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Water-extracted *Perilla frutescens* increases endometrial receptivity though leukemia inhibitory factor-dependent expression of integrins

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ABSTRACT

The leaves and stems of *Perilla frutescens* var. *acuta* Kudo (PF) have been used to prevent threatened abortion in traditional medicine in the East Asian countries. Because reduced receptivity of endometrium is a cause of abortion, we analyzed the action of PF on the endometrial receptivity. PF increased the level of leukemia inhibitory factor (LIF), a major cytokine regulating endometrial receptivity, and LIF receptor in human endometrial Ishikawa cells. The PF-induced LIF expression was mediated by c-jun N-terminal kinase (JNK) and p38 pathways. Adhesion between Ishikawa cells and trophoblastic JAr cells stimulated by PF treatment was abolished by knock down of LIF expression or antagonism of LIFR. In addition, the expressions of integrin β3 and β5 were increased by PF treatment in Ishikawa cells. The PF-induced expression of integrin β3 and β5 was reduced with an LIFR antagonist. Neutralization of both integrins successfully blocked PF-stimulated adhesion of JAr cells and Ishikawa cells. These results suggest that PF enhanced the adhesion between Ishikawa cells and JAr cells by increasing the expression of integrin β3 and β5 via an LIF-dependent pathway. Given the importance of endometrial receptivity in successful pregnancy, PF can be a novel and effective candidate for improving pregnancy rate.

1. Introduction

In spite of the development of diverse assisted reproduction technologies (ARTs), including *in vitro* fertilization, prevention of incomplete embryo implantation remains a major unmet need (1). To solve the problem of embryo implantation, it is crucial to enhance endometrial receptivity toward a properly developed embryo (2). It is well known that diverse biomolecules, such as cytokines and growth factors, play important roles in the process of developing a receptive endometrium (3). Among them, several previous studies have shown that leukemia inhibitory factor (LIF), a member of interleukin-6 family of cytokines, is one of the major factors that regulates endometrial receptivity (4). In addition, LIF expression defects have been shown to be involved in multiple implantations failures in patients with female infertility (5). Therefore, research has focused on developing novel candidates that stimulate embryo implantation rates by enhancing LIF expression, especially by using natural herbal medicines or traditional therapy, including acupuncture (6–8).

The leaves and stems of *Perilla frutescens* var. *acuta* Kudo (Labiatae family), a perennial herb, has being used in traditional medicine of East Asian countries for treating symptoms of the common cold, including shivering fits, fever, chest pains, and cough, and for preventing threatened abortion (9). In addition, previous pharmacological studies have shown that *P. frutescens* has anti-inflammatory and anti-tumor effects (10,11). However, there

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are no reports available on the effects of *P. frutescens* on enhancing endometrial receptivity or pregnancy rate. Because reduced endometrial receptivity is a cause of abortion in early stage of pregnancy (12), we predicted that PF may be involved in the regulation of endometrial receptivity.

In this study, we investigated the effect of *P. frutescens* on the expression of LIF and adhesion between trophoblast and endometrium *in vitro*. The expression of adhesion molecules, including integrins involved in PF-induced endometrial receptivity was also examined.

2. Materials and methods

2.1. Materials

Antibodies against LIF, LIFR, phospho-extracellular signal-regulated kinases (ERK) 1/2, ERK2, p38, integrin αV, β3, and β5, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and β-actin were purchased from Santa Cruz Biotechnology (Dallas, TX). Antibodies against phospho-c-Jun N-terminal kinases (JNK) and phospho-p38 were purchased from Cell signaling (Danver, MA, USA), and the antibody for JNK was purchased from Sigma–Aldrich (St. Louis, MO, USA). Specific inhibitors against signaling pathways, including U0126 (inhibitor for mitogen-extracellular signal-regulated kinase/extracellular signal-regulated kinase; MEK/ERK), SB203580 (inhibitor for p38), and SP600125 (inhibitor for JNK) were obtained from Merck Millipore (Billerica, MA, USA). Recombinant antagonists for human LIFR (hLA) expressed in *Escherichia coli* were prepared as described previously (13).

2.2. Plant material and extract preparation

The leaves and stems of *P. frutescens* were purchased from Omniherb Co. (Daegu, Korea). The plant was collected in Gyeongsangbuk-do Province, Republic of Korea in 2013, and identified by a botanical expert working at Omniherb Co. A boucher specimen (KMRC-DC-H21) was deposited at the Healthy Aging Korean Medicine Research Center, Pusan National University. The sample was extracted as previously described (14). Briefly, the leaves and stems of *P. frutescens* (100 g) were extracted with distilled water (1 L) for 2 h at 100 °C, and then centrifuged at 4000 rpm for 10 min. The supernatant was extracted with 70% ethanol for polysaccharide precipitation at 4 °C. After centrifugation at 4000 rpm for 10 min, the supernatant was evaporated and lyophilized by freeze-drier to give a powder (abbreviated as PF, 7.856 g). PF was freshly dissolved in dimethyl sulfoxide (Sigma–Aldrich) before experiments.

2.3. Fingerprinting high-performance liquid chromatography (HPLC) analysis

Phytochemical characteristics of PF were identified by HPLC analysis as previously described (9,15), with some modifications. HPLC analysis was performed using an Agilent 1200 series system (Agilent Technologies, Santa Clara, CA, USA) and LC solution software (version 1.24) was used for data analysis. AkzoNobel KR100-5C18 column (AkzoNobel, Amsterdam, Netherlands; 4.6 × 250 mm; pore size, 3.5 μm) was used as an analytical column. The mobile phases were solvent A [0.1% formic acid aqueous (v/v)] and solvent B (methanol). The gradient flow was as follows: (A)/(B) = 100/0 (5 min) → (A)/(B) = 50/50 (20 min) → (A)/(B) = 0/100 (30 min) → (A)/(B) = 100/0 (5 min). The column temperature was maintained at 35 °C. The analysis was carried out at a flow rate of 1 mL/min with detection at 254 nm. The column injection volume was 20 μL. A standard solution, containing protocatechuic acid, coumaric acid, ferulic acid, and rosmarinic acid was prepared by dissolving in distilled water (10 μM). The solution was filtered through a 0.45 μm membrane filter before HPLC analysis.

2.4. Cell culture

The human endometrial Ishikawa cell line was generously provided by Dr. Jacques Simard (CHUL Research Center, Quebec, Canada) and human trophoblastic JAr cells were provided from the Korean Cell Line Bank (Seoul, Korea). Ishikawa cells were cultured as monolayers at 37 °C in an atmosphere containing 5% CO_2/air and Dulbecco's Modified Eagle Medium (DMEM; Welgene, Daegu, Korea) with 10% heat-inactivated fetal bovine serum (FBS; Sigma–Aldrich) and 1% penicillin/streptomycin (Gibco, Carlsbad, CA, USA). JAr cells were cultured as monolayers at 37 °C in an atmosphere containing 5% CO_2/air and Roswell Park Memorial Institute 1640 (RPMI1640; Welgene) containing 10% heat-inactivated FBS and 1% penicillin/streptomycin.

2.5. Cell viability assay

Ishikawa cells were cultured in 24-well plates with the indicated concentrations of PF in serum free medium for 24 h. Next, the medium was replaced with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (2 mg/mL) and incubated at 37 °C in a cell culture incubator for 3 h. The formazan crystals formed by MTT reduction were dissolved with DMSO and EtOH solution and measured at 540 nm with a microplate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA).

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from Ishikawa cells using GeneJET RNA Purification Kit (Thermo Fischer Scientific, Waltham, MA, USA). Total RNA of each sample was subjected to reverse transcription with oligo-dT primers by using M-MLV reverse transcriptase (Enzynomics, Daejeon, Korea). The cDNA was amplified by PCR using DiaStarTM Taq DNA Polymerase (Solgent Co., Daejeon, Korea). The primers and PCR conditions used for amplifying *LIF, β-actin, ITGAV, ITGB3,* and *ITGB5* are shown in Table 1. The amplified DNA was separated by electrophoresis in 1% agarose gels containing ethidium bromide and visualized under ultraviolet (UV) light. The images were acquired with the GelDoc-It TS Imaging System (UVP, Upland, CA, USA).

2.7. Western blot analysis

Total proteins were isolated from Ishikawa cells. Equal amount of proteins (25 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked in 5% skim milk in Tris-buffered saline (TBS) and then incubated with each antibody for target proteins, including LIF, LIFR, phospho-ERK1/2, ERK2, phospho-p38, p38, phospho-JNK, JNK, integrin αV, integrin β3, integrin β5, GAPDH, and β-actin, overnight. Membranes were then washed in TBS and incubated with appropriate secondary antibodies conjugated with the horseradish peroxide. The signals were visualized by using the enhanced chemiluminescence system (GE Healthcare, Piscataway, NJ, USA).

2.8. Adhesion assay

Ishikawa cells (1.5×10^6 cells) were seeded in 6 well plates overnight. Media were replaced and incubated in serum free-

Table 1
Primers used for PCR of *LIF, integrins,* and *β-actin*.

Gene	Primer sequences	PCR condition	Size (bp)
LIF	Forward: 5′-GGCCCGGACACCCATAGACG-3′	60 °C	454
	Reverse: 5′-CCACGCGCCATCCAGGTAAA-3′	35 cycles	
ITGAV	Forward: 5′-ATGCTCCATGTAGATCACAAGAT-3′	60 °C	339
	Reverse: 5′-TTCCCAAAGTCCTTGCTGCT-3′	35 cycles	
ITGB3	Forward: 5′-CTGCCGTGACGAGATTGAGT-3′	62 °C	383
	Reverse: 5′-TGCCCCGGTACGTGATATTG-3′	35 cycles	
ITGB5	Forward: 5′-ACCTGGAACAACGGTGGAGA-3′	60 °C	127
	Reverse: 5′-AAAAGATGCCGTGTCCCCAA-3′	35 cycles	
β-actin	Forward: 5′-CAAGAGATGGCCACGGCTGCT-3′	60 °C	275
	Reverse: 5′-TCCTTCTGCATCCTGTCGGCA-3′	30 cycles	

DMEM with the indicated concentration of PF for 48 h. The JAr cells were labeled with 5-chloromethylfluoresceindiacetate (CMFDA) fluorescence dye (CellTracker Green; Invitrogen, Carlsbad, CA, USA) for 10 min at 37 °C. Then, the labeled JAr cells were washed in 1 × phosphate-buffered saline and incubated with Ishikawa cells by gently shaking at room temperature for 30 min. The cells were vigorously washed to remove non-attached JAr cells. The attached JAr cells were visualized by using a fluorescence microscope (Axioimager M1 microscope, Zeiss, Aalen, Germany) and the cell numbers were calculated.

2.9. Knockdown of LIF by short hairpin RNA (shRNA)

To knockdown hLIF expression, five different shRNA clones were purchased from Open Biosystems (Thermo Scientific). The lentiviral vector encoding shRNA (3 μg) was transfected in 293T cells by using Lipofectamine 2000 (Invitrogen). After 48 h, supernatants harboring lentiviruses were collected and infected into Ishikawa cells. The cells were subjected to selection with 3 μg/mL puromycin (Sigma–Aldrich) for 1 week. The knockdown efficiency of the shRNAs against *LIF* expression was checked by western blot analysis.

2.10. Statistical analysis

The data from experiments were indicated as the value of percentage compared to the control and expressed as the mean ± standard deviation (SD). The differences between the mean values of 2 groups were determined by student *t*-test and multicomparisons between groups were analyzed using one-way analysis of variance (ANOVA) with a Turkey's post-hoc test using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). The minimum significance level was set at a *p*-value of 0.05. All experiments were performed at least three times, independently.

3. Results

3.1. HPLC analysis and cytotoxic effect of PF on Ishikawa cells

As shown in Fig. 1A, the PF used in this study seems to be identical to the previously reported PF standard (9,15). To determine the optimal concentration, the cytotoxic effect of PF on Ishikawa cells was determined. PF did not show significant cytotoxicity up to a concentration of 50 μg/mL (Fig. 1B). Thus, for subsequent studies, 50 μg/mL was established as the optimum working concentration.

3.2. PF increased the attachment of JAr onto Ishikawa cells and the expression of LIF and LIFR

To examine the possibility that PF affects implantation of a human embryo onto the endometrium, we performed an *in vitro* adhesion assay using human trophoblastic JAr cells and endometrial Ishikawa cells. Adhesion between Ishikawa cells and, JAr cells

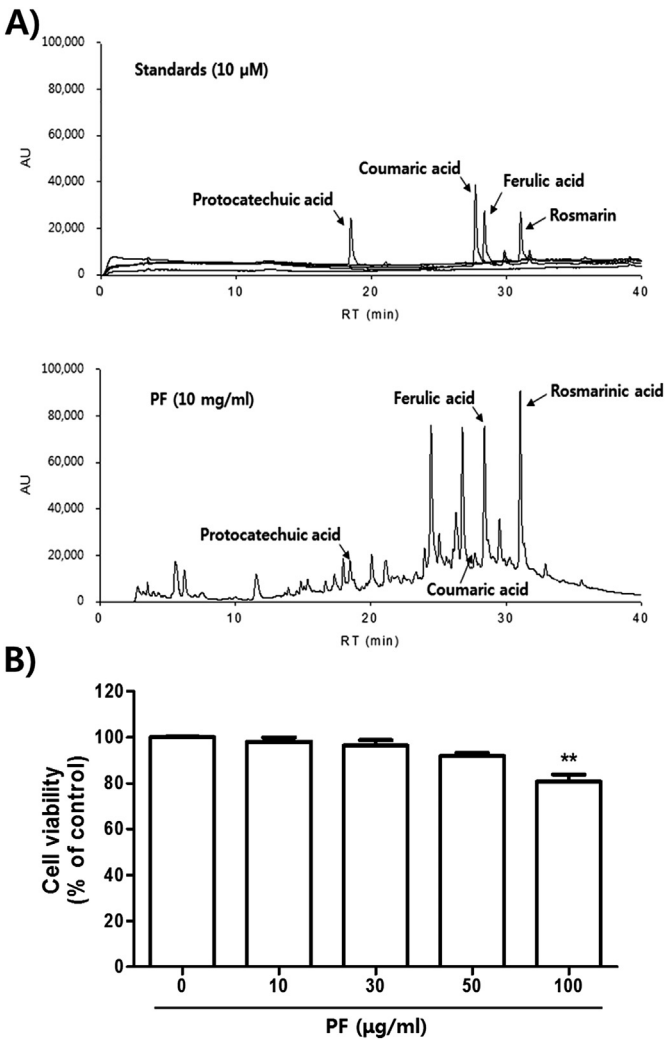

Fig. 1. HPLC chromatogram and cytotoxic effect of leaves and stems of *Perilla frutescens* (PF). **(A)** HPLC chromatogram was performed by using protocatechuic acid, coumaric acid, ferulic acid, and rosmarinic acid as standards. **(B)** Ishikawa cells were treated with indicated concentrations of PF in serum-free culture medium for 24 h. Cell viability was estimated by using MTT assay and calculated as means ± SD of three independent measurements. $^{**}p < 0.05$ in comparison with control group (1st lane).

was increased about 2 fold in PF treated cells compared to control (Fig. 2A), indicating that PF has a positive effect on the adhesion between JAr cells and Ishikawa cells. Because the function of LIF and the LIFR pathway is crucial in embryo implantation process (16), we checked the expression level of LIF and LIFR. Result from western blot and RT-PCR analyses clearly demonstrated that the levels of LIF and LIFR were increased by PF treatment in a dose-dependent manner (Fig. 2B and C).

3.3. PF regulates LIF expression through activating p38 and JNK

Several previous studies have found that the expression of LIF was regulated by MAP kinase signaling pathways (17,18). Therefore, we examined if activation of ERK, JNK, and p38 pathway is involved in PF-induced expression of LIF. The results showed that PF treatment activated p38 and ERK from 2 h. The JNK was activated after 2 h from treatment of PF (Fig. 3A). In addition, phosphorylation of ERK, JNK, and p38 were increased in a dose-dependent fashion (Fig. 3B). To confirm which of these signaling pathways regulated LIF expression, we used specific inhibitors for each of these signaling molecules, such as SP600125 for JNK, SB203580 for p38, and U0126 for MEK/ERK. The results of RT-PCR and western blot analysis clearly indicate that treatment of SP600125 and SB202580 significantly blocked the PF-induced stimulation of LIF expression (Fig. 3C). In addition, as shown in Fig. S1 PF-induced increase in LIFR expression was mediated by JNK, p38, and ERK pathways.

3.4. PF augments adhesion of JAr cells to Ishikawa cells through the LIF/LIFR axis

To demonstrate whether ligand–receptor interaction of LIF/LIFR axis plays an important role in PF-induced adhesion between JAr and Ishikawa cells, we performed the adhesion assay using knocking down of *LIF* expression by shRNA transfection and antagonizing LIFR by hLA treatment. We found that knocking down *LIF* expression in Ishikawa cells abrogated PF-stimulated adhesion between JAr and Ishikawa cells (Fig. 4A). In addition, pretreatment with hLA reduced PF-induced adhesion of JAr cells onto Ishikawa cells (Fig. 4B).

3.5. LIF-dependent expression of integrin β3 and β5 is crucial for the adhesion of trophoblast on endometrial cells

The expressions of adhesion molecules including integrins, plays an important roles in the regulation of endometrial receptivity toward properly developed embryos (19,20). To evaluate which integrins are involved in PF-induced adhesion of trophoblast on endometrial cells, we examined the expression of integrin αV, β3, and β5. We found that the expression of integrin β3 and β5 was increased by PF treatment. The expression of integrin αV was not changed by PF treatment. The PF-stimulated expression of integrin β3 and β5 was reduced by suppression of LIFR function using hLA (Fig. 5A and B). The results demonstrated that PF regulates the expression of integrin β3 and β5 in an LIF-dependent manner.

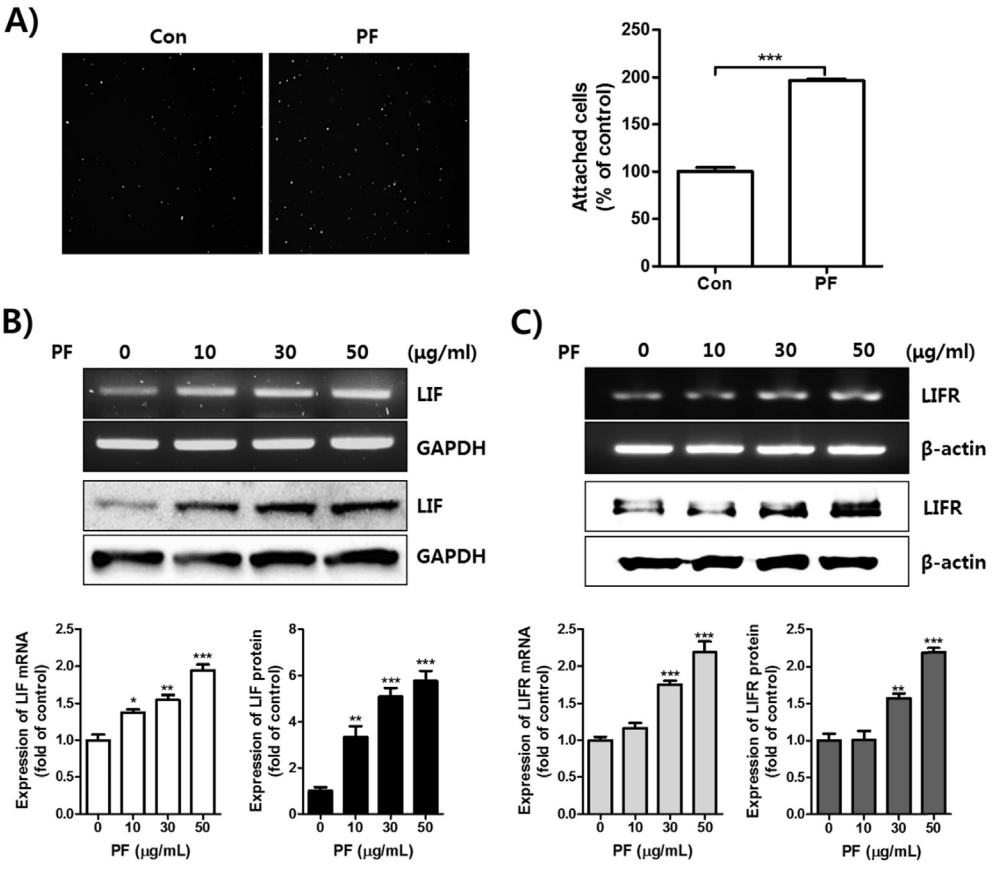

Fig. 2. The effect of PF on adhesion between Ishikawa and JAr cells and on the expression of LIF and LIFR. **(A)** Ishikawa cells were cultured in 6-well plates as monolayer with PF (50 μg/mL) for 48 h. JAr cells labeled with CellTracker were attached onto the Ishikawa cells. The attached cells were fixed and pictured using fluorescent microscopy. Data were calculated as percentage of control and are shown as mean ± SD of three independent experiments. $^{***}p < 0.001$ in comparison between two groups. **(B, C)** Ishikawa cells were treated with indicated concentrations of PF for 24 h. Protein and total RNA were prepared and the expression levels of LIF (B) and LIFR (C) were analyzed by western blotting and RT-PCR. The expressions of GAPDH and β-actin were used as internal controls. Band intensity was calculated by fold of control and shown as mean ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ in comparison with control group (1st lane).

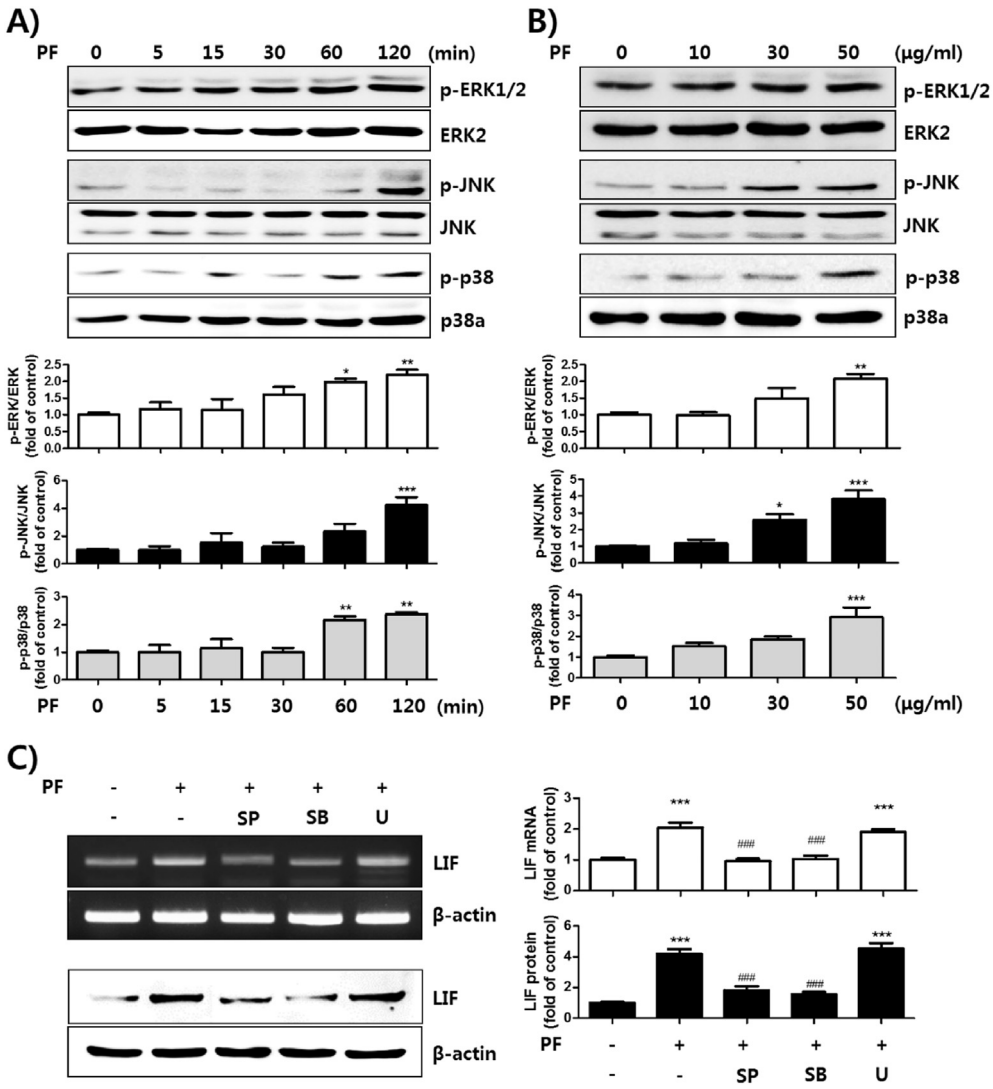

Fig. 3. Analysis of signal pathways responsible for PF-induced LIF expression in Ishikawa cells. **(A, B)** The Ishikawa cells were treated with PF (50 μg/mL) for indicated times (A) or with the indicated concentration of PF for 2 h (B). The phosphorylation of signal molecules was examined by western blot analysis. Band intensity was calculated by fold of control and shown as mean ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ in comparison with control group (1st lane) **(C)** Ishikawa cells were pretreated with 5 μM of specific inhibitors for JNK (SP600125), p38 (SB203580) or ERK (U0126). After 1 h, the cells were treated with PF (50 μg/mL) for 24 h. The protein and mRNA expression levels of LIF were estimated by Western blot analysis and RT-PCR, respectively. The expression of β-actin was used as the internal control. The intensities of bands were calculated by fold of control and shown as mean ± SD of three independent experiments. $^{***}p < 0.001$ in comparison with control group (1st lane) and $###p < 0.001$ in comparison with PF-treated group (2nd lane).

We next examined whether the expressions of integrin β3 and β5 plays a crucial role in PF-enhanced endometrial receptivity toward trophoblast. The treatment of neutralization antibodies against these integrins blocked PF-stimulated attachment of JAr cells onto Ishikawa cells (Fig. 5C). Thus, we concluded that PF-induced expression of integrin β3 and β5 plays a key role in the stimulation of endometrial receptivity.

4. Discussion

Traditional medicine has been used to treat female infertility, and progressively increased efforts have been made to provide evidence that supports its use (7,8,21). Diverse cytokines and growth factors, such as interleukin-1, epidermal growth factor (EGF), calcitonin, amphiregulin, heparin binding-EGF, colony stimulating factor-1, trophinin/tastin, and LIF, are involved in embryo implantation (22). Among them, LIF in particular is regarded as a major factor that regulates endometrial receptivity, because genetic abolishment or functional suppression of LIF impairs embryo implantation (23,24). Thus, we focused on the expression of LIF as part of our ongoing efforts to identify novel agents that enhance embryo implantation and improve pregnancy rates. Here, we demonstrated that PF increases the expression of LIF and improves endometrial receptivity toward trophoblastic cells *in vitro*. As there is no standard drug in use that enhances endometrial receptivity, we were not able to compare our results to reference drug. However, from this point of view, we believe that treatment with PF is a promising strategy for overcoming infertility due to implantation failure.

A well-established *in vitro* model has been used to investigate endometrial receptivity of several human cell lines with, diverse characteristics (25). The Ishikawa cell line, a well-differentiated human endometrial adenocarcinoma, was established by Dr. Nishida in 1980, and it has since been used to

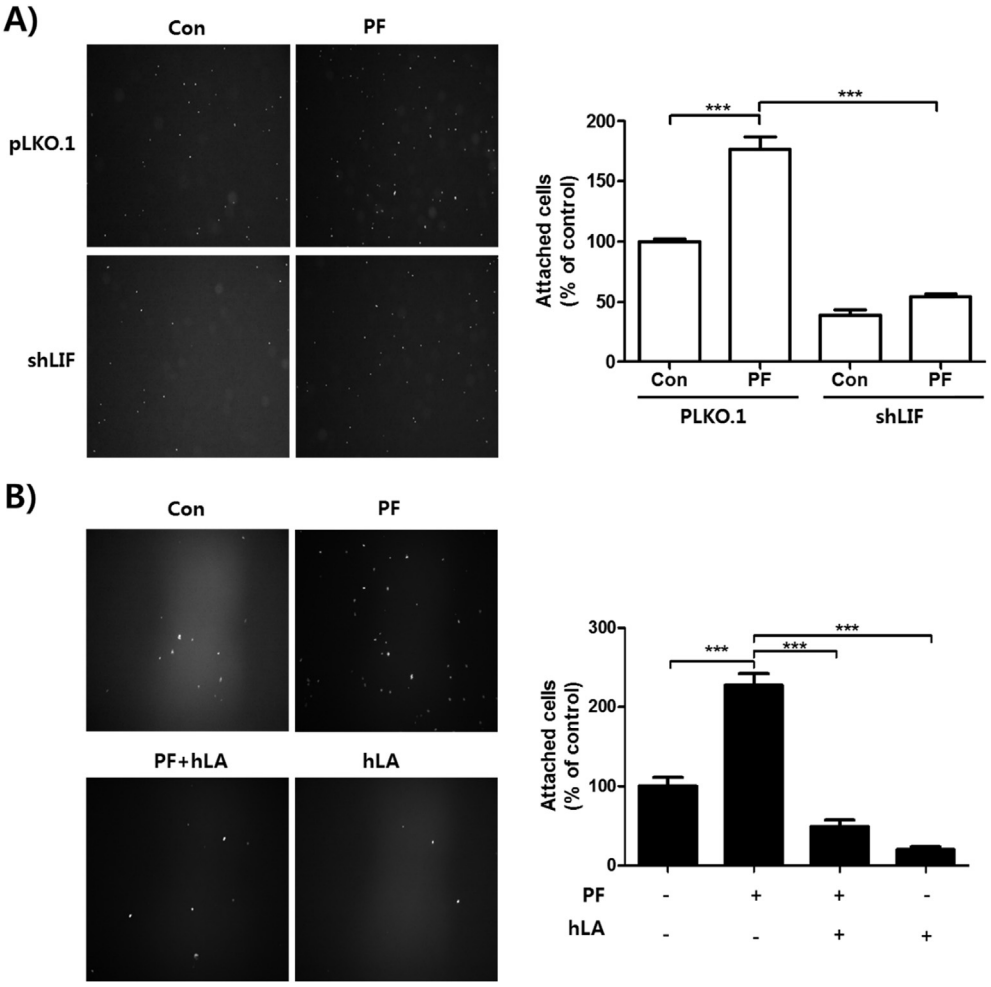

Fig. 4. The expression and function of LIF is crucial for PF-induced adhesion between Ishikawa and JAr cells. **(A)** Ishikawa cells were transfected with pLKO.1 or shLIF, and treated with PF for 48 h. Adhesion assay were performed using fluorescence labeled-JAr cells, Images were acquired, and attached JAr cells were counted. Data were calculated as the percentage of control and are represented as the mean ± SD. ***$p < 0.001$ in comparison between two groups. **(B)** Ishikawa cells were pre-treated with antagonist for LIF-R for 1 h, and then treated with PF (50 μg/mL). Adhesion assay were performed using fluorescence labeled-JAr cells. Attached JAr cells were counted and calculated as percentage of control. Data from three independent experiments were shown as mean ± SD. ***$p < 0.001$ in comparison between two groups.

evaluate both glandular and luminal functions of the endometrium (26). JAr cells, a choriocarcinoma cell line, also have been used as trophoblastic cells to examine embryo implantation (27). Thus, in this study, we chose to use these cells for investigating the expression of LIF and for evaluating endometrial receptivity.

The results clearly showed that PF increases in a dose dependent manner, the expression of not only LIF but also LIFR (Fig. 2). Few studies have examined the regulation of LIF expression. The induction of LIF by IL-1β is mediated by the MEK/ERK and nuclear factor kappa B (NF-κB) pathway in normal articular human chondrocytes (28). In addition, it is known that the p38 MAPK signaling pathway is activated upstream of LIF-dependent neuroprotection (18). Therefore, we determined which of these pathways were involved in PF-induced expression of LIF. The NF-κB pathway was not activated by PF treatment (data not shown). Although all of the MAPK pathways, including ERK, JNK, and p38, were activated by PF, only the JNK and p38 pathways were involved in the PF-stimulated expression of LIF (Fig. 3). Furthermore, the PF-induced expression of LIFR was regulated by JNK, p38, and ERK pathways (Fig. S1). These data suggested that PF regulated the expression of LIF and LIFR expressions via different signaling pathways. Knocking down *LIF* expression or blocking LIFR function decreased the adhesion between trophoblastic JAr cells and endometrial Ishikawa cells (Fig. 4). These results clearly demonstrate that PF-induced endometrial receptivity was directly mediated by increased expression of LIF and by the function of LIFR.

For successful embryo implantation, the interaction between LIF and LIFR must activate the signal transducer and activator of transcription 3 (STAT3), which consequently enhances the expression of adhesion molecules, such as integrin αVβ3 and αVβ5 (29). In addition, integrin αVβ3 and αVβ5 have the motif for cell–cell adhesion that act as receptors for the arginine-glycine-aspartic acid (RGD) domain which is essential in embryo implantation (29,30). Our results also revealed that PF treatment increased the expression of integrin β3 and integrin β5, but the expression of integrin αV was not changed. Treatment with hLA revealed that the expression of integrin β3 and β5 was mediated by LIF expression. Furthermore, inhibition of integrin β3 and β5 using neutralizing antibodies successfully blocked the PF-induced adhesion between JAr and Ishikawa cells (Fig. 5). We previously demonstrated that the expression of integrin β3 and β5 are crucial for the LIF-mediated adhesion of trophoblast and endometrial cells (29). These findings suggest that PF enhances endometrial receptivity through the expression of integrin β3 and β5 in an LIF-dependent manner (Fig. 6).

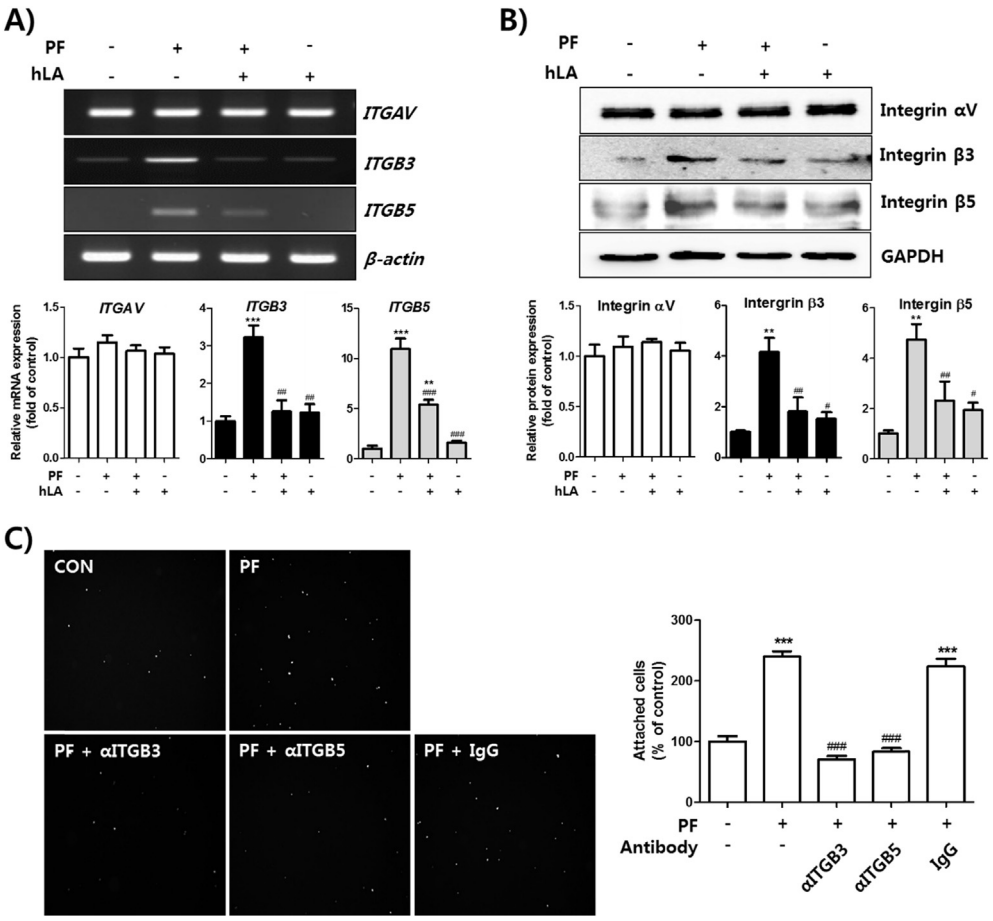

Fig. 5. Antagonist for LIF-R inhibits LIF-induced expression of adhesion molecules in Ishikawa cells. **(A, B)** Ishikawa cells were treated with hLA (50 ng/mL) for 1 h, and then treated with PF (50 μg/mL) for 24 h. The mRNA (A) and protein expressions (B) of adhesion molecules were analyzed by western blotting and RT-PCR, respectively. Band intensity was calculated by fold of control and shown as mean ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ in comparison with control group (1st lane). $\#\#p < 0.01$ and $\#\#\#p < 0.001$ in comparison with PF-treated group (2nd lane). **(C)** Ishikawa cells were pre-treated with neutralizing antibodies against integrin β3 or β5 for 1 h, and then treated with PF (50 μg/mL). Isotype IgG was used for the control. Fluorescence labeled-JAr cells were applied to the adhesion assay. Attached JAr cells were counted, calculated as percentage of control, and shown as mean ± SD. $^{***}p < 0.001$ in comparison with control group (1st lane) and $\#\#\#p < 0.001$ in comparison with PF-treated group (2nd lane).

Major causes of female infertility, such as the problems with ovulation, fertilization, fertilized egg development, and embryo transport, were improved by the development of ARTs (31). However, the imperfection of implantation was not solved by ARTs or other medications, and it remains an unmet need clinically. Thus, several studies using therapeutic manipulation of traditional medicine, such as acupuncture and herbal remedies, were performed to improve implantation rates (7,8). Based on results from these studies and ours, herbal medicines can be a good alternative option for increasing endometrial receptivity. Additional extensive studies are necessary to identify the active compounds of PF, from the known ingredients of PF, such as protocatechuic acid, ferulic acid, rosmarinic acid, and coumaric acid, or unknown compounds. In addition, it is also needed to establish the *in vivo* efficacy of PF.

In conclusion, PF induced the expression of LIF and LIFR through JNK and p38 signaling pathways in endometrium Ishikawa cells. In addition, PF-induced LIF expression increased the attachment of trophoblastic JAr cells to endometrial Ishikawa cells. This adhesion was mediated by the expression of integrin β5 and β3. Taken together, our results suggest that PF is a promising candidate for the development of novel and effective medicines to help female patients suffering from a defect in implantation.

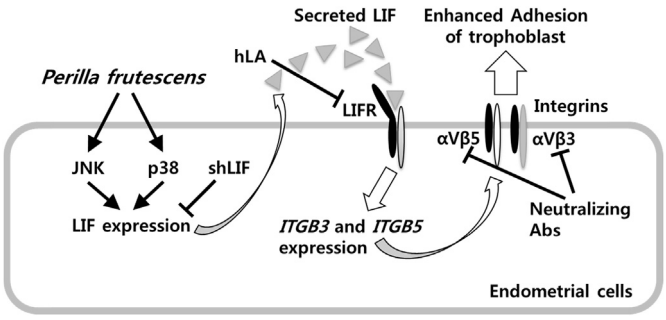

Fig. 6. Schematic representation of the molecular mechanism underlying PF-stimulated endometrial receptivity. PF enhances LIF expression through activation of JNK and p38 pathways. Increased LIF expression stimulates endometrial receptivity toward trophoblast via increasing the expression of *ITGB3* and *ITGB5*.

Conflict of interests

The authors have declared no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jphs.2016.07.004.

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