

# Glycoprotein extraction from *Laminaria japonica* promotes IEC-6 cell proliferation

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**Abstract.** The brown alga *Laminaria japonica* is frequently consumed in Korea, Japan and China, and has been used for more than a thousand years as a drug in traditional Chinese medicine. In this study, we isolated a novel glycoprotein from *L. japonica* that stimulates the growth of the IEC-6 normal murine intestinal epithelial cells. We also identified the mechanism by which this glycoprotein, referred to as LJGP, stimulates cell growth. After 24 h of exposure to LJGP, cell proliferation increased in a dose-dependent manner. To further explore the mechanism associated with LJGP-induced cell proliferation, we treated cells for various times with LJGP. We focused on the epidermal growth factor receptor (EGFR) signaling pathway, which is involved in the regulation of cellular proliferation and differentiation, during LJGP-induced cell growth. The results showed that LJGP induced EGFR and Akt activation. Furthermore, LJGP stimulated Shc/Grb2 binding and ERK activation, but inhibited JNK phosphorylation. These results indicate that LJGP stimulates gastrointestinal cell growth by activating the EGFR signaling pathway.

## Introduction

Algae, including *Laminaria*, *Chlorophyta*, and *Rhodophyta*, have a long history of use in the diets of Pacific and Asian cultures compared to those of Europe, Canada, and the US (1). The biological activities of aqueous extracts from marine algae have been investigated, and some extracts possess potential medicinal agents. One of these algae, the brown alga *Laminaria japonica*, is commonly consumed in Korea, Japan, and China, and has been used for more than 1,000 years as a drug in traditional Chinese medicine. Fucoidan extracted from *L. japonica* has diverse biological properties, including anti-coagulative and antioxidative activities (2-4), and enhances

lipid metabolism. In Huh7 hepatoma cells, fucoidan inhibits cell proliferation through the downregulation of CXCL12 (5). Low-molecular-weight fucoidan extracted from *L. japonica* may have antioxidant activity (4). However, the protein moiety of *L. japonica* has not been investigated thoroughly. Because *L. japonica* contains 60.9% carbohydrate and 10.3% protein (6), we assumed that its medicinal effects are mainly due to the carbohydrate or protein content. Therefore, we extracted functional components, especially glycoprotein, from *L. japonica* and examined its functions.

Cell proliferation is dependent on intracellular signal transduction mediated by enzyme-linked receptors, such as tyrosine kinase receptors. When receptors are activated by phosphorylation, proteins with an src homology 2 (SH2) domain bind to phosphorylated receptors. Growth factor receptor-bound protein2 (Grb2) and Shc also contain SH2 domains. Therefore, these proteins combine tyrosine kinase receptors with Ras, which translocates signals from extracellular to intracellular spaces when mitogen-activated protein kinase (MAPK) is activated. Various extracellular stimuli, including growth factors, hormones, osmolarity shock, stress, and elevated temperature, trigger phosphorylation cascades that utilize MAPK (7-14). MAPK pathways, major signaling cascades downstream of the activated epidermal growth factor receptor (EGFR), are involved in the regulation of cellular proliferation and differentiation. Once activated, MAPK translocates signals to the nucleus, where it presumably regulates the expression of different transcription factors (15).

Regarding intracellular signal transduction mediated by tyrosine kinase receptors phosphatidylinositol 3-kinase (PI3k) also promotes cell growth by phosphorylating Akt, also known as protein kinase B (PKB). The PI3k-Akt pathway is a major intracellular signaling module that regulates multiple cellular processes, including cell proliferation, survival, and cellular responses to insulin and nutrients (16-18). In the PI3k-Akt pathway, growth factors activate PI3k via tyrosine kinase receptors, and activated PI3k phosphorylates phosphatidylinositol 4,5-bisphosphate (PI(4,5)P<sub>2</sub>), converting PI(4,5)P<sub>2</sub> to PI(3,4,5)P<sub>3</sub>, a lipid second messenger (19). Akt binds to PI(3,4,5)P<sub>3</sub> and changes conformation following phosphorylation by 3-phosphoinositide-dependent protein kinase-1 (PDK1). Activated Akt phosphorylates numerous substrates that are involved in various cellular functions (20,21).

Although chemotherapeutic agents, either alone or in combination, are used to treat a variety of diseases or cancers,

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**Key words:** seaweeds, glycoprotein, *Laminaria japonica*, Ras/Raf/MAPK pathway, PI3k/Akt pathway, epidermal growth factor receptor

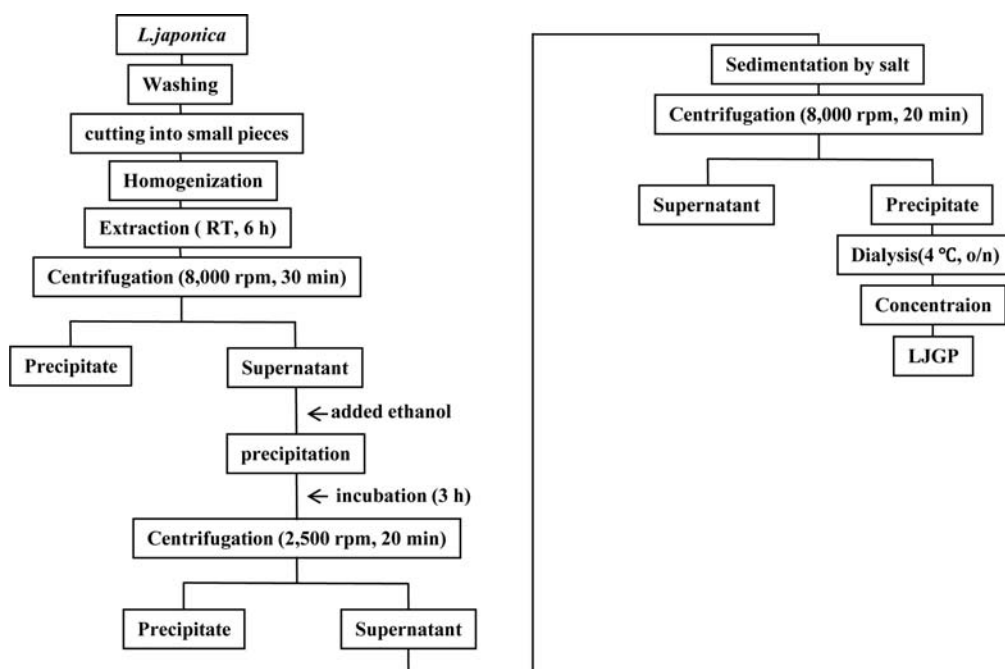


Figure 1. Schematic diagram illustrating the method described in this study to obtain glycoproteins from *L. japonica*.

these treatments often damage normal tissues. Therefore, strategies for preventing normal cell death induced by therapeutic drugs would be very useful in clinical practice. In this study, we used the normal murine small intestinal epithelial cell line IEC-6 to show that glycoprotein from *L. japonica* (LJGP) has promotive effects on the proliferation of these cells. Furthermore, we elucidated the intracellular mechanism involved in LJGP-induced proliferation of IEC-6 cells. We focused on the EGF signaling pathway, which is involved in the regulation of cellular proliferation and differentiation, during LJGP-induced cell growth.

## Materials and methods

**Glycoprotein preparation from *Laminaria japonica* (LJGP).** *L. japonica* was harvested from the coast of Gijang, Busan, South Korea, in May 2008. The sample was washed several times with tap water to remove salt and visible epiphytes and stored at -20°C until use. The sample (160 g) was then cut into small pieces and steeped in 1 l of distilled water for 6 h at room temperature. The aqueous extract was clarified by centrifugation and subsequent filtration through Advantec No. 3 filter paper (Advanced MFS, Inc., Dublin, CA, USA) to remove insoluble materials. The filtrate was then mixed with three volumes of ethanol to precipitate the polysaccharide. The precipitated polysaccharide was removed by filtration, the filtrate was condensed, and ammonium sulfate was added to a final concentration of 80% to precipitate the glycoprotein. The ammonium sulfate-conjugated glycoprotein was then dialyzed against distilled water. Insoluble precipitates were removed by centrifugation and then the supernatant was concentrated using an evaporator. The glycoprotein from *L. japonica* (LJGP) was stored until further use (Fig. 1).

**Cell culture.** Rat small intestine epithelial cells (IEC-6, ATCC CRL-1592) were obtained from the American Type

Culture Collection (Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (HyClone, Inc., South Logan, UT, USA) and antibiotics. The cultures were maintained in a humidified incubator at 37°C under an atmosphere containing 5% CO<sub>2</sub>, and the medium was replaced every other day.

**Cell proliferation assay.** The effects of various LJGP concentrations on cell proliferation were determined colorimetrically after 24 h using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium (MTS) assay with Cell Titer 96 Aqueous One Solution Reagent (Promega, Madison, WI, USA). Cells were seeded onto 96-well plates at 2x10<sup>4</sup> cells per well. After 24 h of incubation, cells were maintained in serum-free medium (SFM) for 12 h. The medium was replaced with fresh SFM medium containing LJGP, and the cells were incubated for an additional 24 h. Cells were exposed to MTS assay solution at 37°C for 30 min, and the optical density at 490 nm was measured using a microplate reader. The OD<sub>490</sub> values of control cells were designated as 100%.

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE).** LJGP was analyzed by SDS-PAGE on 15% gels according to the method of Laemmli (22). After electrophoresis, gels were subjected to Coomassie brilliant blue (CBB) and silver staining for protein bands, and periodic acid-Schiff (PAS) staining for glycoprotein bands (GelCode glycoprotein staining kit, Pierce Co., Ltd., Rockford, IL).

**Immunoprecipitation analysis of EGFR-related signaling proteins.** Cells were plated onto 100-mm dishes at a density of 2x10<sup>5</sup> cells ml<sup>-1</sup> and cultured to 80% confluence at 37°C. Cells were then incubated for 24 h in SFM or SFM containing 20 µg ml<sup>-1</sup> LJGP. After stimulation with EGF (100 ng ml<sup>-1</sup>),

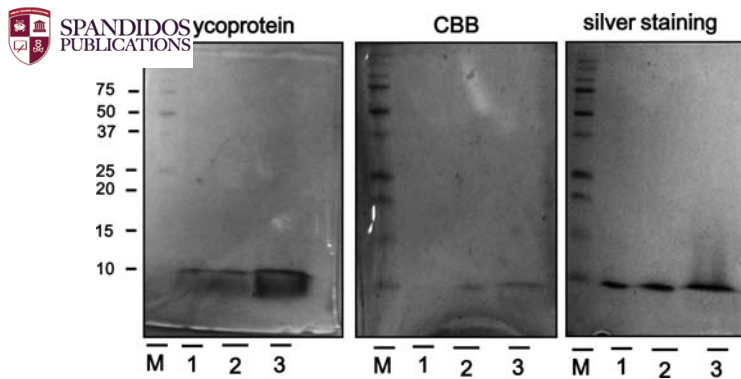


Figure 2. The electrophoresis profiles of LJGP. Each extract (100  $\mu$ g) was applied to a 15% polyacrylamide gel and stained with periodic acid-Schiff, Coomassie blue and silver. 1, water extract; 2, ethanol extract; 3, LJGP.

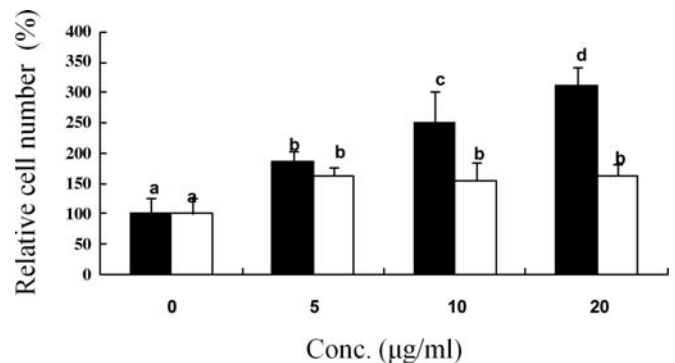


Figure 3. Effect of LJGP treatment on the growth of the IEC-6 small intestine epithelial cells. The cells were treated with the indicated concentrations of LJGP (black bar) and bovine serum albumin (BSA) (white bar), and the relative cell number was determined using the MTS assay. Values are the means  $\pm$ SD; n=6 (p<0.01).

cells were washed in cold phosphate buffer solution, lysed with cold immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 % HP-40) containing protease inhibitors, and centrifuged at 15,000  $\times$  g for 15 min. The resulting supernatant fraction was incubated overnight with primary antibodies (anti-EGFR and anti-Shc) with agitation at 4°C. Protein A-Sepharose beads (Sigma, St. Louis, MO, USA) were then added, and the mixture was incubated for 2 h to capture the immunocomplex. The beads were collected, washed with immunoprecipitation buffer, and boiled to elute the immunocomplex. The eluted proteins were analyzed by SDS-PAGE followed by Western blotting with specific antibodies.

**Western blots.** Proteins (50  $\mu$ g) were separated using 7.5-12.5% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 1% bovine serum albumin (BSA) in TBS-T (10 mM Tris-HCl, 150 mM NaCl, pH 7.5, 0.1% Tween-20) and then incubated overnight with the indicated primary antibodies (diluted 1:1,000) in TBS-T containing 1% BSA with gentle shaking at 4°C. The secondary antibody was a peroxidase-conjugated goat anti-mouse or rabbit antibody (diluted 1:10,000). Signals were detected using an ECL Western blotting kit (Amersham, Piscataway, NJ, USA).

**Statistical analysis.** Multiple mean values were compared for significance using analysis of variance (ANOVA) with SPSS statistical software. Values of p<0.05 were considered significant.

## Results

**Effect of LJGP on IEC-6 cell proliferation.** The glycoprotein extracts from *L. japonica* (Fig. 1) were subjected to SDS-PAGE (Fig. 2). After electrophoresis, the gels were subjected to CBB and silver staining for protein and PAS staining for glycoprotein. We confirmed the presence of a glycoprotein band at ~10 kDa in each lane (Fig. 2) and named it LJGP. LJGP was used in the following assays with the normal murine intestinal epithelial cell line IEC-6. After exposure of the cells to 0-20  $\mu$ g ml<sup>-1</sup> LJGP for 24 h, the cell number increased in a dose-dependent manner (Fig. 3). The cells were

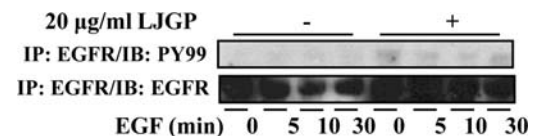


Figure 4. Tyrosine phosphorylation of EGFR in response to LJGP treatment. Cells were treated as mentioned in Materials and methods, protein was extracted with lysis buffer and the intracellular protein expression was analyzed by immunoprecipitation and Western blot analysis. One representative gel from three separate experiments is shown.

also exposed to BSA as a control to confirm the effect of LJGP on IEC-6 cell proliferation. Although BSA treatment slightly stimulated cell growth, the effect was probably due to the protein content of the medium. Therefore, these results demonstrate that LJGP has a consistent acceleration on cell proliferation.

**LJGP stimulates MAPK activation.** Since LJGP induces IEC-6 cell proliferation (Fig. 3), we attempted to determine whether LJGP-induced proliferation was linked to the EGFR signaling pathway, which stimulates cell differentiation and proliferation. IEC-6 cells were treated with LJGP and stimulated with 100 ng ml<sup>-1</sup> EGF for various time periods. LJGP treatment (20  $\mu$ g ml<sup>-1</sup>) for 24 h stimulated EGFR phosphorylation as compared to control cells (Fig. 4). EGFR, a tyrosine kinase receptor, translocates growth signaling from extracellular to nuclear using signal transduction pathways, including the Ras/Raf/MAPK and PI3k/Akt pathways (23). These signaling pathways are known to be important for cell differentiation and proliferation. The Ras/Raf/MAPK pathway is specifically related to cell proliferation and survival.

We also studied the phosphorylation of Shc, an adaptor protein which is known to interact with Grb2 and is involved in signal transduction from the EGFR receptor to the nucleus. We treated IEC-6 cells with LJGP and stimulated them with 100 ng ml<sup>-1</sup> EGF for various time periods. LJGP treatment increased the phosphorylation of Shc in a time-dependent manner (Fig. 5). We also attempted to determine the effect of LJGP on the interaction observed between Shc and Grb2. We detected increased Grb2 binding as compared to control cells

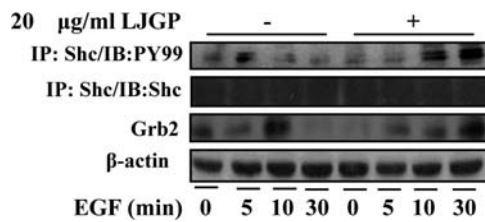


Figure 5. The effect of LJGP on the expression of Shc and Grb2. Cells were treated as indicated in Materials and methods, protein was extracted with lysis buffer and the intracellular protein expression was analyzed by immunoprecipitation and Western blot analysis. One representative gel from three separate experiments is shown.

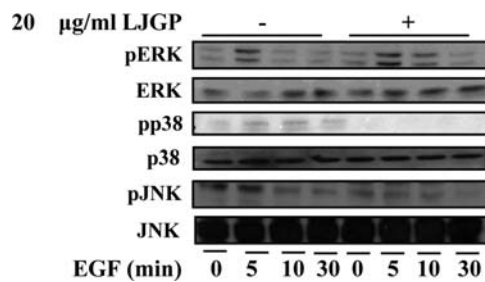


Figure 6. Effect of LJGP on MAPK signaling pathway. Cells were treated as indicated in Materials and methods, protein was extracted with lysis buffer and the intracellular protein expression was analyzed by immunoprecipitation and Western blot analysis. One representative gel from three separate experiments is shown.

(Fig. 5). Interaction between Shc and Grb2 translocates signals to MAPK. Therefore, we examined the phosphorylation levels of MAPKs, including the extracellular signal-regulated kinase (ERK1/2), p38, and c-Jun N-terminal kinase (JNK). Cells were incubated with 20  $\mu\text{g ml}^{-1}$  LJGP for 24 h and stimulated with 100  $\text{ng ml}^{-1}$  EGF at various times. In LJGP-treated cells, ERK1/2 phosphorylation increased 5 min after EGF stimulation (Fig. 6). However, in untreated cells, the phosphorylation decreased significantly in 10 min. Furthermore, the phosphorylation of p38 and JNK decreased in the LJGP-treated cells as compared to untreated cells (Fig. 6).

**LJGP induces phosphorylation in the PI3K-Akt pathway.** The PI3K/Akt pathway is mainly associated with cell growth. We studied the involvement of this pathway in LJGP-induced IEC-6 cell proliferation. PI3k is activated by the growth factor EGF (24), and following activation the recruitment of Akt to the membrane was observed (25,26). We examined whether LJGP stimulates the phosphorylation of p85, a subunit of PI3k. Cells were treated with 20  $\mu\text{g ml}^{-1}$  LJGP for 24 h and stimulated with 100  $\text{ng ml}^{-1}$  EGF for various time periods. LJGP caused increased phosphorylation of p85 (Fig. 7). Moreover, Akt phosphorylation was enhanced in cells treated with LJGP (Fig. 7).

## Discussion

Marine algae, such as seaweeds, are amply present in seawater. Seaweeds contain many useful components and physiologically active substances that remain unidentified (27,28).

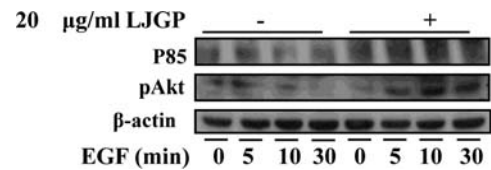


Figure 7. Effect of LJGP on the activation of PI3K and Akt. Cells were treated as indicated in Materials and methods, protein was extracted with lysis buffer and the intracellular protein expression was analyzed by immunoprecipitation and Western blot analysis. One representative gel from three separate experiments is shown.

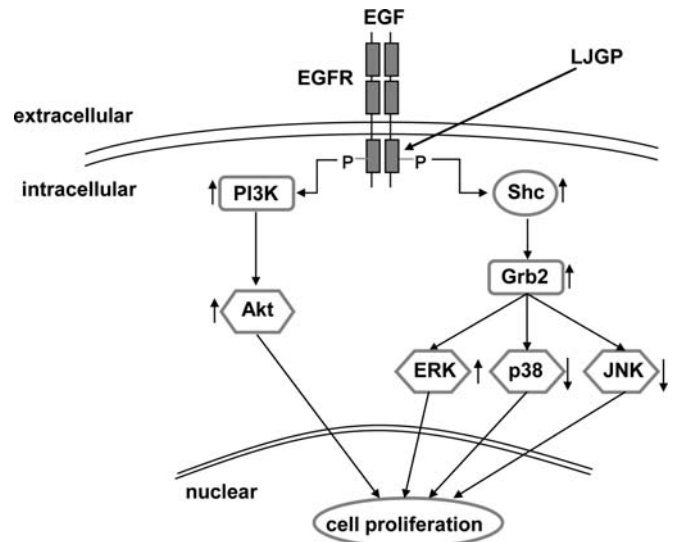


Figure 8. A schematic model of the LJGP-induced signaling pathways. LJGP induced IEC-6 cells proliferation via two signal pathways, the Ras/Raf/MAPK and PI3k/Akt pathways.

Carrageenan and alginic acid, which are isolated from red and brown algae, are known to lower cholesterol levels in the serum and liver (4). We separated an extract from *Laminaria japonica* (Fig. 1) and used SDS-PAGE to confirm that it is a glycoprotein, which we designated LJGP (Fig. 2). To our knowledge, this is the first report on the proliferative activities of this glycoprotein on small murine intestinal epithelial cells (IEC-6). The results from the MTS assay demonstrated that LJGP stimulated cell growth in a dose-dependent manner (Fig. 3).

The stimulation of cell proliferation is dependent on multiple signaling pathways. In this study, we focused on the EGFR signaling pathway. Expression of EGFR was detected in different cell types, including epithelial, mesenchymal, and nerve cells. Its activation leads to cell differentiation and proliferation. Our results showed that LJGP induced EGFR phosphorylation (Fig. 4). Activated EGFR leads to the phosphorylation of specific tyrosine residues within the EGFR cytoplasmic domain that acts as a docking site for effector molecules, triggering downstream signaling pathways (29). One of the downstream signaling pathways is the Ras/Raf/MAPK pathway. The MAPK family in mammalian cells includes extracellular signal-regulated kinase-1 and kinase-2 (ERK-1/2), the c-Jun NH<sub>2</sub> terminal kinase (JNK), and p38 (30). In accordance with the LJGP-induced cell





tion, ERK-1/2 was activated after exposure to LJGP, a potent mediator that regulates cell growth and differentiation (Fig. 6). In contrast, decreased phosphorylation of JNK and p38 was observed (Fig. 6), phenomena that are associated with cell death and oxidative stress. For most cell types, EGFR has been proposed to mediate Ras/Raf/ERK activation and the ERK pathway was implicated in mitogenic signal transduction in response to several stimuli (31-34). Moreover, we confirmed the interaction of the adaptor protein Shc and Grb2 (Fig. 5). The interaction of Shc and Grb2 is an essential step following EGFR activation in MAPK signal translocation.

Our results indicate that EGFR activation also contributes to LJGP-induced proliferation through the activation of the PI3K/Akt pathway. The PI3K/Akt pathway has been identified as a key player in cell survival (35,36). Akt also functions in normal growth as seen in Akt-knockout mice, which show retarded growth (37). PI3k is activated by EGFR through heterodimerization with ErbB3, which contains a docking site for the p85 subunit of PI3k (38). Once the p85 subunit is positioned, the p110 subunit of PI3k generates phosphatidylinositol 3,4,5-triphosphate (PIP3), which activates Akt (38). Consistent with this model, we detected the phosphorylation of Akt (Fig. 7).

In the present study, we extracted a glycoprotein from *L. japonica* that not only has anticancer activity (data not shown), but also enhances cell growth in normal intestinal cells. Furthermore, we studied the intracellular mechanisms involved in cell proliferation through the EGFR signaling pathway. The results showed that LJGP induced Akt/ERK activation and downregulated JNK/p38 (Fig. 8). Although further studies are needed to define its interaction on the cell membrane surface, we suggest that LJGP is a potentially useful agent for understanding intestinal function.

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