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Preclinical analysis of the anti-tumor and anti-metastatic effects of Raf265 on colon cancer cells and CD26⁺ cancer stem cells in colorectal carcinoma

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Abstract

Background: In colorectal carcinoma (CRC), activation of the Raf/MEK/ERK signaling pathway is commonly observed. In addition, the commonly used 5FU-based chemotherapy in patients with metastatic CRC was found to enrich a subpopulation of CD26⁺ cancer stem cells (CSCs). As activation of the Raf/MEK/ERK signaling pathway was also found in the CD26⁺ CSCs and therefore, we hypothesized that an ATP-competitive pan-Raf inhibitor, Raf265, is effective in eliminating the cancer cells and the CD26⁺ CSCs in CRC patients.

Methods: HT29 and HCT116 cells were treated with various concentrations of Raf265 to study the anti-proliferative and apoptotic effects of Raf265. Anti-tumor effect was also demonstrated using a xenograft model. Cells were also treated with Raf265 in combination with 5FU to demonstrate the anti-migratory and invasive effects by targeting on the CD26⁺ CSCs and the anti-metastatic effect of the combined treatment was shown in an orthotopic CRC model.

Results: Raf265 was found to be highly effective in inhibiting cell proliferation and tumor growth through the inhibition of the RAF/MEK/ERK signaling pathway. In addition, anti-migratory and invasive effect was found with Raf265 treatment in combination with 5FU by targeting on the CD26⁺ cells. Finally, the anti-tumor and anti-metastatic effect of Raf265 in combination with 5FU was also demonstrated.

Conclusions: This preclinical study demonstrates the anti-tumor and anti-metastatic activity of Raf265 in CRC, providing the basis for exploiting its potential use and combination therapy with 5FU in the clinical treatment of CRC.

Keywords: Colorectal cancer, Cancer stem cells, Raf265, Raf/MEK/ERK signaling pathway, Metastasis

Background

Colorectal carcinoma (CRC) is a common cancer worldwide [1]. Currently, surgery remains the first-line treatment in localized CRC. Among patients with metastatic CRC (mCRC), only 10-25% are suitable for surgical resection and the five-year survival rate is around 30-40% [2-4]. However, the prognosis remains poor for unresectable mCRC patients and a median survival of up to 24 months was reported despite advances in chemotherapy and molecular targeting drugs [5]. Intrinsic and acquired chemoresistance remains the major reason for

the failure of chemotherapy. A recent study from our team suggests that chemotherapy with fluorouracil (5FU) enriches a subpopulation of CD26⁺ cells and CD26⁺ cells are the cancer stem cells (CSCs) responsible for metastasis, enhanced invasiveness and chemoresistance in CRC [6]. This suggested that conventional chemotherapy treatment is not effective enough to completely eradicate the tumor with metastatic stem cells.

Constitutive activation of the Raf/MEK/ERK pathway is commonly observed in CRC due to overexpression or mutations of the upstream receptor tyrosine kinases (EGFR, PDGFR or VEGFR) or the downstream effectors (Ras, Raf, MEK or ERK). Since this signaling pathway plays a central role in controlling cell proliferation,

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survival, differentiation and metastasis [7], a number of therapeutics targeting on the Raf/MEK/ERK pathway has been established [8]. Raf265, a highly selective inhibitor of Raf (Braf, Craf and mutant Braf) and VEGFR kinase, is an orally bioavailable small molecule [9]. The anti-proliferative effect of Raf265 has been published in melanoma [10] and neuroendocrine tumor cells [11]. The study of the pharmacokinetics and pharmacodynamics of Raf265 has also entered the Phase I/II study in patients with locally advanced or metastatic melanoma.

In this study, our aim is to validate the efficacy of Raf265 on inhibiting tumor growth and preventing metastasis in CRC. A further investigation of the effect of Raf265 on CD26⁺ cells treated with 5FU was carried out. By using an *in vivo* study, we further provide evidence of reduced liver and lung metastasis by Raf265 treatment in combination with 5FU. Therefore, with this study, the pre-clinical anti-tumor and anti-metastatic effects of Raf265 can be demonstrated, which provides the basis for exploiting the use of Raf265 as a potential treatment against mCRC.

Results

Anti-proliferative and apoptotic effects of Raf265 on HT29 and HCT116 cells with the inhibition of Raf/MEK/ERK signaling pathway

The effect of Raf265 on cell proliferation was measured by the MTT cell proliferation assay and the soft agar

colony formation assay. Treatment of Raf265 for 72 hours inhibited cell proliferation in a dose dependent manner with an IC₅₀ of 2.08 μM and 1.83 μM in HT29 and HCT116 cells, respectively (Figure 1A). Dose-dependent reduction in the number and size of colony formed in soft agar was also observed (Figure 1B). When treated with 1 μM Raf265 for 3 weeks, the number of colony formed reduced from 38.6 ± 6.5 and 28.3 ± 3.5 to 1.67 ± 1.15 and 0.67 ± 0.58 colonies for HT29 and HCT116 cells, respectively.

We then determined the apoptotic effect of Raf265 on HT29 (Figure 2A) and HCT116 (Figure 2B) cells with the annexin V/PI assay. After exposing the cells to the indicated concentrations of Raf265 for 48 h, the numbers of apoptotic cells increase with increasing concentrations of Raf265. The percentages of annexin V positive cells increase from 10.5% ± 2.41% at 0 μM Raf265 to 35.1% ± 6.77% at 15 μM Raf265 in HT29 cells and from 20.1% ± 2.99% at 0 μM Raf265 to 42.2% ± 3.58% 15 μM Raf265 in HCT116 cells. To study if the apoptotic effect of Raf265 is a caspase-dependent process, flow cytometry was used to study the activities of caspase 9, caspase 8 and caspase 3 in HT29 (Figure 2C) and HCT116 (Figure 2D) cells. After treatment with 10 μM of Raf265 for 2 days, we found the increases of activities of caspase 9 (HT29: from 2.11% ± 0.33% to 4.52% ± 0.56%; HCT116: 3.25% ± 0.25% to 5.13% ± 0.34%), caspase 8 (HT29: from 2.45% ± 0.40% to 5.07% ± 0.42%; HCT116: 1.34% ± 0.23%

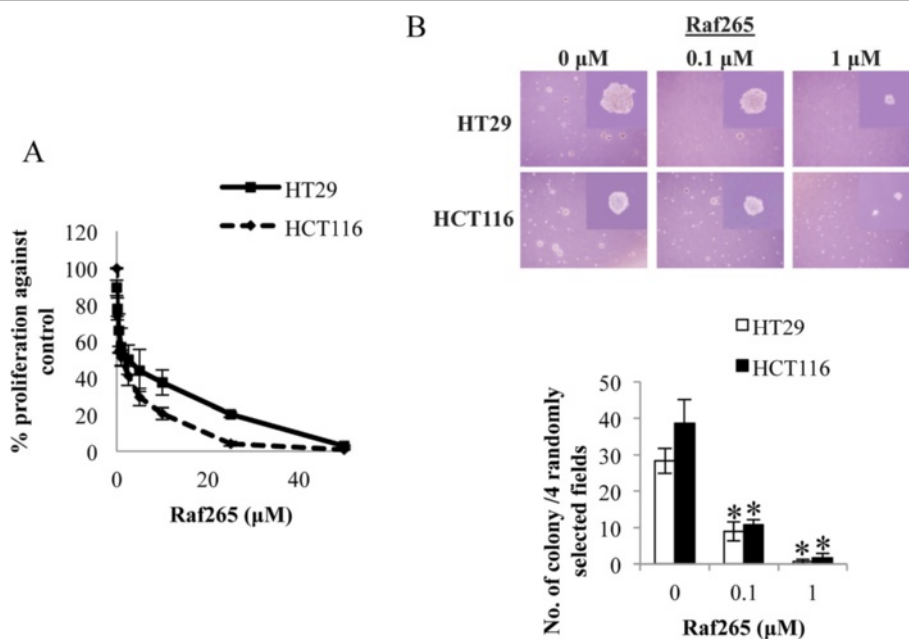
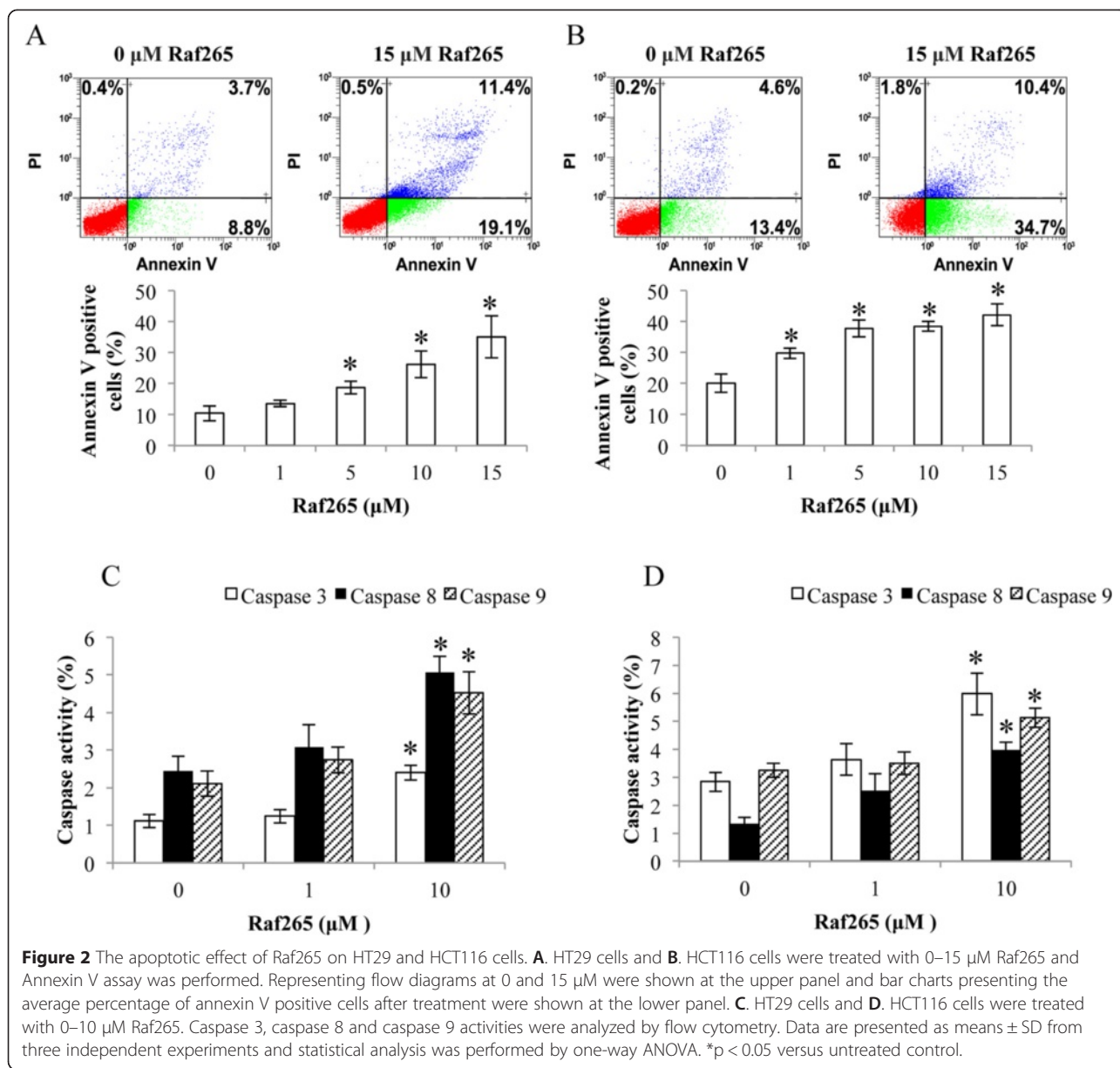


Figure 1 The anti-proliferative effect of Raf265 on HT29 and HCT116 cells. **A**. Cells were treated with Raf265 at 0–50 μM and MTT assay was performed. **B**. Cells were suspended in the solidified agarose at the indicated concentrations of Raf265. Representing images under a phase-contrast microscopy at 40x magnification and an amplified view at 400x magnification were shown at the upper panel. The number of colony formed was then counted and the bar chart presenting the average number of colony formed was shown at the lower panel.



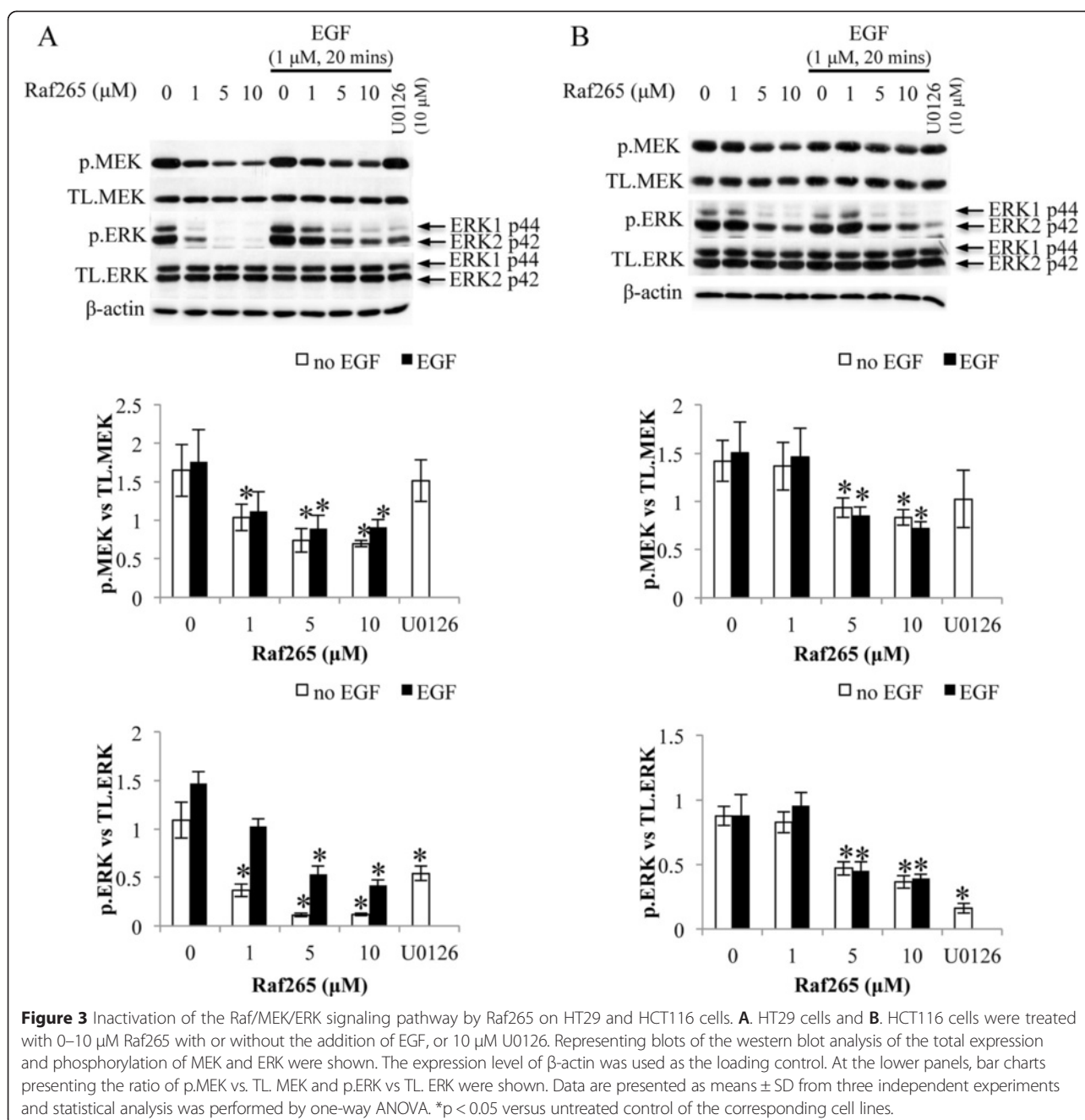
to 3.98% ± 0.29%) and caspase 3 (HT29: from 1.11% ± 0.17% to 2.4% ± 0.20%; HCT116: 2.84% ± 0.35% to 5.98% ± 0.74%).

In order to validate the inhibitory effect of Raf265 on the Raf/MEK/ERK signaling pathway, we further performed the western blot analysis on the total expression and phosphorylation of MEK and ERK after 2 hours treatment with the indicated concentrations of Raf265 in serum free medium. Treatment with increasing concentration of Raf265 inhibited MEK and ERK phosphorylation significantly in both HT29 (Figure 3A) and HCT116 (Figure 3B) cells. With the addition of EGF, which binds EGFR and activates the Raf/MEK/ERK signaling pathway, the inhibition of phosphorylation by

Raf265 was still significant. 1 μM U0126, an MEK inhibitor, was added as a control to demonstrate the inhibition of ERK phosphorylation. The total protein expressions of MEK and ERK were not change with the Raf265 treatment.

Anti-tumor and effect of Raf265 on HT29 and HCT116 cells

An *in vivo* study was used to demonstrate the anti-tumor effect of Raf265. Intraperitoneal injection of Raf265 at 0.2 mg/kg twice a week for 16 weeks significantly reduced the subcutaneous tumor weight starting from week 12 after injection (Figure 4A). The anti-tumor effect was similar between tumors derived from



HT29 and HCT116 with a reduction of tumor weight by 53.8% and 50.4% respectively. The anti-angiogenic effect was also demonstrated by the reduction of CD31 expression expressed by the tumors raised from HT29 and HCT116 cells (Figure 4B).

Activated Raf/MEK/ERK signaling pathway in CD26⁺ cells

As Raf265 targets on the Raf/MEK/ERK signaling pathway, western blotting analysis was performed to compare the Raf expression of CD26⁺ and CD26⁻

cells, in order to predict the efficacy of Raf265 on CD26⁺ CSCs. Five tumor specimens were dissected and sorted for CD26⁺ and CD26⁻ cells. CD26⁺ cells demonstrated a higher expression of Braf than that of the CD26⁻ cells (Figure 5). CD26⁺ cells also demonstrated higher phosphorylation levels of MEK and ERK. The higher Braf expression with enhanced phosphorylation of MEK and ERK in CD26⁺ cells suggested a possible therapeutic effect of Raf265 on CD26⁺ CSCs.

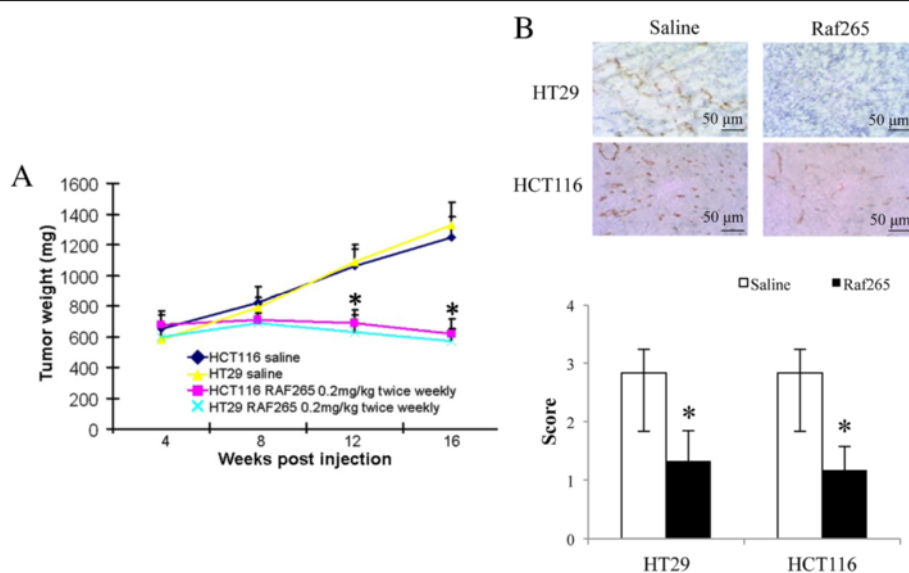


Figure 4 The anti-tumor effect of Raf265 on HT29 and HCT116 cells. **A**. The average tumor weight of mice treated with Raf265 were shown. Data are presented as means \pm SD from 6 nude mice and statistical analysis was performed by one-way ANOVA. * $p < 0.05$ versus the vehicle control group with the xenograft tumors from the corresponding cell lines. **B**. Representing diagrams of the IHC staining of CD31 (magnification: 400 \times) of the primary tumor from HT29 and HCT116 cells of the vehicle control group and treatment group were shown. At the lower panel, a bar chart presenting the scoring of IHC staining of CD31 based on the percentage and intensity of the positively stained cells under high power (400 \times) microscopy was shown. Data are presented as means \pm SD from 6 mice in each group. * $p < 0.05$ vs saline group by one-way ANOVA.

Reduced proliferation and self-renewal ability of CD26⁺ cells by Raf265

As mentioned, treatment with chemotherapy can easily develop multi-drug resistance and enrichment of CD26⁺ subpopulation can also result in chemoresistance and enhanced metastasis. In this study, treatment of HT29 cells with 5FU was found to increase the percentage of CD26⁺ cells by 2 folds whereas treatment with Raf265 did not change the percentage of CD26⁺ cells (Figure 6A). In addition, combined treatment of Raf265 and 5FU significantly restored the percentage of CD26⁺ cells to the baseline level.

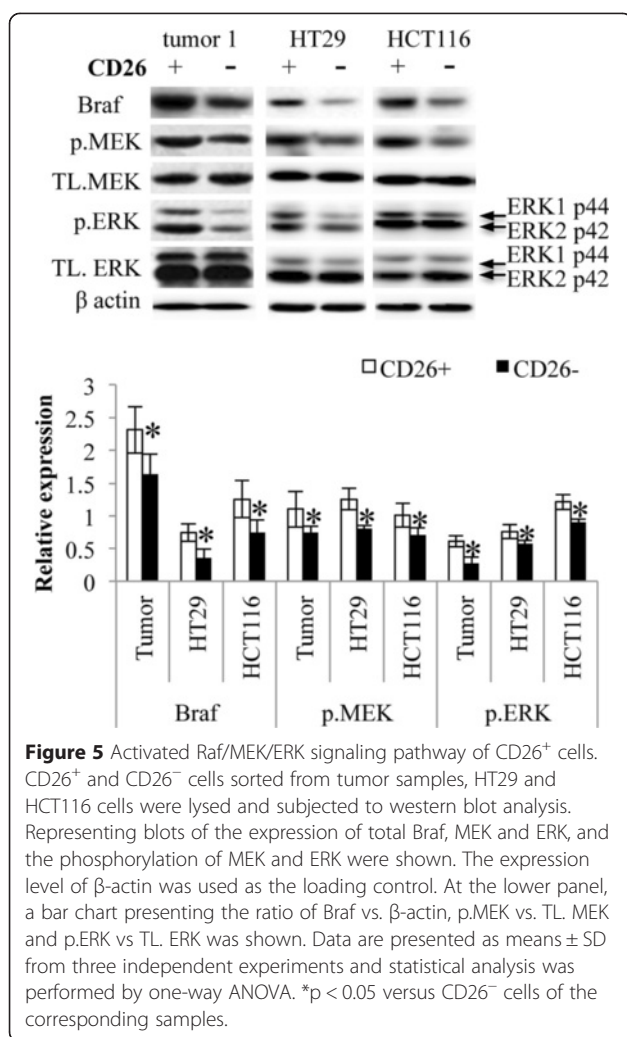
Serial passage of CD26⁺ cells treated with Raf265 was done to study the effect of Raf265 on the ability of self-renewal of CD26⁺ cells (Figure 6B). For the first passage, the number of sphere formed reduced from 25.0 ± 5.0 to 4.0 ± 3.6 when treated with $0.1 \mu\text{M}$ Raf265. For the second passage, the number of sphere formed reduced from 17.7 ± 2.5 to 1.3 ± 0.6 when treated with $0.1 \mu\text{M}$ Raf265. No sphere can be found when treated with $1 \mu\text{M}$ Raf265.

We then developed the 5FU-R cells with an IC₅₀ to 5FU treatment increased from $14.2 \mu\text{M}$ for the CTL cells to $85.7 \mu\text{M}$ for the 5FU-R cells (Figure 6C). Both 5FU-R and CTL cells are sensitive towards Raf265 treatment with an IC₅₀ of $2.43 \mu\text{M}$ and $1.06 \mu\text{M}$, respectively (Figure 6D) and flow analysis of the resistant cells demonstrated an enrichment of the CD26⁺ CSCs when

compared with the CTL cells ($12.9\% \pm 0.7\%$ in 5FU-R cells vs $5.8\% \pm 0.4\%$ in CTL cells).

Enhanced apoptotic effect and reduced migratory and invasive ability of CD26⁺ cells by Raf265

We then further investigate if Raf265 is able to induce apoptosis of CD26⁺ cells by blocking the Raf/MEK/ERK signaling pathway. Cells were first sorted for CD26⁻ and CD26⁺ cells and subjected to different treatments. Cellular apoptosis was determined by annexin V assay (Figure 7A). Treatment with 5FU significantly increased the percentage of annexin V positive cells of CD26⁻ cells to a greater extent than the CD26⁺ cells (2.5 folds in CD26⁻ cells vs 1.4 folds in CD26⁺ cells). On the other hand, treatment of Raf265 significantly increased the percentage of annexin V positive cells in both CD26⁻ (by 1.4 folds) and CD26⁺ (by 1.8 folds) cells. As for the combined treatment of Raf265 and 5FU, the percentage of annexin V positive cells of the CD26⁻ cells was similar to the percentage of annexin V positive cells when treated with 5FU alone (increased by 2.5 folds). On the other hand, the percentage of annexin V positive cells of CD26⁺ cells (increased by 2.7 folds) was higher than the percentage of annexin V positive cells with any of the single drug treatment. Therefore, treatment with Raf265 or combined treatment of Raf265 and 5FU are more effective in enhancing cellular apoptosis of CD26⁺ cells than that of the CD26⁻ cells.



The migratory (Figure 7B) and invasive (Figure 7C) ability of the CD26⁺ cells were also studied after single and combined treatment of Raf265 and 5FU. No significant change in number of migrated and invaded cells when treatment with 5FU alone. A significant decrease in number of migrated cells (by 1.5 folds) and invaded cells (by 2.2 folds) were found when treated with Raf265 and combined treatment of Raf265 and 5FU further reduced the number of migrated cells (by 2.3 folds) and invaded cells (by 5 folds) of the CD26⁺ cells. Therefore, treatment with Raf265 or combined treatment of Raf265 and 5FU reduced the migratory and invasive ability of the CD26⁺ cells.

Anti-tumor effect of Raf265 in combination with 5FU

We further study the effect of combined treatment of Raf265 and 5FU using an animal model (Figure 8). Drugs were administrated 2 weeks after tumor inoculation in the caecal wall. Single or combined treatment was tolerated with no lethality and no significant weight loss

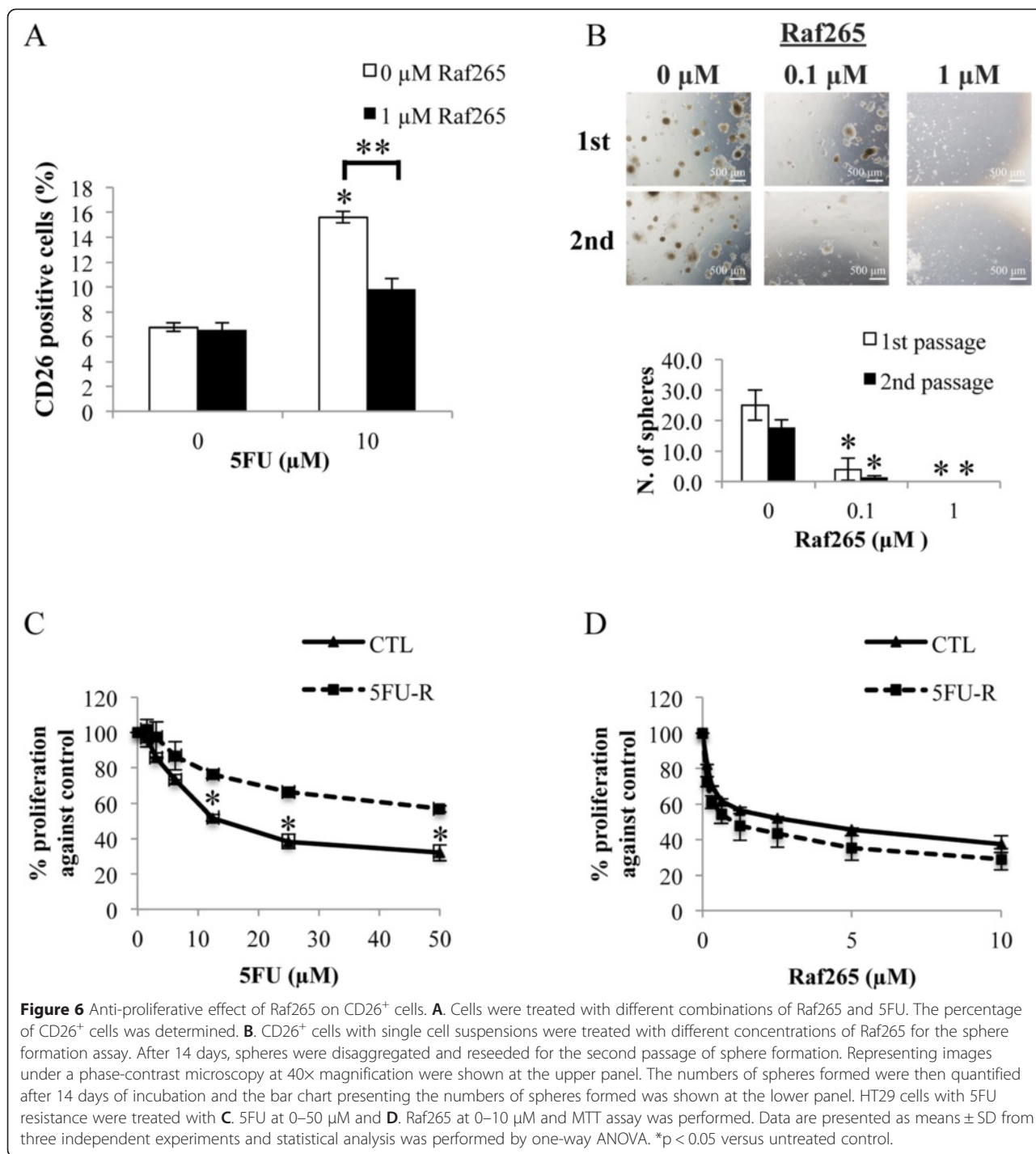
was observed in both tumors derived from HT29 cells (Figure 8A) and HCT116 cells (Figure 8B). For the HT29 cells derived tumor, single treatment with Raf265 at 10 and 20 mg/kg body weight, and 5FU at 20 mg/kg body weight significantly reduced the bioluminescence signals produced from tumor cells by 56.6%, 82.2% and 35.0%, respectively when compared with the control group whereas combined treatment of 5FU with Raf265 at 10 and 20 mg/kg body weight significantly reduced the bioluminescence signals by 76.7% and 92.0%, respectively when compared with the control group (Figure 8C). As for the HCT116 cells derived tumor, single treatment with Raf265 at 10 and 20 mg/kg body weight and 5FU at 20 mg/kg body weight significantly reduced the bioluminescence signals produced from tumor cells by 66.1%, 88.6% and 72.7%, respectively when compared with the control group whereas combined treatment of 5FU with Raf265 at 10 and 20 mg/kg body weight significantly reduced the bioluminescence signals by 89.5% and 96.6%, respectively when compared with the control group (Figure 8D). Therefore as expected, single and combined drug treatment significantly reduced the primary tumor size in this mouse orthotopic model.

Anti-metastatic effect of Raf265 in combination with 5FU

Besides reducing the primary tumor size, we also study if the treatment can reduce the chance of developing metastasis towards liver and lung, which are the common distant metastatic sites for CRC. The presence of liver and lung metastasis after single and combined treatment of Raf265 and 5FU was studied by tracking the bioluminescence signal detected from the specific organ (Figure 9A-D). All mice in the control group developed liver and lung metastasis. In general, single treatment reduced the number of cases and/or the liver and lung metastatic tumor size indicated by the reduction in the bioluminescence signal detected. Combined treatment with different concentrations of Raf265 and 5FU significantly reduced liver and lung metastasis. Treatment with Raf265 at 20 mg/kg body weight and 5FU at 20 mg/kg body weight prevent both the liver and lung metastasis except one case demonstrated a small lung metastasis with HT29 cells derived tumor. Therefore, combined treatment with Raf265 and 5FU is effective in preventing lung and liver metastasis in this mouse orthotopic model.

Discussion

Recent advances in the study of molecular targeted therapy in cancers lead to the development of certain inhibitors or antibodies against specific components in a signaling pathway, which controls cell proliferation and apoptosis. Activation of the Raf/MEK/ERK signaling pathway is commonly found in CRC due to the overexpression



or presence of activating mutations of EGFR, Braf and/or Kras [12-15]. A point mutation at V600E position of Braf occurs in about 10% CRC patients [16]. The presence of BrafV600E mutation leads to a constitutively active protein, which activates the Raf/MEK/ERK signaling pathway [17] and BrafV600E mutation is correlated with poorer overall survival and disease-free survival than tumors with wild type Braf [18-21]. Therefore, in this study, the

targeting effect of Raf265 on HT29 cells with mutant Braf and wild type Kras, and on HCT116 cells with wild type Braf and mutant Kras was demonstrated. RAF265 was found to be highly effective in inhibiting cell proliferation and tumor growth through the inhibition of the RAF/MEK/ERK signaling pathway.

In this study, the anti-proliferative and anti-apoptotic effect of Raf265 was found to be effective in both HT29

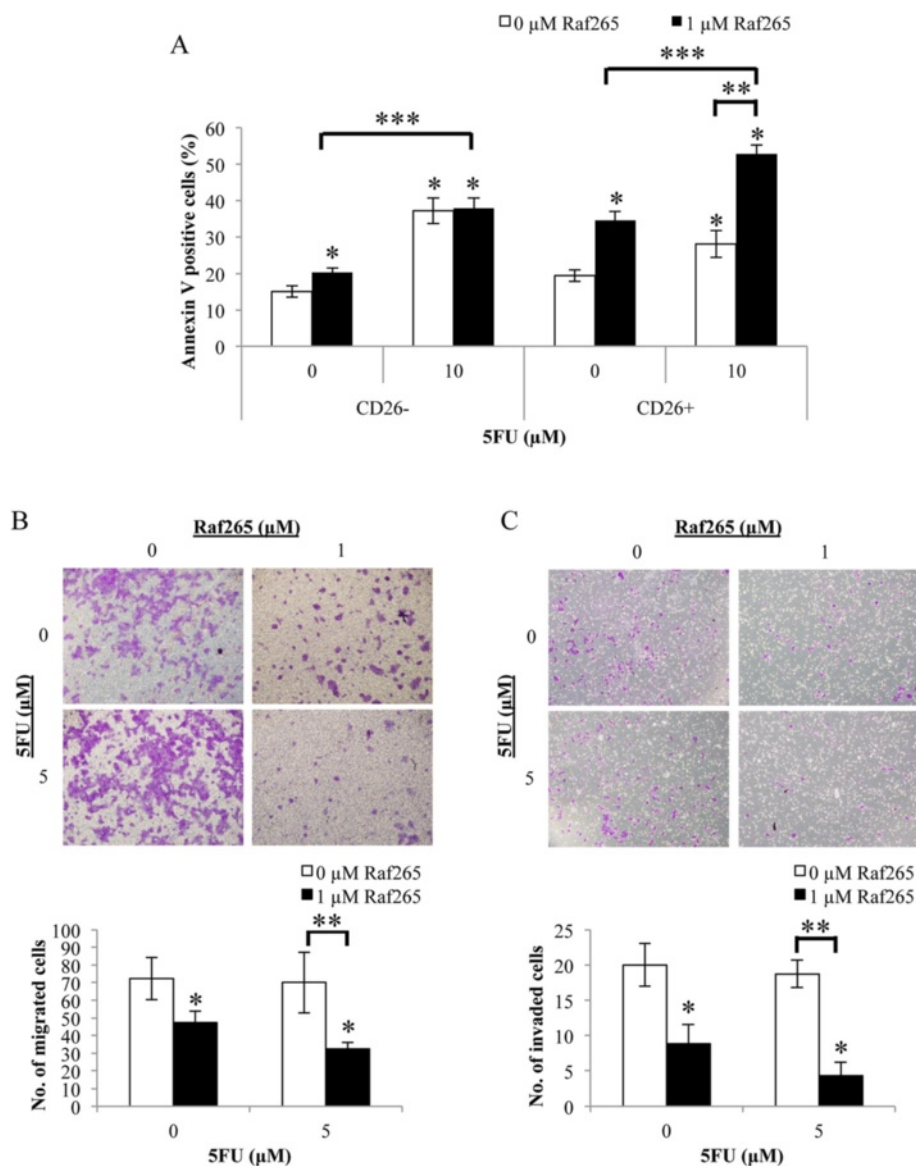


Figure 7 Enhanced apoptotic and reduced migratory and invasive ability of Raf265 by targeting on CD26⁺ CSCs. **A**. CD26⁺ and CD26⁻ cells were treated with different combinations of Raf265 and 5FU and the percentage of annexin V positive cells were analyzed. After treatment, representing view under 100x magnification of the **B**. migrated and **C**. invaded cells were shown at the upper panel and the average numbers of the **B**. migrated and **C**. invaded cells were plotted and shown at the lower panel. Data are presented as means ± SD from three independent experiments and statistical analysis was performed by one-way ANOVA. *p < 0.05 versus untreated control, **p < 0.05 versus 0 μM Raf265 and 10 μM or 5 μM 5FU as indicated and # p < 0.05 versus CTL cells. ***p < 0.05 versus 1 μM Raf265 and 0 μM 5FU.

and HCT116 cells with different Braf and Kras genetic status. However, several studies have demonstrated that Raf inhibitors were only effective in cancers with wild type Kras and mutant Braf. In cancers with wild-type Braf, sorafenib and Raf inhibitors like PLX4720/PLX4032 transactivate Raf dimers and thus activating the Raf/MEK/ERK signaling pathway [22]. Other studies have been carried out to investigate mechanisms of resistance to selective Braf inhibitors in CRC and

suggested that resistance to Braf inhibition is also mediated by EGFR-reactivation of the Raf/MEK/ERK signaling pathway [23]. Mao et al. also demonstrated that presence of activating mutations of PI3K or PTEN activates the PI3K/AKT signaling pathway in CRC cells, which also reduced the sensitivity towards Raf inhibitor treatment [24]. Therefore in order to have a potent anti-tumor effect, a thorough determination of the genetic status [22,25,26] and a personalized combination

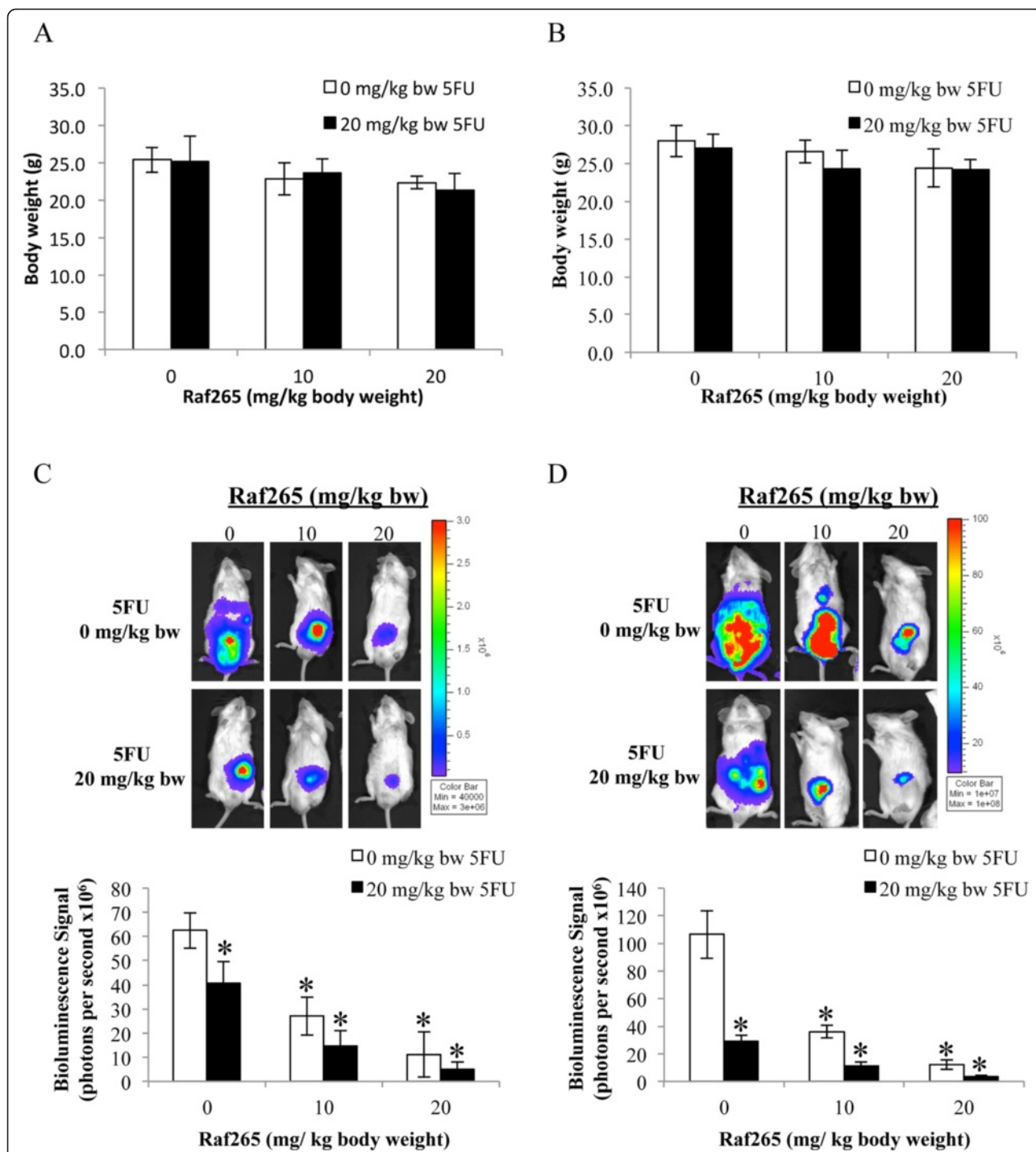


Figure 8 Anti-tumor effect of Raf265 in combination with 5FU. 3 weeks after inoculation of a xenograft tumor to the cecal wall of NOD/SCID mice, Raf265 was administered p.o. twice a week whereas 5FU was administered i.p. once a week for 4 weeks. Mice were sacrificed at week 4 and body weights of mice with tumors from **A**. HT29 and **B**. HCT116 cells were monitored. Representing mice with tumors from **C**. HT29 and **D**. HCT116 cells were shown in the upper panel. The average bioluminescence signals detected were also plotted and shown in the lower panel. Data are presented as means \pm SD from 6 mice and statistical analysis was performed by one-way ANOVA. * $p < 0.05$ versus the vehicle control group with tumors from the corresponding cell lines.

therapy with other inhibitors of the Raf/MEK/ERK and PI3K/AKT signaling pathways are needed. This hindered the application of Raf inhibitors on clinical

studies. As Raf265 was found to be effective in inhibiting the growth of wild-type Braf CRC cells, it is worthwhile to continue the clinical study of the effect of

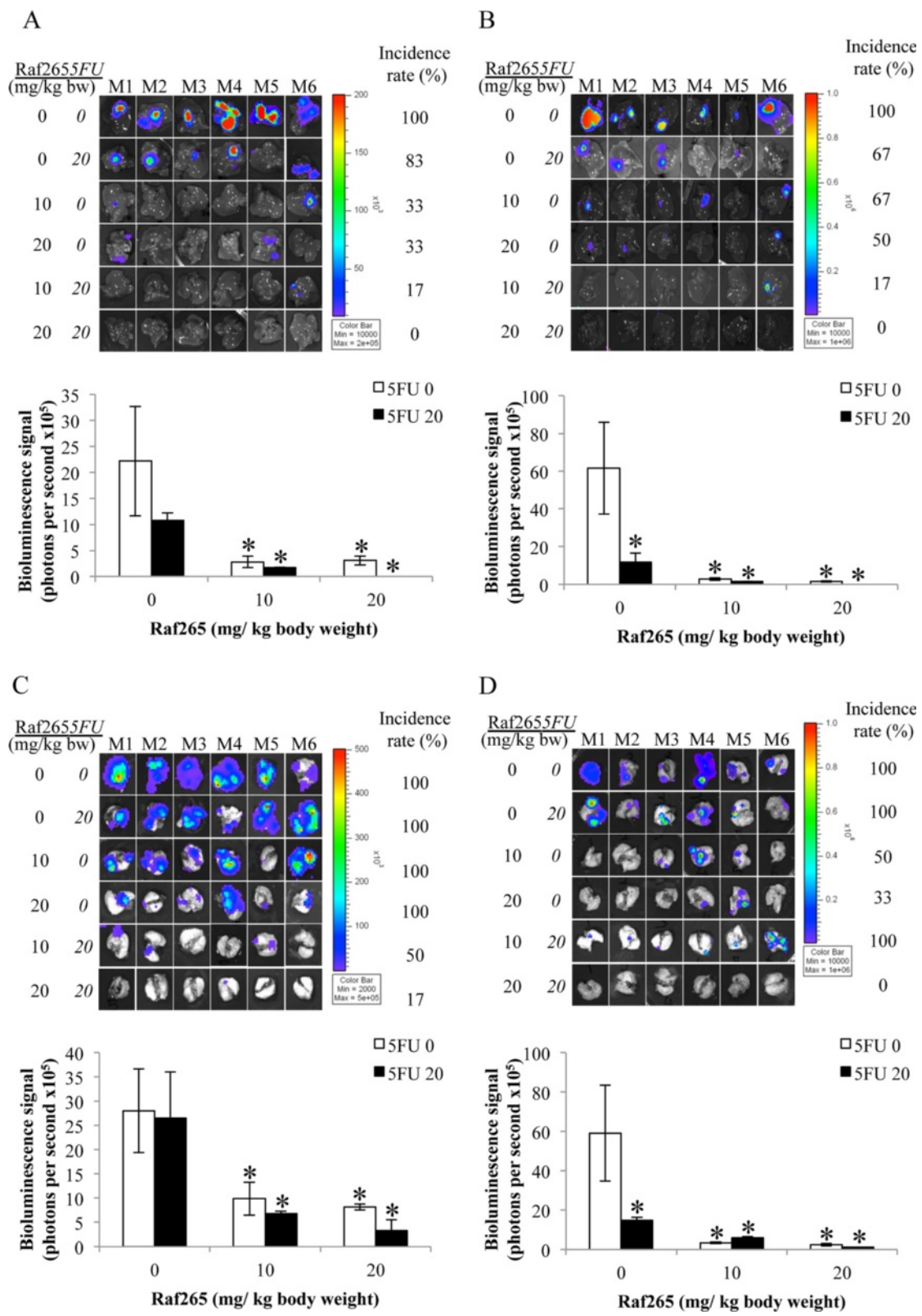


Figure 9 (See legend on next page.)

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Figure 9 Anti-metastatic effect of Raf265 in combination with 5FU. Bioluminescence signals that were detected from the livers of mice with tumors from **A.** HT29 cells and **B.** HCT116 cells, and from the lungs of mice with tumors from **C.** HT29 cells and **D.** HCT116 cells were shown at the upper panel of each graph and the average bioluminescence signals from the corresponding organs with positive signals were plotted and shown at the lower panel of each graph. Data are presented as means \pm SD from 6 mice and statistical analysis was performed by one-way ANOVA. * $p < 0.05$ versus the vehicle group with tumors from the corresponding cell lines.

Raf265 on CRC patients with Braf mutation and Kras mutation.

In mCRC patients, 5FU-based chemotherapy remains the most commonly prescribed anti-cancer therapy. 5FU inhibits the growth of rapidly dividing cells mainly through the inhibition of DNA synthesis. However, it is non-selective and can affect normal fast-growing cells including cells in the hair follicles, bone marrow and the gastrointestinal tract. Intrinsic and acquired chemoresistance can occur which may increase the chance of enriching CSCs population. A recent study from our team suggests that chemotherapy with 5FU enrich a subpopulation of CD26⁺ cells with CSCs properties in mouse xenograft model [6]. In this study, enriched CD26⁺ subpopulation was found in 5FU resistant cells, which are sensitive towards Raf265 treatment. From tumor samples, a higher BRAF expression with an enhanced phosphorylation of MEK and ERK was also demonstrated in CD26⁺ cells when compared with that of the CD26⁻ cells.

We further demonstrated that Raf265 was effective in enhancing the anti-tumor effect of 5FU treatment but preventing metastasis by targeting on the CD26⁺ cells. These findings suggested the possible combination of Raf265 and 5FU in treating mCRC. Other clinical studies also suggested the efficacy of certain molecular targeted agents can be enhanced by combining them with chemotherapy. For example, when cetuximab, a monoclonal antibody directed against EGFR, is combination with irinotecan, response rates in irinotecan-refractory metastatic CRC patients has been improved with the mean survival time increased from 6.9 months (monotherapy with cetuximab alone) to 8.6 months (combined therapy with cetuximab and irinotecan) [27]. When bevacizumab, a monoclonal antibody targeted against VEGF-A, was added to a fluorouracil-based combination chemotherapy, the overall survival rates in patients with metastatic colon cancer can be improved [28].

Conclusions

Raf265 reduced cell proliferation and enhanced cellular apoptosis in CRC cells through the inhibition of the Raf/MEK/ERK signaling pathway. *In vivo* study also demonstrated the anti-tumor effect of Raf265 on mouse xenograft tumor. Although 5FU treatment enriched a subpopulation of CD26⁺ cells, which enhanced the migratory and invasive ability of colon cancer, combination

treatment with Raf265 reversed the 5FU effect by targeting on the CD26⁺ cells and *in vivo* study demonstrated a significant inhibition of liver and lung metastasis. Therefore, this pre-clinical data demonstrate the anti-tumor and anti-metastatic activity of Raf265 in CRC, providing the basis for exploiting its potential use and combination therapy with 5FU in the clinical treatment of CRC.

Methods

Drugs and reagents

Raf265 was provided by Novartis Pharmaceuticals Corporation. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless specified below.

Cell culture and treatment

HT29 (CCL-225) and HCT116 (CCL-247) (obtained from ATCC, Manassas, VA in 2010) were maintained in DMEM (Life Technologies, Carlsbad, CA) containing 10% FBS (Life Technologies) and 1% Penicillin/Streptomycin (Life Technologies), at 37°C humidified incubator with 5% CO₂ in the air and have not been authenticated by the authors. Shortly after purchase, both cell lines were stably transfected with luciferase expressing construct for the ease of detection in the *in vivo* study.

Development of 5FU resistant cells (5FU-R)

For the development of 5FU resistant cells (5FU-R), HT29 cells were treated with 1 μ M 5FU and the concentration of 5FU was increased by 10% every two weeks until the maximum tolerated dose has been reached and 5FU resistance has been developed. Equal volume of DMSO was added in parallel to the control cells (CTL) to eliminate the vehicle effect during the process.

Sample collection and isolation of CD26⁺ and CD26⁻ CSCs

5 fresh tumor specimens were obtained with informed consent from patients who underwent surgical resection of primary colorectal cancer at the Department of Surgery, Queen Mary Hospital, The University of Hong Kong. The study was approved by the Institutional Review Board (HKU/HA HKW). Once obtained, tumor specimens were enzymatically disaggregated as mentioned before [6]. Isolation of CD26⁺ and CD26⁻ CSCs from disaggregated tumor samples, HT29 cells and HCT116 cells was performed by fluorescent-activated cell sorting (FACS) as mentioned before [6]. The sorted

cells were treated with Raf265 in mTeSR™1 medium (STEMCELL Technologies, Vancouver, CA, Canada) for the annexin V analysis.

Cell proliferation and soft agar colony formation assay

The effect of Raf265 on cell proliferation was first examined by MTT assay as previously described [29]. Briefly, cells were plated in 96-well culture plates for 24 hours and media were replaced with culture medium with the indicated concentrations of Raf265. After 72 hours, viability was assessed with the addition of MTT solution (1 mg/ml) (Life Technologies). The percentage of surviving cells was determined by dividing the average absorbance of Raf265-treated cells by the average absorbance of untreated cells.

The proliferation rate of HT29 cells and HCT116 cells was also analyzed using soft agar assay. Cells were suspended in 0.7% microbiology grade agarose in culture medium with the indicated concentrations (0, 0.1 and 1 μ M) of Raf265 and layered on top of a solidified bottom layer of 0.9% agarose with corresponding concentration of Raf265 in culture medium in six-well plates. After 3 weeks of incubation, the numbers of colony in 4 randomly selected fields with 40 \times magnification was counted.

Sphere formation assay

The effect of Raf265 on the self-renewal ability of CD26⁺ cells was examined by serial passage of cells in the sphere formation assay. CD26⁺ cells with single cell suspensions in mTeSR™1 medium were incubated with indicated concentrations (0, 0.1 and 1 μ M) of Raf265 and cultured on ultra low attachment plates (Corning Inc., Corning, NY). The numbers of sphere in each well were quantified after 14 days. Spheres were then disaggregated and reseeded with the indicated concentrations of Raf265 to evaluate self-renewal by formation of secondary passage of spheres and the numbers of sphere were quantified again after 14 days of incubation.

Annexin V analysis by flow cytometry

The effect of Raf265 on cellular apoptosis was examined by Annexin V: PE & PI apoptosis detection kit (BD Biosciences, CA) according to the instructions of the company. Briefly, 1×10^6 cells were grown in 6-well plates. After 24 h, cells were treated with the indicated concentrations (1, 5, 10 and 15 μ M) of Raf265 for 48 h. Cells were harvested, washed with PBS, and stained with annexin V/PI mixture in binding buffer for 15 min at room temperature in dark. Apoptotic cells were determined by Cytomics FC 500 flow cytometer (Beckman Coulter, CA) and analyzed using FlowJo (version 8.7, Tree Star, Inc.).

Caspase 3, 8 and 9 activities by flow cytometry

The effect of Raf265 on caspase activity was examined by flow cytometry using CaspGLOW™ Red Active caspase 3, caspase 8 and caspase 9 staining kits (BioVision, CA) according to the manufacturer's instructions. Briefly, 1×10^6 cells were grown in 6-well plates. After 24 h, cells were treated with the indicated concentrations (1 and 10 μ M) of Raf265 for 48 h. Cells were harvested, washed with PBS, and incubated with specific caspase inhibitor conjugated to sulfo-rhodamine (Red-DEVD-FMK, Red-IETD-FMK, and Red-LEHD-FMK for detection of caspase 3, caspase 8, and caspase 9, respectively) in 300 μ l of culture medium at 37°C incubator with 5% CO₂ for 1 h in dark. After washed with PBS twice, stained cells were analyzed by flow cytometry and analyzed using FlowJo.

Western blotting analysis

Cells were treated with the indicated compounds in DMEM containing 1% BSA for 4 h for the study of phosphorylation of MEK1/2, p44/42 MAPK (ERK1/2), and Akt. 10 μ M U0126, an MEK 1/2 inhibitor, was added as a control. Cell were then lysed by ice-cold RIPA containing 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 0.25% (w/v) sodium deoxycholate, 1 mM PMSE, and protease inhibitor cocktail (Roche Diagnostics, Penzberg, Germany) in 50 mM Tris-HCl buffer, pH 7.4. Equal amount of protein was loaded onto a 12.5% SDS-polyacrylamide gel under reducing condition and transferred to PVDF membrane (Amersham Bioscience, Piscataway, NJ). Blots were probed with the following antibodies: MEK1/2, phospho-MEK1/2, ERK1/2, phospho-ERK1/2, Akt (pan), phospho-Akt, and Braf (Cell Signaling, Danvers, MA) and β -actin (Sigma-Aldrich) was used as loading control. After probing with horseradish peroxidase-conjugated secondary antibodies, membranes were developed with the Immobilon Western Chemiluminescent HRP substrate system (Millipore).

Treatment with Raf265 and 5FU on CD26 distribution

After treatment of 5FU sensitive (CTL) and resistant (5FU-R) cells with Raf265 and 5FU, cells were harvested and stained with CD26-FITC antibody (BD Pharmingen). The percentage of CD26⁺ cells was determined by Cytomics FC 500 flow cytometer (Beckman Coulter, CA) and analyzed using FlowJo (version 8.7, Tree Star, Inc.).

Migration and invasion transwell assay

After treatment with different combinations of drugs for 48 hours, HCT116 cells were plated in top chambers of 24-well transwell plates with 8 μ m pores (Corning) and 24-well BioCoat™ Matrigel™ Invasion chamber (BD Biosciences) at 1×10^5 cells per well in DMEM with 1% FBS, for the study of migration and invasion,

respectively. 10% FBS was used as chemoattractant. After 96 hours incubation, migrated or invaded cells were stained with 0.2% crystal violet. The numbers of migrated and invaded cells in four fields were counted under 100× magnification and the average numbers of migrated and invaded cells were counted.

In vivo study

Animal study was approved by the Committee on the Use of Live Animals for Teaching and Research of the University of Hong Kong (CULTR no. 2407–11). Nude or NOD/SCID mice were maintained in laminar flow cabinets under pathogen-free conditions. For the xenograft model, HT29 or HCT116 cells were harvested from mid-log phase cultures and resuspended in a 50% Matrigel in culture medium. 200 µl cells were injected subcutaneously into the right flank of 6 nude mice of each group to induce xenograft tumor formation. 2 weeks after injection, 0.2 mg/kg Raf265 was administered i.p. twice weekly for 16 weeks. Caliper measurement of tumor dimensions were done every 4 weeks for the estimation of tumor weight by the formula $\pi/6 \times l \times w^2$ where *l* is the length and *w* is the width of the tumor. Mice were sacrificed at week 16 and the xenograft tumor tissue were formalin-fixed and paraffin-embedded.

For the orthotropic model, a xenograft tumor raised from HT29 or HCT116 cells was dissected into 2 mm³. Cecum was exteriorized by laparotomy and the dissected tumor cube was inoculated into the cecal wall of NOD/SCID mice. 3 weeks after inoculation, Raf265 was administered p.o. twice a week whereas 5FU was administered i.p. once a week for 4 weeks. Mice were sacrificed at week 4. Ten minutes before imaging, an intraperitoneal injection of 15 mg/kg body weight D-luciferin (Life Technologies) was administered to the anesthetized mice. Whole body images and organ images of bioluminescent signals were acquired by the *in vivo* imaging system Xenogen IVIS 100 (Xenogen, Alameda, CA). Regions of interest (ROI) were manually selected, and the results were quantified as average radiance of photons emitted per second and area (p/s/cm²) by using the Living Image 2.50.2 software (Xenogen).

Immunohistochemistry (IHC) staining of CD31

The paraffin-embedded tissues were sectioned, deparaffinized and rehydrated through a series of xylenes and ethanol. Antigen retrieval was performed by boiling in sodium citrate buffer (10 mmol/L sodium citrate, pH 6.0). Slides were then incubated with anti-CD31 (Abcam) overnight at 4°C and signal was detected by the LSAB+ System-HRP kits (Dako) according to the manufacturer's instruction. Sections were then counterstained with hematoxylin and dehydrated through a series of ethanol and xylenes.

Statistical analysis

Data are presented as means ± SD from three independent experiments for the *in vitro* study and from *n* = 6 for the *in vivo* study. Data were statistically analyzed with one-way ANOVA, and were considered statistically significant at *p* < 0.05.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AC carried out all the experiments and drafted the manuscript. NC developed and maintained the 5FU resistant cell lines, performed cell proliferation assays on the 5FU resistant cell lines; NC, CL, LN, SW, TW, JM and AC participated in the study in animal models and acquisition and analysis of data; TY, JP, WL and RP designed the experiment and revised the manuscript. All authors read and approved the final manuscript.

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