Research Article

Tumor Necrosis Factor-α Promotes the Tumorigenesis, Lymphangiogenesis, and Lymphatic Metastasis in Cervical Cancer via Activating VEGFC-Mediated AKT and ERK Pathways

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Background. Lymphatic metastasis is a common phenomenon of cervical cancer. Tumor necrosis factor-α (TNF-α) was found to be closely associated with lymphatic cancer metastasis. However, the mechanism through which TNF-α regulates lymphatic metastasis in cervical cancer remains unclear. *Methods*. In this study, cervical cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with or without TNF-α for 48 h, and then the corresponding conditional medium (CM-TNF-α or CM) was collected. The level of vascular endothelial growth factor (VEGFC) in the corresponding CM was then detected using an enzyme-linked immunosorbent assay (ELISA). Next, human lymphatic endothelial cells (HLECs) were cultured in CM-TNF-α or CM for 48 h. Cell viability was measured using the cell counting kit-8 (CCK-8) assay, and angiogenesis was detected using a tube formation assay. Subsequently, the expressions of AKT, p-AKT, ERK, and p-ERK in HLECs were detected using western blotting. In addition, to further investigate the effect of TNF-α on the progression of cervical cancer, a C33A subcutaneous xenograft model was established *in vivo*. *Results*. We found that TNF-α significantly stimulated cervical cancer cells to secrete VEGFC. Additionally, the CM collected from the TNF-α-treated cervical cancer cells notably promoted the proliferation, migration, and angiogenesis of HLECs; however, these changes were reversed by MAZ51, a VEGFR3 inhibitor. Moreover, TNF-α obviously elevated D2-40 and VEGFC protein expressions in tumor tissues, promoting lymphangiogenesis and lymphatic metastasis *in vivo*. Meanwhile, TNF-α markedly upregulated p-AKT and p-ERK expressions in tumor tissues, whereas these changes were reversed by MAZ51. *Conclusion*. Collectively, TNF-α could promote tumorigenesis, lymphangiogenesis, and lymphatic metastasis *in vitro* and *in vivo* in cervical cancer via activating VEGFC-mediated AKT and ERK pathways. These results may provide new directions for the treatment of cervical cancer.

1. Introduction

Cervical cancer remains one of the main cancer in women, particularly in developing countries [1–4]. At present, cervical cancer is the main cause of mortality in women [2, 5, 6]. Lymphatic metastasis has been identified as a risk factor for cervical cancer recurrence [7]. The formation of lymphatic microvessels is the earliest stage of lymphatic metastasis

[8]. At present, clinical treatments for cervical cancer include surgical treatment, radiotherapy, and chemotherapy [9–11]. However, the prognosis of patients with advanced cervical cancer remains relatively poor [12].

Tumor microenvironment (TME) refers to the environment around a tumor, including the surrounding blood vessels, immune cells and son on [13]. A prominent feature of the TME is the recruitment of a large number of inflammatory

cells and the production of inflammatory factors [14]. Inflammatory factors are a double-edged sword for tumors [15–17]. On the one hand, they can directly kill tumor cells or induce immune cells to recognize tumor antigens [18, 19]. On the other hand, they can promote tumor cell invasion and metastasis [20]. Thus, it is necessary to explore the relationships among inflammatory factors, lymphatic microvessel formation, and tumorigenesis in cervical cancer.

The extensively studied pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α) [21], can be secreted by various cells such as adipocytes, activated monocytes, macrophages, B cells, and T cells [22, 23]. Reports have suggested that TNF-α can promote tumor development and metastasis [24] by inducing epithelial-mesenchymal transition (EMT) as demonstrated by Yoshimatsu et al. inoral squamous cell carcinoma [25]. In addition, Fujiki et al. reported that TNF-α was able to facilitate the occurrence and development of gastric cancer [26]. Nevertheless, the relationship among TNF-α, lymphatic microvessel formation, and tumorigenesis in cervical cancer remains unclear. The aim of the present study was therefore to investigate the mechanism through which TNF-α regulates lymphatic microvessel formation in cervical cancer.

2. Materials and Methods

2.1. Cell Culture. HeLa and C33A human cervical cancer cell lines were purchased from the American Type Culture Collection and cultured in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.) with 5% CO_2 at 37°C. HLECs were obtained from Procell and cultured in a medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% endothelial cell growth supplement (ECGS, CELL RESARCH) [27].

2.2. Conditional Medium (CM). HeLa and C33A cells were stimulated with different concentrations of TNF-α (0, 5, and 10 ng/ml) for 48 h at 37°C, and the corresponding CM (CM-TNF-α) was collected. Meanwhile, in the control group, cervical cancer cell lines were cultured in DMEM for 48 h at 37°C, and the corresponding CM was collected [28]. The CM was supplemented with 1% ECGS. After that, HLECs were cultured in CM with or without MAZ51 treatment.

2.3. ELISA Analysis. The level of VEGFC in the CM was detected using a VEGFC assay kit (cat. no. H046; Nanjing Jiancheng Bioengineering) [29]. The VEGFC inhibitor MAZ51 was purchased from MedChemExpress (cat. no. HY-116624).

2.4. CCK-8 Assay. Cell viability was measured using the CCK-8 assay by culturing HLECs in either CM or CM-TNF-α for 48 h, followed by incubation with 10 μl CCK-8 reagent (cat. no. C0047; Beyotime) for 2 h, and then the absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific) [30].

2.5. 5-Ethynyl-2′-Deoxyuridine (EdU) Staining. Cell proliferation was measured using an EdU staining assay. The EdU detection kit was purchased from Guangzhou RiboBio (cat. no. C10310-1). Firstly, HLECs were cultured in CM or CM-TNF-α for 48 h. Next, HLECs were incubated with 100 μl of 50 μm EdU for 1 h, washed with PBS, and incubated with 1 mg/ml DAPI for 10 min. Next, the EdU-positive HLECs were measured using a fluorescence microscope (IX51; Olympus) [31].

2.6. Tube Formation Assay. The number of tube node formed in the HLECs was observed using a tube formation assay. Matrigel-coated 24-well Transwell® (8 μm pore size) was purchased from Corning, Inc. HLECs were cultured in CM or CM-TNF-α for 48 h. Next, HLECs (1×10^5 cells) were placed in the Matrigel®-coated well at 37°C. Next, the number of tube node of HLECs was observed using a microscope (IX51; Olympus) [32].

2.7. Wound Healing Assay. Cell migration was determined using the wound-healing assay by culturing HLECs (5×10^5/cell) in a 6-well plate overnight, creating a wound in the monolayer using a 200 μl pipette tip, washing the cells with PBS, and then observing the scratch widths using a microscope (IX51; Olympus) after 0 and 24 h [31].

2.8. Transwell Migration Assay. Transwell assay was used by adding HLECs to the upper chamber containing serum-free DMEM andDMEM with 10% FBS t the lower chamber. The cells that had migrated to the lower chamber after24 hours of incubation were stained with crystal violet dye (cat. no. AS1086; ASPEN) and observed using a microscope (IX51; Olympus) [33].

2.9. Western Blotting. The total protein from cells and tumor tissues was extracted, the protein concentration was quantified using a BCA Protein Assay Kit (cat. no. AS1086; Aspen Biosciences) and 40 μg per lane of protein was separated by 10% SDS-PAGE and transferred onto PVDF membranes (EMD Millipore). The membranes were then incubated with primary antibodies: anti-AKT (1 : 1,000; cat. no. AF0836), anti-p-AKT (1 : 1,000; cat. no. 28731-1-AP), anti-ERK (1 : 1,000; cat. no. 11257-1-AP), anti-p-ERK (1 : 1,000; cat. no. 28733-1-AP), and anti-GAPDH (1 : 1,000; cat. no. 60004-1-Ig) overnight at 4°C. GAPDH was used as the internal reference. Next, the membranes were incubated with corresponding HRP-conjugated secondary antibodies for 1 h at room temperature. Finally, an enhanced chemiluminescent substrate kit (cat. no. AS1059; Aspen Biosciences) was used to observe the protein bands [34]. Anti-AKT, anti-p-AKT, anti-ERK, anti-p-AKT, and anti-GAPDH antibodies were obtained from Proteintech Group, Inc. The anti-AKT antibody was provided by Affinity Biosciences.

2.10. Animal Study. BALB/c nude mice (4-6 weeks old) were provided by Charles River Laboratories, Inc. All animals were maintained following the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. In addition, the experiments of animal study were approved by the Ethics Committee of HY cell biotechnology (No.
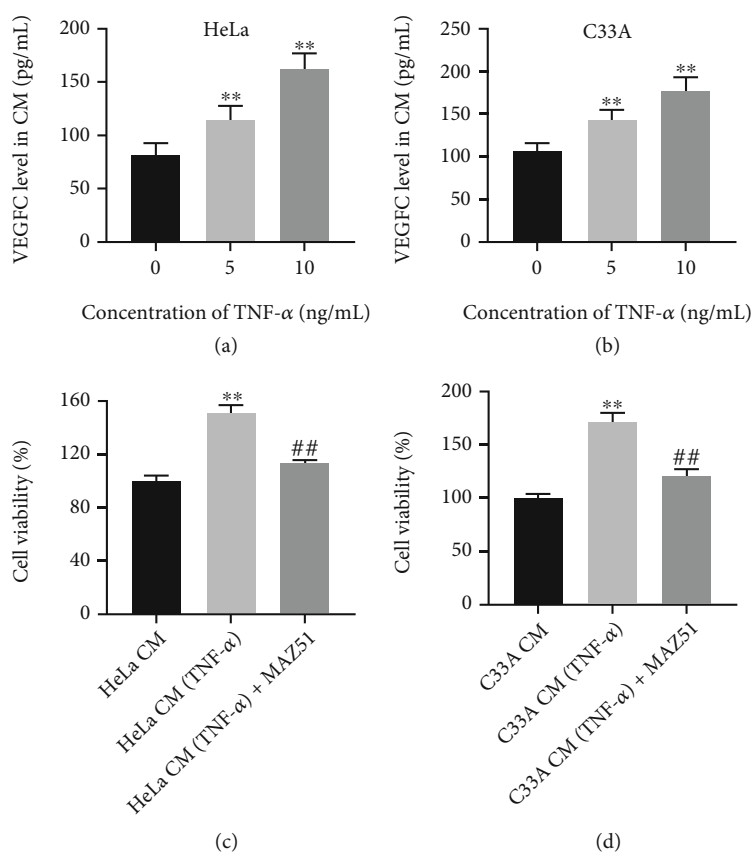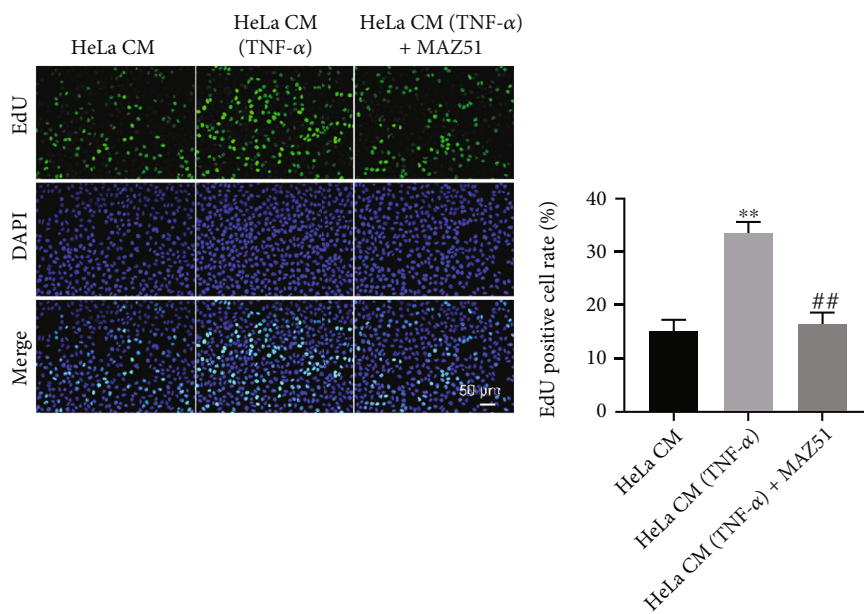
Figure 1: TNF-α stimulates cervical cancer cells to secrete VEGFC and promotes HLEC viability. (a, b) Cervical cancer cells (HeLa and C33A) were stimulated with different concentrations of TNF-α (0, 5, or 10 ng/ml) for 48 h, and corresponding CM was collected. Next, the level of VEGFC in the corresponding CM was detected by ELISA. (c, d) HLECs were cultured in CM with or without MAZ51 treatment. HLEC viability was measured by CCK-8 assay. $^{**}P < 0.01$ compared with CM group; $^{##}P < 0.01$ compared with CM-TNF-α group, $n = 3$.

HY2021-33). C33A cells at the density of 1×10^7 cells were subcutaneously injected into the left flank of nude mice. Next, when the tumor volume reached ~200 mm³, mice were randomly divided into three groups: control, TNF-α, and TNF-α+MAZ251 groups. TNF-α was intraperitoneally injected into mice in TNF-α and TNF-α+MAZ251 groups three times a week at 54 μg/kg for 3 weeks. In addition, MAZ51 was intraperitoneally injected into mice in the TNF-α+MAZ251 group once a day at 8 mg/kg for 15 days. Meanwhile, normal saline was intraperitoneally injected into mice in the control group. The tumor volume was measured weekly according to the following formula: volume = length $\times$ width²/2 [35]. In 3 weeks, all mice were sacrificed using CO_2 (40% volume/min). And the tumors were photographed and weighted. Meanwhile, plasma samples were collected using anticoagulation tubes and then centrifuged for 10 min at 2,000 $\times$ g at 4°C.

2.11. Immunohistochemistry (IHC) Staining. A tumor tissue section was dewaxed with xylene. Antigens from the tumor tissue section were extracted with 0.01 M heated citrate buffers (pH 6.0). Next, 200 μl blocking solution was dropped onto the slices at room temperature for 1 h. The section was then incubated with a primary anti-D2-40 or PDPN antibody overnight at 4°C. Next, the section was washed with

PBS for 3 times. Subsequently, the section was incubated with secondary antibodies for 50 min at room temperature. DAB was then used for chromogenic reactions. In addition, the slices were placed in a hematoxylin solution for redyeing. Finally, the coverslip was placed over the section, and the staining results were observed using a microscope (CX31; Olympus Corporation) [36]. ImageJ software (with IHC Profiler plugins) was used for IHC scoring.

2.12. Real-Time PCR (RT-PCR). The TRIpure Total RNA Extraction Reagent (cat. no. EP013; ELK Biotechnology Co., Ltd.) was used to isolate the RNA from cells. Then, the EnTurbo™ SYBR Green PCR SuperMix kit (cat. no. EQ001; ELK Biotechnology Co., Ltd.) was used to perform RT-PCR. The cycling conditions for qPCR were as follows: 95°C for 3 min, followed by 95°C for 10 s, 58°C for 30 s, and 72°C for 30 s for 40 cycles. The information of primers: GAPDH forward, 5′-CATCATCCCTGCCTCTACTGG-3′ and reverse, 5′-GTGGGTGTCGCTGTTGAAGTC-3′; VEGFC forward, 5′-ACGAGCTACCTCAGCAAGACG-3′ and reverse, 5′-CTCCAGCATCCGAGGAAAAC-3′; D2-40 forward, 5′-CTATAAGTCTGGCTTGACAACTCT-3′ and reverse, 5′-CATCTTTCTCAACTGTTGTCTGTG-3′; VEGFR forward, 5′-GGGCATGTACTGACGATTATGG-3′ and reverse, 5′-
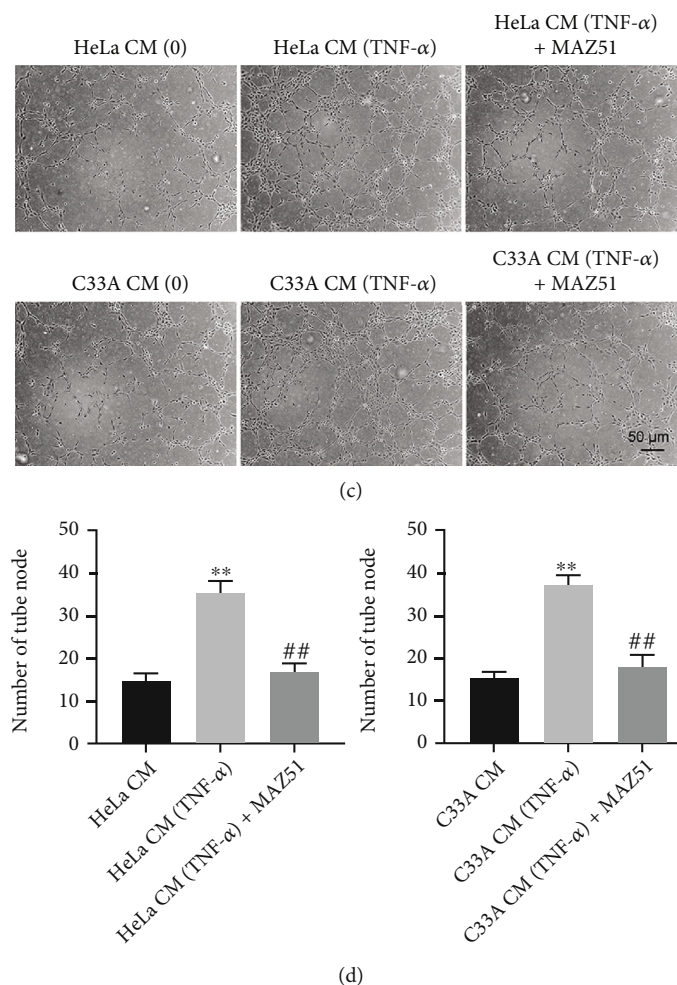
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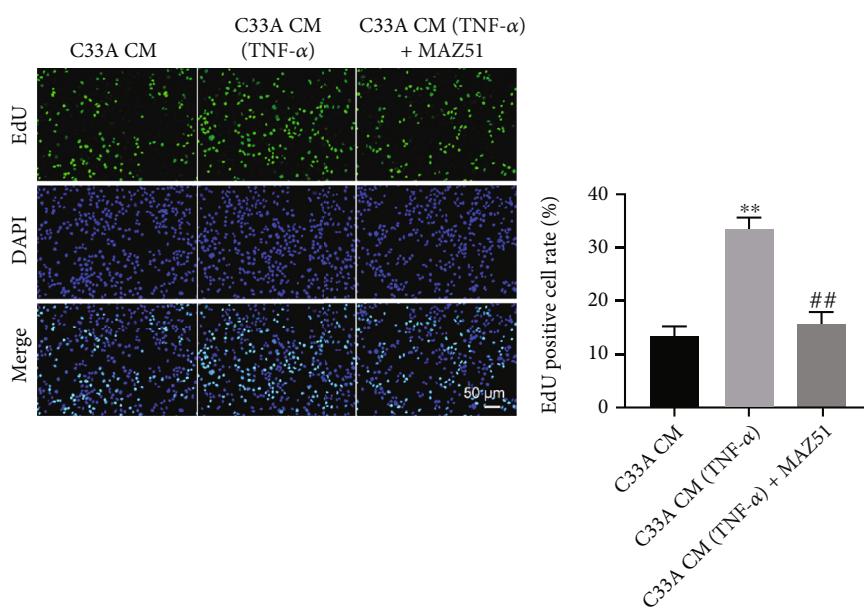
(b)

FIGURE 2: Continued.

(c)

(d)

FIGURE 2: CM-TNF-α promotes the proliferation and angiogenesis of HLECs. Cervical cancer cells (HeLa and C33A) were stimulated with 10 ng/ml TNF-α for 48 h, and corresponding CM was collected. Next, HLECs were cultured in CM with or without MAZ51 treatment. (a, b) The proliferation of HLECs was measured by EdU staining assay. (c, d) The number of tube node of HLECs was observed using a microscope. $^{**}P < 0.01$ compared with CM group; $^{##}P < 0.01$ compared with CM-TNF-α group, $n = 3$.

GGAGGAATGGCATAGACCGTA-3′. The relative level of VEGFC, D2-40, and VEGFR was calculated using the $2^{-\Delta\Delta Ct}$ method [27, 37].

2.13. Statistical Analysis. The statistical analysis was performed using GraphPad Prism software version 7.0 (GraphPad Software, Inc.). Data are presented as the mean ± standard deviation and analyzed using a one-way analysis of variance and Tukey's post hoc test. $P < 0.05$ indicated a statistically significant difference [30, 38].

3. Results

3.1. TNF-α Promotes the Production of VEGFC in Cervical Cancer Cells. It has been reported that VEGFC is the most representative and important factor promoting the formation of tumor lymphangiogenesis [39, 40]. In addition, TNF-α was found to stimulate cell secretion of VEGF [41]. Our results found that TNF-α obviously upregulated the level of VEGFC in the CM of HeLa and C33A cells in a dose-dependent manner (Figures 1(a) and 1(b)). These

results showed that TNF-α promoted the production of VEGFC in cervical cancer cells.

3.2. CM-TNF-α Promotes HLEC Viability, Proliferation, and Angiogenesis. With the aim of investigating the effect of TNF-α on the formation of lymphangiogenesis in cervical cancer, HLECs were cultured in CM-TNF-α. As indicated in Figures 1(c) and 1(d) and 2(a) and 2(b), CM-TNF-α markedly promoted the viability and proliferation of HLECs. However, these phenomena were reversed in the presence of VEGFR3 inhibitor MAZ51 (Figures 1(c) and 1(d) and 2(a)and 2(b)). Meanwhile, CM-TNF-α significantly increased the number of tube node formed in HLECs, and that effect was notably inhibited by MAZ51 (Figures 2(c) and 2(d)). All these results indicated that CM-TNF-α could promote HLEC proliferation and angiogenesis by upregulating VEGFC.
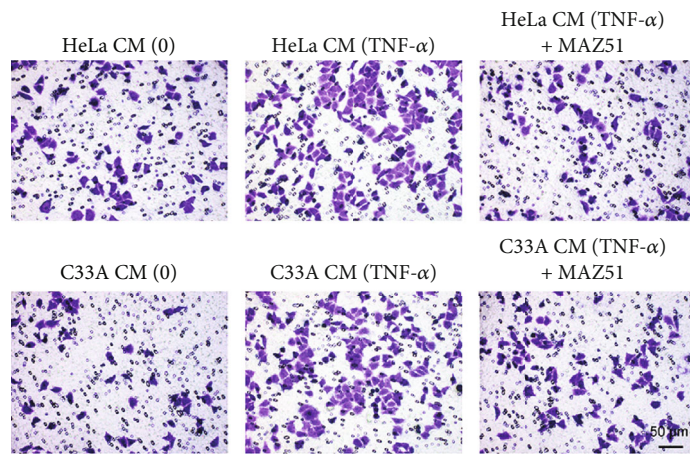
3.3. CM-TNF-α Increases HLEC Migration. In order to study the role of TNF-α on HLEC migration, wound healing, and transwell assays were conducted. The results showed that CM-TNF-α significantly promoted HLEC migration, but
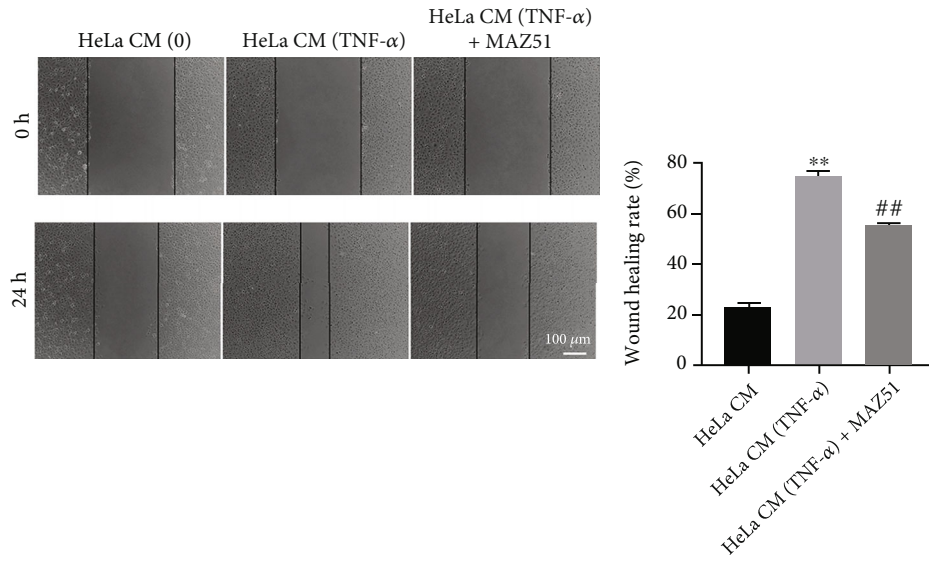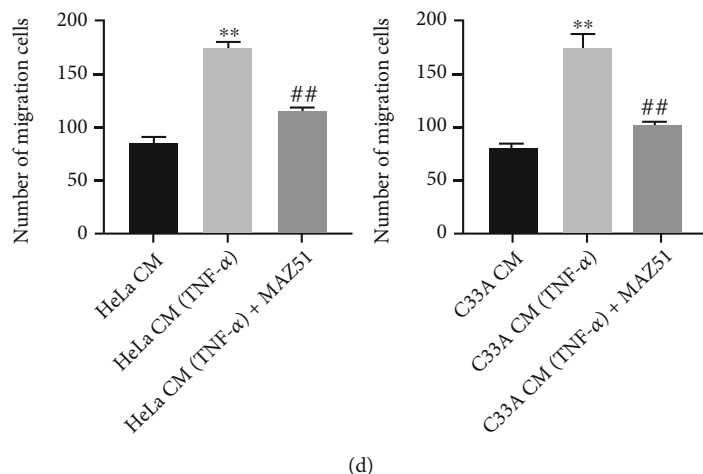
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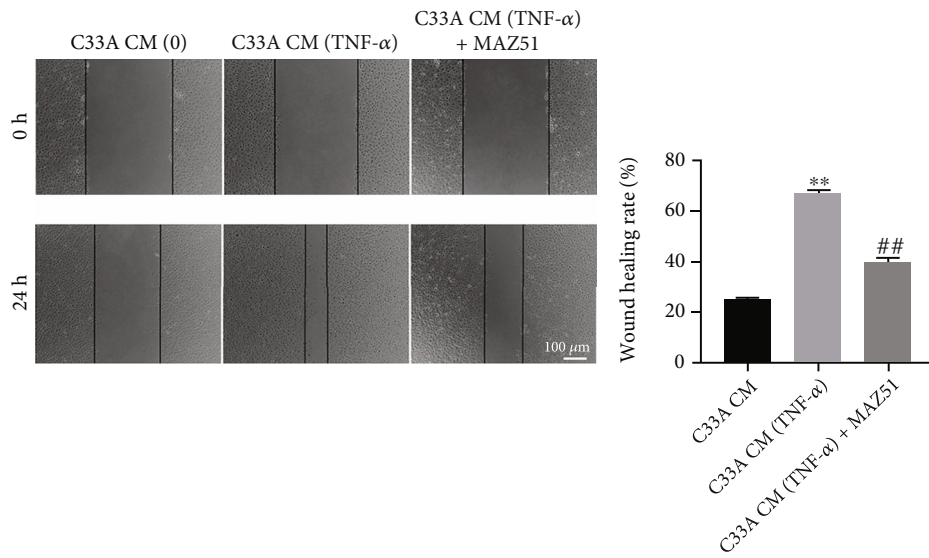
(b)

(c)

Figure 3: Continued.

(d)

FIGURE 3: CM-TNF-α increases the migration of HLECs. Cervical cancer cells (HeLa and C33A) were stimulated with 10 ng/ml TNF-α for 48 h, and corresponding CM was collected. Next, HLECs were cultured in CM with or without MAZ51 treatment. (a, b) The migration of HLECs was measured by wound healing assay. (c, d) The migration of HLECs was measured by transwell migration assay. $^{**}P < 0.01$ compared with CM group; $^{##}P < 0.01$ compared with CM-TNF-α group, $n = 3$.

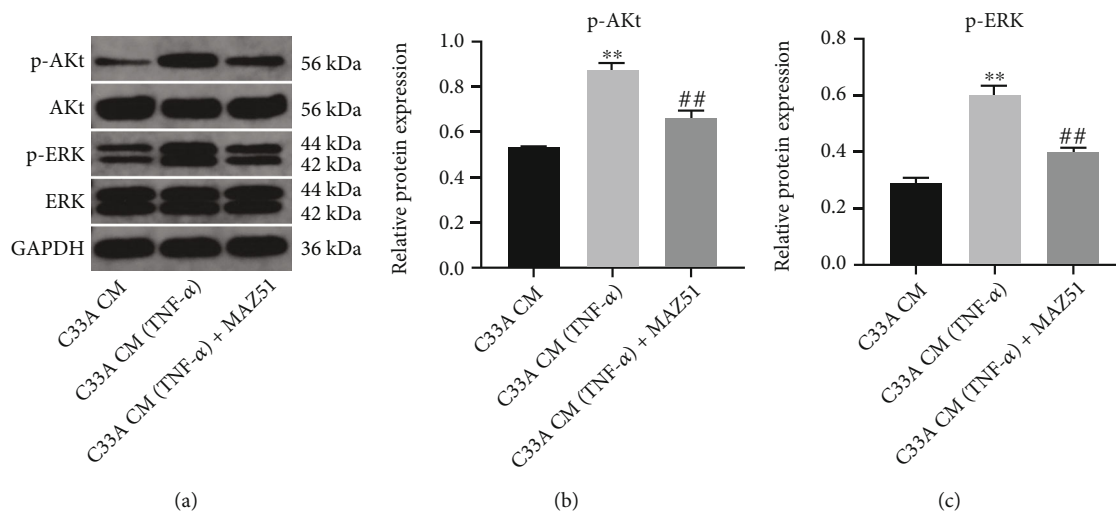(a)   (b)   (c)

FIGURE 4: CM-TNF-α upregulates the expressions of p-AKT and p-ERK of HLECs. C33A cells were stimulated with 10 ng/ml TNF-α for 48 h, and the CM was collected. Next, HLECs were cultured in CM with or without MAZ51 treatment. (a–c) The levels of AKT, p-AKT, ERK, and p-ERK of HLECs were measured by western blot. $^{**}P < 0.01$ compared with CM group; $^{##}P < 0.01$ compared with CM-TNF-α group, $n = 3$.

MAZ51 clearly inhibited this promotion (Figures 3(a)–3(d)). These results showed that CM-TNF-α could increase HLEC migration by upregulating VEGFC.

3.4. CM-TNF-α Upregulates the Expressions of p-AKT and p-ERK of HLECs. The AKT and ERK signaling pathways have been reported to play an important role in cervical cancer progression [42, 43]. In order to explore the mechanism by which TNF-α regulates the lymphangiogenesis of HLECs, the expressions of p-AKT and p-ERK were evaluated by western blotting. The results indicated that CM-TNF-α markedly increased the levels of p-AKT and p-ERK in HLECs, and these increases were markedly suppressed by MAZ51 (Figures 4(a)–4(c)). Collectively, CM-TNF-α could

promote the expressions of p-AKT and p-ERK in HLECs by upregulating VEGFC.

3.5. TNF-α Promotes the Tumorigenesis, Lymphangiogenesis, and Lymphatic Metastasis of Cervical Cancer In Vivo. Finally, to confirm the effect of TNF-α on cervical cancer tumorigenesis and lymph node metastasis, a C33A subcutaneous xenograft model was established in vivo. As shown in Figures 5(a)–5(c), TNF-α remarkably promoted tumor volume and weight in C33A subcutaneous xenografts; however, this promotion was clearly inhibited by MAZ51 treatment. In addition, D2-40 has been reported as a specific marker of lymphatic endothelial cells that can be used in the study of lymph node metastasis [44, 45]. The IHC results suggested that TNF-α visibly increased D2-40 and PDPN levels
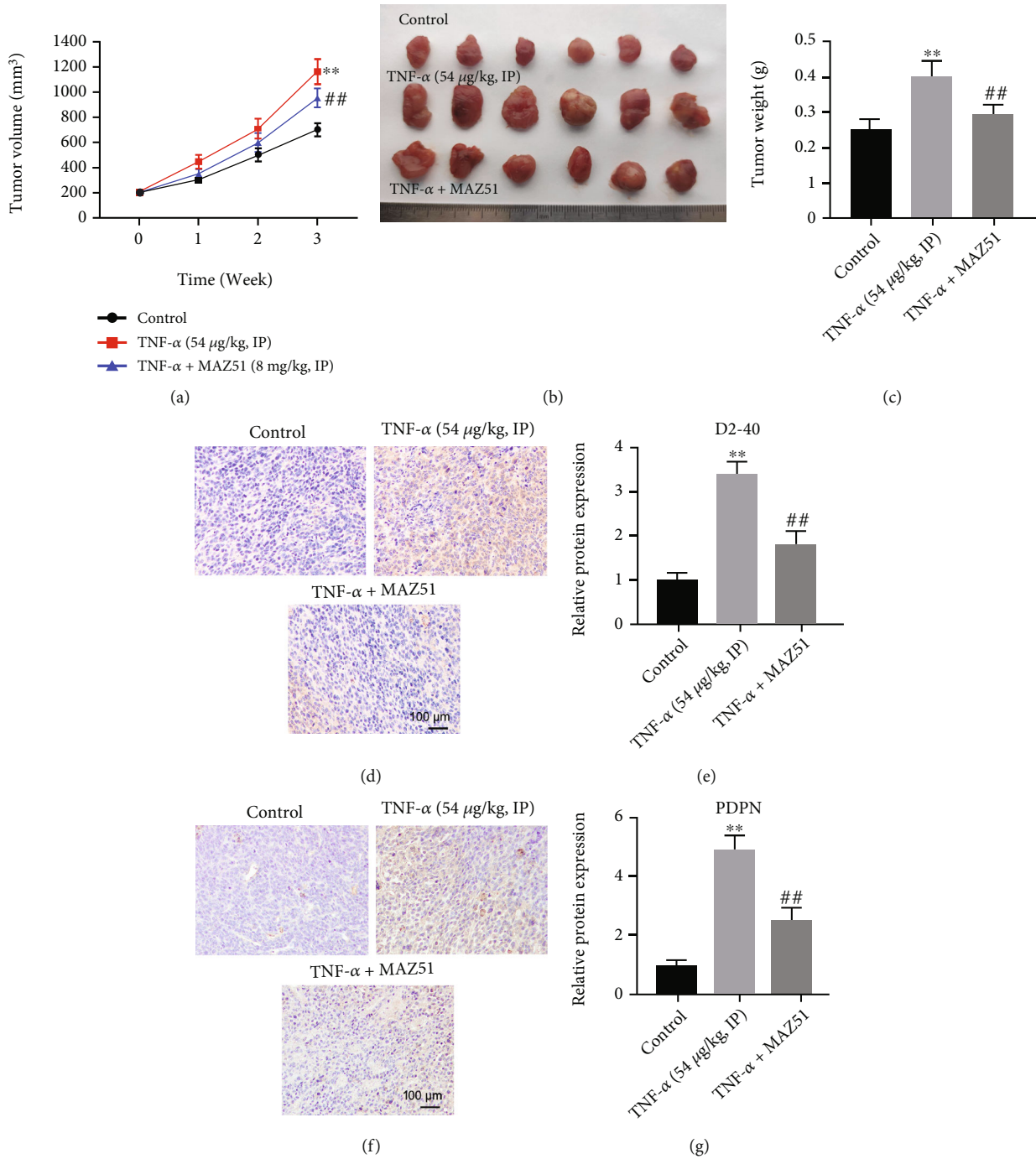
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(g)

FIGURE 5: TNF-α promotes the tumorigenesis of cervical cancer *in vivo*. (a) The tumor volume was measured weekly. (b, c) The tumors were photographed and weighted. (d–g) The level of D2-40 or PDPN in tumor tissue was measured by IHC staining assay. $^{**}P < 0.01$ compared with control group; $^{##}P < 0.01$ compared with TNF-α treated group, $n = 3$.

in tumor tissues, and these increases were reversed by MAZ51 (Figures 5(d)–5(g)).

Furthermore, ELISA results suggested that TNF-α significantly increased VEGFC expression in the plasma *in vivo*, which was reversed by MAZ51 (Figure 6(a)). Meanwhile, TNF-α visibly increased p-AKT and p-ERK expressions in tumor tissues, but these increases were reversed by MAZ51 (Figures 6(b)–6(d)). In addition, TNF-α upreg-

ulated the levels of VEGFC and D2-40 in tumor tissues compared with the control group (Figures 6(e) and 6(f)). Meanwhile, compared with the control group, TNF-α had few effects on VEGFR gene expression in tumor tissues (Figure 6(g)). In general, TNF-α could promote cervical cancer tumorigenesis, lymphangiogenesis, and lymphatic metastasis *in vivo* via activating VEGFC-mediated AKT and ERK pathways.
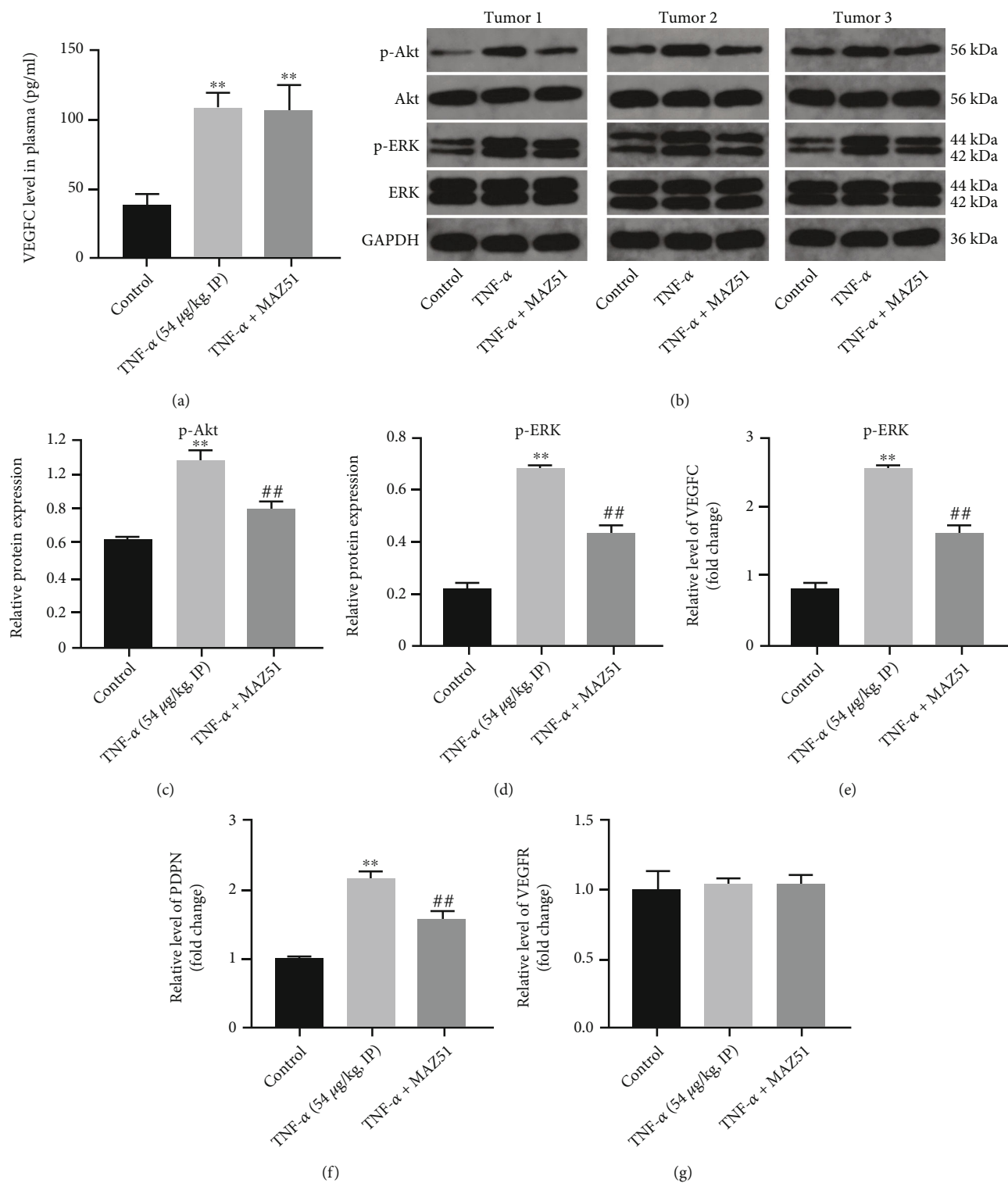
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FIGURE 6: TNF-α upregulates p-AKT and p-ERK expression in tumor tissues via mediating VEGFC signaling *in vivo*. (a) The level of VEGFC in the plasma was measured by ELISA. (b–d) The levels of AKT, p-AKT, ERK, and p-ERK in tumor tissue were measured by western blot. (e–g) The levels of VEGFC, D2-40, and VEGFR were detected with RT-qPCR. $**P < 0.01$ compared with control group; $\#\#P < 0.01$ compared with TNF-α treated group, $n = 3$.

4. Discussion

TNF-α is an important regulator of the inflammatory response [46, 47] that can activate neutrophils and lympho-cytes, increase vascular endothelial cell permeability, and regulate cellular and tissues metabolism [46, 48]. Therefore, studying the mechanism of inflammatory factors to promote tumor progression and metastasis has become a research

hotspot in recent years. For instance, TNF-α has been reported to promote tumor development and metastasis [24, 49]. Forkasiewicz et al. found that TNF-α could promote esophageal cancer cell migration [50]. In addition, Liang et al. showed that TNF-α could enhance gastric cancer cell migration and invasion via activating NF-κB signaling [51]. Consistent with that study, the present data indicated that CM-TNF-α could promote the growth of cervical cancer cells *in vivo*. Furthermore, this study showed that TNF-α could stimulate cervical cancer cells to secrete VEGFC, which in turn promoted the proliferation, migration, and angiogenesis of HLECs. Therefore, this study was the first to explore the effect of TNF-α on the progression of cervical cancer from the perspective of the TME.

The interaction between cancer cells and lymphatic endothelial cells is crucial in promoting tumor growth and metastasis in the TME, as demonstrated by previous studies [52, 53]. One of these studies showed that lymphatic endothelial cells could enhance the proliferation and migration of tumor cells [54], while cancer cells could also accelerate endothelial cell tube formation via activating the PI3K/Akt pathway [55]. Moreover, tumor-induced lymphangiogenesis is known to play a vital role in the initial stages of cancer metastasis [56], andthe link between VEGFC and tumor lymphangiogenesis and metastasis has been extensively investigated [57, 58]. Chen et al. found that cancer cell-derived VEGFC could promote lymphangiogenesis in lymph nodes, which in return promotes cancer metastasis [59]. Meanwhile, He et al. showed that VEGFC could promote cervical cancer metastasis [60]. Besides, researchers have found that TNF-α could upregulate VEGFC expression, promoting lymphangiogenesis and lymphatic metastasis in gallbladder cancer [61]. In our study, we observed that the expression of VEGFC was significantly increased in the CM collected from the TNF-α-treated cervical cancer cells. CM collected from these cells also promoted the proliferation, migration, and angiogenesis of HLECs; and these changes were reversed by MAZ51, a VEGFR3 inhibitor. Furthermore, TNF-αelevated D2-40 and VEGFC protein expressions in tumor tissues, indicating that TNF-α could promote lymphangiogenesis and lymphatic metastasis of cervical cancer *in vivo*. Our findings suggest that TNF-α could be apromising target for cervical cancer treatment, as it promote lymphangiogenesis and lymphatic metastasis by upregulating VEGFC.

TNF-α was found to induce colorectal cancer cell migration and EMT via activating AKT signaling [62]. In addition, TNF-α could promote triple-negative breast cancer cell metastasis through targeting TNFR2-ERK1/2-EZH2 signaling [63]. These findings showed that TNF-α could promote tumor development via modulating AKT and ERK signaling pathways. Additionally, the literature suggested that the AKT and ERK signaling pathways are extensively involved in cervical cancer development [64–66]. For example, exosomal miR-221-3p secreted by cervical squamous cell carcinoma promoted the formation and metastasis of HLECs by upregulating the AKT/ERK pathway [67]. In addition, protein tyrosine phosphatase receptor M can induce lymphangiogenesis and lymph node metastasis through the AKT signaling pathway in a VEGFC-dependent manner [68]. In the present study, the CM collected from the TNF-α-treated cervical cancer cells was found to increase p-AKT and p-ERK expressions in HLECs. Moreover, TNF-α could upregulate p-AKT and p-ERK expressions in tumor tissues. However, inhibition of VEGFR3 obviously reversed these changes. All these data suggested that the AKT and ERK signaling pathways are involved in the lymphangiogenesis in cervical cancer.

In this study, we only determined that TNF-α could inhibit cervical cancer progression by targeting VEGFC-mediated AKT and ERK pathways. Thus, further study is needed to investigate whether TNF-α could affect the progression of cervical cancer via targeting other pathways, such as AMPK/mTOR or the NF-κB signaling pathway [69, 70].

5. Conclusion

To sum up, our studyrevealed that TNF-αactivatesVEGFC-mediated AKT and ERK pathways, leading to tumorigenesis, lymphangiogenesis, and lymphatic metastasis *in vitro* and *in vivo* in cervical cancer. Wehope that our research will provide new directions for the treatment of cervical cancer.

Data Availability

All data, models, and code generated or used during the study are available from the corresponding author upon reasonable request.

Conflicts of Interest

These authors declared no competing interests in this research.

Acknowledgments

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