ORIGINAL ARTICLE

Ping-Chong-Jiang-Ni Formula effectively inhibits migration and invasion of endometriosis 12Z cell line via TRIP13

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Abstract

The Ping-Chong-Jiang-Ni Formula (PCJNF), a compound of Traditional Chinese Medicine (TCM), has demonstrated remarkable clinical effectiveness and is widely utilized in the treatment of endometriosis (EMs). Previous studies have shown that PCJNF inhibits the proliferation and induces the apoptosis of ectopic endometrial stromal cells (EESCs) by modulating the JNK signaling pathway. In our most recent study, published in January 2024, we found that PCJNF effectively alleviates EMs-associated pain by reducing inflammatory mediators, including tumor necrosis factor-alpha (TNF-α) and nerve growth factor (NGF). Furthermore, PCJNF down-regulates the expression of transient receptor potential vanilloid 1 (TRPV1), phosphorylated TRPV1 (p-TRPV1), and protein kinase C (PKC). To further investigate the therapeutic potential of PCJNF, we conducted a comprehensive tandem mass tag (TMT) proteomics analysis, identifying 4984 proteins co-expressed in human EESCs treated with PCJNF and control serum. Based on these findings, this study explored the inhibitory effects of PCJNF on the migration and invasion of EMs cells *in vitro*. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of 12 genes revealed a significant upregulation of FER and TRIP13 in ectopic lesions, which was further validated by immunohistochemistry. Given the crucial role of TRIP13 in EMs, we employed small interfering RNA (siRNA) to knock down its expression, observing a marked reduction in the migration and invasion abilities of EMs cells. Conversely, overexpression of TRIP13 using short hairpin RNA (shRNA) enhanced these processes. Additionally, after PCJNF treatment, we observed significant alterations in the expression of migration- and invasion-related proteins, such as vimentin and E-cadherin. In conclusion, these findings suggest that TRIP13 may serve as a potential therapeutic target for EMs and that PCJNF offers a promising new approach for integrating Traditional Chinese Medicine with modern medical treatments.

Keywords: Invasion, Mechanism study, Migration, PCJNF, TRIP13

1. Introduction

Endometriosis (EMs) is a chronic systemic disease [1], that affects approximately 5%−10% of women of childbearing age worldwide [2]. The hallmark of EMs lies in the abnormal growth and persistence of endometrial-like tissue outside the uterus, leading to a variety of clinical symptoms. Up to 80% of patients report experiencing pain, while 40%−60% also struggle with infertility [3,4]. In recent years, the incidence of EMs has steadily increased, signaling an expanding global health burden, with its occurrence rate rising annually. Despite extensive research efforts, the exact cause of

Received 9 September 2024; revised 12 January 2025; accepted 25 March 2025.
Available online 13 June 2025

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https://doi.org/10.38212/2224-6614.3544
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EMs remains unclear, and current treatment modalities primarily rely on surgical interventions [5] and hormonal therapies [6]. However, these treatments often prove insufficient, with high recurrence rates observed following intervention. Not only does EMs impose a significant physical and psychological toll on patients, but it also places a substantial economic burden on healthcare systems. The lifetime treatment cost per patient is estimated at approximately $85,530 annually [7]. As a result, EMs is increasingly recognized as a chronic health issue requiring urgent attention, emphasizing the pressing need for more effective therapeutic strategies and comprehensive management solutions [1].

As is all know, the pathogenesis of EMs has been accounted for by several hypotheses, such as coelomic metaplasia, Müllerian duct remnants, lymphatic or vascular metastasis, retrograde menstruation, and the endometrial stem cell implantation theory [6]. Among these, Sampson's retrograde menstruation theory is widely accepted. This theory proposes that retrograde menstruation serves as a critical initiating factor in the development of EMs. During this process, menstrual blood, along with viable endometrial fragments, flows backward through the fallopian tubes into the pelvic cavity, where it implants on the peritoneal surfaces and pelvic organs [8,9]. The presence of menstrual debris in the peritoneal cavity during menstruation further supports this hypothesis, providing a robust foundation for understanding the genesis of this complex disease [8].

Traditionally, EMs has been categorized as a benign disease. However, it exhibits characteristics similar to malignant tumors in several critical biological processes, including adhesion, proliferation, invasion, local inflammation, immune dysregulation, and angiogenesis [10]. The adhesion, invasion, and growth of retrograde endometrial fragments outside the uterine cavity are closely linked to the aberrant biological behaviors of ectopic endometrial stromal cells (EESCs) [11]. Although retrograde menstruation occurs in up to 90% of women of reproductive age, the prevalence of EMs is comparatively low, at approximately 20% [12]. This discrepancy raises questions about whether the retrograde menstruation theory sufficiently explains the pathogenesis of EMs, particularly in cases where lesions are located deep within abdominal organs or outside the abdominal cavity [13]. Given the tumor-like properties of EMs, Lang's “In-Situ Intimal Decision Theory” expands upon the retrograde menstruation hypothesis [14]. This theory posits that the development of EMs involves a pathological process characterized by adhesion, invasion, and angiogenesis, collectively referred to as the “3A model” [14]. According to this model, as endometrial stromal cells (EESCs) invade and migrate to ectopic sites, they initiate periodic tissue repair, leading to the infiltration and migration of stromal tissue. This transition promotes cell adhesion, collagen aggregation, and the eventual formation of fibrous tissue [15]. Furthermore, prolonged exposure to elevated estrogen levels exacerbates the transition from stromal fibrosis to fibrosis in endometrial stromal cells, enhancing their invasive and migratory capabilities [16]. This process is closely associated with clinical manifestations such as chronic inflammation, progressive pelvic pain, and infertility [17]. As a result, migration and invasion are recognized as pivotal steps in the initiation of EMs and are key contributors to the formation of ectopic lesions [18].

Numerous studies have demonstrated the efficacy of Traditional Chinese Medicine (TCM) in the treatment of EMs, with notable benefits such as improved fertility, pain reduction, and prevention of recurrence [17,19]. PCJNF is a TCM compound developed based on our clinical experience and specifically formulated for the management of EMs. It consists of nine herbal constituents, namely Gui Zhi, Xue Jie, Wu Ling Zhi, Pu Huang, Jiang Xiang, Shui Zhi, Mai Dong, Bai Shao, Gan Cao. This formulation integrates classical TCM

Abbreviations

PCJNF	Ping-Chong-Jiang-Ni Formula
EMs	Endometriosis
EESCs	Endometriosis interstitial cells
TMT	Tandem mass tag
TCM	Traditional Chinese medicine
VAS	Visual analogue scale
PRL	Reduce serum prolactin
CA_{125}	Cancer Antigen 125
JNK	c-Jun N-terminal kinase
TRIP13	Thyroid Hormone Receptor Interactor 13
SiRNA	Small interfering RNA
shRNA	Short hairpin RNA
RAB2B	Recombinant Protein
C6orf120	chromosome 6 open reading frame 120
GLUL	Glutamine synthetaseGlutamine synthetase
SLC44A2	Choline transporter-like protein 2
DAP	Death-Associated Protein
GCLC	Recombinant Glutamate Cysteine Ligase, Catalytic
ASNS	Asparagine synthetase
FER	Ferritin
BPTF	Bromodomain PHD finger Transcription Factor
APOB	Apolipoprotein B
IGFALS	Recombinant Insulin Like Growth Factor Binding Protein

principles and is further refined through our clinical expertise. The chemical compositions of the components within PCJNF have been extensively characterized [20]. In clinical settings, PCJNF has demonstrated efficacy in alleviating EMs-related symptoms, reducing ovarian ectopic cyst size, lowering visual analog scale (VAS) pain scores, and decreasing serum CA125 and prolactin (PRL) levels. Additionally, it has shown improvements in TCM syndrome differentiation in patients [21,22]. Previous studies have shown that a 10% concentration of PCJNF significantly suppresses cell proliferation, migration, and invasion while promoting apoptosis. These anti-proliferative and pro-apoptotic effects are mediated via the activation of the JNK signaling pathway [23]. Furthermore, research indicates that PCJNF reduces lesion size in endometriosis animal models and modulates critical inflammatory processes. Despite these findings, the precise mechanisms by which PCJNF affects cell migration and invasion remain unclear. To address this gap, the current study employs the 12Z cells line, derived from human endometriotic tissue, as an experimental model to investigate the effects of PCJNF. While our earlier research focused on EESCs to explore the pathophysiological mechanisms of EMs and the therapeutic potential of PCJNF, the incorporation of the 12Z cell line in this study broadens the scope of our investigation. This dual-cell-line approach facilitates a more comprehensive evaluation of PCJNF's therapeutic effects in distinct cellular contexts, providing a robust framework for assessing its potential in EMs treatment (Fig. 1).

2. Materials and methods

2.1. Animal and ethical statements

Non-pregnant, pathogen-free female Sprague–Dawley (SD) rats (n = 40; weight, 200–230 g) were procured from the Laboratory Animal Science and Technology Center of Jiangxi University of Chinese Medicine, Nanchang, Jiangxi, China (SCXK(Gan) 2018–0002). The animals were housed and cared for in accordance with internationally recognized guidelines, including the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1985), as well as the Regulations on the Administration of Laboratory Animals in the People's Republic of China.

2.2. Reagents and instruments

All the herbs used in the preparation of PCJNF were sourced from Jiangxi Jiangzhong Traditional Chinese Medicine Co., Ltd. (Nanchang, Jiangxi, China). Comprehensive details about the herbs are presented (Table 1). All herbs were thoroughly dried before processing. Furthermore, on July 20, 2024, we accessed http://www.worldfloraonline.org to confirm that the plant names align with the latest taxonomic updates.

2.3. Preparation of normal saline PCJNF containing serum and control containing serum

All medicinal plants utilized for the preparation of PCJNF were sourced from the Affiliated Hospital of Jiangxi University of Chinese Medicine (Nanchang, Jiangxi). The PCJNF extract was prepared following a preliminary experimental protocol, yielding a crude drug concentration of 18.2 g/kg for PCJNF [23]. After a five-day acclimatization period, female Sprague–Dawley (SD) rats were randomly assigned to a PCJNF group (n = 20) and a control group (n = 20). Each rat in the PCJNF group received oral administration of PCJNF twice daily for 14 consecutive days, while the control group was administered an equivalent volume of 0.9% normal saline

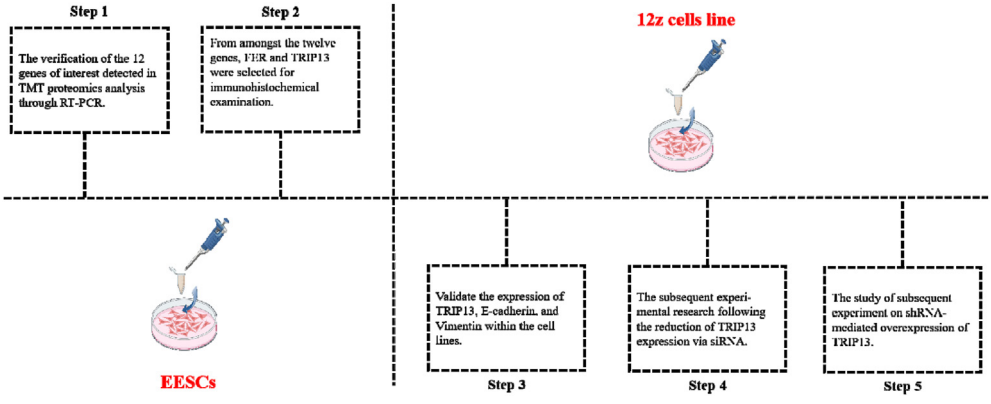

Fig. 1. General flow of the experiment.

Table 1. Chinese herb drugs contained in PCJNF.

Chinese name	Scientific name	*Family*	*Plant part*	Doses (g)	Stored at	Batch Number
Gui Zhi	Neolitsea cassia (L.) Kosterm	*Lauraceae*	*Twigs*	15	Herbarium of South China Botanical Garden, Chinese Academy of Sciences (IBSC)	0046270
Xue Jie	Calamus draco Willd.	*Palmaceae*	*Resin*	6	/	/
Wu Ling Zhi	Trogopterus xanthipes	*Petauristinae*	*Feces*	10	/	/
Pu Huang	Typha angustifolia L.	*Typhaceae*	*Pollen*	6	Herbarium of the Institute of Botany, Chinese Academy of Sciences (PE)	02308864
Jiang Xiang	Dalbergia odorifera T.C. Chen	*Leguminosae*	*Root*	6	Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (KUN)	0607325
Shui Zhi	Whitmania pigra Whitman	*Hirudinidae*	*Body*	3	/	/
Mai Dong	Ophiopogon japonicus (Thunb.) Ker Gawl.	*Asparagaceae*	*Root*	10	Herbarium of the Institute of Botany, Chinese Academy of Sciences (PE)	02230303
Bai Sha	Cynanchumotophyllum C.K. Schneid	*Apocynaceae*	*Root*	10	Herbarium of the Institute of Botany, Chinese Academy of Sciences (PE)	02247250
Gan Cao	Glycyrrhiza uralensis Fisch.	*Leguminosae*	*Root*	6	Herbarium of the Institute of Botany, Chinese Academy of Sciences (PE)	02246883

(NS). Two hours following the final dose, a trained experimenter anesthetized the rats with isoflurane (Lot: I40690, Shanghai Acmec Biochemical Co., Ltd., Shanghai, China) and collected blood samples via cardiac puncture under sterile conditions. The experimenter ensured that the rats were euthanized promptly and with minimal discomfort. Blood samples were centrifuged at 3000 rpm for 10 minutes at 4 °C to separate the serum, which was subsequently filtered through a 0.22 μm Millipore filter and stored at −80 °C until further analysis.

2.4. Drug-containing serum culture of cells

Based on prior research, cells were cultured in 10 cm culture dishes. Once the cultures reached approximately 90% confluence, as confirmed via microscopic observation, the EESCs were treated with serum supplemented with 10% PCJNF or control serum for 48 hours [23].

2.5. TMT proteomic analysis

After incubation with drug-containing serum for 48 hours, the EESCs were harvested, and total protein was extracted for subsequent TMT analysis (Zhongke Protein Technology Co., Ltd., Shanghai, China). The extracted proteins were digested and labeled with TMT reagents following the manufacturer's protocol (Applied Biosystems). Each group consisted of three biological replicates, with each replicate representing a pooled mixture of EESCs from three independent samples.

2.6. RT-PCR determination

RT-PCR was performed to validate the gene expression data obtained from proteomics. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and then reverse transcribed into complementary DNA (cDNA). The RT products were amplified using SYBR Green on a 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Each sample was tested in triplicate, and gene expression levels were analyzed using the $2^{-\Delta\Delta Ct}$ method for relative quantification.

2.7. Immunohistochemical staining detection

Endometrial tissues from non-EM patients and lesion tissues from EM patients were fixed in 4% paraformaldehyde. Following antigen retrieval, the tissues were incubated overnight with the primary antibody at 4 °C, followed by a 1 hour incubation

with the secondary antibody at room temperature in the dark. Staining was performed using Diaminobenzidine (DAB), and the sections were mounted with neutral resin. The primary antibodies used were TRIP13 (Catalog No. ab81289, 1:10,000, Abcam, USA) and FER (Catalog No. 10727-1-AP, 1:500, Proteintech, China). The average optical density was quantified using ImageJ software. Each group consisted of three samples for analysis.

2.8. Lentiviral construction

The lentiviruses were purchased from Kaiji Technology Co., Shanghai, China, and used according to the manufacturer's instructions. The TRIP13 lentiviral vector sequences are provided below.

Ubi-F (3756–3778): GGGTCAATATGTAATTTTCAGTG.

FLAG-R (3940–3919): CCTTATAGTCCTTATCATCGTC.

2.9. Gene silencing

The siRNA was purchased from Guangzhou Ruibo Biotechnology Co., Ltd. and used according to the manufacturer's instructions. The catalog numbers for the products are provided below.

siRNA-001: GGGACAGCTTGGTATACGA,
siRNA-002: GTACCGATATGGCCAATTA,
siRNA-003: GCAAGCTGGTAACCAAGAT.

2.10. Transwell chamber method

Cell migration and invasion were evaluated using a Transwell assay (Millipore, Billerica, MA, USA). Cultured ESCs were digested with trypsin, resuspended in serum-free DMEM/F12 medium, and then seeded into the upper chamber. For invasion assays, 50 μL of Matrigel (BD, USA) was pre-coated in the upper chamber. The cell suspension was adjusted to a concentration of 1×10^4 EESCs per 200 μL, with different treatment groups established accordingly. A total of 700 μL of medium containing 10% FBS was added to the lower chamber. After 48 hours of incubation, the cells were fixed with 4% paraformaldehyde for 30 minutes and stained with 0.5% crystal violet for 30 minutes. The cells were counted by visualization under a light microscope.

2.11. Western blotting

Protein expression was analyzed using Western blotting. Proteins from the different treatment groups were extracted, and concentrations were determined. Subsequently, 20 μg of protein were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore Sigma). After blocking with 5% nonfat milk for 50 minutes at room temperature, the membranes were incubated overnight at 4 °C with the following primary antibodies: rabbit anti-TRIP13 (Cat. No. ab32351, 1:2000, Abcam, USA), rabbit anti-E-cadherin (Cat. No. ab40772, 1:50,000, Abcam, USA), rabbit anti-vimentin (Cat. No. ab92547, 1:5000, Abcam, USA), and rabbit anti-GAPDH (Cat. No. ab9485, 1:1000, Abcam, USA). Membranes were then incubated with pre-adsorbed goat anti-rabbit IgG H&L (HRP) (Cat. No. ab7097, 1:1000; Abcam, Cambridge, UK) for 1 hour at room temperature. Bands were detected using an enhanced chemiluminescence kit (Thermo Fisher Scientific), with GAPDH serving as the loading control.

3. Results

3.1. Quality control of PCJNF

We previously employed LC-MS/MS to assess the quality and stability of PCJNF, and the results have been published. Three separate samples (PCJNF-1, PCJNF-2, and PCJNF-3) were decocted individually. Each portion of PCJNF was pre-soaked in Shuizhi for 30 minutes before being decocted with the full complement of Chinese herbs to obtain the medicinal extract. The results are presented as total ion chromatograms. In our study, we identified 636 chemical components in PCJNF-1, 639 in PCJNF-2, and 658 in PCJNF-3. Of these, 456 components were common to all three samples. A subsequent comparison with the mzCloud database allowed for the identification of five key compounds in PCJNF: linoleic acid, adenosine, oleic acid, D-(+)-proline, and L-phenylalanine [20].

3.2. RT-PCR validation of proteomic differences after PCJNF treatment

To investigate the molecular mechanisms underlying the effects of PCJNF on cell migration and invasion, we employed TMT labeling [24,25], a high-throughput proteomics strategy, to identify differentially expressed proteins following PCJNF treatment. The TMT results revealed that, compared to the serum-treated stromal cells from the NS control group, the PCJNF-treated cells expressed 4984 unique proteins. Based on a fold change (FC) ≥ 1.5 (or ≤ 0.67) and a p-value <0.05, we identified 3 upregulated and 9 downregulated genes of interest, as shown in Table 2,

Table 2. Details of the 3 upregulated genes and 9 downregulated genes

	Accession	Protein Name	Gene Name	Description	FC Value	P Value
UP	Q8WUD1	Ras-related protein Rab-2B	RAB2B	Ras-related protein Rab-2B OS=*Homo sapiens* OX = 9606 GN=RAB2B PE = 1 SV = 1 - [RAB2B_HUMAN]	2.4431	0.0492
	Q7Z4R8	UPF0669 protein C6orf120	C6orf120	UPF0669 protein C6orf120 OS=*Homo sapiens* OX = 9606 GN=C6orf120 PE = 1 SV = 1 - [CF120_HUMAN]	1.5679	0.0081
	P15104	Glutamine synthetase	GLUL	Glutamine synthetase OS=*Homo sapiens* OX = 9606 GN = GLUL PE = 1 SV = 4 - [GLNA_HUMAN]	1.5094	0.0258
DOWN	Q8IWA5	Choline transporter-like protein 2	SLC44A2	Choline transporter-like protein 2 OS=Homo sapien s OX = 9606 GN=SLC44A2 PE = 1 SV = 3 - [CTL2_HUMAN]	0.8278	0.0317
	P51397	Death-associated protein 1	DAP	Death-associated protein 1 OS=*Homo sapiens* OX = 9606 GN = DAP PE = 1 SV = 3 - [DAP1_HUMAN]	0.8214	0.0179
	P48506	Glutamate–cysteine ligase catalytic subunit	GCLC	Glutamate–cysteine ligase catalytic subunit OS=*Homo sapiens* OX = 9606 GN = GCLC PE = 1 SV = 2 - [GSH1_HUMAN]	0.7822	0.0470
	Q15645	Pachytene checkpoint protein 2 homolog	TRIP13	Pachytene checkpoint protein 2 homolog OS=*Homo sapiens* OX = 9606 GN = TRIP13 PE = 1 SV = 2 - [PCH2_HUMAN]	0.7677	0.0409
	P08243	Asparagine synthetase [glutamine-hydrolyzing]	ASNS	Asparagine synthetase [glutamine-hydrolyzing] OS=*Homo sapiens* OX = 9606 GN = ASNS PE = 1 SV = 4 - [ASNS_HUMAN]	0.7651	0.0299
	P16591	Tyrosine-protein kinase Fer	FER	Tyrosine-protein kinase Fer OS=*Homo sapiens* OX = 9606 GN=FER PE = 1 SV = 2 - [FER_HUMAN]	0.7163	0.0230
	Q12830	Nucleosome-remodeling factor subunit BPTF	BPTF	Nucleosome-remodeling factor subunit BPTF OS=*Homo sapiens* OX = 9606 GN=BPTF PE = 1 SV = 3 - [BPTF_HUMAN]	0.6485	0.0125
	P04114	Apolipoprotein B-100	APOB	Apolipoprotein B-100 OS=*Homo sapiens* OX = 9606 GN = APOB PE = 1 SV = 2 - [APOB_HUMAN]	0.6012	0.0261
	P35858	Insulin-like growth factor-binding protein complex acid labile subunit	IGFALS	Insulin-like growth factor-binding protein complex acid labile subunit OS=*Homo sapiens* OX = 9606 GN=IGFALS PE = 1 SV = 1 - [ALS_HUMAN]	0.5457	0.0488

and validated the expression of these 12 genes using RT-PCR. Following PCJNF treatment, the mRNA levels of RAB2B and GLUL were significantly increased ($p < 0.05$), while the mRNA levels of SLC44A2, TRIP13, ASNS, FER, BPTF, APOB, and IGFALS were significantly reduced ($p < 0.05$) (Fig. 2), demonstrating consistency with the TMT results. Additionally, TMT analysis indicated that C6ORF120, DAP, and GCLC were downregulated in response to PCJNF treatment. However, although the mRNA levels of C6ORF120, DAP, and GCLC showed a downward trend, no statistically significant differences were observed ($p > 0.05$).

3.3. First confirmation of the presence of TRIP13 in endometriosis

Based on the results from RT-PCR, FER and TRIP13 were selected for immunohistochemical (IHC) analysis. A total of five non-EMs patients and five EMs patients, aged 25–40 years and with regular menstrual cycles, were enrolled in the study. None of the patients had received hormonal therapy within three months prior to surgery. For the non-EMs group, proliferative-phase endometrial tissue was collected, whereas for the EMs group, ovarian endometriotic lesions were excised during the proliferative phase and subsequently embedded in paraffin. IHC staining was conducted on these tissue sections to evaluate the expression levels of FER and TRIP13 in endometriotic lesions. The results confirmed the expression of FER and TRIP13 in EMs tissue (Fig. 3). Notably, this study represents the first detection of TRIP13 in endometriosis, as no prior reports in the literature have documented its presence. In subsequent experiments, TRIP13 was selected as a key research target to further explore its role in the pathogenesis of EMs. All human

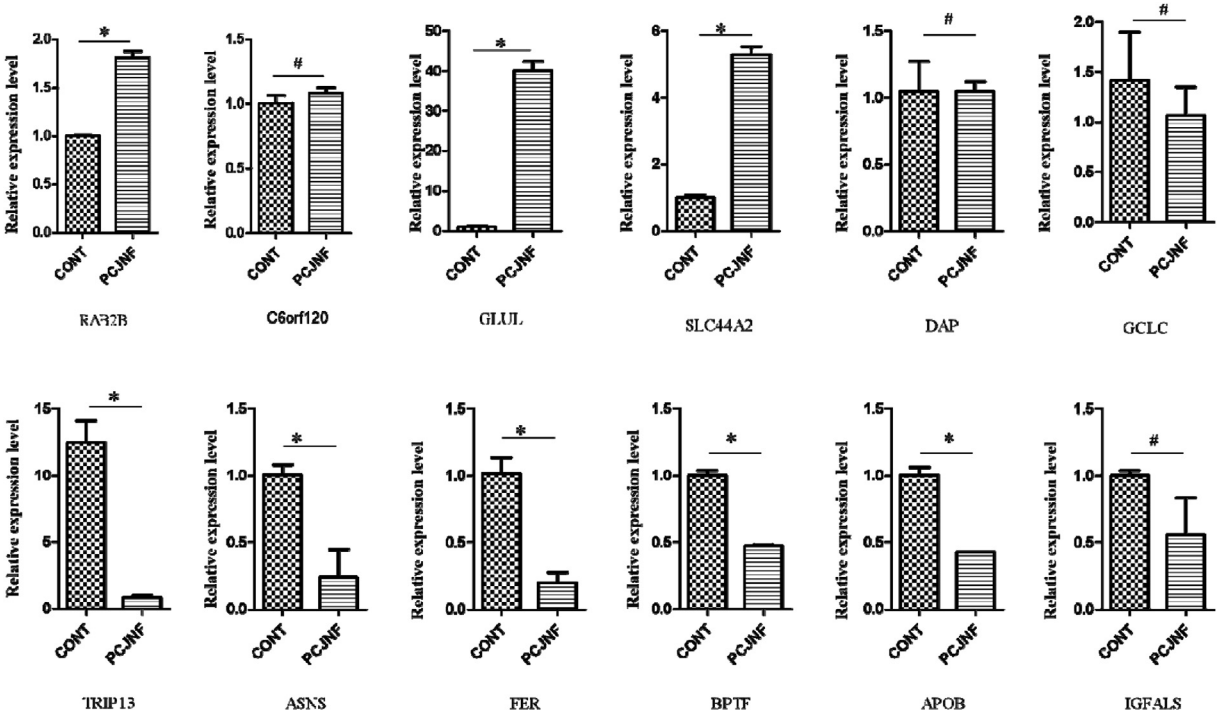

Fig. 2. Expression of 12 gene mRNAs after intervention with PCJNF containing serum in EESCs.

samples were collected in compliance with ethical guidelines and have been approved by the Institutional Review Board of The Second Affiliated Hospital of Jiangxi University of Chinese Medicine (IRB approval number: ky20181212008).

3.4. PCJNF-containing serum can reduce the expression of TRIP13 in 12Z cells

To ensure the stability of subsequent experimental data, the upcoming experiments were conducted using the 12Z cells line, which is derived from EMs. In this study, we evaluated the mRNA and protein expression levels of TRIP13 in 12Z cells using. RT-PCR analysis revealed a significant increase in TRIP13 mRNA expression in cells treated with FBS and control serum, whereas its expression was notably reduced in the PCJNF-treated serum group (Fig. 4(E), $p < 0.05$). This observation was further validated by Western blot analysis, which demonstrated a marked reduction in TRIP13 protein expression in the PCJNF group compared to the FBS and control serum groups (Fig. 4 (A–B), $p < 0.05$). Additionally, we analyzed the expression of E-cadherin and vimentin in 12Z cells. Following PCJNF treatment, E-cadherin expression was significantly upregulated, while vimentin expression was downregulated (Fig. 4 (C- D), $p < 0.05$). Western blotting

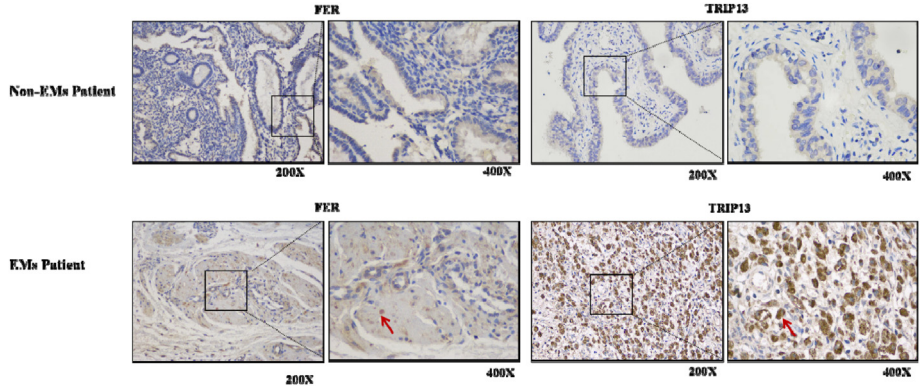

Fig. 3. Through IHC detection, the expression of FER and TRIP13 in human endometriosis lesions was found to be positive.

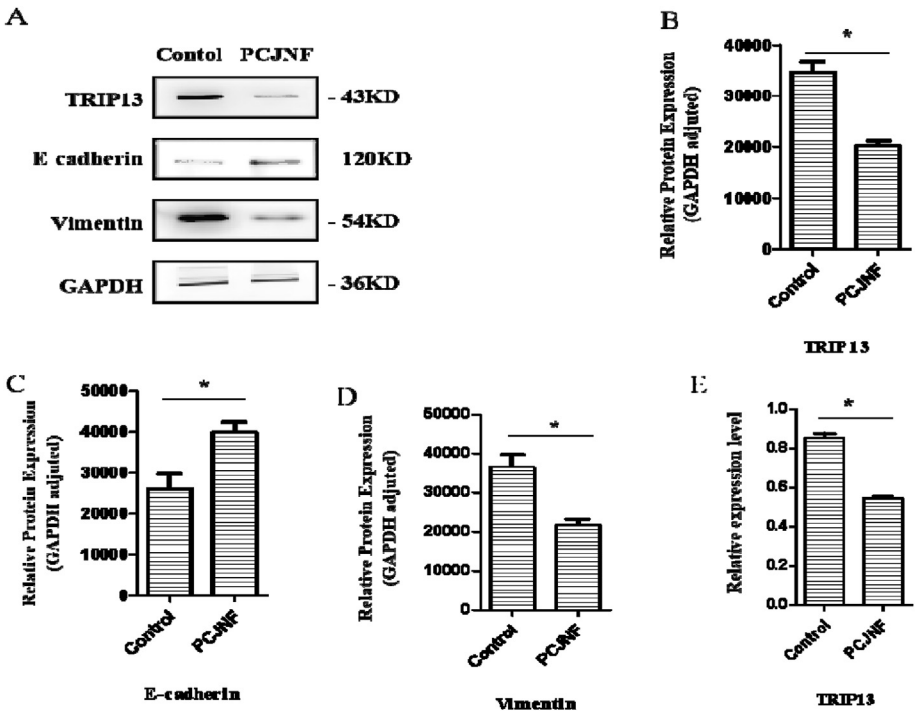

Fig. 4. (A–D) Western blot analysis of TRIP13, E-cadherin and vimentin in PCJNF-containing serum groups compared to control serum groups ($p < 0.05$). Bars show mean ± SEM ($n = 3$). (E) RT-PCR analysis confirmed that, mRNA levels of TRIP13, were decreased in the PCJNF-containing serum group compared to the control serum group (*$p < 0.05$). Bars show mean ± SEM ($n = 3$).*

was performed with GAPDH as a reference protein, and all experiments were conducted in triplicate to ensure reproducibility.

3.5. siRNA reduces the expression of TRIP13 protein and inhibits the migration and invasion of EMs 12Z cells

To assess PCJNF's impact on the migration and invasion capabilities of 12Z cells via TRIP13 regulation, a series of experiments were conducted. Initially, TRIP13 expression was knocked down using siRNA for 48 hours, with a mix of 50 nM siRNAs (001 + 002 + 003) identified as the most effective (Fig. 5A, $p < 0.05$) following RT-PCR analysis. Comparative analysis of TRIP13, E-cadherin, and vimentin expression levels post siRNA treatment versus siRNA NC demonstrated significant downregulation of TRIP13, upregulation of E-cadherin, and downregulation of vimentin (Fig. 6 (A-D), $p < 0.05$). Subsequently, PCJNF was administered to the siRNA-treated cells, resulting in further suppression of TRIP13 and vimentin expression,

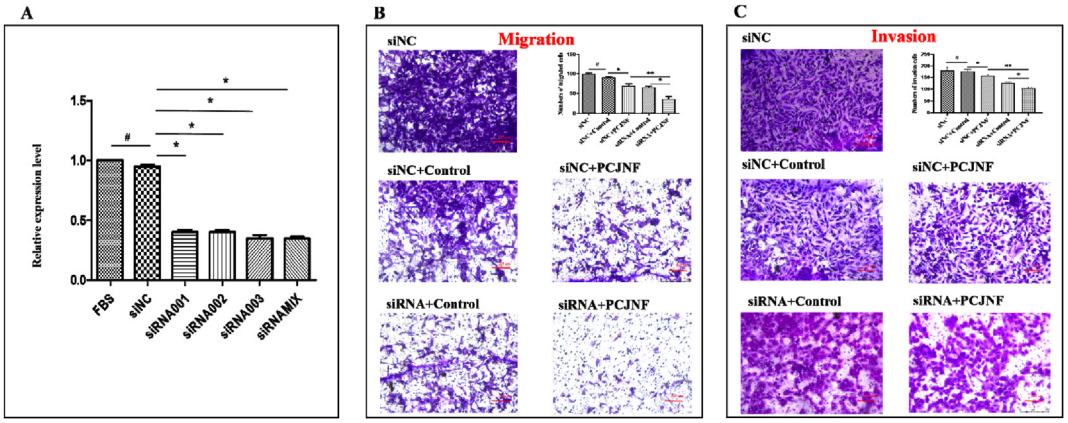

Fig. 5. (A) The best group intervened by siRNA was selected using RT-PCR. (B–C) The migration and invasion after siRNA intervention were detected using Transwell.

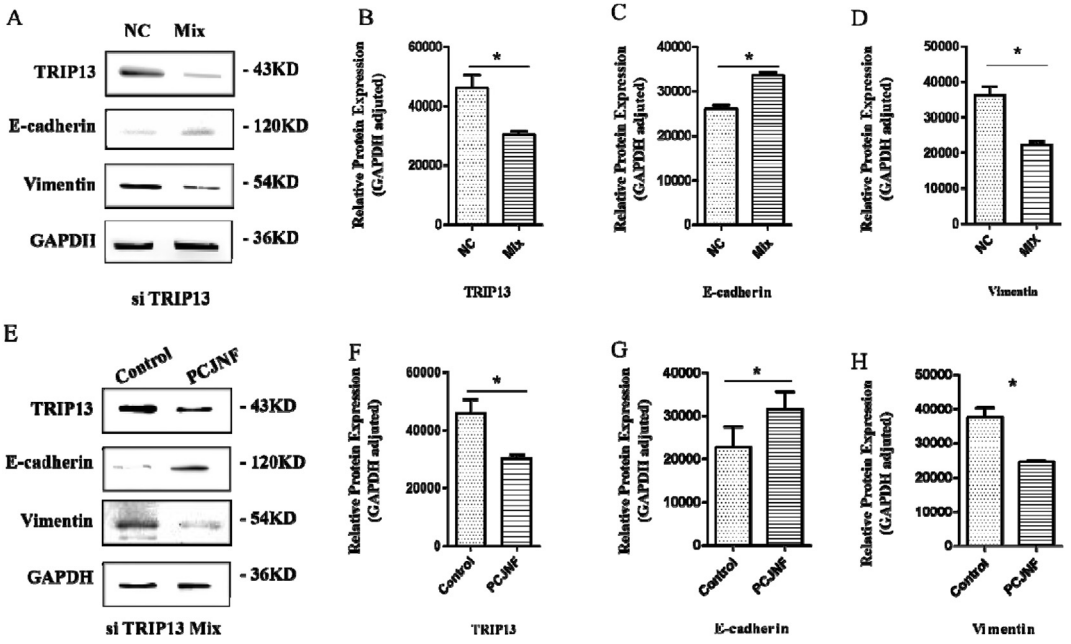

Fig. 6. (A–D) Western blot analysis showed that after PCJNF action on 12Z cells, the expression of TRIP13 and vimentin decreased, while the expression of E-cadherin increased. (E–H) After the intervention of 12Z cells using siRNA, Western blotting was performed to detect the effect of PCJNF on TRIP13, E-cadherin and vimentin. The results showed that PCJNF decreased TRIP13, which affected the expression of E-cadherin and vimentin.

coupled with increased E-cadherin levels (Fig. 6 (E-H), $p < 0.05$). Additionally, transwell chamber assays revealed a notable reduction in cell migration and invasion in PCJNF-treated, TRIP13-knockdown cells compared to the control group (Fig. 5 (B–C), $p < 0.05$). All experiments were performed in triplicate.

3.6. PCJNF can reduce shRNA overexpression of TRIP13 protein and inhibit the migration and invasion of EMs 12Z cells

To further investigate the role of TRIP13, short hairpin RNA (shRNA) was employed to induce its overexpression. Successful lentiviral transduction was confirmed at 48 hours post-infection (Fig. 7A). Since no significant difference was observed between a multiplicity of infection (MOI) of 50 and 100, the optimal MOI for the YANG group was determined to be 50, and this concentration was used in subsequent experiments. Following lentiviral infection in the YANG group, TRIP13 and vimentin expression levels were significantly upregulated, while E-cadherin expression was markedly downregulated compared to the YING control group (Fig. 8 (A-D), $p < 0.05$). Treatment with PCJNF resulted in a significant decrease in TRIP13 and vimentin expression, accompanied by a notable increase in E-cadherin levels (Fig. 8 (E-H), $p < 0.05$). Transwell migration and invasion assays demonstrated that TRIP13 overexpression in the YANG group enhanced cell migration and invasion, whereas PCJNF effectively suppressed these processes (Fig. 7 (B–C), $p < 0.05$).

4. Discussion

For millennia, TCM has been a cornerstone of China's healthcare system, renowned for its ability to enhance therapeutic efficacy, address drug resistance, and reduce adverse effects [26]. Its enduring significance was particularly evident during the COVID-19 pandemic, when TCM demonstrated notable clinical success and gained increasing international recognition. At the heart of TCM's effectiveness lies its unique integration of bioactive compounds derived from a rich variety of herbal sources [27]. While over 200,000 compounds have been identified within the TCM pharmacopeia, only a select few, such as artemisinin and arsenic trioxide, have been thoroughly studied to uncover their pharmacological properties and mechanisms of action. The vast majority remain largely uncharacterized, leaving their molecular targets and mechanisms a mystery. This knowledge gap highlights the critical need to leverage advanced molecular biology tools to decode the intricate interactions underlying TCM's therapeutic potential and unlock new frontiers in modern medicine.

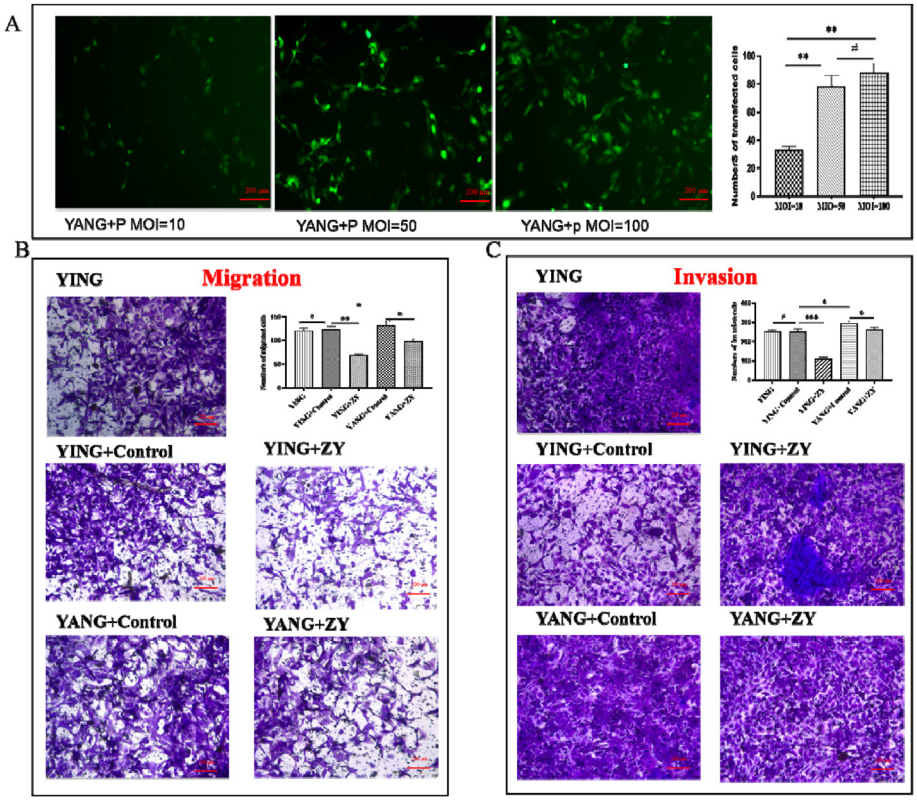

Fig. 7. (A) The situation observed under a reversed fluorescence microscope for the intervention of TRIP13-positive slow viruses. (B−C) Transwell method employment to assess the migration and invasion of shRNA-mediated intervention.

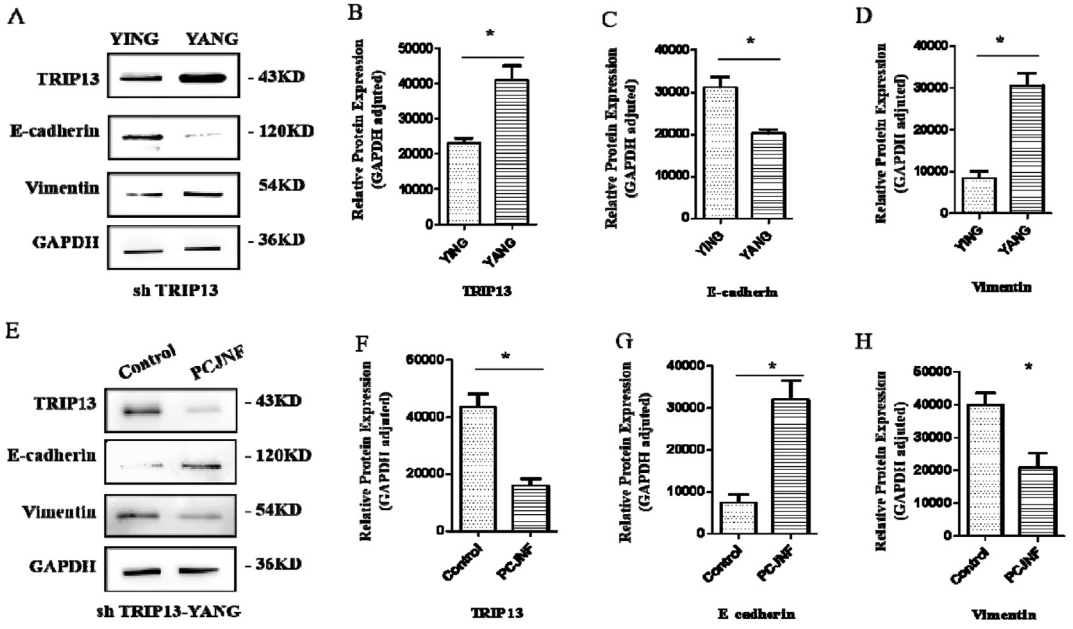

Fig. 8. (A−D) Compared to the YING group, the YANG group exhibited enhanced expressions of TRIP13 and vimentin, while the E-cadherin expression was diminished. (E-H) Within the positive lentiviral group the incorporation of PCJNF led to a decline in TRIP13 and vimentin expression, while augmenting the manifestation of E-cadherin.

This study highlights the critical clinical and biological significance of investigating TRIP13. As a member of the ATPase family, TRIP13 is broadly expressed across species [28], and its upregulation has been observed in numerous human cancers. The central focus of this research is to determine whether PCJNF can exert therapeutic effects on lesions associated with EMs through its interaction with TRIP13. While historically regarded as a benign condition from clinical and histopathological perspectives, recent molecular genetic advancements have identified somatic mutations in cancer-associated genes within specific deep infiltrating EMs lesions, such as deep infiltrating endometriosis (DIE), ovarian endometriosis, and iatrogenic endometriosis [29]. Notably, mutations in genes like Wnt4 [30], KRAS [31], PIK3CA [32], and ARID1A [33], which are frequently implicated in endometrial and ovarian cancers, have been detected in the glandular epithelium of these lesions. This finding underlines the urgent necessity to explore cancer-related genes and their potential roles in the pathogenesis of EMs. Moreover, EMs exhibits behaviors such as abnormal proliferation, migration, invasion, resistance to apoptosis, and angiogenesis—hallmark features of tumorigenesis. These parallels suggest that the molecular mechanisms underlying EMs may overlap with those driving cancer progression. By leveraging a cancer research-oriented approach, this study seeks to unravel the molecular pathways involved in EMs, providing a deeper understanding of its pathogenesis and identifying novel therapeutic targets. This interdisciplinary exploration bridges cancer biology and EMs research, paving the way for innovative and more effective treatment strategies. Given the complex and multifaceted nature of EMs, such an approach holds immense promise for advancing clinical management and alleviating the significant burden it poses to patients and clinicians alike.

Another critical rationale for investigating TRIP13 in this study lies in its pivotal role in the migration and invasion of affected cells [28]. Notably, studies conducted by Zhang, Zhu, and others [19] has demonstrated that TRIP13 enhances the proliferation, migration, and invasion of glioblastoma cells by activating the FBXW7/c-MYC pathway [34]. Depletion of TRIP13 significantly impairs the proliferative, migratory, and invasive capacities of tumor cells. Additionally, in 2016 [35], underscored the contribution of TRIP13 to the migration and invasion of colorectal cancer cells. Studies have further indicated that inhibiting TRIP13 expression can suppress the migration and invasion of liver cancer cells [36]. Moreover, Cai et al. reported elevated TRIP13 expression in lung adenocarcinoma tissues, where its high expression was associated with enhanced proliferation, migration, and invasion of lung adenocarcinoma cells, correlating with poorer patient survival outcomes [37]. TRIP13 also promotes the proliferation and invasion of epithelial ovarian cancer cells through the Notch signaling pathway [38]. These findings collectively position TRIP13 as a central mediator of the aggressive behaviors characteristic of malignancies, reinforcing the need to explore its molecular mechanisms and biological implications. In the context of EMs, which exhibits tumor-like features such as abnormal proliferation, migration, and invasion, the investigation of TRIP13 could provide transformative insights. Deciphering its role in EMs pathogenesis not only deepens our understanding of the disease but also holds the promise of identifying novel therapeutic strategies that target this critical driver of pathological behavior.

Building upon the aforementioned research background, we hypothesized that PCJNF inhibits the migration and invasion of endometriotic stromal cells by modulating the expression of TRIP13, either through downregulation or upregulation. To validate this hypothesis, we employed protein blotting to confirm the differential expression of TRIP13 identified in the TMT experiment and observed its alteration following treatment with serum containing PCJNF. Our findings indicated that PCJNF significantly suppressed TRIP13 expression. To further substantiate whether PCJNF inhibits cell migration and invasion via TRIP13, we conducted additional loss-of-function and overexpression experiments. Specifically, we introduced 50 nM siRNA targeting TRIP13 for knockdown in the EMs 12Z cells line and used a 50-fold concentrated shRNA targeting TRIP13 for overexpression in the same cell line. We observed that siRNA-mediated knockdown of TRIP13 mitigated the effects of PCJNF on cell migration and invasion, while shRNA-mediated overexpression of TRIP13 exacerbated these effects. These results strongly suggest that TRIP13 plays a pivotal role in PCJNF-induced modulation of cell migration and invasion. In conclusion, TRIP13 has emerged as a critical protein in the intervention of EMs by PCJNF, and its regulation may play a significant role in the onset and progression of EMs through the modulation of cell migration and invasion.

Furthermore, we investigated the changes in the protein levels of E-cadherin and vimentin following TRIP13 silencing and overexpression. E-cadherin, a crucial protein for mediating cell adhesion, plays a key role in preserving the morphological integrity and tissue structure of epithelial cells [39,40]. In the context

of endometriosis (EMs), reduced E-cadherin expression leads to impaired cell adhesion, increased invasiveness of lesion tissues, and promotes disease progression. vimentin, a major component of the intermediate filament (IF) protein family, directly facilitates cell migration and is frequently upregulated in various cancers, where it is associated with tumor growth, invasion, and poor prognosis. Several studies have shown that vimentin overexpression in invasive breast cancer cell lines contributing to an excessive increase in cellular migration and invasion [41,42]. In the context of EMs, the overexpression of E-cadherin and vimentin may reflect similar aberrations observed in cancerous tissues [43,44]. Our results showed that PCJNF treatment upregulated E-cadherin expression while downregulating vimentin levels. Notably, silencing TRIP13 further amplified the effect of PCJNF on increasing E-cadherin and reducing vimentin. In contrast, overexpression of TRIP13 resulted in decreased E-cadherin and increased vimentin levels, but these changes could be reversed by PCJNF treatment. This suggests that TRIP13 regulation significantly influences the expression of key migration- and invasion-related proteins like E-cadherin and vimentin, thereby modulating the migratory and invasive behaviors of cells.

5. Conclusion

In conclusion, our findings demonstrate that PCJNF significantly suppresses the migration and invasion of EMs cells by modulating TRIP13, thereby mitigating the onset and progression of the disease. These results underscore TRIP13 as a promising therapeutic target in the context of PCJNF treatment for EMs, presenting a novel and potentially impactful discovery that warrants further exploration in subsequent research.

Author contributions

Ruining Liang (jack169@sina.com) and Jiahua Peng (397090311@qq.com) conceived and designed the study. Yunxiang Xie (1906399533@qq.com) collected clinical samples. Shuzhen Liu (17865582187@163.com) and Min Xiao (xwq102@163.com) prepared PCJNF decoction, carried out data acquisition and interpretation. Chichiu Wang (chenxiaoyan@cuhk.edu.hk) modify the papers. Ruining Liang and Jiahua Peng wrote the paper. All authors approved the final manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (No. 81760878, No. 81160447), Jiangxi Provincial Key Laboratory of TCM Female Reproductive Health and Related Diseases Research and transformation (No. 2024SSY06311), the Science and Technology Research Project of Jiangxi Provincial Department of Education (No. GJJ2200915), National Natural Science Foundation of Jiangxi Provincial (No. 20202BAB206073, 20212BAB216066), The Jiangxi Province Postgraduate Innovation Fund Project (No. YC2023-B220), The Science and Technology Project of the Jiangxi Province Traditional Chinese Medicine Administration (No. 2019A085), and the Scientific and Technological Innovation Team Development Plan of Jiangxi University of Chinese Medicine (No. CXTD22013).

Conflict of interest

No competing interests declared.

Acknowledgments

We would like to express our sincere gratitude to Dr. Yang Zou and Dr. Yong Luo from Jiangxi Maternal and Child Health Hospital. They provided clinical cells for the pre-experiment and also furnished guidance, laboratory facilities, and experimental equipment for the experiment. Our thanks also go to Dr. He Bin from Jiangxi Science and Technology Normal University for guiding the revision of the article.

Data availability

Data will be made available on request.

Supplementary material

https://doi.org/10.38212/2224-6614.3544

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