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Anticancer activity of emodin is associated with downregulation of CD155

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ABSTRACT

Emodin is a Chinese herb-derived compound that exhibits a variety of pharmacological benefits. Although emodin has been shown to inhibit growth of cancer cells, its antineoplastic function is incompletely understood. CD155 is a member of poliovirus receptor–related (PRR) family of adhesion molecules; it is constitutively expressed on many tumor cell lines and tissues and has diverse functions. CD155 has been reported to mediate activation of T cells via CD226 or inhibition of T cells via T-cell immunoreceptor with Ig and ITIM domains (TIGIT). In addition, CD155 may play a critical role through non-immunological mechanisms in cancer. In this study, we tested the ability of emodin to modulate CD155 expression in cancer cells. We found that emodin significantly decreased the expression of CD155 in tumor cells and inhibited tumor cell proliferation and migration, and induced cell-cycle arrest at G2/M phase. The tumor inhibitory effects of emodin were lost with CD155 knockdown. Furthermore, emodin was used to treat mice bearing B16 melanoma. It was shown that emodin attenuated tumor growth accompanied by suppressing CD155 expression. Therefore, we propose that emodin could inhibit tumor growth, and the antineoplastic properties of emodin are at least partially CD155 dependent. Our study provides new insights into the mechanisms by which emodin inhibits tumor growth.

1. Introduction

Adhesion molecules are transmembrane proteins responsible for a variety of biological signaling in cell regulation. Many studies have documented that alterations in expression of adhesion molecules correlate with the growth of primary or metastatic tumors. The adhesion molecule CD155, also called PVR or necl-5, is broadly distributed on epithelial and endothelial cells in many tissues [1]. Notably, CD155 is overexpressed on various tumors, including colorectal cancer [2], gastric cancer [3], ovarian cancers [4], neuroblastoma [5], myeloid leukemias [6], multiple myeloma [7] and melanoma [8]. Interactions between CD155 on tumor cells and CD226 on NK or T cells augment cell-mediated cytotoxicity and cytokine production [9–11]. However, TIGIT competes with CD226 for binding to CD155 and exerts potent inhibitory action in various subsets of immune cells [12–14]. In addition, CD155 is localized in the cell–matrix and cell–cell junctions, and it could inhibit cell adhesion and enhance cell migration [15]. Knockdown of CD155 resulted in markedly decreased invasion of glioblastoma [16]. Therefore, CD155 may be an attractive target for cancer therapy.

Emodin is a naturally occurring anthraquinone derivative that is found in several Chinese herbs, particularly in their roots and barks. These herbs have been wildly used as traditional medicines in many countries, especially in eastern Asia [17]. Emodin has been shown to possess a wide spectrum of pharmacological effects, such as anti-tumor, anti-inflammatory, antiviral, antibacterial, anti-allergic, anti-osteoporotic, anti-diabetic, immunosuppressive, neuroprotective and hepatoprotective activities [17]. Currently, a number of researchers are investigating its anti-tumor effects [18]. It has been shown to affect many different tumor cell lines and inhibit proliferation of leukemia, breast, colon, and lung carcinoma cells [19,20]. However, the molecular targets of emodin in tumor cells are elusive, and thus the mechanisms of its

Abbreviations: TME, the tumor microenvironment; TIGIT, T-cell immunoreceptor with Ig and ITIM domains; PRR, poliovirus receptor-related family

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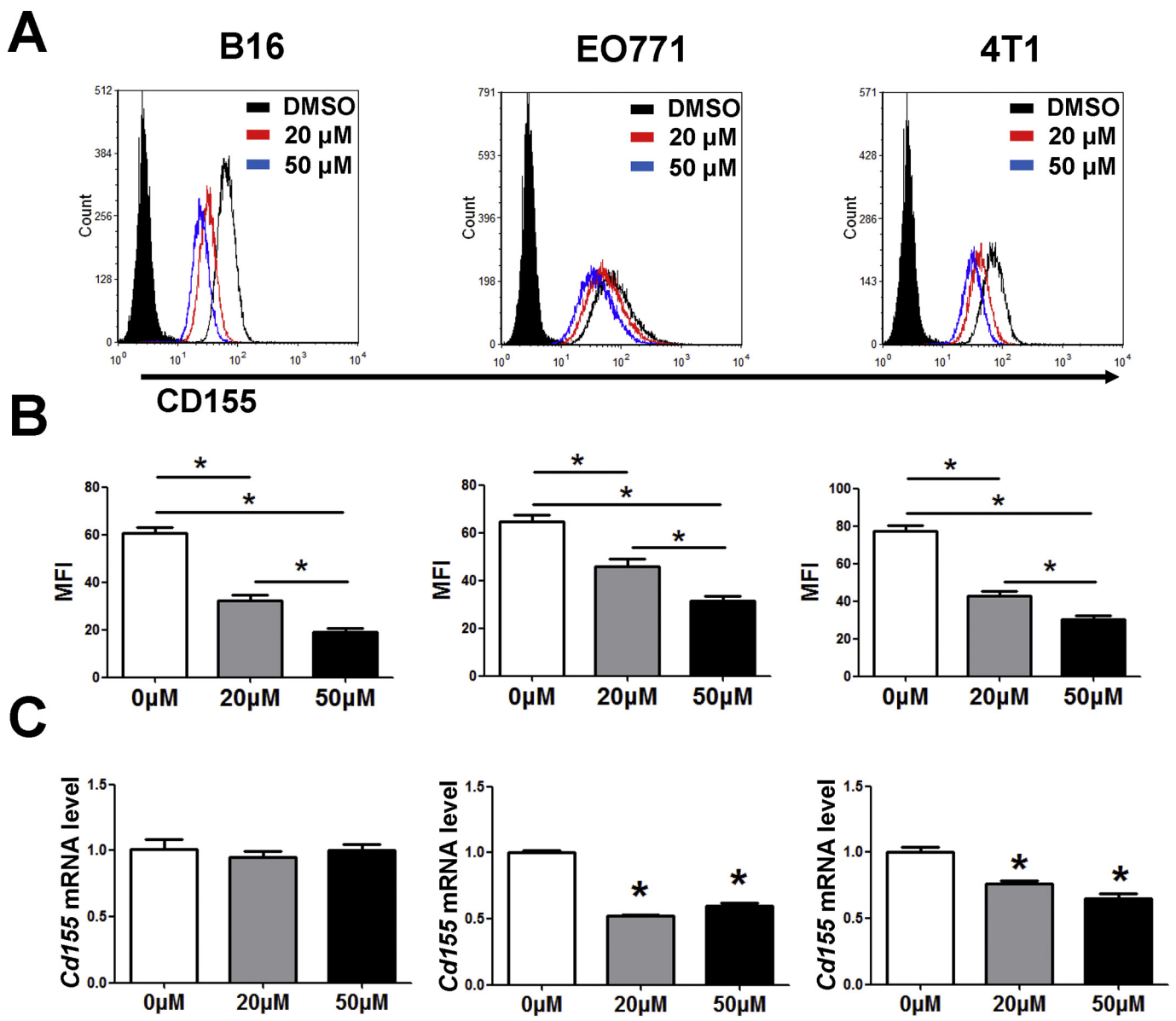

Fig. 1. Emodin decreased the expression of CD155 in cancer cells. (A) Emodin inhibited CD155 expression in B16-F10, 4T1 and EO771 cells. Cells were treated with or without 20 or 50 μM emodin for 24 h and then analyzed using flow cytometry. (B) Quantification of MFI is shown. *p < 0.05. (C) Cells were treated with or without 20 or 50 μM emodin for 24 h, and CD155 expression was analyzed by qPCR. Data were presented as means ± SEM of one of three independent experiments; n = 3. *p < 0.05 vs Control.

inhibitory effects on tumors are not completely understood.

Our laboratory has previously shown that emodin inhibited the growth and metastasis of breast tumors through inhibition of the macrophage-tumor cell feedforward tumor-promoting loop and macrophage M2-like polarization [21,22]. In this study, we demonstrated that emodin may also inhibit tumor growth via downregulation of CD155 in cancer cells. We provide new insights into the mechanisms by which emodin inhibits tumor growth.

2. Materials and methods

2.1. Reagents

Emodin, a trihydroxy-anthraquinone, was purchased from Nanjing Langze Medicine and Technology Co. Ltd. and verified by NMR spectroscopy and mass spectrometry as we previously described [23].

2.2. Cell culture and siRNA transfection

B16-F10 melanoma and 4T1 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). EO771 cells, developed from an ER^+ spontaneous mammary adenocarcinoma [24,25], were maintained in culture as previously described [22]. The cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and a combination of penicillin/streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Mouse CD155 siRNA and control siRNA (Qiagen, USA) were transfected into B16-F10, 4T1 and EO771 cells in 24-well plates using Lipofectamine 3000 (Invitrogen, CA, USA), according to the manufacturer's protocol.

2.3. Flow cytometric analysis (FACS)

Cells were stained with anti-CD155 PE mAb, anti-CD3 APC mAb, and anti-NK1.1 APC mAb (all from eBioscience, San Diego, CA) in

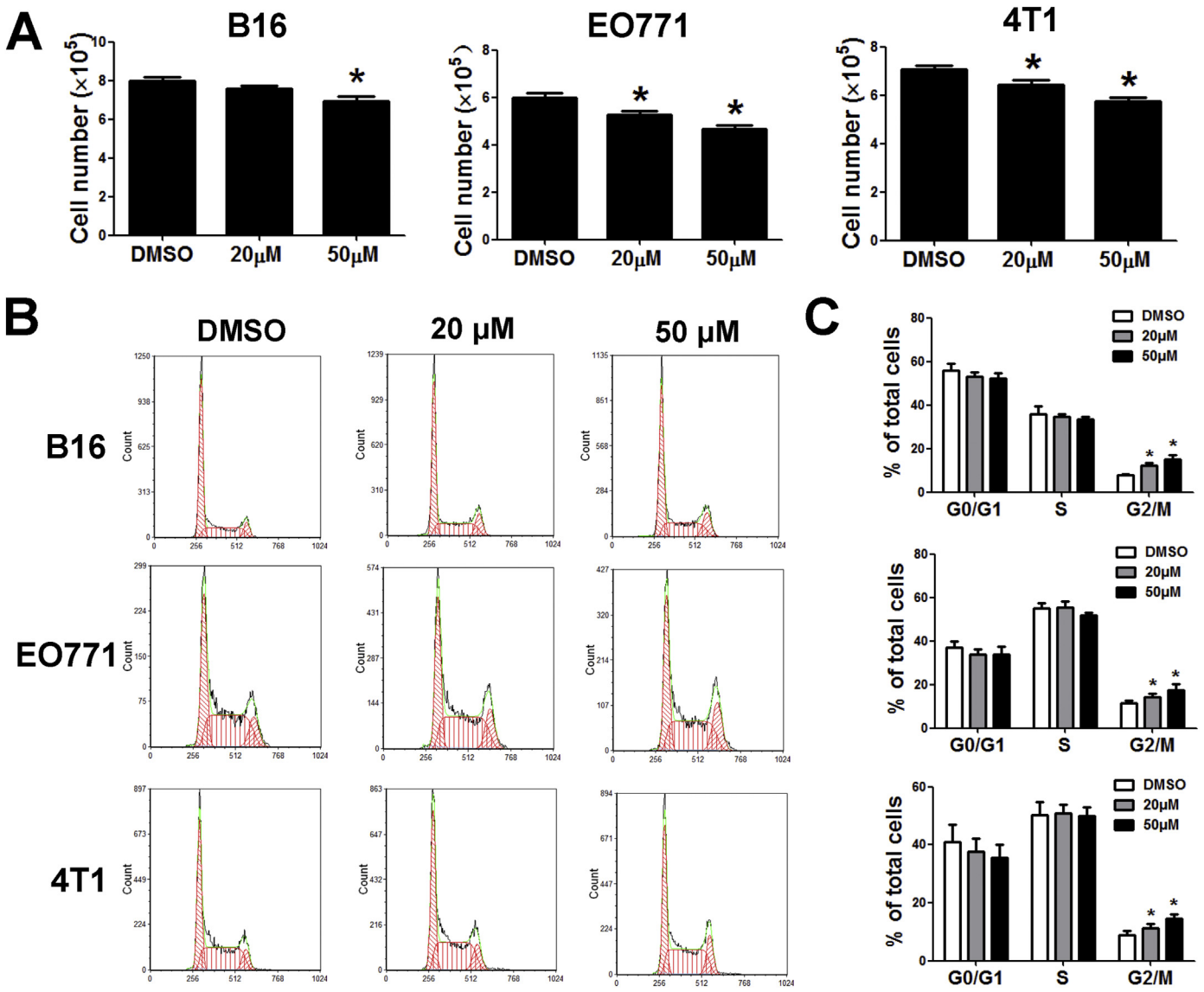

Fig. 2. Emodin suppressed cell proliferation and induced G2/M-phase arrest. (A) Relative number of cells after 24 h of 20 and 50 μM emodin treatment. Living cells were counted and compared with controls. Data were presented as means ± SEM of one of three independent experiments; n = 3. *p < 0.05 vs Control. (B) Emodin induced G2/M-phase arrest in tumor cells. The cell cycle distribution was monitored by flow cytometry. (C) Cell cycle results as means ± SEM of one of three independent experiments; n = 3. *p < 0.05 vs Control.

staining buffer (PBS containing 2% FBS) for 30 min on ice in the dark. Samples were washed twice with staining buffer, the mean fluorescence intensity (MFI) was analyzed by flow cytometry using a FACSAria flow cytometer and the FCS Express 4 software (De Novo Software, Glendale, CA, USA).

2.4. Cell isolation

Tumors were weighed, cut into small fragments (< 3 mm) and digested in 5 ml of dissociation solution (RPMI 1640 medium supplemented with 10% FBS, Collagenase type I (200 U/ml) and DNase I (100 μg/ml)) for 60 min at 37 °C. Erythrocytes were lysed with red blood cell lysing buffer (Sigma, St. Louis, MO). Cell suspensions were passed through 70-μm cell strainers, then washed and resuspended in staining buffer.

2.5. Quantitative real-time PCR (qPCR)

Total RNA was extracted using the TRIzol reagent (Invitrogen). RNA (1 μg) was reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad, Life Science). qPCR was conducted on a CFX96 system (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). All primers used for qPCR analysis were synthesized by Integrated DNA Technologies. All assays were conducted following the manufacturer's instructions. The relative amount of target mRNA was determined using the comparative threshold (Ct) method by normalizing target mRNA Ct values to those of 18S RNA. PCR thermal cycling conditions were 3 min at 95 °C, and 40 cycles of 15 s at 95 °C and 58 s at 60 °C. Samples were run in triplicate.

2.6. Wound healing assay

B16-F10, 4T1 cells and EO771 cells were seeded in 24-well plates and cultured until 70–80% confluent. A straight scratch was made using a pipette tip to form an artificial wound. The cells were treated with emodin and/or siRNA transfection, and the migration of cells across this artificial wound was assessed.

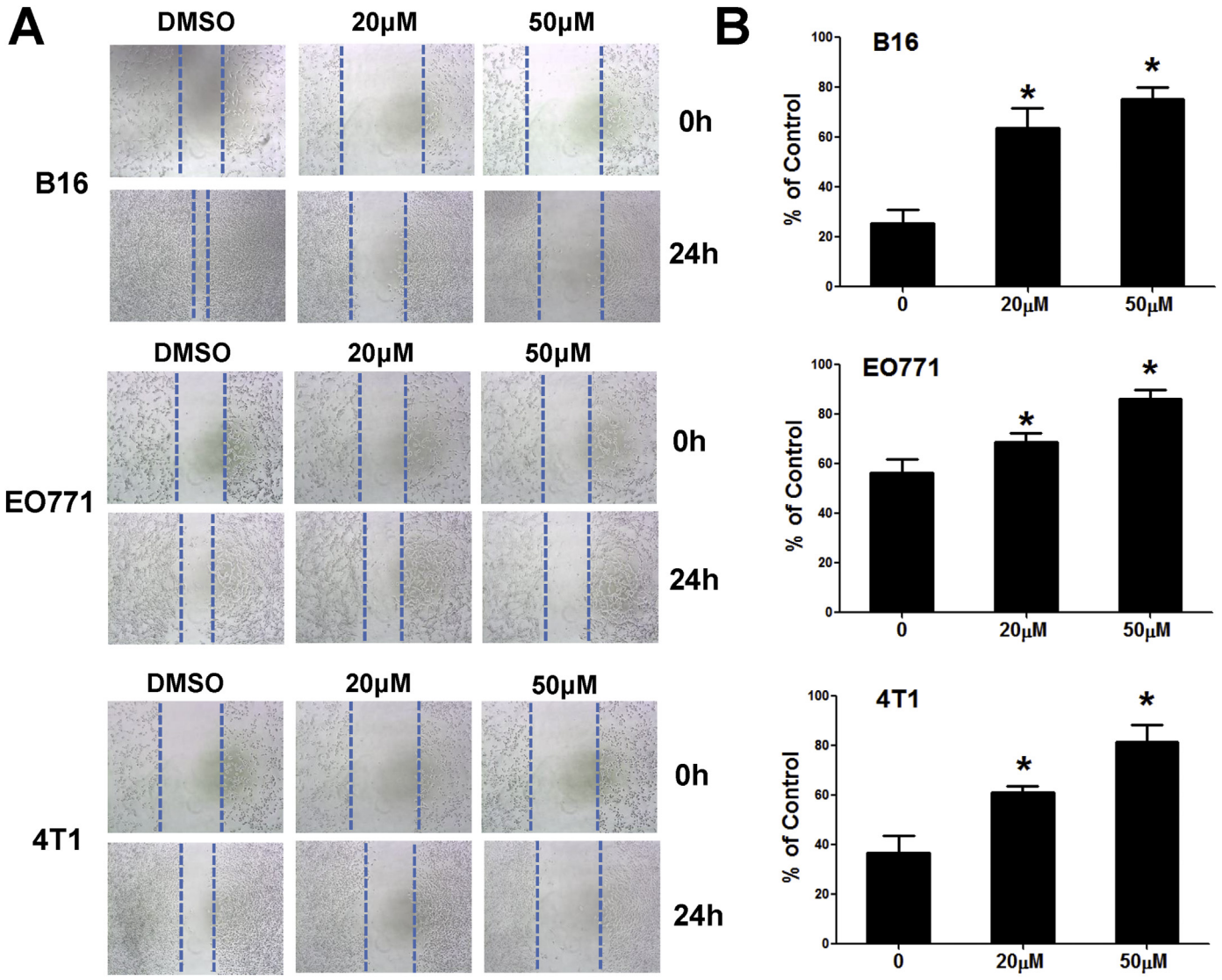

Fig. 3. Emodin inhibited migration of cancer cells. Effects of emodin on cancer cell migration were examined by a wound-healing assay. Representative images (A) and quantification of migration distance (B) were shown. Data were presented as means ± SEM of one of three independent experiments; n = 3. *p < 0.05 vs Control.

2.7. Proliferation and cell cycle analysis

B16-F10, 4T1 cells and EO771 cells (50%–60% confluent) were synchronized for overnight with the methods of serum starvation, and were treated with emodin (20 or 50 μM) for 24 h. Proliferation was evaluated by counting relative number of living cells after 24 h of 20 and 50 μM emodin treatment , and trypan blue staining was used to count the live cells. After washed twice with chilled PBS, cell pellet was resuspended in 150 μl cold PBS to which cold ethanol (450 μl) was added, then were incubated for 12 h at 4 °C. After centrifuged at 1000 rpm for 5 min, the pellet was washed twice with chilled PBS, and incubated with 100 μl RNase A (20 μg/ml) at 37 °C for 30 min. The cells were then cooled down on ice for 10 min and incubated with 400 μl PI (50 μg/ml) for 30 min in the dark and were analyzed by flow cytometry. Data were further analyzed by using ModiFitLT software (Verity Software House) for cell cycle analysis.

2.8. Tumor models

C57BL/6 (8–12 weeks, female) were purchased from The Jackson Laboratory. They were housed at the University of South Carolina Animal Research Facility, and all procedures were approved by the Institutional Animal Care and Use Committee. To establish subcutaneous tumors in mice, 5×10^6 B16-F10 cells in 200 μl of PBS were implanted into the rear flanks of mice. Starting on Day 1, emodin (40 mg/kg) or vehicle (1% DMSO) was injected intraperitoneally in 1 ml PBS once daily. Tumor growth was monitored by measurement of tumor size with a caliper every other day. Tumor volume was determined by the formula: length × width^2 / 2. At the experimental end point (15 days), mice were sacrificed, and tumors were removed, weighed and processed for FACS analysis.

2.9. Statistical analysis

Data were presented as mean ± SEM as indicated. Statistical significance was calculated using the Students' *t*-test (two-group comparison) or one-way ANOVA followed by post hoc Dunnett test (multi-group comparison) using the GraphPad Prism statistical program (GraphPad Prism; GraphPad Software, Inc.). $p < 0.05$ was considered significant.

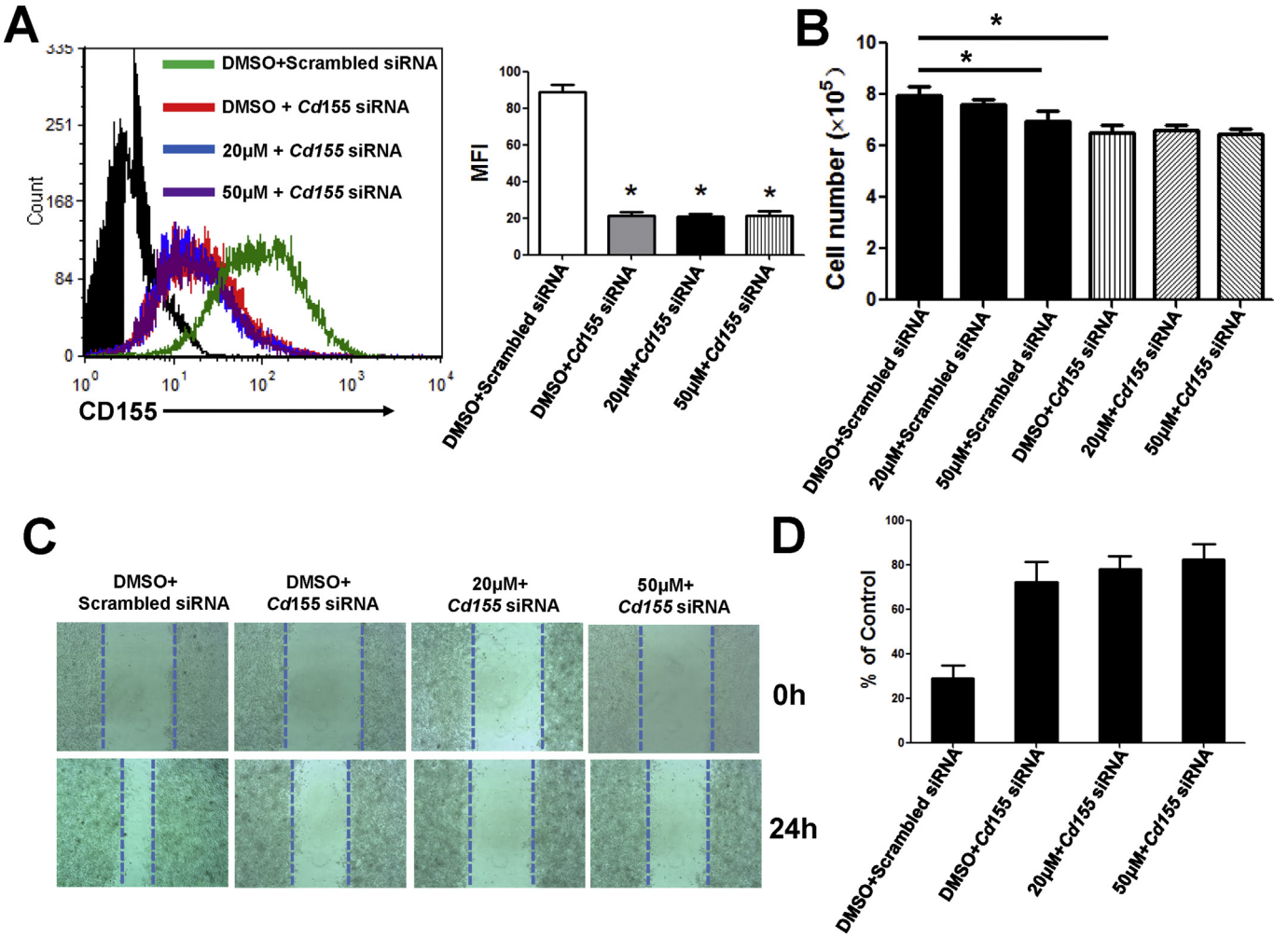

Fig. 4. Knockdown of CD155 abolished the effects of emodin on cancer cells. Cells were transfected with scrambled siRNA or siRNA targeting CD155 for 48 h, and treated with or without 20 or 50 μM emodin for 24 h. (A) Cell surface CD155 expression in B16-F10 cells was measured by flow cytometry. Representative histograms (left) and quantification of MFI (right) are shown. Data were presented as means ± SEM of one of three independent experiments; n = 3. *p < 0.05 vs Control. (B) Relative numbers of cells are shown. Living cells were counted and compared with controls. Data were presented as means ± SEM of one of three independent experiments; n = 3. *p < 0.05 vs Control. Cells were transfected with scrambled siRNA or siRNA targeting CD155 for 48 h, and then subjected to wound healing assays with or without 20 or 50 μM emodin for 24 h. Representative images (C) and quantification of migration distance (D) are shown. Data were presented as means ± SEM of one of three independent experiments; n = 3. *p < 0.05 vs all other groups.

3. Results

3.1. Emodin inhibits CD155 expression in multiple cancer cell lines

The alterations in expression of adhesion molecules have been shown to correlate with the growth of primary and metastatic tumors [26]. We hypothesized that emodin might regulate the expression of adhesion molecules in cancer cells, which may at least partially contribute to the tumor inhibitory effects of emodin observed in our previous studies [21,22]. We examined the expression of CD155 in mouse B16-F10 melanoma, EO771 and 4T1 breast cancer cells. The results showed that CD155 expression in the cells treated with emodin was significantly lower than that in control group (Fig. 1A and B). The results also showed that both emodin treatment at 20 μM and 50 μM decreased CD155 mRNA in EO771 and 4T1 cells, but had no effect on the expression of CD155 mRNA in B16-F10 cells (Fig. 1C).

3.2. Emodin suppresses cell proliferation and induces G2/M-phase arrest in cancer cells

Emodin has been shown to have detrimental effects on tumor cells in culture; we thus evaluated the response of cancer cell lines to emodin. Fig. 2A showed that emodin at 50 μM significantly reduced proliferation of B16, EO771 and 4T1 cells by 10–20%, while at 20 μM it only reduced proliferation of EO771 and 4T1. Cell cycle progression plays an important role in proliferation of cancer cells. Thus, we investigated cell phases of cancer cell lines in order to determine whether the inhibition of emodin on tumor cell proliferation was mediated by dysregulation of cell cycle. Fig. 2B showed the changes in the cell cycle of cancer cells induced by emodin. The proportion of cancer cells in the G2/M-phase of the cell cycle was increased significantly by emodin in a dose-dependent manner as compared with the untreated cells (Fig. 2C). Our data demonstrated that emodin causes G2/M-phase arrest in the cell cycle of cancer cells.

3.3. Emodin inhibits the migration of cancer cells

To examine the effects of emodin on tumor cell migration, cancer cells were treated with emodin. The motilities of B16-F10, EO771, and 4T1 cells were examined using wound-healing assays. At 24 h after treatment, the wound in the emodin group had healed less compared to that in the control group (Fig. 3).

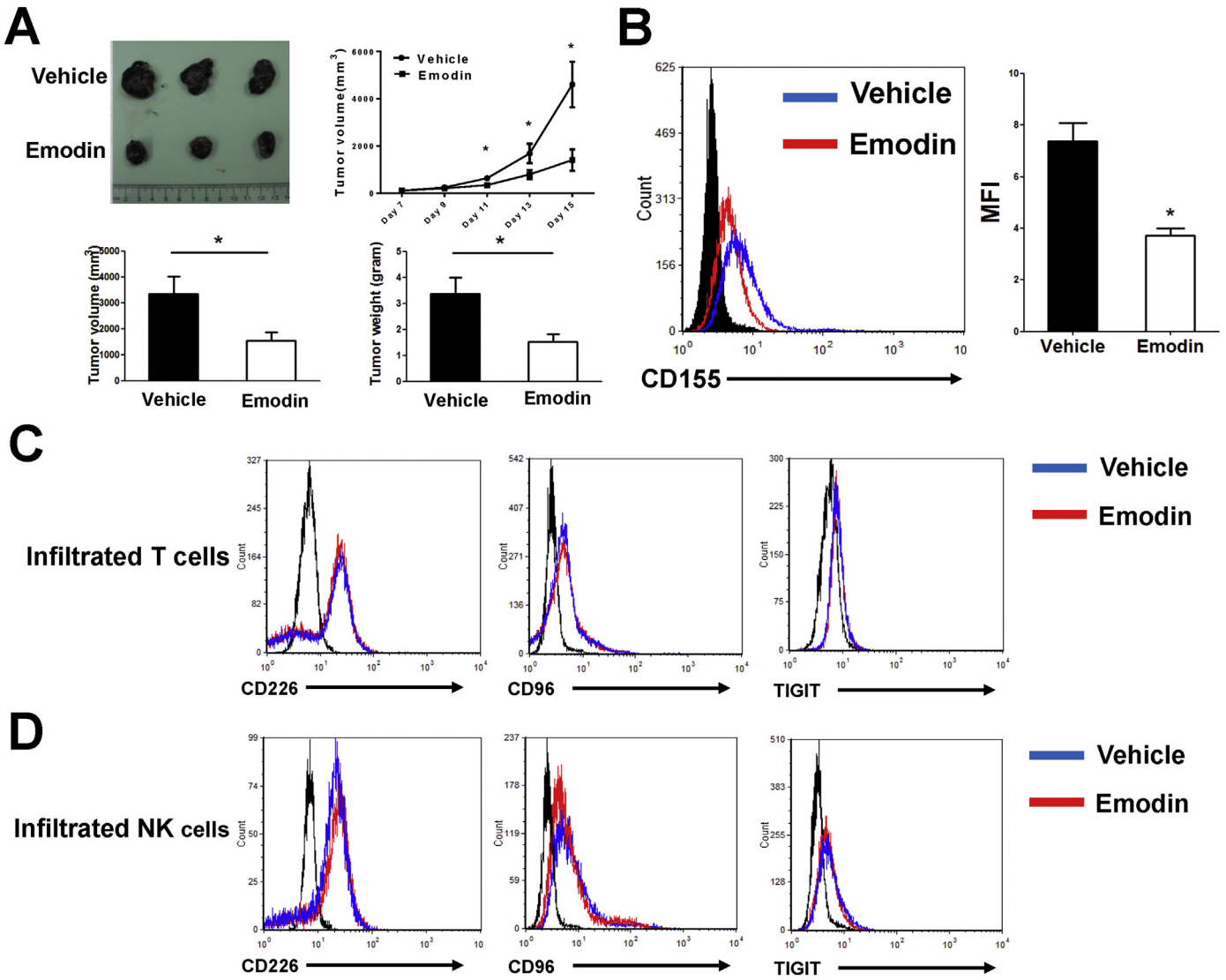

Fig. 5. Emodin decreased the expression of CD155 in tumors in vivo. (A) Emodin inhibited growth of B16-F10 melanoma. Representative tumors (upper), tumor sizes (lower left) and weights (lower right) are shown. (B) Cell surface CD155 expression in B16-F10 cells. Cancer cells were acquired from B16-F10 tumors at the experimental endpoint and measured by flow cytometry. Data were presented as means ± SEM of one of two independent experiments; n = 5. *$p < 0.05$. (C and D) Cell surface CD226, CD96 and TIGIT expression in tumor-infiltrating T cells (C) and NK cells (D) from control and emodin treatment mice were analyzed by flow cytometry. Tumor-infiltrating T cells were gated from $CD3^+$ population, and tumor-infiltrating NK cells were gated from $NK1.1^+$ population.

3.4. CD155 is involved in emodin-mediated tumor-suppressive effects

To examine the involvement of CD155 in anticancer activity of emodin, we transfected B16-F10 cells with CD155 small interfering RNAs (siRNA) for 48 h to knock down CD155 expression in the cells. We then treated the cells with 20 or 50 μM emodin for 24 h. Transfection of CD155 siRNAs significantly reduced CD155 expression in the cells, and emodin treatment did not further reduce CD155 expression (Fig. 4A). Silencing of CD155 in B16-F10 cells decreased the cell numbers, while emodin had no further effects (Fig. 4B). Furthermore, emodin could not further inhibit migration of the cancer cells with CD155 silencing (Fig. 4C–D). These results suggested that emodin's inhibitory effects on cancer cell growth and migration are dependent on CD155 expression.

3.5. Emodin inhibited CD155 expression in vivo

To examine if emodin affects tumor development in vivo through acting on CD155, B16-F10 melanoma cells were subcutaneously inoculated into the rear flanks of WT mice. Emodin treatment (40 mg/kg i.p. once daily) began 1 day after tumor cell injection. Emodin caused a significant inhibition of primary tumor growth, and reduced tumor weight and tumor size at the endpoints when the mice were sacrificed on Day 15 post-inoculation (Fig. 5A). Moreover, emodin treatment resulted in decrease of CD155 in the tumor cells (Fig. 5B), which is similar to the results obtained from the in vitro study. Furthermore, we examined if emodin affects the expression of CD155 binding partners in NK cells and T cells in the tumors, including the costimulatory receptor CD226 and the coinhibitory receptors CD96 and TIGIT. We isolated T cells and NK cells from the tumors at the experimental endpoint and found that there was no difference in the expression of CD226, CD96 and TIGIT in the tumor infiltrated T cells (Fig. 5C) and NK cells (Fig. 5D) between the two groups.

4. Discussion

Emodin has been reported to induce cancer cell apoptosis [27], inhibit metastasis [22], induce cell cycle arrest [28], and reverse multidrug resistance [29]. Our previous study showed that emodin could significantly inhibit the growth of early-stage breast cancer and effectively suppress metastatic tumor growth at the late stage by modulating

the tumor microenvironment (TME) [21,22,30]. Although there were important reports that revealed multiple signaling pathways and molecules involved in the anti-cancer functions of emodin [18], and emodin in combination with chemotherapy could increase the therapeutic efficacy [31,32], the detailed molecular mechanisms remained to be elucidated. In this study, our data showed emodin inhibited the tumor growth also through downregulation of CD155 in cancer cells. These results suggest that emodin inhibits tumor development by multiple mechanisms.

The majority of previous studies on emodin have been focused on emodin's direct toxicity to tumor cells. In this study, we found that emodin could decrease some adhesion molecules which are normally associated with cell migrations and often up-regulated in tumors. Growing evidences indicate that alterations in the adhesion properties of tumor cells play a pivotal role in the development and progression of cancer [33]. CD155 is an immunoglobulin-like molecule which is barely detected in most normal tissues, but highly expressed in different type of tumors, including colon cancer, lung adenocarcinoma, melanoma, pancreatic cancer and glioblastoma [2,8,34,35]. It has been reported to be involved in cell–cell adhesion via a heterophilic trans-interaction with nectin-3 and thus plays important roles in cell adhesion and migration [36–38]. CD155 overexpression contributes to tumor growth and metastasis [34,39]. CD155 downregulation suppresses tumor cell proliferation and induces cell-cycle arrest at G2/M phase [35]. In this study, we examined if emodin inhibits CD155 expression in multiple cancer cell lines. We used 3 tumor cell lines, B16, 4T1 and EO771, in all of which CD155 is highly expressed. We found that emodin could significantly decrease the expression of CD155 in these cells and thereby inhibited their proliferation and migration. Therefore, we hypothesized that the anticancer activity of emodin is at least partially CD155 dependent. In support of this hypothesis, we observed that knockdown of CD155 by siRNA significantly abolished the effect of emodin on cancer cell proliferation in vitro. To further validate the involvement of CD155 in the inhibitory effects of emodin on tumor growth in vivo, we used a B16 melanoma model in mice. We found emodin could decrease CD155 expression in implanted B16 tumors and inhibit tumor growth. Taken together, these results indicated that CD155 is a new target of emodin in tumors.

CD155 can ligate with costimulatory molecule CD226, or coinhibitory molecules TIGIT and CD96, to exert a dual function in oncoimmunity [40]. Interaction of CD226 with CD155 triggers NK or T cell-mediated cytotoxicity [9,41], while CD155/TIGIT or CD155/CD96 ligation could mediate inhibitory signaling in immune cells [12,13,42]. Emodin has been reported to exert a broad range of actions on the immune system. While our study in vivo showed that emodin did not have an effect on the expression of all above three CD155 receptors in T cells and NK cells, it is conceivable that emodin may affect T cell and NK cell function through reducing the binding of CD155 to these receptors, especially the coinhibitory molecules TIGIT and CD96. This aspect warrants further investigation.

The mechanism by which emodin suppresses the suppression of CD155 in tumor cells is currently unknown. Emodin has been reported to inhibit LPS-induced expression of proinflammatory cytokines in macrophages through suppressing Erk1/2 and p38 signaling [23]. It is also reported regulation of CD155 expression involves Raf-MEK-ERK-AP1 signaling [43]. Therefore, it is likely that emodin might use one of these signaling axes to regulate CD155 expression. However, we cannot exclude other mechanisms. For example, emodin treatment significantly suppressed the TWIST1-induced upregulation of CD44, which is associated with tumor initiation [44].

In conclusion, we propose a new mechanism by which emodin suppresses tumor growth. CD155 expression is upregulated in some cancers, and some chemotherapeutic agents can also upregulate CD155 on tumor cells [45]. Emodin can inhibit tumor cell proliferation and migration through decreasing their expression of CD155. Considering that emodin can act on both cancer cells and macrophages in the TME, our study provides a more comprehensive mechanistic insight into the anti-tumor activities of emodin.

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Declaration of Competing Interest

Authors declare no conflicts of interest.

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