Original Article

Frankincense ameliorates endometriosis via inducing apoptosis and reducing adhesion

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ABSTRACT

Background: Frankincense, a resin derived from trees of the Boswellia genus, has been used as an incense and a type of herbal medicine for treating inflammatory diseases such arthritis, chronic bowel illness, and asthma. While endometriosis is a well-known inflammatory gynecological illness caused by the ectopic attachment and development of uterine tissue over the menstrual cycle, the impact of frankincense on this illness is poorly understood. The purpose of this study was to explore the effects of frankincense on endometriosis.
Methods: We used a network pharmacological assessment, *in vitro* and *in vivo* investigations with a human endometriotic cell line as well as a syngeneic uterine transfer mouse model. High-performance liquid chromatographic analysis was used to compare water-extracted frankincense (Fr) to its reference compounds and validate the sample.
Results: A network pharmacological analysis suggested a positive effect of Fr on endometriosis. Fr relieved endometriosis by reducing ectopic endometrial adherence and development, according to both *in vivo* and *in vitro* models. We suggested that the ER stress/p53-apoptosis and chemokine-migration/adhesion pathways underlie Fr's anti-endometriotic action using RNA sequencing and bioinformatic analysis.
Conclusion: This study revealed the potential effect of Fr on endometriosis using an experimental investigation. Fr may have the potential to be an effective and safe treatment for endometriosis.

1. Introduction

A common gynecological illness known as endometriosis occurs when endometrial tissue grows outside of the uterus.[1] It develops inflammation, bleeding, visceral discomfort, fibrosis, and infertility in response to hormonal cues like the eutopic endometrium.[2,3] Several multistep mechanisms, such as endometrial cell migration, adhesion, proliferation, and invasion, are crucial for the pathogenesis of endometriosis even if its pathophysiology is not entirely understood.[4-6] An initial step in the development of an endometriotic lesion is the adhesive implantation of migrating endometrial tissue, which is controlled by adhesion molecules.[7,8] In addition, a dysregulated apoptotic pathway also generally upregulates the growth of ectopic endometrial tissue.[9,10] As a result, apoptosis induction and adhesion reduction are seen as attractive new therapeutic targets.[11-14]

Since ancient times, frankincense, a resin derived from trees of the Boswellia genus, has been used as an incense and a type of herbal medicine.[15] Three principal species of frankincense are used for collection: *B. sacra* Flueck. from East Africa, the Middle East, and China; *B. frereana* Birdw. from Somalia; and *B. serrata* Roxb. ex Colebr. from India.[15-17] It is currently recognized that *B. carteri* Birdw., which were widely used in China, is a synonym

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for *B. sacra*.[16,17] Frankincense has been used to treat inflammatory diseases such arthritis, chronic bowel illness, and asthma, despite species differences.[15,18]

A recent systematic review found that the chemical components in Boswellia species and their anti-inflammatory and anti-cancer effects occur through a variety of mechanisms, including reducing oxidative stress, controlling immune cells, modifying cell cycle, and preventing angiogenesis, invasion, and metastasis.[19] Due to its ability to increase blood flow and reduce discomfort,[15] frankincense is also used in herbal formulations that are typically combined with myrrh or ginger to treat a number of gynecological disorders, such as dysmenorrhea and menstrual bleeding.[20,21] However, to the best of our knowledge, there isn't any direct experimental proof that frankincense has an inhibitory effect on the etiology of endometriosis.

In this study, we investigated the potential anti-endometriosis effects of frankincense using bioinformatic analyses of network pharmacology and RNA-sequencing data, as well as *in vitro* and *in vivo* experiments. As a result, we discovered that frankincense could prevent the development of endometriosis by decreasing adhesive implantation and increasing apoptotic cell death. For the development of novel pharmaceutical treatments, we thus propose frankincense as a potential anti-endometriosis agent.

2. Methods

2.1. Preparation of frankincense extracts

Frankincense water-extract (Fr) was purchased from the Korea Plant Extract Bank at the Korea Research Institute of Bioscience and Biotechnology (Chungju, Korea). The original plant, *B. sacra*, were cultivated in China and the lot number is CW04–036. The extract was dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, Mo), and immediately diluted with culture media or corn oil immediately before use *in vitro* experiments or animal study, respectively.

2.2. High performance liquid chromatography (HPLC) analysis of Fr extract

Standard compounds, including β-boswellic acid (97.1% purity), 3-acetyl-11-keto-beta-boswellic acid, and 11-keto-beta-boswellic acid (100% purity), were provided by Cayman Chemical (Ann Arbor, MI) as standard chemicals according to previous studies.[22,23] For usage as a standard stock solution, the compounds were dissolved in methanol at a concentration of 500 μg/ml. This solution was then diluted further for analysis. The Fr was filtered through a 0.45 μm syringe filter (Merck Millipore, Burlington, MA) after being dissolved in methanol and sonicated using ultrasound for 20 min. An Agilent 1200 series system (Agilent Technologies, Santa Clara, CA) with a C18 reverse phase column (Capcell Pak C18 UG120 4.6 $\times$ 250 mm, 5 μm; Shiseido, Tokyo, Japan) maintained at 30 °C was used for the HPLC study. For all three substances, the mobile phase was acetonitrile (95%): 0.5% acetic acid (5%). Beta-boswellic acid was determined from samples (20 L) using an ultraviolet detector at 205 nm and 11-keto-beta-boswellic acid and 3-acetyl-11-keto-beta-boswellic acid at 250 nm, respectively.

2.3. Network pharmacologic analysis

The databases of the traditional Chinese medicine integrative database (TCMID, http://bidd.group/TCMID/)[24] and DisGeNet (https://www.disgenet.org/)[25] were used to gather the possible target genes for Fr and endometriosis, respectively. The 81 genes of targets of Fr were analyzed for finding out potential pathways by Cytoscape (version 3.9.1, https://cytoscape.org/) with the aid of the JEPPETO plugin.[26] Visualization was performed by constructing a

scatter plot with XD-score and q value as axes. XD-score > 0.5 and q value < 0.25 was used as the cutoff criterion. A Venn diagram was created to display the 38 genes that are shared by the prospective targets of Fr and endometriosis. Cytoscape with the STRING application[27] was used for analyzing the protein-protein interaction network of the 38 common targets.

2.4. Cell culture

Human pleural mesothelial Met-5A cells and immortalized human endometriotic epithelial 12Z cells were obtained from American Type Culture Collection (#CRL-9444; Manassas, VA) and Applied Biological Materials (#T0764; Richmond, Canada), respectively. Platinum A cells were provided by Cell Biolabs Inc. (RV-102; San Diego, CA). Met-5A cells or Platinum A cells were maintained in M199 (Welgene, Daegu, Korea) or DMEM (Welgene), respectively, with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA) and 1% penicillin/streptomycin supplemented. 12Z cells were maintained in Precoat T75 flask (Applied Biological Materials) with DMEM/F12 (Welgene) containing 10% non-inactivated charcoal-stripped FBS and 1% penicillin. All cell lines were grown at 37 °C in a humid environment with 5% CO_2.

2.5. Cell viability

Cell viability was examined using 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich). The stated concentrations of Fr were applied to 12Z cells that had been seeded into 96 wells. Using a Spectramax M2 spectrofluorometer (Molecular Devices, San Jose, CA), absorbance at 450 nm was measured to determine the vitality of the cells. The dose of 50% growth inhibition (GI_{50}) was calculated with the assistance of GraphPad Prism software (GraphPad, San Diego, CA).

2.6. Animal care

Female C57BL/6 mice aged five weeks were acquired from Orient Bio (Seongnam, Korea), and housed in an animal facility managed by Pusan National University's Institute for Laboratory Animals. The animal facility was regularly kept at a room temperature of 22 $\pm$ 2 °C, a humidity level of 50–60%, and a day/night cycle of 12 hrs. Inhaling CO_2 or isoflurane was applied for anesthesia or sacrifice, respectively. The Institutional Animal Care and Use Committee of Pusan National University granted its approval to all experimental methods (Pusan, Republic of Korea; PNU-2020–2679).

2.7. Endometriosis mouse models

Based on a recently published version of our protocol, endometriosis was induced.[28] Briefly, female C57BL/6 mice that were six weeks old had their ovaries removed and then recovered. Isoflurane inhalation was used for anesthesia. Mice were subcutaneously injected every week with 100 mg/kg of β-estradiol (Santa Cruz Biotechnology; Dallas, TX) fourteen days after ovariectomy. The uteri of donor mice were extracted and chopped using Gentle Max (Miltenyi Biotec, Bergisch Gladbach, Germany) before being inoculated intraperitoneally into recipient mice at a 1:1 ratio. After one day, the mice were given Fr orally 5 days a week, and 100 mg/kg β-estradiol was subcutaneously injected once a week for 3 weeks. Based on GI_{50} of Fr in 12Z cells, the doses of Fr given to mice were estimated. Two dosages, such as 0.3 mg/kg/day and 1.5 mg/kg/day, were lower than the usual dose, 1.321 g/kg/day, that was derived from the general human equivalent dose (HED)

(0.2 g/kg/day) of Fr using the FDA-provided formula presented below.[29]

$$Animal\ dose\ (mg/kg) = HED(0.2\ mg/kg) \times Multiplying\ index\ (12.3)$$
$$\times yield\ of\ the\ extraction\ (0.537)$$

The mice were dissected under CO_2 inhalation 1 day following the final injection of β-estradiol, and the number and weight of endometriotic lesions were assessed. There were five mice per group.

2.8. Apoptosis analysis

Apoptosis of the cells was examined propidium iodide (PI)/Annexin V (BD Biosciences, Franklin Lakes, NJ) techniques. 12Z cells were plated into 6 wells for PI/Annexin V detection. Fluorescence-activated cell sorting (FACS) analysis using Attune X (Thermo Fisher Scientific) was used to assess the proportion of PI/Annexin V positive cells at excitation (Ex) 494/emission (Em) 525 nm for Annexin V and Ex 535/Em 617 nm for PI. The FlowJo application was used for the analysis of FACS data (BD Bioscience).

2.9. Immunoblot analysis

Immunoblot analysis was used to investigate cell apoptotic signal proteins. RIPA buffer (Thermo Fisher Scientific) containing a protease inhibitor cocktail (Roche, Basel, Switzerland) was used to extract total proteins, and the concentration of proteins were then measured using the Bradford assay. Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the proteins were separated and then transferred to a polyvinylidene fluoride membrane (Amersham Bioscience, Uppsala, Sweden). The membranes were incubated with primary antibody, including anti-human poly (ADP-ribose) polymerase (PARP, #9542 s; Cell Signalling, Danvers, MA), caspases-3 (#9665 s; Cell Signalling), caspase-9 (#9508 s; Cell Signalling), bax (NB100–56095; Novus Biologicals, Centennial, CO), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; sc-32233; Santa Cruz), at 4 °C overnight. After vigorous washing with tris-buffered saline, the horseradish peroxidase-conjugated secondary antibodies, such as anti-rabbit IgG or anti-rat IgG (Invitrogen, Waltham, MA), were applied to the membranes at room temperature for 1 hrs. The specific bands were developed using the ImageQuant LAS 4000 chemiluminescence imaging equipment (GE Healthcare, Chicago, IL) and a western blot detection kit from Bio-Rad (Hercules, CA).

2.10. Plasmid transfection and retroviral infection in Met-5A cells

Using a retroviral packaging technique, pMX mCherry(N)-IRES plasmid DNA was transfected into Met-5A cells to produce red fluorescent cell lines. Our team member Professor Bae (Kosin University, Korea) developed the plasmid DNA designated as pMX mCherry(N)-IRES. Briefly, retroviral packaging was carried out by transfecting Platinum A with linear polyethylenimine (Polysciences, Warrington, PA) and retroviral vector. The media was replaced with new media a day after transfection, and transfected Platinum A cells were grown for the following 48 hrs. The virus particles in the cultured media were then collected and purified through a 0.45 μm syringe filter. Then, Met-5A cells were grown in medium containing viruses and polybrene (5 μg/ml; Sigma-Aldrich). Blasticidin (10 μg/ml; Sigma-Aldrich) was used to select transduced cells for 3 days.

2.11. Cell adhesion assay

A cell adhesion experiment was performed in accordance with a prior study.[30] Met-5A cells were grown in 6-well plates, while 12Z cells were seeded in 100 mm cell culture dishes. The next day, 12Z cells were incubated with prescribed dosage of Fr for 24 hrs and were labeled with chloromethylfluorescein diacetate (CMFDA; Invitrogen) for 15 min at 37 °C. After being washed with phosphate-buffered saline (PBS), CMFDA-labeled 12Z cells were then carefully transferred onto a monolayer of Met-5A cells. For the purpose of removing non-adhered 12Z cells, cells were gently shaken at 20 rpm for 90 min at 37 °C. Following this, attached 12Z cells were observed under a fluorescent microscope (200x magnification; Axio Imager M1, Carl Zeiss, Oberkochen, Germany), and four randomly selected areas' numbers of adherent 12Z cells were determined for statistical analysis.

2.12. RNA-Sequencing

The 12Z cells were cultured at a density of 5×10^5/well in 6-well plates and treated with Fr (60 μg/ml) or vehicle (DMSO) for 12 h. Three wells of each treatment group were employed for RNA sequencing, and all sequencing processes were performed using the services of DNAlink Inc. (Seoul, South Korea). The quality of total RNA was verified using 2100 Expert Bioanalyzer with RNA 6000 Nano kit (Agilent Inc., Santa Clara, CA, USA) for ensuring the RNA integrity number of all samples > 7.0.

2.13. Gene set enrichment analysis (GSEA)

The GSEA was carried out using GSEA software from the Broad Institute (version 4.3.0, available at http://www.gsea-msigdb.org/gsea/downloads.jsp) as described previously.[31,32] Analysis of the biological processes of DEGs was done using hallmark, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Ontology (GO). Cytoscape was used to display the results of the network analysis of the GO gene with values of $p < 0.001$ and $q < 0.5$.

2.14. Differentially expressed gene (DEG) and Heatmap analysis

The R program DESeq2 (version 4.1.1, available at https://www.r-project.org/) was used to perform the transcriptome analysis as described previously.[32, 33] Genes having p-values and Log2|FoldChange| (Log|FC|) values under and above 0.05 and 1, respectively, were deemed to be DEGs. Morpheus, a flexible matrix visualization and analysis program from the Broad Institute (https://software.broadinstitute.org/morpheus/), was used to create a heatmap of 88 discovered DEGs (50 up- and 37 down-regulated), which are engaged in the endometriosis-related pathways using GSEA analyses.

2.15. Statistical analysis

The mean and SEM are used to express values. For comparisons between two distinct groups, a two-tailed Student's *t*-test was employed, and for comparisons between multiple groups, a one-way analysis of variance followed by a Tukey's post-hoc test was utilized. These were completed with the use of GraphPad Prism software.

3. Results

3.1. Standardization of frankincense water-extract (Fr)

Using high-performance liquid chromatography with UV detection, the Fr used in this study was examined for validation and quantification. Boswellic acid derivatives, such as β-boswellic acid (97.1% purity), 3-acetyl-11-keto-beta-boswellic acid, and 11-keto-beta-boswellic acid (100% purity), were utilized according to
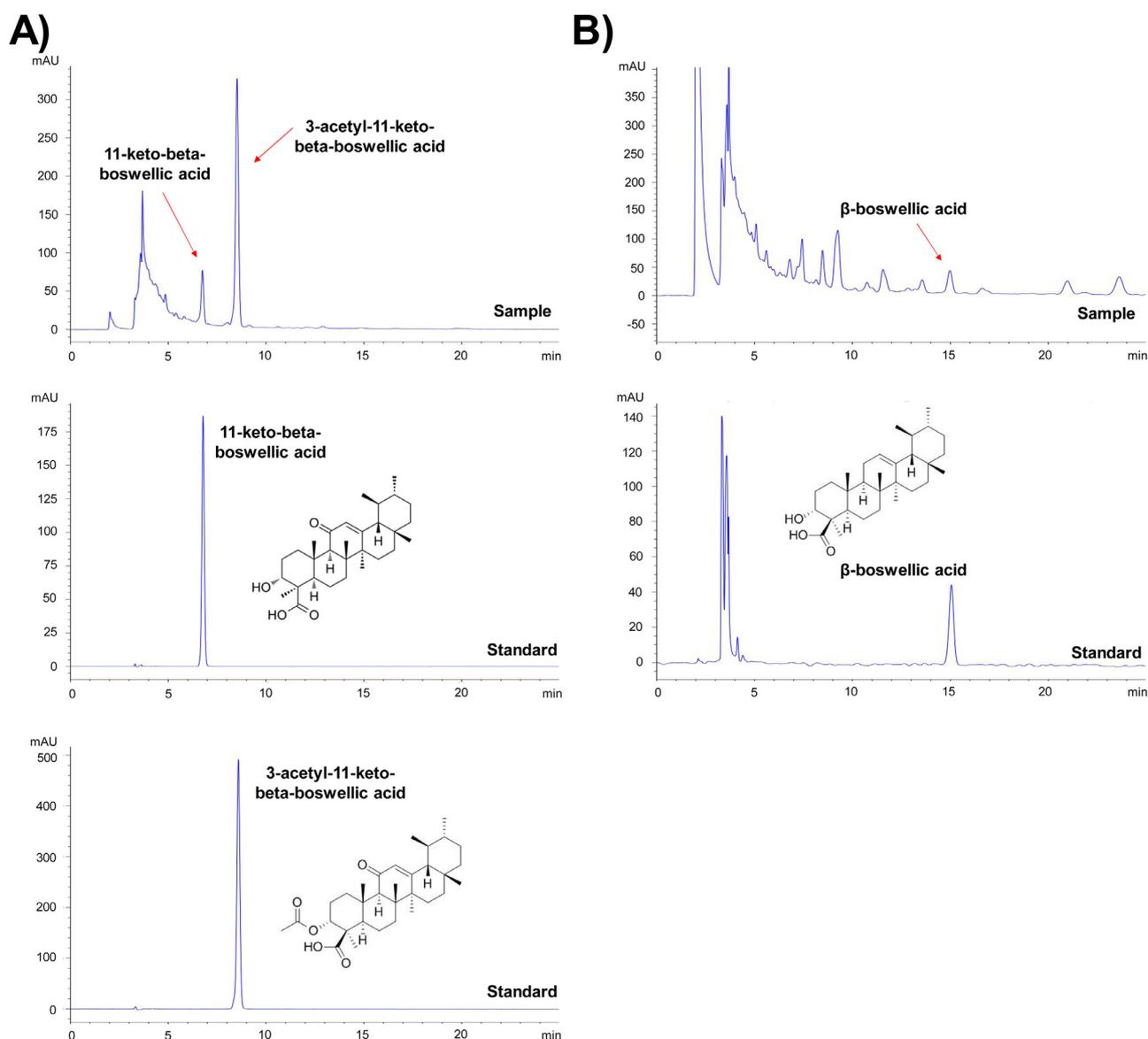
Fig. 1. Fr was validated by high performance liquid chromatography. Fr was subjected to 20 min of ultrasound sonication before being passed through a 0.45 μm syringe filter. Beta-boswellic acid, 3-acetyl-11-keto-beta-boswellic acid, and 11-keto-beta-boswellic acid were employed as reference materials. The samples were injected with 20 μL and examined using an ultraviolet detector at 205 nm for β-boswellic acid and 3-acetyl-11-keto-beta-boswellic acid **(A)**, and at 250 nm for 11-keto-beta-boswellic acid and **(B)**.

Table 1
The concentration of each compound within Fr.

Compounds	Retention time (min)	Concentration (mg/g)
11-keto-beta-boswellic acid	6.785	9.225 $\pm$ 0.151
3-acetyl-11-keto-beta-boswellic acid	8.587	47.191 $\pm$ 0.162
β-boswellic acid	15.054	20.789 $\pm$ 0.148

earlier studies [22, 23]. As shown in Fig. 1, Fr was well separated within 20 min, and we estimated concentrations by the retention times of the peaks with those of three boswellic acid-derived standard solutions. To evaluate the reproducibility of the extraction efficiency, HPLC analysis was repeated three times using same samples. According to the data shown in Table 1, there was 9.225 $\pm$ 0.151 mg, 47.191 $\pm$ 0.162 mg, and 20.789 $\pm$ 0.148 mg of 11-keto-beta-boswellic acid, 3-acetyl-11-keto-beta-boswellic acid, and β-boswellic acid in 1 g of Fr were, respectively (Table 1).

3.2. Network pharmacological predicted Fr as a potential anti-endometriotic agent

The list of potential Fr targets obtained from the TCMID (Supplementary Table S1) was employed in the network analysis of the JEPPTO plugin on the Cytoscape software. The findings showed that steroid hormone, inflammatory, migratory, adhesion, angiogenesis, and autophagic pathways were significantly altered, which were linked to endometriosis[34, 35] (Supplementary Fig. S1 and Supplementary Table S2-S5). Thus, we hypothesized that endometriosis could be a potential target disease of Fr. The notion was tested by comparing the Fr targets to endometriosis-related genes from the DisGeNet database (listed in Supplementary Table S6). There were 38 genes identified as shared targets between endometriosis and Fr (Fig. 2A). With the exception of GBA, 37 of them have robust protein-protein interactions (PPI). Higher PPI levels were seen in the genes *AR, CXCL8, CYP1A1, CYP1A2, CYP3A4, EGFR, ESR2, GSK3B, HIF1A, HSD17B2, MAPK1, NFKB, NR3C1, PGR, PPARG, PTGS2,* and *UGT1A1* (Fig. 2B).
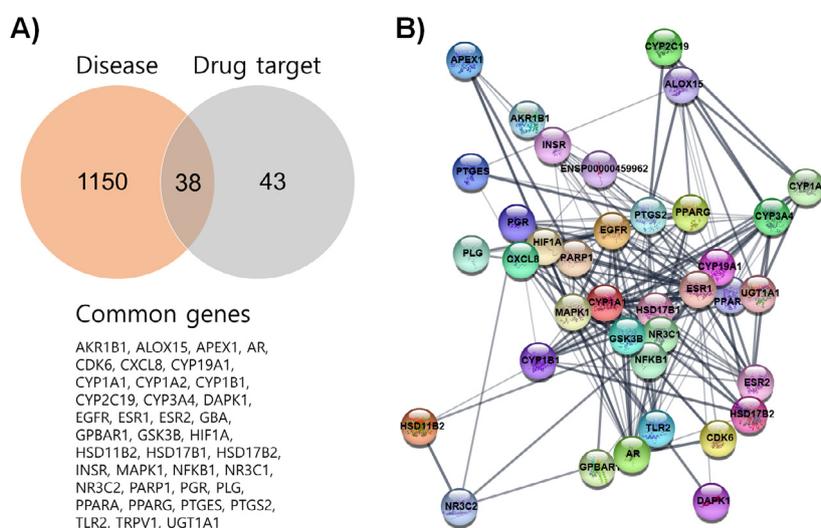
Fig. 2. The potential therapeutic effect of Fr on endometriosis is presented via network pharmacological analysis. The DisGeNet database was used to compile the list of endometriosis-related genes. A Venn diagram analysis was performed utilizing endometriosis-related genes and Fr targets. 38 common genes were listed (**A**). Cytoscape with the STRING plugin was used to show the protein-protein interaction network of the 38 common genes (**B**).
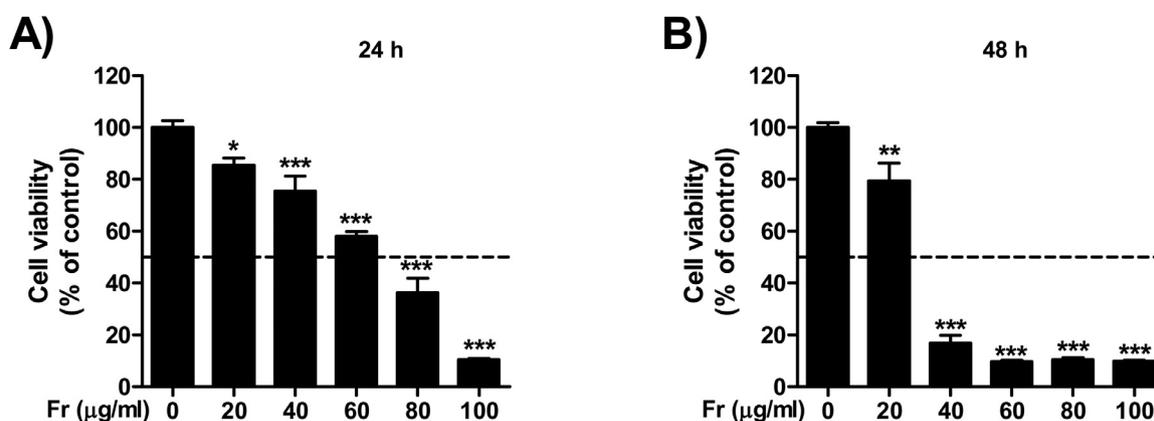

Fig. 3. Fr reduced human endometriotic cell proliferation. Fr was dissolved in DMSO and diluted in DMEM-F12 medium. Indicated concentrations of Fr was treated to human endometriotic epithelial (12Z) cells. After 24 h (**A**) and 48 h (**B**), the cell viability was analyzed by measuring at a wavelength of 450 nm using MTT. Data shown as mean $\pm$ SEM of quintuplicated and are representative of three independent experiment. Statistical analysis was conducted using a Dunnet one-way ANOVA (*; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$).

3.3. Fr reduced the viability of 12Z cells

To first confirm the plausibility of an anti-endometriotic effect, we used an *in vitro* colorimetric cell viability assay. Fr was treated for 24 and 48 h at the stated concentrations (0, 20, 40, 60, 80, and 100 g/mL). The results indicated that Fr treatment decreased the viability of 12Z cells in a dose-dependent manner (Fig. 3A-B). The GI_{50} for 24 and 48 h is 78.46 μg/ml and 26.34 μg/ml, respectively. In the subsequent experiments, including the animal research, the apoptosis analysis, and the adhesion assay, we used the dose as a reference.

3.4. Fr decreased the number and weight of mouse endometriotic lesion

To validate the *in vivo* effectiveness of Fr in endometriosis, we applied the endometriosis mouse model using transfer of syngeneic uterus. After experimentally inducing endometriosis, Fr (0.3 or 1.5 mg/kg/day) was administered orally five times per week. We examined the quantity and weight of cyst-like ectopic lesions, *i.e.* endometrial foci, adherent to intestinal or abdominal tissues

three weeks after Fr administration. When compared to the vehicle, Fr significantly reduced the number and volume of ectopic endometrial foci (Fig. 4). Under microscopic examination of H&E-stained sections of kidney and liver tissues, the amounts of Fr employed in the *in vivo* experiment were determined to be nontoxic (Supplementary Fig. S2). These findings suggest that administering Fr orally prevented the adhesion or development of ectopic endometriotic tissues.

3.5. Fr induced apoptosis in 12Z cells

We investigated the possibility of apoptotic cell death to identify the mechanism explaining the reduced viability of 12Z cells treated with Fr. The PI/Annexin V apoptosis assay demonstrated that Fr dose-dependently promoted apoptosis of 12Z cells, similar to the cell viability analyses. At a concentration of 40 μg/ml, apoptotic cells (Annexin V^+), including necroptic and early apoptotic cells, were first noticed. At an increase of 80 μg/mL, these apoptotic cells were noticeably more prevalent. More than 50% of the dead cells (PI$^+$) were seen at a concentration of 100 μg/ml. (Fig. 5A and B). Following that, we evaluated the expression of pro-
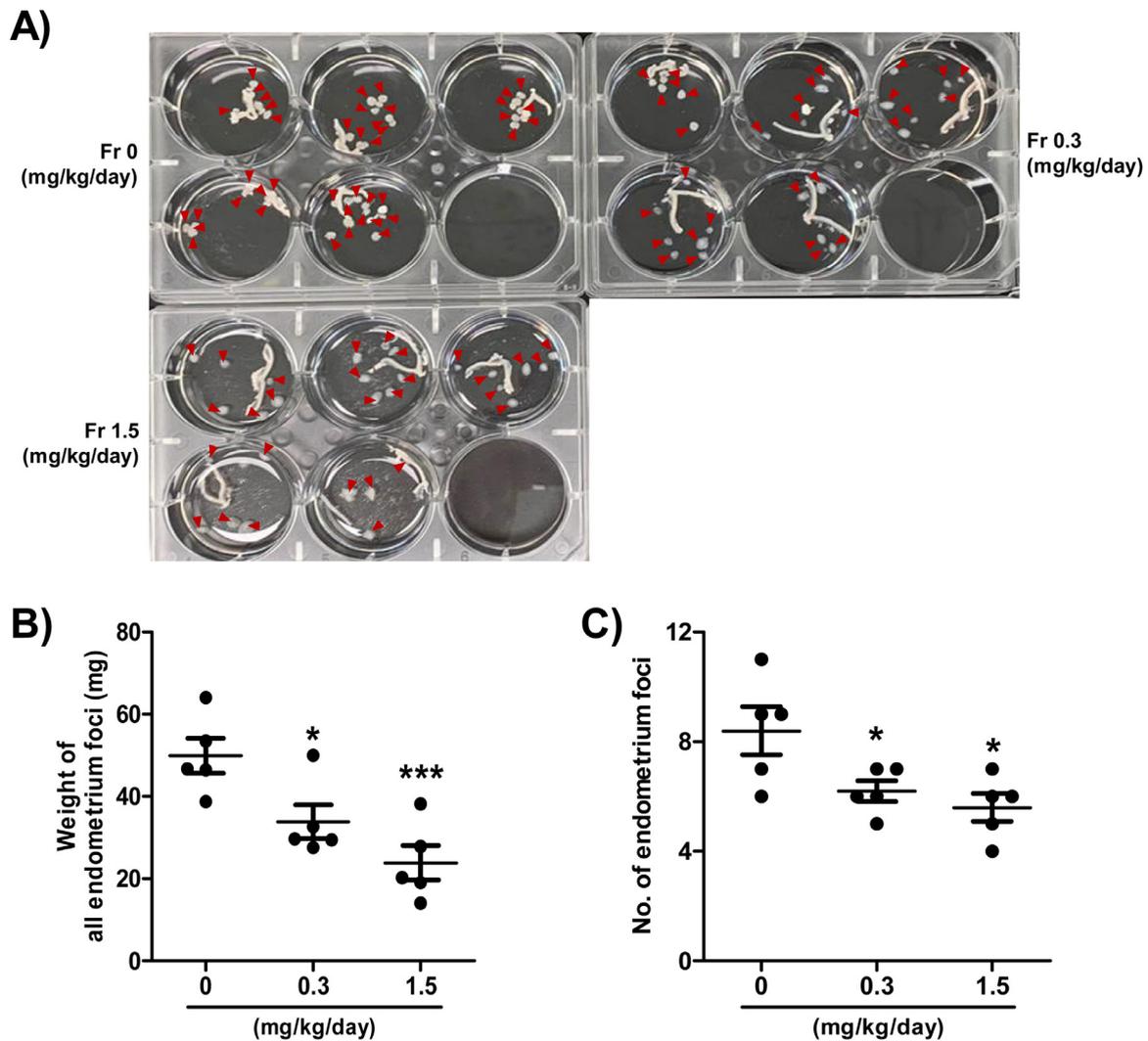
Fig. 4. Fr reduces the size and number of murine ectopic endometrial lesions. The pictures of the ectopic endometrial tissues and uterus. Red arrows indicate endometrial tissues **(A)**. The weight and number of ectopic endometrial tissues **(B-C)**. Data are expressed as mean ± SEM. Statistical analysis was conducted using a *t*-test (*; $p < 0.05$, ***; $p < 0.001$).

teins implicated in intracellular apoptotic signaling in 12Z cells. As illustrated in Fig. 5C, the Fr treatment decreased the expression of the bcl-2 protein while increasing the expression of the bax protein. Fr also regulated the conversion of caspases-3, −9, and PARP from pro- to cleaved active-form (Fig. 5D). These findings suggest that Fr induces apoptosis in 12Z cells by activating intracellular apoptosis pathways and modulating the bcl2/bax balance.

3.6. Fr prevented 12Z from attaching to Met-5A cells

According to the amount of endometriotic foci in an animal investigation, we speculated that Fr could prevent endometriotic tissue from adhering to abdominal tissues. Thus, using Met-5A and 12Z cells, we conducted an *in vitro* adhesion assay to test the potential. As seen in Fig. 6, Fr prevented transferred 12Z cells (green) from adhering to the Met-5A cells underneath them (red). In a dose-dependent way, there was a considerable decrease in the amount of 12Z cells adhering to Met-5A cells. The amount of attached 12Z cells was decreased to 50.49 percent of control with 20 μg/ml of Fr-treatment. Overall, the findings suggest that Fr's potential function in limiting the initial stage of endometriosis by regulating the endometrial-mesothelial adhesion.

3.7. RNA sequencing identified potential mechanisms for Fr's anti-endometriotic activity

We employed RNA sequencing to investigate how Fr regulates cell death and adhesion at the gene expression level. According to a GSEA analysis using KEGG and hallmark, p53 and apoptosis pathways were increased whereas chemokine signaling and cellular adhesion pathways were downregulated (Fig. 7A, B, and Supplementary Fig. S3). Proteolytic activities such as unfolded protein response (UPR), proteasome, lysosome, and autophagy were elevated. Additionally, the network analysis of the GO analysis revealed downregulated immune response and migration pathways, and elevated ER stress signature (Fig. 7C). The mapping of KEGG pathways, including p53, apoptosis, chemokine signaling, ECM-receptor, and cell adhesion molecule, was shown in Supplementary Fig. S4. According to DEG analysis, 949 genes were considerably elevated, whereas 284 genes were significantly downregulated (Fig. 7D). The 88 genes (38 downregulated and 50 upregulated) were chosen based on their involvement in the pathways depicted in Fig. 7D and Supplementary Fig. S3. shows the symbols of such chosen genes separately, and heatmap analysis was used to summarize the relative expression levels of 88 genes (Fig. 7E).
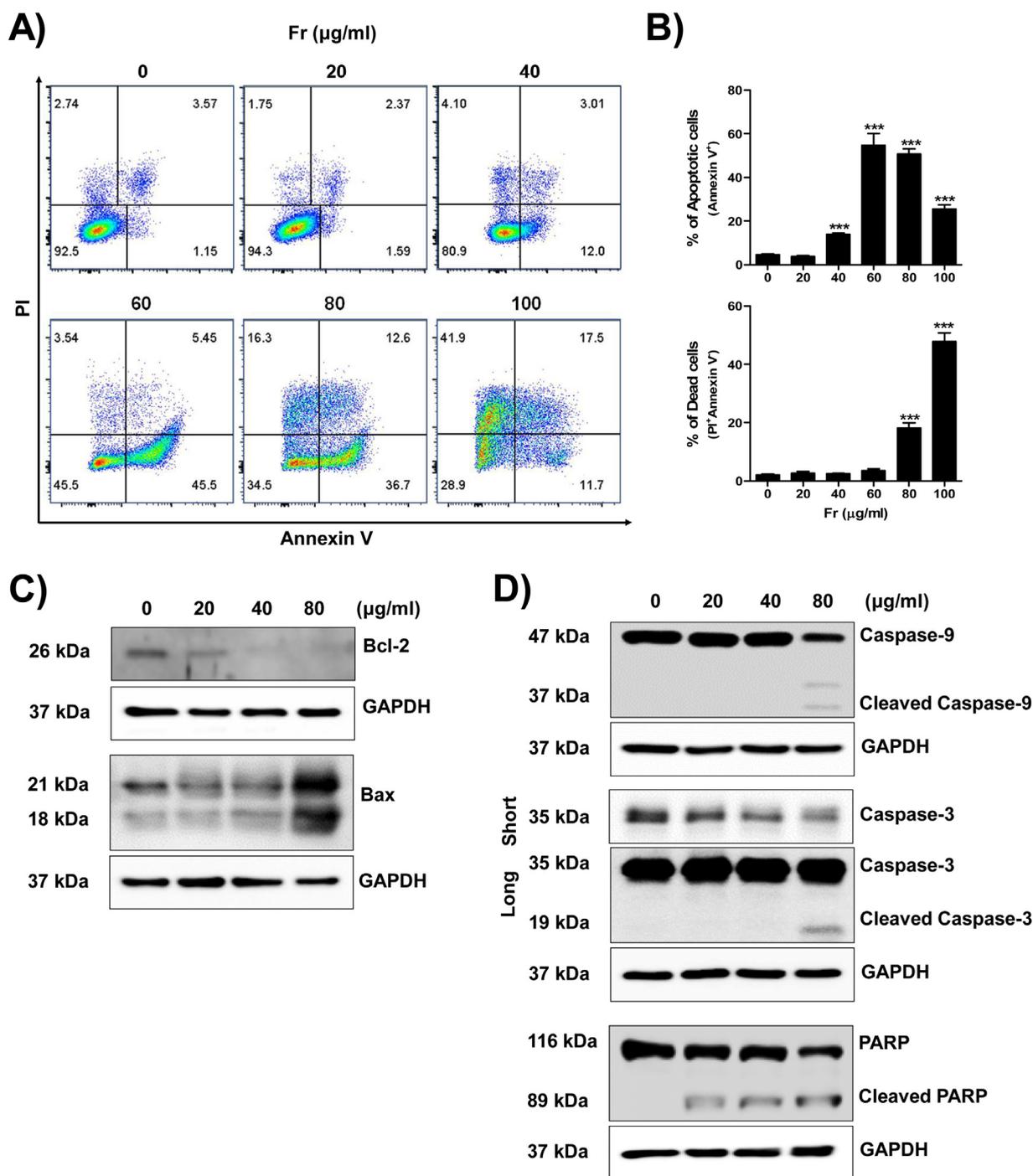
Fig. 5. Fr activates the apoptosis-death signaling pathway in endometriotic cells. For analysis of frequency of apoptotic or dead cell populations, 12Z cells were incubated with the increasing doses of Fr for 24 h, and then the percentage of Annexin[+] or PI^{+} cells were measured at excitation (Ex) 494/emission (Em) 525 nm for Annexin V and Ex 535/Em 617 nm for PI (**A, B**). Mitochondria-relative apoptosis molecules, Bax and Bcl-2 (**C**) and the cleavage forms of caspase −3 and −9, and PARP, main intracellular apoptosis signaling proteins (**D**), were detected 24 hrs after Fr treatment. GAPDH was used as control, and the specific molecular weight is represented on the left.

4. Discussion

Several frankincense-containing herbal remedies, including Bushen Huoxue prescription, Qu Yi Kang, and Qing Yi decoction, have been used to treat endometriosis.[36,37] In addition, due to its anti-proliferative, anti-nociceptive, and anti-inflammatory activities, frankincense was already proposed as a possible herbal therapy for treating endometriosis.[38] However, there is no concrete evidence to support the review paper's claim that frankincense has an anti-endometriotic effect. In this investigation, we validated

the possibility by employing network pharmacologic analysis using a list of probable target genes from TCMID and several signaling pathways associated to steroid hormone, inflammation, migration, and cell-cell adhesion, were discovered. These pathways are recognized as having a close connection to the pathogenesis of endometriosis.[34,39,40] Additionally, GO biological process analysis established the vascular endothelial growth factor and autophagy pathways, which are emerging pathways for the development of endometriosis.[41-43]
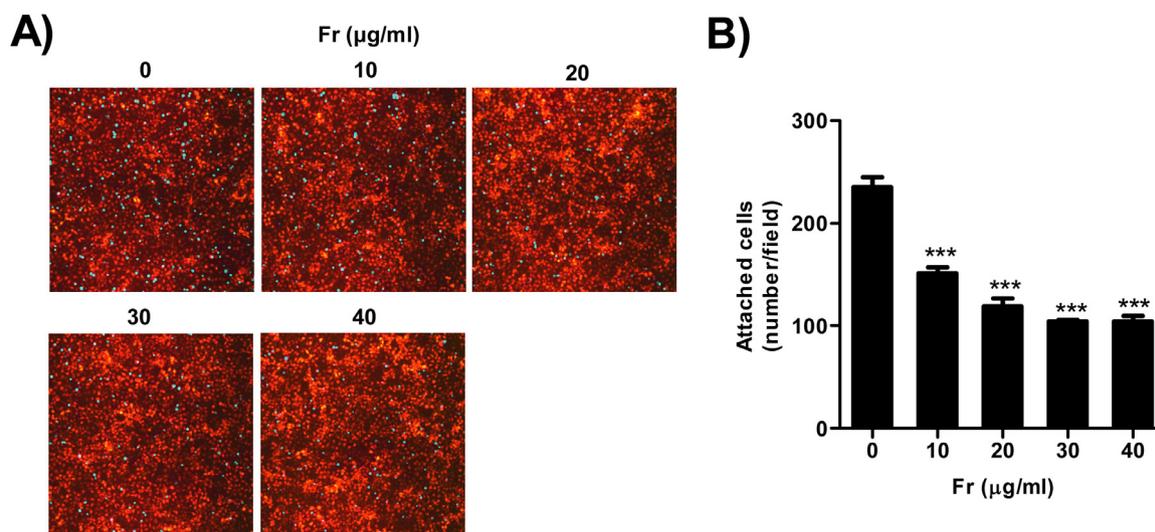
Fig. 6. Fr decreased the ability of endometrial cells to adhere to mesothelial cells. 12Z cells were stained with CMFDA after 24 h of Fr treatment and then gently transferred onto a red fluorescent Met-5A cell monolayer. After 90 min with gentle shaking at 20 rpm, unbound 12Z cells were removed, and cells were then visualized using a fluorescent microscope (200 × magnification) **(A)**. The number of 12Z cells bound to Met-5A cells was manually counted in four randomly chosen areas for statistical analysis **(B)**. Data are representative of one of five independent experiments and values are expressed in mean ± SEM. Statistical analysis was conducted using a Dunnet one-way ANOVA (***; $p < 0.001$).

The *in vivo* experiments employing syngeneic uterine transfer mice model showed that Fr effectively reduced the number and weight of ectopic endometriosis foci. Based on the findings, we hypothesized that Fr might decrease the attachment of injected uterine cells to peritoneal myometrium and subsequent proliferation of the ectopic endometrial cells. Increased adhesion and resistance to apoptosis are thought to be crucial stages in the development of endometriosis and promising therapeutic targets.[8,13,44] *In vitro* experiments using a FACS-based apoptosis assay, western blot analysis, and cell-cell adhesion assay were used to conform the possibilities. In multiple previous studies, frankincense was identified as a therapeutic herb that promoted apoptosis and prevented tumor migration in a number of cancer types, particularly estrogen-dependent breast cancer.[45-47] Despite differences in cell types, the prior publications agree with our observations.

Herbal medications are generally recognized to have multi-activity due to the multi-component nature of its constituent phytochemicals and secondary metabolites.[48,49] As a consequence, it is challenging to understand the mode of action (MoA) that drives the effects of herbal medications. Several prior studies used bioinformatic analysis based on omics data, including transcriptome, to address the issue.[31,50,51] Frankincense contains a wide range of phytochemicals, including volatile oil, polysaccharides, monoterpenes, diterpenes, and lipophilic pentacyclic triterpene acids.[19,22] In order to understand the molecular mechanisms behind the pro-apoptotic and anti-adhesive effects of Fr, we also used the transcriptome and bioinformatic analyses in this study. Based on the results, we hypothesized that the anti-adhesive activity of Fr may be related to cytokine and chemokine signaling. Additionally, the p53-DNA damage response and ER stress-mediated UPR may serve as a model for its pro-apoptotic activity (Fig. 7F). However, more extensive investigation is required to identify the main active ingredients and assess potential MoA of Fr pathways.

Among the numerous constituent compounds in frankincense, boswellic acids and triterpenoids are well-known main constituents.[52] Although we have not examined each substance's activity, based on previous research, 3-acetyl-11-keto-β-boswellic acid is expected to be the most effective endometriosis inhibitor. There are three main reasons why 3-acetyl-11-keto-β-boswellic acid is the most effective ingredient. To begin, *in vitro* experiments

with tumor cells show that 3-acetyl-11-keto-β-boswellic acid and 11-keto-β-boswellic acid initiate an apoptotic cascade by increasing the activity of caspase-8, −9, −3, and PARP cleavage. However, β-boswellic acid has no effect on the induction of cell apoptosis.[53] Beta-boswellic acids, in particular, act as an apoptosis inhibitor in normal cells but induce apoptosis in cancer cells. Furthermore, 3-acetyl-11-keto-β-boswellic acid has antitumor effects *in vivo* by inhibiting cell growth, proliferation, and migration/invasion potential.[54,55] Finally, in HPLC analysis, the content of 3-acetyl-11-keto-β-boswellic acid in Fr is the highest of the three types of boswellic acids (Table 1).

In an animal investigation, mice with endometriosis were given two different dosages of Fr that were calculated from an *in vitro* cytotoxic assay. The doses are substantially lower than those predicted by the typical human use of Fr. Moreover, histological examination of the liver and kidney at the levels used in the animal study revealed no abnormal morphological alterations. A number of previous researches carried on the other species, such as rat, dog, horse, and human, have shown that general human ingestion is safe across the board.[56-58] According to these publications and our results, Fr may be a favorable candidate for endometriosis treatment in terms of safety.

In conclusion, here we firstly revealed the potential effect of Fr on endometriosis using an experimental investigation. The results from both *in vivo* and *in vitro* models showed that Fr relieved endometriosis by decreasing ectopic endometrial adherence and development. Using RNA sequencing and bioinformatic analysis, we proposed the ER stress/p53-apoptosis and chemokine-migration/adhesion pathways as the mechanisms underlying the anti-endometriotic action of Fr. As a result, we recommend Fr, a water-extract of frankincense, as an efficient and safe option for treating endometriosis.

CRediT authorship contribution statement

Min Kyoung Cho: Methodology, Investigation, Validation, Formal analysis, Writing original draft, Writing review & editing. **Jung-Sook Jin:** Methodology, Investigation, Writing review & editing. **Yunju Jo:** Formal analysis, Writing review & editing. **Jung Ho Han:** Investigation, Writing review & editing. **Su Shin:** Investiga-
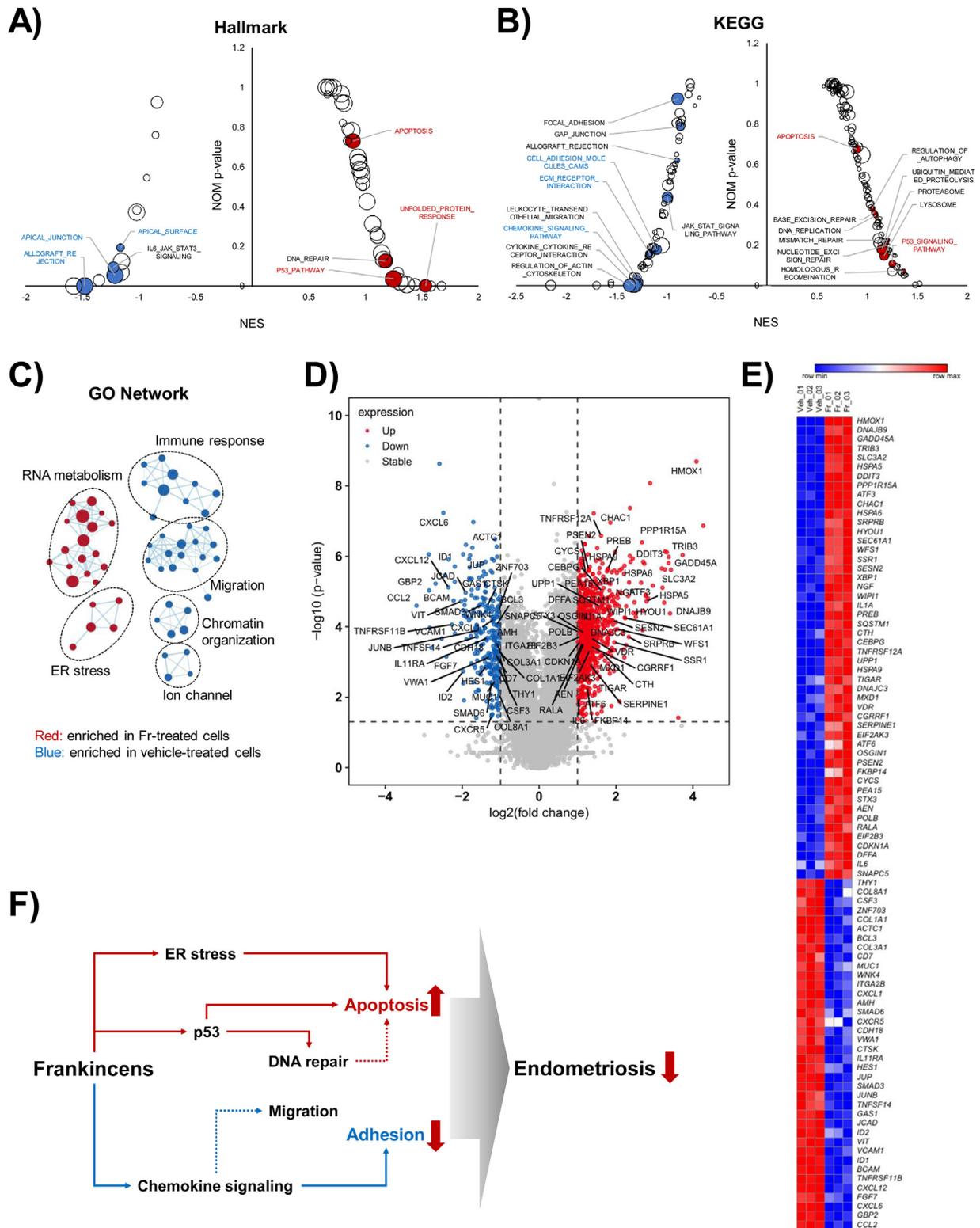
Fig. 7. RNA-sequencing analysis suggests possible mode of action (MoA) underlying anti-endometriosis effect of Fr. The 12Z cells were treated with Fr (70 μg/ml) for 12 h. The RNA-sequencing dataset supplied by commercial service was applied to GSEA. The results from hallmark and KEGG analysis were visualized by bubble plot. The major pathways related with endometriosis was indicated by red (upregulated) or blue (downregulated) characters **(A, B)**. The data from GO analysis was described by Cytoscape with enrichment map visualization. The cutoff value was set as $p > 0.001$ and $q > 0.5$ **(C)**. The DEG analysis visualized as log2 fold change and -log10 p-value. Genes that were noticeably up- or down-regulated were denoted by red or blue characters, respectively. The genes participating in endometriosis-related pathways, as shown in the A and B panels, were indicated by a symbol for each gene **(D)**. Heatmap analysis was used to display the relative expression levels of genes connected to endometriosis-related pathways **(E)**. A graphical scheme was used to outline the potential mechanisms of action (MoA) underlying the relieving effects of Fr on endometriosis **(F)**.

tion, Writing review & editing. **Sung-Jin Bae:** Methodology, Writing review & editing. **Dongryeol Ryu:** Formal analysis, Writing review & editing. **Jongkil Joo:** Writing review & editing. **Jang-Kyung Park:** Conceptualization, Funding acquisition, Writing review & editing. **Ki-Tae Ha:** Conceptualization, Funding acquisition, Writing review & editing, Supervision.

Conflict of interest

The authors declare that they have no conflict of interest.

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Ethical statement

This research was approved by the guidelines of Institutional Animal Care and Use Ethical Committee of Pusan National University (Pusan, Republic of Korea; PNU-2020–2679).

Data availability

The GEO datasets was uploaded privately on the GEO homepage, and the accession number is GSE217151. All data generated during the current study are available from the corresponding author upon reasonable request.

Supplementary materials

Supplementary information on methods, supplementary tables, and supplementary figures associated with this article can be found, in the online version, at doi:10.1016/j.imr.2023.100947.

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