

Brucein D suppresses breast cancer proliferation and migration via targeting the FAK/LRG1 signaling pathway

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Abstract

Background

Breast cancer (BC) ranks as one of the most prevalent cancers in women globally and stands as a primary cause of cancer-induced death. The scarcity of effective BC treatments prompts the urgent need for innovative therapeutic approaches and new agents. Focal adhesion kinase (FAK), a critical non-receptor intracellular tyrosine kinase, has garnered significant attention as a viable target for cancer therapy. Bruceine D (BD), an active compound isolated from *Brucea javanica*, has demonstrated efficacy in inhibiting the proliferation of various cancer cells. However, its impact on BC through FAK modulation has not been established.

Methods

The MTT and transwell were used to determine the cell proliferation and migration ability. Furthermore, mitochondrial biological function is assayed by ROS release, ATP production, and mitochondrial membrane potential. In addition, RNA-seq explored the involvement of FAK in regulating the LRG1 signaling pathway and revealed its role in mediating apoptosis in BC cells.

Results

The experimental results revealed that BD exerted remarkable inhibitory effects on BC cell proliferation and migration. Furthermore, BD treatment induced substantial metabolic alterations in BC cells, characterized by reduced ATP production, increased ROS accumulation, and decreased MMP. Clinical research demonstrated significantly elevated FAK expression levels in BC patient tissue samples, highlighting its potential as a promising therapeutic target. Mechanistic investigations elucidated that BD exerts its anti-cancer effects through dual modulation of FAK/LRG1 signaling pathway, ultimately triggering apoptotic cell death in BC cells.

Conclusion

These results elucidate a potential mechanism of BD action and underscore its promise as a small-molecule FAK inhibitor, potentially valuable in BC treatment.

Background

BC is still the most commonly diagnosed malignancy and the leading cause of cancer-related mortality among women [1]. The etiology of BC involves a complex interplay of genetic, hormonal, environmental, and lifestyle factors [2]. However, the precise pathomechanism is unknown. According to the latest global cancer data published by the International Agency for Research on Cancer (IARC) of the World

Health Organization, lung cancer has been replaced by BC as the most commonly diagnosed cancer worldwide, with a global incidence of 2.26 million new cases in 2020 [3]. Although conventional treatments such as chemotherapy, surgery, hormone therapy, radiation therapy, and biological therapy are widely used to treat BC with efficacy [4], they may eventually lead to a poor prognosis. Target therapy, which overcomes the resistance mechanisms that cancer cells develop to avoid chemotherapy, has received widespread attention in recent years [5]. Target therapy has been used to treat various types of cancer, including BC. However, targeted therapy is prone to drug resistance, and searching for new effective targets and drugs to treat BC is particularly urgent and necessary in the clinical treatment of BC.

Focal adhesion kinase (FAK), a non-receptor tyrosine kinase, is a key signal transduction protein involved in various signaling pathways [6]. Recent studies strongly suggested that FAK is significantly upregulated in numerous invasive and metastatic malignancies, including BC [7, 8]. Considering its wide variety of functions, frequent overexpression in many types of cancer, and correlation with negative prognoses, FAK is identified as a potential target for anti-cancer therapy, and the development of FAK inhibitors is a promising therapeutic strategy for malignant tumors [9]. Numerous FAK small molecular inhibitors have been developed, and some are progressing to the clinical research stage [10]. In contrast, FAK kinase function inhibitors have demonstrated safety and efficacy, but no clinically approved FAK inhibitors [11]. Developing FAK inhibitors for treating BC will help provide new therapeutic strategies and drugs for BC patients.

Leucine-rich alpha-2-glycoprotein 1 (LRG1) was initially identified and characterized in human serum [12]. Emerging evidence indicates that LRG1 plays a crucial role in diverse biological processes, including immune regulation, cell proliferation, metastasis, and apoptosis [13]. Notably, LRG1 has been implicated in the pathogenesis of various diseases, particularly cancer. Elevated circulating levels of LRG1-either alone or in combination with other biomarkers have been associated with disease progression, tumor burden, and unfavorable clinical outcomes [14]. Furthermore, LRG1 is frequently overexpressed in multiple malignancies, such as pancreatic, colorectal, bladder, and notably, breast cancer [15, 16]. Given the pivotal involvement of both FAK and LRG1 in breast cancer progression, therapeutic strategies aimed at inhibiting their expression may hold significant promise for improving treatment outcomes.

Active ingredients and natural products of Chinese medicines are important sources for discovering and developing new drugs. BD, is one of the active compounds isolated from the Chinese herb *Brucea javanica*, which has been widely used for treating inflammation, malaria, and warts for many years [17]. Furthermore, BD exhibits significant anti-cancer activity, such as lung cancer [18], liver cancer [19], osteosarcoma [20], and breast cancer [21]. Although studies have reported the anti-cancer potential of BD, a connection between FAK and BD in breast cancer has not been explored. In our study, BD is identified as an FAK inhibitor, and BD exerts anti-breast cancer effects by targeting the FAK/LRG1 signaling pathway, which promotes breast cancer cell apoptosis. All these findings suggest that BD has great potential as a novel and potent therapeutic agent for the treatment of BC.

Methods

Cell culture

The human breast cancer cell lines MCF-7 and MDA-MB-231 were acquired from the Chinese Academy of Sciences and Shanghai Institute of Biological Sciences. These cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA), which was supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin-streptomycin antibiotic solution (Beyotime, China). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Reagents and antibodies

Brucein D (TWS2045) was purchased from Innochem and dissolved in DMSO for use. The ROS Assay Kit (S0033M) and ATP Assay Kit (S0026) were obtained from Beyotime (Shanghai, China). The MTT (GC307004), Annexin V-FITC Apoptosis Assay Kit (G1511), and Mitochondrial Membrane Potential Assay Kit were obtained from Solarbio (Beijing, China). The primary antibodies against p-FAK (3283S) were purchased from CST. FAK (12636-1-AP) was obtained from proteintech. The E-cadherin (YT1454), BAX (YT0455), Cleaved-caspase 3 (YC0006), p-AKT (YP0006), AKT (YT0185), and mTOR (YT2913) were obtained from Immunoway. The primary antibodies against p-mTOR (AF5869), 53BP1 (AF6111), Snail (AF8013), and BCL-2 (AG1222) were purchased from Beyotime. The p-PI3K (AP0427), PI3K (A11177), and LRG1 (A7850) were obtained from (ABclonal). Secondary antibodies, including anti-rabbit IgG (No. 7074) and anti-mouse IgG (No. 7076), were purchased from Cell Signaling Technology (USA).

Cell viability assay

To evaluate the inhibitory effects of BD on breast cancer cell proliferation, cells were plated in 96-well plates at a density of 8×10^3 cells per well and allowed to adhere overnight. Following attachment, the cells were treated with varying concentrations of BD for 48 h. After the incubation period, 20 µL of MTT solution (5 mg/mL) was added to each well, and the plates were further incubated at 37°C in a 5% CO₂ atmosphere for 4 h. The culture medium was then carefully aspirated, and 100 µL of DMSO was added to solubilize the formazan crystals. Finally, the OD at 490 nm was measured using a microplate reader to determine cell viability [22].

Clone formation assay

For colony formation analysis, MCF-7 and MDA-MB-231 cells were seeded in 12-well plates at a density of 1×10^3 cells per well and cultured overnight to ensure proper attachment. The cells were then exposed to increasing concentrations of BD (0.5, 1.0, and 2.0 µM) and maintained in a 37°C, 5% CO₂ incubator for 7 days to allow colony development. Following incubation, the resulting colonies were gently washed with PBS, fixed with 4% paraformaldehyde solution for 15 min, and subsequently stained with 0.1% crystal violet solution for 10 min at ambient temperature [23].

Transwell assay

Cell migration was assessed using a 24-well Transwell system (Corning, USA). Following treatment, breast cancer cells were trypsinized, resuspended, and diluted in serum-free medium to a final concentration of 1×10^6 cells/mL. The lower chamber was filled with 600 μ L of medium containing 10% FBS as a chemoattractant, while 100 μ L of cell suspension was added to the upper chamber. After 24 h of incubation at 37°C, non-migrated cells were removed from the upper chamber, and the membrane was washed three times with PBS. Migrated cells on the lower surface were fixed with 4% paraformaldehyde (15 min, RT) and stained with 0.1% crystal violet. Excess stain was rinsed with distilled water, and the retained crystal violet was solubilized with 30% glacial acetic acid. Absorbance was measured at 560 nm to quantify cell migration [24].

Immunofluorescence (IF) assay

Breast cancer cells grown on coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.25% Triton X-100 in 1 $\times$ PBS. After blocking, the cells were incubated with primary antibodies against FAK and 53BP1, followed by corresponding DyLight-conjugated secondary antibodies. Nuclei were counterstained with DAPI, and the coverslips were mounted onto slides. Fluorescence signals were captured using an Olympus fluorescence microscope.

Determination of intracellular reactive oxygen species (ROS)

Intracellular ROS levels were assessed using the Reactive Oxygen Species Assay Kit (Beyotime, China). Briefly, breast cancer cells were seeded in 6-well plates at a density of 4×10^5 cells per well and allowed to adhere overnight. Following attachment, the cells were treated with BD at varying concentrations (0.5, 1.0, and 2.0 μ M) for 2 h. Subsequently, the cells were incubated with 10 μ M DCFH-DA at 37°C for 20 min. ROS production was visualized and quantified using an Olympus fluorescence microscope.

ATP content detection assay

To quantify cellular ATP levels in breast cancer cells, we performed measurements using a commercial firefly luciferase-based ATP assay kit (Beyotime, China) according to the manufacturer's protocol. Briefly, cells were lysed with ice-cold lysis buffer, followed by centrifugation to collect the supernatant. Both standard ATP solutions and cellular supernatants were aliquoted into 96-well white opaque plates. Subsequently, 100 μ L of detection buffer was added to each well. Luminescence signals were measured using a multi-mode microplate reader, and ATP concentrations were determined by interpolation from a standard curve generated with known ATP concentrations."

Assessment of mitochondrial membrane potential (MMP)

Mitochondrial membrane potential ($\Delta\Psi$ m) was assessed using the JC-1 Mitochondrial Membrane Potential Assay Kit (Beyotime, China) following the manufacturer's instructions. Briefly, breast cancer cells treated with BD were washed twice with ice-cold dilution buffer and then incubated with JC-1 dye (5 μ g/mL) at 37°C for 30 min in the dark. After two additional washes with dilution buffer, fluorescence images were acquired using an Olympus fluorescence microscope. The ratio of red-to-green

fluorescence intensity was quantified to determine $\Delta\Psi_m$ changes, where a decrease indicates mitochondrial depolarization.

Cell apoptosis

Apoptosis was analyzed using a FITC Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA). Briefly, breast cancer cells were cultured in 6-well plates and treated with BD at varying concentrations (0.5, 1.0, and 2.0 μM) for 48 h. After treatment, cells were harvested, washed twice with ice-cold PBS, and resuspended in 1 $\times$ Binding Buffer. Subsequently, 5 μL of propidium iodide (PI) and 5 μL of FITC Annexin V were added to the cell suspension, followed by a 15-minute incubation in the dark at room temperature. Finally, 400 μL of 1 $\times$ Binding Buffer was added to each sample, and apoptotic cell populations were quantified using flow cytometry (BD FACSCanto™ II) [25].

Western blot (WB) analysis

The MCF-7 and MDA-MB-231 cells and tumor tissues were homogenized and then lysed in RIPA lysis buffer containing 1% phosphatase inhibitor and PMSF for 10 mins at 4°C. The lysates were collected and measured by using the Bradford assay (Bio-rad, Hercules, CA). Protein samples were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride transfer membranes and then transferred to polyvinylidene difluoride (PVDF) membrane. The blots were blocked for 2 h at room temperature with 5% nonfat milk in TBST and then incubated with specific primary antibodies overnight at 4°C. After washing, the membranes were incubated with HRP-conjugated secondary antibody for 1.5 h at RT. Finally, the bands were visualized and photographed using the ECL substrate for detection [24].

Human subjects

Human breast cancer tissue samples were obtained from the Department of Breast Surgery at Ningbo Medical Center Lihuili Hospital. All participating patients provided written informed consent prior to sample collection. This study was conducted in strict accordance with the ethical principles outlined in the Declaration of Helsinki (1975) and received formal approval from the Institutional Ethics Committee of Ningbo Medical Center Lihuili Hospital (Approval No: KY2023PJ289).

Immunohistochemistry (IHC)

Tumor specimens were fixed in 10% neutral buffered formalin for 48 h at room temperature, then processed and embedded in paraffin. Serial sections (5 μm) were prepared using a microtome. For IHC staining, tissue sections were incubated overnight at 4°C with primary antibodies against FAK (dilution 1:200), followed by treatment with HRP-conjugated secondary antibodies for 1.5 h at 37°C. Immunoreactivity was visualized using DAB chromogen, resulting in characteristic brownish-yellow deposition in positive cells. Stained sections were examined under a light microscope (Nikon Eclipse E200), with FAK expression localized to either nuclear or cytoplasmic compartments.

Drug affinity responsive target stability (DARTS)

Breast cancer cells were harvested and lysed using RIPA lysis buffer (containing protease and phosphatase inhibitors) on ice for 10 min. Following centrifugation at $12,000 \times g$ for 15 min at 4°C , the clarified cell lysates were collected and aliquoted. The lysates were then treated with varying concentrations of BD (0.5, 1.0, and $2.0 \mu\text{M}$) for 40 min at room temperature with gentle agitation. Subsequently, samples were subjected to thermolysis at 37°C for 10 min to complete protein denaturation. After a final centrifugation step ($12,000 \times g$, 10 min, 4°C), the supernatants were collected and analyzed by Western blotting assay [26].

Solvent-induced protein precipitation (SIP) assay

The breast cancer cells' lysates were collected and incubated with $10 \mu\text{M}$ of BD for 50 min. Then, the cell lysates were incubated with the indicated solvent volume containing acetone-ethanol-acetic acid (AEA) (50:50:1) for 30 min. After centrifugation, the supernatant was collected and analyzed by western blotting assay.

Molecular docking

The Protein Data Bank (PDB ID: 4gu9) provided the crystal structure of FAK. Molecular docking studies were performed using Discovery Studio 4.5 Client software.

Transient transfection of small interfering RNA (siRNA)

The siRNA targeting human FAK was purchased from Tsingke Biotechnology (Beijing, China) with the following sequence: siFAK: 5-GGGCAUCAUUCAGAAGAUATT-3. Human breast cancer cells were transfected with 50 nM siRNA using Lipofectamine RNAiMAX (Invitrogen, CA, USA) for 12 h.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 9.0 software. All the results were performed at least three times. One-way ANOVA followed by Dunnett's post hoc test is used when comparing more than two data groups. A p-value < 0.05 was considered statistically significant [27].

Results

3.1 BD inhibits breast cancer cell proliferation and migration

To determine the cytotoxicity of BD, the MCF-7 and MDA-MB-231 cells were subjected to BD treatment of various concentrations, and cell viability was examined via the MTT assay. The structure of BD is shown in Fig. 1A. The MTT results confirmed that BD is capable of reducing cell viability in a dose-dependent manner (Fig. 1B and 1C). Additionally, colony formation results suggested that the number of breast cancer cell colonies in the BD-treated group was lower than in the control group (Fig. 1D). Cell metastasis is a complex tumor development process involving cell migration. Here, the effects of BD on the migration of MCF-7 and MDA-MB-231 cells were examined by transwell assay. Our results found that BD dose-dependently decreased the migration of breast cancer cells through the transwell filter (Fig. 1E).

EMT could decrease intercellular adhesion and enhance cell motility, leading to cancer cell metastasis and promoting cancer development. Therefore, we detected the changes in such protein expressions in breast cancer cells. We found that the expression of E-cadherin was significantly upregulated while Snail was downregulated (Fig. 1F). These data demonstrated that BD altered the breast cancer EMT process, thereby changing cell migration capacities.

3.2 BD promotes DNA damage and disrupts mitochondrial biological function

Natural products often exert their anti-cancer effects by inducing DNA damage and disrupting mitochondrial biological function. Therefore, we conducted an IF assay using 53BP1 antibody, which has been used as a marker for DNA double-stranded breaks to verify BD could promote DNA damage. Our results suggested that BD dose-dependently increased the number of 53BP1 foci in the breast cancer cells (Fig. 2A to 2C). Mitochondria provide an energetic basis for the unlimited proliferation of cancer cells. Therefore, we examined the effect of BD on ATP production in breast cancer cells. We found that ATP production was significantly reduced in breast cancer cells after BD treatment (Fig. 2D and 2E). Insufficient ATP production generates excess ROS and destroys mitochondrial membrane potential. Then, we used DCFH-DA and JC-1 fluorescent staining to detect ROS accumulation and MMP changes in breast cancer cells. As shown in Fig. 5F-5H, we found that after IATL treatment, the ROS of liver cancer cells was up-regulated dose-dependent. Additionally, treatment with BD decreased the MMP (Fig. 2I and 2J).

3.3 BD induces breast cancer cell apoptosis by disrupting the mitochondrial biological functions

Disruption of mitochondrial function leads to cell apoptosis. We further detected cell apoptosis in breast cancer cells by Hoechst staining, we found that BD-treated exhibited significant nuclear shrinkage and nuclear fragmentations, which implies that BD caused apoptosis of breast cancer cells (Fig. 3A). Consistent with the Hoechst staining results, we also found that BD treatment promoted cell apoptosis dose-dependently and 2.0 μ M BD caused apoptosis in about 30% of breast cancer cells (Fig. 3B-3D). Meanwhile, the well-known apoptosis-related proteins, including cleaved-caspase 3, BAX, and Bcl2, which are widely regarded as apoptosis markers, were chosen in the western blot assay. We found that BD decreased the expression of Bcl2 and increased the expression of cleaved-caspase 3 and BAX (Fig. 3E and 3F). The results further indicated that BD induces cell apoptosis by disrupting the mitochondrial biological functions.

3.4 FAK is a predictive marker of breast cancer progression and prognosis

To investigate the anti-liver cancer mechanisms of BD, we performed RNA sequencing (RNA-seq) to analyze the global gene expression profiles in BD-treated MCF-7 cells compared to untreated controls. KEGG pathway analysis identified cell adhesion molecular and Focal adhesion as key pathways modulated by BD treatment (Fig. 4A). Interestingly, FAK exhibits close interactions with both molecules. Several studies reported that FAK was associated with the development and progression of various human cancers, whereas inhibition of FAK could attenuate cancer progression. Therefore, it is reasonable to assume that FAK could be a potential therapeutic target against breast cancer. To validate the hypothesis, we analyzed data from the TCGA database. Our results revealed that breast cancer

patients with high FAK expression exhibited a lower overall survival rate (Fig. 4B and 4C). Importantly, we found the distribution and expression of FAK in patient-matched tissue samples than in tumor-adjacent tissues after analyzing the clinical samples of breast cancer via IHC and Western blot assay, which were consistent with the TCGA database results (Fig. 4D and 4E).

3.5 BD suppresses breast cancer progression by targeting the FAK/LRG1 axis

FAK is highly expressed in breast cancer patients, but whether BD exerts its anti-breast cancer effects by targeting FAK remains unclear. In this study, we use immunofluorescence and western blotting analyses to examine the effect of BD on the expression of FAK. Compared with those in the untreated cells, the expression levels of FAK were downregulated and localized to the nucleus in the BD-treated breast cancer cells (Fig. 5A), and BD dose-dependently decreased the expression of phosphorylated FAK in breast cancer cells (Fig. 5B). By further analyzing the differential genes, we found that the transcript level of LRG1 was significantly down-regulated (Fig. 5C), and thus speculated that LRG1 might be a downstream regulatory protein of FAK. The WB results similarly found that the expression level of LGR1 decreased with the increase of BD concentration, which further verified our speculation (Fig. 5D). Evidence suggested that the PI3K/AKT/mTOR signaling pathway is the key intracellular signaling pathway of LGR1 [28]. As shown in Figs. 5E, the phosphorylated PI3K, AKT, and mTOR were decreased with an increasing BD concentration, while the PI3K, AKT, and mTOR levels remained unchanged. These results suggested that BD may inhibit breast cancer cell progression by blocking the FAK/LRG1 signaling pathway.

3.6 FAK was a therapeutic target in breast cancer cells

Given that BD significantly reduced FAK phosphorylation and its expression effectively inhibited breast cancer cell proliferation and migration, we hypothesized that BD exerts its anti-breast cancer activity through direct binding to FAK. To test this hypothesis, we first employed molecular docking analysis, which revealed that BD forms stable hydrogen bonds with key FAK residues (LYS-454, GLN-432, and GLU-430; Fig. 6A). To further validate this interaction, we performed DARTS and SIP assays. The DARTS results demonstrated dose-dependent binding between BD and FAK (Fig. 6B), while SIP analysis indicated that BD treatment enhanced FAK stability in BC cells (Fig. 6C).

To elucidate the functional significance of this interaction, we employed FAK siRNA knockdown experiments. Silencing FAK expression led to significant inhibition of colony formation and migration capacity in breast cancer cells. Notably, the combination of FAK knockdown with BD treatment produced synergistic inhibitory effects on these malignant phenotypes (Fig. 6D and 6E). At the molecular level, FAK silencing resulted in decreased FAK expression and phosphorylation, accompanied by reduced LRG1 expression (Fig. 6F and 6G). We also observed compensatory downregulation of PI3K/AKT/mTOR phosphorylation following LRG1 decreased. Most importantly, the combination of FAK silencing with BD treatment produced more pronounced inhibition of LRG1 and PI3K/AKT/mTOR pathway activation compared to BD treatment alone. Collectively, these findings demonstrate that BD exerts its anti-breast cancer effects primarily through targeting the FAK/LRG1 axis, leading to subsequent inhibition of PI3K/AKT/mTOR signaling pathway activation in breast cancer cells.

Discussion

BC is the most prevalent female cancer, and its morbidity is the highest in the world and has become a huge burden for patients' families and society [4]. BC can be categorized into three groups; BC expressing hormone receptor (estrogen receptor (ER+) or progesterone receptor (PR+)), BC expressing human epidermal receptor 2 (HER2+), and triple-negative breast cancer (TNBC) based on both molecular and histological evidence [29]. Despite the various treatments that have been developed for BC, the survival rate of patients remains low, with a median survival time of 21 months and a 3-year survival rate of only 15.5% due to the severe side effects and drug resistance [30, 31]. Thus, the development of effective modalities and drugs for the treatment of BC is particularly urgent.

In recent years, traditional Chinese medicine (TCM) has been increasingly used and has become well-known for its significant role in preventing and treating cancer due to its high effects, few side effects, and low cost [32]. *Brucea javanica* (L.) Merr. belong to the liver and large intestine channels, its fruits and leaves can be used as TCM with cold in property, and bitter in prescription. In China, it is called "Yadanzi", which has heat-clearing, detoxifying, and preventing malaria [33]. BD is one of the main active ingredients of *Brucea javanica* (L.) Merr. which has been demonstrated in multiple biological activities, especially anti-cancer [34]. Our current study also found that BD exhibited significant anti-proliferative and anti-migration activities in vitro.

ROS play an important role in cell proliferation, differentiation, apoptosis, and survival. Excessive accumulation of ROS elicits protein oxidation, lipid peroxidation, cellular DNA damage, and ultimate cell death or apoptosis [35]. Studies showed that BD can promote ROS accumulation and cause cancer cell apoptosis [17]. Our results revealed that BD reduced ATP production, leading to ROS accumulation, decreasing mitochondrial membrane potential, disrupting the biological function of mitochondria in BC cells, and ultimately promoting BC cell apoptosis, consistent with previous studies. However, the mechanism of ROS development is a complex process and can be regulated by a variety of factors.

FAK is a tyrosine kinase overexpressed in cancer cells and plays an important role in the progression of cancers to a malignant phenotype. Evidence has indicated that the dissociation of FAK promotes its autophosphorylation, participates in the form of ROS, and subsequently promotes lung cancer cell proliferation and migration [36, 37]. Thus, we hypothesized that FAK could promote the production of large amounts of ROS in BC cells by targeting FAK. Emerging evidence has highlighted the multifaceted role of Leucine-rich alpha-2-glycoprotein 1 (LRG1) in various biological processes, including signal transduction, immune regulation, and cellular proliferation and metastasis. Notably, LRG1 has been found to be significantly upregulated in multiple malignancies, particularly in pancreatic, ovarian, and colorectal cancers [14].

Conclusion

Consistent with these findings, our study demonstrates that BD specifically targets FAK and inhibits its phosphorylation activity, subsequently suppressing the expression level of the downstream LRG1 protein. These results suggest that this compound holds therapeutic potential for development as a novel FAK inhibitor.

Abbreviations

BC

Breast Cancer

BD

Brucein D

FAK

Focal Adhesion Kinase

LRG1

Leucine-Rich Alpha-2-Glycoprotein 1

Declarations

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Contributions

LL: conceptualization, methodology, validation, data curation, resources, writing—original draft preparation, and funding acquisition. SH: methodology, validation, and data curation. QY: validation and investigation. CY: validation and formal analysis. WW: conceptualization, writing-review and editing, and supervision.

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Ethics approval and consent to participate

Human clinical breast cancer samples were collected from the Department of Breast Surgery Ningbo Medical Center LiHuiLi Hospital. Patients included were all signed with an informed consent form. The research protocol was approved by the Ethical Committees of the Ningbo Medical Center LiHuiLi Hospital (KY2023PJ289) based on the ethical guidelines of the 1975 Declaration of Helsinki.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Additional information

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Figures

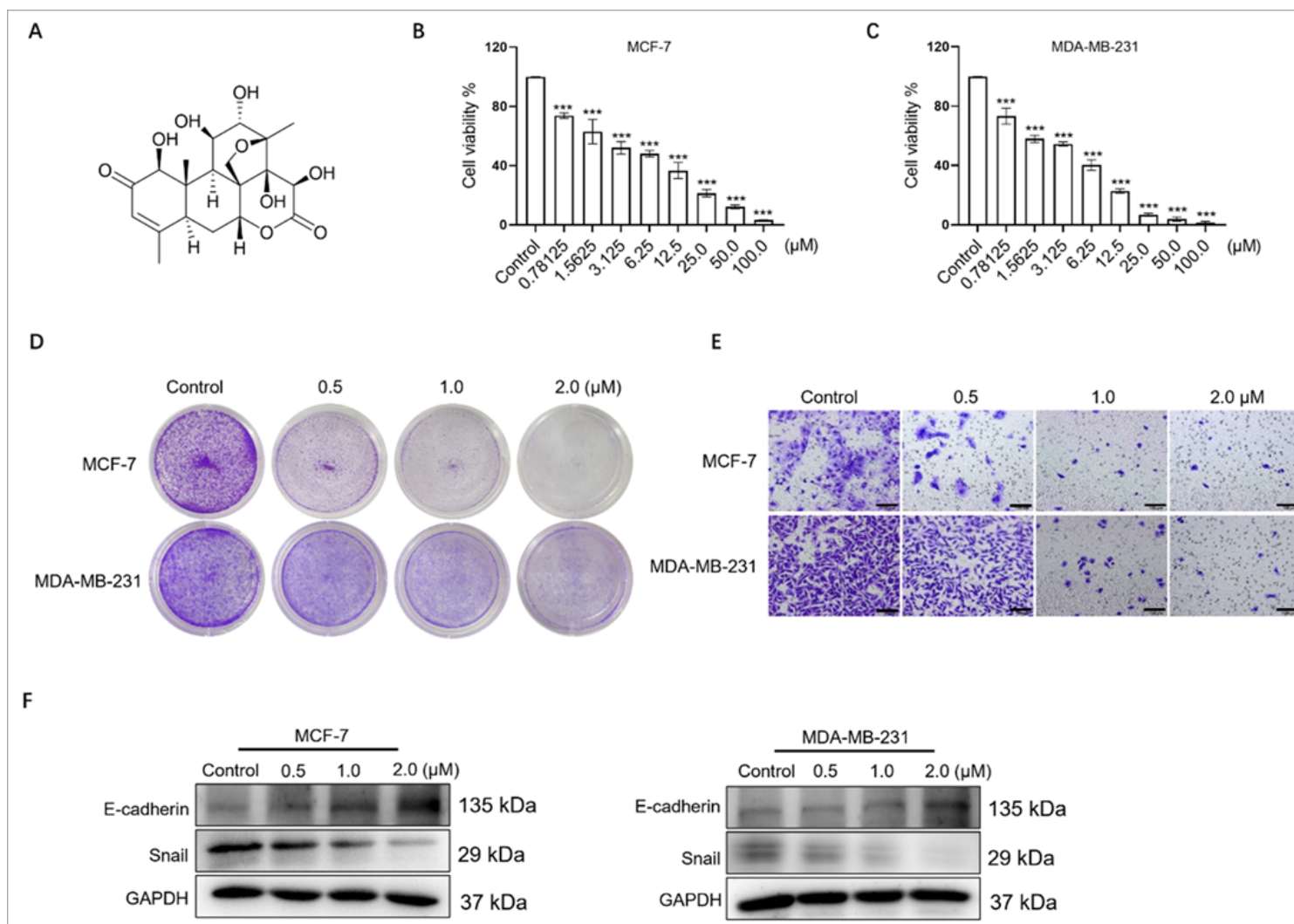


Figure 1

BD inhibits breast cancer cell proliferation and migration. (A) The chemical structure of BD. (B, C) The effect of BD on BC cell viability in the indicated cell lines. (D) Colony formation assay in BC cells treated with the indicated concentrations of BD. (E) Transwell assay was used to measure the effect of BD on the migration of BC cells (200×). (F) Western blot assays of E-cadherin and Snail protein expression levels in BD-treated BC cells and normal controls at indicated doses (0.5, 1.0 and 2.0 μM) for 48 h. GAPDH was set as an internal loading control. Full-size blots are presented in Supporting Information. The average ± SD of three independent assessments was used to calculate the values. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control group.

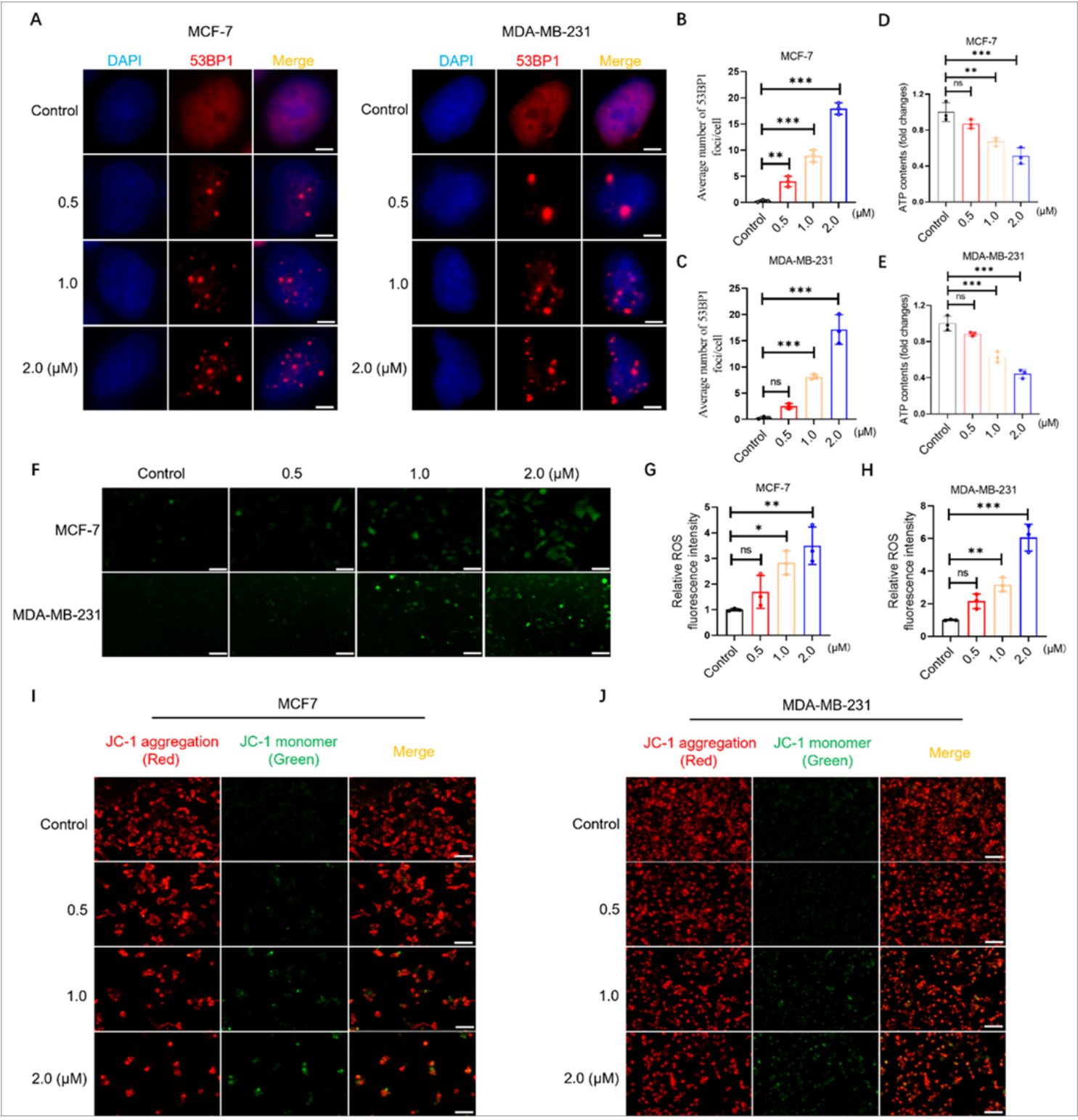


Figure 2

BD promotes DNA damage and disrupts mitochondrial biological.(A) Representative images of 53BP1 foci in BC cells treated with the indicated concentrations of BD. (B, C) Quantification of the 53BP1 foci per cell. More than 100 cells were counted in each group. (D, E) The effect of ATP levels in BC cells after BD treatment. (F) Intracellular ROS as detected by DCFH-DA staining (green). (G, H) Quantification of F. (I, J) JC-1 was used to detect the mitochondrial membrane potential of BC cells. J-aggregates (red) and

JC-1 monomers (green) were detected. The average $\pm$ SD of three independent assessments was used to calculate the values. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control group.

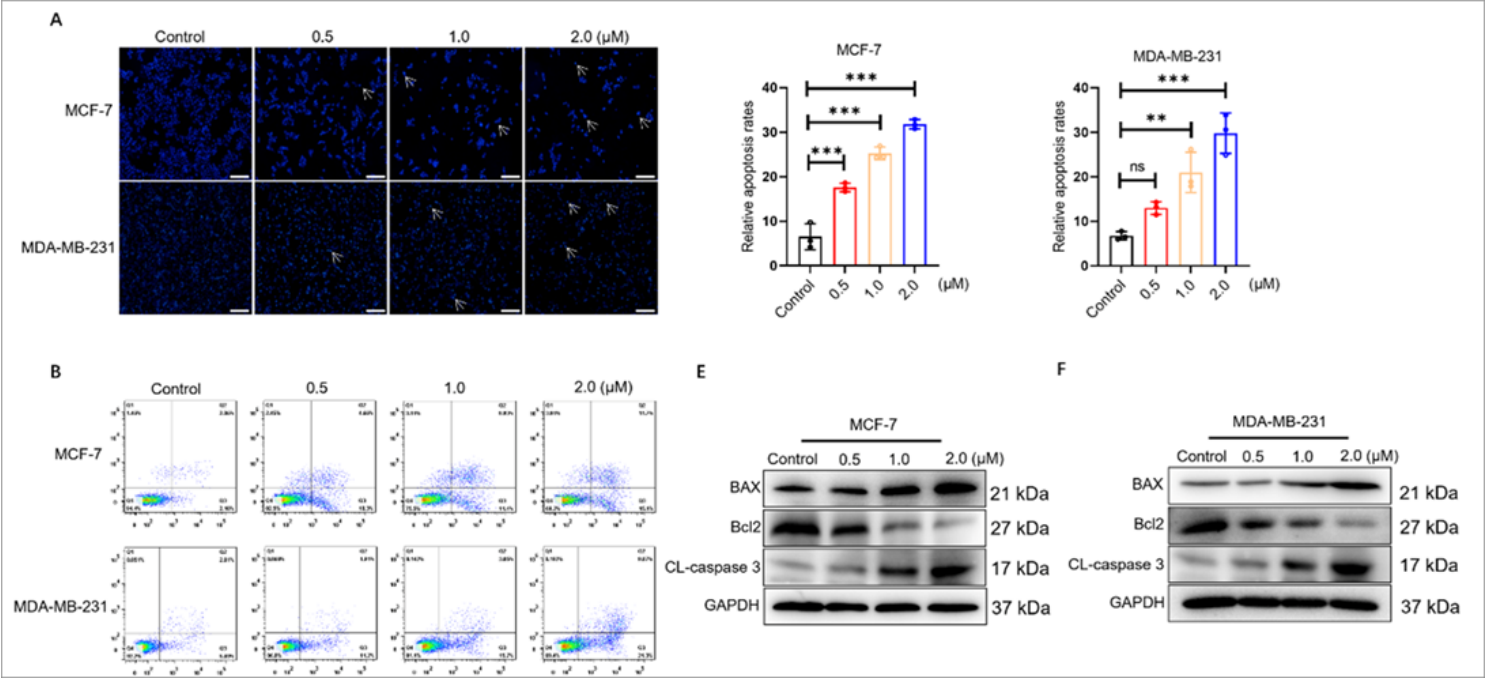


Figure 3

BD induces breast cancer cell apoptosis by disrupting the mitochondrial biological functions. (A) The Hoechst staining was used to detect BC cell apoptosis levels. (B) BC cells were treated with BD at indicated concentrations for 48 h, apoptotic cells were assayed by Annexin V/PI staining and FACS analysis. (C, D) Quantification of B. (E, F) Western blot determined the abundance of BAX, Bcl-2, and CL-caspase 3 in control and BD-treated BC cells at indicated concentrations. Full-size blots are presented in Supporting Information. The average $\pm$ SD of three independent assessments was used to calculate the values. ** $P < 0.01$, *** $P < 0.001$ vs. control group.

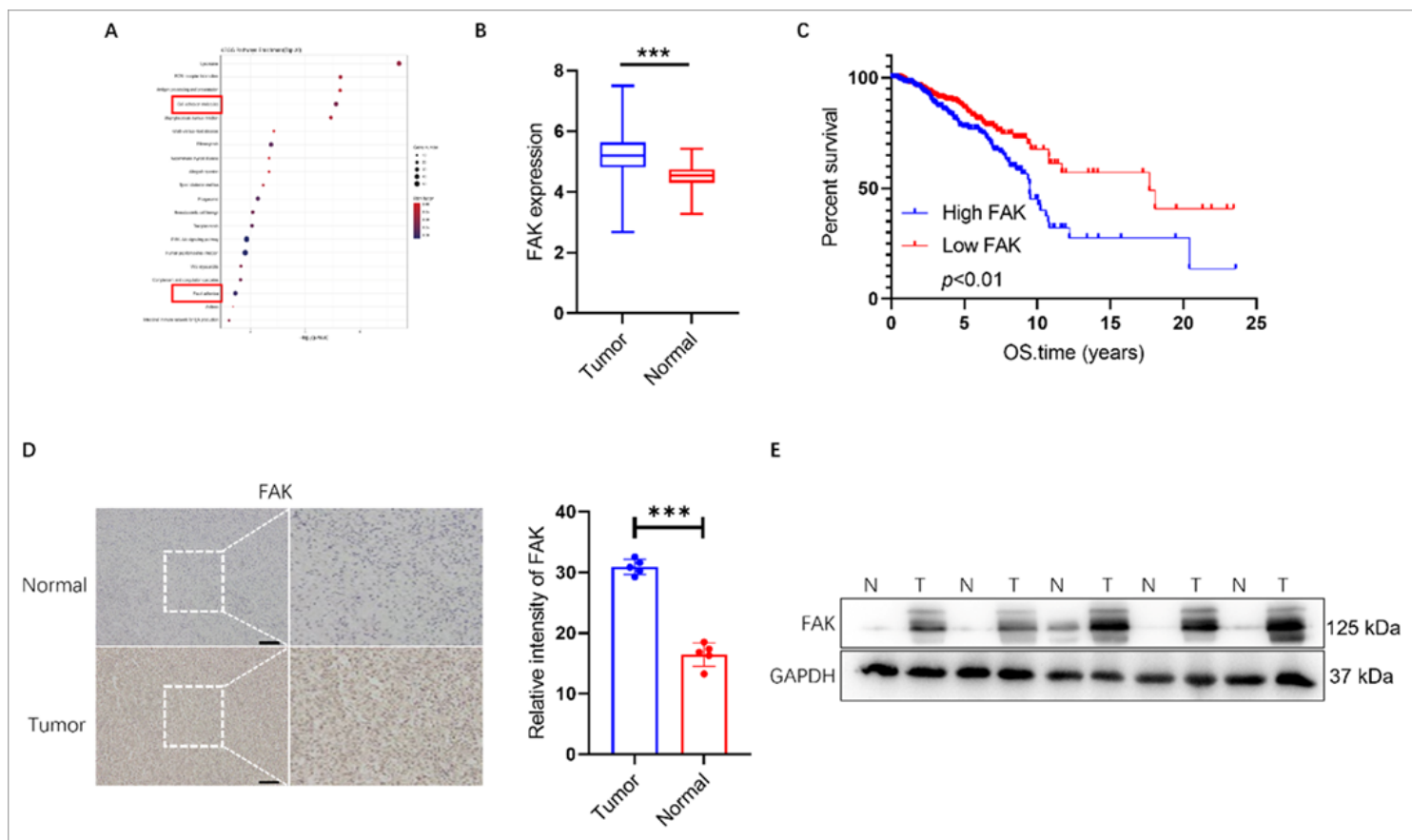


Figure 4

FAK is a predictive marker of breast cancer progression and prognosis. (A) KEGG enrichment analysis of the control group and the IATL treatment group. (B) Relative gene expression of FAK in BC samples. (C) Overall survival of BC patients with up- and down-regulated FAK expression levels. (D) Immunohistochemical staining of FAK levels in patient-matched and tumor-adjacent normal controls of BC (100×). (E) Western blot analysis of FAK protein levels in different BC tissues. Full-size blots are presented in Supporting Information. *** $P < 0.001$ vs. normal group.

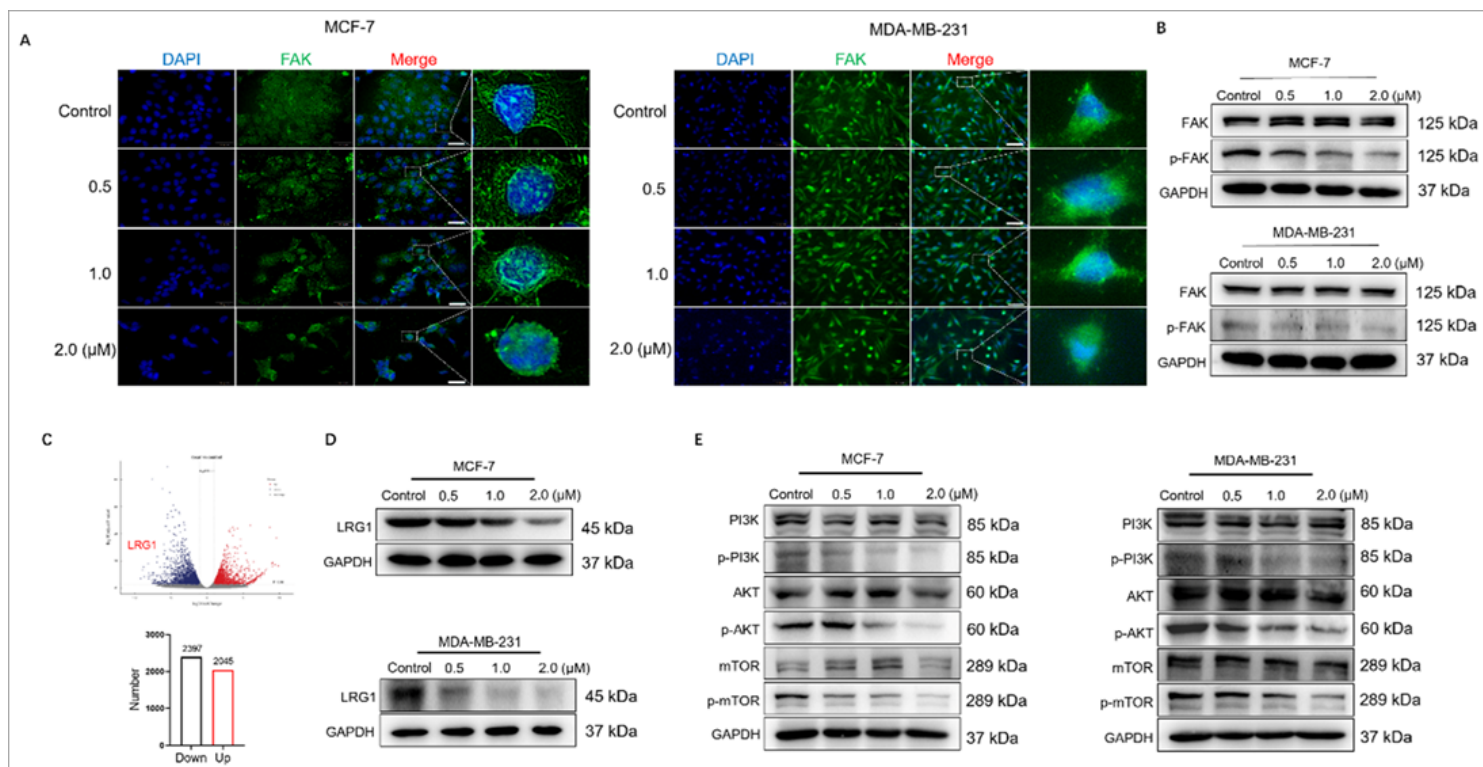


Figure 5

BD suppresses breast cancer progression by targeting the FAK/LRG1 axis. (A) BC Cells were treated with indicated concentrations of BD (0.5, 1.0 and 2.0 μM) for 48 h and subjected to immunofluorescence analysis. Fluorescence of DAPI (blue), anti-FAK antibody (green). (B) Western blot assays of p-FAK and FAK protein expression levels in BD-treated BC cells and normal controls at indicated doses (0.5, 1.0 and 2.0 μM) for 48 h. GAPDH was set as an internal loading control. (C) Volcano plot of up-regulated and down-regulated differentially expressed genes after RNA-seq analysis. (D) Western blot assays of LRG1 protein expression levels in BD-treated BC cells and normal controls at indicated doses (0.5, 1.0 and 2.0 μM) for 48 h. GAPDH was set as an internal loading control. (E) Western blot analysis of PI3K/AKT/mTOR proteins in BC cells treated with BD for 48 h. GAPDH was used as the loading control. Full-size blots are presented in Supporting Information. The average $\pm$ SD of three independent assessments was used to calculate the values. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control group.

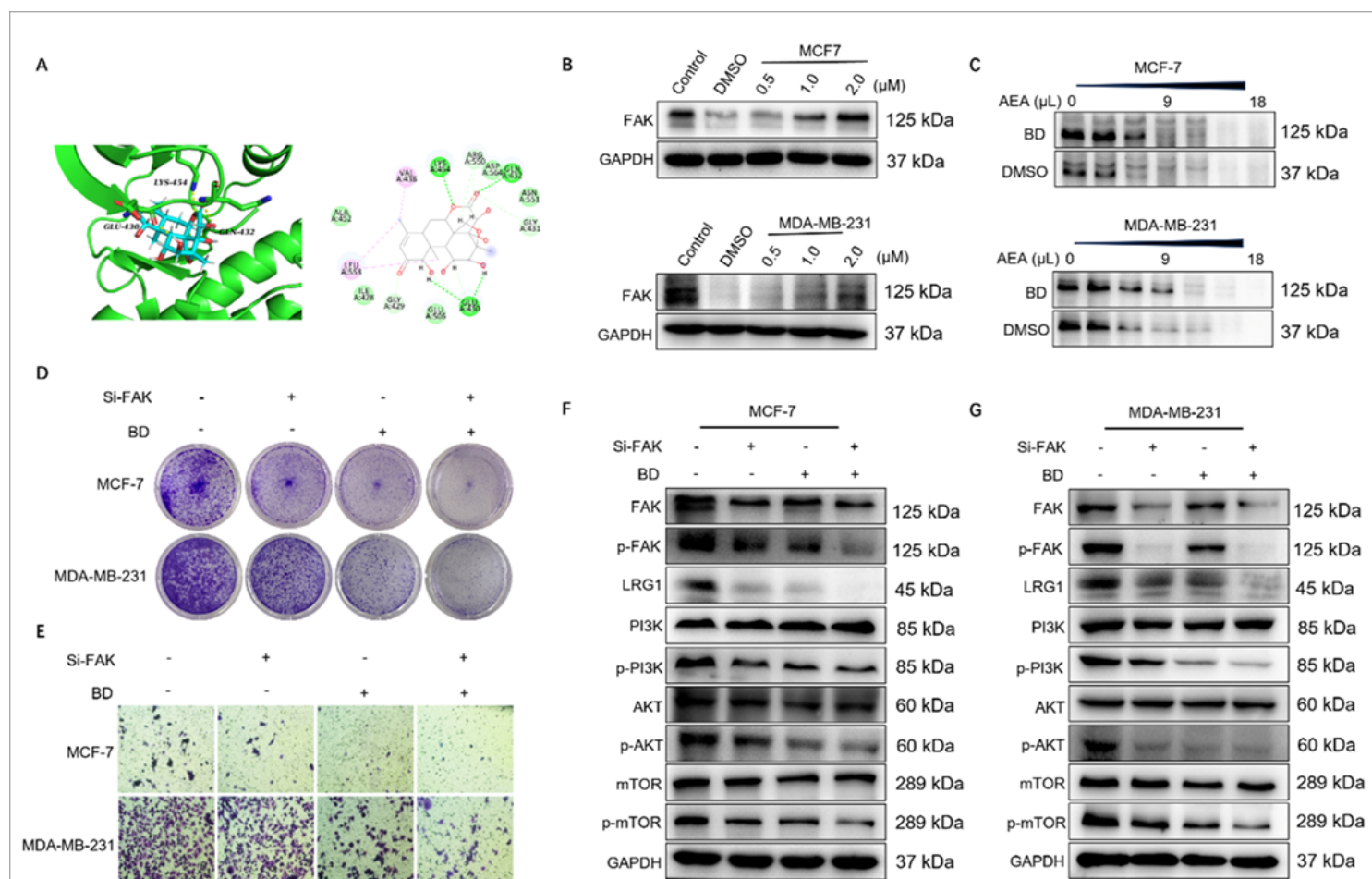


Figure 6

FAK was a therapeutic target in breast cancer cells. (A) Putative binding modes of BD with FAK (4gu9). (B) Immunoblotting for quantification of FAK level in drug affinity responsive target stability (DARTS) assay. (C) Immunoblotting for quantification of FAK level in solvent-induced protein precipitation (SIP) assay. (D) BC cells were treated with si-FAK combined with BD for 48 h, and the effect on colony formation was assessed. (E) BC cells were treated with si-FAK combined with BD for 48 h, and the effect of migration ability was assessed. (F, G) Western blot analysis was used to detect the effect of FAK silencing and BD treatment in BC cells on FAK downstream LRG1, PI3K/AKT/mTOR signaling pathway. Full-size blots are presented in Supporting Information.

Supplementary Files

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