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Arctigenin, a natural ER β ligand occurring in edible burdock, accelerates focal adhesion disassembly of colon epithelial cells *via* enhancing autophagic degradation of paxillin to facilitate mucosal healing in ulcerative colitis

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Burdock is consumed as a vegetable in the Asian region and is also used in medicinal dishes or health soups. Our previous studies demonstrated that arctigenin, a natural agonist of estrogen receptor β (ER β) occurring in burdock, can promote colonic mucosal healing in the context of ulcerative colitis (UC). Whether and how arctigenin intervenes in focal adhesion disassembly, a critical step for migration and wound healing of colon epithelial cells, were examined within this research. The results showed that arctigenin substantially accelerated focal adhesion disassembly and therefore facilitated migration and wound healing of colonic epithelial cells. Mechanistically, arctigenin reduced excitatory amino acid transport to specifically enhance autophagic degradation of paxillin, thereby accelerating focal adhesion disassembly through an ER β -dependent pathway. In colitis-induced mice, ER β knockout significantly attenuated promotion of colonic mucosal healing by arctigenin. These findings indicate that arctigenin is a promising mucosal healing promoter, which functions by accelerating focal adhesion disassembly in an ER β -dependent manner.

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1. Introduction

Ulcerative colitis (UC) is a chronic nonspecific inflammatory bowel disease. The primary goals of UC therapies are to rapidly alleviate symptoms and maintain clinical remission.¹ For patients who are unresponsive to conventional pharmacological treatments or experience severe complications, surgical treatments such as colectomy may be required.² Mucosal healing is defined as the restoration process by which the colonic mucosa recovers from inflammation and ulceration to a normal physiological state, which can be assessed through endoscopic examination and the use of endoscopic scoring systems to evaluate the degree of mucosal inflammation and healing.^{3,4} Patients with mucosal healing typically exhibit the disappearance of mucosal ulcers, bleeding, and inflammation,

while histological examination shows a significant reduction or absence of inflammatory cell infiltration and restoration of crypt architecture.⁵ Clinical observations have indicated that patients are more likely to achieve sustained clinical remission without the need for corticosteroid treatment, which is of significant importance for long-term disease management and control.⁶ Commonly used clinical medications include 5-aminosalicylic acid, corticosteroids, immunosuppressants, and biologics, which alleviate UC symptoms by reducing inflammation and suppressing abnormal immune responses.⁷ To date, no mucosal healing promoters are available.

As a complex biological process, mucosal healing involves the migration and proliferation of epithelial cells, along with inflammation resolution and crypt repair.^{8,9} Among them, cell migration stands as a pivotal step. During cell migration, focal adhesion disassembly is required to detach cells from the extracellular matrix, enabling the cells to migrate into new regions.^{10,11} In the early stages of wound healing, focal adhesion disassembly accelerates epithelial cell migration to initiate the repair process.¹² Dysregulation of focal adhesion disassembly would lead to abnormal wound healing. For example, in chronic wounds, excessive stabilization of focal adhesions hinders cell migration and wound healing.¹³

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Despite its importance in mucosal healing and other cell migration-related diseases, the underlying mechanisms of focal adhesion disassembly are poorly understood, and agents that accelerate focal adhesion disassembly are still in their infancy.¹⁴

Burdock is a nutrient-rich vegetable commonly used in dishes in the Asian region. It is packed with dietary fiber, vitamin C, and minerals and offers various health benefits, including promoting digestion, enhancing immunity, and maintaining gut microbiota balance.¹⁵ Arctigenin, a major active component of burdock, exhibits diverse bioactivities ranging from inhibiting inflammation and regulating immune responses to alleviating oxidative stress.¹⁶ Previous studies of our research team have demonstrated that arctigenin could promote colonic epithelial cell migration to accelerate colonic mucosal healing of mice with colitis.¹⁷ In this study, we explored whether and how arctigenin functions by regulating focal adhesion disassembly to provide new evidence for the development of arctigenin as a mucosal healing promoter.

2. Methods and materials

2.1 Reagents

Arctigenin (purity > 99%) was purchased from Chengdu Pushi Biotechnology Co., Ltd (Chengdu, China); 3-methyladenine (purity > 99%), chloroquine (purity > 99%), and MG132 (purity > 99%) were purchased from MedChemExpress (Shanghai, China); Novozymes qPCR master mix and Novozymes reverse transcription mix were purchased from Novozymes Biotech Co., Ltd (Nanjing, China); Trizol total RNA extraction reagent was purchased from Nanjing Shengxing Biotechnology Co., Ltd (Nanjing, China); NP-40 lysis buffer was purchased from Shanghai Yansheng Industrial Co., Ltd (Shanghai, China); vinculin antibody, paxillin antibody, LC3 antibody, and p-mTOR antibody were purchased from Wuhan Sanying Biotechnology Co., Ltd (Wuhan, China); EpCAM antibody, P62 antibody, and GAPDH antibody were purchased from Abcam Biotech Co. (Wuhan, China); mTOR, Kindlin, and Talin antibodies were purchased from Santa Cruz Biotechnology (Dallas, USA); glutamate detection kit was purchased from Shanghai Enzyme-Linked Biotech Co., Ltd (Shanghai, China); aspartate detection kit was purchased from Nanjing HZ Biotech Co., Ltd (Nanjing, China); fetal bovine serum was purchased from Suzhou Ecos Bio-Tech Co., Ltd (Suzhou, China); dextran sulfate sodium (DSS) was purchased from MP Biomedicals (OH, USA); human-source siER β was purchased from Suzhou Hongxun Biotech Co., Ltd (Suzhou, China); and ER β antibody was purchased from Nanjing Baode Biotechnology Co., Ltd (Nanjing, China).

2.2 Animals

ER β ^{-/-} mice were purchased from Shanghai Model Organisms Biotechnology Co., Ltd (Shanghai, China). Male and female ER β ^{+/-} mice were crossed to obtain ER β ^{-/-} mice, and tail tissue samples from their offspring were obtained from geno-

typing. Wild-type mice from the same litter were retained as a control. All animals were kept in an environment with a 12 h alternating light and dark cycle and a temperature of 22 ± 2 °C, with free access to food and water. Offspring were used for experiments at 6–8 weeks of age. Animal experiments were carried out after authorization by the Animal Ethics Committee of China Pharmaceutical University (2024-11-032).

2.3 Cell culture

NCM460 cells (normal human colonic epithelial cells) and HT-29 cells (human colon adenocarcinoma cells) were purchased from the Shanghai Institute of Life Sciences, Chinese Academy of Sciences (Shanghai, China). NCM460 and HT-29 cells were cultured in RPMI 1640 (Nanjing Senbeijia Biotechnology Co., Ltd, Nanjing, China) under a culture environment set at 37 °C and 5% CO₂.

2.4 Establishment of a mouse colitis model and treatments

ER β ^{-/-} mice (6–8 weeks old) were assigned at random to the ER β ^{-/-} group and the arctigenin + ER β ^{-/-} group, while wild-type control mice from the same litter were assigned at random to the normal group, the DSS model group, and the arctigenin group, each group containing 6 mice. A 3.5% (w/v) DSS solution was administered in drinking water to trigger colonic mucosal injury in mice for 5 days. Afterward, the mice were provided with plain water *ad libitum* for 5 days, and therapeutic treatment was initiated starting from the 6th day of modeling. The mice in the arctigenin group and the arctigenin + ER β ^{-/-} group received gavage of arctigenin (25 mg kg⁻¹) for 5 consecutive days, while mice in the normal group and the DSS model group were administered the same volume of the corresponding vehicle. On day 10, 1 h after the final dose, blood was obtained from the venous network of the eyes, and the mice were humanely sacrificed. The serum and colon tissues were collected for subsequent experiments.

2.5 Disease activity index (DAI)

Starting from the day of modeling, the body weight, diarrhea, and occult blood of the mice were monitored and documented daily. The disease activity index (DAI) was computed using a standard formula: DAI = (body weight loss + diarrhea + rectal bleeding)/3. This index was applied to evaluate the severity of colitis.

2.6 Myeloperoxidase (MPO) activity

Colon tissues (10 mg) were weighed and rinsed with PBS. The tissues were then homogenized in an ice-cold glass homogenizer with homogenization buffer to prepare a 5% tissue homogenate. MPO activity was measured following instructions provided with the MPO assay kit (Nanjing Jiancheng Reagent Co., Ltd, Nanjing, China).

2.7 Histological evaluation

After being fixed in 4% formaldehyde, colon tissues underwent dehydration, paraffin embedding, sectioning, hematoxylin-eosin (H&E) staining and then mounting. Light microscopy



was used by a pathologist to observe pathological changes in the colon tissues, and histological scores were assigned based on the following criteria:

Inflammation severity of the intestinal wall: 1 point (mild), 2 points (moderate), and 3 points (severe). Extent of lesion involvement: 1 point (mucosal layer), 2 points (mucosal and submucosal layers), and 3 points (transmural injury). Crypt damage: 1 point (1/3 crypts damaged), 2 points (2/3 crypts damaged), 3 points (crypt loss with intact epithelium), and 4 points (crypt and epithelium loss).

The scores for these three parameters were multiplied by the proportion of the affected area in the tissue to obtain a total score. The proportion was scored based on the length of the affected region in the colon section: 0 points (0%), 1 point (1–25%), 2 points (26–50%), 3 points (51–75%), and 4 points (76–100%). No lesions were scored as 0 points. Each mouse's final score was obtained by averaging these scores.

Ulcer score: 0 points (0% ulceration), 1 point (1–25% ulceration), 2 points (26–50% ulceration), 3 points (51–75% ulceration), and 4 points (76–100% ulceration). The average score for each mouse in each group was calculated, and the final pathological scores for all mice were obtained.

2.8 FITC-dextran intestinal permeability assay

To evaluate the changes in colonic barrier permeability in DSS-induced colitis mice, on the 10th day after modelling, each group of mice was orally administered with fluorescein isothiocyanate–dextran (FITC-dextran, 440 mg kg⁻¹) (Sigma-Aldrich, St. Louis, USA). After 4 h, mice were given Avertin for anesthesia, sacrificed by cervical dislocation, and serum was collected. The serum was placed into a 96-well culture plate, and absorbance was recorded at wavelengths of 485/530 nm using a microplate reader.

2.9 Extraction of primary colonic epithelial cells of mice

After euthanasia, approximately 1.5 cm of colon tissue of mice was excised and placed into a 5 mL centrifuge tube. The colon tissue was minced into pieces smaller than 1 mm³ and washed several times with PBS containing 1× penicillin–streptomycin to remove any obvious impurities. The tissue pieces were then placed in a neutral protease (Sigma-Aldrich, St. Louis, USA)/collagenase XI (Sigma-Aldrich, St. Louis, USA) mixture and digested at 37 °C for 1 h in a water bath with shaking. After digestion, the solution became turbid. The tissue digestion solution was filtered twice using a 70 µm cell strainer, and the filtrate was centrifuged at 300g at 4 °C for 10 min. The cell pellet was harvested for subsequent experiments, and the supernatant was discarded.

2.10 Immunofluorescence staining of colon tissue sections

The tissue sections were transferred to a fume hood for deparaffinization and gradient dehydration with ethanol. The sections were then placed in the prepared antigen retrieval solution and incubated at 95 °C in a water bath for 15 min. Thereafter, the sections were treated with 5% BSA for blocking in the wet box for 1.5 h. The primary antibody was added to

the wet box and incubated overnight at 4 °C. The sections were then treated with a fluorescence-conjugated antibody and incubated at 37 °C for 1.5 h while protected from light. The sections were stained with an appropriate amount of Hoechst anti-fluorescence quenching mounting solution and incubated in the dark for 15 min. Images were obtained using an inverted fluorescence microscope.

2.11 Real-time QPCR assay

For total RNA extraction, colon tissues or cells were processed with Trizol reagent, and the RNA was reverse transcribed into cDNA using the 5XNovozymes reverse transcription mix according to the instructions. The cDNA was then amplified using the 2XNovozymes qPCR master mix. The threshold cycle (Ct) values were normalized to the Ct value of the housekeeping gene GAPDH, and analysis was performed using the 2^{-ΔΔCt} method.

2.12 Western blot assay

Total cellular protein was extracted using a mixture of protease inhibitor PMSF and NP-40 lysis buffer (PMSF:NP-40 = 1:100). The BCA protein assay kit was used for protein quantification. They were resolved on SDS-PAGE gels and transferred to a nitrocellulose membrane. After blocking with 5% skim milk for 2 h, the membrane was treated with a specific primary antibody at 4 °C overnight. Afterward, the membrane was probed with a fluorescence-conjugated antibody for 2 h. The membrane was visualized using enhanced chemiluminescence (ECL) reagents and detected on a gel imaging system.

2.13 Wound healing assay

When the NCM460 or HT-29 cells reached 90% confluency, the cell monolayer was gently scraped with a sterile pipette tip. Images were acquired using a fluorescence microscope and the wound area was calculated as B0. After imaging, the medium was exchanged for serum-free medium containing the treatment, and cells were incubated under 5% CO₂ at 37 °C for 24 h. The wound area was measured again as B24. The cell wound healing rate was determined *via* the following formula: Wound healing% = (B0 – B24)/B0 × 100%.

2.14 Cell migration assay

NCM460 cells (5 × 10⁴ cells per mL) were seeded into the upper chamber of a Transwell insert. The cells were cultured for 24 h. Afterward, cells remaining in the upper chamber were wiped away with a cotton swab. The migrated cells were immobilized in 4% paraformaldehyde for 15 min, followed by staining with 0.1% crystal violet for 20 min under light protection. The stained cells were observed under a fluorescence microscope, and over three arbitrary fields were counted.

2.15 Immunofluorescence staining analysis

NCM460 cells (5 × 10⁴ cells per mL) were fixed with 4% paraformaldehyde for 25 min. The cells were made permeable with



0.5% Triton-X100 for 8 min and incubated with 3% BSA for 1.5 h. The primary antibody was incubated under a 37 °C oven for 1.5 h. Then, a fluorescence-conjugated antibody was treated at 37 °C for 1.5 h. Hoechst anti-fluorescence quenching mounting solution was supplemented, and the cells were incubated in darkness for 20 min. Images were acquired using a fluorescence microscope.

2.16 Co-immunoprecipitation assay

Protein A/G/M magnetic beads (80 µL) were supplemented with a 2 mL EP tube, followed by the incorporation of 1 µg of antibody (with IgG as a negative control). The mixture was incubated for 12 h at 4 °C using a rotary mixer. The tube was then placed on a magnetic rack to recover the antibody. The extracted protein samples were mixed with Protein A/G/M magnetic beads and incubated at 4 °C for 6 h. A mixture of NP-40 (50 µL) and loading buffer (loading buffer : NP-40 = 1 : 4) was supplemented and heated at 100 °C for 15 min, and then the supernatant was collected. Western blot analysis was performed.

2.17 Nocodazole washout assay

NCM460 cells (5×10^4 cells per mL) were treated with nocodazole (10 µM) and incubated at 37 °C for 3.5 h. The medium was then exchanged for serum-free medium containing arctigenin, and the cells were incubated at 37 °C for 0, 15, 30, and 60 min, respectively. Then, the cells were fixed with 4% paraformaldehyde for 20 min. The cells were permeabilized with 0.5% Triton-X100 for 8 min, blocked with 3% BSA for 1.5 h, and then incubated with primary antibody overnight at 4 °C. After the cells were treated with a fluorescence-conjugated antibody at 37 °C for 1.5 h, the cells were mounted in Hoechst anti-fluorescence quenching mounting solution, incubated for 15 min, and captured using a fluorescence microscope.

2.18 Glutamate content detection

NCM460 cells were harvested into a 2.0 mL Eppendorf tube and the supernatant was discarded. They were re-suspended with reagent 1 (cell count: 1 mL reagent = 5×10^7 cells) and sonicated (ice bath, power 20% or 200 W, sonication for 3 seconds, interval of 10 seconds, repeated 30 times). Then, they were centrifuged at 8000 g for 10 min, and the supernatant was collected to be used for further analysis. Glutamate content was measured in accordance with the operating guidelines furnished by the glutamate detection kit.

2.19 Aspartate content assay

NCM460 cells (2×10^6 cells per mL) were collected, and 0.4 mL of reagent 1 was added for sonication. An aliquot of 50–350 µL of the supernatant was moved to a 3 KD ultrafiltration tube (activated in 75% ethanol), then centrifuged at 12 000g for 10 min, and the filtrate was harvested for analysis. Aspartate content was measured in accordance with the operating guidelines furnished by the aspartate detection kit.

2.20 Transient transfection

siERβ- and SLC1A6-plasmids were transfected according to the Lipofectamine 6000 transfection reagent protocol: 5 µL of overexpression plasmid and 5 µL of Lipofectamine 6000 were separately supplemented into 125 µL of serum-free, antibiotic-free medium, followed by incubation for 5 min. The overexpression plasmid was blended with Lipofectamine 6000 solution, followed by incubation for 18 min. The two solutions were then combined and gently mixed by pipetting, followed by incubation for 18 min. 130 µL of the mixed transfection solution were supplemented into each well containing cells, mixed gently, and incubated at 37 °C for 6–8 h. The cells were then used for subsequent experiments.

2.21 Metabolomics assay

NCM460 cells were seeded in a 10 cm dish and grown to 90% confluence, then treated with arctigenin-containing serum-free medium for 24 h. The cells were digested with trypsin and transferred to a centrifuge tube, followed by centrifugation to remove residual medium and halt cell metabolism. Pre-chilled 80% methanol was added to extract the cellular polar metabolites *via* repeated cycles of freezing and thawing. After centrifugation, the supernatant was collected and submitted to Meiji Bio-Technology Co., Ltd (Shanghai, China) for further processing. The following criteria were set: $|\log_2 \text{FoldChange}| > 1$ and $P < 0.05$, to accurately select differentially expressed metabolites between the two groups and perform functional enrichment analysis.

2.22 Statistical analysis

Data were analyzed using SPSS software and are presented as means \pm S.E.M. The Student's *t*-test was utilized for comparisons between two groups, and one-way ANOVA was employed for those involving more than two groups. $P < 0.05$ was regarded as statistically significant. Correlations among diverse factors were assessed *via* Pearson correlation analysis.

3. Results

3.1 Arctigenin promotes cell migration and wound healing in colonic epithelial cells by accelerating focal adhesion disassembly

Focal adhesion disassembly is essential for a range of physiological processes, encompassing wound healing and immune responses, as it regulates extracellular matrix connections, controls cell morphology and migration, and facilitates the restoration and reconstruction of tissue architecture at the wound site.¹⁸ We examined the impact of arctigenin on focal adhesion disassembly of colonic epithelial cells and its importance in promoting cell migration and wound healing. The results showed that arctigenin (3, 10 µM) significantly diminished the count of focal adhesions in either NCM460 or HT-29 cells treated with nocodazole at 15 and 30 min, suggesting that arctigenin accelerates focal adhesion disassembly in colonic epi-



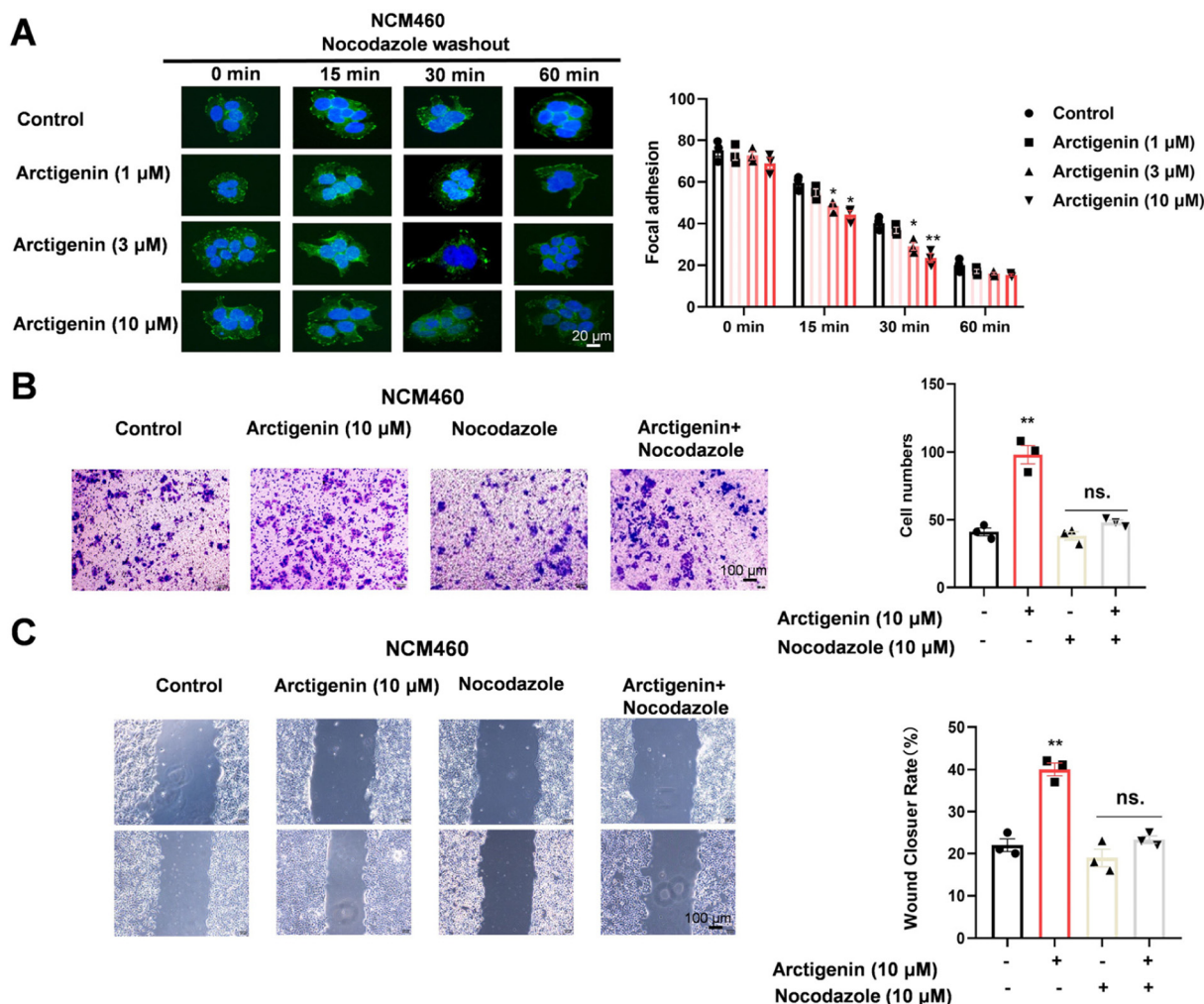


Fig. 1 Promotion effect of arctigenin on focal adhesion disassembly and its importance in acceleration of cell migration and wound healing in NCM460 cells. NCM460 cells were treated with arctigenin (10 μM) for 24 h in the presence or absence of nocodazole (10 μM). (A) Focal adhesion disassembly of NCM460 cells assessed by confocal immunofluorescence using an antibody against vinculin (plotting scale: 20 μm). (B) Migration of NCM460 cells measured using the transwell assay (plotting scale: 100 μm). (C) Rate of wound healing of NCM460 cells measured using the scratch assay (plotting scale: 100 μm). The data are presented as means ± S.E.M. Significant differences are indicated as follows: * $P < 0.05$ and ** $P < 0.01$ compared to the control group. ^{ns} $P > 0.05$ compared to the nocodazole group. The results shown are representative of three independent experiments.

thelial cells (Fig. 1A and SI Fig. S1). Subsequently, we used the focal adhesion disassembly inhibitor nocodazole in combination with arctigenin to investigate the importance of focal adhesion disassembly in arctigenin-mediated promotion of colonic epithelial cell migration and wound healing. The results showed that nocodazole significantly attenuated the effect of arctigenin on the migration and wound healing of NCM460 and HT-29 cells (Fig. 1B, C and SI Fig. S1). Moreover, we treated NCM460 cells with 100 ng mL⁻¹ TNF-α to mimic the inflammatory environment of colonic epithelial cells in the context of UC and further evaluated the impact of arctigenin on wound healing and cell migration. The results showed that arctigenin effectively promoted wound healing and cell migration in NCM460 cells under an inflammatory environment (SI Fig. S2). Taken together, arctigenin drives cell

migration and accelerates wound healing in colonic epithelial cells by accelerating focal adhesion disassembly.

3.2 Arctigenin accelerates focal adhesion disassembly, cell migration and wound healing by enhancing autophagic degradation of paxillin in colonic epithelial cells

Paxillin, talin, and kindlin, the structural proteins that form the scaffold of focal adhesions, play essential roles in maintaining focal adhesion stability, thereby influencing cell motility and stress transmission.^{19,20} We used western blotting and q-PCR assay to examine the expression of these three proteins in colon epithelial cells and found that arctigenin (10 μM) did not appreciably alter the mRNA expression levels of paxillin, talin, and kindlin in NCM460 cells (Fig. 2A). In contrast, arctigenin significantly inhibited the protein expression of paxillin



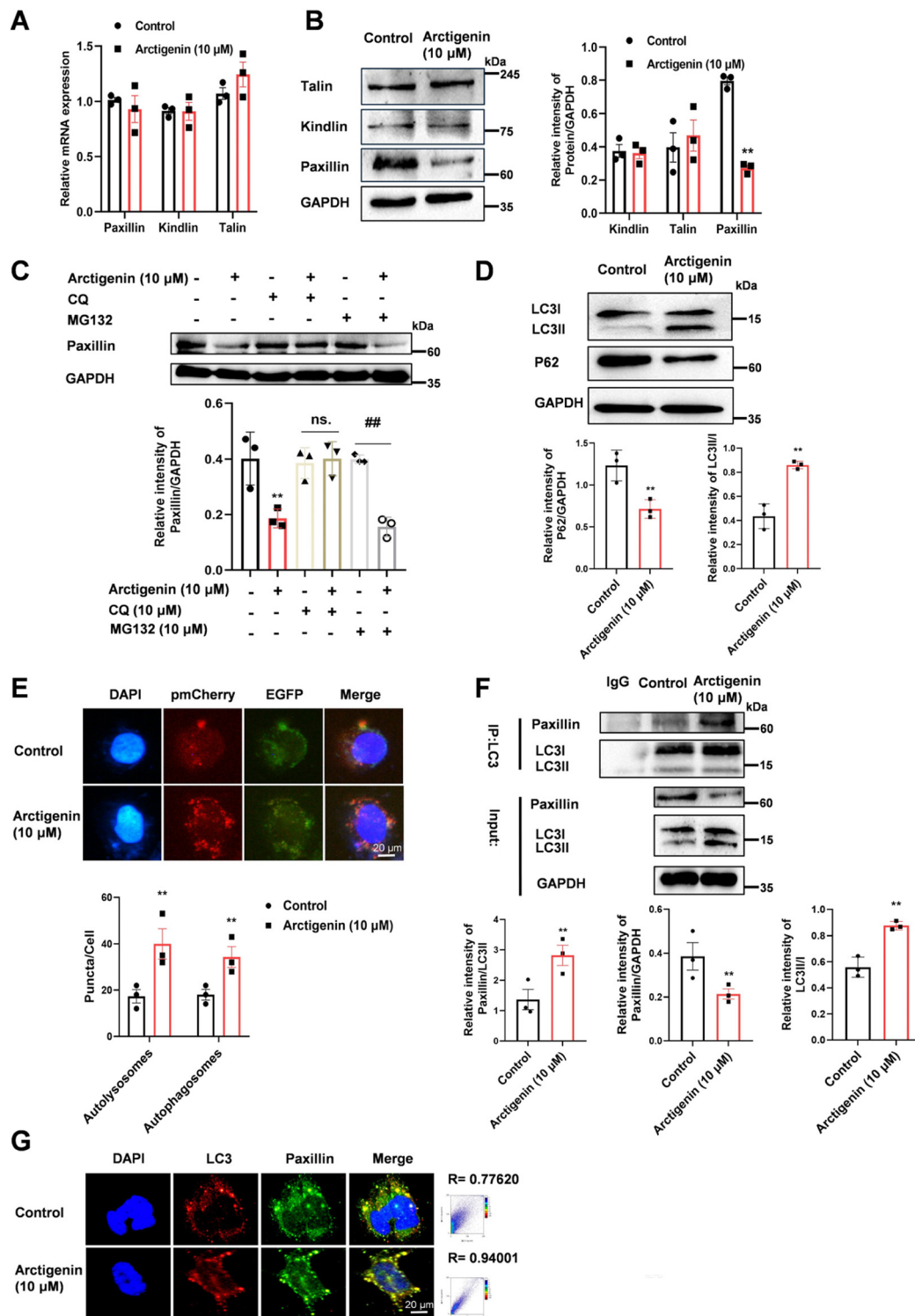


Fig. 2 Arctigenin promotes focal adhesion disassembly by enhancing autophagic degradation of paxillin in colonic epithelial cells. NCM460 cells were treated with arctigenin (10 μ M) for 24 h. (A) mRNA expression levels of paxillin, talin, and kindlin measured using real-time qPCR. (B) Protein expression levels of paxillin, talin, and kindlin in NCM460 cells measured using western blotting. (C) Protein expression levels of paxillin in NCM460 cells measured using western blotting. (D) Protein expression levels of LC3 and P62 in NCM460 cells measured using western blotting assay. (E) Autophagosomes (yellow fluorescence) and autolysosomes (red fluorescence) visualized by confocal microscopy (scale bar: 20 μ m). (F) Total protein isolated from NCM460 cells and immunoprecipitated with an anti-LC3 antibody, and the protein expression levels of LC3 and paxillin analyzed by western blotting. (G) Representative immunostaining images of LC3 (red), paxillin (green), and DAPI (blue) in NCM460 cells. The R represents Pearson's correlation CR coefficient value showing the signal colocalization status (plotting scale: 20 μ m). The data are presented as means \pm S.E.M. Significant differences are indicated as follows: ** P < 0.01 compared to the control group. ^{ns} P > 0.05 compared to the CQ group, indicating no significant difference. ^{##} P < 0.01 compared to the MG132 group. The results shown are representative of three independent experiments.



rather than talin and kindlin in NCM460 cells (Fig. 2B), suggesting that paxillin may be the target protein through which arctigenin accelerates focal adhesion disassembly. Meanwhile, the fact that arctigenin reduced the protein expression but not mRNA expression of paxillin in colonic epithelial cells implied that it may act by promoting the protein degradation of paxillin. Protein degradation occurs mainly *via* the ubiquitin-proteasome pathway and the autophagy-lysosome pathway. To identify the unique pathway involved in arctigenin-induced paxillin degradation, specific inhibitors were used. Chloroquine (CQ), a common autophagy inhibitor, blocks autophagosome-lysosome fusion, while MG132 is a proteasome inhibitor. The results showed that co-treatment with CQ nearly fully negated the ability of arctigenin to inhibit paxillin protein expression in NCM460 cells (Fig. 2C), while co-treatment with MG132 had no significant effect on paxillin protein expression. These results suggest that arctigenin accelerates focal adhesion disassembly to specifically promote autophagic degradation of paxillin protein in colonic epithelial cells.

Next, we investigated how arctigenin influences autophagy and autophagic flux in colonic epithelial cells. Western blot assay revealed that arctigenin significantly promoted LC3I-to-LC3II conversion in NCM460 cells and accelerated the degradation of Sequestosome 1 (P62) (Fig. 2D). To assess alterations in autophagic flux, we transfected the mCherry-EGFP-LC3 plasmid into NCM460 cells. Under fluorescence microscopy, yellow fluorescent spots were observed in the autophagosomes. Once autolysosomes formed, the acidic lysosomal environment quenched EGFP fluorescence, while pmCherry fluorescence remained unaffected, resulting in red fluorescence in the autolysosomes. Arctigenin significantly upregulated the quantity of autophagosomes and autolysosomes in NCM460 cells (Fig. 2E), indicating that arctigenin enhances autophagy and autophagic flux in colonic epithelial cells.

Subsequently, we investigated if arctigenin-induced autophagy in colonic epithelial cells was linked to the encapsulation of paxillin by autophagosomes. Immunoprecipitation results revealed that arctigenin notably augmented the binding of LC3 and paxillin in NCM460 cells (Fig. 2F). Additionally, co-localization immunofluorescence assay also showed that arctigenin significantly enhanced the co-localization of LC3-labeled autophagosomes (red dots) and paxillin (green dots) (Fig. 2G). These results suggest that arctigenin substantially promotes autophagic degradation of paxillin in colonic epithelial cells.

Furthermore, the correlation between arctigenin-induced autophagic degradation of paxillin in colonic epithelial cells and acceleration of focal adhesion disassembly, cell migration and wound healing was examined. Arctigenin was used in combination with two classical autophagy inhibitors 3-methyladenine (3-MA, a suppressor of autophagosome biogenesis by blocking the PI3K pathway) and CQ (a suppressor of autolysosome biogenesis), respectively. The results showed that co-treatment with either 3-MA or CQ significantly reversed arctigenin-induced promotion of LC3-paxillin binding and co-local-

ization, as well as acceleration of focal adhesion disassembly, cell migration and wound healing in NCM460 cells (Fig. 3A–E). These findings indicate that enhancing autophagic degradation of paxillin is crucial for arctigenin accelerating focal adhesion disassembly, cell migration and wound healing in colonic epithelial cells.

3.3 Arctigenin enhances autophagy in colonic epithelial cells through reducing excitatory amino acid transport

Cellular energy metabolism is essential for triggering autophagy. Under conditions of nutrient deprivation, autophagy is activated to clear damaged organelles or unnecessary cellular components, providing amino acids and fatty acids to maintain cellular energy and survival.²¹ Subsequently, we explored the mechanism by which arctigenin activates autophagy in colonic epithelial cells based on cellular metabolism. Non-targeted metabolomics analysis was performed to assess the different metabolites in NCM460 cells between the arctigenin-treated group and the untreated control group. The results illustrated a clear separation between the sample populations of the arctigenin-treated group and the untreated control group, demonstrating notable metabolic differences between the two groups (SI Fig. S3). After arctigenin treatment, the differentially expressed metabolites in the NCM460 cells were primarily enriched in amino acids, peptides, and their analogs (Fig. 4A). The most notable downregulation was observed in glutamate and aspartate levels in the arctigenin-treated group (Fig. 4B).

Next, we validated the conclusions from the non-targeted metabolomics analysis. The results showed that arctigenin significantly downregulated the levels of glutamate and aspartate in NCM460 cells (Fig. 4C and D). As non-essential amino acids, glutamate and aspartate undergo synthesis, transport, and degradation processes. Therefore, we investigated the impact of arctigenin on the enzymes responsible for the synthesis and metabolism of glutamate and aspartate. The findings revealed that arctigenin showed no significant impact on the mRNA expression of GLDH, GPT, GLS, CPS, GAD, DDO, GOT1, and APSH in NCM460 cells (Fig. 4E), suggesting that arctigenin does not significantly affect the degradation and synthesis of glutamate and aspartate in colonic epithelial cells, and it is possible to intervene in the transport of the two amino acids.

Amino acid transport into cells depends on specific transporters, and excitatory amino acid transporters regulate the concentrations of glutamate and aspartate inside cells to maintain normal cellular function.²² The main excitatory amino acid transporters include SLC1A1, SLC1A2, SLC1A3, SLC1A6, and SLC1A7. We evaluated the impact of arctigenin on the expression of these transporters. The q-PCR assay revealed that arctigenin treatment markedly downregulated the mRNA levels of SLC1A6 in NCM460 cells, whereas it exerted no statistically significant impact on the mRNA levels of SLC1A1, SLC1A2, SLC1A3, and SLC1A7 (Fig. 4F). Western blot analysis yielded the identical conclusion that arctigenin markedly decreased the protein expression of SLC1A6 in NCM460 cells (Fig. 4G).



These observations suggest that arctigenin downregulates the levels of glutamate and aspartate in colonic epithelial cells through blocking the expression of the excitatory amino acid transporter SLC1A6.

Intracellular amino acid concentrations play a direct regulatory role in the autophagy process. When amino acid levels

decrease, the activity of mTORC1 is reduced, thereby promoting the initiation of autophagy.²³ Our findings align with previous studies, demonstrating that arctigenin exerts no notable impact on mTORC1 protein expression but significantly suppresses p-mTORC1 protein expression in NCM460 cells (Fig. 4H). The results suggest that arctigenin reduces excitatory

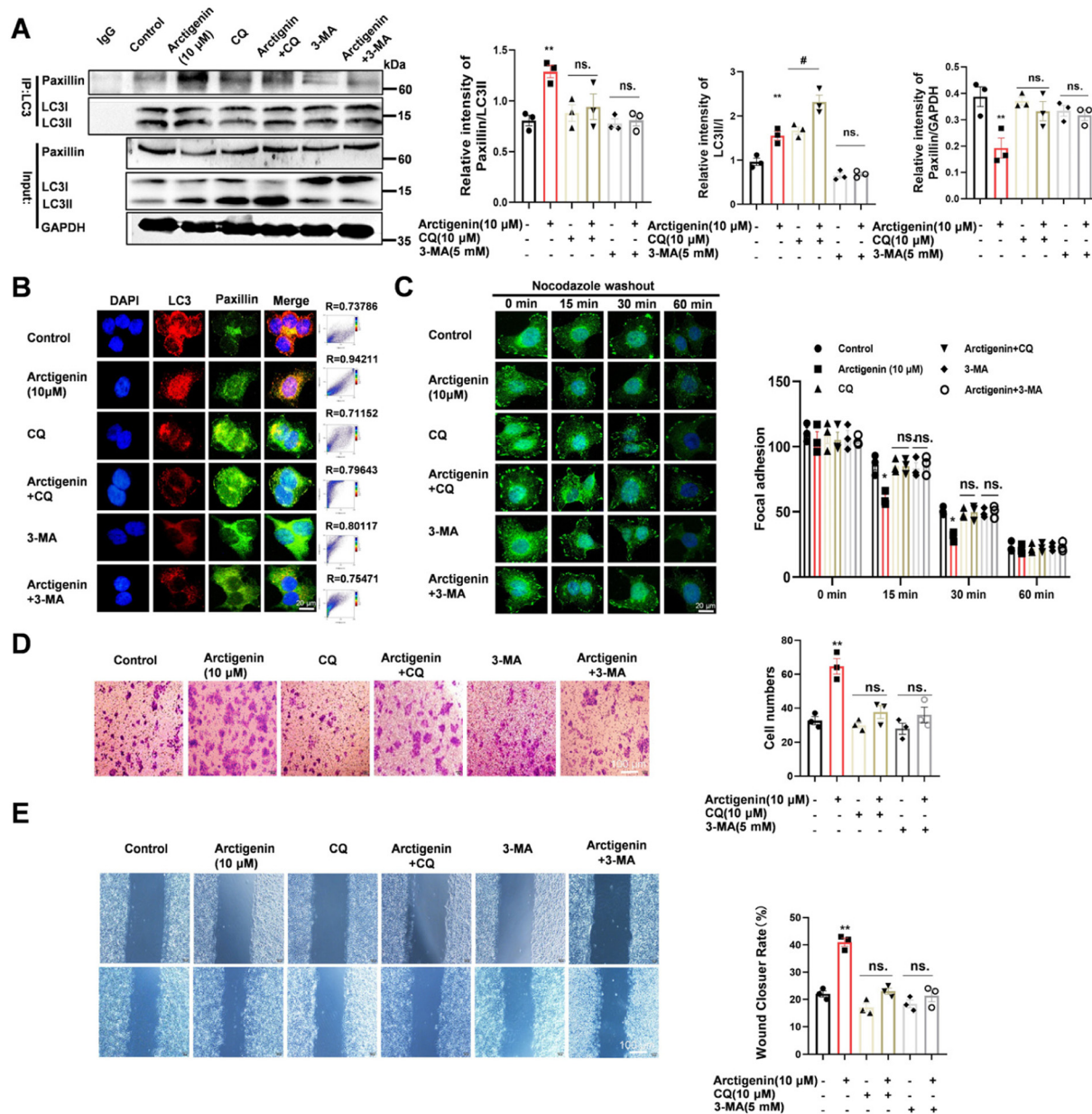


Fig. 3 Arctigenin accelerates focal adhesion disassembly, cell migration and wound healing in colon epithelial cells by promoting autophagic degradation of paxillin. NCM460 cells were treated with arctigenin (10 μ M) for 24 h in the presence or absence of 3-MA (5 mM) and CQ (10 μ M). (A) Total protein isolated from NCM460 cells and immunoprecipitated with an anti-LC3 antibody, and the protein expression levels of LC3 and paxillin analyzed by western blotting. (B) Representative immunostaining images of LC3 (red), paxillin (green), and DAPI (blue) in NCM460 cells. The R represents Pearson's correlation CR coefficient value showing the signal colocalization status (plotting scale: 20 μ m). (C) Focal adhesion disassembly assessed by confocal immunofluorescence using an antibody against vinculin (plotting scale: 20 μ m). (D) The migration of NCM460 cells measured using the transwell assay (plotting scale: 100 μ m). (E) Rate of wound healing of NCM460 cells measured using the scratch assay (plotting scale: 100 μ m). The data are presented as means \pm S.E.M. Significant differences are indicated as follows: * P < 0.05 and ** P < 0.01 compared to the control group. # P < 0.05 compared to the CQ group. ^{ns} P > 0.05 compared to the 3-MA or the CQ group, indicating no significant difference. The results shown are representative of three independent experiments.



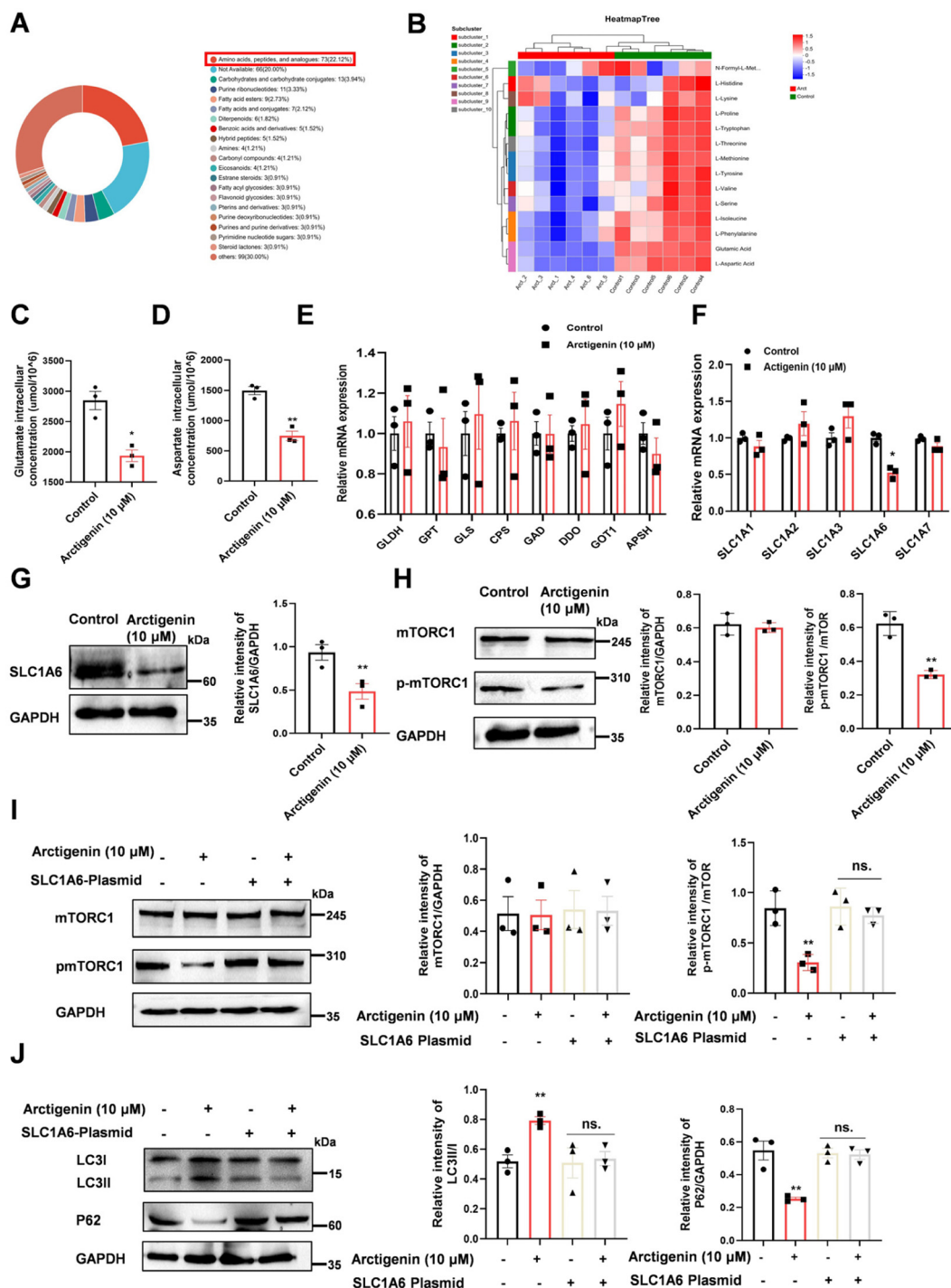


Fig. 4 Arctigenin enhances autophagy in colon epithelial cells by reducing excitatory amino acid transport. The metabolites of NCM460 cells with or without arctigenin treatment were detected by a non-targeted metabolomics assay. (A) A pie chart showing the main metabolic differences between arctigenin-treated and untreated NCM460 cells. (B) Heatmap showing the levels of 14 amino acids in arctigenin-treated NCM460 cells compared to untreated NCM460 cells. (C and D) The levels of glutamate and aspartate in NCM460 cells measured using ELISA kits. (E) mRNA expression levels of GLDH, GPT, GLS, CPS, GAD, DDO, GOT1 and APSH in NCM460 cells measured using real-time qPCR. (F) mRNA expression levels of SLC1A6 in NCM460 cells measured using real-time qPCR. (G) Protein expression levels of SLC1A6 in NCM460 cells measured using western blotting. (H) Protein expression levels of p-mTORC1 and mTORC1 in NCM460 cells measured using western blotting. (I) Protein expression levels of p-mTORC1 and mTORC1 in NCM460 cells measured using western blotting. (J) Protein expression levels of LC3 and P62 in NCM460 cells measured using western blotting. The data are presented as means \pm S.E.M. Significant differences are indicated as follows: * $P < 0.05$ and ** $P < 0.01$ compared to the control group; ^{ns} $P > 0.05$ compared to the SLC1A6-plasmid group, indicating no significant difference. The results shown are representative of three independent experiments.



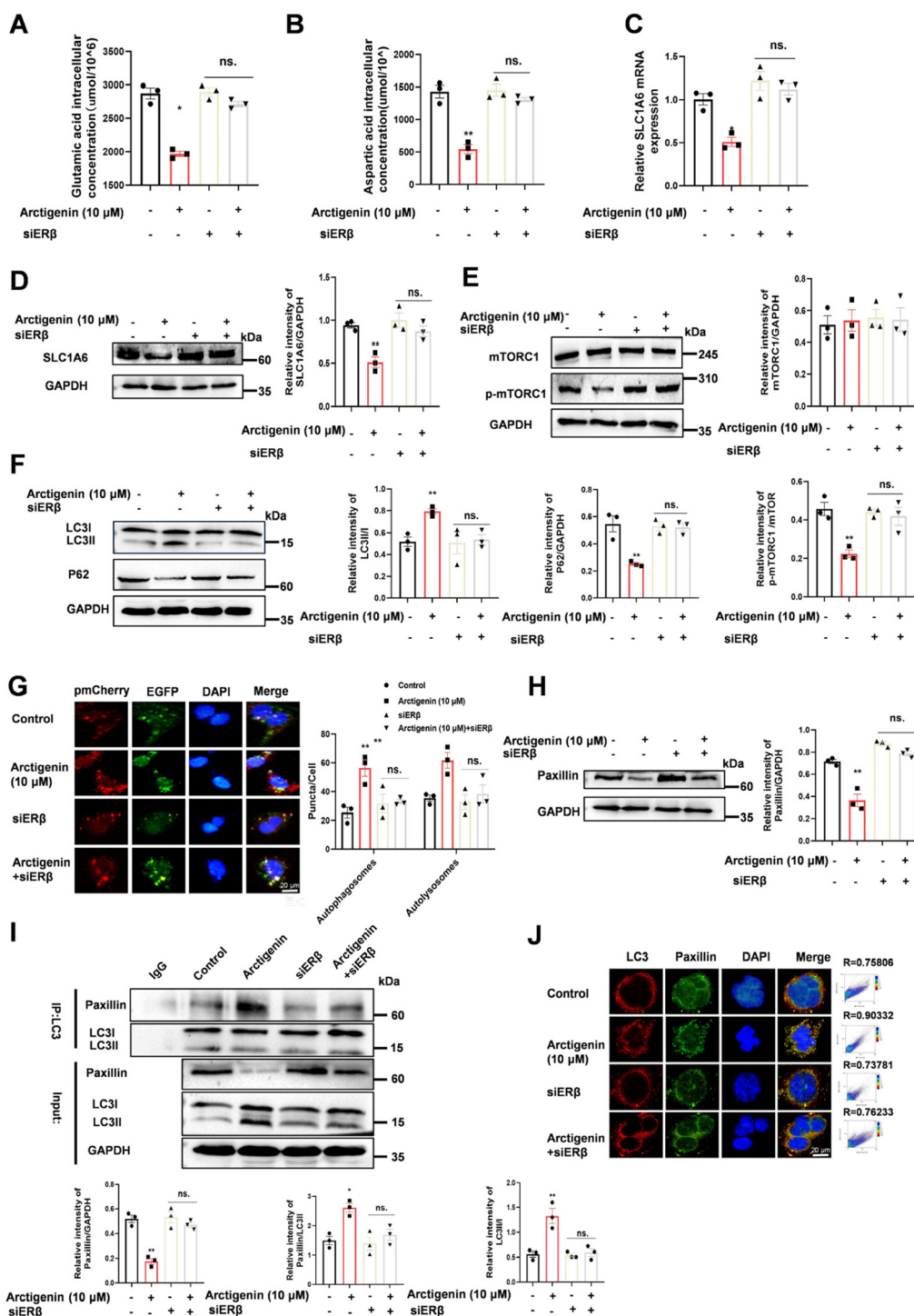


Fig. 5 Arctigenin reduces amino acid transport, enhances autophagy, and accelerates focal adhesion disassembly and wound healing of colonic epithelial cells in an ER β -dependent manner. NCM460 cells were treated with arctigenin (10 μ M) for 24 h in the presence or absence of siER β . (A and B) The levels of glutamate and aspartate in NCM460 cells measured using ELISA kits. (C) mRNA expression levels of SLC1A6 in NCM460 cells measured using real-time qPCR. (D) Protein expression levels of SLC1A6 in NCM460 cells measured using western blotting. (E) Protein expression levels of p-mTORC1 and mTORC1 in NCM460 cells measured using western blotting. (F) Protein expression levels of LC3 and P62 in NCM460 cells measured using western blotting assay. (G) Autophagosomes (yellow fluorescence) and autolysosomes (red fluorescence) visualized by confocal microscopy (scale bar: 20 μ m). (H) Protein expression levels of paxillin in NCM460 cells measured using western blotting. (I) Total protein isolated from NCM460 cells and immunoprecipitated with an anti-LC3 antibody, and the protein expression levels of LC3 and paxillin analyzed by western blotting. (J) Representative immunostaining images of LC3 (red), paxillin (green), and DAPI (blue) in NCM460 cells. The R value represents the Pearson's correlation coefficient, indicating the degree of signal colocalization (plotting scale: 20 μ m). The data are presented as means \pm S.E.M. Significant differences are indicated as follows: * P < 0.05 and ** P < 0.01 compared to the control group. ^{ns} P > 0.05 compared to the siER β group, indicating no significant difference. The results shown are representative of three independent experiments.



amino acid transport, inhibiting mTORC1 activation, and thereby enhancing autophagy in colonic epithelial cells.

To validate the above-mentioned findings, we transfected NCM460 cells with SLC1A6-plasmid to investigate its effect on

the inhibition of mTORC1 activation by arctigenin and the subsequent enhancement of autophagy. Co-treatment with SLC1A6-plasmid reversed the inhibitory impact of arctigenin on mTORC1 activation and the enhancement of autophagy

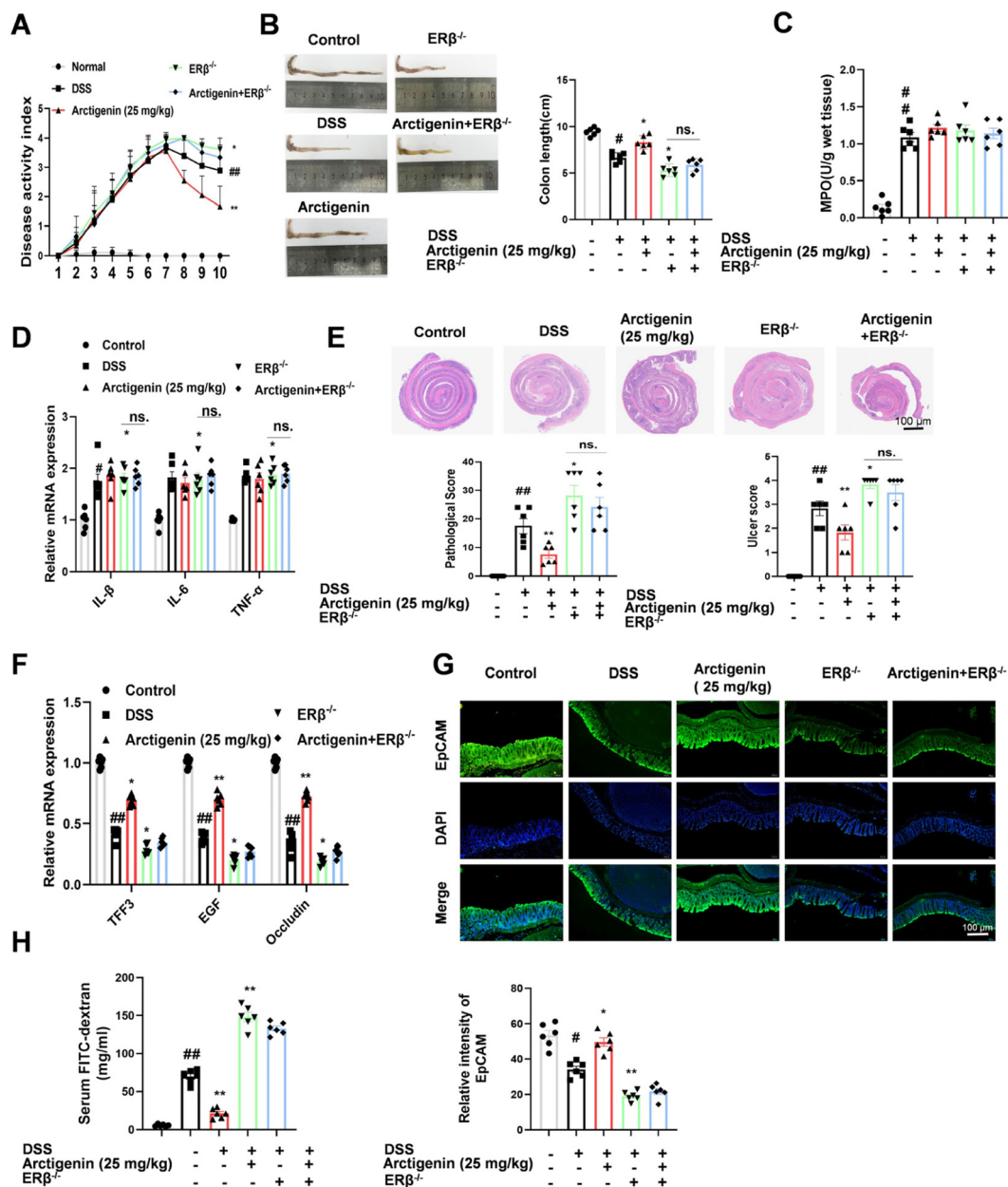


Fig. 6 Arctigenin promotes colonic mucosal healing of mice with DSS-induced colitis in an ERβ-dependent manner. Colitis was induced in wild-type and ERβ^{-/-} mice by drinking 3.5% (w/v) DSS solution *ad libitum* for 5 days, followed by normal plain water for 5 days. Starting from the 6th day after colitis induction, mice in the arctigenin group and the arctigenin + ERβ^{-/-} group were orally administered with arctigenin (25 mg kg⁻¹) for 5 consecutive days, and mice in the normal group and the DSS model group received an equal volume of vehicle. (A) The DAI score. (B) The lengths of colons. (C) mRNA expression of IL-β, IL-6, and TNF-α in colon tissues quantified using real-time qPCR. (D) MPO activity of colon tissues measured using a commercial kit. (E) Representative images of Swiss roll colon sections stained with H&E, along with pathological and ulcer scores (scale bar: 100 μm). (F) mRNA expression of TFF3, EGF, and occludin in colon tissues quantified using real-time qPCR. (G) Representative immunofluorescence images of epithelial recovery of colon tissues for Ep-CAM (green) and DAPI (blue) (scale bar: 100 μm). (H) Detection of FITC-dextran levels in serum. The data are presented as means ± S.E.M. Significant differences are indicated as follows: **P* < 0.05 and ***P* < 0.01 compared to the DSS group. #*P* < 0.05 and ##*P* < 0.01 compared to the control group. ^{ns}*P* > 0.05 compared to the ERβ^{-/-} group, indicating no significant difference. The results shown are representative of six mice.



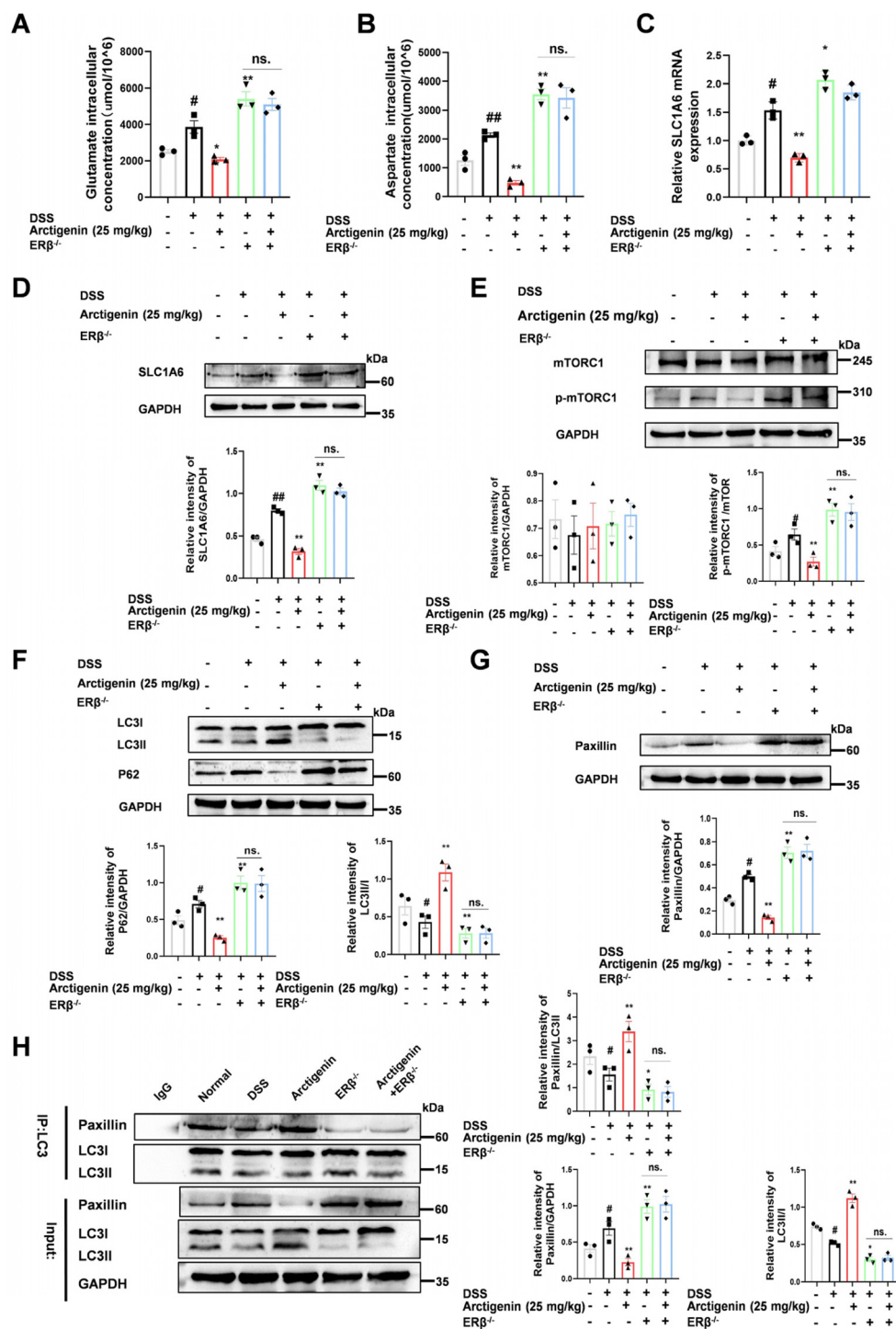


Fig. 7 Arctigenin reduces excitatory amino acid transport, suppresses mTORC1 activation, and enhances autophagic degradation of paxillin in primary colonic epithelial cells of mice with DSS-induced colitis in an ERβ-dependent manner. Colitis was induced in wild-type and ERβ^{-/-} mice by drinking 3.5% (w/v) DSS solution *ad libitum* for 5 days, followed by normal plain water for 5 days. On the 10th day after colitis induction, the primary colonic epithelial cells were isolated. (A and B) The levels of glutamate and aspartate in primary mouse colonic epithelial cells measured using ELISA kits. (C) mRNA expression levels of SLC1A6 in primary mouse colonic epithelial cells measured using real-time qPCR. (D) Protein expression levels of SLC1A6 in primary mouse colonic epithelial cells measured using western blotting. (E) Protein expression levels of p-mTORC1 and mTORC1 in primary mouse colonic epithelial cells measured using western blotting. (F) Protein expression levels of LC3 and P62 in primary mouse colonic epithelial cells measured using western blotting assay. (G) Protein expression levels of paxillin in primary mouse colonic epithelial cells measured using western blotting. (H) Total protein isolated from primary mouse colonic epithelial cells and immunoprecipitated with an anti-LC3 antibody, and the protein expression levels of LC3 and paxillin analyzed by western blotting. The data are presented as means ± S.E.M. Significant differences are indicated as follows: **p* < 0.05 and ***p* < 0.01 compared to the DSS group. #*p* < 0.05 and ##*p* < 0.01 compared to the control group. *ns* *p* > 0.05 compared to the ERβ^{-/-} group, indicating no significant difference. The results shown are representative of three independent experiments.



(Fig. 4I and J). This indicates that inhibition of excitatory amino acid transport represents a crucial pathway through which arctigenin suppresses mTORC1 activation and activates autophagy in colonic epithelial cells.

3.4 Arctigenin reduces amino acid transport, enhances autophagy, and accelerates focal adhesion disassembly and wound healing of colonic epithelial cells in an ER β -dependent manner

Estrogen receptor beta (ER β) is an estrogen-dependent nuclear transcription factor that undergoes a conformational change upon binding with estrogen, which in turn interacts with co-activators or corepressors to initiate or inhibit the transcription of target genes.²⁴ Accumulating evidence indicates that ER β activation plays a key role in wound healing and angiogenesis by stimulating cell proliferation, inhibiting inflammation, and accelerating endothelial cell migration.^{25,26} Our previous studies demonstrated that arctigenin, a phytoestrogen, exerts an anti-colitic effect through activating ER β .²⁷ Therefore, we investigated the significance of ER β in the effect of arctigenin on reducing amino acid transport, enhancing autophagy, promoting focal adhesion disassembly, and facilitating wound healing in colonic epithelial cells.

We transfected siER β into NCM460 cells, and the results showed that siER β -3 achieved the highest knockdown efficiency, so it was selected for subsequent experiments (SI Fig. S4). ER β knockdown reversed the downregulation of glutamate and aspartate levels, as well as the inhibition of SLC1A6 mRNA and protein expression by arctigenin (Fig. 5A–D). Furthermore, ER β knockdown significantly diminished the ability of arctigenin to promote autophagy *via* the inactivation of mTORC1 (Fig. 5E–G). Co-localization immunofluorescence staining and co-immunoprecipitation results demonstrated that ER β knockdown reversed the promotion of arctigenin on autophagic degradation of paxillin (Fig. 5H–J), focal adhesion disassembly, cell migration, and wound healing in NCM460 cells (SI Fig. S5). These findings suggest that ER β activation exerts a crucial function in arctigenin-mediated migration and wound healing in colonic epithelial cells.

3.5 Arctigenin promotes colonic mucosal healing of colitis mice in an ER β -dependent manner

ER β knockout mice were generated to identify the ER β dependency for arctigenin facilitating colonic mucosal healing in colitis mice. Tail tissue samples were obtained from ER β knockout mouse offspring to perform genotype identification, while wild-type littermates served as a control (SI Fig. S4). To induce colitis, wild-type and ER β ^{-/-} mice were provided with drinking water containing 3.5% (w/v) DSS for 5 days, followed by a 5-day period of normal water supply. Starting from the 6th day after colitis induction, mice in the arctigenin group and the arctigenin + ER β ^{-/-} group were orally administered with arctigenin for 5 consecutive days, and mice in the normal group and DSS model group were administered the same volume of vehicle. The results showed that ER β knockout significantly attenuated the effect of arctigenin on reducing DAI

scores and inhibiting colon shortening (Fig. 6A and B) but did not modify the impact of arctigenin on MPO activity and mRNA expression of pro-inflammatory cytokines in the colon tissues of mice (Fig. 6C and D). Histopathological examination of colonic tissues indicated that ER β knockout led to a further increase in mucosal damage and significantly diminished the improvement of arctigenin (25 mg kg⁻¹) on colonic histopathological changes of mice, including epithelial destruction, ulcer bleeding, and crypt loss (Fig. 6E). Additionally, ER β knockout significantly attenuated the promotion of arctigenin on the mRNA expression of mucosal healing-related factors TFF3, EGF and occludin in the colon tissues of mice (Fig. 6F). Immunofluorescence staining of Ep-CAM, a marker of colonic epithelial cells, showed that ER β knockout significantly attenuated the repair effect of arctigenin (25 mg kg⁻¹) on the colonic epithelial layer injury in mice (Fig. 6G). The FITC-dextran permeability assay showed that ER β knockout substantially impaired the protective capacity of arctigenin against disruptions to intestinal barrier integrity (Fig. 6H). Collectively, these findings indicate that arctigenin enhances colonic mucosal healing in mice with colitis through an ER β -dependent pathway.

3.6 Arctigenin reduces excitatory amino acid transport, suppresses mTORC1 activation, and enhances autophagic degradation of paxillin in primary colonic epithelial cells of mice with DSS-induced colitis in an ER β -dependent manner

To validate *in vitro* findings in NCM460 cells, the primary colonic epithelial cells were isolated from wild-type and ER β ^{-/-} mice with DSS-induced colitis. As shown in Fig. 7A–H, ER β knockout nearly completely reversed the effects of arctigenin on downregulating the levels of glutamate and aspartate, inhibiting the mRNA and protein expression of SLC1A6, suppressing mTORC1 activation, enhancing autophagy, reducing paxillin protein expression, and promoting the interaction between paxillin and LC3 in primary colonic epithelial cells isolated from mice with colitis. These findings further verify that arctigenin ER β -dependently reduces excitatory amino acid transport, suppresses mTORC1 activation, and enhances autophagic degradation of paxillin in colonic epithelial cells in the context of colitis.

4. Discussion

Under physiological conditions, the intestinal mucosa has self-repair capability. In a chronic inflammatory environment, persistent inflammation and immune responses hinder complete mucosal repair.²⁸ Among individuals with severe UC, the structure and function of the intestinal mucosa are obviously impaired, which can result in complications such as colorectal cancer and intestinal fibrosis.²⁹ Protecting the intestinal mucosa and promoting its repair are critical for managing UC and preventing relapse.³⁰ Although numerous drugs are available for the clinical treatment of UC, only a small proportion of patients achieve mucosal healing. Clinical investigations



have indicated that attainment of mucosal healing in patients is associated with lower relapse rates, lower surgery rates, and reduced colorectal cancer risks compared to those who achieve only symptom relief without mucosal healing.³¹ Development of mucosal healing promoters that directly target mucosal repair is of significant importance.

Adhesion and migration of epithelial cells play crucial roles in wound closure following mucosal injury. After injury, epithelial cells migrate toward the wound site to form a new epithelial layer that covers the wound.³² The process of cell migration requires the release of the traction forces formed by epithelial cells, which is mediated by focal adhesion disassembly.³³ There are reports indicating that focal adhesion disassembly participates in various processes such as cell migration, cytoskeletal remodeling, and tissue repair regulation, and is closely associated with angiogenesis and wound healing,^{34,35} which may serve as a new target for the treatment of mucosal injury-related diseases, including UC, oral ulcers and skin ulcers. The findings of this study demonstrate that arctigenin promotes focal adhesion disassembly, thereby enhancing cell migration and wound healing in colonic epithelial cells to alleviate mice with colitis, suggesting that it is a potential agent for promoting colonic mucosal healing in UC therapy.

When the protein complexes in focal adhesions are dissociated through the action of proteases or protein–protein interactions, the adhesion force between the cell and the extracellular matrix weakens, leading to accelerated focal adhesion disassembly. Proteins such as talin, kindlin, and paxillin play crucial roles in maintaining the structure and function of focal adhesions. This study demonstrates that arctigenin specifically enhances the autophagic degradation of paxillin in colonic epithelial cells, thereby accelerating focal adhesion disassembly. The findings elucidate the importance of autophagy in focal adhesion disassembly and provide new insights into the regulatory crosstalk between autophagy and cell migration. It is well established that autophagy plays a critical role in maintaining intracellular homeostasis and serves as an adaptive response to metabolic stress and environmental stressors.^{36,37} Through untargeted metabolomics analysis, we show that arctigenin induces the activation of autophagy of colonic epithelial cells by decreasing intracellular levels of excitatory amino acids, such as glutamate and aspartate. Arctigenin functions by specifically downregulating the expression of the excitatory amino acid transporter SLC1A6 and inhibiting the activation of mTORC1 to enhance autophagy.

The gender differences in UC are evident in epidemiology, disease manifestations, disease progression and complications, compliance, and mental health.³⁸ According to epidemiological surveys, no significant gender difference was found in the incidence of UC, but men exhibit higher disease activity and severity. Exogenous estrogen supplementation has been shown to alleviate symptoms in UC patients.³⁹ Studies have indicated that activation of ER β plays an important role in wound healing and angiogenesis by promoting cell proliferation, inhibiting inflammatory responses, and accelerating

endothelial cell migration.^{40,41} Therefore, we investigated whether the mucosal healing-promoting effect of arctigenin is dependent on ER β activation. The results demonstrate that ER β knockdown notably attenuates the impact of arctigenin, including reducing excitatory amino acid transport, enhancing autophagy, accelerating focal adhesion disassembly, and promoting wound healing in colonic epithelial cells. In mice with colitis, ER β knockout reverses the promoting effect of arctigenin on mucosal healing in colitis mice. These findings offer new evidence to support the utilization of arctigenin and ER β agonists as mucosal promoters.

Author contributions

Mianjiang Zhao: conceptualization, data curation, formal analysis, and writing – original draft. Jiafeng Zhang: methodology. Yilei Guo: data curation and formal analysis. Lei Gao: methodology. Zhifeng Wei: resources, methodology, and supervision. Yue Dai: conceptualization, funding acquisition, supervision, project administration, and writing – review and editing. Yufeng Xia: funding acquisition, methodology, and supervision.

Conflicts of interest

The authors declare no competing financial interest.

Abbreviations

ASPH	Aspartate β -hydroxylase
CPS	Carbamoyl phosphate synthetase
CQ	Chloroquine
DAI	Disease activity index
DSS	Dextran sulfate sodium
ER β	Estrogen receptor β
GAD1	Glutamate decarboxylase 1
GLS	Glutaminase
GOT1	Glutamate-oxaloacetate transaminase 1
GPT	Glutamate-pyruvate transaminase
3-MA	3-Methyladenine
SLC1A1	Solute carrier family 1 member a1
SLC1A2	Solute carrier family 1 member a2
SLC1A3	Solute carrier family 1 member a3
SLC1A6	Solute carrier family 1 member a6
SLC1A7	Solute carrier family 1 member a7
UC	Ulcerative colitis

Data availability

Data will be made available on request.

Supplementary information (SI) is available. See DOI: <https://doi.org/10.1039/d5fo04195c>.



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