Ezrin/NF-κB Pathway Regulates EGF-induced Epithelial-Mesenchymal Transition (EMT), Metastasis, and Progression of Osteosarcoma

Background: Epithelial-mesenchymal transition (EMT) is responsible for metastasis of cancers, and NF-κB can promote tumor progression. Ezrin is an important molecule participating in EMT. However, whether Ezrin mediates NF-κB in EGF-induced osteosarcoma is unknown.

Material/Methods: Ezrin phosphorylation, NF-κB activation, and EGF-induced EMT were studied in MG63 and U20S cells with NF-κB inhibition, silencing, or over-expressing Ezrin. Cell morphology, proliferation, migration, and motility were analyzed. An osteosarcoma model was established in mice by injecting MG63 and U20S and reducing Ezrin.

Results: With EGF induction in vitro, Ezrin Tyr353 and Thr567 were phosphorylated, and EMT, proliferation, migration, and motility of osteosarcoma cells were promoted. Silencing Ezrin suppressed and over-expressing Ezrin promoted the nuclear translocation of p65 and phosphorylated IκBα (p-IκBα) in EGF-induced osteosarcoma cells. NF-κB inhibitor blocked EGF-induced EMT in both cell types, as well as reserving cell morphology and suppressing proliferation, migration, and motility. In vivo, reducing Ezrin significantly suppressed metastasis of osteosarcoma xenografts, increased liver and lung weights, and activated NF-κB, which were both induced by EGF.

Conclusions: Ezrin/NF-κB regulated EGF-induced EMT, as well as progression and metastasis of osteosarcoma in vivo and in vitro. Ezrin/NF-κB may be a new therapeutic target to prevent osteosarcoma from deterioration.

MeSH Keywords: Disease Progression • Epithelial-Mesenchymal Transition • Lymphatic Metastasis • Neurofibromin 2 • Osteosarcoma • Receptor Activator of Nuclear Factor-kappa B

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Background

Osteosarcoma is one of the most common malignant cancers, and is the most common primary malignant neoplasm of bone, with rapid progression and poor prognosis clinically [1,2]. Surgery is the primary treatment for osteosarcoma, and multi-agent chemotherapy and postoperative chemotherapy are adjuvant therapies [3].

Previous studies demonstrated that tumor metastasis is associated with EMT as the biological process in which epithelial cells lose cell-cell contact, with enhanced E-cadherin expression, reduced vimentin, and developing mesenchymal properties, leading to promotion of proliferation, invasion, and viability [4,5]. During the pathogenesis and progression of malignant tumors, many signaling pathways are involved as the regulators triggered by various members of the transforming growth factor-β (TGF-β) superfamily [4,6]. NF-κB is one of the transcription factors demonstrated to suppress EMT and metastasis by blocking its activation and suppressing the nuclear translocation of p65 [7,8]. In addition, activation of NF-κB is important in transmission of signals in responses to extracellular factors [13,14]. Ezrin has many functions in tumor metastasis, including modulating the formation of microvilli [16], maintaining cell shape [17], cell-cell adhesion [18], cell motility [19], and invasion [20]. EGF can stimulate Ezrin activation in tumor cells, which can result in tumor metastasis [17].

According to Gavert et al. [21], NF-κB and Ezrin are both essential for L1-mediated metastasis of colon cancer cells. Lim et al. [22] suggested that NF-κB activity is responsible for Ezrin-induced actin polymerization. Tang et al. [23] found that Ezrin interacts with p65 in breast cancer cells, and could be a promoter in tumor metastasis and proliferation. Increasing evidence suggests that Ezrin is important in EMT by activating NF-κB. However, whether Ezrin can mediate NF-κB activation in EGF-induced osteosarcoma cells is still obscure.

In the present study, we investigated the role of Ezrin in regulating EGF-induced EMT in MG63 and U2OS cell lines in vitro. Tumor xenografts were constructed from mouse models of osteosarcoma. The phosphorylation of Ezrin in response to EGF and the effect on the activation of NF-κB were studied to understand the role of Ezrin/NF-κB regulation in EMT and metastasis of osteosarcoma.

Material and Methods

Cell culture and EGF induction

Human osteosarcoma cell lines MG63 and U205 were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). MG63 and U205 were cultured in Dulbecco’s minimum essential medium (DMEM, Thermo Fisher Scientific, Inc. Shanghai, China) containing 10% fetal bovine serum (FBS, Thermo), 100 U/ml penicillin, and 1% streptomycin (Sigma Aldrich, Co. LLC, Shanghai, China). All the cells were maintained at 37°C, 5% CO₂, and saturated humidity. When they reached 90% confluence, the cells were digested with 0.25% trypsin-EDTA (Thermo) and sub-cultured (1:3).

For inhibiting NF-κB, inhibitor of cytokine-induced IκBα phosphorylation (BAY 11-7082, 10μM, Sigma) was used to treat the 90% confluent cells. For EGF induction, 90% confluent cells were starved for 4 h and then stimulated by EGF (25 nM) for 30 min.

Transfection

Small interfering RNA (siRNA) silencing Ezrin (5’-GUG GGA UGC UCA AAG AUA ATT-3’) was designed and synthesized by GenePharma (Shanghai, China). An expression construction was a kind gift from Prof. Sanbao Hu. Lipofectamine® 3000 transfection reagents (Thermo) was used to transfect cells with Opti-MEM™ reduced serum media (Thermo) without penicillin- streptomycin for 4 h, according to the manufacturer’s specifications.

Western blotting (WB)

Proteins were extracted from cell lysate with nuclear and cytoplasmic protein extraction kit (Beyotime Biotechnology, Shanghai, China), and then quantified with BCA protein assay kit (Beyotime). Proteins were analyzed by 10% sodium dodecyl sulfate (SDS) polyacrylamide gel and 5% stacking gel, and then transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millpore Corporation, Shanghai, China). Protein bands were incubated with E-cadherin (1: 1000, Proteintech Group, Inc. Wuhan, China), vimentin (1: 1000, Proteintech), Ezrin (1: 1000, Proteintech), p-Ezrin Tyr353 (1: 1000, Abcam, Shanghai, China), p-Ezrin Thr567 (1: 1000, Abcam), NF-κB p65 (1: 3000, Abcam), p-IκBα Ser32/36 (1: 1000, CST), and GAPDH (1: 10000, Proteintech) primary antibodies at 4°C overnight, and then incubated with goat anti-rabbit and goat anti-mouse secondary antibodies (1: 3000, Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA) at 25°C for 1 h. An electrochemiluminescence (ECL, Millipore) system was used to expose proteins.
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Proliferation assay

Cells were cultured in a 96-well plate (Corning, New York, USA) for 72 h. We used the Cell Counting Kit-8 (CCK-8) liquid (Thermo) and incubated cells for 20 min. Optical density (OD) value was determined by use of a Synergy NEO Microplate Spectrophotometer (BioTek instruments, USA).

Transwell assay

A total of 1×10⁵ cells were cultured in the upper chamber of the polycarbonate Transwell filter chamber (Corning) containing Matrigel (Becton, Dickinson and Company, Shanghai, China) and incubated for 16 h. Cells on the lower surface of the chamber were fixed with 4% paraformaldehyde and stained with 0.25% crystal violet (Solarbio Technology Co., Ltd, Beijing, China). Cells on the upper surface were cleaned. Cells were observed and counted in 5 random 100× fields per well using a microscope (Olympus Corporation, Beijing, China).

Wound-healing assay

A total of 1×10⁶ cells were cultured in a 6-well plate (Corning) until confluence reached 90%. Linear wounds were created with pipette tips and the cells were cultured without serum. Cells were cultured for 10 h and we observed the cells under a microscope (Olympus).

Mouse models of osteosarcoma

From Shanghai Jia Ke Biotechnology Co., Ltd. (China), we obtained 40 male BALB/c-nu mice, age 4 to 6 weeks, weight 14–20 g, and used them to construct an osteosarcoma model (n=5 each group). Mice were kept in a specific pathogen-free (SPF) animal room maintained at 25°C and 60~70% humidity. Mice ate and drank freely. Animal experiment protocols were according to guidelines of Ethical Issues in Animal Experimentation [24] and were approved by the Ethics Committee of Beijing Anzhen Hospital. MG63 and U20S cells and then detected E-cadherin and vimentin. To investigate the effects of EGF on proliferation, migration, and motility of osteosarcoma cells, we used EGF to treat MG63 and U20S cell lines and then detected Ezrin Thr567, Ezrin Tyr353, E-cadherin, and vimentin.

Results

EGF promoted Ezrin phosphorylation and EMT, while silencing Ezrin suppressed EGF-induced EMT in osteosarcoma cells

As shown in Figure 1, with EGF induction, Ezrin Tyr353 and Ezrin Thr567 were both significantly (P<0.05) elevated in MG63 and U20S cells compared to control. These results indicated the promotion of EGF on Ezrin phosphorylation. In addition, E-cadherin decreased with EGF while vimentin significantly (P<0.05) increased in MG63 and U20S cells comparing to the control, which suggested that EMT of osteosarcoma cells was promoted by EGF induction.

EGF induced morphological change and promoted proliferation, migration, and motility of osteosarcoma cells

To investigate the effects of Ezrin on EMT, we silenced Ezrin in MG63 and U20S cell lines and then detected E-cadherin and vimentin. By silencing Ezrin, on the one hand, Ezrin Tyr353 and Ezrin Thr567 both significantly (P<0.05) decreased in MG63 and U20S cells compared to the EGF treatment. On the other hand, E-cadherin significantly (P<0.05) increased while vimentin decreased in MG63 and U20S cells compared to the EGF treatment.

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Statistical analysis

All data analyses were performed using SPSS 21.0 software. Data are expressed as mean ± standard deviations (X±SDs). All experiments were performed at least in triplicate. One-way ANOVA or t test were used to analyze differences among groups. Levene test was used for homogeneity test of variance, and the LDS was performed for homogenous data, while Dunnett’s T3 was performed for heterogeneous data. P<0.05 was considered a significant difference.

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Figure 1. EGF promoted Ezrin phosphorylation and EMT, and silencing Ezrin suppressed EGF-induced EMT in osteosarcoma cells. GAPDH was used as internal control (* compared to the Con., \( P<0.05 \); # compared to EGF, \( P<0.05 \)).

Figure 2. EGF induced morphological change and promoted proliferation, migration, and motility of osteosarcoma cells (* comparing to the Con., \( P<0.05 \)).
As shown in Figure 2 and Table 1, without EGF induction, MG63 and U20S were round in shape with tight cell-cell junctions, whereas both of them induced with EGF displayed spindle shape and were separated from one another (Figure 2-Morphology).

Without EGF induction, CCK-8 assay demonstrated that the proliferation of MG63 and U20S cells were significantly (P<0.05) lower than those with EGF induction (Figure 2-Proliferation). Transwell assay demonstrated that the invasive ability of MG63 and U20S cells with EGF stimulation were significantly (P<0.05) enhanced, appearing as more cells on the lower surface of the membrane, comparing to that without EGF stimulation (Figure 2-Migration). With EGF treatment, the motility of MG63 and U20S cells was significantly (P<0.05) strengthened compared to those without EGF treatment (Figure 2-Motility). These data suggest that the proliferation, migration, and motility of osteosarcoma cells were promoted by EGF induction.

**Ezrin was responsible for NF-κB activity in osteosarcoma cells**

To investigate the role of Ezrin in regulating NF-κB activity in osteosarcoma, we silenced Ezrin by RNA interfering (RNAi) assay or over-expressed Ezrin with expression construction, both with EGF induction, in MG63 and U20S cell lines. The protein levels of nuclear p65 and p-IκBα were detected.

As shown in Figure 3, WB assay showed that with EGF treatment, nuclear p65 and p-IκBα levels were both significantly (P<0.05) suppressed compared to EGF stimulation only. With over-expressing Ezrin and EGF induction, nuclear p65 and p-IκBα levels were both significantly (P<0.05) promoted compared to the control, but without significance compared to EGF induction. These data indicate that Ezrin was responsible for translocation of nuclear p65, suggesting the activation of NF-κB in EGF-induced osteosarcoma cells.

**EGF induced EMT by Ezrin/NF-κB in osteosarcoma cells**

To study the role of Ezrin/NF-κB in EMT in osteosarcoma, we used IκBα phosphorylation inhibitor (BAY) to suppress NF-κB activity, as well as silencing Ezrin in EGF-induced MG63 and U20S. E-cadherin and vimentin levels were detected.

As shown in Figure 4 and Table 2, with inhibiting NF-κB activation and silencing Ezrin, E-cadherin significantly (P<0.05) decreased, while vimentin increased in EGF-induced MG63 and U20S. E-cadherin and vimentin levels were detected.

Additionally, to investigate the effect of Ezrin/NF-κB on the progression of osteosarcoma cells, we observed the morphology and detected the proliferation, migration, and motility of MG63 and U20S cell lines. As shown in Figure 5, suppressing NF-κB and silencing Ezrin reversed the mesenchymal morphology of EGF-induced MG63 and U20S, appearing rounded, with tight cell-cell junctions, similar to the control. With BAY and siRNA treatments, CCK-8 assay showed that the proliferation of MG63 and U20S cells was not significantly different from the control. The wound-healing assay showed that

|                     | EGF   | MG63  | U20S  | F     | P     | P     |
|---------------------|-------|-------|-------|-------|-------|-------|
| Proliferation (OD value) | –     | 0.121±0.018 | 0.120±0.007 | 33.241 | <0.001 | <0.001 |
|                     | +     | 0.185±0.007* | 0.182±0.012* | 38.937±1.730 | <0.001 | <0.001 |
| Migration (%)       | –     | 40.827±3.857 | 38.937±1.730 | 157.640 | 191.214 | <0.001 |
|                     | +     | 95.470±6.477* | 95.727±6.900* | 209.958 | <0.001 | <0.001 |
| Motility (%)        | –     | 3.177±0.293 | 2.770±0.543 | 347.000 | 209.958 | <0.001 |
|                     | +     | 7.803±0.315* | 8.617±0.440* | 209.958 | <0.001 | <0.001 |

* Comparing to the “–”, P<0.05
motility of MG63 and U2OS were not significantly different from the control. However, Transwell assay showed that after suppressing NF-κB and silencing Ezrin, the invasive ability of MG63 and U2OS cells were significantly (P<0.05) weakened compared to the control.

These data suggest that Ezrin/NF-κB was responsible for the EMT of osteosarcoma, as well as the progression and pathogenesis.

Reduction of Ezrin suppressed metastasis of EGF-induced osteosarcoma xenografts

To investigate the effect of reducing Ezrin on metastasis of EGF-induced osteosarcoma xenografts, we injected mice with MG63 and U2OS with silencing Ezrin to construct osteosarcoma models (Figure 6A); 40 mice received osteosarcoma xenografts successfully and no mice died.

As shown in Table 3 and Figure 6B, with both MG63 or U2OS osteosarcoma xenografts, liver weights of the tumorigenic mice with EGF injection increased significantly (P<0.05) compared to tumorigenic mice without EGF induction. Silencing Ezrin obviously and significantly (P<0.05) reduced liver weight of tumorigenic mice with EGF induction, and similar results were found for lung weight.

As shown in Table 4 and Figure 6C, in both MG63 and U2OS osteosarcoma xenografts, HPRT in livers and lungs of tumorigenic mice injected with EGF were obviously higher than in the tumorigenic mice without EGF injection, while silencing Ezrin significantly (P<0.05) reduced HPRT in livers and lungs.

As shown in Figure 6D, reduction of Ezrin obviously suppressed the down-regulation of E-cadherin and up-regulation of vimentin, as well as the up-regulation of p65 expression. All these results suggest that reducing Ezrin can suppress EMT and metastasis of osteosarcoma by regulating the NF-κB signaling pathway in vivo.

Discussions

In the present study, we demonstrated that Ezrin/NF-κB was responsible for EGF-induced EMT and progression in osteosarcoma. Reducing Ezrin reversed mesenchymal features of EGF-treated osteosarcoma cells and suppressed proliferation, migration, and motility of osteosarcoma cells by inhibiting NF-κB activity, as well as inhibiting osteosarcoma metastasis in mice with tumor xenografts.

Silencing Ezrin was demonstrated to suppress EMT [14], while over-expression of Ezrin changed cell morphology, adhesion,
Figure 4. EGF induced EMT by Ezrin/NF-κB in osteosarcoma cells (* compared to the Con., P < 0.05).

Table 2. Proliferation, migration and motility of MG63 and U20S with NF-κB inhibitor, silencing Ezrin and EGF treatments (X±SDs).

| Treatment            | MG63    | U20S    |
|----------------------|---------|---------|
| Proliferation (OD value) | 0.109±0.007 | 0.115±0.005 |
|                      | 0.103±0.007 | 0.107±0.006 |
|          +           | 0.138 ±0.007 |
|          +           | 3.238    |
| F         | 1.338    | 3.238   |
| P         | 0.312    | 0.146   |
| Migration (%)          | 31.360±2.098 | 30.727±3.252 |
|                      | 20.970±1.304* | 21.513±2.214* |
|          +           | 53.055   |
|          +           | 16.451   |
| F         | 0.002    | 0.015   |
| P         | 0.002    | 0.015   |
| Motility (%)          | 8.380±0.884 | 7.930±0.665 |
|                      | 7.693±0.754 | 6.953±0.085 |
|          +           | 1.048    |
|          +           | 6.374    |
| F         | 0.364    | 0.065   |
| P         | 0.364    | 0.065   |

* Comparing to the “–”, P<0.05
metastasis, motility, and apoptosis in different kinds of human tumors [15,16,18,27]. Ezrin phosphorylation was responsible for the activity of many cell lines, especially Ezrin Tyr353 and Ezrin Thr567, as the 2 most common types of activated Ezrin [14]. According to Crepaldi et al. [28], EGF can activate Ezrin by phosphorylation. In our study, we used EGF to induce MG63 and U2OS cells. Results showed that Ezrin Tyr353 and Ezrin Thr567 were both activated in vitro.

By detecting E-cadherin and vimentin levels, we found that EMT in osteosarcoma cells and xenografts were both promoted by EGF. CCK-8, Transwell, and wound-healing assays showed the promotion of proliferation, migration, and motility in osteosarcoma cells, which was consistent with results of previous studies.

Previous studies [27,29] demonstrated that several signal pathways, such as Akt, ERK1/2, and ROCK1 were related to the

**Figure 5.** Suppressing Ezrin/NF-κB reversed EGF-induced morphological change and promotion of proliferation, migration, and motility of osteosarcoma cells (* compared to the Con., *P*<0.05).

**Table 3.** Liver and lung weight of mice with osteosarcoma (X±SDs) (g).

|          | Con.+PBS | siRNA+PBS | Con.+EGF | siRNA+EGF | F      | P      |
|----------|----------|-----------|----------|-----------|--------|--------|
| **Liver** |          |           |          |           |        |        |
| MG63     | 0.479±0.055 | 0.412±0.032 | 0.625±0.029** | 0.468±0.036a | 16.135 | 0.001  |
| U2OS     | 0.508±0.023 | 0.448±0.029* | 0.626±0.032** | 0.510±0.013** | 26.359 | <0.001 |
| **P**    | 0.697     |           |          |           |        |        |
| **Lung** |          |           |          |           |        |        |
| MG63     | 0.164±0.006 | 0.184±0.007** | 0.298±0.007** | 0.194±0.008** | 217.230 | <0.001 |
| U2OS     | 0.171±0.006 | 0.183±0.008 | 0.302±0.010** | 0.192±0.012** | 129.802 | <0.001 |
| **P**    | 0.011     |           |          |           |        |        |

Con.+PBS – tumor xenografts and PBS injection; siRNA+PBS – tumor xenografts with silencing Ezrin and PBS injection; Con.+EGF – tumor xenografts and EGF injection; siRNA+EGF – tumor xenografts with silencing Ezrin and EGF injection; * comparing to Con.+PBS, P<0.05; ** comparing to siRNA+PBS, P<0.05; # comparing to Con.+EGF, P<0.05.
**Figure 6.** Reducing Ezrin suppressed metastasis of EGF-injected MG63 and U20S xenografts of BALB/c-nu mice. (A) BALB/c-μu mouse with osteosarcoma xenografts. (B) Liver and lung weights of tumorigenic mice. (C) HPRT mRNA level in livers and lungs of tumorigenic mice. (D) E-cadherin, vimentin, and p65 protein levels in livers and lungs of tumorigenic mice. (HPRT, hypoxanthine guanine phosphoribosyl transferase; Con.+PBS, tumor xenografts and PBS injection; siRNA+PBS, tumor xenografts with silencing Ezrin and PBS injection; Con.+EGF, tumor xenografts and EGF injection; siRNA+EGF, tumor xenografts with silencing Ezrin and EGF injection; * compared to Con.+PBS, \( P < 0.05 \); # compared to siRNA+PBS, \( P < 0.05 \); & compared to Con.+EGF, \( P < 0.05 \).)
activation of Ezrin. Wang et al. [30] showed that the Akt/Ezrin Tyr353/NF-κB pathway regulated EGF-induced EMT and metastasis in tongue squamous cell carcinoma. In our investigations, we silenced Ezrin and found that nuclear p65 and p-IκBα, as the direct activators of NF-κB, were both lower, while over-expression of Ezrin increased nuclear p65 and p-IκBα in vitro. In vivo, reducing Ezrin also suppressed p65 expression in lungs and livers of osteosarcoma mice. All of these results suggest that phosphorylated Ezrin is required for the activation of NF-κB in EGF-induced osteosarcoma.

EMT was demonstrated to be crucial in progression in metastasis of many tumors, and EGF is an important inducer of EMT [31,32]. Gan et al. [33] showed that EGF promoted EMT by activating the Akt pathway. Gupta et al. [34] found that carcinoma metastasis was a major issue in human cancer, and EMT is known to be essential for initiation of metastasis. In our study, we used lxBα inhibitor to suppress NF-κB activation, and then silenced Ezrin in EGF-induced MG63 and U2OS cells. Our results suggest that suppressing NF-κB activity and silencing Ezrin can inhibit EMT induced by EGF in osteosarcoma cells. Additionally, suppressing NF-κB activity and silencing Ezrin obviously reversed osteosarcoma cell morphology and motility but weakened cell migration. In vivo, reducing Ezrin suppressed EGF-induced EMT of osteosarcoma xenografts. These results indicate that Ezrin/NF-κB plays a pivotal role in EMT in EGF-induced metastasis of osteosarcoma. The molecular mechanisms we found agree with previous reports.

Cell and animal experiments both confirmed that Ezrin promotes osteosarcoma metastasis by regulating NF-κB pathway activity, which suggests the importance of the relationship between Ezrin and the NF-κB pathway in regulating osteosarcoma metastasis and progression.

Conclusions

Cell and animal experiments both confirmed Ezrin promotes osteosarcoma metastasis by regulating NF-κB pathway activity, which has a critical role in regulating EGF-induced EMT and progression of osteosarcoma in vivo and in vitro. Our study provides clinical treatments and prevention of osteosarcoma using molecular mechanisms and therapeutic targets.

Conflict of interest

None.

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Table 4. Liver and lung HPRT of mice with osteosarcoma (X±SDs).

|          | Con.+PBS  | siRNA+PBS | Con.+EGF   | siRNA+EGF | \( F \)   | \( P \)  |
|----------|-----------|-----------|------------|-----------|----------|--------|
| Liver    |           |           |            |           |          |        |
| MG63     | 1.125±0.008 | 1.128±0.003 | 5.074±0.034** | 1.363±0.014*** | 31367.710 | <0.001 |
| U2OS     | 1.119±0.014 | 1.125±0.013 | 5.100±0.037** | 1.348±0.011*** | 25445.074 | <0.001 |
| Lung     |           |           |            |           |          |        |
| MG63     | 1.255±0.013 | 1.177±0.015* | 6.873±0.021** | 1.668±0.017*** | 84049.804 | <0.001 |
| U2OS     | 1.286±0.012 | 1.174±0.027* | 6.812±0.090** | 1.653±0.032*** | 8905.229  | <0.001 |

HPRT – hypoxanthine guanine phosphoribosyl transferase; Con.+PBS – tumor xenografts and PBS injection; siRNA+PBS – tumor xenografts with silencing Ezrin and PBS injection; Con.+EGF – tumor xenografts and EGF injection; siRNA+EGF – tumor xenografts with silencing Ezrin and EGF injection; * comparing to Con.+PBS, \( P <0.05 \); ** comparing to siRNA+PBS, \( P <0.05 \); * comparing to Con.+EGF, \( P <0.05 \).
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