

Role of *EtMIC4* EGF-like in regulating the apoptosis of *Eimeria tenella* host cells via the EGFR pathway

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ABSTRACT This study aimed to explore the role and key point of *EtMIC4* EGF-like recombinant protein in regulating the apoptosis of *Eimeria tenella* host cells via the epidermal growth factor receptor (**EGFR**) pathway. The cells were treated with *EtMIC4* EGF-like protein, EGFR-specific siRNA, or both. Infection and apoptosis rates as well as dynamic changes in the key genes and proteins of the EGFR signaling pathway in the host cells were determined. Results showed that the *E. tenella* and *EtMIC4* EGF-like group had the highest infection rate ($P < 0.01$). In cells treated with *EtMIC4* EGF-like for 4 to 24 h, the apoptosis rate was significantly decreased ($P < 0.01$) and the relative mRNA expression and protein phosphorylation levels of EGFR,

protein kinase B (**AKT**), and extracellular regulated protein kinases (**ERK**) were significantly increased ($P < 0.01$). In *E. tenella* sporozoites infected for 4 to 96 h, the rate of host cell apoptosis induced by *E. tenella* infection was significantly ($P < 0.01$) reduced by *EtMIC4* EGF-like. The relative mRNA expression and protein phosphorylation levels of EGFR, AKT, and ERK in the host cells of *E. tenella* + *EtMIC4* EGF-like group were significantly increased ($P < 0.01$). These results indicated that *E. tenella* could activate the EGFR pathway through *EtMIC4* EGF-like and regulate the expression of key genes in the AKT and ERK signaling pathways, thereby inhibiting cell apoptosis.

Key words: *Eimeria tenella*, *EtMIC4* EGF-like, apoptosis, EGFR pathway

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INTRODUCTION

Chicken eimeriosis is an intracellular parasitic protozoal disease that seriously endangers the chicken industry, and its infection is very common, that is, chicken coccidia are often present wherever chickens are raised. Worldwide losses due to coccidiosis in chickens (chicken death, reduced productivity, and cost of preventive medicine, etc.) amount to £10.4 billion annually (Blake et al., 2020). At present, the control of coccidiosis mainly relies on anti-coccidial drugs and live coccidiosis vaccines. However, drug resistance and the pathogenicity of live vaccines have limited its application widely. To solve this problem, we cannot rely solely on research of anticoccidial drugs and vaccines. The interaction

between coccidia and the organism and the mechanism of damage to the host should be determined.

Epidermal growth factor receptor (**EGFR**) is a family of receptor tyrosine kinases that are widely distributed in the cell membranes of human and animal tissues. It is activated by binding to its specific ligands (such as EGF, etc.) and then recruits downstream cell signaling molecules to complete the process. The transduction of cell signals from outside to the inside of the membrane precisely regulates a series of complex physiological processes, such as cell proliferation, apoptosis, migration, adhesion, and survival. Several apicomplexan microneme proteins have been reported to have tandem arrays of epidermal growth factor-like repeats. These proteins include *Toxoplasma gondii* MIC3, MIC6, and MIC8 (Muniz-Feliciano et al., 2013); *Cryptosporidium parvum* MIC1 (Putignani et al., 2008); *Eimeria tenella* MIC4 (Periz et al., 2005); and *Eimeria maxima* TFP250 (Witcombe et al., 2003). Other studies have shown that a microneme protein from *E. tenella* (*EtMIC4*) has the characteristics of EGF-like region and can bind to EGFR in chicken cells (Tomley et al., 2001;

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Yang, 2014). However, whether *E. tenella* can regulate host cell apoptosis through the *EtMIC4* EGF-like region remains unclear.

In this study, we used chick embryonic cecal epithelial cells with EGFR-specific siRNA interference and noninterfering EGFR pathway as an in vitro model to detect the key factors of the pathway in *E. tenella* host cells at different developmental stages with or without the *EtMIC4* EGF-like protein. Dynamic changes in mRNA expression and kinase phosphorylation were measured to explore the effect of *EtMIC4* EGF-like protein on the EGFR pathway in *E. tenella* host cells.

MATERIALS AND METHODS

Ethics Statement

All experiments involving animals were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC). This study was approved by the Animal Protection and Utilization Committee of Shanxi Agricultural University, China.

Experimental Animals, Parasite, and Recombinant Protein

In brief, 15-day-old SPF chicken embryos were obtained from Beijing Meri Avigon Laboratory Animal Technology Co., Ltd. (Beijing, China). *E. tenella* Shanxi virulent strain (Tsx202106) and recombinant *EtMIC4* EGF-like protein were provided by the Laboratory of Veterinary Pathology in the College of Veterinary Medicine, Shanxi Agricultural University.

Preparation of *E. tenella* sporozoites

Sporocysts were released from the sporulated *E. tenella* oocysts by mechanical rubbing with a glass homogenizer and then centrifuged at 1800 r/min for 5 min. The precipitate was added with suitable quantum spore digestion solution (0.75% trypsin and 10% chicken bile in a ratio of 1:1) and digested with shaking at 41°C and 150 r/min until 80% of the sporozoites were released. The sporozoites were purified by filtration through a 1800-mesh sieve (10 μm) and centrifuged at 3,000 r/min for 10 min to collect free sporozoites, which were diluted with MEM199 (Gibco, California) to 2.0×10^5 sporozoites/mL.

Primary Culture of Chicken Embryo Cecal Epithelial Cells

The primary cecal epithelial cells of the chicken embryo were cultured using primary culture technique as described previously (Zhang et al., 2019; Zhang et al., 2020). The cecum was carefully removed and placed in PBS (pH 7.4). The cecum was then cut into smaller fragments (about 1 mm³) with a sterile scalpel blade and

transferred into 50 mL tubes with thermolysin (50 mg/L, 0.25 mL/embryo) (Sigma, CA). Under constant gentle shaking (80 r/min), the embryo cecal fragments were digested at 37°C for 95 to 110 min. The sample was rinsed with PBS and centrifuged at 1,000 r/min for 5 min. The cells were cultivated at 41°C in a humidified, 8% CO₂ air incubator for 70 min on the basis of each cell's adherence speed to separate different cells. Non-adherent cells were collected and centrifuged to remove the cell culture medium. The cells were then resuspended and counted in a cell culture medium (1% glutamine, 1% heparin sodium, 1% sodium pyruvate, 1% dual antibodies, 0.02% hyperinsulin, and 2.5% fetal bovine serum). The live cell aggregates were diluted to 1.5×10^5 /mL. The cells were seeded on 96-well and 6-well cell culture plates at volumes of 200 μL and 2 mL per well, respectively. Chamber slides were placed in some of the 6-well plates to allow cell growth. The cells were used for subsequent experiments when the adherence rate reached about 95%.

Proliferative Activity of *EtMIC4* EGF-like Recombinant Protein

The 96-well cell culture plates were divided into 6 groups. The cells were treated with different concentrations of *EtMIC4* EGF-like protein (0, 0.02, 0.05, 0.1, and 1 μg/mL). We also used 0.02 μg/mL EGF (PeproTech, NJ) as a positive control (Duan et al., 2019). The experiment was conducted five times. The medium was discarded and replaced with a medium containing the corresponding *EtMIC4* EGF-like protein concentration. At 4, 24, and 48 h after the addition of *EtMIC4* EGF-like protein, the medium was carefully discarded and cell proliferation activity was detected by MTT method. Cell proliferation rate was determined by the following equation: [(OD₄₉₀ of test group - OD₄₉₀ of control group) / OD₄₉₀ of control group] × 100.

EGFR-Specific siRNA (siRNA EGFR) Transfection

Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China) was commissioned to design and synthesize siRNA *EGFR* based on chicken *EGFR* mRNA (GenBank NM_205497.2). Lipofectamine RNAiMAX (Invitrogen, CA) was used as the transfection reagent. The siRNA sequence is shown in Table 1, and the transfection condition of siRNA was 40 nM for 48 h.

Table 1. siRNA sequence.

Name		Sequence
siRNA <i>EGFR</i>	Sense	CCCUUGCUGUUGUUAACUUTT
	Antisense	AAGUUAACAACAGCAAGGGTT
NC siRNA	Sense	UUCUCCGAACGUGUCACGUTT
	Antisense	ACGUGACACGUUCGGAGAATT

Experimental Protocol in Vitro

The chicken embryo cecal epithelial cells (adherence rate was reached about 95%) in the 6-well cell culture plates (some of chamber slides) were randomly divided into siRNA *EGFR* + *E. tenella* group, siRNA *EGFR* + *Etmic4* EGF-like group, siRNA *EGFR* + *Etmic4* EGF-like + *E. tenella* group, *E. tenella* group, *Etmic4* EGF-like group, *Etmic4* EGF-like + *E. tenella* group, siRNA *EGFR* group, NC siRNA group, EGF group, and control group. After inoculation of sporozoites (4.0×10^5 sporozoites/per well) for 4 h, the culture medium in each group was changed. The medium was then changed every 48 h. For culture plates sampled at 4 and 24 h, 0.2 μ g of *Etmic4* EGF-like protein per well was added while inoculating sporozoites. At 24 h before sampling, *Etmic4* EGF-like protein was added to the culture plate cells sampled at 96 h. The cell culture plates in each group were cultured at 41°C and 8% CO₂. Samples were taken at 4, 24, and 96 h after inoculation of sporozoites.

Hematoxylin and Eosin Staining

The chamber slides in siRNA *EGFR* + *E. tenella* group, siRNA *EGFR* + *Etmic4* EGF-like + *E. tenella* group, *E. tenella* group, *Etmic4* EGF-like + *E. tenella* group, and control group were taken out at indicated times of infection, respectively. The slides were stained with H&E according to the method of Zhang et al. (2019). Sample *E. tenella* infections were observed by light microscopy in 200 randomly selected cells. Infection rate (%) was calculated using the following equation: number of infected parasite cells/200 \times 100.

Apoptosis Detection

At indicated times of infection, cell culture medium was collected and 0.25% trypsin was added to the cell culture plate. The cells were digested at 37°C and 80 r/min for 5 min. After the digestion was terminated with the collected cell culture medium, all liquids were collected and centrifuged at 1000 r/min for 5 min. The cells were resuspended in 1 mL of precooled PBS, centrifuged at 1,000 r/min for 5 min, and washed. The cells were then resuspended in 1 \times binding buffer, added with 100 μ L of Hoechst 33342 Stain (Beyotime, Shanghai, China), and incubated at 37°C for 20 min in the dark. After cooling in ice water, the cells were centrifuged at

1000 r/min at 4°C for 5 min. The cell precipitate was stained with annexin V-FITC/PI double-staining flow cytometry kit (BD Biosciences, San Diego, CA) and centrifuged at 1000 r/min for 5 min. The precipitate was collected and resuspended in 1 \times binding buffer. The cells were observed under a fluorescence microscope (OLYMPUS, Hatagaya, Japan). The results were assessed as follows: all cell nuclei were stained by Hoechst 33342 (blue fluorescence); normal cells were low fluorescence in annexin V-FITC (green fluorescence) and PI (red fluorescence); early apoptotic cells were highly stained by annexin V-FITC and low in PI; and late apoptosis and necrosis cells were highly stained with annexin V-FITC and PI. More than 200 cells were observed and used for calculation: apoptotic rate = number of early apoptotic cells/total number of observed cells \times 100; and late apoptosis and necrosis rate = late apoptotic and necrotic cells/total number of observed cells \times 100.

RT-qPCR

The culture medium of each group of cells was discarded, and the cells were added with RNAiso Plus (Takara Bio, Japan) to digest. The cells were collected, and total RNA was extracted by Trizol method. RT-qPCR detection was performed on each group of samples by using a reverse transcription kit (Takara Bio, Japan) and fluorescence quantitative kit (Takara Bio, Japan). The primer sequences are listed in Table 2.

Western Blot Analysis

At indicated times of infection, the culture medium in the cell plate was discarded. The cells were washed three times with PBS and lysed in RIPA buffer containing protease phosphatase inhibitors (Beyotime, Shanghai, China) for 30 min on ice under shaking. The cells were centrifuged at 4°C and 12000 r/min for 10 min, and the supernatant was collected. Protein concentration was determined using a bicinchoninic acid protein assay kit (Thermo Scientific, MA). The protein sample was mixed with 5 \times protein loading buffer (Boster biological, Wuhan, China) at a ratio of 4:1 and denatured in a boiling water bath for 10 min. The protein samples (40 μ g/lane) were separated using 10% polyacrylamide gels and transferred to 0.45 μ m nitrocellulose membranes (NC membranes) by using the wet transfer system (Junyi,

Table 2. RT-qPCR primer sequences.

Gene name	Primer sequences	Amplicon size (bp)	GenBank accession No.
β -actin-F	5'-CACCACAGCCGAGAGAGAAAT-3'	135	L08165.1
β -actin-R	5'-TGACCATCAGGGAGTTCATAGC-3'		
EGFR-F3	5'-TATCCGAGGCCGAACCAAGC-3'	148	NM_205497.2
EGFR-R3	5'-GGTGTCCAGCATAGCAGAGGTTCTT-3'		
ERK-F	5'-GCTCTCCAACAGACCCATCTT-3'	146	NM_204150.1
ERK-R	5'-TGTGTGGTAGGAAAGCAAATAGT-3'		
Akt-F3	5'-AACGGAGGGGAGTTGTTTTT-3'	149	AF039943.1
Akt-R3	5'-ATGTGCCCGTCTTTATCCAG-3'		

Beijing, China). After blocking with 5% BSA in TBST for 1 h at 37°C, the NC membranes were incubated at 4°C overnight with primary antibodies for p-EGFR (CST, MA), EGFR (CST, MA), protein kinase B (AKT) (Abmart, Shanghai, China), p-Akt (Abmart, Shanghai, China), extracellular regulated protein kinases (ERK) (Abmart, Shanghai, China), and p-ERK (Abmart, Shanghai, China). After washing off the primary antibody, the membranes were exposed to goat anti-rabbit IgG-HRP-conjugated antibody (Abmart, Shanghai, China) as secondary antibody for 2 h at 28°C. The secondary antibody was washed, and the ECL chemiluminescence reaction was carried out. Imaging was conducted with a high-sensitivity chemiluminescence imager (BIO RAD, ChemiDOC XRS + Imaging System, CA). We used β -Actin (CST, MA) as an internal reference protein.

Image and Statistical Analyses

Results are presented as arithmetic mean \pm standard error. Each experiment was repeated at least three times. The SPSS 22.0 statistical software package (Chicago) was used to perform ANOVA of all data. Histograms were prepared with GraphPad Prism 6.0 software (San Diego, CA). Grayscale value was analyzed by ImageJ software (National Institutes of Health, USA).

RESULTS

Concentration of *EtMIC4* EGF-Like Protein to Promote Cell Proliferation

The cells were treated with *EtMIC4* EGF-like protein for 4 and 24 h. The cell activity increased with increasing protein concentration and reached the peak when the protein concentration was 0.1 $\mu\text{g}/\text{mL}$. The proliferation rates were 11.37% and 18.09% at 4 and 24 h, respectively, which were significantly ($P < 0.01$) higher than those in the blank control group. Subsequently, the cell

proliferation rate decreased. In the cells treated with the *EtMIC4* EGF-like protein for 48 h, the cell proliferation rate was lower than that in the 24 h group. When the protein concentration was 0.1 $\mu\text{g}/\text{mL}$, the cell proliferation rate in each group was not significantly different from that in the EGF group ($P > 0.05$) (Figure 1). According to the results of MTT, we selected the conditions of *EtMIC4* EGF-like protein concentration of 0.1 $\mu\text{g}/\text{mL}$ for 48 h of treatment for subsequent experiments.

The *E. tenella* Infection Rate of Chick Embryo Cecal Epithelial Cells was Increased due to the *EtMIC4* EGF-like Protein

At 4 and 24 h after sporozoite inoculation, the infection rate in the *E. tenella* group was significantly higher than that in the siRNA *EGFR* + *E. tenella* group ($P < 0.01$) and that in the *E. tenella* + *EtMIC4* EGF-like group was significantly higher than that in the *E. tenella* group ($P < 0.05$) and the siRNA *EGFR* + *E. tenella* + *EtMIC4* EGF-like group ($P < 0.01$). After sporozoite inoculation for 96 h, the infection rate in the *E. tenella* group was not significantly different from that in the siRNA *EGFR* + *E. tenella* group ($P > 0.05$) and that in the *E. tenella* + *EtMIC4* EGF-like group was significantly higher than that in the siRNA *EGFR* + *E. tenella* group ($P < 0.05$) and the siRNA *EGFR* + *E. tenella* + *EtMIC4* EGF-Like group ($P < 0.05$) (Figure 2). Based on the results, the infection rate of *E. tenella* host cells decreased after *EGFR* silencing and increased upon the addition of the *EtMIC4* EGF-like protein.

The Early Apoptosis Rate of Chick Embryo Cecal Epithelial Cells was Reduced by the *EtMIC4* EGF-like Protein

The early apoptosis rate of host cells in the *EtMIC4* EGF-like group was significantly lower than that in the

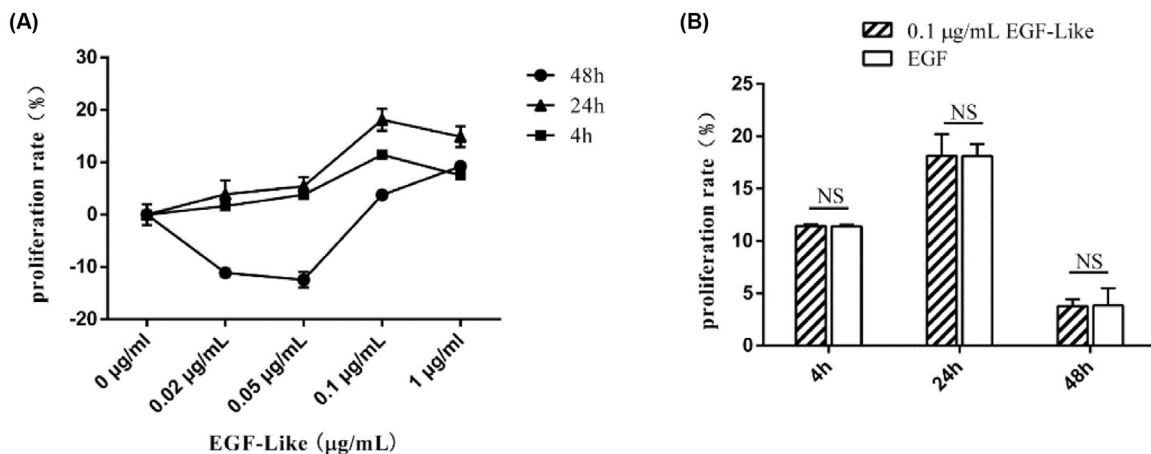


Figure 1. *EtMIC4* EGF-Like recombinant protein concentration on cell proliferation. (A) Effects of different *EtMIC4* EGF-Like recombinant protein concentrations on cell proliferation for 4, 24, and 48 h, respectively. (B) The *EtMIC4* EGF-Like recombinant protein concentration at 0.1 $\mu\text{g}/\text{mL}$ was consistent with the effect of 0.02 $\mu\text{g}/\text{mL}$ EGF.

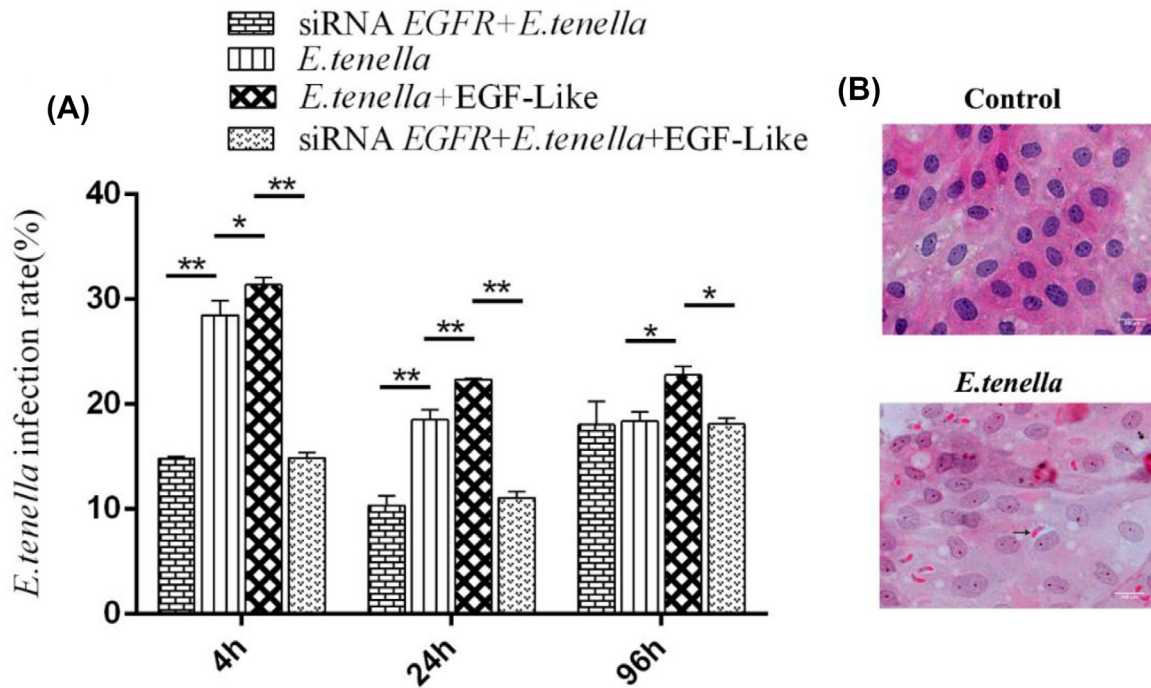


Figure 2. *E. tenella* infection of host cells. (A) Infection rate of cells at 4, 24, and 96 h after inoculation with *E. tenella*. "*" indicates $P < 0.05$; "**" indicates $P < 0.01$. The same as the figure below. (B) HE staining of cells. The arrow points to the sporozoite. Scale bar = 200 μ m. The same as the figure below.

control group ($P < 0.01$) and the siRNA *EGFR* + *EtMIC4* EGF-Like group at 4 to 96 h ($P < 0.01$). The early apoptosis rate in the siRNA *EGFR* + *EtMIC4* EGF-like group was significantly higher than that in the control group ($P < 0.01$). Hence, early apoptosis was inhibited by the *EtMIC4* EGF-like protein and promoted by *EGFR* silencing.

After 4 h of inoculation with sporozoites, the early apoptosis rate of host cells in the *E. tenella* group was significantly lower than that in the control group ($P < 0.01$) and the siRNA *EGFR* + *E. tenella* group ($P < 0.05$). The early apoptosis rate in the *E. tenella* + *EtMIC4* EGF-like group was significantly lower than that in the *E. tenella* group ($P < 0.01$) and the siRNA *EGFR* + *E. tenella* + *EtMIC4* EGF-like group ($P < 0.01$). At 24 and 96 h after inoculation with sporozoites, the early apoptosis rate of host cells in the *E. tenella* group was significantly lower than that in the siRNA *EGFR* + *E. tenella* group ($P < 0.05$) and significantly higher than that in the control group ($P < 0.05$, $P < 0.01$). The early apoptosis rate of host cells in the *E. tenella* + *EtMIC4* EGF-like group was significantly lower than that in the *E. tenella* group ($P < 0.01$) and the siRNA *EGFR* + *E. tenella* + *EtMIC4* EGF-like group ($P < 0.01$) but was not different from that in the control group ($P > 0.05$) (Figure 3 a1-2, c, d, e1). We found that the early apoptosis rate was reduced by the inoculation of sporozoites for 4 h and increased by the inoculation for 24 to 96 h. After the inoculation of sporozoites for 4 to 96 h, the early apoptosis rate was reduced by the addition of the *EtMIC4* EGF-like protein and increased by *EGFR* silencing.

The Late Apoptosis and Necrosis Rates of Chick Embryo Cecal Epithelial Cells Were Also Reduced by the *EtMIC4* EGF-like Protein

The late apoptosis and necrosis rates of host cells in the *EtMIC4* EGF-Like group were significantly lower than those in the control group ($P < 0.01$) and the siRNA *EGFR* + *EtMIC4* EGF-like group at 4 to 96 h ($P < 0.01$). The rates in the siRNA *EGFR* + *EtMIC4* EGF-Like group were significantly higher than those in the control group ($P < 0.01$). The results of the late apoptosis and necrosis were consistent with the early apoptosis.

After 4 h of inoculation with sporozoites, the late apoptosis and necrosis rates of host cells in the *E. tenella* group were significantly lower than those in the control group ($P < 0.01$) and the siRNA *EGFR* + *E. tenella* group ($P < 0.05$). The rates in the *E. tenella* + *EtMIC4* EGF-like group were significantly lower than those in the *E. tenella* group ($P < 0.01$) and the siRNA *EGFR* + *E. tenella* + *EtMIC4* EGF-like group ($P < 0.01$). At 24 and 96 h after inoculation with sporozoites, the late apoptosis and necrosis rates of host cells in the *E. tenella* group were significantly lower than those in the siRNA *EGFR* + *E. tenella* group ($P < 0.05$) and significantly higher than those in the control group ($P < 0.05$, $P < 0.01$). The early apoptosis rate of host cells in the *E. tenella* + *EtMIC4* EGF-like group was significantly lower than that in the *E. tenella* group ($P < 0.01$) and the siRNA *EGFR* + *E. tenella* + *EtMIC4* EGF-like group ($P < 0.01$) but was not different from that in the control group ($P > 0.05$) (Fig. 3 b1-2, c, d, e2). The results were similar to the early apoptosis.

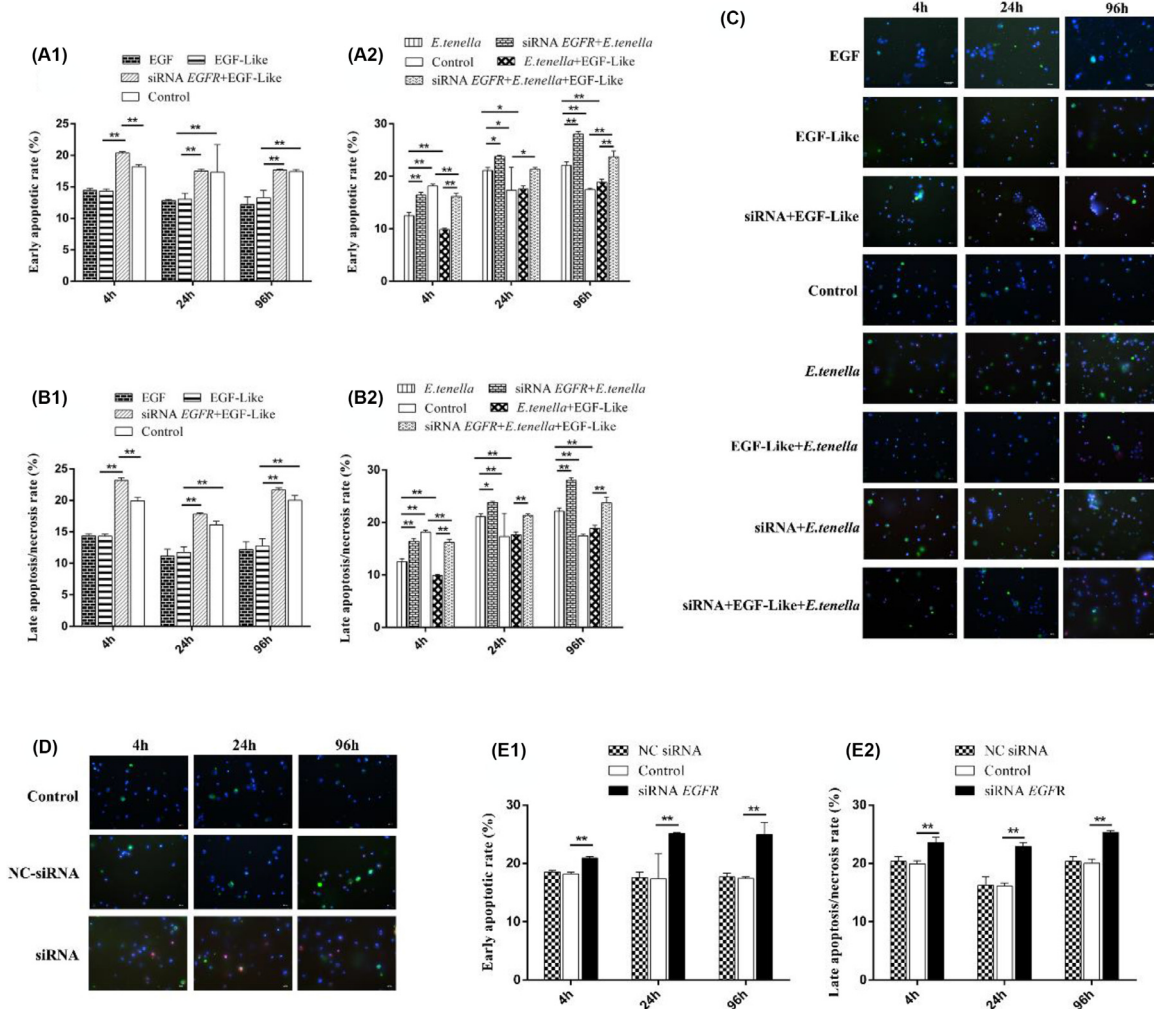


Figure 3. Apoptosis rate of chick embryo cecal epithelial cells. (A1) Early apoptosis rate of cells added with *EtMIC4* EGF-Like recombinant protein. (A2) Early apoptosis rate of cells with *E. tenella* or both *EtMIC4* EGF-Like recombinant protein and *E. tenella*. (B1) Late apoptosis and necrosis rate cells with *EtMIC4* EGF-Like recombinant protein. (B2) Late apoptosis and necrosis rate of cells with *E. tenella* or both *EtMIC4* EGF-Like recombinant protein and *E. tenella*. (C,D) All cell nuclei were stained by Hoechst 33342 (blue fluorescence); normal cells were low in AnnexinV-FITC (green fluorescence) and PI (red fluorescence) (Annexin V+/PI+); early apoptotic cells were highly stained by AnnexinV-FITC and low in PI (Annexin V++/PI+); late apoptosis and necrosis cells were highly stained with AnnexinV-FITC and PI (Annexin V++/PI++). (e1, e2) The apoptosis rate of cells in EGFR-specific siRNA control group.

Dynamic Changes in the Gene Expression of Key Factors in the EGFR Pathway

After treatment with the *EtMIC4* EGF-like protein for 4 h, the relative expression levels of *EGFR*, *AKT*, and *ERK* mRNA in the host cells in the *EtMIC4* EGF-like group were significantly higher than those in control group ($P < 0.01$) and the siRNA *EGFR* + *EtMIC4* EGF-like group ($P < 0.01$). The levels in the siRNA *EGFR* + *EtMIC4* EGF-like group were significantly lower than those in the control group ($P < 0.01$). After 4 h of inoculation with sporozoites, the relative expression levels of *EGFR*, *AKT*, and *ERK* mRNA in the host cells in the *E. tenella* group were significantly higher than those in the control group ($P < 0.01$) and the siRNA *EGFR* + *E. tenella* group ($P < 0.01$). The levels in the *E. tenella* + *EtMIC4* EGF-like group were significantly higher than those in the *E. tenella* group ($P < 0.01$) and the siRNA *EGFR* + *E. tenella* + *EtMIC4* EGF-like group ($P < 0.01$) (Figure 4 a1-3).

The results of the addition of the *EtMIC4* EGF-like protein for 24 h were consistent with the test results after 4 h. After 24 h of inoculation with sporozoites, the relative expression levels of *EGFR*, *AKT*, and *ERK* mRNA in the host cells in the *E. tenella* group were significantly higher than those in the siRNA *EGFR* + *E. tenella* group ($P < 0.01$) but were not different from those in the control group ($P > 0.05$). The levels in the *E. tenella* + *EtMIC4* EGF-like group were significantly higher than those in the *E. tenella* group ($P < 0.01$), the control group ($P < 0.01$), and the siRNA *EGFR* + *E. tenella* + *EtMIC4* EGF-like group ($P < 0.01$) (Figure 4 b1-3).

The same experimental results were obtained in the *EtMIC4* EGF-like group after 4, 24, and 96 h. After inoculation with *E. tenella* for 96 h, the relative expression levels of *EGFR*, *AKT*, and *ERK* mRNA in the host cells in the *E. tenella* group were significantly lower than those in the control group ($P < 0.01$). The relative expression levels of *EGFR* mRNA was significantly

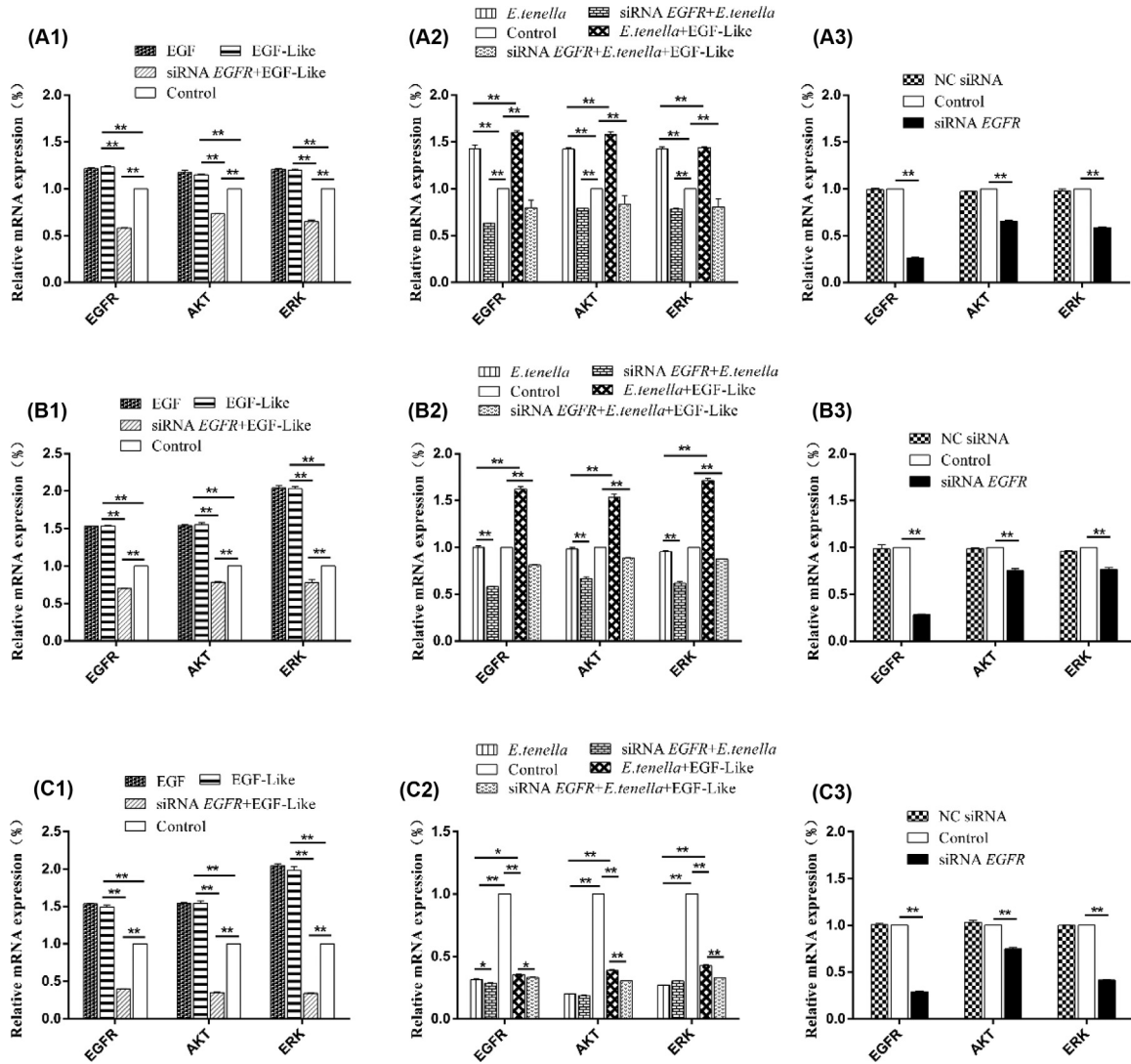


Figure 4. Dynamic changes of gene expression of key factors in the EGFR pathway. (A1-3) The relative expression levels of *EGFR*, *AKT* and *ERK* mRNA in host cells for 4 h. (B2-3) The relative expression levels of *EGFR*, *AKT* and *ERK* mRNA in host cells for 24 h. (C1-3) The relative expression levels of *EGFR*, *AKT* and *ERK* mRNA in host cells for 96 h.

higher than those in the siRNA *EGFR* + *E. tenella* group ($P < 0.05$). The relative expression levels of *EGFR*, *AKT*, and *ERK* mRNA in the *E. tenella* + *EtMIC4* EGF-like group were significantly higher than those in the *E. tenella* group ($P < 0.01$) and the siRNA *EGFR* + *E. tenella* + *EtMIC4* EGF-like ($P < 0.01$) group and significantly lower than those in the control group ($P < 0.01$) (Figure 4 c1-3). These findings demonstrated that *E. tenella* and *EtMIC4* EGF-like protein influenced the relative mRNA expression of *EGFR*, *AKT*, and *ERK*.

Dynamic Changes in the Protein Expression of key Factors in the EGFR Pathway

After treatment with *EtMIC4* EGF-like for 4h, in the *EtMIC4* EGF-like group, the phosphorylation levels of EGFR, AKT, and ERK proteins in the host cells were significantly higher than those in the control group ($P < 0.01$) and the siRNA *EGFR* + *EtMIC4* EGF-like group

($P < 0.01$). The levels in the siRNA *EGFR* + *EtMIC4* EGF-like group were significantly lower than those in the control group ($P < 0.01$). After 4 h of inoculation with sporozoites, the phosphorylation levels of EGFR, AKT, and ERK proteins in the host cells in the *E. tenella* group were significantly higher than those in the control group ($P < 0.01$) and the siRNA *EGFR* + *E. tenella* group ($P < 0.01$). The levels in the *E. tenella* + *EtMIC4* EGF-like group were significantly higher than those in the *E. tenella* group ($P < 0.01$) and the siRNA *EGFR* + *E. tenella* + *EtMIC4* EGF-Like group ($P < 0.01$) (Figure 5).

The results of adding *EtMIC4* EGF-like protein for 24 h were consistent with the test results after 4 h. After 24 h of inoculation with sporozoites, the phosphorylation levels of EGFR, AKT, and ERK proteins in the host cells in the *E. tenella* group were significantly higher than those in the siRNA *EGFR* + *E. tenella* group ($P < 0.01$) and significantly lower than those in the control group ($P < 0.01$). The levels in the *E. tenella* + *EtMIC4* EGF-like group were significantly higher than those in

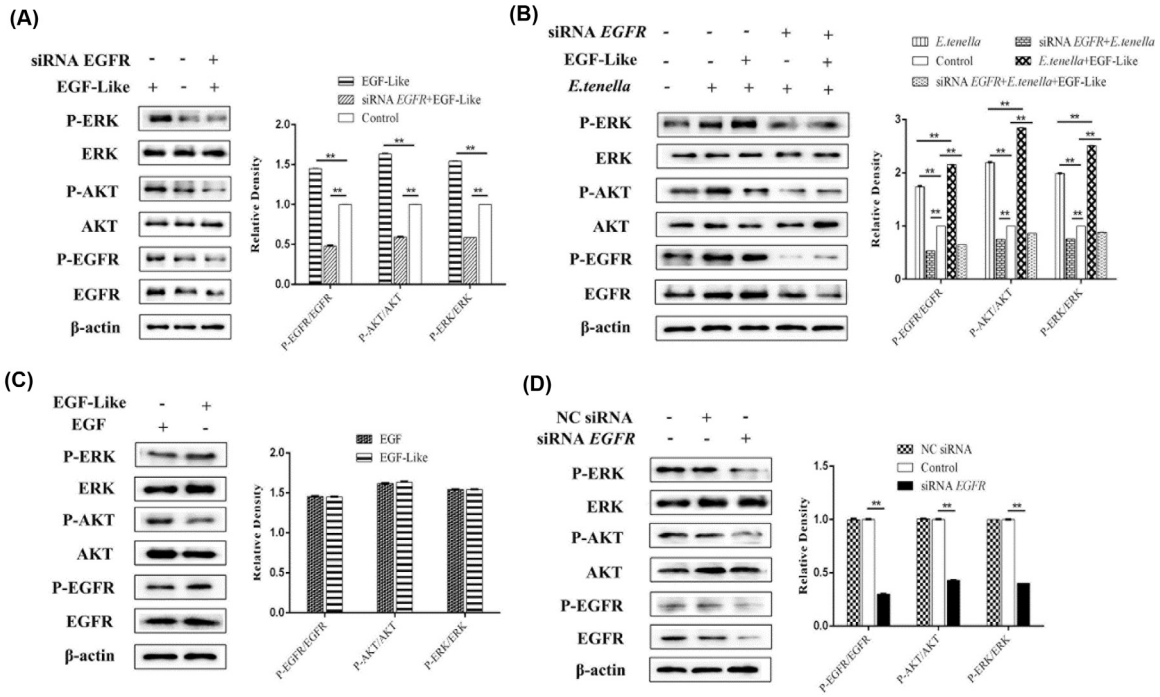


Figure 5. Dynamic changes of protein expression of key factors in the EGFR pathway for 4 h. (A) The phosphorylation levels of EGFR, AKT and ERK proteins in host cells with *E.t*MIC4 EGF-Like recombinant protein. (B) The phosphorylation levels of EGFR, AKT and ERK proteins in host cells with *E. tenella* or both *E.t*MIC4 EGF-Like recombinant protein and *E. tenella*. (C) The phosphorylation levels of EGFR, AKT and ERK proteins in host cells with EGF or *E.t*MIC4 EGF-Like recombinant protein. (D) The phosphorylation levels of EGFR, AKT and ERK proteins of cells in EGFR-specific siRNA control group.

the *E. tenella* group ($P < 0.01$), the control group ($P < 0.01$), and the siRNA *EGFR* + *E. tenella* + *E.t*MIC4 EGF-like group ($P < 0.01$) (Figure 6).

The experimental results were the same in the groups added with the *E.t*MIC4 EGF-like protein for 4, 24, and

96 h. After inoculation with *E. tenella* for 96 h, the phosphorylation levels of EGFR, AKT, and ERK proteins in the host cells in the *E. tenella* group were significantly lower than those in the control group ($P < 0.01$) and significantly higher than those in the siRNA *EGFR* + *E.*

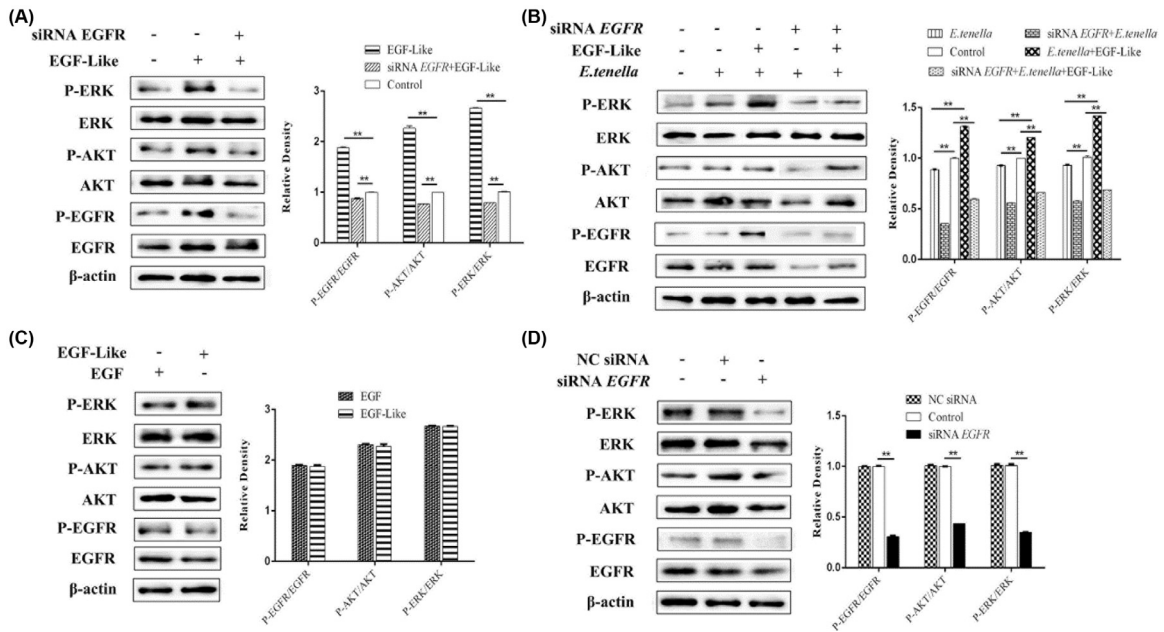


Figure 6. Dynamic changes of protein expression of key factors in the EGFR pathway for 24 h. (A) The phosphorylation levels of EGFR, AKT and ERK proteins in host cells with *E.t*MIC4 EGF-Like recombinant protein. (B) The phosphorylation levels of EGFR, AKT and ERK proteins in host cells with *E. tenella* or both *E.t*MIC4 EGF-Like recombinant protein and *E. tenella*. (C) The phosphorylation levels of EGFR, AKT and ERK proteins in host cells with EGF or *E.t*MIC4 EGF-Like recombinant protein. (D) The phosphorylation levels of EGFR, AKT and ERK proteins of cells in EGFR-specific siRNA control group.

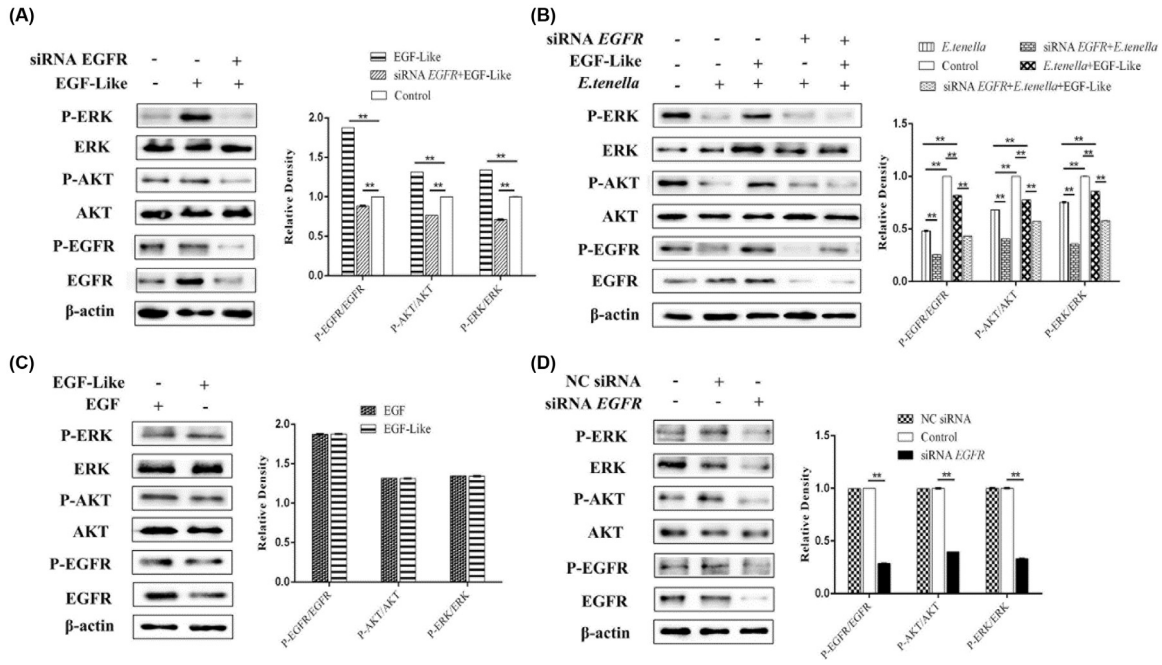


Figure 7. Dynamic changes of protein expression of key factors in the EGFR pathway for 96 h. (A) The phosphorylation levels of EGFR, AKT and ERK proteins in host cells with *EtMIC4* EGF-Like recombinant protein. (B) The phosphorylation levels of EGFR, AKT and ERK proteins in host cells with *E. tenella* or both *EtMIC4* EGF-Like recombinant protein and *E. tenella*. (C) The phosphorylation levels of EGFR, AKT and ERK proteins in host cells with EGF or *EtMIC4* EGF-Like recombinant protein. (D) The phosphorylation levels of EGFR, AKT and ERK proteins of cells in EGFR-specific siRNA control group.

tenella group ($P < 0.01$). The levels in the *E. tenella* + *EtMIC4* EGF-like group were significantly higher than those in the *E. tenella* group ($P < 0.01$) and the siRNA *EGFR* + *E. tenella* + *EtMIC4* EGF-like group ($P < 0.01$) but significantly lower than those in the control group ($P < 0.01$) (Figure 7). Western blot detection revealed that the protein phosphorylation of EGFR, AKT, and ERK was activated by *E. tenella* and *EtMIC4* EGF-like protein.

DISCUSSION

The infection rate in the *E. tenella* group was significantly higher than that in the siRNA *EGFR* + *E. tenella* group after sporozoite inoculation for 4 to 24 h. The infection rate in the *E. tenella* + *EtMIC4* EGF-like group was significantly higher than that in the *E. tenella* group and the siRNA *EGFR* + *E. tenella* + *EtMIC4* EGF-like group after sporozoite inoculation for 4 to 96 h. We confirmed that the *EtMIC4* EGF-like protein could increase the infection rate of *E. tenella* through the EGFR signaling pathway. Jin Xiaoxia's research has proved that siRNA interferes with *EGFR* expression, which inhibiting the cell infection of *N. caninum*, and the addition of EGF-like recombinant protein could improve the infection rate of *Neospora* (Jin, 2017). Corcino et al. demonstrated that transgenic mice that inhibited EGFR signaling had reduced parasitic infection rates in the brain and retina following *T. gondii* infection (Corcino et al., 2019). Yang et al. used gefitinib to target inhibition *EGFR* and significantly reduced the infection

and proliferation of *T. gondii* in cells (Yang, et al., 2014).

EtMIC4 EGF-like treated for 4 to 24 h, the results of Hoechst/Annexin V-FITC/PI immunofluorescence triple staining assay demonstrated that apoptosis of host cells could be inhibited by *EtMIC4* EGF-like through EGFR signaling pathway. After inoculation with sporozoites for 4 h, the early apoptosis rate and late apoptosis / necrosis rate of host cells in *E. tenella* group were significantly lower than those in control group and siRNA *EGFR* + *E. tenella* group; and the early apoptosis rate and late apoptosis/necrosis rate of host cells in *E. tenella* + *EtMIC4* EGF-like group were significantly lower than those in *E. tenella* group and siRNA *EGFR* + *E. tenella* + *EtMIC4* EGF-like group. Inoculated with sporozoites for 24 h to 96 h, the early apoptosis rate and late apoptosis/necrosis rate of host cells in the *E. tenella* group were significantly lower than those in siRNA *EGFR* + *E. tenella* group, and significantly higher than those in control group. We demonstrated that the apoptosis of host cells caused by *E. tenella* infection would be inhibited by *EtMIC4* EGF-like through the EGFR pathway. Jin also proved that the apoptosis rate of *N. caninum* infection at the early stage (the first 12 h) was not significantly different from that of the negative control group, but the apoptosis rate increased significantly 24 h after *N. caninum* infection (Jin, 2017). The previous experiments of our team also certified that *E. tenella* inhibited the apoptosis of host cells in the early stage of infection, which was conducive to the growth and reproduction of parasites. However, in the middle and late stages of infection, the apoptosis of host cells was promoted (Xu et al., 2016; Xu et al., 2017;

Zhang et al., 2019; Zhang et al., 2020; Xu et al., 2021). The EGF-like region of *EtMIC4* of *E. tenella* is similar to EGF in the host cell and can bind to EGFR in chicken cells to regulate cell proliferation, apoptosis, and vital movement (Yang, 2014).

The binding of EGF to EGFR induces the activation of the latter. The activation of EGFR leads to the autophosphorylation of tyrosine in the intracellular domain and thus has kinase activity. After EGFR binds to the ligand, the mechanism was by activating intracellular phosphatase C-y, phosphatidylinositol-3 kinase and ras GTPase activating protein, then catalyzes the corresponding substrates, which produce secondary messengers of diacylglycerol (DAG), inositol triphosphate (IP3), and phosphorylated ras, respectively, thereby regulating cell growth metabolism (Ullrich and Schlessinger, 1990).

After *EtMIC4* EGF-like treatment for 4 to 24 h, the relative mRNA expression and protein phosphorylation levels of *EGFR*, *AKT*, and *ERK* genes in the host cells in the *EtMIC4* EGF-Like group were significantly higher than those in the control group and the siRNA *EGFR* + *EtMIC4* EGF-Like group; the levels in the siRNA *EGFR* + *EtMIC4* EGF-Like group were significantly lower than those in the control group. The experiment results suggested that *EtMIC4* EGF-like protein could up-regulate the relative mRNA expression and protein phosphorylation of *AKT* and *ERK* by up-regulating the relative mRNA expression and protein phosphorylation of *EGFR* in host cells. After the inoculation of sporozoites for 4 h, the relative mRNA expression and protein phosphorylation levels of *EGFR*, *AKT*, and *ERK* in the *E. tenella* group were significantly higher than those in the control group, and the levels in the *E. tenella* + *EtMIC4* EGF-like group were significantly higher than those in the *E. tenella* group. After silencing *EGFR*, the relative mRNA expression and protein phosphorylation levels of *EGFR*, *AKT* and *ERK* in siRNA *EGFR* + *E. tenella* group and siRNA *EGFR* + *E. tenella* + *EtMIC4* EGF-like were significantly lower than those in the control group. After inoculation with *E. tenella* for 96 h, the relative mRNA expression and protein phosphorylation levels of *EGFR*, *AKT*, and *ERK* in the *E. tenella* group and the *E. tenella* + *EtMIC4* EGF-like group were significantly lower than those in the control group; the levels in the siRNA *EGFR* + *E. tenella* group and the siRNA *EGFR* + *E. tenella* + *EtMIC4* EGF-like group were significantly lower than those in the *E. tenella* group and the *E. tenella* + *EtMIC4* EGF-like group. The results indicated that *E. tenella* and *EtMIC4* EGF-like protein could affect the expression and protein phosphorylation of *EGFR*, thereby affecting the expression and protein phosphorylation of *AKT* and *ERK*. The studies have shown that EGF-MICs of *T. gondii* play a role in mediating EGFR-Akt activation, and the addition of recombinant EGF-MICs (MIC3 and MIC6) induced EGFR-Akt activation; meanwhile, MICs lacking the EGF region did not cause significant EGFR and Akt activation (Muniz-Feliciano et al., 2013). EGF can reduce the infection rate of *Cryptosporidium andersoni* and inhibit

its mediated apoptosis (Buret et al., 2003). EGFR is a glycoprotein that belongs to a family of tyrosine kinase-type receptors. It consists of an extracellular ligand binding region, a single-chain transmembrane region with a single hydrophobic anchor sequence, and a highly conserved intracellular region with tyrosine kinase activity. Ligands bound to the extracellular region can cause a conformational change in the kinase region that activates EGFR via the trans-autophosphorylation of tyrosine residues in the carboxy-terminal tail (Endang et al., 2017). These phosphorylated residues recruit signaling molecules downstream of EGFR, such as PI3K/AKT and Ras/Raf/MEK/ERK (Hartman et al., 2020). After autophosphorylation, EGFR directly binds or acts indirectly on the GRB2 complex to activate the Ras protein. The activated Ras activates the downstream serine/threonine protein kinase, and Raf can phosphorylates ERK-1/2 (Chen et al., 2021).

Collectively, our results confirmed that *E. tenella* could activate the EGFR signal pathway in host cells through *EtMIC4* EGF-like protein and regulates the expression of key factors in its downstream AKT and ERK signaling pathways, thereby inhibiting cell apoptosis and increasing the infection rate of *E. tenella*. After using siRNA to block the EGFR pathway, the inhibition of apoptosis by *E. tenella* was significantly reduced, and the infection rate of *E. tenella* was also reduced. Hence, *E. tenella* could inhibit the apoptosis of its host cells by activating the EGFR signal pathway, thereby increasing the infection rate of the host cells.

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DISCLOSURES

Xue-song Zhang carried out most of the experiments, wrote the manuscript, and should be considered as primary author. Ming-xue Zheng critically revised the manuscript and the experiment design. Yong-juan Zhao, Yu Zhang, Tong Xu, Kai-ling Cui, Bu-ting Duan, Xiao-ling Lv, Li Zhang, Zhi-yong Xu and Rui Bai helped with the experiment. All the authors read and approved the final version of the manuscript.

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