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RESEARCH ARTICLE

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β-Elemene inhibits peritoneal metastasis of gastric cancer cells by modulating FAK/Claudin-1 signaling

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Peritoneal metastasis is common in advanced gastric cancer patients and is typically associated with a worse prognosis. β-Elemene is a natural compound that can be isolated from the *Curcuma wenyujin* plant and has been widely used in China to treat a variety of cancers. However, the anti-metastatic impacts of β-elemene on gastric cancer remain unknown. In our study, we found that β-elemene significantly inhibited the migration and invasive capacity of gastric cells in vitro and inhibited the capacity of gastric cancer cells to peritoneally diffuse and metastasize in vivo. Mechanistically, we demonstrated that the anti-metastatic effects of β-elemene were exerted by downregulating the expression of Claudin-1. Furthermore, β-elemene was found to inhibit the metastatic capacity of cells by downregulating FAK phosphorylation, which regulated Claudin-1. Overall, our result revealed that β-elemene inhibited peritoneal metastases from gastric cancer by modulating the FAK/Claudin-1 pathway.

KEYWORDS

Claudin-1, FAK, gastric cancer, peritoneal metastasis, β-Elemene

1 | INTRODUCTION

Gastric cancer is a worldwide problem, leading to a multitude of deaths each year (Van Cutsem, Sagaert, Topal, Haustermans, & Prenen, 2016). Despite improvements in diagnosis and treatment, the overall survival of gastric cancer patients remains low, largely due to many patients having inoperable disease at diagnosis, the occurrence of distant metastases, or recurrent disease (Edwards, Davidson, Calamai, Cunningham, & Starling, 2018; Van Cutsem et al., 2016). Peritoneal metastasis is a common form of metastasis in advanced gastric cancer patients, which is typically associated with a worse prognosis (Dahdaleh & Turaga, 2018; Mikula-Pietrasik, Uruski, Tykarski, & Ksiazek, 2018). Therefore, developing pharmaceutical interventions that target peritoneal metastasis is a new focus for gastric cancer therapy.

β-Elemene can be isolated from the *Curcuma wenyujin* plant and has been widely used in China for the treatment of various cancer types (Y. Liu et al., 2017; Zhai et al., 2018; G. N. Zhang, Ashby, Zhang, Chen, & Guo, 2015). Several studies have revealed that β-elemene can inhibit cell proliferation and induce apoptosis in a variety of cancers, including breast (B. Zhang, Zhang, Tang, Zheng, & Zhang, 2012) and lung cancers (J. Liu et al., 2012; Y. Liu et al., 2017). Recent studies have demonstrated that β-elemene can reduce B16F10 melanoma cell invasion (Shi et al., 2014) and reduce the metastatic potential of MCF-7 breast cancer cells (X. Zhang, Zhang, & Li, 2013). However, the anti-metastatic effects of β-elemene on gastric cancer have not been shown.

Claudin-1 is a critical component of tight junctions and plays a crucial role in regulating proliferation and metastasis (Zeisel, Dhawan,

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& Baumert, 2018). Several studies have indicated that Claudin-1 is upregulated in colorectal (de Oliveira, de Oliveira, De Souza, & Morgado-Diaz, 2005), melanoma (Leotlela et al., 2007), and gastric cancer (Eftang et al., 2013) and can promote the epithelial-mesenchymal transition (EMT; Stebbing, Filipović, & Giamas, 2013). Efforts to target Claudin-1 have become a novel strategy to inhibit tumor metastasis (Hashimoto, Fukasawa, Kuniyasu, Yagi, & Kondoh, 2017). Focal adhesion kinase (FAK) is a nonreceptor protein tyrosine kinase that can promote tumor invasion and metastasis (Kleinschmidt & Schlaepfer, 2017). Previous studies have emphasized that FAK plays a crucial role in modulating tight junctions by regulating occludin and ZO-1 expression (Linlin, Mruk, Wing-Yee, Lee, & Yan, 2011; Ma et al., 2013). However, the regulation of β-elemene on FAK and Claudin-1 has not been investigated.

In this study, we demonstrated that β-elemene significantly prevented gastric cancer cell peritoneal metastasis through the FAK/Claudin-1 pathway. Thus, β-elemene could be an effective treatment for gastric cancer patients with suspicious peritoneal metastasis.

2 | MATERIALS AND METHODS

2.1 | Reagents

β-Elemene (molecular formula of $C_{15}H_{24}$ and molecular weight of 204.35) was purchased from Sigma-Aldrich (St. Louis, MO, USA). An injectable solution of β-elemene was obtained from Jingang Pharmaceuticals (#081152, Dalian, China).

2.2 | Cell culture

The gastric cancer cell lines, BGC823 and SGC7901, were obtained from the Cell Bank of the Type Culture Collection of the Chinese Academy (Shanghai, China) in June 2016. In January 2018, cell lines used in this study were sent to the Chinese Academy of Sciences and authenticated using short tandem repeat profiling. All cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum.

2.3 | Cell viability assay

BGC823 and SGC7901 cells were seeded overnight in 96-well cell culture plates. Cells were treated with various concentrations of β-elemene for 24 hr. MTT solution (20 μl, 5 mg/ml) was added to each well and incubated for 4 hr at 37°C. The supernatant was removed, and 200 μl of dimethyl sulfoxide was added to each well to dissolve the precipitate. Absorbance at 570 nm was measured using a spectrophotometer.

2.4 | Cell migration and invasion assay

Cell migration and invasion assays were performed using Transwell chambers (Corning Scientific, Corning, NY, USA). For migration assays, cells were placed in the upper chamber of the Transwell insert and incubated with various concentrations of β-elemene. RPMI-1640 containing 2% fetal bovine serum was added to the lower chamber. After 12 or 24 hr, adherent cells were removed from the upper surface of the chamber, whereas migrated cells on the lower surface of the chamber were stained with Wright–Giemsa dye and imaged with a brightfield light microscope at 200 *g* magnification. Cell invasion experiments were performed identically to cell migration assays, except the Transwell membranes were precoated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA).

2.5 | Animal experiments

Four-week-old female BALB/c nude mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and housed in a pathogen-free environment in the Animal Laboratory Unit of China Medical University. Mice were inoculated peritoneally with BGC823 cells and randomized to receive either phosphate-buffered saline (q3dx7, ip, $n = 5$) or β-elemene (25 mg/kg, q3dx7, ip, $n = 5$). The dosage for animal depends upon our previous report (Zhang et al., 2013). In our previous studies about gastric cancer, we confirmed that 25 mg/kg of β-elemene could not significantly inhibit the growth of tumor xenografts. All mice were sacrificed 16 days post cell line inoculation, and metastatic peritoneal nodules were counted and analyzed. All animal experiments were preapproved by the Committee on Animal Care of China Medical University (approval code: 2018113).

2.6 | Western blot analysis

Western blot analysis was performed as previously described (Deng et al., 2018). The following primary antibodies were used: rabbit anti-Claudin-1 polyclonal antibody (1:1,000; #13255S, Cell Signaling Technology, Boston, MA, USA), rabbit anti-FAK polyclonal antibody (1:1,000; #3285S, Cell Signaling Technology), rabbit anti-p-FAK polyclonal antibody (1:1,000; #3281S, Cell Signaling Technology), rabbit anti-ZO-1 polyclonal antibody (1:1,000; #13663, Cell Signaling Technology), mouse anti-Occludin polyclonal antibody (1:1000; #sc-133256, Santa Cruz Biotechnology, Dallas, TX, USA), and mouse anti-Actin polyclonal antibody (1:250; #sc-47778, Santa Cruz Biotechnology, Dallas, TX, USA). Immunoreactive bands were analyzed with ImageJ software.

2.7 | RNA-seq

Total RNA was isolated from cultured cells. RNA integrity was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples with RNA integrity numbers ≥7 were subjected to subsequent analyses. Libraries were constructed using TruSeq Stranded Total RNA with Ribo-Zero Gold, according to the manufacturer's instructions. Libraries were then sequenced on an Illumina sequencing platform (HiSeq™ 2500), and 150/125-BP paired-end reads were generated.

2.8 | RNA isolation and real-time polymerase chain reaction

Isolation and reverse transcription of RNA was performed as previously described (Deng et al., 2018). The comparative cycle threshold (Ct) method was used to calculate the relative expression of Claudin-1, and the expression of the 18S small nuclear RNA was used as the reference. The polymerase chain reaction (PCR) conditions included 10 min at 95°C, followed by 45 cycles of 95°C for 15 s, and 58°C for 34 s.

2.9 | Plasmid transfections

The overexpression plasmids containing whole coding sequence of FAK, Claudin-1, and pcDNA 3.1 vector served as the NC were purchased from GeneChem (Shanghai, China), and we use pcDNA3.1–Claudin-1 and pcDNA3.1–FAK to represent overexpressed Claudin-1 and FAK. Cells were seeded at 2×10^5 cells/well in six-well plates overnight and then transfected by Lipofectamine 2000 reagent with plasmid. After transfections for 48 hr, the expression of FAK and Claudin-1 was evaluated by western blot.

2.10 | Statistical analysis

All data are presented as the mean ± standard deviation and are representative of at least three independent experiments. Statistical analyses were performed using Statistical Package for the Social Sciences 13.0 software (International Business Machines, Armonk, NY, USA). Statistical comparisons were calculated using a two-tailed Student's *t* test, with $p < .05$ being considered statistically significant.

3 | RESULTS

3.1 | β-Elemene inhibits human gastric cancer cell migration and invasion

We first measured the cytotoxicity of β-elemene on BGC823 and SGC7901 gastric cancer cell lines (Figure 1b). Concentration gradient experiments demonstrated minimal cytotoxic effects of β-elemene at 5 μg/ml. Therefore, β-elemene was used at 1 or 5 μg/ml in the remainder of the study to investigate the influence of β-elemene on migration and invasion only.

After treatment of cells with β-elemene (1 or 5 μg/ml) for 12 and 24 hr, migration and invasion were significantly decreased in Transwell experiments (Figure 1c,d). These results showed that β-elemene significantly decreased gastric cancer cell migration and invasion in a dose-dependent manner.

3.2 | β-Elemene inhibits peritoneal metastasis of gastric cancer cells

The peritoneum is a common site for the metastasis of gastric cancer (Mikula-Pietrasik et al., 2018). Peritoneal involvement is also a sign of disease recurrence and is associated with a worse prognosis (Dahdaleh & Turaga, 2018). To investigate whether β-elemene can inhibit gastric cancer peritoneal metastasis, a nude mouse peritoneal metastasis model was developed. BGC823 cells were intraperitoneally injected with or without concurrent β-elemene treatment. Metastatic tumors were measured on the peritoneal surface and specifically observed around the mesenteric area (Figure 2a). All tumors were confirmed pathologically with hematoxylin and eosin (H&E) staining (Figure 2b). The total number of disseminated peritoneal tumors was quantified (Figure 2c) and found to be significantly decreased with β-elemene treatment. Additionally, β-elemene significantly reduced the number of nodules observed on the liver, spleen, and kidney (Figure 2d). Above all, these results demonstrated that β-elemene inhibited the ability of gastric cancer cells to peritoneally diffuse and metastasize in vivo.

3.3 | β-Elemene prevents the metastatic capacity of gastric cancer cells by downregulating Claudin-1 expression

To further investigate the regulatory mechanisms of the anti-metastatic effect of β-elemene, we treated BGC823 cells with β-elemene and performed a comprehensive transcriptomic analysis of treated cells with RNA-seq. Compared with control cells, 1,166 significantly upregulated and 1,241 significantly downregulated genes were identified in β-elemene–treated cells (Figure 3a). These genes were further investigated using gene set enrichment analysis (Subramanian et al., 2005). The "Cell_Adhesion_Molecules_CAMs" and "Tight Junction" pathways were identified as being differentially regulated (Figure 3b), as well as biologically relevant because of the importance of these pathways in regulating cancer cell metastasis. Next, we verified several key proteins in these pathways including ZO-1, Occludin, and Claudin-1 by quantitative real-time PCR and western blotting. In Figure S1, the change of Claudin-1 was most significant after treatment with β-elemene. Therefore, we try to explore the role of Claudin-1 in the anti-metastasis process of β-elemene. Claudin-1 is a tight junction protein that takes part in EMT and metastasis. Next, we explored the relationship between β-elemene and Claudin-1. Quantitative real-time PCR (Figure 3c) and western blotting (Figure 3 d) analyses showed that β-elemene downregulated the expression of Claudin-1 in gastric cancer cell lines. Overexpressing Claudin-1 in the presence of β-elemene restored Claudin-1 expression (Figure 3e). The overexpression of Claudin-1 also increased the migratory and invasive ability of gastric cancer cells. In addition, the overexpression of Claudin-1 in the presence of β-elemene also partially rescued the migratory and invasive capacity of gastric cancer cells (Figure 3f,g).

3.4 | β-Elemene inhibits Claudin-1 expression by attenuating FAK phosphorylation

We sought to explore the relationship between β-elemene and Claudin-1. Activation of FAK has been shown to increase tumor

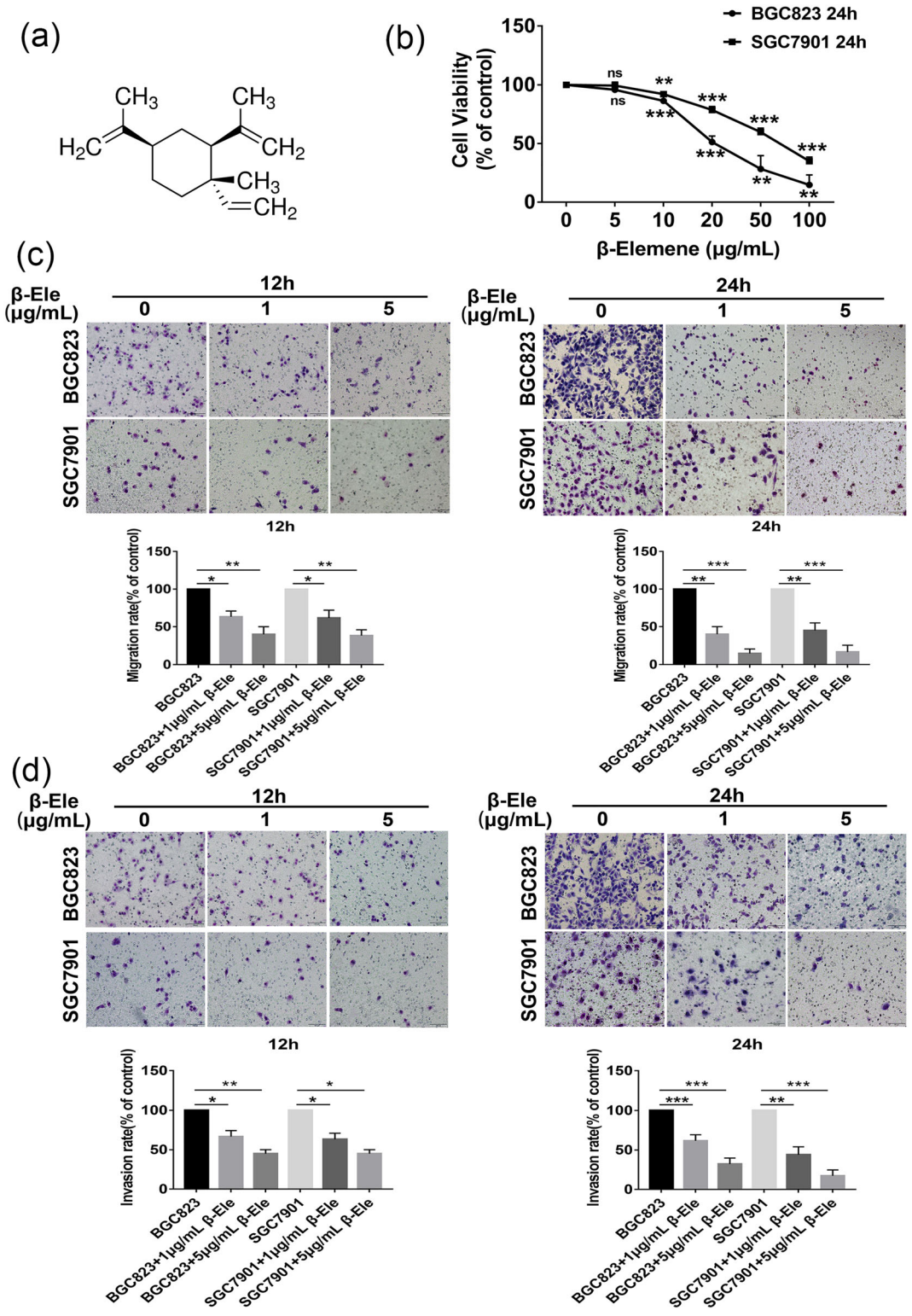

FIGURE 1 β-Elemene inhibits the migration and invasion of gastric cancer cells in vitro. (a) The structure of β-elemene. (b) The gastric cancer cell lines, BGC823 and SGC7901, were treated with different β-elemene concentrations for 24 hr. (c,d) The effect of β-elemene treatment on cell migration and invasion was determined after 12 and 24 hr of treatment. Representative images of migratory and invasive cells on Transwell membranes (magnification, 200 *g*) are shown on the upper. Panels on the lower display the quantification of cell migration and invasion. Scale bar = 100 μm. All experiments were performed in triplicate, and data are represented as the mean ± SD. ns: not significant (versus the control group). $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ [Colour figure can be viewed at wileyonlinelibrary.com]

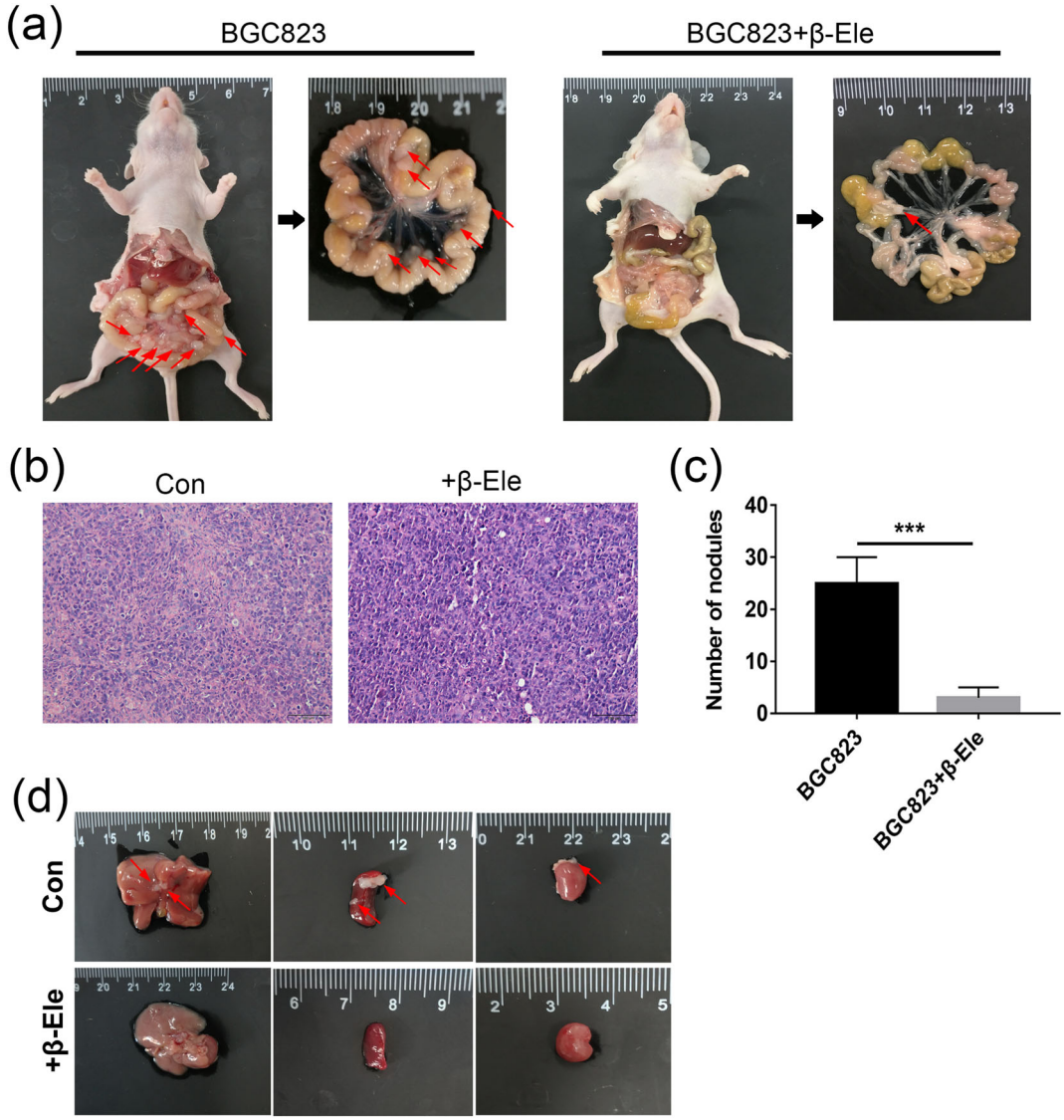

FIGURE 2 β-Elemene inhibits peritoneal metastasis of gastric cancer cells in vivo. (a) Images of the macroscopic appearance of peritoneal metastatic nodules (red arrows) in mice treated with phosphate-buffered saline or β-elemene (n = 5 per group). (b) Images of pathological H&E staining assessments per tumor in each group. (c) The average number of peritoneal nodules is displayed. Data are represented by the mean ± SD. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ (versus control group). (d) Representative images of the liver, spleen, and kidneys from treated mice [Colour figure can be viewed at wileyonlinelibrary.com]

invasion and metastasis (Kleinschmidt & Schlaepfer, 2017). Several studies have shown FAK to be involved in modulating tight junctions, by altering the expression of occludin and ZO-1 (Linlin et al., 2011). However, it has not been determined whether FAK regulates Claudin-1 expression. Moreover, it has not been determined whether β-elemene regulates Claudin-1 expression via FAK.

To investigate the relationship between FAK and Claudin-1, we blocked FAK activity with the FAK inhibitor, PF-573228. We found that Claudin-1 expression decreased with PF-573228 treatment (Figure 4a), indicating that FAK regulated Claudin-1 expression. Finally, Claudin-1 overexpression in the presence of PF-573228 treatment restored the migratory (Figure 4b) and invasive (Figure 4c) ability of gastric cancer cells. Thus, the FAK/Claudin-1 pathway is involved in the malignant progression of gastric cancer by regulating metastasis.

Finally, we investigated whether β-elemene treatment repressed the metastasis of gastric cancer cells by inhibiting the FAK/Claudin-1 pathway. We found that β-elemene treatment significantly inhibited FAK phosphorylation in a dose-dependent manner (Figure 4d). Additionally, the overexpression of FAK in the presence of β-elemene restored Claudin-1 expression (Figure 4e), thus revealing that β-elemene could regulate the FAK/Caludin-1 pathway.

4 | DISCUSSION

Peritoneal metastases are common in advanced gastric cancer patients and typically confer a poor prognosis (Dahdaleh & Turaga, 2018; Edwards et al., 2018; Mikula-Pietrasik et al., 2018). Thus, exploring

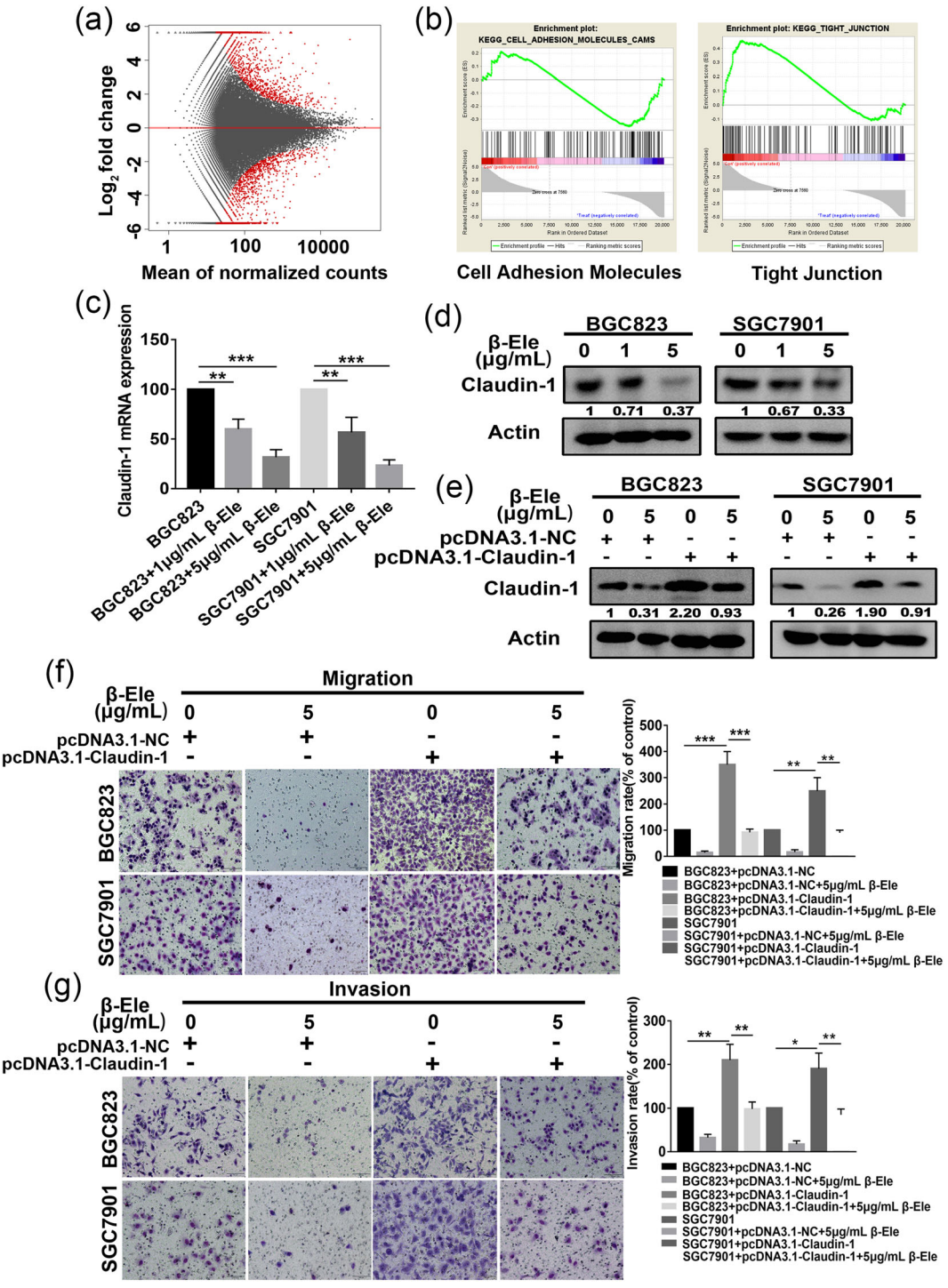

FIGURE 3 β-Elemene inhibits gastric cancer cell metastasis by inhibiting Claudin-1. (a) The M-versus-A plot of genes that were differentially expressed in SGC7901/ADR cells after β-elemene treatment. (b) Gene set enrichment analysis results showing that "Cell_Adhesion_Molecules_CAMs" and "Tight Junction" pathways were significantly associated with β-elemene treatment. (c) Quantitative real-time polymerase chain reaction analysis of Claudin-1 in human gastric cancer cells treated with increasing concentrations of β-elemene (5 μg/ml) for 24 hr. (d) Western blot analysis of Claudin-1 in human gastric cancer cells treated with increasing concentrations of β-elemene (5 μg/ml) for 24 hr. (e) BGC823 and SGC7901 cells were transiently transfected with Claudin-1 for 48 hr, followed by 5-μg/ml β-elemene treatment for 24 hr. Protein expression was analyzed by western blotting. (f,g) Cell migration (upper) and invasion (lower) were quantified after β-elemene (5 μg/ml) treatment for 24 hr, post-Claudin-1 transfection. Data are represented by the mean ± SD. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ (versus control group) [Colour figure can be viewed at wileyonlinelibrary.com]

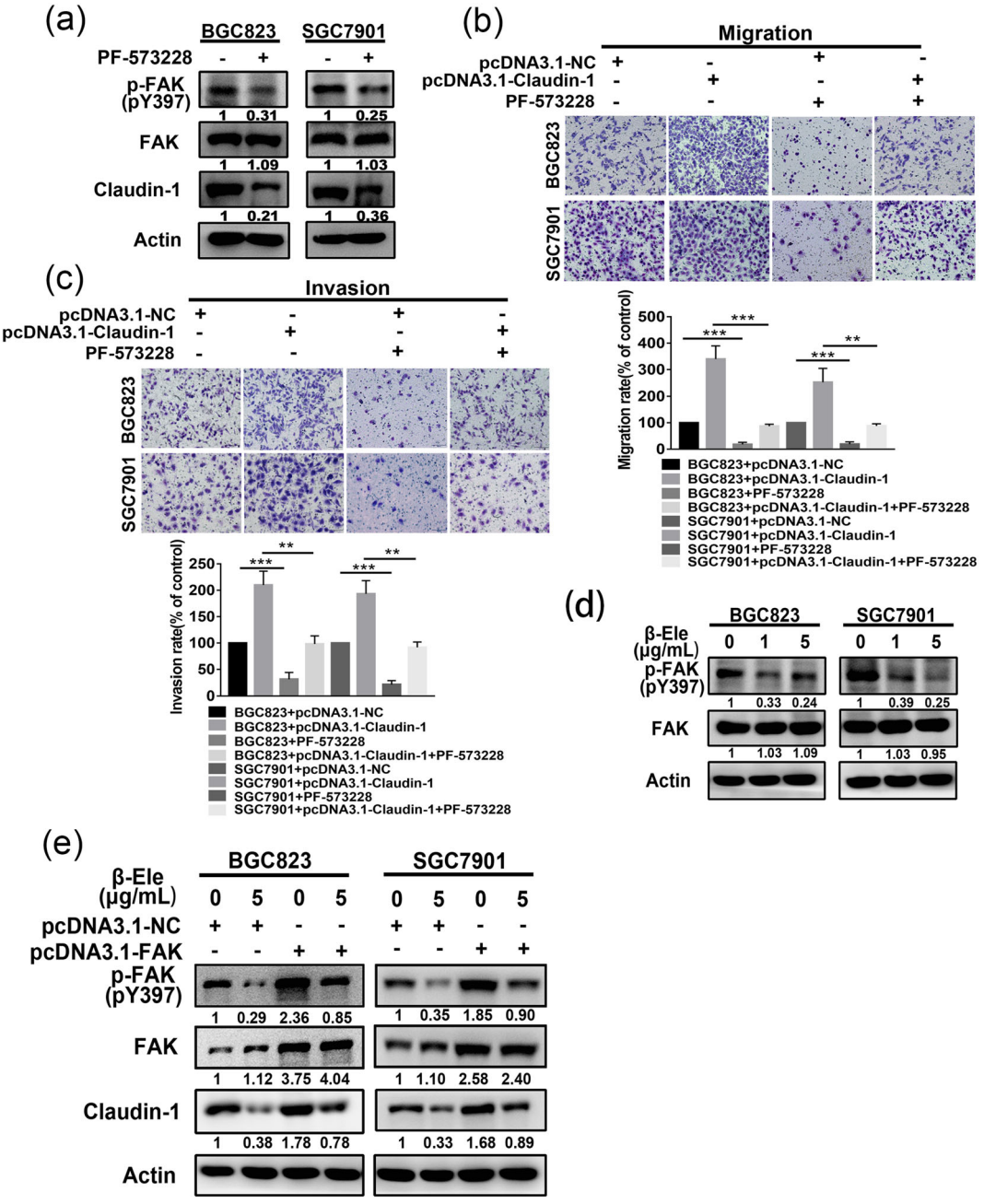

FIGURE 4 β-Elemene represses the metastatic ability of cells by inhibiting the FAK/Claudin-1 pathway. (a) The effect of PF-573228 (10 μM) treatment for 24 hr on the expression of Claudin-1 was detected by western blotting. β-actin was used as an internal loading control. (b,c) Cell migration (upper) and invasion (lower) were quantified after PF-573228 (10 μM) treatment for 24 hr, following Claudin-1 transfection. (d) The effect of β-elemene (5 μg/ml) treatment for 24 hr on the expression of Claudin-1 was measured using western blotting. β-actin was used as the internal loading control. (e) Cells were transiently transfected to overexpress FAK for 48 hr, followed by 5-μg/ml β-elemene treatment for 24 hr. Protein expression was analyzed by western blotting. Data are represented by the mean ± SD. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ (versus control group) [Colour figure can be viewed at wileyonlinelibrary.com]

treatments for peritoneal metastases in gastric cancer patients is urgently needed due to the poor efficacy of systemic chemotherapies in such patients.

β-Elemene is a promising novel plant–derived compound with broad-spectrum anticancer activity and has the potential to be further developed as an anti-metastatic drug (Shi et al., 2014; Zhang et al., 2013; Zhang, Zhang, & Li, 2013). However, the anti-metastatic impact of β-elemene on gastric cancer cells has not previously been elucidated. This study is the first to report that β-elemene inhibited the invasive ability of gastric cancer cells in vitro, in a dose-dependent manner. Additionally, we are the first to demonstrate that β-elemene sufficiently inhibited the ability of gastric cancer cells to peritoneally diffuse and metastasize in vivo. Thus, these results suggest the therapeutic potential of β-elemene in gastric cancer patients with peritoneal metastasis.

The anti-metastatic activity of β-elemene has been confirmed in melanoma and breast cancer; however, the mechanism by which it

occurs had not been determined. Recent studies have identified the molecular targets of β-elemene that may have a role in cancer therapy, which include kinases, transcription factors, and growth factors and their receptors, among other proteins. However, the real target of β-elemene remains unknown. To elucidate the anti-metastasis mechanism of β-elemene in gastric cancer, RNA-seq was used to explore differentially expressed genes after β-elemene treatment. Claudin-1 has previously been identified as a potential target for anti-metastatic treatments (Cherradi et al., 2017; Hashimoto et al., 2017). Claudin-1 is a tight junction protein whose overexpression is seen in several cancers (de Oliveira et al., 2005; Eftang et al., 2013; Leotlela et al., 2007). Previous studies have demonstrated that Claudin-1 promotes EMT in human bronchial epithelial cells via the Notch signaling pathway (Jing, Sun, Mai, Jiang, & Du, 2017). Other studies have indicated that Claudin-1 activates the c-Abl-ERK signaling pathway to induce EMT in human liver cells (Suh et al., 2013). In addition, Claudin-1 is a promoter of EMT in liver cancer (Stebbing et al., 2013). Claudin-1 also stimulates the activation of pro-matrix metalloproteinase-2 to enhance invasiveness (Miyamori et al., 2001). Thus, targeting Claudin-1 could potentially inhibit tumor metastasis (Hashimoto et al., 2017). Our study showed that β-elemene downregulated Claudin-1 mRNA and protein expression. Overexpression of Claudin-1 reversed the inhibition of invasion and migration by β-elemene. Together, these results indicated that β-elemene inhibited the migratory and invasive ability of gastric cancer cells by decreasing Claudin-1 expression.

Another important finding was that β-elemene regulated the expression of Claudin-1 through FAK phosphorylation. FAK is a key regulator of cell adhesion and adherens junctions (Ma et al., 2013). Recent studies have indicated that FAK can modulate tight junctions (Guo et al., 2015; Lie et al., 2012). One study showed that FAK was an integral component of the occludin/ZO-1 complex that regulated cell adhesion and that the tight adherens connection was compromised upon FAK downregulation (Linlin et al., 2011). Our study showed that FAK regulated metastatic ability via Claudin-1 and that β-elemene inhibited Claudin-1 expression by inhibiting FAK phosphorylation. We therefore demonstrated that β-elemene inhibited gastric cancer cell metastasis by regulating the FAK/Claudin-1 pathway.

There are some limitations to this study. The findings of this study were based on in vitro and animal experiments. Therefore, clinicopathological validations are required and are the focus of future studies.

In conclusion, our results strongly suggested that β-elemene inhibited gastric cancer cell metastasis by modulating FAK/Claudin-1 signaling. Thus, it would be beneficial for patients with suspected peritoneal metastases to use β-elemene at an early stage.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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