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EGF enema and EGFR monoclonal antibody injection alleviate the inflammatory bowel disease in AMO/DSS induced mice model

Original Study

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Abstract

Introduction. Inflammatory bowel disease (IBD) is a chronic condition that significantly impacts the gastrointestinal system. Using an AMO/DSS-induced IBD mouse model, we evaluated the therapeutic potential of epidermal growth factor (EGF) enema and epidermal growth factor receptor (EGFR) monoclonal antibody (mAb) injection as a treatment for IBD.

Materials and Methods. Twelve male C57BL/6 mice were divided into 4 groups (n=3 in each group), including a control group, model group, model group treated with EGF, and model group treated with EGF and EGFR mAb. Mice were sacrificed 9 weeks after disease symptoms first appeared. Colon tissues were then analyzed for histological changes, and protein expression levels of EGFR, Ki-67, p-AKT, and p-ERK were evaluated using immunohistochemistry and Western blotting.

Results. The AOM/DSS model exhibited significant weight loss and inflammation-driven colon damage compared to baseline at week 2; however, by the end of the study, body weight showed a trend toward recovery, with no significant differences observed between the groups. Histological analysis indicated partial improvement in the EGF group, and the combination treatment reduced inflammation compared to the model group. Both treatments significantly reduced Ki67 expression, with no significant difference between the EGF and combination treatments. Additionally, both treatments led to a decrease in p-Akt and p-ERK1/2

Conclusion. EGF enema and EGFR mAb injection exhibited significant therapeutic effects against AOM/DSS-induced IBD *in vivo*. Hence, EGFR-targeted therapy represents a promising approach for treating IBD-related complications.

Keywords

inflammatory bowel disease • AOM/DSS model • EGF • EGFR • monoclonal antibody

1. Introduction

Inflammatory bowel disease (IBD) is a chronic condition with increasing prevalence that severely affects the gastrointestinal tract via recurring episodes [1]. The disease is predominantly manifested as ulcerative colitis or Crohn's disease, conditions with recurring episodes of inflammation and epithelial injury, leading to poor quality of life and significant morbidity [2]. The pathogenesis of IBD results from a complex interplay of genetic predisposition, environmental factors, immune dysregulation, and gut microbiota alterations [3]. Notably, IBD is associated with an increased risk of colorectal cancer over time [4]. The molecular bases of inflammatory bowel disease-driven colorectal cancer are somewhat different from those of sporadic colon cancer. Mutations in tumor suppressor genes, including P53, K-ras, bcl-2, and APC, are crucial in initiating, progressing, and driving the invasion of IBD-associated and sporadic colorectal cancers. However, the distinction lies in the varying roles these gene mutations play at different stages of the carcinogenesis process [5,6]. In particular, inflammation in IBD plays an important role in the progression towards cancer, as long-term chronic inflammatory stimulation increases the likelihood of genetic alterations within cells, thereby driving carcinogenesis [7]. Therefore, therapeutic strategies aimed at limiting inflammation and preventing the progression of cancer are critical areas of ongoing research.

Among the cellular pathways implicated in the progression of IBD, the epidermal growth factor receptor (EGFR) signaling has been identified as a key regulator of intestinal epithelial homeostasis [8]. EGFR is a transmembrane receptor tyrosine kinase that, upon activation by epidermal growth factor (EGF) ligand, regulates cell proliferation, differentiation, survival, and repair. The activation of EGFR-mediated signaling, including PI3K/AKT and RAS/RAF/MEK/ERK, plays an essential role in mucosal healing and the maintenance of intestinal barrier integrity [9]. However, in the context of IBD, EGFR expression is often downregulated in inflamed intestinal tissues due to chronic cytokine exposure, epithelial damage, and epigenetic alterations, thereby impairing mucosal repair [10]. In addition, EGFR expression is significantly lower in inflammatory tissues compared to normal specimens and decreases further in mild ulcerative colitis compared to specific colitis or mild Crohn's colitis [11].

Based on EGF's ability to significantly promote the growth of epithelial tissues, its recombinant form has been successfully used in treating wounds, burns, and ulcers, highlighting its

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potential for epithelial repair in IBD [12]. EGF treatment mediated hyperactivation of EGFR signaling has been shown to induce significant therapeutic effects in the short term in animal models of chronic colitis, where it promotes mucosal restitution and colonic ion transport responses [13]. A large clinical trial also confirmed that EGF enemas effectively treat patients with left colonic ulcerative colitis [14]. These findings opened up a new path for the biological treatment of inflammatory bowel disease. However, concerns about the long-term safety of EGF therapy, particularly its potential to promote inflammation-associated colorectal cancer via prolonged activation of EGFR signaling [15], limit its widespread use. To address these challenges, a combinatorial approach using EGFR monoclonal antibodies (EGFR mAb) alongside EGF may offer a safer and more effective therapeutic strategy by mitigating oncogenic risks while preserving the benefits of EGF-induced mucosal repair.

Here, we aimed to evaluate the therapeutic potential of EGF enema and EGFR mAb injection in an azoxymethane (AOM)/dextran sodium sulfate (DSS)-induced mouse model of IBD. By investigating their effects on inflammation, epithelial repair, and cancer-associated signaling pathways, we seek to elucidate the therapeutic balance between mucosal regeneration and oncogenic safety in IBD treatment.

2. Materials and methods

2.1. Reagents and equipment

The following reagents and equipment were used in the study: recombinant human epidermal growth factor (Shanghai Haohai Biotechnology Co., Ltd., National Medicine Standard S20010099), Cetuximab injection (Merck, Germany, product lot number 264637), fecal occult blood (OB) reagent (pilamid), Hole semi-quantitative detection method) (Zhuhai Bezo Biotechnology Co., Ltd., production batch number: B191001), Rabbit Anti EGFR (ab52894, Abcam, 1/2000), Rabbit Anti p-AKT (AF0016, Affnity, 1/1000), Rabbit Anti p-ERK1/2 (AF1015, Affnity, 1/1000), Mouse Monoclonal Anti-GAPDH (TA-08, Zhongshan Jinqiao, 1/2000), horseradish enzyme labeled goat anti-mouse IgG (H+L) (ZB- 2305, Zhongshan Jinqiao, 1/2000), EGFR (AF6043 Affinity), Ki67 (ab15580 abcam), horseradish enzyme labeled goat anti-rabbit IgG (H+L) (ZB-2301, Zhongshan Jinqiao), hematoxylin staining solution (AR11800-1, BOSTER), eosin staining solution (AR11800-2, BOSTER), automatic microplate reader (WD-2102B, Beijing Liuyi Instrument Factory), ultra-high sensitivity chemiluminescence imaging system (Chemi DocTM XRS+, protein vertical electrophoresis instrument (DYY-6C, Beijing Liuyi Instrument Factory), Biorad Life Medical Products of Shanghai Co., Ltd.), optical microscope (CX41, OLYMPUS), microtome (BQ-318D, Bernard).

2.2. Animal experiments

Twelve 8-week-old C57BL/6 male mice (about 25g) were purchased from Hunan Slack Jingda Experimental Animal Co., Ltd. (License number: SCXK (Xiang) 2019-0004). The mice were divided into four groups, each having 3 mice: a control group, a model group, model mice treated with EGF, and model mice treated with EGF in combination with EGFR monoclonal antibody. To develop the model, mice were injected with a small dose of 7.5mg/kg of AOM intraperitoneally, which was followed by 2% DSS in drinking water for 7 days, then switched to purified water for 14 days. This DSS feeding cycle was repeated 3 times. The mice developed symptoms such as diarrhea and bloody defecation from the first cycle. Mice in the EGF treatment group were given an EGF enema from the onset of symptoms. In contrast, the monoclonal antibody intervention group received an intraperitoneal injection of EGFR monoclonal antibody after EGF enema. Mice were weighed weekly and sacrificed 9 weeks after the symptoms appeared. The colon tissue was carefully excised. The intestinal cavity was emptied, and the colon was weighed and photographed.

2.3. Hematoxylin and eosin (H&E) staining

Tissues were first fixed with 4% paraformaldehyde and rinsed with running water for several hours. The samples were then dehydrated sequentially in 70%, 80%, and 90% ethanol, followed by treatment with a 1:1 mixture of absolute alcohol and xylene for 15 minutes, xylene I for 15 minutes, and II for 15 minutes (until it became transparent). The samples were infiltrated with a 1:1 mixture of xylene and paraffin for 15 minutes. Later, samples were embedded in paraffin I and II for 50-60 minutes each. Later, tissue sections were cut, dewaxed, and hydrated. For staining, sections were immersed in hematoxylin solution for 3 minutes, differentiated with hydrochloric acid ethanol for 15 seconds, rinsed, and treated with bluing solution for 15 seconds. After rinsing with running water, sections were stained with eosin for 3 min, again rinsed with running water, dehydrated, cleared, and sealed with neutral resin. Prepared slides were inspected by microscopy.

2.4. Immunohistochemistry (IHC)

Tissue sections were heated at 65 °C for 2 hours, then immersed in xylene twice for 10 minutes each. The sections were subsequently placed in 100% ethanol twice, 95% ethanol, 80% ethanol and purified water for 5 minutes each. The sections were then incubated with pepsin repair solution at 37°C for 30 minutes, then rinsed with PBS. The sections were treated with 3% hydrogen peroxide to block endogenous peroxidase. After washing the sections with PBS thrice for five minutes each, the sections were incubated with 5% BSA at 37 °C for 30 minutes, followed by primary antibody incubation overnight at 4 °C. The Sections were then incubated at room temperature for 45 minutes, then washed with PBS thrice for three minutes each. After washing, sections were incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (H+L 1:100 1:100)at 37 °C for 30 min, followed by rinsing thoroughly with PBS, developed with DAB for

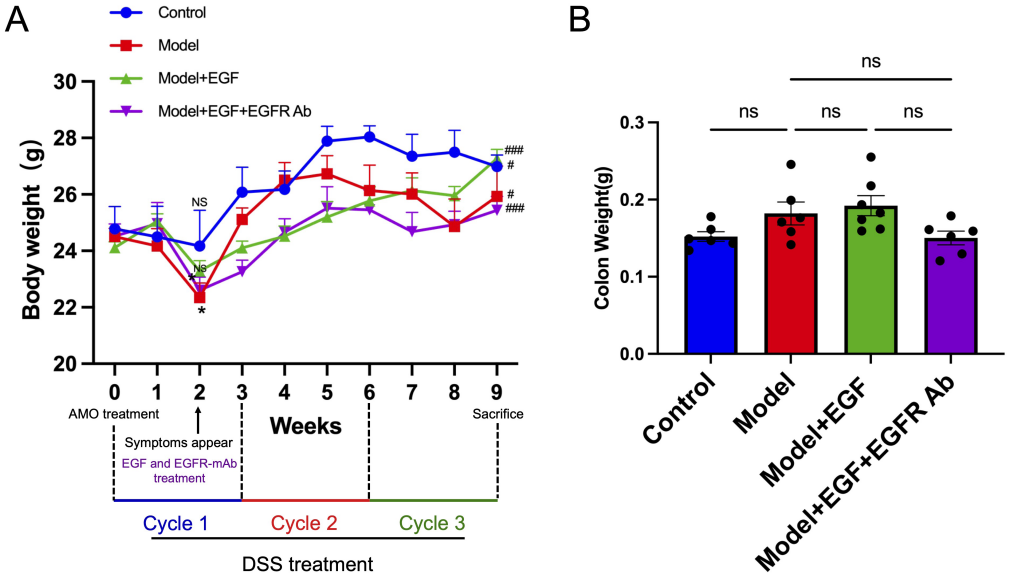

*Figure 1. The effect of EGFR mAb treatment combined with EGF enema in the IBD model in vivo. (A) Line-graph showing changes in body weight of mice (n=3 per group) in control, AOM/DSS model, model with EGF enema and model with EGF enema and EGFR mAb treatment. Treatment time and cycles are mentioned. (B) Bar-graph showing changes in weight of colon tissues (grams) among control, AOM/DSS model, model with EGF enema and model with EGF enema and EGFR mAb treatment. Data is presented as mean ± SEM. * indicates comparison to the initial body weight (Week 0), and # denotes comparison to the body weight at Week 2. ns; non-significant. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$*

5-10 min, and examined under a microscope. After rinsing with PBS or tap water for 1 minute, sections were counterstained with hematoxylin for 3 minutes, differentiated with hydrochloric acid and alcohol, and treated with blueing solution. The sections were then rinsed with tap water for 1 minute, dehydrated, cleared, mounted, and examined under a microscope.

2.5. Western blot

Lysis buffer was used to lyse the cells at 4°C for 30 min. Samples were then centrifuged at 10,000 rpm/min for 10 minutes, and the supernatant was carefully collected as total protein. Protein concentration was determined using the BCA Kit. Proteins were then denatured and separated by electrophoresis for 1-2 hours, followed by wet transfer for 30-50 minutes. Membranes were incubated with primary antibodies overnight at 4°C. The next day, the membranes were washed thrice with 1X TBST and incubated with secondary antibody at room temperature for 1-2 hours. After washing with 1X TBS-T thrice, ECL exposure solution was used for chemiluminescence, which was detected under a BioRad ChemiDoc imaging system. Protein band intensity was measured through "Quantity One" software.

2.6. Statistical analyses

The Shapiro-Wilk test assessed the normality of data in each group. Data conforming to a normal distribution are expressed as mean ± standard deviation (mean ± SD). The Levene's test was employed to evaluate the homogeneity of variance, with $P > 0.05$ indicating equal variances. When both normality and variance homogeneity assumptions were satisfied, comparisons among multiple groups were performed using one-way analysis of variance (one-way ANOVA), followed by Bonferroni post hoc tests. For data that met normality but exhibited unequal variances (heteroscedasticity), the Dunnett T3 test was used for post hoc comparisons among groups. For body weight comparison over time (throughout the experiment), the paired t-test was used. Statistical significance was considered at a *P*-value of < 0.05. All data are presented as mean ± standard error of the mean (SEM). GraphPad Prism software (Version 9.0) performed all the statistical analyses.

3. Results

3.1. The effect of EGFR mAb treatment combined with EGF enema in the IBD model *in vivo*

To evaluate the therapeutic potential of EGF enema and EGFR mAb injection against IBD, we developed an AOM/DSS-induced mouse model of IBD. By the second week, all non-control groups developed diarrhea and hematochezia, with significant initial weight loss observed in the model and EGF+EGFR Ab groups ($P<0.05$, compared to baseline) (Figure 1A). EGF enema with and without EGFR mAb injection was started at that point. Although all groups showed significant weight recovery from the second week by the end of the intervention ($P<0.001$, $P<0.05$, $P<0.05$, $P<0.001$, compared to the second week), mice treated with EGF or EGF+EGFR Ab exhibited no notable increase in body weight compared to the model group (Figure 1A). In addition, we also

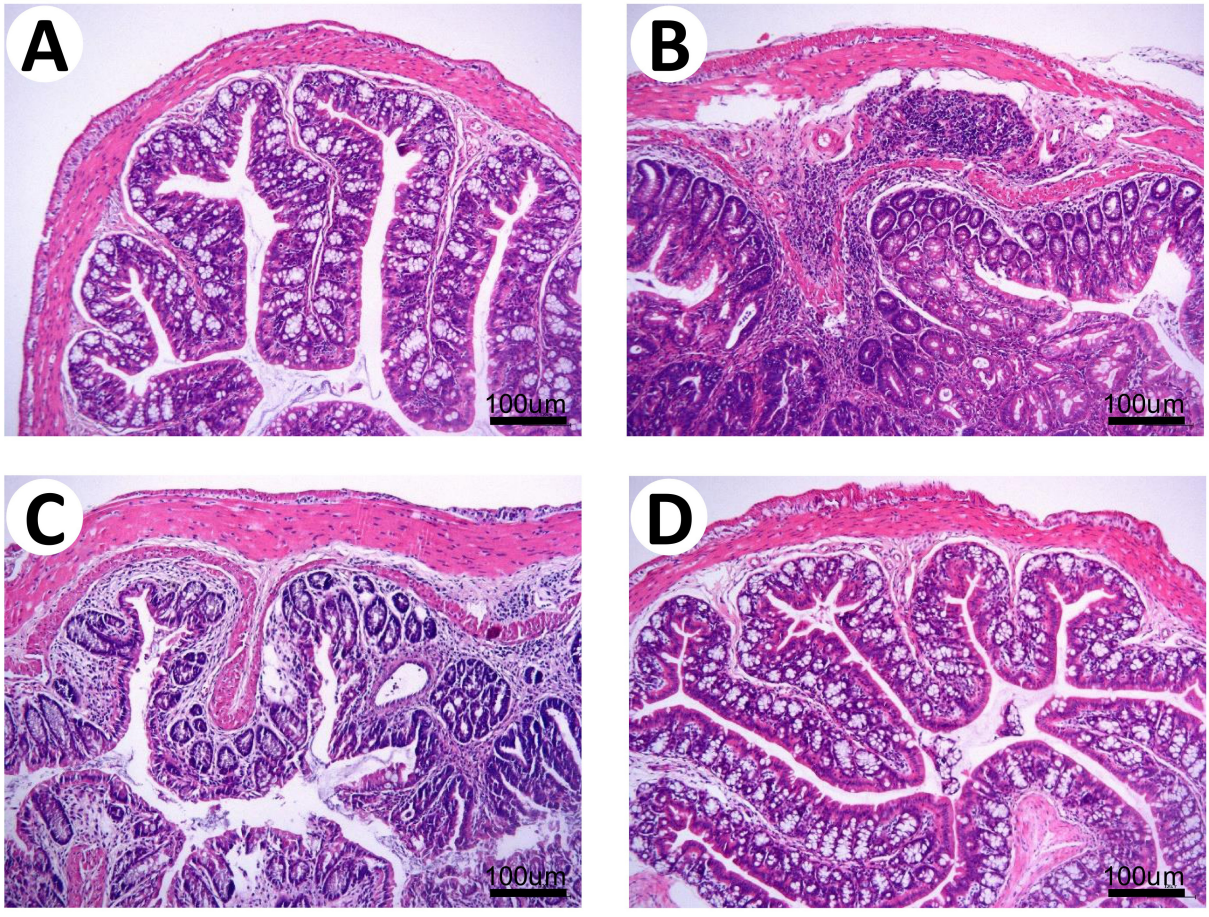

Figure 2. EGFR mAb treatment combined with EGF enema alleviates colonic inflammation in the IBD model in vivo. (A-D) Representative H&E staining images (100x) of colon tissue from control (A), AOM/DSS model (B), model with EGF enema (C) and model with EGF enema and EGFR mAb treatment (D)

compared the changes in the weights of colon tissue among the four groups. Although the average weight of colon tissues in the model group was higher than that of the control group, it was not statistically significant. On the other hand, EGFR mAb treatment decreased the average weight of colon tissue compared to the EGF enema group, though it was also not significant (Figure 1B). Overall, the AOM/DSS model was successfully established; however, neither EGF monotherapy nor combination therapy with an EGFR mAb significantly affected body weight.

3.2. EGFR mAb treatment combined with EGF enema alleviates colonic inflammation in the IBD model *in vivo*

Next, we performed H&E staining on colon tissues to assess histological changes across the experimental groups (Figure 2) and made the following observations. In the control group, the colon structure was intact and clearly defined. Goblet cells were abundant between the mucosal and intestinal gland epithelial cells. There were no signs of swelling, congestion, or structural disruption. The glands were well-developed and orderly, with a systematically arranged mucosal muscularis, indicating normal histological features (Figure 2A). In the AOM/DSS model group, significant pathological changes were observed. The intestinal mucosa showed congestion and edema, while the lumen was significantly narrowed. The glandular epithelial cells exhibited proliferation, and the glands were disorganized in their arrangement. A large infiltration of inflammatory cells was evident, with increased lamina propria cellular components. The muscle fibers of the muscularis mucosa were disrupted, and the gap between the muscularis and submucosa was widened. The submucosa contained large, loose connective tissue infiltrated with many inflammatory cells (Figure 2B). In the EGF enema group, partial alleviation of inflammation was observed, but pathological changes were still prominent. The mucosal muscular layer was thickened, and inflammatory cell infiltration persisted in the lamina propria. Detached epithelial cells were visible, having shed into the intestinal lumen, suggesting incomplete recovery

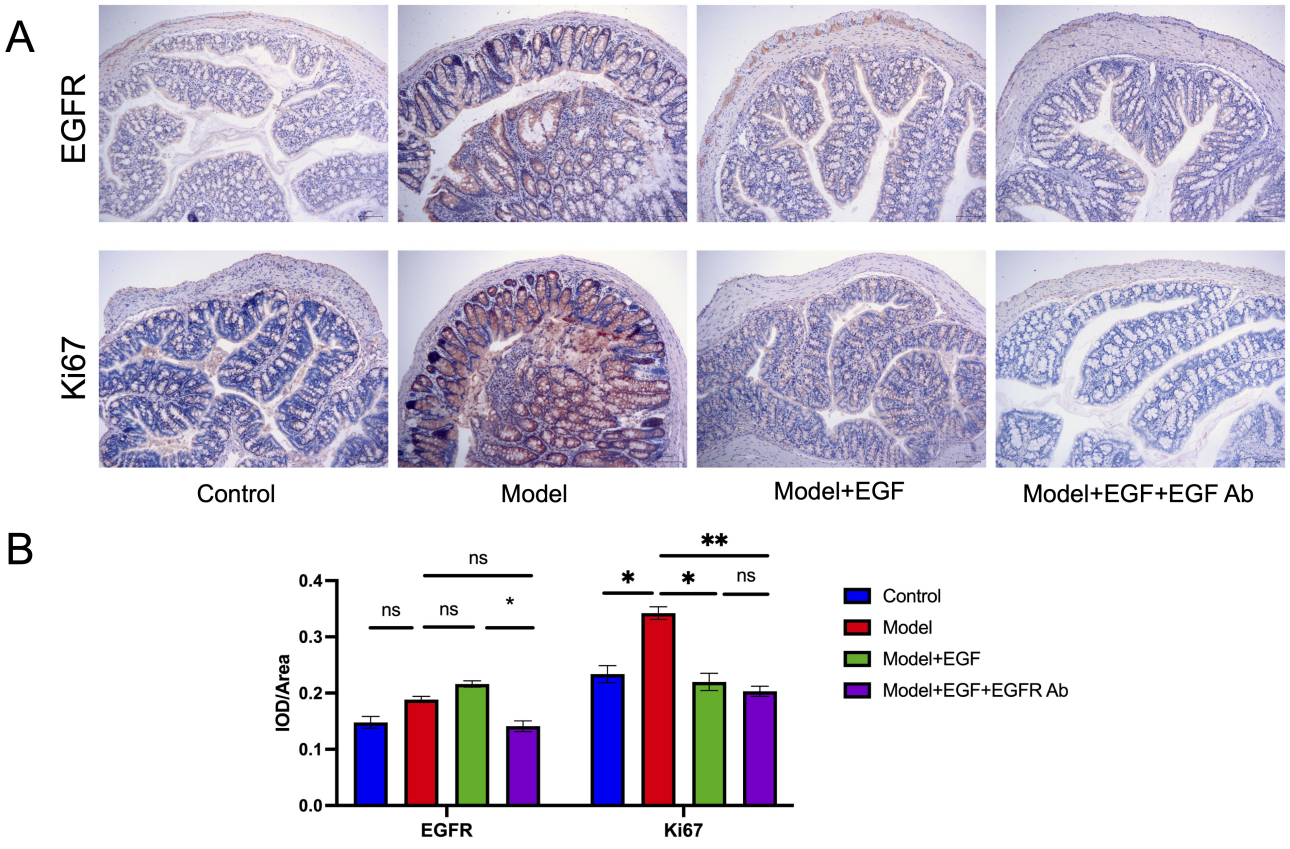

*Figure 3. EGFR mAb treatment combined with EGF enema reduces proliferation in the IBD model in vivo. (A) IHC images (100x) showing changes in expression of Ki67 and EGFR among control, AOM/DSS model, model with EGF enema and model with EGF enema and EGFR mAb treatment. (B) Bar-graph showing quantification of staining in (A). Data is presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001*

from the AOM/DSS-induced damage (Figure 2C). The colon structure showed significant improvement in the EGF enema combined with EGFR mAb group, with histology returning close to normal. The lamina propria structure remained intact, and the infiltration of inflammatory cells was greatly reduced. No congestion or edema was observed in the intestinal mucosa; only a few epithelial cells detached from the mucosal surface (Figure 2D). These findings highlight the therapeutic potential of EGFR mAb treatment in reducing inflammation and restoring normal colon histology in the AOM/DSS-induced inflammatory carcinogenesis model.

3.3. EGFR mAb treatment combined with EGF enema reduces proliferation in IBD model *in vivo*

Ki67 is a widely recognized marker of cellular proliferation, as it is expressed in actively dividing cells but absent in quiescent, non-dividing cells. Elevated levels of Ki67 indicate increased proliferation, which is often associated with inflammation, tissue repair, and carcinogenesis [16]. We performed IHC to evaluate the expression of Ki67 and EGFR across the experimental groups (Figure 3) and made the following observations. In the model group, Ki67 expression was significantly increased compared to the control group, reflecting enhanced epithelial cell proliferation (Figure 3A and B). Compared to the model group, the EGF group and the EGF+EGFR Ab group exhibited significantly reduced Ki67 protein expression levels ($P<0.05$, $P<0.01$); however, the combination of EGF and EGFR Ab did not further decrease Ki67 levels compared to the EGF group alone (Figure 3A and B). These findings demonstrate that both EGFR mAb and EGF enema treatments reduce the pathological upregulation of Ki67 and effectively inhibit proliferation.

3.4. EGFR mAb treatment combined with EGF enema reduces oncogenic signaling in IBD model *in vivo*

Next, we aimed to investigate whether EGFR mAb treatment combined with EGF enema impacts oncogenic signaling in the IBD model *in vivo*. Earlier studies have shown that elevated expression of phosphorylated AKT (p-AKT) and phosphorylated ERK (p-ERK) positively correlates with the degree of tumor tissue differentiation [17,18]. As expected, EGFR expression was

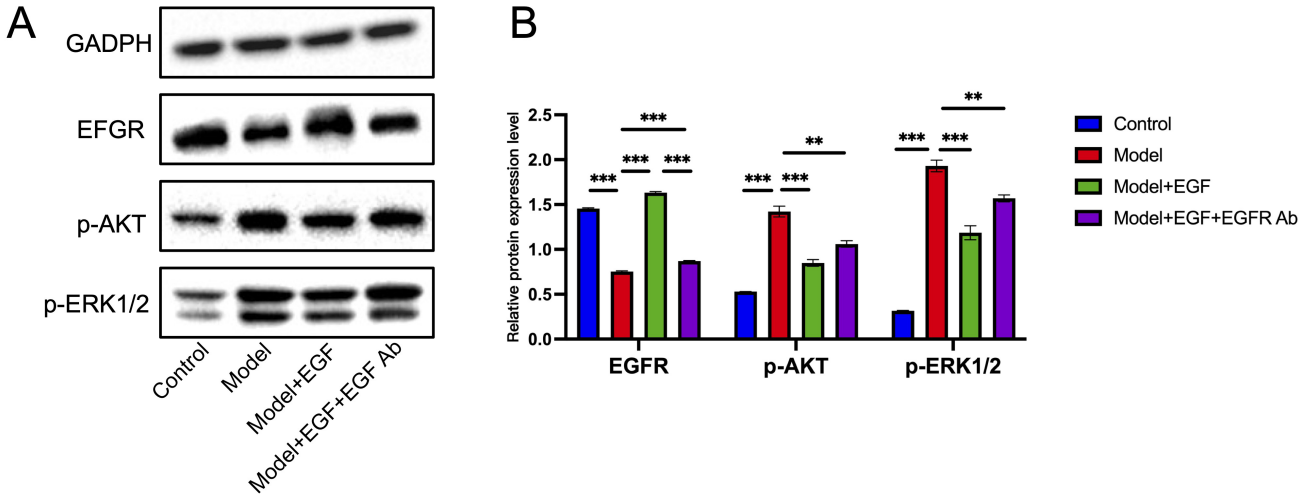

*Figure 4. EGFR mAb treatment combined with EGF enema reduces oncogenic signaling in the IBD model in vivo. (A) Immunoblots showing changes in expression of p-AKT, p-ERK and EGFR among control, AOM/DSS model, model with EGF enema and model with EGF enema and EGFR mAb treatment. (B) Bar-graph showing quantification of changes in protein expression in (A). Data is presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001*

significantly reduced in the model compared to control, aligning with the notion that EGFR expression is generally downregulated in inflammatory tissues in IBD [10,11]. However, EGF enema led to a significant increase in EGFR expression, suggesting the involvement of a compensatory mechanism where hyperactive EGFR signaling due to elevated EGF levels in the microenvironment further induces EGFR expression [9]. Notably, Compared to the model group, EGFR protein expression was downregulated in both the EGF-treated (enema) group and the EGF+EGFR Ab combination group (*P*<0.001, *P*<0.001); furthermore, the combination group exhibited a further decrease in EGFR levels compared to the EGF group alone (*P*<0.001)(Figure 4A and B). On the other hand, expression of oncogenic proteins, p-Akt and p-ERK1/2, was significantly elevated in the model group compared to the control group, indicating increased activation of oncogenic signaling pathways in response to AOM/DSS-induced inflammation (Figure 4A and B). Most importantly, both EGF enema with and without EGFR mAb treatment groups showed marked decreases in the expression of oncogenic proteins, p-Akt and p-ERK1/2, compared to the model group (*P*<0.001, *P*<0.01) (Figure 4A and B). These results suggest that combining EGFR mAb treatment with EGF enema effectively reduces oncogenic signaling pathways and keeps a check on hyperactive EGFR signaling by limiting EGFR expression, eventually mitigating inflammation-driven tumorigenesis.

4. Discussion

IBD is a complex condition characterized by chronic inflammation of the gastrointestinal tract, and it is known to increase the risk of colorectal cancer over time [4]. The progression from IBD to colorectal cancer involves multiple molecular pathways, including the activation of oncogenic signaling cascades such as those mediated by EGFR [19]. Here, we evaluated the therapeutic potential of EGF enema combined with EGFR mAb treatment in the AOM/DSS-induced IBD model to examine the balance between mucosal healing and oncogenic signaling inhibition. Our results indicate that EGF enema alone and in combination with EGFR mAb exert similar effects in suppressing oncogenic signaling, with no significant differences observed between the two treatments, and without compromising mucosal repair.

The AOM/DSS-induced IBD model is widely recognized as an effective method for studying the link between chronic inflammation and carcinogenesis in the colon. As demonstrated in previous studies, the dual exposure to AOM and DSS triggers a cascade of inflammatory responses and increases susceptibility to tumorigenesis (Figure 2) [20]. In our study, the therapeutic effects of EGF enema on inflammation and epithelial repair were confirmed, consistent with prior reports [13]. EGFR is a central player in IBD-related carcinogenesis, as its activation drives several key cellular processes, including proliferation, survival, and tissue repair. While EGFR signaling plays an essential role in mucosal healing, chronic activation of EGFR in inflammation is also associated with cancer development [15]. In this study, we found that EGF enema led to the reactivation of EGFR signaling, as evidenced by increased EGFR expression in inflamed colon tissues. This compensatory upregulation of EGFR in response to EGF treatment is consistent with previous reports, highlighting the delicate balance between EGFR's role in tissue repair and its potential to drive tumorigenesis in chronic inflammation [9]. Meanwhile, treatment with EGFR mAb alone or combined with

EGF reduced EGFR protein expression and downregulated key oncogenic mediators, including p-AKT and p-ERK1/2. Although the combination group exhibited lower EGFR levels than the EGF group alone, both treatments comparably suppressed downstream oncogenic signaling and significantly reduced Ki67 expression, a marker of aberrant epithelial proliferation. Notably, there was no significant difference in Ki67 levels between the EGF and combination groups, indicating that the anti-proliferative effect of EGFR mAb does not necessarily enhance the benefit achieved with EGF alone. These findings suggest that EGF enema and its combination with EGFR mAb can mitigate inflammation-induced epithelial proliferation and oncogenic signaling. However, the combination does not confer an additional advantage over EGF alone in reducing proliferative activity in this IBD model.

Ki67 is a widely used marker of cellular proliferation and is often associated with active cell division during inflammation and cancer progression [16]. Our findings showed a marked increase in Ki67 expression in the AOM/DSS model group, reflecting heightened epithelial cell proliferation in response to inflammation. These findings are in line with those where elevated Ki67 has been found in colitis samples from patients [21,22]Treatment with EGF enema and its combination with EGFR mAb led to a reduction in Ki67 expression, suggesting some degree of inhibition of proliferation. This effectively reduced the proliferative response in inflamed tissues. This reduction in Ki67 levels indicates that EGFR mAb treatment, in combination with EGF enema, can attenuate excessive cell proliferation, which is crucial in preventing the progression of IBD to cancer.

Our analysis of oncogenic signaling pathways revealed that EGFR mAb treatment combined with EGF enema reduced the activation of both p-AKT and p-ERK, key downstream effectors of EGFR signaling [17,18]. The elevated expression of p-AKT and p-ERK in the model group reflects the activation of these pro-tumorigenic pathways in response to chronic inflammation. In contrast, treatment with both EGF enema and EGFR mAb led to a significant reduction in the levels of these phosphorylated proteins, suggesting that this combination therapy not only restores mucosal repair but also mitigates the oncogenic signaling pathways that contribute to IBD-associated colorectal cancer. This finding aligns with previous studies showing that EGFR-targeted therapies can reduce the activation of oncogenic pathways in the context of inflammation-driven carcinogenesis [23].

In conclusion, our study highlights the therapeutic potential of EGFR mAb treatment combined with EGF enema in mitigating the oncogenic risks associated with IBD while promoting mucosal healing. By targeting both the inflammatory and oncogenic pathways, this combinatorial strategy provides a balanced approach to treating IBD with a reduced risk of cancer progression, offering new insights into the management of IBD-associated colorectal cancer.

5. Limitations of the study

While the present study provides valuable insights into the potential therapeutic effects of combining EGF enema and EGFR mAb treatment for IBD, several limitations must be considered when interpreting the results. The small sample size of only three mice per group limits the generalizability of the results. Additionally, the AOM/DSS mouse model does not fully represent human IBD, limiting the direct applicability of the findings to human patients. Furthermore, we did not specifically focus on analyzing any potential side effects of this combination treatment. We observed delayed alleviation of loss in body weight in mice who received combined EGF enema and EGFR mAb treatment, which could have been due to stress related to the combination treatment. Furthermore, the study did not explore the combined effects of EGF enema and EGFR monoclonal antibody with other treatments or assess potential adverse effects, which are important for clinical translation.

Abbreviations

AKT	Protein kinase B
ANOVA	Analysis Of Variance
AOM	Azoxymethane
DSS	Dextran Sodium Sulfate
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
H&E	Hematoxylin and Eosin
IBD	Inflammatory Bowel Disease
mAb	Monoclonal Antibody
p-ERK	Phosphorylated Extracellular Signal-Regulated Kinase
p-AKT	Phosphorylated Protein kinase B

Authors' contribution

XZ: Conceptualization, Supervision, Project administration, Writing—review and editing. **CW:** Methodology, Formal analysis, Investigation, Writing—original draft, Writing—review and editing.

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Conflict of interest

The authors have no potential conflicts of interest to declare.

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