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RESEARCH ARTICLE

Biglycan up-regulated vascular endothelial growth factor (VEGF) expression and promoted angiogenesis in colon cancer

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Abstract Biglycan is an important component of the extracellular matrix, which belongs to the small leucine-rich proteoglycan family. Recent studies have shown that biglycan expression is elevated in many tumor tissues and implies poor prognosis, such as colon cancer. However, the molecular mechanism of biglycan in colon cancer has not been investigated. The present study aimed to investigate the effects of biglycan on vascular endothelial growth factor (VEGF) expression in colon cancer cells and on tumor angiogenesis in vivo. Biglycan overexpression vectors were constructed, and the stable biglycan overexpression in human colon cancer cell lines (HCT116 cells) was established by G418 screening. The stable cell clones were subsequently used to initiate tumor xenografts in nude mice. Our results showed that biglycan overexpression notably up-regulated the levels of VEGF in colon cancer cells, which was further confirmed by immunohistochemistry analysis in the xenograft colon tumors. Moreover, high levels of biglycan promoted angiogenesis and colon tumor growth, as evidenced by the increased cell viability, colon tumor size, and weight, as well as the CD34 expression. Additionally, we found that the extracellular signal-regulated kinase (ERK) signaling pathway was activated by biglycan in colon cancer cells. The ERK inhibitor PD98059 dramatically reversed the increased expression of VEGF induced by biglycan. Taken together, our results indicated that biglycan up-regulated VEGF expression in colon cancer cells and promoted tumor angiogenesis. Biglycan-mediated VEGF regulation may correlate with the activation of the ERK signaling pathway. Therefore, biglycan may be a promising target for anti-angiogenic therapy for cancer.

Keywords Biglycan · Colon cancer · VEGF · ERK signaling pathway

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Introduction

The main causes of death in patients with cancer are due to the uncontrolled growth, dispersion, and metastasis of the tumor [1]. Angiogenesis plays an important role in tumor invasion and metastasis [2]. Inhibition of tumor neovascularization has been an important target for cancer therapy. The process of angiogenesis is complicatedly mediated by the interactions of multiple factors, including various growth factors such as vascular endothelial growth factor (VEGF) and cytokines, which induce vascular remodeling in normal tissue and neovascularization and triggering tumor metastasis [3–5]. Therefore, it is important to further elucidate the roles of angiogenesis-related molecules and the possible mechanisms they participate in tumor angiogenesis.

Biglycan is ubiquitously expressed in extracellular matrix. It is a key member of the small leucine-rich proteoglycan family [6]. Clinical studies have shown that biglycan expression is elevated in hepatocellular carcinoma [7], ovarian carcinoma [8], endometrial cancer [9], pancreatic cancer [10], gastric cancer [11], and colon cancer [12] and that biglycan up-regulation is associated with poor prognosis [13, 14]. Recent studies have demonstrated that biglycan expression is significantly higher in tumor vascular tissue than in normal vascular tissue; therefore, biglycan can be used as a marker of

tumor endothelial cells involved in neovascularization [15]. However, whether it involves in and the underlying mechanism of tumor angiogenesis requires further investigation. In this study, we sought to determine the effect of biglycan on VEGF expression, and the possible mechanism in angiogenesis was evaluated.

Methods

Cell culture

HCT116 human colon cancer cells were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in McCoy's 5A medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10 % fetal bovine serum (FBS, Hyclone, Logan, UT, USA). The cells were cultured in a 37 °C, 5 % CO_2 incubator. The medium was changed every 2–3 days. Cell passaging was performed using 0.25 % trypsin digestion after the cells reached 80 % confluence.

Plasmid construction and screening the stably transfected cell lines

The human biglycan messenger RNA (mRNA) sequence (NM_001711.4) was searched through the NCBI database. Reverse transcription polymerase chain reaction (RT-PCR) was used to amplify the complete coding sequence, which was then ligated into the pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA, USA) using the EcoRI and XhoI restriction sites. After sequencing, the resulting construct was named pcDNA3.1-biglycan. HCT116 cells (1.0×10^5 cells/well) were seeded in six-well plates and cultured overnight to attain 70–80 % confluence. The cells were then transfected with pcDNA3.1-biglycan or pcDNA3.1-empty vector using Lipofectamine 2000 (Invitrogen), according to the kit instructions. After 48 h, G418 (Invitrogen) solution was added to the culture to screen for stably transfected cell lines. G418-resistant colonies were selected after 4 weeks. After confirming biglycan expression, the cells were continuously cultured in G418-containing medium for subsequent experiments. The resulting cell lines were designated as HCT116/biglycan and HCT116/pcDNA3.1, respectively. The non-transfected cells served as control.

Cell viability assay

Transfected or non-transfected HCT116 cells were harvested, counted, and transferred into 96-well plates at a density of 1×10^4 cells/well. Subsequently, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) was added to the cultures 24–96 h, then the cell-free supernatants were removed, and the resulting formazan was dissolved in DMSO. The optical density was measured at 490 nm on a microplate reader [16, 17].

Real-time PCR

Total RNA from the cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). The RNA was reverse transcribed into complementary DNA (cDNA) using the cDNA first-strand synthesis kit (Takara, Dalian, China) in strict accordance with the kit instructions. The primers were utilized as follows: biglycan, 5′-GGTCTCCAGCACCTCTACGCC-3′ (upstream) and 5′-AACACTCCCTTGGGCACCTT-3′ (downstream). The length of this amplified fragment was 203 bp; VEGF, 5′-CCTGAAATGAAGGAAGAGGAGACT-3′ (upstream) and 5′-CTCGGTGATTTAGCAGCAAGA-3′ (downstream). The length of this amplified fragment was 150 bp; β-actin, 5′-CTTAGTTGCGTTACACCCTTTCTTG-3′ (upstream) and 5′-CTGTCACCTTCACCGTTCCAGTTT-3′ (downstream). The length of this amplified fragment was 156 bp. Real-time PCR was performed using an ExicyclerTM 96 fluorescence quantitative instrument (Bioneer, Daejeon, Korea). The PCR master mix included SYBR-Green Master Mix, the template cDNA, the forward primer, and the reverse primer. The PCR reactions were carried out as the following: 95 °C for 10 min; 40 cycles of 95 °C for 10 s, 58 °C for 20 s, and 72 °C for 30 s; and 4 °C for 5 min.

Cytokine analysis

The culture supernatants from different group cells were harvested, and the VEGF content was determined by ELISA according to the manufacturer's instructions (USCN Life Science. Inc., Wuhan, China).

Western blot analysis

HCT116 cells were treated with vehicle or extracellular signal-regulated kinase (ERK) inhibitor PD98059 (50 μM; R&D Systems, Wiesbaden-Nordenstadt, Germany) for 2 h. The total proteins from cells were extracted by RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Protein concentration was measured by BCA method. A total of 40 μg of protein from each group was analyzed by SDS-PAGE. After electrophoresis, the samples were electronically transferred to a PVDF membrane (Millipore, Bedford, MA, USA). Subsequently, 5 % skim milk powder was added at room temperature to block the membranes for 1 h. Next, diluted primary antibodies (Biglycan 1:100, p-ERK 1:100, and ERK 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA; VEGF 1:1000, Wanleibio, Shenyang, China) were added and incubated overnight at 4 °C. A 1:5000 dilution of the horseradish peroxidase (HRP)-labeled secondary antibody

(Beyotime Institute of Biotechnology) was added, and the membranes were incubated at 37 °C for 1 h. The targeted proteins were visualized with ECL substrate and analyzed by Image J software. The β-actin was used as an internal reference.

Tumor xenograft study

Female BALB/c (nu/nu) mice (6 weeks old) were purchased from the Experimental Animal Center of China Medical University. All animal care and experiment procedures were approved by the Ethics Committee on experimental animals of Liaoning Cancer Hospital and Institute. To initiate tumor xenografts, nu/nu mice were randomly divided into three groups: HCT116 group, mice injected with HCT116 cells; pcDNA3.1 group, mice injected with pcDNA3.1-empty-transfected cells; and biglycan group, mice injected with pcDNA3.1-biglycan-transfected cells (n=21 in each group); 1×10^7 HCT116 cells or transfected cells were injected into the armpit of the right forelimb of the mouse, respectively. The tumors were isolated every 3 days, and the tumor volumes were determined. All mice were euthanized at 21 days after inoculation; the tumors were removed and weighed, then fixed in 4 % paraformaldehyde fixation solution, and paraffin embedded for immunohistochemistry staining.

Immunohistochemical analysis

The expression of VEGF and CD34 in tumor tissues was assessed by immunohistochemical analysis. Briefly, paraffin-embedded tumor tissue was sliced into 5-μm sections. The sections were dewaxed to hydrate using graded alcohol. Sections were placed in 0.01 mol/L citrate buffer for antigen retrieval for 10 min and then incubated with 0.3 % H_2O_2 at room temperature for 15 min to exhaust endogenous peroxidase activity. After being blocked with normal goat serum, a 1:100 dilution of the CD34 and VEGF antibodies (Wanleibio, Shenyang, China) was added to the slice respectively and incubated overnight at 4 °C. The slides were then washed with PBS and incubated with biotinylated goat anti-rabbit IgG (1:200 dilution) for 30 min at room temperature. After that, the sections were again washed and incubated with HRP-labeled avidin for 30 min at room temperature. After DAB staining, the hematoxylin was used for re-staining. The immunohistochemistry changes were observed under a light microscope.

Statistical analysis

All of the experiments were performed in triplicate. All of the data are presented as the mean±standard deviation. One-way analysis of variance was used for the analyses between the groups; Bonferroni's post hoc test was used for multiple comparisons. The results were considered statistically significant when $P<0.05$.

Results

Establishment of stable biglycan-overexpressing colon cancer cell lines

To investigate the role of biglycan in colon cancer angiogenesis, we constructed a biglycan overexpression plasmid and transfected it into HCT116 cells. The mRNA and protein levels of biglycan in the transfected cells were determined. As shown in Fig. 1, low levels of biglycan were detected in the pcDNA3.1-empty-transfected cells and non-transfected cells, whereas the mRNA level of biglycan in the pcDNA3.1-biglycan-transfected cells was significantly elevated compared with the pcDNA3.1-empty-transfected cells or non-transfected cells ($P<0.01$, Fig. 1a). Western blot results also confirmed that overexpression of biglycan presented in pcDNA3.1-biglycan-transfected cells ($P<0.01$, Fig. 1b, c). Furthermore, a significant increase in cell viability was seen at 48 h ($P<0.01$, Fig. 1d), and the increase rate persisted higher than that in the pcDNA3.1-empty-transfected or non-transfected cells at 96 h. These results indicated that the stably transfected cell clones which overexpressed biglycan were successfully established and biglycan overexpression promoted the HTC116 cell viability; the stable cell clones could be used for subsequent in vivo studies.

Biglycan up-regulated VEGF expression in colon cancer cells

Abundant evidence has demonstrated that VEGF plays an important role in cell proliferation and tumor angiogenesis. To test whether overexpression of biglycan affects the VEGF expression in colon cancer cells, we determined the VEGF expression in mRNA and protein levels. The results showed that overexpression of biglycan significantly enhanced the VEGF transcription (Fig. 2a), as well as the protein expression of VEGF in pcDNA3.1-biglycan-transfected cells or in the cell culture supernatants ($P<0.01$, Fig. 2b–d). The results indicate that biglycan up-regulates the VEGF expression in colon cancer cells.

Biglycan increased VEGF expression and promoted xenograft tumor growth and angiogenesis

The effects of biglycan on colon tumor growth and angiogenesis in vivo were investigated subsequently. Tumor volume and weight were measured to index tumor growth. As illuminated in Fig. 3a–c, the xenograft colon tumor volume and weight were dramatically increased by overexpression of

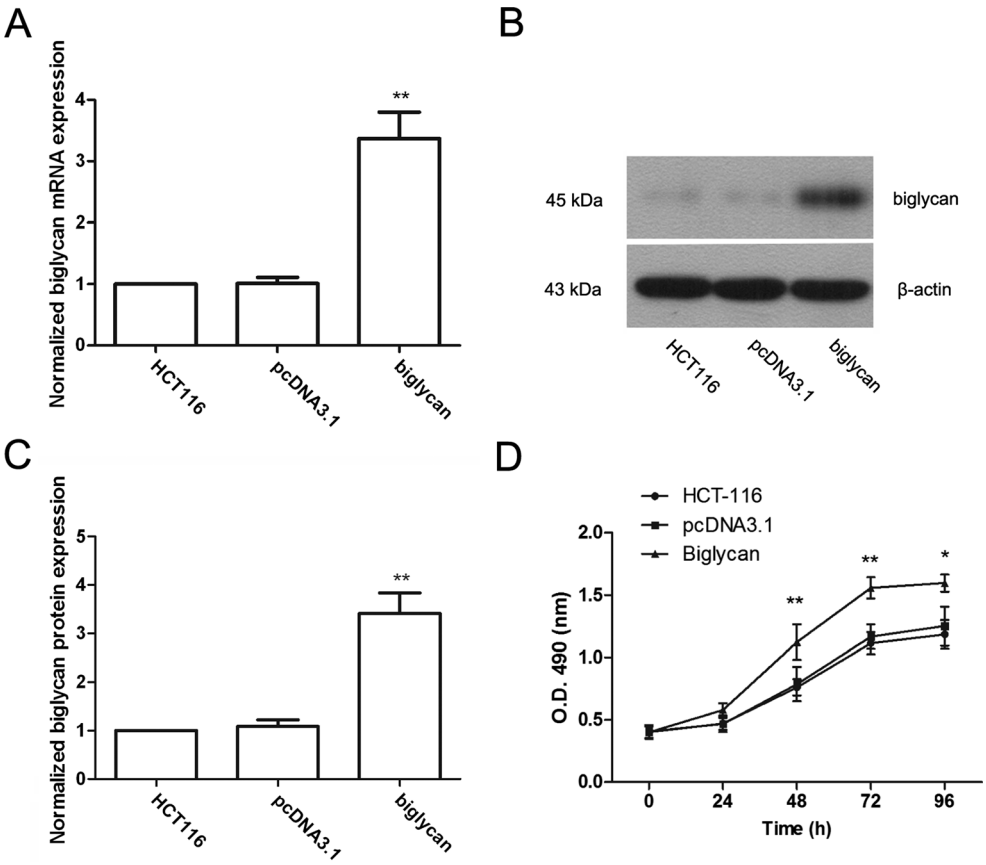

Fig. 1 Establishment of stable biglycan-overexpressing cell lines. The mRNA (**a**) and protein (**b**, **c**) expression of biglycan from pcDNA3.1-biglycan, pcDNA3.1-empty, or non-transfected cell groups was measured by RT-PCR or Western blot. The β-actin was used as an internal reference to normalize the relative protein expression of biglycan. Overexpression of biglycan increases HCT116 cell viability (**d**). The cell viability was determined by MTT assay. All data are presented as the mean±standard deviation. Compared with the control group, $*P<0.05$ or $**P<0.01$

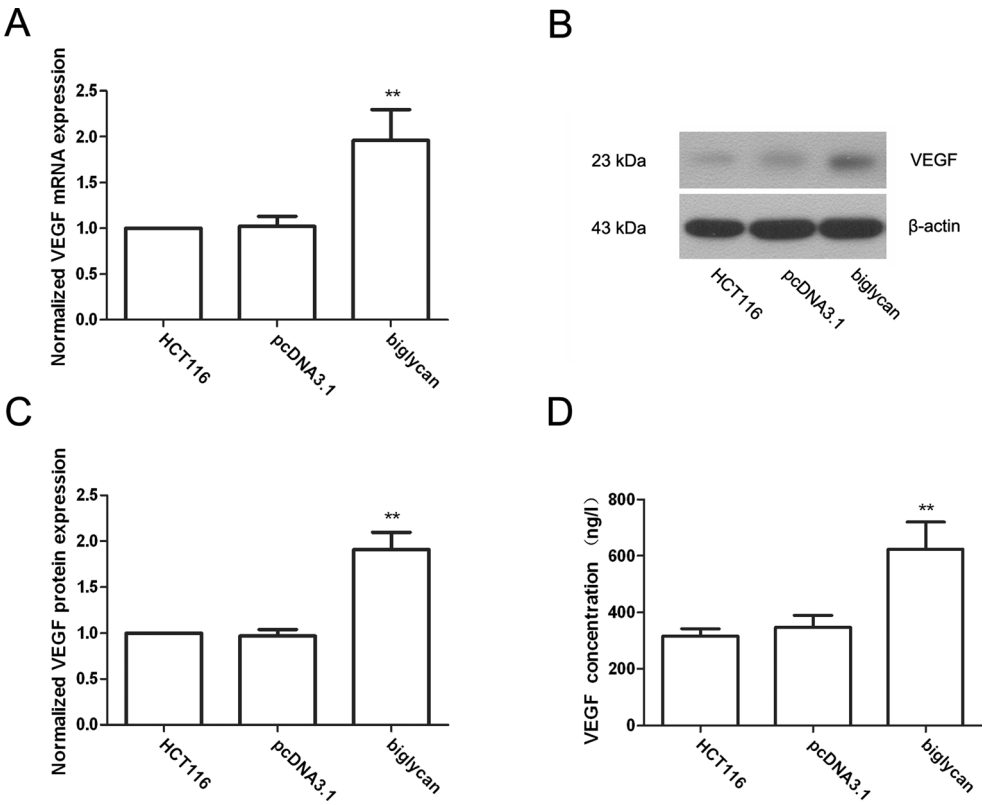

Fig. 2 Effects of biglycan on VEGF expression in colon cancer cells. VEGF **a** mRNA transcription and **b**, **c** protein expression in pcDNA3.1-biglycan, pcDNA3.1-empty, and non-transfected cells or **d** in the cell culture supernatants were determined by RT-PCR, Western blot, and ELISA, respectively. The β-actin was used as an internal reference to normalize the relative protein expression of biglycan. All of the experiments were repeated three times, and the data are presented as the mean±standard deviation. Compared with the control group, $**P<0.01$

biglycan compared to those in the control or pcDNA3.1 groups ($P<0.01$), which indicate that high level of biglycan promotes colon cancer cell proliferation and tumor growth in vivo. CD34 is a marker of small vessel endothelial cells; CD34 expression in the xenograft tumor tissues was detected by immunohistochemical analysis. Figure 3d showed a widespread expression of CD34 in the tumor tissues of pcDNA3.1-biglycan group. Moreover, overexpression of biglycan promoted VEGF expression in the xenograft tumors (Fig. 3e). Take together, the results above indicated that biglycan was able to promote the tumor growth and angiogenesis possibly through up-regulation of VEGF.

Biglycan increased VEGF expression through the ERK signaling pathway

Activation of ERK is critical in the regulation of VEGF expression. To test whether biglycan up-regulate VEGF expression through the ERK signaling pathway, ERK inhibitor PD98059 was added into the cell cultures. Western blot results (Fig. 4a–d) showed that VEGF expression and the phosphorylation of ERK were markedly increased in pcDNA3.1-biglycan-transfected cells. However, the increase was suppressed by administration of ERK inhibitor PD98059 ($P<0.05$), parallel with this, ERK inhibitor PD98059 administration notably

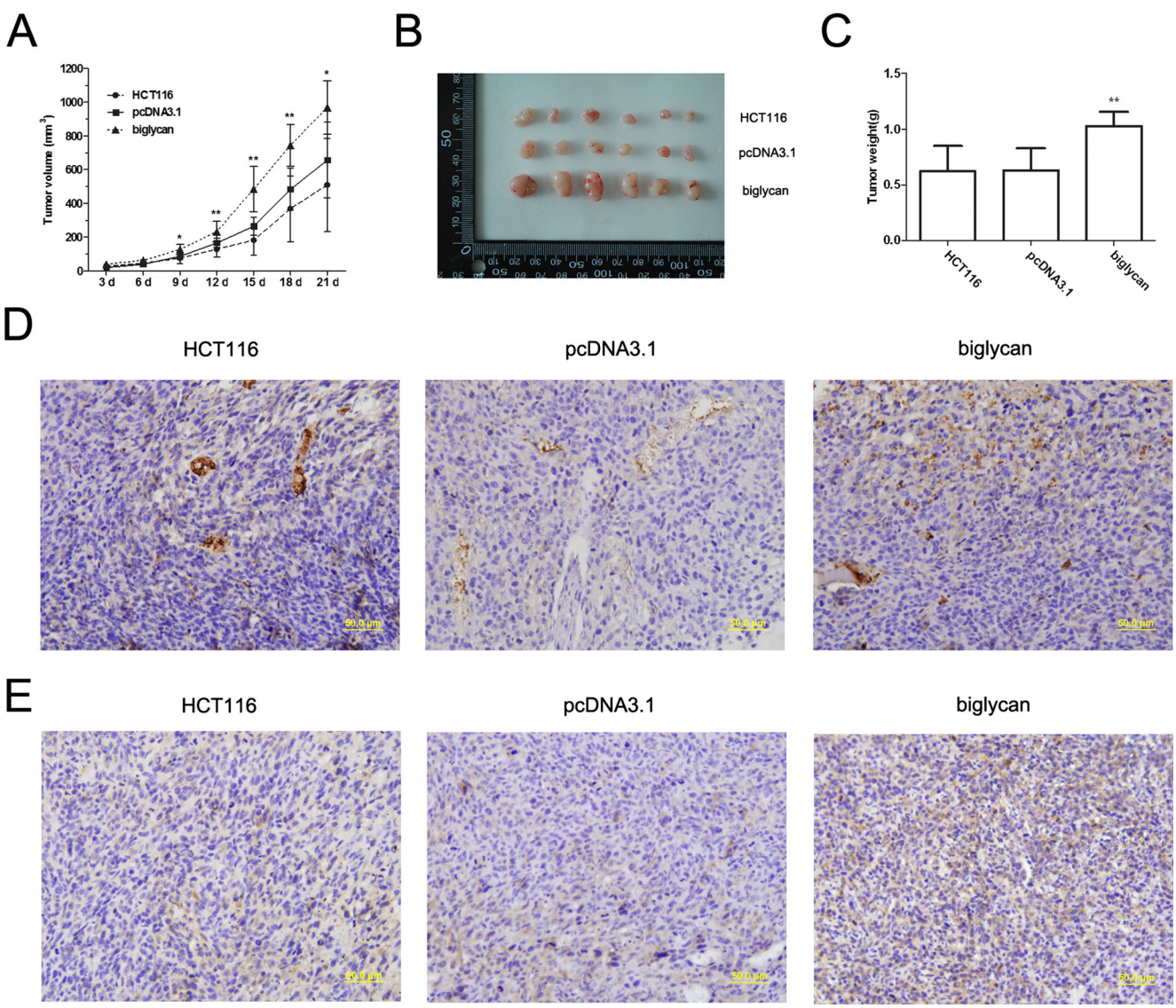

Fig. 3 Biglycan promoted xenograft tumor growth and angiogenesis (**a**) Tumor volume measurement. Transfected or non-transfected cells (1 × 10) were injected into the armpit of the right forelimb of nude mice, respectively. The tumor volume from biglycan, pcDNA3.1 or control groups was measured every 3 days; (**b**) Visualization of tumor size. (**c**) Comparison of tumor weight from biglycan, pcDNA3.1, and control groups. (**d**) Immunohistochemical analysis of CD34 expression in the xenograft tumors. (**e**) Immunohistochemical analysis of VEGF expression in the xenograft tumors. All of the experiments were c and the data are presented as the mean±standard deviation. Compared with the control group, $*P<0.05$, $**P<0.01$

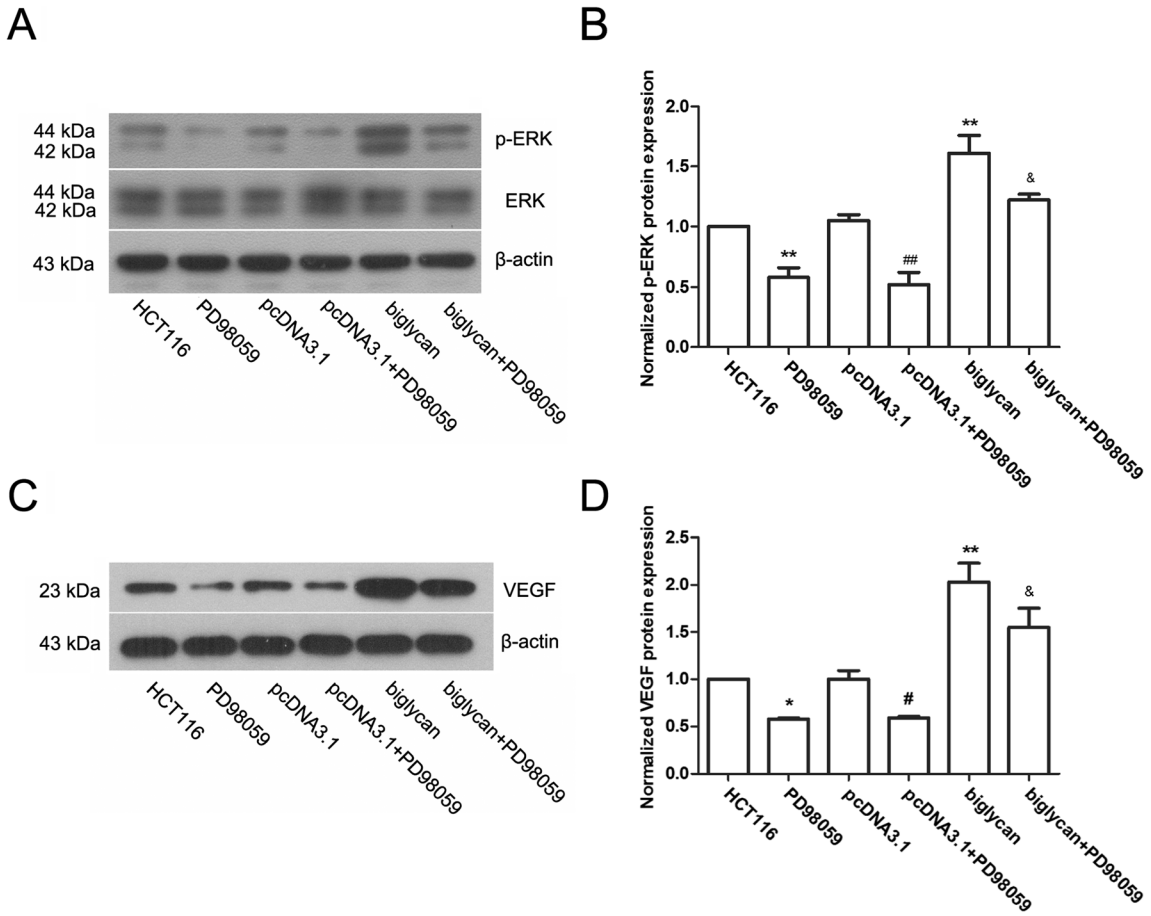

Fig. 4 Biglycan regulated VEGF expression through the ERK signaling pathway. **a** The activation of the ERK signaling pathway was detected by Western blot. **b** Total ERK expression levels were used to normalize the relative expression of p-ERK. **c** VEGF protein expression was measured by Western blot. **d** β-actin was used as an internal reference to normalize VEGF expression. All of the experiments were repeated three times, and the data are presented as the mean±standard deviation. Compared with the control group, $*P<0.05$ or $**P<0.01$; compared with the pcDNA3.1 group, $\#P<0.05$ or $\#\#P<0.01$; compared with the biglycan group, $\&P<0.05$

inhibited the phosphorylation of ERK and VEGF expression in the non-transfected or pcDNA3.1-transfected cells ($P<0.01$). The results revealed that biglycan up-regulates VEGF expression possibly through activating the ERK signaling pathway.

Discussion

We previously reported that the expression of biglycan was up-regulated in human colorectal cancers. Hence, biglycan may be a useful molecular target for colorectal cancer [18]. In the present study, we detected the effect of biglycan on VEGF expression in pcDNA3.1-biglycan-transfected colon cancer cells and xenograft tumors. The results showed that biglycan overexpression promoted angiogenesis and colon tumor growth, as well as increased the VEGF expression in colon cancer cells and in xenograft colon tumors. However, the up-regulation of VEGF was suppressed by ERK inhibitor PD98059. Our data indicate that biglycan overexpression promotes angiogenesis and colon tumor growth, and biglycan-mediated VEGF up-regulation may correlate with the activation of the ERK signaling pathway.

Biglycan has been reported to play an important role in tumor progress, and a high level of biglycan implies poor prognosis [13, 14]. Yamamoto et al. indicated that biglycan was highly expressed in tumor vascular tissue, and knockdown biglycan inhibited the tumor endothelial cell migration and tube formation [15]. A recent study showed that overexpression of biglycan enhanced the gastric cancer cell invasion [19]. Our lab previously reported that biglycan up-regulation was closely correlated with poor tumor differentiation and lymph node metastasis in colorectal cancers. To assess the effects of biglycan overexpression in colon cancer, we first constructed the biglycan overexpression vectors and established the stable biglycan overexpression in human colon cancer cell lines (HCT116 /biglycan cells), which were confirmed by Western blot and RT-PCR results. Interestingly, biglycan overexpression caused a significant up-regulation of VEGF both in biglycan-transfected colon cells and in the

xenograft colon tumors. Moreover, high biglycan expression obviously promoted the xenograft colon tumor growth, as indicated by the increased tumor size and weight, as well as the colon cancer cell viability. Our results were in line with the previous data and indicate that biglycan participates in the development of colon cancer.

Angiogenesis plays an important role in tumorigenesis. New blood vessels supply nutrients during the tumor cell proliferation and migration [20]. CD34 mainly presents on the surface of hematopoietic stem cells, progenitor cells, and small vessel endothelial cells [21]. It is used as a marker of microvessel density in tumors [22]. VEGF is another critical mediator of angiogenesis especially in cancer tissue [23]. Massive evidence has demonstrated that VEGF prevents tumor cell apoptosis and induces cell proliferation and migration [24, 25]. High levels of VEGF are correlated with many malignant tumors with poor prognosis, such as colon cancer [26]. In the present study, we found that biglycan increased VEGF expression and secretion in the pcDNA3.1-biglycan-transfected colon cancer cells. In tumor xenograft study, we also confirmed that biglycan overexpression up-regulated the VEGF levels in the xenograft tumors and promoted angiogenesis. Our results revealed that biglycan is involved in regulating malignant transformation by up-regulation of VEGF. The results were consistent with previous findings, which reported that the VEGF expression is reduced in biglycan knockout mice [27].

Previous studies have shown that the ERK signaling pathway participates in regulation of endothelial cell proliferation, apoptosis, the formation of new blood vessels, and VEGF expression [28, 29]. The ERK pathway has also been implicated in mediating biglycan signaling in other cell types [30, 31]. To further investigate the possible mechanisms of biglycan in colon cancer, we detected the ERK pathway in colon cancer cells. The results showed that biglycan overexpression up-regulated the VEGF levels and the phosphorylation of ERK, whereas the ERK inhibitor PD98059 effectively reversed the increase of VEGF induced by biglycan. The results indicate that biglycan may up-regulate the VEGF expression by activating the ERK signaling pathway.

Conclusion

Our results showed that biglycan overexpression promoted angiogenesis and colon tumor growth, as well as increased the VEGF expression in colon cancer cells and in xenograft colon tumors. The mechanism by which biglycan exerts its effects is partially through activating the ERK signaling pathway. Therefore, biglycan may be a promising target for anti-angiogenic therapy for cancer.

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Conflicts of interest None

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