

# EGFL6 promotes colorectal cancer cell growth and mobility and the anti-cancer property of anti-EGFL6 antibody

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## Research

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# Abstract

## Background

A reliable cancer biomarker will be critical for advanced colorectal cancer (CRC) therapeutic approaches since current treatments are limited to certain patient characteristics, such as age, sex, comorbidities and patients received self-expandable metal stents implantations. The association of epidermal growth factor-like domain 6 (EGFL6) with cancer development has been reported. Here, we focused on the role of EGFL6 in CRC progression and its clinical relevance. An anti-EGFL6 antibody was generated by phage display technology to investigate the potential therapeutic efficacy in CRC. Student t test, Kruskal-Wallis test and multiple comparisons were used to statistical analysis results.

## Results

Significant EGFL6 expression was found in colon tissues from patients and spontaneous tumorigenesis mouse but not in normal tissue. Under hypoxia condition, EGFL6 expression were enhanced in protein and transcript-level. Furthermore, we found EGFL6 could enhance cancer cell migration, invasion and proliferation in CRC via up-regulating ERK/ AKT pathway, as well as reducing ADAMTS1 and Snail expression. We also found EGFL6 regulates cell abilities through EGFR/ $\alpha$ v $\beta$ 3 integrin receptors. By conducting animal experiments, our anti-EGFL6 antibody, EGFL6-E5-IgG, showed tumor inhibition and anti-metastasis ability. Furthermore, no impact on angiogenesis and wound healing by using EGFL6-E5-IgG were observed.

## Conclusions

We demonstrated that EGFL6 plays a role in CRC tumorigenesis and tumor progression, indicating that EGFL6 is a potential cancer biomarker and therapeutic target worth further investigation.

## Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed malignant cancer with high morbidity and mortality in the world. Approximately 50% CRC patients will develop to metastasis CRC, and about 25% patients develop metastasis already while being diagnosed [1]. Bevacizumab (Avastin®, Genentech) is a monoclonal antibody targeting vascular endothelial growth factor (VEGF), broadly combine with 5-fluorouracil (5-FU)-based chemotherapy regimens as first and second-line treatment for metastatic CRC (mCRC). Although significantly improvement of progression-free survival (PFS) and overall survival (OS) showed in mCRC patients receiving bevacizumab plus chemotherapy compared to chemotherapy alone [1, 2], disease progression still arises within 9 months in patients [3]. The incidence of some side effects, such as perforation, hemorrhage, and wound healing complications associated with VEGF inhibition, also increased when adding bevacizumab to chemotherapy, especially in elderly and female patients [4, 5]. CRC patients who received stent placement to treat malignant bowel obstruction may also have higher

possibility occurring colon perforation [6]. Therefore, an effective therapeutic agent with mild adverse effect is an urgent medical need for CRC.

Cancer cells release factors to conserve a microenvironment which is conducive to growth [7]. It has been demonstrated that some proteins such as CB-EGF-like repeat-containing proteins, were expressed highly during development and then were often reactivated under pathological conditions like cancer [8]. EGFL6, a member of the epidermal growth factor (EGF) repeats protein superfamily, is suggested as a secreted form protein due to its putative signal peptide and implied the potential binding activities with integrins via its RGD motif [9]. Previous studies have shown that EGFL6 is highly expressed in fetal tissues but the expression is dramatically reduced in adult tissues [9, 10]. The role of EGFL6 was previously shown to promote endothelial cell migration and angiogenesis during bone development [11], and promote adipose tissue-derived stromal vascular cells' proliferation [12].

Recent evidence from human tumor biopsy transcription analysis has shown that EGFL6 mRNA is expressed at high levels in some cancers including CRC, while levels in normal tissues were nearly undetectable [9] [13] [14] [15]. However, EGFL6's role in promoting CRC tumorigenesis and progression is still need to study in-depth. We focus on investigating the clinicopathologic role of EGFL6 in CRC patients and its involvement of tumorigenesis, as well as its function in regulating cell proliferation, migration and the signaling pathways in CRC. Further, we developed an anti-EGFL6 antibody, EGFL6-E5-IgG, to verify the anti-tumor, anti-metastasis and anti-angiogenesis properties *in vivo* to validate the potential of EGFL6 as a therapeutic target in CRC.

## Results

### *EGFL6 increased abundance in CRC*

Many oncogenic proteins were highly expressed during development and then were reactivated in adult under pathological conditions, EGFL6's aberrant expression in adult might be related to tumorigenesis. To investigate our hypothesis, we analyzed the EGFL6 expression in patients' tumor tissues. It showed that EGFL6 expression was significantly detected in each CRC stage but not in normal tissue in CRC patients, without significantly difference between stages (Fig. 1A-B). Besides, EGFL6 expression was associated with neither tumor size, nearby lymph nodes involved, nor metastasis (Table 1). We developed an experimental azoxymethane (AOM)-induced mouse model to validate the role of EGFL6 in tumor progression *in vivo* (Fig. 1C). AOM-induced mice showed aberrant crypt foci at 8 and 18 weeks after the last AOM injection, which are putative precursor lesions for CRC, by methylene blue staining (Fig. 1D). We further demonstrated that the expression of EGFL6 was notably accompanied with CRC progression during spontaneous tumorigenesis (Fig. 1E).

### *EGFL6 plays a role in promoting cell proliferation*

To investigate EGFL6's role in CRC *in vitro*, we first confirmed the EGFL6 expression in different CRC cell lines and the normal colon epithelial cell as well. We found that in protein level, EGFL6 were highly

detected in CRC cells except SW480, and very low expression in normal colon epithelial cells. Since HCT116 and HT29 express EGFL6 more abundantly compare to other CRC cell lines, we use HCT116 and HT29 for our further investigation (Fig. 2A-B, Additional file 1: Fig. S1). EGFL6 was also detected in culture medium, implying EGFL6 is a secreting protein (Fig. 2C). Molecular weight of EGFL6 slightly shifted in culture medium because of two N-linked glycosylation sites that EGFL6 contains [9]. Specific glycosylation site described in GeneCards is at Asn397. To investigate EGFL6's role in tumorigenesis, we first evaluated the cell proliferation abilities. EGFL6 induced CRC cells proliferation when treated with human recombinant EGFL6 (Fig. 2D). In addition, EGFL6 activated ERK and AKT phosphorylation, which signal controls cell proliferation and is associated with CRC progression (Fig. 2E). We next evaluated the impact of EGFL6 knockdown on the growth of CRC cells. EGFL6 siRNA showed a significant silencing effect ( $P < 0.001$ ) and knocked down about 50% EGFL6 mRNA in CRC cells in comparison of scrambled siRNA (Fig. 2F-G). EGFL6 knockdown was associated with obviously reduced tumor cell viability as well as ERK and AKT phosphorylation (Fig. 2H). These data indicated that EGFL6 potentially promotes cancer cells proliferation through ERK and AKT pathways.

#### *EGFL6 plays roles in cancer cells migration and invasion and is inducible under hypoxia environment*

Next, we test whether silencing EGFL6 in CRC cells affects the clonogenic potential. The data showed that CRC cells treated with EGFL6 siRNA exhibited fewer colony numbers compared with cells treated with the scramble siRNA (Fig. 3A-B). Clonogenic potential decreasing usually relates to the loss of migration and invasion abilities of cancer cells. Since cell migration and invasion are critical properties for the dissemination of cancer cells and metastasis; therefore, *in vitro* migration and invasion assays were performed to investigate the effect of EGFL6 on cell invasiveness. There were significantly fewer migrated cells in EGFL6 siRNA transfected groups compared with scrambled siRNA-treated groups (Fig. 3C). Consistent with the finding in migration assay, EGFL6 siRNA treated cancer cells reaching the lower matrigel-free well was significantly reduced in comparison with scrambled siRNA-treated groups (Fig. 3D).

Hypoxia is a key regulatory factor in tumor growth, survival and proliferation when cancer cells experience genetic changes in toxically hypoxic environment. We examined whether EGFL6 could be induced under hypoxia condition and both transcript and protein levels of EGFL6 was enhanced by hypoxia condition (Fig. 3E-F). Moreover, hypoxia-induced EGFL6 expression was abrogated under hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) siRNA treatment. To corroborate this result, CRC cells were treated with CoCl<sub>2</sub>, a hypoxia mimicking agent reported to stabilize HIF-1 $\alpha$ . CoCl<sub>2</sub> treatment enhanced EGFL6 expression, whereas EGFL6 expression was reduced when given HIF-1 $\alpha$  siRNA treatment in CoCl<sub>2</sub> condition (Fig. 3E). Furthermore, a disintegrin and metalloproteinase with thrombospondin motif (ADAMTS) is an acute hypoxia-inducible gene, our data showed that ADAMTS1's expression was enhanced under hypoxia and reduced when HIF-1 $\alpha$  siRNA treatment. ADAMTS1 expression was also inhibited when EGFL6 siRNA treatment in hypoxia (Fig. 3E). Therefore, EGFL6, as well as downstream pathway such as ADAMTS1, can response to hypoxia condition, which also suggests the potential role of EGFL6 in regulating cancer cell migration.

In addition, matrix metalloproteinase (MMP), ADAMTS1 and Snail are factors associated with cancer migration and invasion, thus we analyzed MMP-2, MMP-9, ADAMTS1, and Snail's expressions in EGFL6 siRNA treated CRC cells. It showed that EGFL6 knockdown did not affect the gene expression of MMP2 and MMP9 while ADAMTS1 and Snail was significantly reduced in EGFL6 siRNA treated cells in mRNA level (Fig. 3G). Taken together, these results indicate that EGFL6 plays roles in invasive and migrate properties of CRC cells.

#### *EGFL6 activates cancer cell signaling through EGFR and integrin receptors*

Since EGFL6 harbors EGF repeats domain and RGD motif, EGFL6 might induce cell proliferation through its EGF repeats coupling with EGFR or through its RGD motif binding to integrin receptors. We demonstrated that EGFL6-induced cell proliferation was inhibited when cells were treated with Erlotinib, a RTKs acting on the EGFR. It has been reported that many cancer cells express  $\alpha\beta3$  integrins. Similar results were shown that EGFL6-induced cell proliferation ability was reduced in the presence of  $\alpha\beta3$  integrin inhibitor SB273005. The inhibitory ability was obvious when combine Erlotinib and SB273005 (Fig. 4A). Hence, we supposed that EGFL6 affects cell abilities through EGFR/integrin receptors.

Transmembrane growth factor receptors, such as EGFR, and integrin receptors form multi-protein signaling complexes with focal adhesion kinase (FAK) [16]. FAK auto-phosphorylation at Tyr397 creates a binding site for the Src-homology 2 (SH2) domain, which promotes Src family kinases phosphorylate FAK at Tyr925 [17, 18]. We found that EGFL6 was associated with FAK-Src signaling in which phosphorylated FAKs (Tyr397 and Tyr925) and Src phosphorylation at Try416 was reduced after treated siEGFL6 (Fig. 4B-C). In addition, AKT, FAK and Src kinase's collaboration was involved in CRC cells' migration and invasiveness [19]. Here, we showed that phosphorylation of ERK, AKT and Snail's expression were associated with EGFL6 induction (Fig. 2E,4B). FAK plays a role in the regulation of cell cycle progression, correlating with changes in cyclin D1's expression, and cyclin D1's deregulation may promote tumor development [20, 21]. EGFL6 can affects cyclin D1 in which cyclin D1 was enhanced with EGFL6 treatment but decreased in the presence of EGFL6 siRNA (Fig. 4B-C). Further, STAT3, FAK and Src are known to regulate cancer stem cell proliferation and self-renewal [22, 23]. In our result, STAT3 phosphorylation was enhanced after EGFL6 treatment but was reduced after EGFL6 siRNA treated (Fig. 4B-C). From this result, we found EGFL6 expression may associate with cancer cell self-renewal ability, and colony forming ability is related with the cell self-renewal ability [24] (Fig. 3A-B), we further explored the mechanism of EGFL6 in keeping the cell stemness by detecting the expression of several associated genes, including POU5F1, NANOG, and LIN28. The mRNA level of POU5F1, NANOG, and LIN28 all had obvious reductions when EGFL6 was knocked down in both HCT116 and HT29 (Fig. 4D-E). Taken together, we found that EGFL6 could regulate cancer cell migration, invasion, proliferation and self-renewal by affecting EGFR and integrin receptor signaling.

#### *EGFL6 antibody demonstrated anti-cancer and anti-metastasis properties without interfering wound healing in vivo*

According to above data, we assume that EGFL6 might be a possible therapeutic target for CRC treatment. In order to address the *in vivo* function of EGFL6, EGFL6-E5-IgG, a humanized antibodies specific targeting to EGFL6 were generated using phage display system.

To verify the anti-cancer activity of EGFL6-E5-IgG, HCT-116 xenograft model was established. EGFL6-E5-IgG inhibited tumor growth in HCT-116 xenograft model with TGI 36.2% (\*\*,  $P < 0.01$ ), without significant body weight change (Fig. 5A), indicate EGFL6-E5-IgG's anti-tumor capability in CRC. Along with the association ( $k_{on}$ ) and dissociation ( $k_{off}$ ) rates, the affinity ( $K_D$ ) of EGFL6-E5-IgG was calculated to be  $1.91 \times 10^{-8}$  M (Table 2 and Additional file 1: Fig. S2).

EGFL6-E5-IgG was then used to evaluate its anti-metastatic property in a syngeneic lung metastasis model. Both EGFL6-E5-IgG and bevacizumab significantly inhibited CT-26 metastasis compared to Control group (\*  $P < 0.05$  and \*\*  $P < 0.01$ , respectively) without significant differences between two treated groups (Fig. 5B), indicating that EGFL6 blockade can suppress CRC cell metastasis *in vivo* and EGFL6-E5-IgG's potential effectiveness.

Protracted wound healing is one of the major side effects interfering the treatment efficacy of bevacizumab. EGFL6-E5-IgG-treated animals has similar wound healing closure speed compared to the Control group. In day 6, wounds of Control and EGFL6-E5-IgG groups already formed scabs, but not in bevacizumab-treated group (Fig. 5C), suggesting that bevacizumab has the potential affecting wound repairing. Although EGFL6-E5-IgG exhibited no impact on wound healing, wound healing ability is associated with angiogenesis function, and angiogenesis function relates to tumor growth. Here we use angiogenesis assay to demonstrated EGFL6-E5-IgG has potential to inhibit angiogenesis, despite no statistically significant showed, hemoglobin concentration still lower than Control group (Fig. 5D). These data indicated the anti-cancer, anti-metastasis, anti-angiogenesis capability as well as no impact on wound healing, suggested that EGFL6-E5-IgG has potential becoming a therapeutic agent for CRC.

## Discussion

The current CRC first-line treatment is bevacizumab combined with chemotherapy, such as 5-FU, oxaliplatin or irinotecan, the regimens evidently improve overall and progression-free survival in CRC patients. However, it worth to noted that in a meta-analysis review article, there are some statistically significant increase in severe adverse events associated with bevacizumab, for example, severe hypertension and gastrointestinal perforation [25]. In addition, Koichi Taira et al. reported that patient received bevacizumab occurred skin ulcer and wound healing delayed, recommended the treatment of bevacizumab should be suspended at least 5 weeks before patient undergo an operation [26]. Furthermore, drug resistance also occurs using bevacizumab [27], leading to treatment failure. Although these adverse events don't happen often, but they are potentially life threatened, which need to be considered into any decision. Therefore, finding new targets and therapeutic strategy towards CRC is still an important issue.

EGFL6 is proved to relate to the progression of several cancers [28-31]; however, the function and mechanism of EGFL6 in CRC has not yet been elucidated in detail. Recently, a research found EGFL6 increased abundance in human CRC tissues by gene analysis, but were nearly undetected in normal colorectal tissues [32]. EGFL6 also significantly express in oral squamous cell carcinoma tumor part but not express in normal part, with significantly higher expression in stage IV compare to stage I [28]. Our data exhibited statistically significant EGFL6 expression in stage I-IV patient tumor tissues, without expression in non-tumor tissue, in accordance with previous researches. Notably, in our AOM-induced mice model, EGFL6 started to express when colorectal polyps occurred, and the expression of EGFL6 increased as colorectal polyps progressed. Hence, we speculated that EGFL6 expression could be detected in precancerous stage of CRC. As colorectal polyps usually progress to colorectal tumors [33], EGFL6 could be as a suitable biomarker for early CRC detection. According to these results, we assumed that EGFL6 functioned as a tumor-specific protein and could promote tumorigenesis.

Recently, some studies showed EGFL6 could modulate cancer cell proliferation, and metastasis in colorectal cancer and different cancer types [13, 15, 29, 31]. In our data, knockdown EGFL6 associated with tumor cell viability reduction as well as ERK and AKT phosphorylation, indicates EGFL6 promotes CRC cells survival and proliferation through ERK and AKT pathways, this result was consistent with a research which investigated nasopharyngeal carcinoma [13]. The same result was also found in zebrafish model [34], but was different from a research in which EGFL6 recombinant protein did not change p-ERK or p-AKT level in CRC cell HCT116 and SW480 [15]. The possible explanation of this difference might due to different treatment procedure from our study. In Zhang's study, CRC cells were treated with 500 ng/mL EGFL6 for 48 h, whereas we treated only 10 ng/mL for 60 min and the ERK and AKT phosphorylation were obviously activated. In Zhang's study, they found that EGFL6 modulates cell proliferation through WNT/ $\beta$ -catenin pathway. Here, we discovered that p-AKT was increased activity by EGFL6, which means EGFL6 may contribute to cell proliferation pathway. Whereas, EGFL6 was found contributing to CRC cell proliferation in Zhang's study, which is consistent with our result.

To further understand the EGFL6 mechanism in CRC, we conducted series of experiments to investigate the EGFL6 downstream pathways. Our *in vitro* studies demonstrated that under hypoxia condition, EGFL6 binds to EGFR, activates EGFR/ $\alpha$ v $\beta$ 3 integrin receptors to affect cancer cell proliferation. Furthermore, Src, FAK and STAT3, which kinases are related to cell proliferation, migration and invasion, were phosphorylated by EGFL6 in CRC cell. This result coincides with the research that collaboration of AKT, FAK and Src was associated with migration and invasion of CRC cells [19]. Cyclin D1, activated by ERK, was also found associated with EGFL6 expression. We demonstrated in detail that EGFL6 modulates CRC cell proliferation, self-renewal ability, cell migration and invasion via EGFR/ $\alpha$ v $\beta$ 3 integrin signaling.

Hypoxia is a condition that could be observed during tumorigenesis, which is regulated by HIFs. HIFs maintain cancer stem cells stemness, and cancer stem cells could induce colon tumorigenesis [35]. EGFL6 has been mentioned that could be induced under hypoxia [36]. In our research, we found that hypoxia not only induce EGFL6 expression, but also ADAMTS1, an acute hypoxia-inducible gene [37]. In our results, EGFL6 regulates STAT3, FAK and Src, which correlate to cancer stem cell proliferation (Fig 4B-

C) and self-renewal [22, 23]. A research also demonstrated that EGFL6 promotes the asymmetric division of cancer stem-like cells in ovarian cancer [29]. Consistent with our result that EGFL6 in keeping the cell stemness by regulating the expression of cancer stem cell associated genes, POU5F1, NANOG, and LIN28 (Fig. 4D-E). These provide evidence that EGFL6 expression correlate with CRC cancer stem cell.

One of the bevacizumab's adverse events is wound healing complications; therefore, whether EGFL6-E5-IgG could contribute to better efficacy but exert favorable adverse effect profile become crucial. In our wound healing model, the wound almost recovered in EGFL6-E5-IgG-treated group, which showed similar closer rate compared to Control group. In contrast to these two groups, bevacizumab-treated group exhibited impaired healing, which wounds still moist and had inflammatory response, with high risk of infection due to long-term recovery. Indicated that EGFL6-E5-IgG had no effect on wound healing also showed significantly tumor growth inhibitory capability in HCT-116 xenograft model. Similar results had also been obtained by Noh et al. [36]. In addition, EGFL6-E5-IgG exhibited significant anti-metastasis ability, without significant difference compared to bevacizumab-treated group. Therefore, we conclude that EGFL6-E5-IgG has ability to inhibit CRC growth, metastasis and has no visibly toxicity in *in vivo* model, which worth further investigation and development.

There are limitations in our study. The characteristic of CRC patients didn't include treatment type and comorbidities, it's hard to validate the unknown background factors that may potentially influence EGFL6 expression. In addition, whether EGFR or  $\alpha\beta3$  integrin is more primarily to EGFL6 still need further investigation. EGFL6-E5-IgG still need to optimize, current data showed the potential efficacy to treat colorectal cancer. While the animal model using EGFL6-E5-IgG compare to biologic targeted therapy drugs, such as bevacizumab plus chemotherapy, need to conduct. The side effects of EGFL6-E5-IgG now is not clear enough, still need further experiments to validate.

## Conclusions

In summary, we demonstrated that hypoxia-induced EGFL6 activated EGFR/ $\alpha\beta3$  integrin signaling, brought FAK binding to Src, further activated ERK, AKT, Cyclin D1 to promotes cell proliferation, induced STAT3, ADAMTS1 and Snail to influence cell migration and invasion function, as well as promoted cell self-renewal activity in CRC. EGFL6 could be detected in early stage of CRC patients and tumorigenesis mouse. Moreover, anti-EGFL6 antibody EGFL6-E5-IgG showed tumor growth inhibition and significantly anti-metastasis property *in vivo* without affecting wound healing, indicated EGFL6-E5-IgG is worthy of further development as a potential therapeutic agent against CRC. Taken together, our study demonstrated that EGFL6 could be a potential biomarker in CRC.

## Methods

### *Cell culture and reagents*

Human colorectal carcinoma cell lines HCT-116 and HT-29, and mouse colon carcinoma cell line CT-26 were purchased from Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). HCT-116 and HT-29 cells were grown in McCoy's 5A (Sigma-Aldrich, Darmstadt, Germany) and CT-26 was grown in RPMI-1640 (Gibco, Dublin, Ireland) supplemented with 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate. All cells were maintained in humidified air containing 5% CO<sub>2</sub> incubator at 37°C and cultured every 2-3 days.

EGFL6 recombinant protein was purchased from Sino Biological (Beijing, China). EGFL6 therapeutic antibody (EGFL6-E5-IgG) was generated and provided by Dr. Yu-Ching Lee using phage display technology. Bevacizumab (Avastin<sup>®</sup>) was purchased from Genentech (California, USA). 5-FU (Fluorouracil<sup>®</sup> Injection) was purchased from Pfizer (New York City, USA).

### *Patients and colon tissue specimens*

Tissue biopsy samples used in fig. 1A were collected after prior informed written consent as part of a study (no. 106IRB0417) approved by the human ethics committee of Taipei Medical University Joint Institutional Review Board. No chemotherapy or radiation therapy were given to the enrolled patients before surgical therapy. The TNM stages were determined based on the American Joint Committee on Cancer/International Union Against Cancer TNM staging system. Tissue biopsy used in fig. 1B are approved by Taipei Medical University Joint Biobank (TMU-JBB) (no. N201703080).

### *In vivo animal models*

All experiments on mice were performed in accordance with institutional and national guidelines and regulations. Protocols have been reviewed and approved by Animal Use and Management Committee of Taipei Medical University (IACUC approved No. TMU LAC-2016-0034). Mice were maintained under a 12 h:12 h light: dark cycle and fed with standard diet and water ad libitum.

For Azoxymethane (AOM)-induced mutagenesis assay, a total of 35 eight-week-old A/J mice (Jackson Laboratories, Bar Harbor, ME) were randomly divided into Control or AOM treatment group. Mice were injected with 10 mg/kg AOM intraperitoneally (i.p.) once a week for 6 consecutive weeks as previously described [38, 39]. Mice were sacrificed and colons were collected at 1, 2, 4, 8, and 18 weeks after the last AOM challenge to assess for methylene blue, H&E staining, and immunohistochemistry (IHC) staining of EGFL6.

For anti-cancer activity xenograft model, a total of 24 six-week-old nude mice (National Laboratory Animal Center (NLAC), Taipei, Taiwan) were injected subcutaneously with the same volume of Matrigel (BD bioscience, San Jose, California, USA), and  $1 \times 10^7$  of HCT-116 cells into the right flank of each animal. When tumors had grown to around 300 mm<sup>3</sup>, the treatment started. Tumor size was measured twice weekly and calculated from  $V = \text{length} \times \text{width}^2 / 2$ . Tumor growth inhibition (TGI%) =  $[1 - (T_t - T_0) / (C_t - C_0)] \times 100$ , where  $C_0$  and  $C_t$  are mean tumor volumes of Control group by first data point and day  $t$ , respectively; while  $T_0$  and  $T_t$  are mean tumor volumes of treatment group by first data point and day  $t$ .

For lung metastasis animal model, eight-week-old balb/c mice (NLAC, Taipei, Taiwan) were injected with CT-26 cells ( $2 \times 10^5$  cell/mouse) intravenously and then divided into three groups for treatment. After 3 weeks of treatment, animals were sacrificed and lung nodules were counted for data analysis.

For wound healing animal model, after given narcotic, 4.0-mm circular full-thickness skin excision wound were created on the dorsal site of each nude mice using biopsy punch. A Tegaderm film was attached to protect and avoid scab generation. Mice were then randomly divided into three groups for treatments. The wound area and body weight were measured every other day until wound closure.

For angiogenesis animal model, mice were injected with 500  $\mu$ l matrigel subcutaneously mixed with endothelial growth factor (EGF, 150 ng/ml) and heparin (10  $\mu$ l), treated anti-EGFL6 antibody (15 mg/kg, q5D) through intravenous injection immediately after subcutaneous injection. After seven-day treatment, animals were sacrificed and the matrigel were carefully dissected. Hemoglobin content was then analyzed by Drabkin's reagent kit (Sigma Chemical, St. Louis, MO, USA) to quantify the blood vessel formation.

#### *RNA isolation and Quantitative real-time PCR*

The RNA was isolated using the TRIzol reagent and the Direct-zol RNA MiniPrep (ZYMO research, Irvine, CA, USA) according to the manufacturer's instruction. Reverse transcription reactions were performed using the RT Kit (Takara, USA) with 2  $\mu$ g of total RNA according to the manufacturer's instruction. Quantitative real-time PCR was performed with ABI StepOnePlus Real-Time PCR Systems using SYBR Green dye (Life Technologies, Grand Island, NY, USA). Relative RNA abundance was calculated using the  $2^{-\Delta\Delta C_T}$  formula and normalized to the transcript levels of the housekeeping gene GAPDH. Primer sequences used for quantitative real-time PCR were listed in Additional file 2: Table S1.

#### *Western blotting*

Total protein was extracted by homogenization in ice-cold RIPA buffer containing protease and phosphatase inhibitor cocktail. Equal amounts of protein extracts were heated in sample buffer and then separated by SDS-polyacrylamide gel electrophoresis. Separated proteins were then transferred to PVDF membrane. The membranes were subsequently probed using the following primary antibodies listed in Additional file 2: Table S2-3. Immunoreactive bands were visualized with an enhanced chemiluminescence substrate detection kit (Amersham, Buckinghamshire, UK).

#### *Colony Formation Assay*

For determination of colony forming units (CFUs), cells were plated at a density of 300 cells/9.01  $\text{cm}^2$  culture dish. After 8 days of incubation, the colonies formed were fixed with ice-cold methanol for 10 min, and then stained with Giemsa solution for 15 min. After washing and drying, the colony numbers were calculated.

#### *siRNA transfection*

Cells were transfected with the siRNA targeting EGFL6, HIF-1 $\alpha$  and scrambled siRNA (#1299003, #42840, #4390847, Thermo Fisher Scientific) using RNAiMAX Transfection Reagent according to the manufacturer's protocol.

### *Migration and invasion assays*

Cell migration and invasion abilities were evaluated in transwell with 8- $\mu$ m pore size polycarbonate membranes in 24-well plates (Corning Inc., Corning, NY, USA). The  $1 \times 10^5$  cells were seeded to each transwell insert, and filled each well with culture medium. For invasion assays, the membranes in transwell were pre-coated with 50  $\mu$ g of Matrigel to form matrix barriers. After incubation for 16 h, the cells remaining on the upper surfaces of the membrane were cleaned. The cells moving to the lower surfaces of the membrane were fixed with ice-cold 10% formalin for 10 min, stained with 0.2% crystal violet for 15 min and counted under a light microscope.

### *Construction of chicken scFv library and biopanning*

Chicken scFv library was constructed according to the published protocol with minor modifications [40]. Detail methods are described in Additional file 3: supplementary materials.

### *Statistical analysis*

Each experiment was performed independently at least three times and the data were presented as mean  $\pm$  standard error of the mean. Student t test was used to analyze the data between two groups. Kruskal-Wallis test and multiple comparisons were used to analysis data between more than 3 groups. A P value of less than 0.05 was defined as a statistically significant difference.

## **Abbreviations**

EGFL6: epidermal growth factor-like domain 6; CRC:Colorectal cancer; mCRC:metastasis colorectal cancer; VEGF:vascular endothelial growth factor; 5-FU:5-fluorouracil; AOM:Azoxymethane.

## **Declarations**

### **Ethics approval**

All samples were collected after prior informed written consent as part of a study (no. N201703080) approved by the human ethics committee of Taipei Medical University Joint Institutional Review Board.

### **Consent for publication**

All authors reached an agreement to publish the study in this journal.

### **Availability of data and materials**

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

### **Conflicts of interest/Competing interests**

The authors have declared that no competing interest exists.

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### **Author Contributions**

Conceptualization, HLH, CCC, YCL, WCHF and SLP; methodology, TYS, HLH, CCC, FLC, YCL and SLP; validation, TYS, HLH, CCC, and FLC.; formal analysis, TYS, HLH, CCC and SLP; investigation, TYS, HLH and CCC.; resources, YCL, YWC, PLW, CCH, WCHF, and SLP; data curation, TYS, HLH, CCC, FLC, CCH, PLW, YWC and SLP; writing—original draft preparation, Original Draft, TYS, HLH, and CCC.; writing—review and editing, TYS, HLH, CCC, YCL and SLP; supervision, SLP; project administration, TYS and HLH; funding acquisition, CCH and SLP. All authors have read and agreed to the published version of the manuscript.

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## Tables

**Table 1. Association of EGFL6 expression and clinical parameters in tumor tissues of CRC patients.**

EGFL6			
Parameters	Low	High	p value
	(n=73)	(n=119)	
Age (years)			
≤65	32	62	
>65	41	57	0.299
Gender			
Female	30	60	
Male	43	59	0.235
T factor			
1	3	4	
2	10	18	
3	43	67	
4	17	30	0.968
N factor			
0	31	52	
1+2	42	67	0.882
M factor			
0	58	101	
1	15	18	0.333
Stage			
I	10	13	
II	18	35	
III	29	53	
IV	16	18	0.555

T factor: tumor size; N factor: lymph nodes; M factor: metastasis.

Table 2. $k_{on}$ and $k_{off}$ rate constants of E5 IgG targeting to EGFL6					
Ligand	Analyte (IgG)	$k_{on}(10^3 M^{-1}S^{-1})$	$k_{off}(10^{-5}S^{-1})$	$K_D(10^{-8}M)$	$\chi^2$
EGFL6	E5	$2.3 \pm 0.00251$	$4.38 \pm 0.00177$	$1.91 \pm 0.0792$	67.39
$k_{on}$ : association rate; $k_{off}$ : dissociation rate; $K_D$ : affinity binding constants; $\chi^2$ : Chi-squared test.					

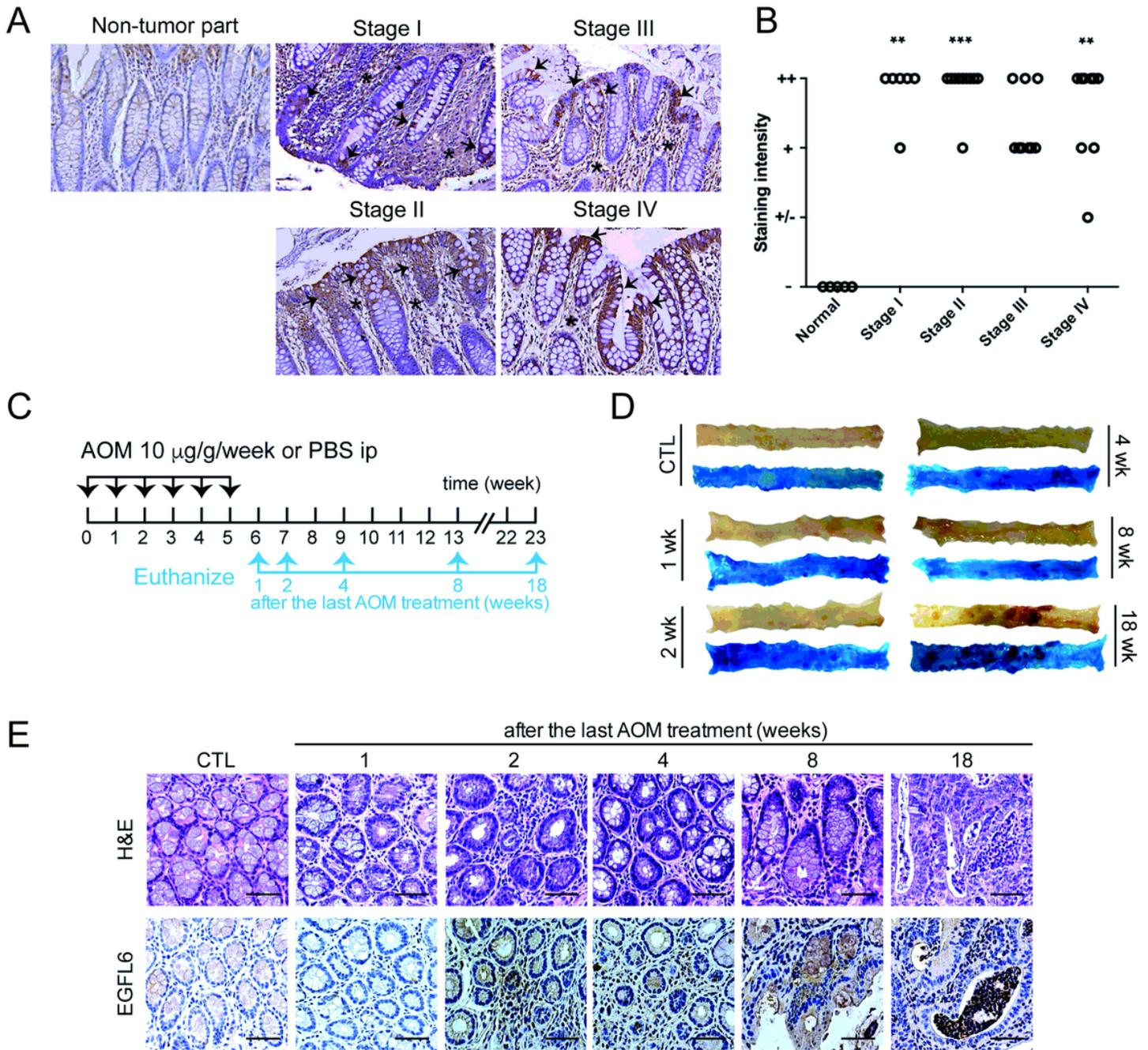
## Supplementary Information

**Additional file 1: Fig. S1. EGFL6 expresses in CRC cells instead of normal colon epithelial cell.** The protein expression of EGFL6 in normal colon epithelial cell and CRC cells. **Fig. S2. The binding affinity of EGFL6-E5-IgG.** Binding curves (black thin line) and the sensor gram traces (blue, red and black thick line) exemplifying association / dissociation kinetics of scFv E5 to the immobilized EGFL6 recombinant protein as the graph shown. The scFv E5 concentrations are 50  $\mu\text{g/mL}$  (red), 100  $\mu\text{g/mL}$  (black), and 400  $\mu\text{g/mL}$  (blue). Data was fit with 1:1 binding interaction model with errors from TraceDrawer.

**Additional file 2: Table S1. Primers sequences for quantitative real-time PCR, Table S2. Primary antibodies for western blot, Table S3. Secondary antibodies for western blot.**

**Additional file 3: Supplementary Materials and Methods:** *Surface plasmon resonance, Construction of chicken scFv library and biopanning, Methylene blue staining, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay.*

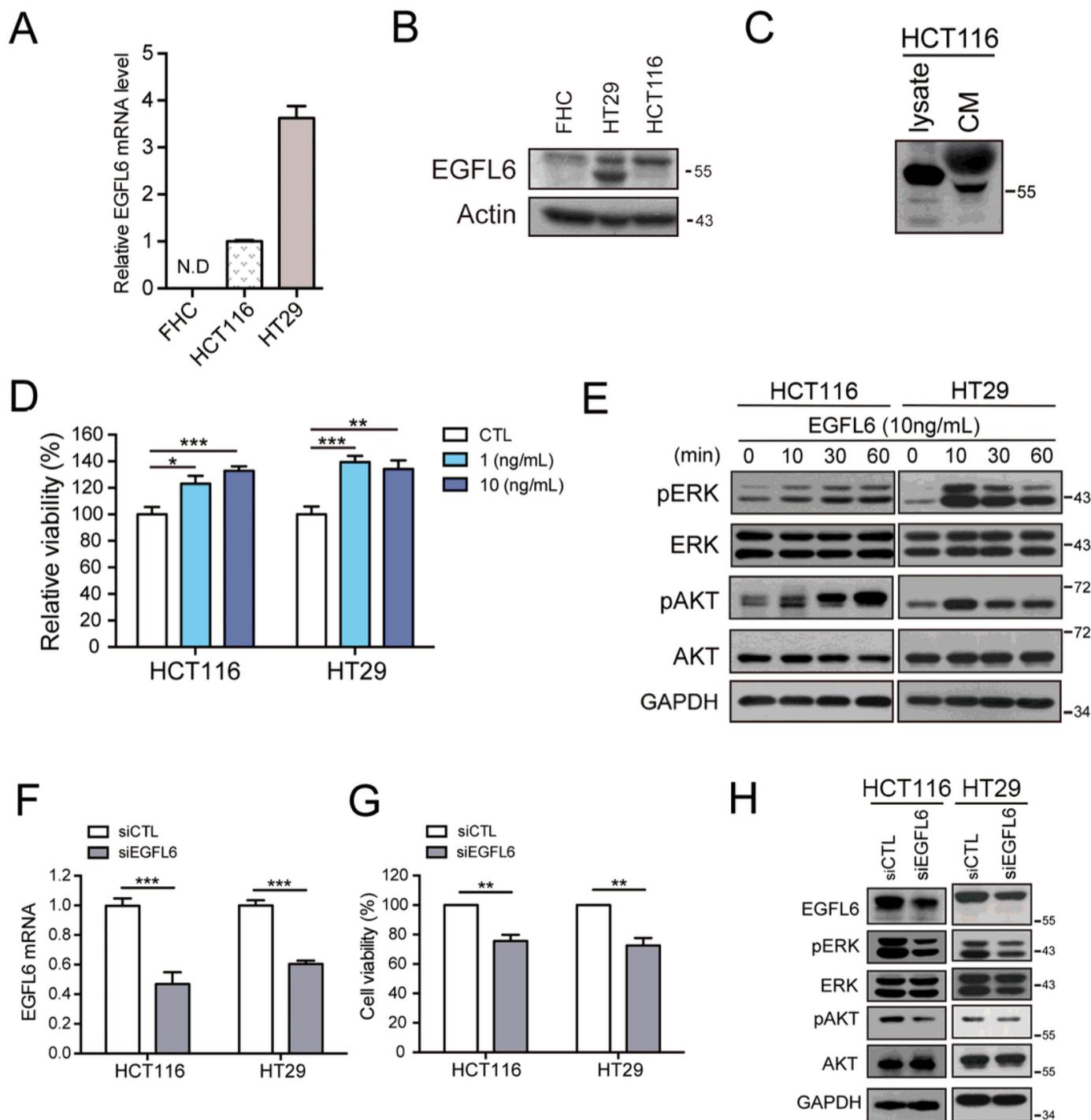
## Figures



**Figure 1**

EGFL6 expresses in CRC patients and in early-developed colon carcinogenesis animal model. (A) EGFL6 expression in human CRC tissue with indicated stages under 100X magnification. Arrow: cytoplasmic EGFL6. Asterisk: secreted form of EGFL6. (B) The statistical analysis of normal group (n=5) and human CRC group stage I (n=6), stage II (n=10), stage III (n=9), stage IV (n=9). \*P<0.05, \*\*P<0.01, \*\*\*P<0.005. (C) AOM mouse model schedule. Total number of 35 eight-week-old A/J mice were randomly divided into control or AOM treatment group. Mice were sacrificed and colons were collected at 1, 2, 4, 8, and 18 weeks after the last AOM challenge to assess for methylene blue, H&E staining, and immunohistochemistry (IHC) staining of EGFL6. (D) Methylene blue staining of intestinal tissue (from

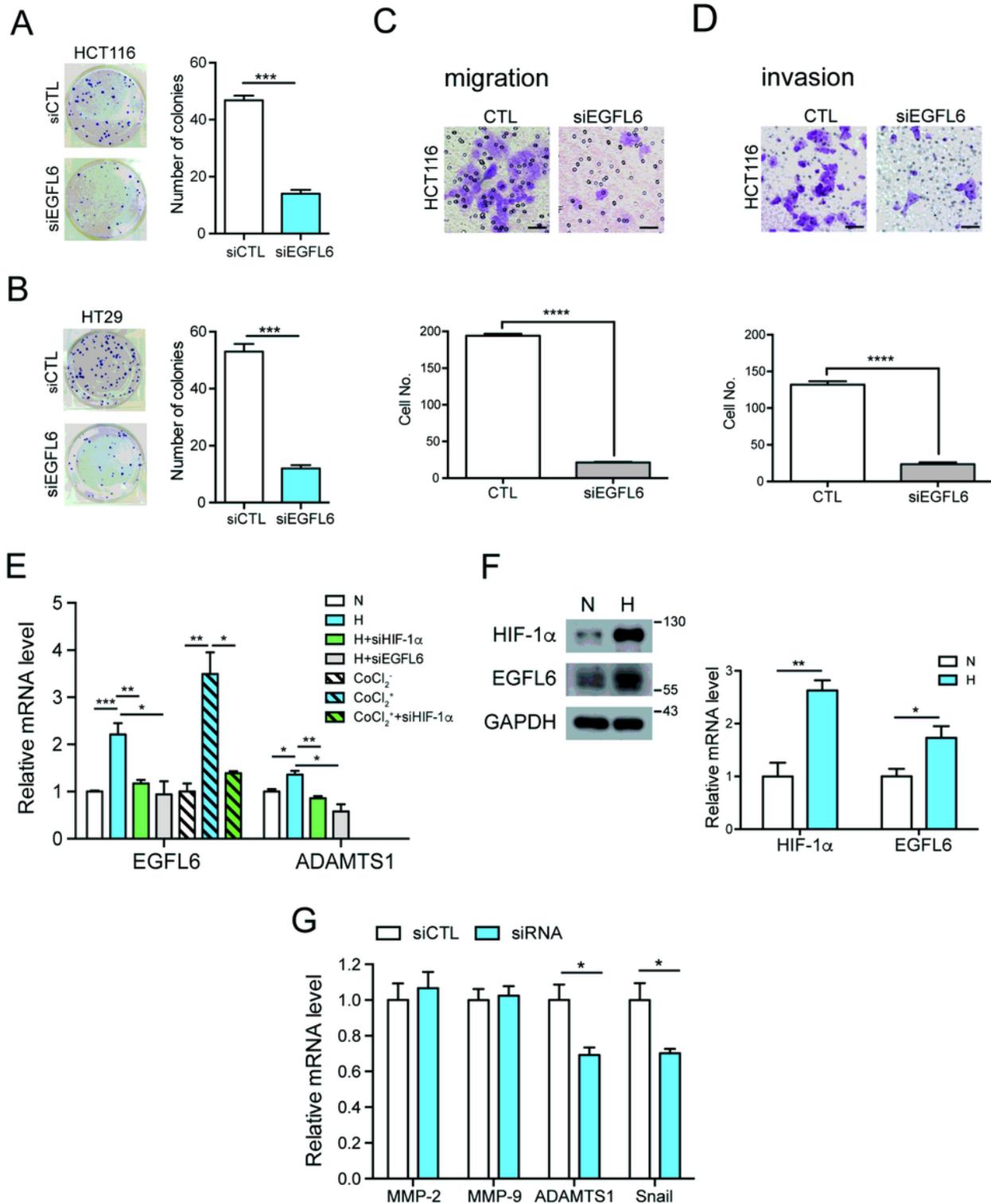
anus to cecum) to observe changes in the intestines. Stained with 0.05% methylene blue. (E) Immunohistochemistry staining of intestinal tissues in different time points after AOM stimulation. Scale bar represents 50  $\mu$ m.



**Figure 2**

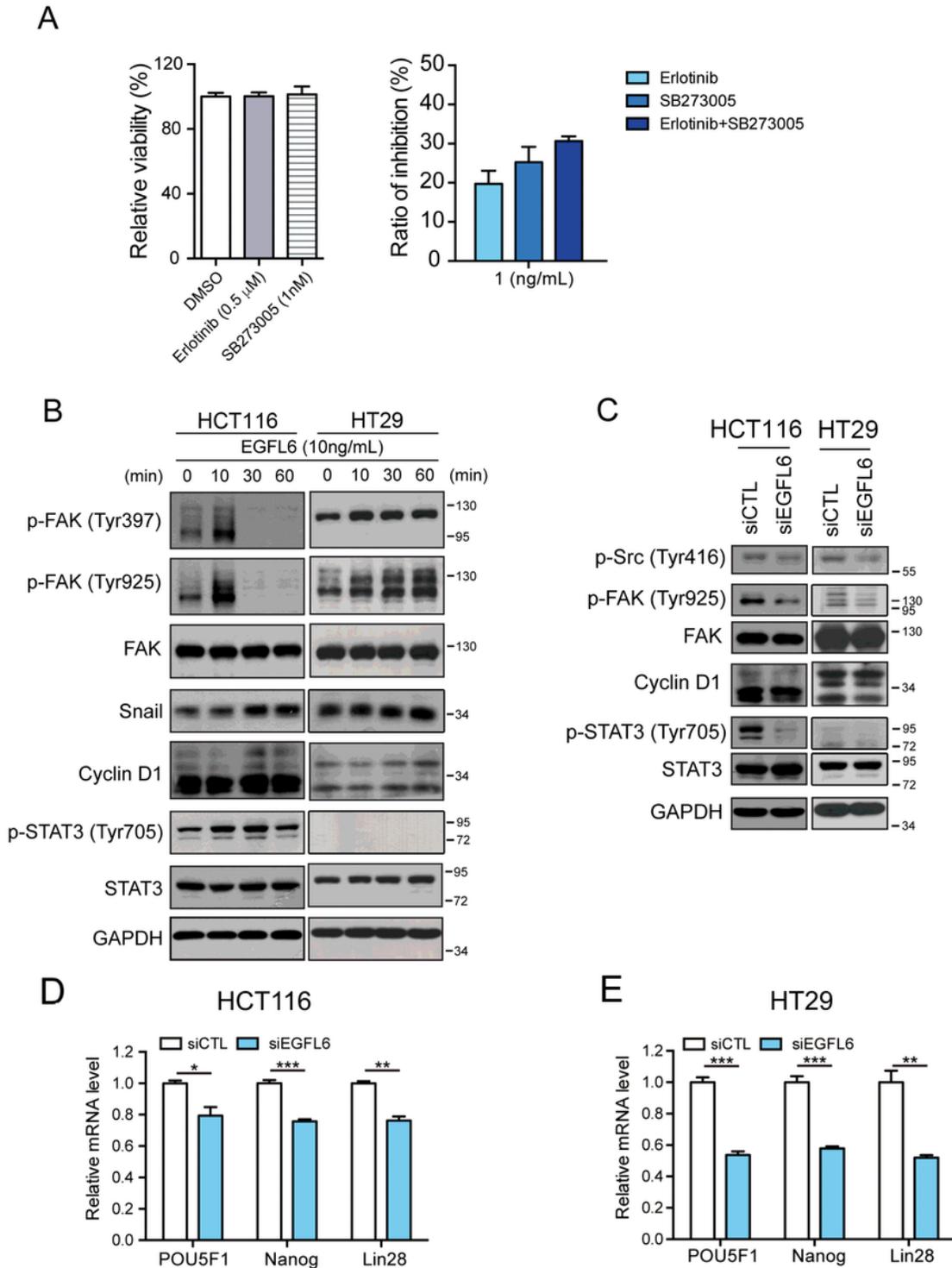
EGFL6 promotes CRC cell proliferation. (A, B) Relative EGFL6 mRNA level and protein expression of FHC (human normal colon epithelial cell), HCT-116 (human colorectal carcinoma epithelial cell) and HT29 (human colorectal epithelial adenocarcinoma cell). (C) EGFL6 expression of HCT-116 culture medium

presenting in western blot. CM, condition medium. (D) The relative viability of HCT-116 and HT29 treated from different concentration of human recombinant EGFL6 (1 ng/mL or 10 ng/mL), incubated 5 days for SRB. (E) Cell proliferation signals (p-ERK, p-AKT) protein expression after EGFL6 (10 ng/mL) treatment by different time point. (F) EGFL6 mRNA expression of HCT-116 and HT29 after knockdown EGFL6. Incubated 48 h after siRNA treatment. (G, H) Tumor cell viability as well as ERK and AKT phosphorylation of HCT-116 and HT29 after EGFL6 knockdown. Incubated 48 h after siRNA treatment. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$ .



### Figure 3

EGFL6 regulates CRC cell migration and invasion. (A, B) Colony formation assay to test the proliferation of HCT116 and HT29 after silencing EGFL6. (C, D) The migration and invasion assay of HCT116 cell after silencing EGFL6. Scale bar represents 100  $\mu\text{m}$ . (E) The mRNA level of EGFL6 and ADAMTS1 under normoxia and hypoxic conditions to validate the signaling relationship between EGFL6 and HIF-1 $\alpha$ . N = normoxia, H = hypoxia. (F) HIF-1 $\alpha$  and EGFL6 expression under hypoxia condition. N = normoxia, H = hypoxia. (G) The mRNA expression of invasion and migration-associated MMP-2, MMP-9, ADAMTS1 and Snail after silencing EGFL6. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$ , \*\*\*\* $P < 0.001$ .

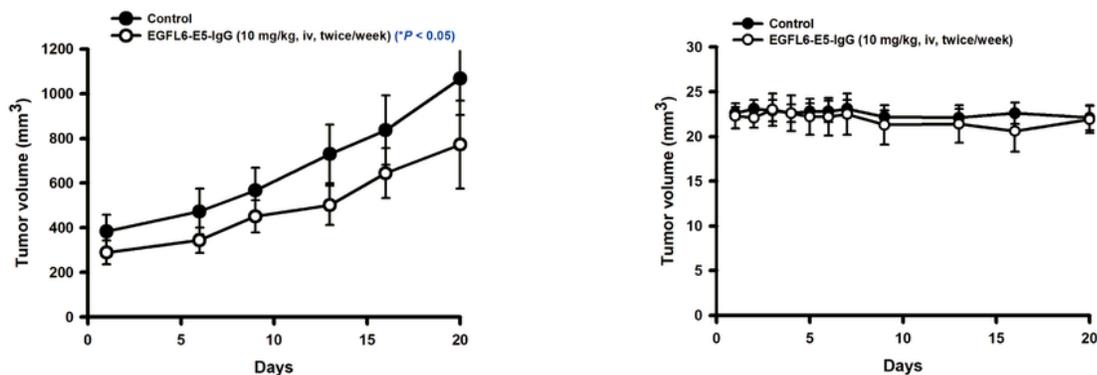


**Figure 4**

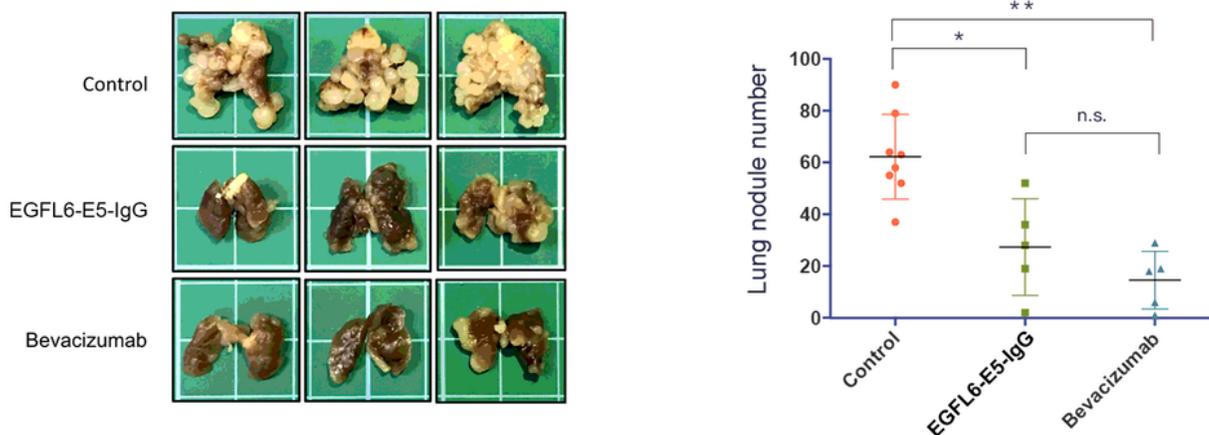
EGFL6 activates EGFR and integrin signaling in CRC cells. (A) EGFL6-induced cell proliferation ability under SB273005 (an  $\alpha v \beta 3$  integrin inhibitor), and Erlotinib (a receptor tyrosine kinase inhibitor on EGFR) treatment. (B, C) The migration, invasion and proliferation-associated protein signaling of HCT116 and HT29 after EGFL6 treatment by time and after silencing of EGFL6. (D, E) The mRNA expression of cell

stemness maintenance associated gene, POU5F1, NANOG and LIN28 after EGFL6 silencing in HCT116 and HT29. \*P<0.05, \*\*P<0.01, \*\*\*P<0.005.

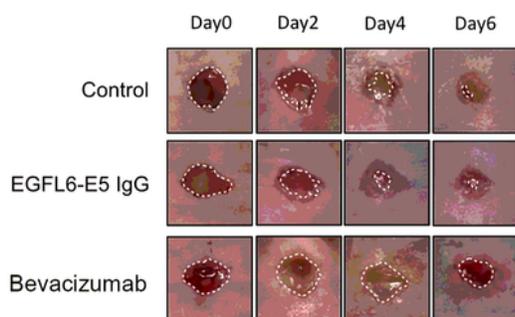
A



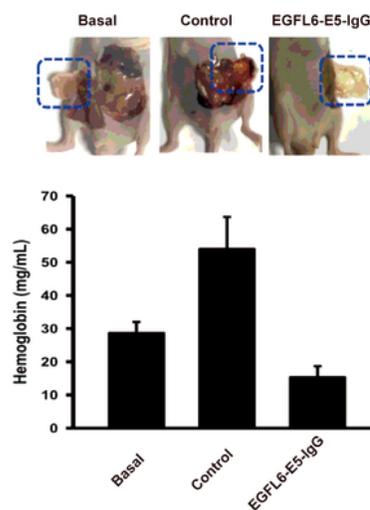
B



C



D



**Figure 5**

In vivo efficacy evaluation of EGFL6 antibody. (A) A total of 16 six-week-old nude mice were injected subcutaneously with the same volume of Matrigel, and  $1 \times 10^7$  of HCT-116 cells into the right flank of each animal. The tumor volume and body weight observation in HCT-116 xenograft model treated with

three groups: control (IgG, i.p, twice/week, n=8) and EGFL6-E5-IgG (10 mg/kg, iv, twice/week, n=8). (B) Eight-week-old balb/c mice (NLAC, Taipei, Taiwan) were injected with CT-26 cells ( $2 \times 10^5$  cell/mouse) intravenously and then divided into three groups for treatments: control (IgG, 20 mg/kg, n = 8), EGFL6-E5-IgG (20 mg/kg, n = 6) and bevacizumab (20 mg/kg, n = 6). Antibodies were given every 5 days by tail vein injection. Body weight was monitored every 2 or 3 days. Scale bar represents 1 cm. (C) A total of 11 seven-week-old nude mice (NLAC, Taipei, Taiwan) were used in wound healing model, 3 groups of mice treated with control (IgG, 20 mg/kg, n = 3), EGFL6-E5-IgG (20 mg/kg, n = 4), and bevacizumab (20 mg/kg, n = 4). Treatments were given intravenously for a consecutive five-on-two-off regimen. Observed for 1 week until Control healed. (D) A total of twelve nude mice (NLAC, Taipei, Taiwan) were divided into four groups. For basal group (n=2), mice were injected with 500  $\mu$ l matrigel subcutaneously. For control group (n=5), mice were injected with 500  $\mu$ l matrigel subcutaneously mixed with endothelial growth factor (EGF, 150 ng/ml) and heparin (10  $\mu$ l). For indicated antibody group (n=5), mice were injected with 500  $\mu$ l matrigel subcutaneously mixed with endothelial growth factor (EGF, 150 ng/ml), heparin (10  $\mu$ l), and treated anti-EGFL6 antibody (15 mg/kg, iv, q5D). \*P<0.05, \*\*P<0.01.

## Supplementary Files

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