressed the expression and secretion of Sema4D in Colo-205 cells and tissues. Interestingly, overexpression of Sema4D significantly weakened the regulatory effects of DMY on oxidative stress. Furthermore, overexpression of Sema4D significantly attenuated the anti-inflammatory effects of DMY. Collectively, we drew a conclusion that the anti-colon cancer effect of DMY was attributed to its negative modulation on oxidative stress and inflammation *via* suppression of Sema4D. The findings broaden the width and depth of molecular mechanisms involved in the DMY action, facilitating the development of DMY in anti-colon cancer therapies.

1. Introduction

Colon cancer (CC) has become the second highest cause of deaths among females and the third highest cause of deaths among males with malignant neoplasms around the world [1]. Unfortunately, currently approved therapeutic agents have rather limited efficacies [2]. Surgery [3] is still given the first priority for treating CC, but only suitable for the radical resection of lesions or focal lesions at the early and middle stages, as well as the palliative treatment of lesions at the advanced stage. However, the effects are limited for metastatic patients [4]. In addition, chemoradiotherapy is one of the essential means for CC treatment. Chemotherapeutic drugs travel through most organs and tissues as the blood circulates, so chemotherapy remains the main strategy for tumors prone to dissemination throughout the body and those that have metastasized to the advanced stage. Nevertheless, the clinical applications of chemotherapeutic reagents are largely restricted owing to toxicity [5] and drug resistance [6] of chemotherapeutic drugs greatly limit their clinical application. Recently, new therapies, such as immunotherapy [7], have shown excellent effect on CC, but these drugs have only been tested in clinical trials, without being commercially available yet. Therefore, it is challenging and urgent to find effective anticancer drugs with fewer side effects.

Although the underlying mechanisms remain elusive, oxidative stress and inflammation have been demonstrated to play crucial roles in regulating CC. Oxidative stress is a negative effect produced by free radicals [8], and leading to various chronic diseases including cancers [9], which can be attributed to damage of the main components of cells (lipids, proteins and DNA). There are two kinds of antioxidant systems

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in the body. One is the enzymatic antioxidant system [10], including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and heme oxygenase 1(HO-1). The other is the non-enzymatic antioxidant system [11], including ergot sulfur, vitamin C, vitamin E, glutathione, melatonin, alpha lipoic acid, etc. It is well-documented that regulating oxidative stress helped to control the progression of CC [12,13]. Interestingly, various natural compounds show different regulatory effects, some of which promote oxidative stress [14] while the others inhibit this process [12].

Inflammation plays a crucial role in regulation of a wide range of cellular processes involved in induction, initiation, progression, and aggravation of colon cancer [15,16]. Improvement of the inflammatory environment helps to control the progression of colon cancer [17]. Zong, et al. reported that macrophage scavenger receptor class A1 (SR-A1), as a pattern recognition receptor primarily expressed in macrophages, could suppress colon inflammation through negative regulation of NF-κB signaling [18]. Zhu, et al. showed that inhibition of inflammation by catalpol could significantly suppress the proliferation and growth of colon cancer [19]. Actually, colon cancer was treated as an inflammation-associated cancer, and continuous colitis could lead to the development of colon cancer [20]. In addition, many inflammatory factors, such as interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-1β (IL-1β), tumor necrosis factor (TNF-α), cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS), are often aberrantly expressed in CC cells, tissues and clinical samples [19,21]. Therefore, CC can be effectively combated by suppressing the expressions of inflammatory factors to relieve inflammation.

Sema4D, a member of class 4 semaphorins and also known as CD 100, is up-regulated in aggressive cervical, pancreatic, prostate, colon, lung and ovarian cancers [22]. It also has a previously unrecognized function, as a compensatory angiogenic factor which promotes tumor growth and angiogenesis [23]. Sema4D functions its role by binding to its high-affinity receptor plexin-B1 [24]. Studies showed that Plexin-B1 was a transmembrane protein with a sema ligand binding domain in its extracellular domain. Upon ligand binding, Plexin-B1 extracellular domain undergoes proteolysis by subtilisin-like proprotein convertases to further increase its affinity for Sema4D [25]. Higher Sema4D level enhances angiogenesis by increasing blood supply, providing more resources for involved dividing cells. Tumors with lower Sema4D expressions are less capable of driving angiogenesis in the tumor microenvironment, so the blood supply is insufficient, failing to support a large number of cells in a limited space [23]. Thus, targeting Sema4D can effectively regulate tumor progression. However, except for the promotion of tumor angiogenesis [23], the precise role of Sema4D in CC has not yet been fully clarified till now.

As an important complementary and alternative therapies, natural products have attracted considerable attention worldwide because of abundant resources, low cost and easy availability [26,27]. Notably, ingredients from natural resources have milder side effects than those of synthetic drugs [28]. Extracted from Chinese herbal medicine *Ampelopsis grossedentata*, dihydromyricetin (DMY) (Fig. 1) [29], has evident pharmacological effects on oxidation [30,31], inflammation [32], cardiac fibrosis [31], improves type 2 diabetes-induced cognitive impairment [33], ameliorates nonalcoholic fatty liver, depression [34] and cancers [35,36]. Moreover, DMY shows inhibitory effects on various cancers, including lung cancer, gastric cancer, ovarian cancer and liver cancer. However, the *in vitro* and *in vivo* pharmacological effects of DMY on colon cancer have seldom been evaluated.

In the present study, the anti-proliferation and anti-growth effects of DMY on Colo-205 colon cancer cell were first determined *in vitro* and *in vivo*, respectively. Secondly, the effect of DMY on oxidative stress and inflammation were studied. Thirdly, the effects of DMY on the expressions of Sema4D were examined *in vitro* and *in vivo*, respectively. Finally, the mechanisms were clarified by detecting the levels of oxidative stress factors and inflammatory factors after overexpression of Sema4D. Therefore, a new mechanism for the antitumor effects of DMY was confirmed. By suppressing Sema4D-dependent oxidative stress and inflammation, DMY inhibited the proliferation and growth of colon cancer.

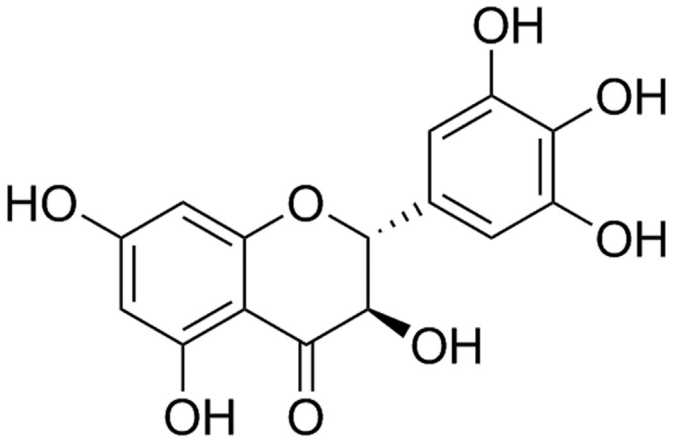

Fig. 1. Chemical structure of dihydromyricetin.

2. Materials and methods

2.1. Chemicals and reagents

Dihydromyricetin (DMY), HPLC purity 98%, was purchased from Xi'an Lincao Biological Engineering Co. (Xi-an, Shanxi, China); Primary antibodies against COX-2(12375-1-AP, 1:500) and GAPDH (10494-1-AP) were purchased from Proteintech; Semaphorin 4D (ab231961, 1:1000) and PlexinB1(ab90087, 1:500) were obtained from abcam; iNOS(#13120, 1:1000) was purchased from cell signaling. Malondialdehyde (MDA) examination kit was obtained from Beyotime Biotechnology Co., Ltd. (No. S0131); glutathione reductase (GR) and glutathione peroxidase (GPx) examination kits were purchased from Nanjing Jiancheng Bioengineering Institute (No. A030-2); Total Superoxide dismutase (SOD) activity detection kit (S0101) and CuZn/Mn-SOD activity detection kit were purchased from Beyotime (S0103). Glutathione (GSH) and catalase (CAT) detection kits were purchased from Beijing Leagene Biotechnology Co., Ltd. (No. 0731A17); ELISA kit (PeproTech, USA); Matrigel (BD, USA); all horseradish peroxidase-conjugated secondary antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). All the other reagents used were of highest purity and commercially available.

2.2. Cell lines, cell culture and animals

Human colon cancer cell lines Colo-205 was obtained from the Chinese Academy of Sciences Cell Bank of Type Culture Collection (CBTCCCAS, Shanghai, China). The colon cancer cell lines were cultured in DMEM supplemented with 10% fetal bovine serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin and were maintained in an incubator with a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Male Balb/c nude mice (6 weeks, 18–22 g) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). All experimental procedures were approved by the hospital and local committee on the care and use of animals and all animals received humane care according to the National Institutes of Health (USA) guidelines. All mice were maintained under a 12 h light/dark cycle at a controlled temperature (25 °C) with free access to food and tap water until the day of the experiment.

Cell viability and cytotoxicity assays.

Cells were seeded in 96-well plates (8×10^3 cells/well, 200 μL) and cultured in DMEM medium with 10% FBS for 24 h. Cells were treated with DMY at various concentrations ranging from 2 to 64 μM for 24 and

48 h. After treatment, MTS solution (5 mg/mL) was added (10 μL/well) and the cells were further incubated for 3 h at 37 °C. The spectrophotometric absorbance at 490 nm was measured by a SPECTRAmaxTM microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). For cytotoxicity assay, lactate dehydrogenase (LDH) activity in culture medium was determined with a LDH release assay kit according to the protocol.

2.3. The xenograft tumor transplant model

The xenograft tumor transplant model was performed as described previously [19]. The animals were injected subcutaneously with Colo-205 colon cancer cells (2×10^6 per mouse) into the abdomen and allowed 1 week to establish the tumors. Later, the xenografted mice were randomly divided into four groups of five mice each group. DMY of different concentration (25, 50, and 100 mg/kg body weight), dissolved in saline, was then given by intragastric administration daily. The model group was daily administered with the same volume of saline only by oral. The tumor size was measured by vernier caliper every three days and the tumor volume was calculated as $0.5 \times L \times W \times H$, where L is the tumor dimension at the longest point, W is the tumor dimension at the widest point, and H is the tumor dimension at the highest point. Besides, the body weight of each mouse was recorded every three days. After 21 days, all the mice were sacrificed, subcutaneous tumor tissues and livers were peeled off. The liver weight and tumor weight were measured using an analytical balance. Then the tumors were fixed in 10% formalin and paraffin-embedded for immunohistochemical staining. For the hepatic index was calculated as m_0/m_1 (m_0 is the liver weight and m_1 is the body weight).

2.4. Immunohistochemical assay

Histochemical staining of tissues was performed as described [37]. Tumor tissues were fixed and processed by conventional paraffin-embedded method. The tumor sections (5 μm thick) were heat-immobilized, deparaffinized, and rehydrated. Antigen retrieval was done by incubation in 10 mM citrate buffer (pH 6.0) for 10 min, followed by the incubation with 5% block serum for 1 h. Sections were incubated with sema4D, plexinB1, COX-2 and iNOS antibodies at 4 °C overnight, respectively. After washes, the sections were incubated for 1 h with goat anti-mouse secondary IgG, and detected by incubation with streptavidin-biotin-horseradish peroxidase complex. The tissue sections were stained with hematoxylin for a short time, and subsequently detected under a microscope. Three middle-power microscopic fields (×200) were randomly selected for each section to determine the positive staining intensities by IPP software (Image-Pro Plus 6.0, Media, Cybernetics) and the integrated optical density (IOD) was calculated. The expression levels of the proteins were presented as the average IOD of the 3 fields.

2.5. Immunofluorescence analysis

Colo-205 cells were seeded in 6-well plates and cultured in DMEM supplemented with 10% FBS for 24 h, followed by treatment with drugs for the indicated time periods. Cells were incubated with antibody against Interleukin-1β (IL-1β) (1:200 dilution) at 4 °C overnight followed by incubation with FITC-labeled Goat Anti-Rabbit IgG (1:50 dilution; Abcam, USA) for 1 h at room temperature. Cellular nuclei were stained with DAPI. Immunofluorescent staining was visualized with a fluorescence microscope (Nikon, Tokyo, Japan).

2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of each factor in blood and Colo-205 culture supernatant were determined with an ELISA kit (Nanjing Jiancheng Bioengineering Institute) according to the protocol [38,39]. Briefly, for the experiments *in vitro*, Colo-205 (500,000 cells/well) were seeded in six-well plates and cultured in DMEM with 10% FBS for 24 h and then in low-serum medium (0.5%, v/v) for an additional 12 h. Colo-205 were treated with various reagents as indicated in specific experiments for 24 h. For the blood, samples of 100 μL were added to each well of the 96-well plates coated with antibody, followed by incubation for 2 h at room temperature. Working detector solution of 100 μL was loaded into each well, and the plates were incubated for an additional 1 h at room temperature before the addition of substrate solution of 100 μL. The reaction was stopped by adding stop solution of 50 μL. The absorbance was read at 450 nm wavelength.

2.7. Measurement of ROS

ROS were measured using the oxidation sensitive fluoroprobe 20, 70-dichlorofluorescin diacetate (DCFHDA, Beyotime) [40]. DCFH-DA is a non-fluorescent compound that is freely taken up by cells and hydrolyzed by esterases to 20, 70-dichlorofluorescin (DCFH). DCFH is then oxidized to the fluorescent 20, 70-dichlorofluorescin (DCF) in the presence of peroxides, thereby indicating the level of intracellular ROS. Cells from the tissue were harvested and incubated with 10 mM DCFH-DA dissolved in cell-free medium at 37 °C for 30 min and then washed three times with PBS. Cellular fluorescence was quantified using fluorescence microplate reader (BioTek, America) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. During the whole procedure with DCFH-DA, the plate was kept out of light to minimize the fading of the fluoroprobe.

2.8. Measurement of MDA and GSH levels and antioxidant enzymes activity

Measurement the levels of MDA and GSH and the activities of antioxidant enzymes including CAT, GPx, GR and HO-1 in Colo-205 cells were detected by using examination kits according to the protocols, respectively. Activity of SOD in Colo-205 cell was assessed with WST-1 method (Beyotme, Shanghai, China) and was expressed as U/mg protein.

2.9. Plasmid transfection

Sema4D plasmid constructs and negative control vectors were transfected into Colo-205 cells using MegaTran 1.0 transfection reagent according to manufacturer's instructions [41]. The transfection efficiency was confirmed by Western blot analysis.

2.10. Western blot analyses

The whole cell protein extracts were prepared from treated Colo-205 cells. Cells were lysed in ice-cold RIPA lysis buffer and subjected to centrifugation (10,000 *g*) at 4 °C for 15 min. Then, the supernatants were used for quantification of the total protein concentration by using a BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's protocol. Western blot analysis was performed as we described previously [42]. Briefly, Colo-205 cells lysate (50 μg/well) was separated by SDS-PAGE and the proteins were transferred onto the nitrocellulose membrane. GAPDH was used as an invariant control for the target proteins. Representative blots were shown.

2.11. Statistical analysis

The level of significance between different groups was analyzed by Student-Newman-Keuls *t*-test after the application of one-way analysis of variance (ANOVA) by InStat software. Values of $P < 0.05$ were considered statistically significant.

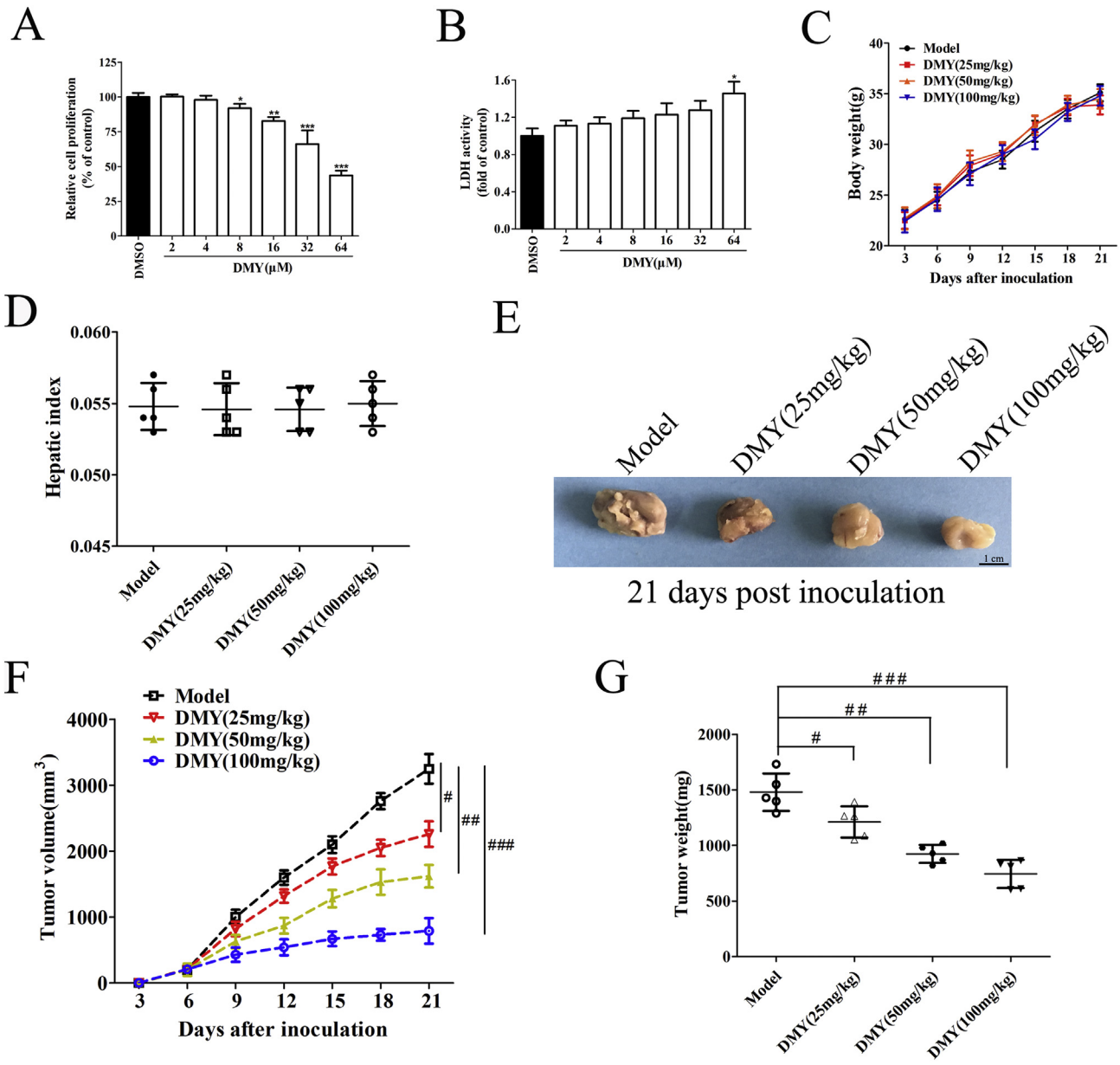

Fig. 2. DMY inhibited tumor proliferation and growth in Colo-205 cells. (A) The effect of DMY on cell viability of Colo-205 cells *in vitro* ($n = 3$). (B) The toxicity of DMY on Colo-205 cells by LDH activity assay ($n = 3$). $^{*}P < 0.05$ *versus* the DMSO group; $^{**}P < 0.01$ *versus* the DMSO group; $^{***}P < 0.001$ *versus* the DMSO group. (C) Body weights were recorded every three days. Body weights are plotted, showing changes observed every three days until the killing of the animals. (D) Hepatic index. (E) The solid tumor photograph of Colo-205 cells in mice. (F) Tumor volumes were recorded every three days until the killing of the animals. (G) Tumor weight was recorded in different groups. The data are presented as mean ± SD, and each group had five mice. $^{\#}P < 0.05$ *versus* the model group; $^{\#\#}P < 0.01$ *versus* the model group; $^{\#\#\#}P < 0.001$ *versus* the model group.

3. Results

3.1. DMY inhibited cell proliferation and growth in CoLo-205 cells

We first studied the inhibitory effects of DMY on the proliferation and growth of CoLo-205 cells *in vitro* and *in vivo*. The proliferation of CoLo-205 cells was significantly inhibited by DMY in a dose-dependent manner compared to the control group (DMSO) *in vitro* (Fig. 2A), and showing cytotoxicity at 64 μM (Fig. 2B). A mouse xenograft tumor model was then established to test the effects of DMY on CoLo-205 cell growth. As shown in Fig. 2C and D, the hepatic indices and weights of mice treated and untreated with DMY are similar throughout the experiment, indicating that DMY did not result in systemic toxicity. DMY significantly inhibited the CoLo-205 tumor growth in a dose-dependent manner (Fig. 2E). The mean tumor volumes of the model group and DMY (25, 50, 100 mg/kg) groups were (3250.57 ± 106.35) mm^3, (2261.82 ± 87.17) mm^3, (1623.79 ± 109.37) mm^3 and (790.46 ± 78.63) mm^3 after four 21 days, respectively (Fig. 2F). The average tumor weight of the model group and DMY (25, 50, 100 mg/kg) groups were (1479.46 ± 98.48) mg, (1211.82 ± 93.84) mg, (924.68 ± 63.59) mg and (744.93 ± 59.70) mg, respectively (Fig. 2G). Collectively, DMY inhibited cell proliferation and growth in CoLo-205 cells.

3.2. DMY suppressed oxidative stress in Colo-205 cells

Increased generation of reactive oxygen species (ROS) and cellular oxidative stress have been strongly linked to malignant tumor

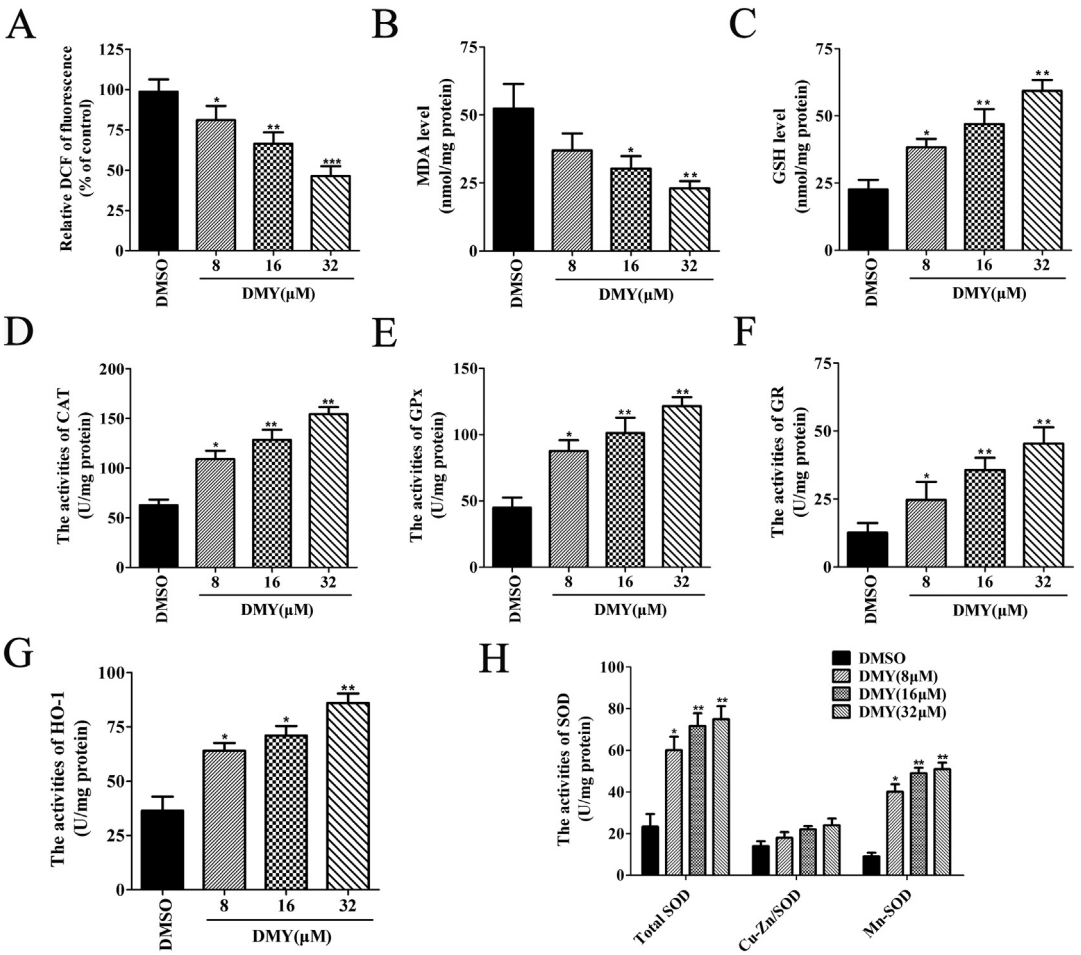

Fig. 3. DMY suppressed oxidative stress in Colo-205 cells. (A) The DCFH-DA staining was used to detect ROS production in the indicated groups ($n = 3$). (B) The effect of DMY on MDA level in Colo-205 cells. (C) The effect of DMY on GSH level in Colo-205 cells. (D-H) The effect of DMY on the enzyme activities of CAT, GPx, GR, HO-1, total SOD, Cu-Zn/SOD and Mn-SOD were checked, respectively. The data are presented as mean ± SD, and the experiment was repeated three times. $^{*}P < 0.05$ *versus* the DMSO group; $^{**}P < 0.01$ *versus* the DMSO group; $^{***}P < 0.001$ *versus* the DMSO group.

transformation, metastasis and resistance to anticancer therapy [43]. Inhibiting ROS and antioxidant systems can effectively combat cancers, sparing normal cells due to lower intracellular ROS levels [44]. Thus, we then investigated the effect of DMY on oxidative stress in Colo-205 cells. DMY significantly inhibited the accumulation of ROS compared to the untreated group (DMSO) (Fig. 3A). Lipid peroxidation is regarded as one of the key mechanisms of cellular damage caused by ROS [45]. MDA is one of the end products of lipid peroxidation, the level of which in Colo-2015 cells was detected to evaluate the effect of DMY. As shown in Fig. 3B, compared to the untreated group, DMY inhibited the MDA level in a concentration-dependent manner in Colo-205 cells. GSH is the major endogenous antioxidant *in vivo*, the decrease of which is bound to pose greater oxidative damage to cells. DMY increased the GSH level significantly compared to the untreated group (Fig. 3C). In short, treatment with DMY significantly suppressed oxidative stress in Colo-205 cells. Then how DMY exert the anti-oxidative stress effect were further investigated. Oxidative stress can be prevented by activation of the antioxidant enzymatic system. Thus, we then studied the regulation effect of DMY on the antioxidant enzymatic system. Compared to the untreated group, DMY significantly increased the activities of antioxidant enzymatic CAT, GPx, GR, HO-1 and total SOD (especially in Mn-SOD but not in Cu-Zn/SOD) (Fig. 3D–H). Altogether, these findings indicated that DMY exerted its anti-oxidative stress effect by increasing the activities of antioxidant enzymes in Colo-205 cells.

3.3. DMY inhibited the inflammation in Colo-205 cancer tissues

Inflammation has been causally linked to carcinogenesis and participates in the whole process of colon cancer progression [19]. In addition, chronic inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease are associated with an increased risk of developing CC [20]. Therefore, in this study, we postulated that inhibition of inflammation may be one of the main effects of DMY on colon cancer. Firstly, the effects of DMY on the serum levels of inflammatory factors IL-1β, IL-6, IL-8 and TNF-α were studied. The serum levels of IL-1β, IL-6, IL-8 and TNF-α increased significantly in the model group in comparison with the control group (Fig. 4A–D). Treatment with DMY decreased the levels of these inflammatory factors in dose-dependent manners (Fig. 4A–D). Secondly, the expressions of two important inflammatory factors COX-2 and iNOS in colon cancer tissues were further investigated. The expressions of COX-2 and iNOS decreased significantly compared to the model group *in vivo* (Fig. 4E and F). The immunohistochemical analyses also showed the same results as Western blot (Fig. 4G and H). Taken together, these results indicated that DMY showed potent anti-inflammatory effect on colon cancer *in vivo*.

3.4. DMY suppressed the expression of Sema4D in vivo and in vitro

As Sema4D is closely related to tumor growth [46], we firstly studied the effects of DMY on Sema4D expression *in vitro* and *in vivo*. DMY significantly inhibited the expression of Sema4D compared to the

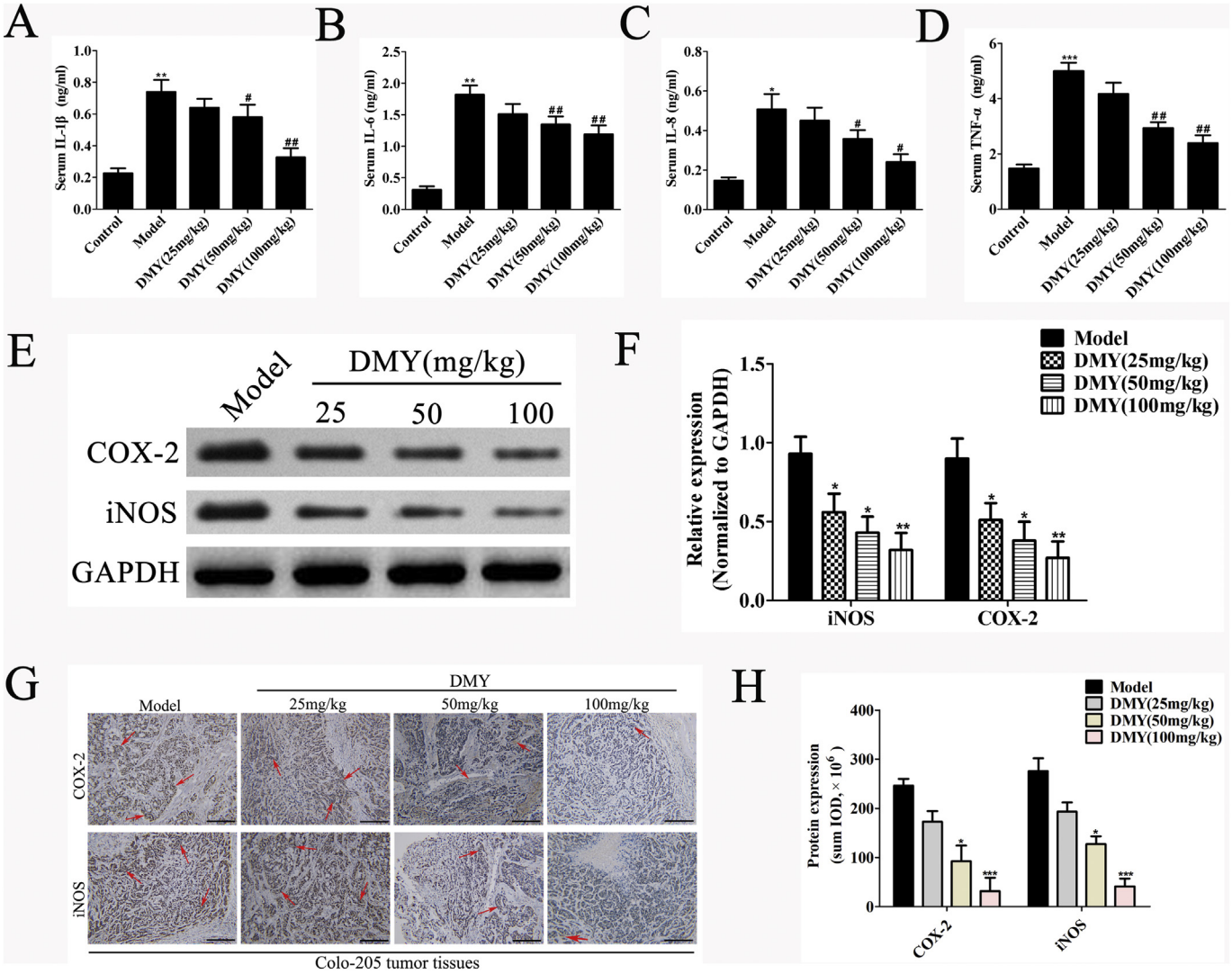

Fig. 4. DMY inhibited the inflammation in Colo-205 cancer tissues. (A–D) The effect of DMY on the serum levels of IL-1β, IL-6, IL-8 and TNF-α in mice, respectively. The data are presented as mean ± SD, and the experiment was repeated three times. $^{*}P < 0.05$ *versus* the control group; $^{**}P < 0.01$ *versus* the control group; $^{***}P < 0.001$ *versus* the control group. $^{\#}P < 0.05$ *versus* the model group; $^{\#\#}P < 0.01$ *versus* the model group. (E) Protein expressions of COX-2 and iNOS in tumor tissues were measured by Western blot assays. GAPDH was used as an invariant control for equal loading. Representative blots are shown with densitometry. (F) Quantitation of the result of western blot. (G) Immunohistochemical assay of protein expressions of COX-2 and iNOS in HT29 transplanted tumor tissues. (H) Quantitation of the result of positive expressions rate of COX-2 and iNOS. The data are presented as mean ± SD, and the experiment was repeated three times. $^{*}P < 0.05$ *versus* the model group; $^{**}P < 0.01$ *versus* the model group; $^{***}P < 0.001$ *versus* the model group.

untreated group (0 μM) in CoLo-205 cells (Fig. 5A and B). In addition, as Sema4D is a secretory protein, we then investigate the effect of DMY on Sema4D secretion. As expected, DMY suppressed the secretion of Sema4D in the supernatant of CoLo-205 culture medium (Fig. 5C). Secondly, the effects of DMY on the expression of Sema4D and its receptor plexin-B1 were evaluated in CoLo-205 tumor tissues. DMY inhibited the expressions of Sema4D and plexin-B1 in dose-dependent manners compared to the model group (Fig. 5D and E). Immunohistochemical assay further showed that DMY could down-regulate the expressions of Sema4D and plexin-B1 *in vivo* (Fig. 5F and G). Collectively, DMY suppressed the expression of Sema4D *in vivo* and *in vitro*.

3.5. Sema4D was essential for the antioxidant stress effects of DMY on CoLo-205 cells in vitro

To study the role of Sema4D in mediating the antioxidant effect of DMY on Colo-205 cells, Sema4D plasmid was constructed to over-expression the Sema4D. As was shown in Fig. 6A and B, Sema4D plasmid significantly increased the expression of sema4D compared with the control vector, indicating that the overexpression plasmid was successfully constructed. Overexpression of sema4D significantly attenuated the inhibitory effect of DMY on the accumulation of ROS (Fig. 6C) as well as MDA (Fig. 6D). Moreover, overexpression of sema4D significantly attenuated the enhancement effect of DMY on GSH (Fig. 6E). Then we further studied the effect of DMY on the antioxidant enzymes. DMY significantly augmented the activities of antioxidant enzymes CAT, GPx, GR and total SOD (especially in Mn-SOD but not in Cu-Zn/SOD) (Fig. 6F–I) compared to the untreated group (0 μM), however, were reversed by overexpression of Sema4D (Fig. 6F–I). Collectively, Sema4D was essential for the antioxidative effects of DMY on CoLo-205 cells *in vitro*.

3.6. Sema4D was required for the induction of anti-inflammatory effects of DMY in CoLo-205 cells

We further studied the role of Sema4D in regulating the anti-inflammatory effect of DMY in Colo-205 cells. We employed two methods

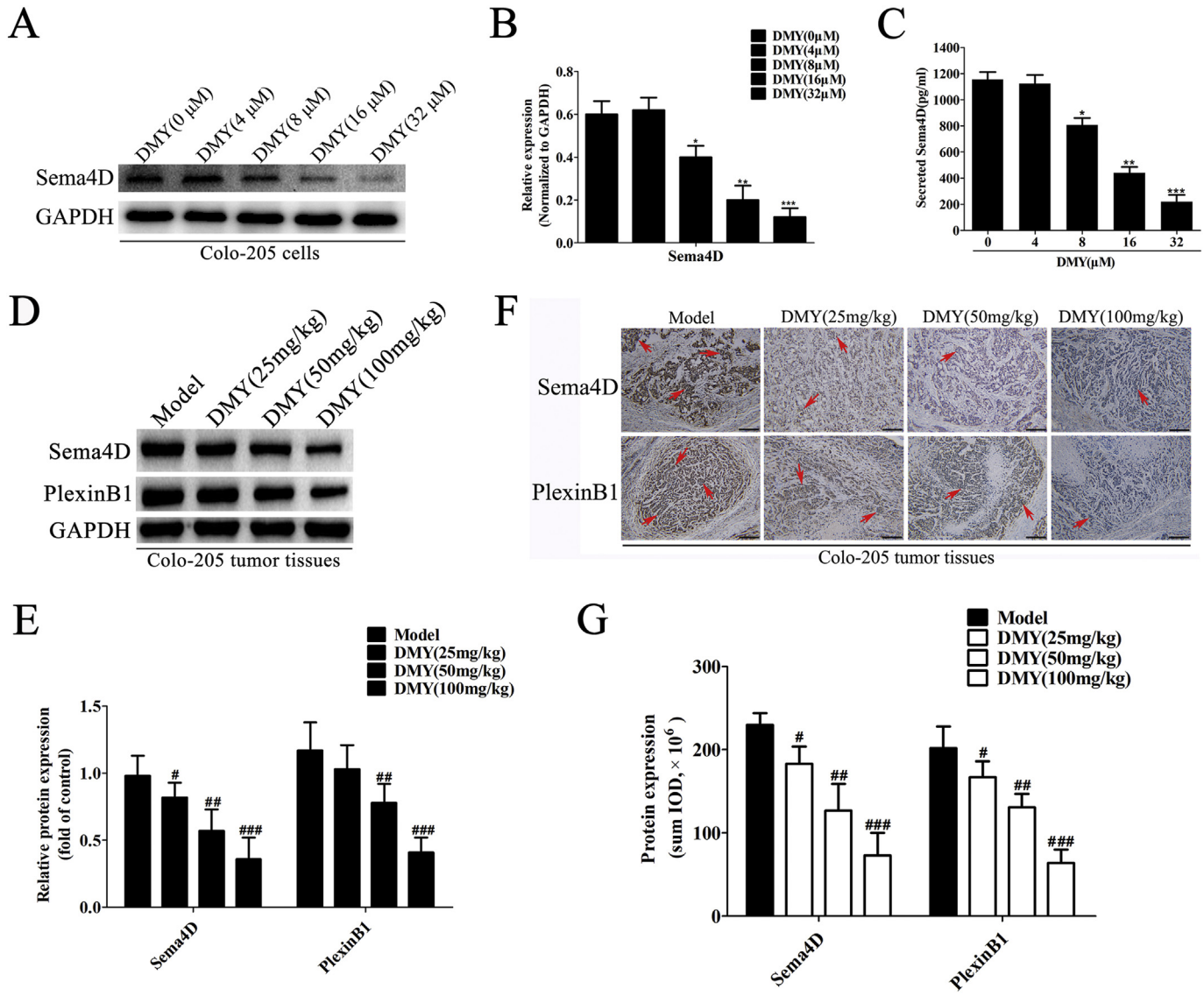

Fig. 5. DMY suppressed the expression of Sema4D *in vivo* and *in vitro*. (A) Protein expression of Sema4D was measured by Western blot assays. GAPDH was used as an invariant control for equal loading. Representative blots are shown with densitometry. (B) Quantitation of the result of western blot. (C) The effect of DMY on the secretion of Sema4D in the supernatant of Colon-205 culture medium. The data are presented as mean ± SD, and the experiment was repeated three times. $^{*}P < 0.05$ *versus* the DMY (0 μM) group; $^{**}P < 0.01$ *versus* the DMY (0 μM) group; $^{***}P < 0.001$ *versus* the DMY (0 μM) group. (D) Protein expressions of Sema4D and PlexinB1 in tumor tissues were measured by Western blot assays. GAPDH was used as an invariant control for equal loading. Representative blots are shown with densitometry. (E) Quantitation of the result of western blot. (F) Immunohistochemical assay of protein expressions of Sema4D and PlexinB1 in Colo-205 transplanted tumor tissues. (G) Quantitation of the result of positive expressions rate of Sema4D and PlexinB1. The data are presented as mean ± SD, and the experiment was repeated three times. $^{\#}P < 0.05$ *versus* the model group; $^{\#\#}P < 0.01$ *versus* the model group; $^{\#\#\#}P < 0.001$ *versus* the model group.

to determine whether overexpression of Sema4D was able to attenuate the MDY-induced anti-inflammatory effect on Colo-205 cells. We first measured the levels of IL-6, IL-8 and TNF-α in the supernatant of Colo-205 cells by ELISA. DMY significantly decreased the levels of the above inflammatory factors compared to the untreated group, which were significantly reversed by overexpression of Sema4D (Fig. 7A). Then immunofluorescence was further used to check the expression of IL-1β after overexpression of Sema4D. Overexpression of Sema4D significantly attenuated the inhibitory effect of DMY on the expression of IL-1β (Fig. 7B). Overall, Sema4D was required for the induction of anti-inflammatory effects of DMY in CoLo-205 cells.

4. Discussion

Here we have identified and characterized a novel mechanism of DMY-induced anti-oxidative stress and anti-inflammatory effects in colon cancer, and also identified Sema4D as a potential target for the treatment of colon cancer. DMY inhibited not only the expression of Sema4D in CC tissues, but also its secretion in the supernatant of CoLo-205 cell culture medium. In particular, we found that overexpression of Sema4D could attenuate the inhibition effects of DMY on oxidative stress and inflammation in colon cancer, indicating that Sema4D played key mediating roles in the pathogenesis of colon cancer. To our knowledge, the antitumor effects of DMY have seldom been evaluated from the viewpoint of Sema4D-dependent oxidative stress and inflammation.

In previous studies, DMY showed anticancer efficacies on various cancers. Fan et al. reported that DMY inhibited the proliferation of human lung cancer by targeting stromal fibroblasts, and further showed that targeting the activation of Erk1/2 and Akt may be the potential mechanism [35]. Ji et al. found that DMY inhibited the proliferation of AGS human gastric cancer cells by inducing cell apoptosis through a

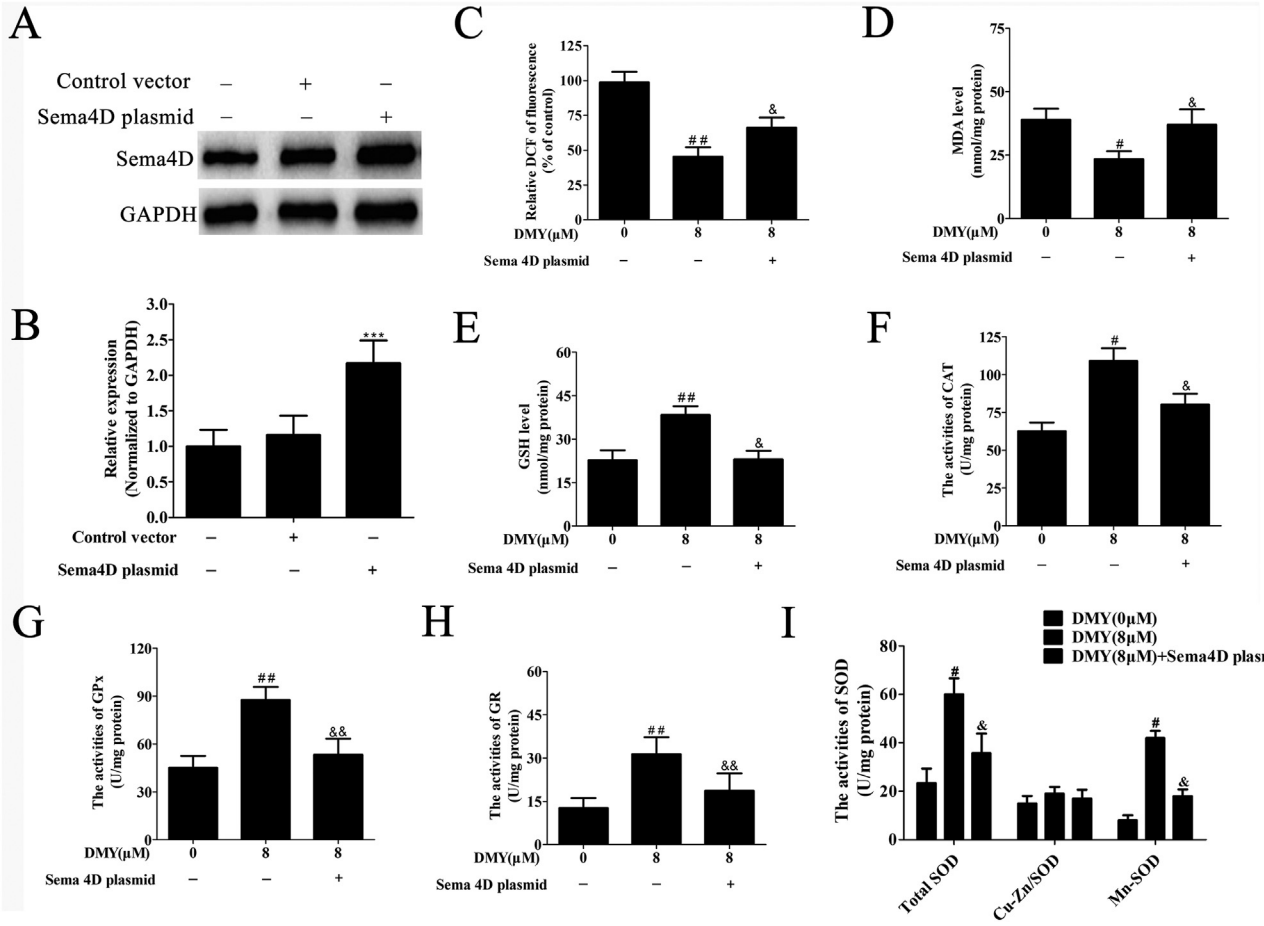

Fig. 6. Sema4D was essential for the antioxidant stress effects of DMY on CoLo-205 cells *in vitro*. (A) Protein expression of Sema4D in Colo-205 was measured by Western blot assays to detect the efficiency of Sema4D plasmid. GAPDH was used as an invariant control for equal loading. Representative blots are shown with densitometry. (B) Quantitation of the result of western blot. (C) The effect of overexpression of Sema4D on the ROS production in Colo-205 cells. (D) The effect of overexpression of Sema4D on MDA level in Colo-205 cells. (E) The effect of overexpression of Sema4D on GSH level in Colo-205 cells. (F–I) The effect of overexpression of Sema4D on the enzyme activities of CAT, GPx, GR, total SOD, Cu-Zn/SOD and Mn-SOD in Colo-205 cells, respectively. The data are presented as mean ± SD, and the experiment was repeated three times. $^{***}P < 0.001$ *versus* the control vector group; $^{\#}P < 0.05$ *versus* the DMY (0 μM) group; $^{\#\#}P < 0.01$ *versus* the DMY (0 μM) group; $^{\&}P < 0.05$ *versus* the DMY (8 μM) group; $^{\&\&}P < 0.01$ *versus* the DMY (8 μM) group.

p53-related pathway [47]. Kao, et al. showed that DMY could suppress the proliferation of in A549 and H1975 human NSCLC cell lines, and demonstrated that inhibition of reactive oxygen species-mediated ERK and JNK activation could sensitize dihydromyricetin-induced mitochondrial apoptosis in the above two NSCLC cell lines [48]. Besides, Liu et al. found that DMY could inhibit proliferation and promote apoptosis in HepG2 cells, mainly by reducing ROS production in HepG2 cells [49]. In the current study, DMY showed potent antitumor effect on colon cancer, which further enriched the anticancer spectrum of DMY.

Accumulating evidence identified that oxidative stress is widely participate in the onset and progression of tumors [50]. Oxidative stress can be induced by excessive ROS, and actually, almost all tumor cells share a common characteristic, *i.e.* an imbalanced intracellular redox system. Thus, recovering the balance of redox system can effectively control cancer progression. In previous studies, DMY showed potent anti-oxidative stress effects in multiple disease models [51]. Chen, et al. reported that DMY could attenuate transverse aortic constriction-induced myocardial Hypertrophy mainly by suppressing oxidative stress [30]. Zhang, et al. found that DMY could inhibit osteoclastogenesis and bone loss by inhibiting the production of ROS and nitric oxide (NO), and promote the activity of the antioxidative system [52]. Besides, Song, et al. showed that DMY attenuated Ang II induced cardiac fibroblasts proliferation mainly through inhibition of oxidative stress. Consistent with previous studies, our data showed that DMY inhibited oxidative stress in colon cancer, and inhibited the production of ROS and enhanced the activities of antioxidative enzymes, which highlighting antioxidant stress as an underlying mechanism that may contribute to the anti-colon cancer effect of DMY.

Now it is recognized that various types of inflammation, such as colitis, infection and autoimmune diseases, can increase cancer incidence including colon cancer [17]. Actually, inflammatory cells, cytokines and chemokines can be found in all early tumor microenvironments [53]. COX-2 and iNOS are two key inflammation-associated proteins, which play important roles in colon cancer. COX-2 is a bifunctional enzyme, *i.e.* cyclooxygenase and catalase activities [54]. Studies found that COX-2 and iNOS were highly expressed in colon cancer, and could be regarded as the markers for the diagnosis of colon cancer. Besides, the expressions of COX-2 and iNOS were also related with lymph node metastasis, differentiation and infiltration depth of colon cancer, thus had certain clinical significance [55,56]. Moreover, targeting inflammatory mediators (such as COX-2 and iNOS) and transcription factors could reduce the occurrence and spread of tumors. In previous studies, DMY showed strong anti-inflammatory bioactivities on various of diseases. Liu, et al. reported that DMY showed potent anti-inflammatory effect on lead-induced cognitive impairments and inflammation, mainly by decreasing the level of inflammatory cytokines in the brains of Pb-treated mice [57]. Feng, et al. found that DMY could inhibit neuroinflammation by suppressing

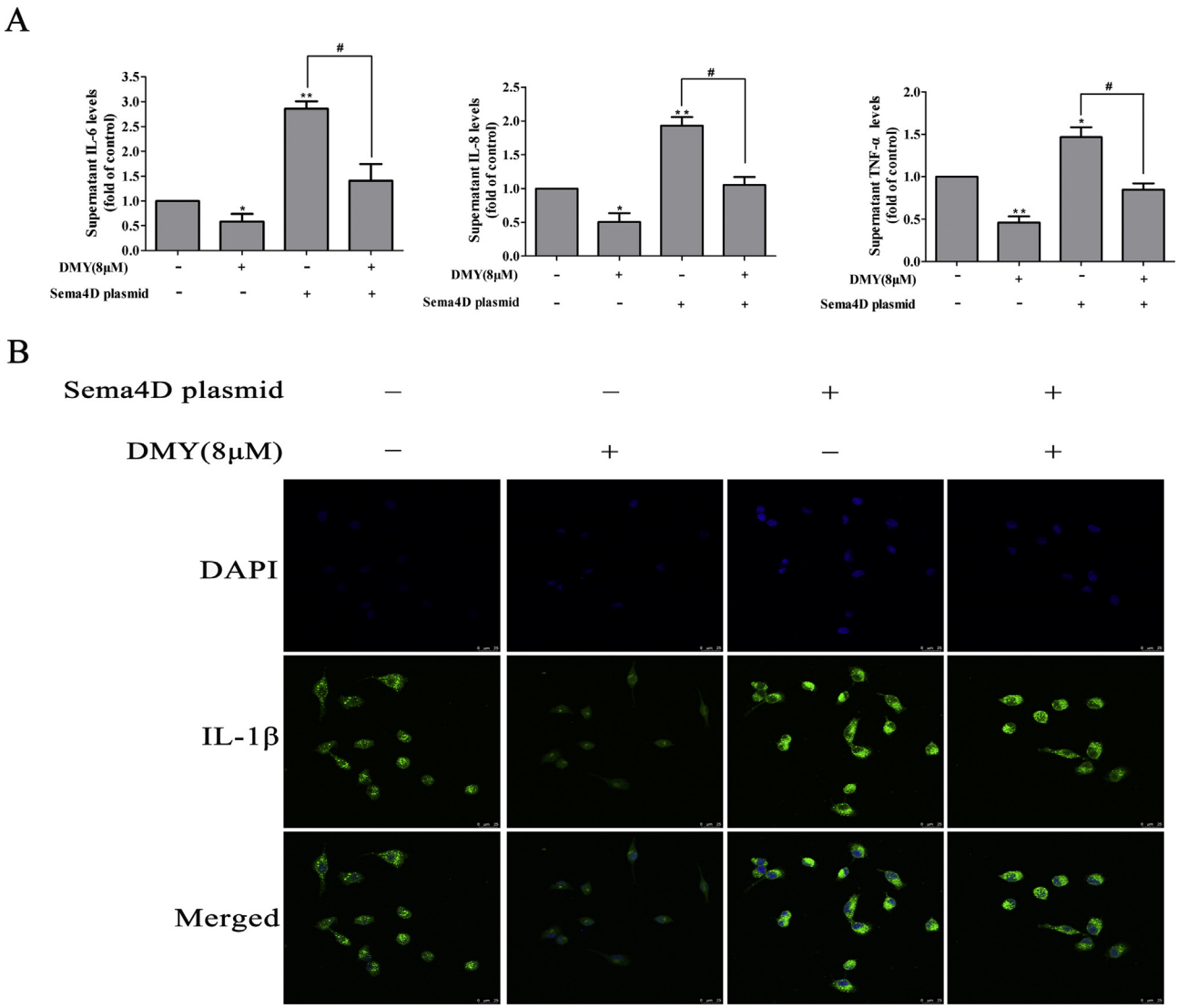

Fig. 7. Sema4D was required for the induction of anti-inflammatory effects of DMY in CoLo-205 cells. (A) The effect of overexpression of Sema4D on the supernatant of IL-6, IL-8 and TNF-α levels in Colo-205 cells was examined by ELISA, respectively. (B) The effect of overexpression of Sema4D on the expression of IL-1β was examined by immunofluorescence. The data are presented as mean ± SD, and the experiment was repeated three times. $^{*}P < 0.05$ *versus* the untreated group; $^{**}P < 0.01$ *versus* the untreated group; $^{\#}P < 0.05$, Sema4D plasmid treated alone group *versus* Sema4D plasmid combined with DMY treated group.

NLRP3 inflammasome activation in APP/PS1 transgenic mice [32]. Besides, Wang, et al. showed that DMY ameliorated the lung pathological changes and suppressed the inflammation response in lung tissues after LPS challenge. Consistent with the previous studies, DMY down-regulated the expression of inflammatory factors COX-2 and iNOS *in vivo*, and inhibited the secretion of inflammatory factors IL-1β, IL-6, IL-8 and TNF-α, showing potent anti-inflammatory effect on colon cancer.

Previous studies have demonstrated that Sema4D played important role in the progression of colon cancer. Ding et al. reported that Sema4D was high expressed in human CC samples, and Sema4D alone can elicit a significant angiogenic response to promote tumor growth independently of VEGF, showing potent proangiogenic effect [23]. Therefore, Sema4D has been regarded as a potential biomarker for antiangiogenic therapy in CC. Ikeya, et al. found that the expression of Sema4D and PlexinB1 respectively were both found to be significantly related to stage, depth of tumor invasion, lymph node metastasis, lymphatic invasion, and venous invasion, respectively [1]. Moreover, the positive expressions of both Sema4D and PlexinB1 were found to be an independent risk factor for a worse survival, which highlighting that the combination of Sema4D and PlexinB1 protein was useful for predicting disease recurrence in CC patients. However, besides the roles of Sema4D in tumor angiogenesis and growth, the precise regulation role of Sema4D in CC remains to be determined. Thus, in the present study, we further investigated the roles of Sema4D in oxidative stress and inflammation in CC. We found that DMY could inhibit the expression and secretion of Sema4D in Colo-205 cells *in vivo* and *in vitro*. More interesting, overexpression of Sema4D significantly attenuated the anti-oxidant stress and anti-inflammatory effects in Colo-205 cells, highlighting the important regulation effects of Sema4D on oxidative stress and inflammation in CC. Therefore, our data further enriched the effects of Sema4D in CC, making Sema4D an attractive target for CC treatment. Besides the role in CC, Sema4D also play important roles in other diseases. Lu et al. [58]. reported that increased levels of plasma soluble Sema4D was seen in heart failure (HF) patients compared to those in healthy controls, suggesting that Sema4D involved in the development and progression of HF and making Semd4D a potential target for treatment of HF. Consistent result was also obtained by other study [59]. Zhou, et al. found that Sema4D synchronously increased with blood-brain barrier (BBB) permeability and accumulated in the perivascular area after stroke. In addition, suppressing Sema4D/PlexinB1 signaling in the periinfarct cortex significantly decreased BBB permeability and thereby improved stroke outcome, which highlighting

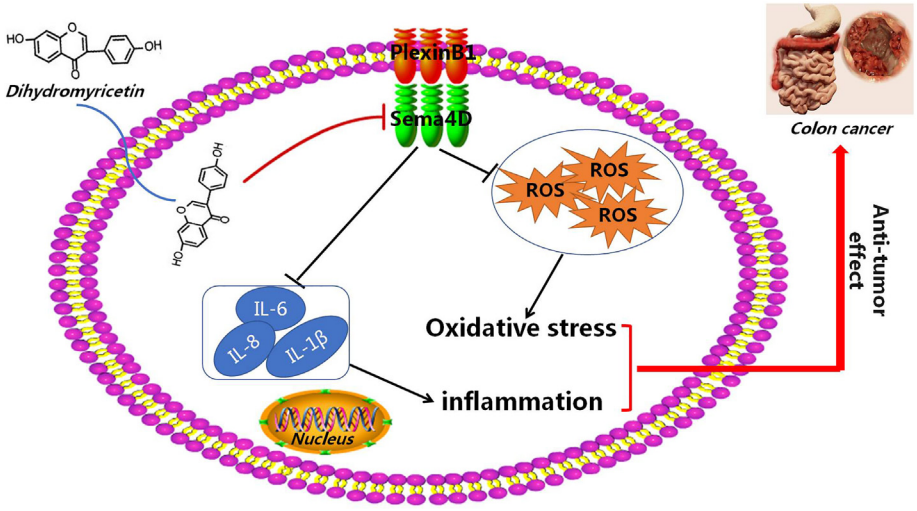

Fig. 8. Schema of the underlying mechanism of DMY inhibition of Colo-205 colon cancer. Sema4D functions its role by binding to its high-affinity receptor plexin-B1. DMY could inhibit the levels of inflammatory factors, such as IL-6, IL-8 and IL-1β and exerts its inhibition effect on inflammation. In addition, DMY could inhibit the accumulation of ROS and lead to the inhibition on oxidative stress. Most importantly, Sema4D is required for the inhibition effect of DMY on both oxidative stress and inflammation, indicating the essential role of Sema4D in it. DMY inhibits the tumor growth of Colo-205 colon cancer, at least partly, by suppressing Sema4D-dependent oxidative stress and inflammation.

that Sema4D may be a novel therapeutic target for treatment in the acute phase of stroke [60]. Nishide, et al. showed that Sema4D could inhibit neutrophil activation and involved in the pathogenesis of neutrophil-mediated autoimmune vasculitis (AAV), making Sema4D a promising biomarker and potential therapeutic target for AAV. Thus, Sema4D has wide pharmacological effects on varieties of diseases.

In summary, we demonstrated that DMY exerted antitumor effects by inhibiting oxidative stress and inflammation, possibly based on the regulation of Sema4D expression (Fig. 8). Understanding the action of mechanism of natural dietary products, such as DMY, offers further insight into developing drugs for the prevention and treatment of CC.

Conflict of interest

The authors declare that they are no conflicts of interest.

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