이학석사 학위논문

효모 유래 글루칸에 의한 HDAC5 유도 혈관신생성 활성화 연구

Yeast beta-glucan mediates histone deacetylase 5 induced angiogenesis in vascular endothelial cells

울 산 대 학 교 대 학 원

의 과 학 과

최 민

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지 도 교 수　하 창 훈

이 논문을 이학석사학위 논문으로 제출함

2022년 2월

울 산 대 학 교 대 학 원

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최　민

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Abstract

β-glucan is a polysaccharide found in large amounts in of bacteria, fungi, and yeast cell walls. The biological and physiological effects of β-glucan have been reported as anti-tumor, anti-diabetic, lowering cholesterol levels, and immunity enhancement. However, the role of yeast-derived β-glucan on angiogenesis has not been elucidated. There have been few specific studies on the clinical and physiological significance of yeast-derived β-glucan. Therefore, this study showed the correlation between β-glucan and histone deacetylase5 (HDAC5) in human umbilical vein endothelial cells (HUVECs), revealing the role of β-glucan on the activity of angiogenesis. We confirmed that HDAC5 was phosphorylated by β-glucan stimulation and released from the nucleus to the cytoplasm. In this study, we found that β-glucan-stimulated HDAC5 mediates the transcriptional activation of MEF2. As a result, the expression of KLF2, EGR2, and NR4A2 genes that are dependent on MEF2 and involved in angiogenesis increased. Thus, we showed the angiogenesis activity of β-glucan through cell migration, tube formation, and aortic ring analysis as *in vitro* and *ex vivo* angiogenesis assays. Specifically, inhibition of HDAC5 by an HDAC5 inhibitor repressed transcriptional activation of MEF2, *in vitro* and *ex vivo* angiogenesis. However, it was shown that β-glucan treatment restored MEF2 transcriptional activation, which had been inhibited. These findings suggest that HDAC5 is essential for angiogenesis and that β-glucan induces angiogenesis activity. In conclusion, this study demonstrates that β-glucan induces the activity of angiogenesis through HDAC5. It also suggests that β-glucan has potential value as a novel therapeutic agent modulating angiogenesis.

Key Words: β-glucan; HUVEC; HDAC5; MEF2; Angiogenesis

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Introduction

Angiogenesis, the formation of new blood vessels, is essential in the development of embryos and physiological repair damage such as wound healing and post-ischemic tissue restoration (1, 2). Also, this event is essential in pathological processes such as diabetic retinopathy, rheumatoid arthritis, and atherosclerosis (2). Therefore, the regulation of angiogenesis is considered an important part physiologically and pathologically. Angiogenesis occurs by various types of angiogenic growth factors produced in a variety of different cells, which are critical for angiogenesis (1). As these angiogenic growth factors, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and angiopoietin (Ang)-1, are well known representatively (1). In addition to VEGF and FGF, animal model studies have revealed that other factors implicated in angiogenesis include tumor necrosis factor (TNF) and insulin like growth factor-1 (1).

The cell wall of *S.cerevisiae* accounts for 30% of the dry weight of the cell and is composed of β-glucan, mannan, protein, lipid and chitin (3, 4). β-glucan is one of the most common polysaccharide types found in the cell walls of bacteria, yeast, and fungi (5). β-glucan is made up of a backbone of β-(1→3)-glucan with a side chain of β-(1→6)-glucan connected (4). The molecular weight and degree of branching, including the structural complexity of β-glucan, also determine the biological activity (6). β-glucan has the following properties: It significantly lowers total cholesterol in plasma and increases HDL-cholesterol concentration (7). And β-glucan has the effect of inhibiting the growth of melanoma in mice (6). Many studies have published that β-glucan lowers glucose levels and improves diabetes symptoms in diabetes models (8). In addition, β-glucan stimulates immunity by releasing cytokines such as interleukins (ILs) and TNF-α in macrophages and dendritic cells and binding to Dectin-1 or TLRs to secrete IgG immunoglobulin from B cells (4). Like this, β-glucan possesses biological properties that include reducing cholesterol levels, as well as anti-tumor and anti-diabetic

capabilities. On the other hand, research shows that mannoprotein, another yeast component, enhances immunity and induces angiogenesis (4, 9). Also, a study shows barley-derived β-glucan induces angiogenesis in endothelial cells under oxidative conditions (10). However, angiogenesis studies on yeast-derived β-glucan, which have a large proportion of yeast components, have not been conducted yet.

When VEGF binds to VEGF receptor-2 (VEGFR2), various intracellular signaling pathways are mediated, among which phosphatidylinositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling pathways are well known (11). Akt is a serine/threonine protein kinase activated by PI3K, and extracellular signal-regulated kinase (Erk1/2) is activated through the MAPK cascades (12, 13). Therefore, the activation of the Akt and Erk1/2 signaling pathways is entailed in many of the biological functions ascribed to VEGF (12, 14, 15). Recently, it has been reported that yeast-derived β-glucan phosphorylates Akt and Erk1/2 (16).

The acetylation and deacetylation of histones in the nucleosome, composed of octamers of four core histones, are pivotal for gene expression regulation (2, 17). Because histone acetylation and deacetylation cause chromatin relaxation or condensation, modulation is the primary mechanism in gene expression (2, 17, 18). Human HDACs are divided into three classes: class , , and , of which class a HDACs (HDAC 4, 5, 7 and 9) are expressed in about a few cell types (18, 19). HDAC5, which belongs to class a HDAC, has been reported to be most expressed in heart and skeletal muscle (18). Furthermore, HDAC5 has been shown to repress angiogenesis in endothelial cells in previous investigations. For example, silencing the HDAC5 gene induced angiogenesis in mouse and resulted in cell migration and tube formation in endothelial cells (19). Meanwhile, when VEGF or other external sources stimulate endothelial cells, HDAC5 induces angiogenesis (20, 21). Indeed, when endothelial cells are stimulated to VEGF, HDAC5 is phosphorylated via the VEGFR2-PLCγ-PKC-PKD

pathway, resulting in angiogenesis (21). As such, the relationship between VEGF and HDAC5 has been clarified, but the correlation between yeast-derived β-glucan and HDAC5 has not yet been elucidated.

Class a HDACs have diverse biological functions through many interactions with transcriptional regulators (18). Structurally, there is a myocyte enhancer factor 2 (MEF2) binding domain at the N-terminus of class a HDACs (22, 23). MEF2, a DNA binding transcription factor, plays a crucial role in the myogenesis of skeletal and cardiac muscles (17, 22). When class a HDACs interact with MEF2, MEF2 activity is suppressed (23). It has been reported that HDAC5 through the PKD pathway activates MEF2 to induce angiogenesis (21). Thus, the interaction of class a HDACs with MEF2 is significant in angiogenesis (18). Interestingly, previous studies have shown that phosphorylated HDAC5 increases the expression of the Krüppel-like factor 2 (KLF2) gene (21, 24). KLF2 expression is regulated by MEF2 expression because the KLF2 promoter contains a single consensus MEF2 binding site (24). Thus, these studies found the role of HDAC5 as an inducer of MEF2 transcriptional activation and angiogenesis. Despite the relevance of HDAC5 in vascular functions, the mechanisms of β-glucan-induced HDAC5 activation and angiogenesis remain unknown.

In this study, we evaluate the physical and functional interaction of β-glucan with HDAC5 as β-glucan stimulates the phosphorylation of HDAC5 at the Ser259 residue in endothelial cells. Our results show that phosphorylated HDAC5 induces nuclear export and MEF2 transcriptional activation. In addition, the β-glucan-induced HDAC5 pathway mediates cell migration and the formation of tubes and microvessels *in vitro* and *ex vivo*. These findings establish an important role for β-glucan and HDAC5 in the regulation of angiogenesis.

Materials and Methods

Plasmid, antibodies, and Reagents

Plasmids 3X-MEF2-Luc was a gift from Ron Prywes (Addgene plasmid, #329767). Recombinant Human $VEGF_{165}$ was purchased from R&D System (Minneapolis, MN, USA). Antibodies against phospho-Akt Ser^{473} (p-Akt), Akt, Erk1/2 and phospho-HDAC5 Ser^{259} (p-HDAC5) were obtained from Cell Signaling Technologies (Beverly, Massachusetts). Mouse monoclonal antibodies against HDAC5, phospho-Erk1/2 (p-Erk1/2), and β-actin were from Santa Cruz Biotechnology (Santa Cruz, Califonia) (25). LMK235, a specific inhibitor for HDAC4, 5, was from Selleck Chemicals. β-glucan from baker's yeast was purchased from Sigma (10).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh human umbilical veins (Asan Medical Center, Seoul, Korea). HUVECs were grown in medium 200 supplemented with fetal bovine serum, hydrocortisone, human epidermal growth factor, basic fibroblast growth factor, heparin, ascorbic acid, 1% and antibiotic-antimycoticum (Gibco Life Technologies, Camarillo, CA, USA), as described previously (9). Bovine aortic endothelial cells (BAECs) were purchased from Clonetics and were grown in Dulbecco's Modification of Eagle's Medium (DMEM) with 10% fetal bovine serum and 1% antibiotic-antimycotium. Cells were maintained at 37 in a humidified atmosphere of 5% CO_2 (10). All assays were conducted using cell passage cells (6-8 passages) (10).

Western blotting

For Western blot analysis, HUVECs were lysed with lysis buffer containing protease inhibitors (GenDEPOT, USA) on ice. After centrifugation for 20 minutes at 13,000rpm (4). The protein

content of the samples was determined according to the Bradford Protein Assay Reagent (Bio-Rad). Equal amounts of protein were separated by 6% and 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes and polyvinylidene fluoride (PVDF). The membrane was blocked with 5% BSA in Tris-buffered saline and Tween-20 (TBS-T) for 1 hour 30 minutes at RT. And incubated with anti-phopho-HDAC5 (1:1000; Cell Signaling Technology, USA), HDAC5 (1:1000, Santacruz, USA), phospho-Akt (1:1000; Cell Signaling Technology), Akt (1:1000; Cell Signaling Technology), phospho-Erk1/2 (1:1000; Cell Signaling Technology), Erk1/2 (1:1000; Santacruz), and β-actin (1:1000, Santacruz) for overnight at 4 . After being washed four times with TBS-T, the samples were incubated with an HRP-conjugated secondary antibody (Jackson ImmunoResearch Inc, USA) (9, 19). Western blot analysis was performed according to standard procedures using the ECL detection kit (Youngin frontier, Korea), and visualized using ChemiDoc imaging system (Bio-Rad, USA) (26).

Immunofluorescence

HUVECs were plated at 3-well chamber removable slides (ibidi GmbH Am Klopferspitz, Germany). After that VEGF (20ng/ml) was treated for 6 hours and β-glucan (1μg/ml) was treated from 30 minutes to 24 hours, respectively. Cells were fixed with 4% paraformaldehyde in 1X PBS for 5 minutes, and permeabilized in 0.1% Triton X-100. To monitor the subcellular localization of HDAC5, cells were incubated in anti-HDAC5 antibody at 1:500 dilution in PBS containing 0.25% Tween-20, 1% normal donkey serum, and 1% bovine serum albumin overnight at 4 . The next day cells were washed and followed by incubation with Alexa Fluor^{TM} 488 goat anti-mouse IgG (H+L) (Invitrogen) at 1:300 dilution for 2 hours at room temperature. Afterwards the slides mounted with a cover slip using Duolink® In Situ Mounting Medium with DAPI (Sigma) (27). Fluorescence images were captured using a fluorescent microscope EVOS M5000 (Thermo Fisher scientific).

Luciferase assay

To determine the MEF2 promoter activity in response to β-glucan, BAECs were seeded in 12-well plates overnight and transiently co-transfected using lipofectamine 3000 (Invitrogen) with 3xMEF2-luciferase plasmids and SV40 vectors for 48 hours. Transfected cells were incubated 16 hours followed by exposure to VEGF (20ng/ml), β-glucan (1μg/ml), and LMK235 (1μM). Firefly and Renilla luciferase activities in cell extracts were measured using Dual-Luciferase Reporter Assay System (Promega). The relative luciferase activity was then calculated by normalizing MEF2 promoter-driven Firefly luciferase activity to control Renilla luciferase activity. Data from all experiments are presented as the relative luciferase activity (mean ± SD) from at least three independent sets of experiments, each with triplicate measurements (25).

RNA isolation and RT-PCR

Total RNA was isolated from cultured HUVECs using a Trizol reagent (Invitrogen) and purified with use of a NanoDrop 2000 Spectrophotometer system (Thermo Fisher Scientific, Waltham, MA, USA) (23). First-strand cDNA was synthesized with the PrimeScript^{TM} 1st strand cDNA synthesis kit (Takara, Japan). PCR was performed 30 cycles with primer sets as follows (9). The forward and reverse primers for human KLF2 are 5′-GCACGCACACAGGTGAGAAG -3′ and 5′- ACCCAGTCACAGTTTGGGAGGG -3′; for human EGR2 are 5′- CCTTCACTTACATGGGCAAG -3′ and 5′-ACGGATTGTAGAGAGTGGAG -3′; for human NR4A2 (Nurr1) are 5′-TCTAGTTGCCAGATGCGC -3′ and 5′- GTGAGGGCACCTGCGTGTCG -3′. Human GAPDH served as an internal control (2).

Scratched wound healing assay

Migration of HUVECs was detected using a "scratched wound assay" (19). HUVECs were seeded on 6-well plates coated with 0.2% gelatin and grown to confluence. HUVECs were scratched with a 200ml pipette tip across the plates. After the plates were washed with PBS, HUVECs were treated with VEGF (20ng/ml), β-glucan (1μg/ml), and LMK235 (1μM), respectively. They were treated for 10 hours, but when LMK235 and β-glucan were treated together, LMK235 was pretreated for 30 minutes. HUVECs were grown for 24hours and photographed at 0 hour and 10 hours in a light microscope (9, 21).

Tube formation assay

Matrigel Basement Membrane Matrix (Corning, USA) was pipetted into cold 24-well culture plates (120μl/well) and polymerized for 30 minutes at 37 . HUVECs were treated with VEGF (20ng/ml), β-glucan (1μg/ml), and LMK235 (1μM), respectively, were seeded onto the surface of the Matrigel. At this time, the group treated with LMK235 and β-glucan was pre-treated with LMK235 for 30 minutes. HUVECs were stained with 1Mm Cell Tracker™ Green-CMFDA (Invitrogen). After 6 hours of incubation, tube formation was observed and digitized with a fluorescent microscope EVOS M5000 (Thermo Fisher Scientific). The tubular networks were quantified using ImageJ.

Rat aortic ring assay for *ex vivo* angiogenesis

Thoracic aortas were removed from 10- to 12-week-old male Spargue-Dawley rats immediately transferred to a cold PBS. The periaortic fibroadipose tissue was carefully removed with fine micro dissecting forceps and Castroviejo iridectomy scissors paying special attention not to damage the aorta walls. One-millimeter-long aortic rings were sectioned. Ring-shaped explants of rat aorta were then placed into 24-well plate was coated with 120μl Matrigel and polymerized in an incubator. VEGF (20ng/ml), β-glucan (1μg/ml), and LMK235 (1μM)

in 400μl of serum-free medium 200 was added into each well (28).The aortic ring were incubated in an incubator at 37 , 5% CO_2 for 10 days for optimal microvessel sprouting (2). For analysis, the aortic ring was stained with 1mM calcein AM (Invitrogen), and the images of aortic rings were taken using a fluorescent microscope EVOS M500 (Thermo Fisher Scientific).

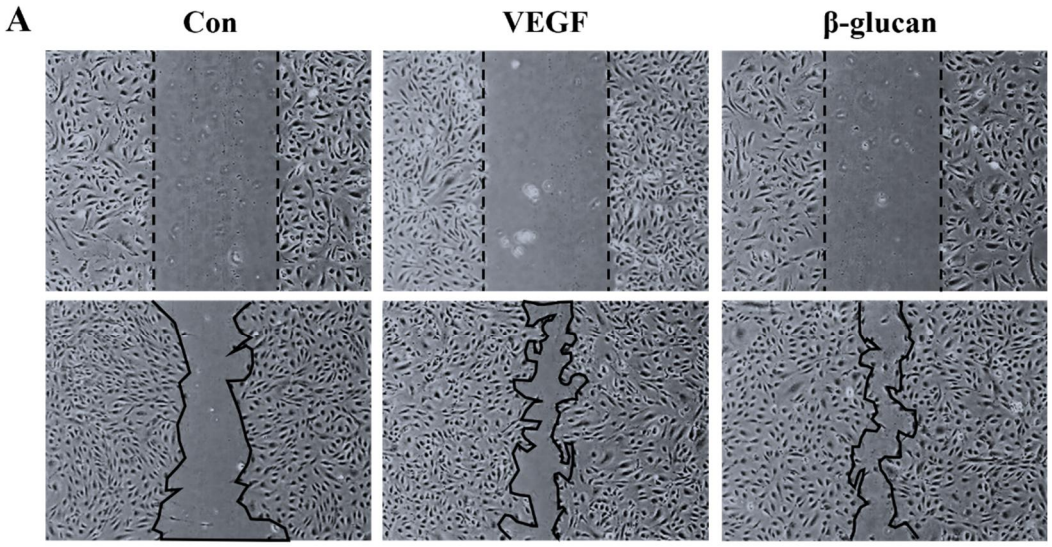
Statistical analysis

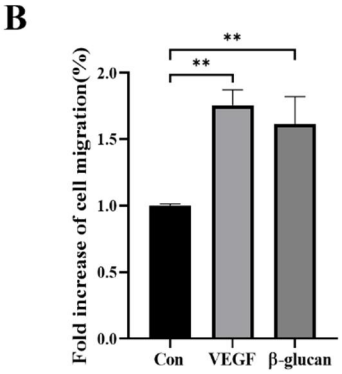
Data are presented as means ± SDs from triplicate experiments, using the GraphPad Prism ver. 8. A paired t-test between two groups was used to determine the significance of the findings. Statistical significance was set at the * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ level.

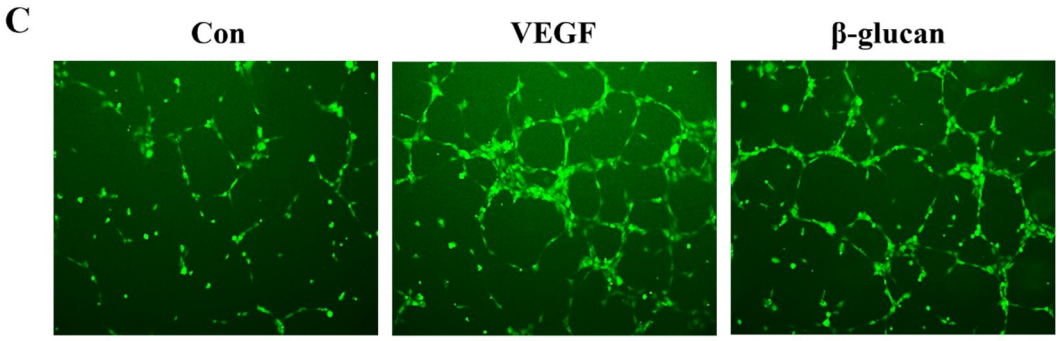
Result

1. Promotion of angiogenic function by β-glucan in HUVECs.

To determine whether β-glucan induces angiogenesis, we studied an angiogenic functional assay on HUVECs. HUVECs were treated with VEGF or β-glucan, respectively. And we confirmed the angiogenesis process in the following way. First, in the scratched wound assay, VEGF (20ng/ml) and β-glucan (1μg/ml) were treated and incubated for 10 hours (Figure 1. A). In this experiment, the migration of cells in response to VEGF or β-glucan was observed. When β-glucan was treated, cell migration showed similar results to VEGF. We quantified HUVECs migration by measuring the width of the cell-free region immediately after scratch and after 10 hours (Figure 1. B). Next, to observe tube formation of HUVECs by β-glucan were performed a tube formation assay. In this experiment, VEGF (20ng/ml) and β-glucan (1μg/ml) were treated in HUEVCs. After incubation for 6 hours, the number of bridges in the tube formed by a fluorescent microscope using a cell tracker was confirmed (Figure 1. C). As a result, HUVECs formed tubes in response to β-glucan, and the number of bridges in the formed tubes was similar to that of VEGF treatment. Quantitative analysis was performed by measuring the number of bridges in the tube-like structures (Figure 1. D). So, based on the results of these two experiments, we concluded that β-glucan induces angiogenesis.


A
Con
VEGF
β-glucan


B
**
**
Fold increase of cell migration(%)
2.0
1.5
1.0
0.5
0.0
Con
VEGF
β-glucan


C
Con
VEGF
β-glucan

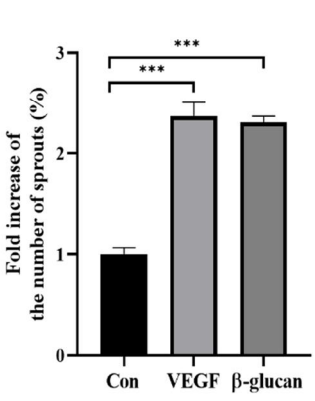

D
***
***
Fold increase of the number of sprouts (%)
3
2
1
0
Con
VEGF
β-glucan

Figure 1. β-glucan promotes angiogenic function in HUVECs.

(A) HUVECs were exposed to VEGF (20ng/ml) and β-glucan (1μg/ml). After 10 hours of observation, HUVECs showed that β-glucan promotes cell migration. (B) Quantitative analysis was performed by measuring the width of the cell-free region promptly after scratch and after 10 hours, respectively. (C) HUVECs showed that β-glucan formed tubes after 6 hours. (D) Quantitative analysis was done by counting the number of bridges extending from the tube after 6 hours. The representative images and quantitative statistics are provided (n=3). * $P <$ 0.05, ** $P < 0.01$, and *** $P <0.001$.

2. Regulation of phosphorylation of Akt by β-glucan.

There are several other pro-angiogenic growth factors, such as FGF, VEGF, and platelet-derived growth factor (PDGF), and we selected VEGF among them (29). Among the many biological activities attributed to VEGF is the activity of PI3K-Akt (14). Although there are studies on angiogenesis by VEGF-dependent PI3K/Akt signaling, the results of studies on angiogenesis by β-glucan are still unknown (30). So, we performed western blot to confirm activation of Akt by β-glucan.

First, we treated HUVECs with β-glucan at a concentration of 1μg/ml for 2, 5, 15, and 30 minutes, respectively. Then, Western blot was performed using an antibody in which Ser473 of Akt was phosphorylated (Figure 2. A). Akt started to be phosphorylated when β-glucan was treated for 2 minutes. After that, it showed the maximum expression level at 5 minutes and began to decrease from 15 minutes (Figure 2. B). Thus, after confirming phosphorylation of Akt by β-glucan in a time-dependent manner, we confirmed the concentration-dependent phosphorylation of Akt. Next, we treated HUVECs with β-glucan at concentration of 0.1, 1, 2, and 5μg/ml for 5 minutes, respectively (Figure 2. C). As a results of Western blot, the expression level of phosphorylation of Akt increased from the treatment of 0.1μg/ml and reached the maximum at 1μg/ml (Figure 2. D).

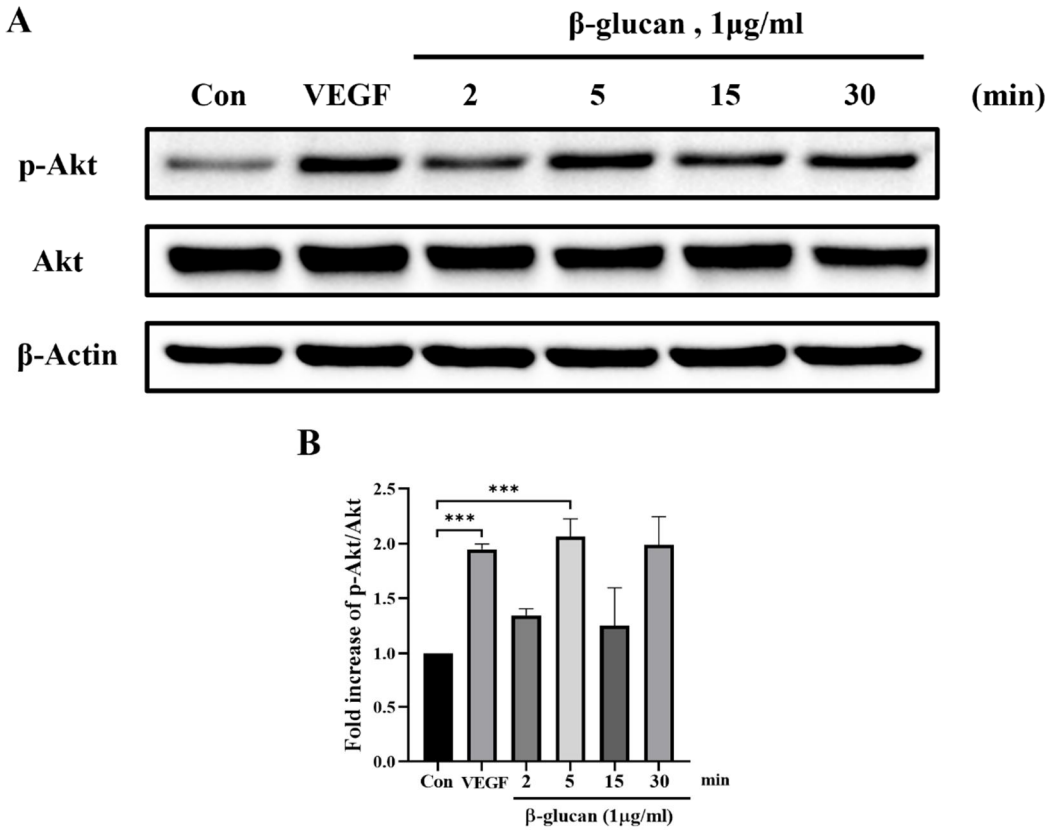

A
β-glucan , 1μg/ml
Con
VEGF
2
5
15
30
(min)
p-Akt
Akt
β-Actin
B
Fold increase of p-Akt/Akt
***
***
2.5
2.0
1.5
1.0
0.5
0.0
Con VEGF 2 5 15 30 min
β-glucan (1μg/ml)
C
β-glucan
Con
VEGF
0.1
1
2
5
(μg/ml)
p-Akt
Akt
β-Actin
D
Fold increase of p-Akt/Akt
*
*
4
3
2
1
0
Con VEGF 0.1 1 2 5 μg/ml
β-glucan

Figure.2. β-glucan regulates phosphorylation of Akt.

(A) HUVECs were exposed to VEGF (20ng/ml) and β-glucan (1μg/ml) for various times. (B) Quantitative data of Akt phosphorylation normalized with the level of Akt. (C) HUVECs were exposed to β-glucan for 5 minutes with the indicated concentrations. (D) Measurement of the level of p-Akt expression normalized with the level of Akt. Each experiment was conducted in triplicate. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

3. Modulation of phosphorylation of Erk1/2 by β-glucan.

VEGF induces angiogenesis through the MAPK signaling pathway in addition to the PI3K/Akt signaling pathway. The classical MAPK cascades include Erk1/2, c-Jun NH2-terminal kinase (JNK), and stress activated protein kinase-2 (p38), which are well known elements (13). Through its receptor VEGFR2, VEGF activates the MAPK signaling pathway (15). There are studies on angiogenesis by VEGF-dependent Erk1/2 signaling but the results of studies on angiogenesis by β-glucan are not yet known. So, we confirmed the activation of Erk1/2 by β-glucan by Western blot.

HUVECs were treated with 1μg/ml of β-glucan for 2, 5, 15, and 30 minutes, respectively. After that, the expression of phosphorylated Erk1/2 was confirmed using an antibody in which Erk1/2 was phosphorylated (Figure 3. A). Erk1/2 started to be phosphorylated when β-glucan was treated for 2 minutes. Subsequently, the maximum expression level was at 5 minutes, and the expression level started to decrease gradually from 15 minutes (Figure 3. B). The expression level of phosphorylated Erk1/2 by β-glucan was examined in a time-dependent manner, and then dose-dependent phosphorylation of Erk1/2 was confirmed. We treated HUVECs with β-glucan at concentration of 0.1, 1, 2, and 5μg/ml for 5 minutes, respectively (Figure 3. C). As a results of Western blot, the expression level of phosphorylation of Erk1/2 increased from the treatment of 0.1μg/ml and reached the maximum at 1μg/ml (Figure 3. D).

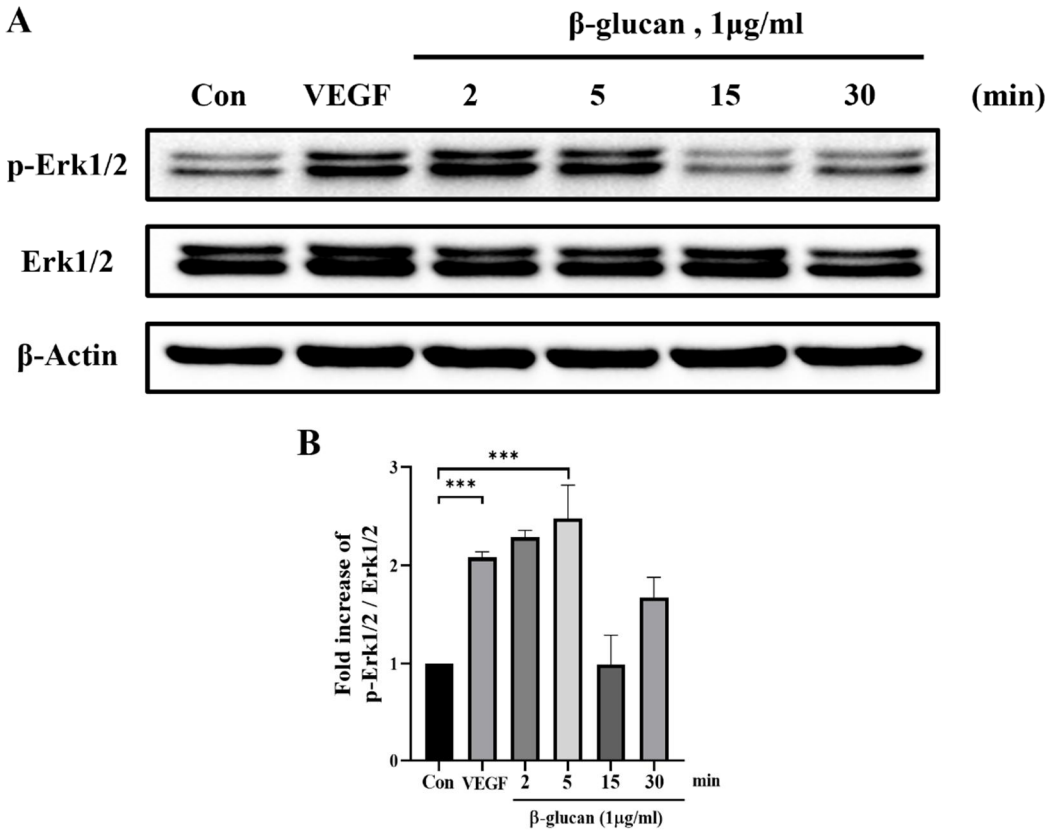

A
β-glucan , 1μg/ml
Con
VEGF
2
5
15
30
(min)
p-Erk1/2
Erk1/2
β-Actin
B
***
***
Fold increase of p-Erk1/2 / Erk1/2
Con VEGF 2 5 15 30 min
β-glucan (1μg/ml)
C
β-glucan
Con
VEGF
0.1
1
2
5
(μg/ml)
p-Erk1/2
Erk1/2
β-Actin
D
*
**
*
Fold increase of p-Erk1/2 / Erk1/2
Con VEGF 0.1 1 2 5 μg/ml
β-glucan

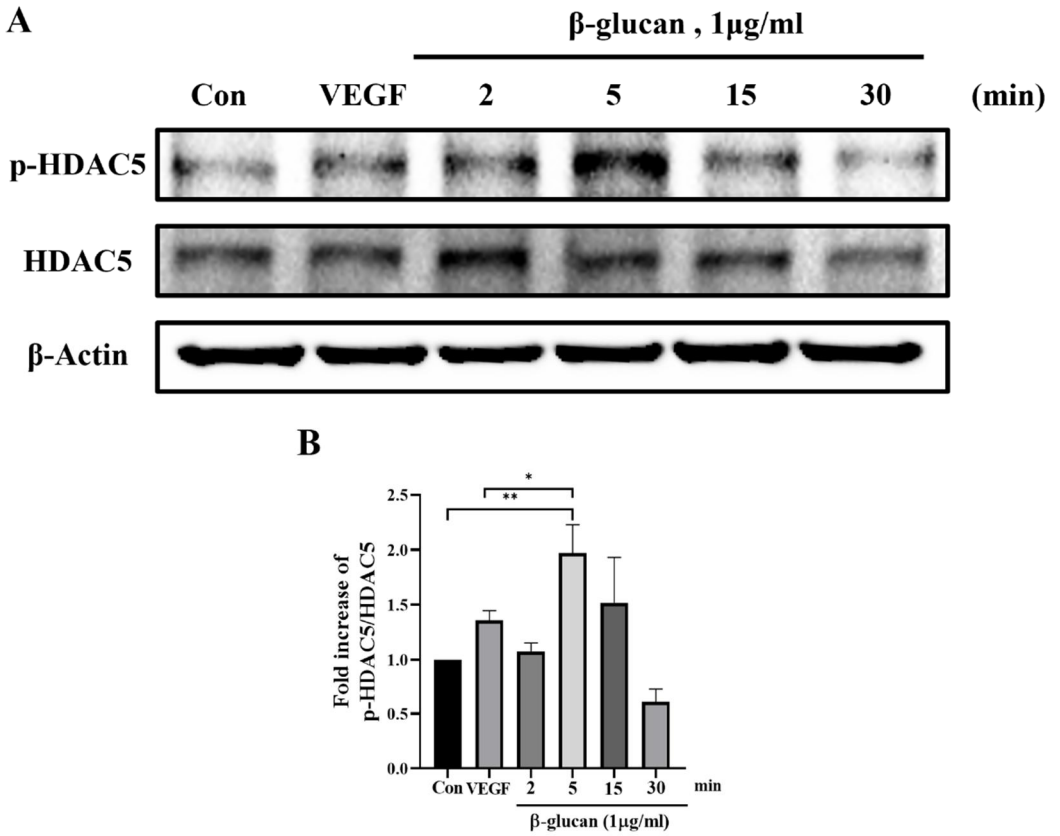
Figure.3. β-glucan modulates phosphorylation of Erk1/2.

(A) HUVECs were exposed to VEGF (20ng/ml) and β-glucan (1μg/ml) for various time. (B) Quantitative data of Erk1/2 phosphorylation normalized with the level of Eri1/2. (C) HUVECs were exposed to β-glucan for 5 minutes with the indicated concentrations. (D) Measurement of the level of p-Erk1/2 expression normalized with the level of Erk1/2. Each experiment was conducted in triplicate. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

4. Phosphorylation of HDAC5 by β-glucan.

Previously, we found that β-glucan phosphorylates and activates VEGF-dependent Akt and Erk1/2. Therefore, in addition to Akt and Erk1/2, we also studied the VEGF-dependent HDAC5. HDAC5 is phosphorylated by VEGF to induce angiogenesis (21). However, studies on phosphorylation of HDAC5 by β-glucan have not yet been conducted. Therefore, we hypothesized that β-glucan would induce angiogenesis by also being involved in HDAC5 activity. And to confirm this hypothesis, first, the phosphorylation of HDAC5 by β-glucan was confirmed by Western blot.

We exposed HUVECs to 1μg/ml of β-glucan for 2, 5, 15, and 30 minutes, respectively. Then, western blot was performed using an antibody in which Ser259 of HDAC5 is phosphorylated (Figure 4. A). As a result, HDAC5 began to be phosphorylated 2 minutes after exposure to β-glucan. After showing the maximum expression level at 5 minutes immediately, it decreased from 15 minutes (Figure 4. B). After confirming the time-dependent phosphorylation of HDAC5, the concentration-dependent phosphorylation of HDAC5 was confirmed. We exposed HUVECs with β-glucan at concentration of 0.1, 1, 2, and 5μg/ml for 5 minutes, respectively (Figure 4. C). As a results of Western blot, the expression level of phosphorylation of HDAC5 increased from 0.1μg/ml and reached the maximum at 1μg/ml (Figure 4. D).


A
β-glucan , 1μg/ml
Con
VEGF
2
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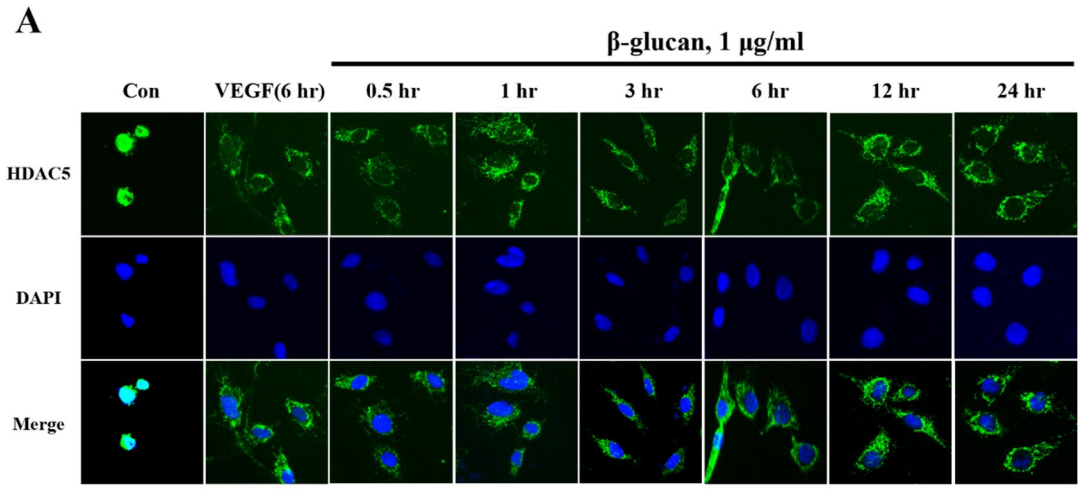
15
30
(min)
p-HDAC5
HDAC5
β-Actin
B
Fold increase of p-HDAC5/HDAC5
Con VEGF 2 5 15 30 min
β-glucan (1μg/ml)
C
β-glucan
Con
VEGF
0.1
1
2
5
(μg/ml)
p-HDAC5
HDAC5
β-Actin
D
Fold increase of p-HDAC5/HDAC5
Con VEGF 0.1 1 2 5 μg/ml
β-glucan

Figure.4. β-glucan mediates phosphorylation of HDAC5.

(A) HUVECs were exposed to VEGF (20ng/ml) and β-glucan (1μg/ml) for various time. (B) Quantitative data of HDAC5 phosphorylation normalized with the level of HDAC5. (C) HUVECs were exposed to β-glucan for 5 minutes with the indicated concentrations. (D) Measurement of the level of p-HDAC5 expression normalized with the level of. Each experiment was conducted in triplicate. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

5. Nuclear export of HDAC5 in response to β-glucan

Originally, HDAC5 is located in the nucleus, but when it receives a phosphorylation signal, it is exported out of the nucleus (31). Previous studies revealed that phosphorylated HDAC5 by VEGF is exported out of the nucleus (21). To confirm HDAC5 nuclear export by β-glucan, β-glucan was treated HUVECs for 0.5, 1, 3, 6, 12, and 24 hours. In this way, we showed the importance of phosphorylation of HDAC5 by β-glucan while time-dependently investigating HDAC5 nuclear export by β-glucan. In the control group not treated with β-glucan, HDAC5 was present in the nucleus (Figure 5. A). 30 minutes after treatment with β-glucan, HDAC5 was exported from the nucleus to the cytoplasm. After 6 hours, the exporting level of HDAC5 export to the cytoplasm showed a maximum. However, after 12 hours, HDAC5, exported to the cytoplasm, started to return to the nucleus (Figure 5. B). For these results, we can conclude that phosphorylated HDAC5 by β-glucan can shuttle in and out of the nucleus.


A
β-glucan, 1 μg/ml
Con
VEGF(6 hr)
0.5 hr
1 hr
3 hr
6 hr
12 hr
24 hr
HDAC5
DAPI
Merge

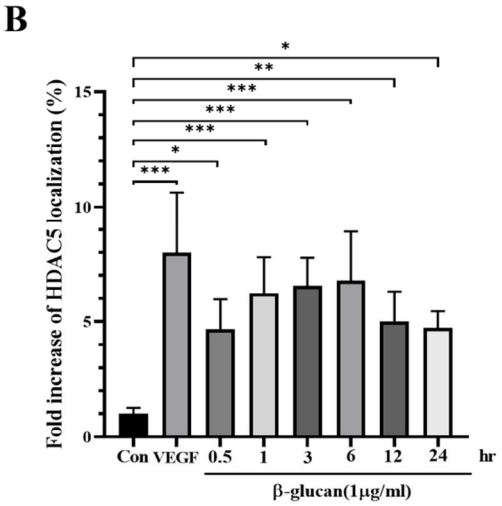

B
Fold increase of HDAC5 localization (%)
15
10
5
0
*
**
***
***
***
*
***
Con
VEGF
0.5
1
3
6
12
24
hr
β-glucan(1μg/ml)

Figure.5. β-glucan induces nuclear export of HDAC5

HUVECs were exposed to VEGF (20ng/ml) for 6 hours and β-glucan (1μg/ml) for the time as indicated. Nuclei were stained with DAPI (blue). Nuclei and HDAC5 (green) were visualized by fluorescent microscope. (A, B) Representative images of HDAC5 nuclear localization in HUVECs with the indicated treatments. Each experiment was conducted in triplicate. * $P <$ 0.05, ** $P < 0.01$, and *** $P <0.001$.

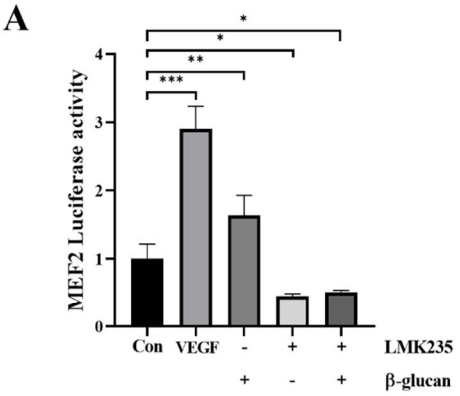
6. Expression of MEF2-dependent gene through phosphorylated HDAC5 in response to β-glucan

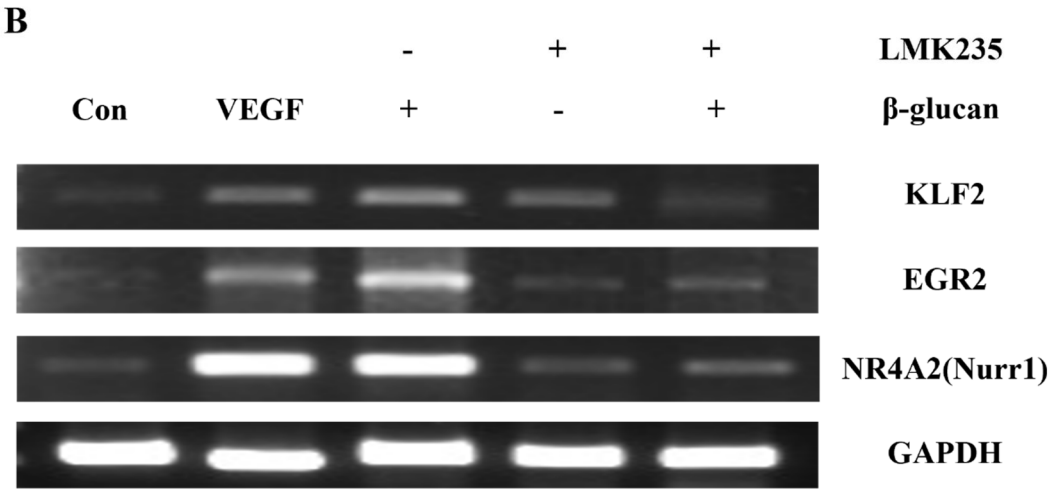
HDAC5, which is bound to the transcription factor MEF2, regulates the expression of MEF2-dependent genes by phosphorylation (21). Based on the previous results, we performed a luciferase assay to determine whether phosphorylated HDAC5 by β-glucan regulates the transcriptional activity of MEF2. We transiently co-transfected BAECs with 3xMEF2-promoter-luciferase constructs and SV40 vectors. Then, we measured the transcriptional activity of MEF2 by luciferase assay by exposure to VEGF or β-glucan, respectively. Our data indicate that the transcriptional activity of MEF2 increased 2.9 folds when exposed to VEGF than in the control group not treated with VEGF or β-glucan. In addition, when β-glucan was treated, it shows that the transcriptional activity of MEF2 is increased 1.6 folds compared to the control.

Since the transcriptional activity of MEF2 is regulated by phosphorylation of HDAC5, we measured the transcriptional activity of MEF2 using 1μM LMK235, an inhibitor against phosphorylation of HDAC5. As a result, when LMK235 was treated, the transcriptional activity of MEF2 decreased 2.5 folds compared to the control. Next, the transcriptional activity of MEF2 was investigated by treatment with LMK235 and β-glucan. Our data show that when β-glucan was treated, the transcriptional activity of MEF2, which was reduced by LMK235, increased by 1.13 folds compared to that of LMK235 alone (Figure 6. A). From these results, we concluded that HDAC5, which regulates the transcriptional activity of MEF2, is activated by β-glucan.

MEF2-dependent pro-angiogenic genes include KLF2, EGR2, and NR4A2 (32-36). We performed RT-PCR to confirm that MEF2 activated by β-glucan expresses these genes (Figure 6. B). First, in the case of KLF2, when VEGF was treated, the expression level increased 1.7 folds compared to the control group, and when β-glucan was treated, it increased 1.8 folds.

Next, the expression level of EGR2 increased 2.5 folds compared to the control group when treated with VEGF and 2.2 folds increased in the case of β-glucan. Finally, the expression level of NR4A2 was increased 2.2 folds for VEGF and 2.5 folds for β-glucan. Since HDAC5 regulates MEF2, HDAC5 was inhibited through LMK235 to confirm the expression of MEF2-dependent pro-angiogenic gene. Expression levels of KLF2, EGR2 and NR4A2 decreased in response to LMK235. However, when LMK235 and β-glucan were treated together, the expression levels of these genes were similar to those of the control group (Figure 6. C). In conclusion, phosphorylated HDAC5 by β-glucan activates MEF2 to express MEF2-dependent pro-angiogenic genes.


A
MEF2 Luciferase activity
Con VEGF - + + LMK235
+ - + β-glucan


B
- + + LMK235
Con VEGF + - + β-glucan
KLF2
EGR2
NR4A2(Nurr1)
GAPDH

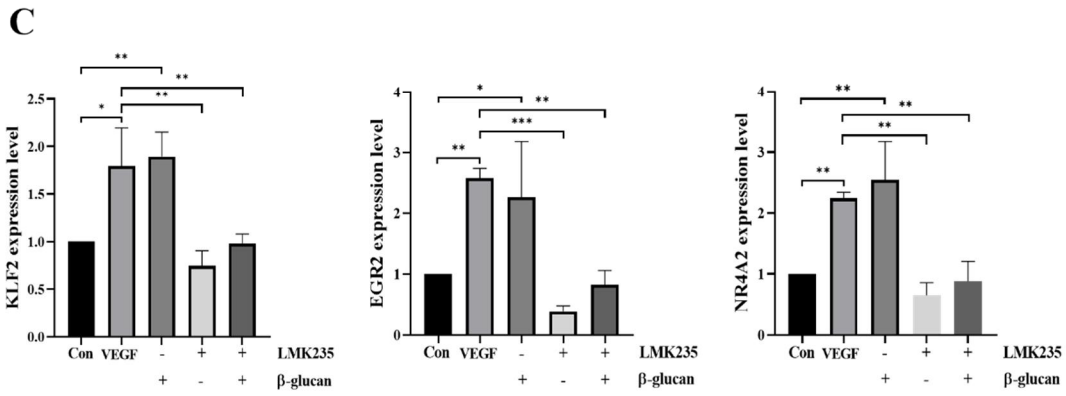

C
KLF2 expression level
EGR2 expression level
NR4A2 expression level
Con VEGF - + + LMK235
+ - + β-glucan

Figure.6. β-glucan-induced phosphorylated HDAC5 expresses the MEF2-dependent gene

(A) BAECs were co-transfected with a plasmid containing a 3xMEF2-luciferase reporter gene and a SV40 vector for 48 hours. After that, VEGF (20ng/ml) and β-glucan (1μg/ml) were treated for 16 hours and LMK235 (1μM) was treated for 30 minutes. (B) Phosphorylation of HDAC5 by β-glucan regulates MEF2-dependent KLF2, EGR2, and NR4A2 expression in HUVECs. HUVECs were exposed to VEGF and β-glucan for 8 hours and LMK235 was exposed to 30 minutes. The mRNA was purified cell lysates, and the RT-PCR with primers of KLF, EGR2, and NR4A2 are described in Materials and Methods. (C) Quantitative analysis of the level of mRNA expression normalized control (GAPDH). Each experiment was conducted in triplicate. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

7. Functional significance of HDAC5 in β-glucan-induced angiogenesis.

Finally, to confirm angiogenesis by β-glucan-induced HDAC5 activity, we performed a scratched wound assay, and tube formation assay of *in vitro* angiogenesis.

HUVECs migrated from scratched wound assay in response to β-glucan. However, when LMK235 was treated it decreased 1.75 folds compared to the control, showing an inhibitory effect on cell migration. But when LMK235 and β-glucan were treated together, there was a 0.73 folds increase compared to the LMK235 treatment group (Figure 7. A and B). The same results were also shown in the tube formation assay. β-glucan promoted capillary-like tube formation on Matrigel. As a result of counting the number of bridges in the formed tube, treatment with β-glucan increased 1.48 folds compared to the control group. However, when treated with LMK235, the number of bridges formed decreased 1.45 folds compared to the control group. And when treated with LMK235 and β-glucan, it increased by 1.3 folds compared to the LMK235 treated (Figure7. C and D). Collectively, these findings suggest that HDAC5 plays a vital role in the control of β-glucan-induced *in vitro* angiogenesis.

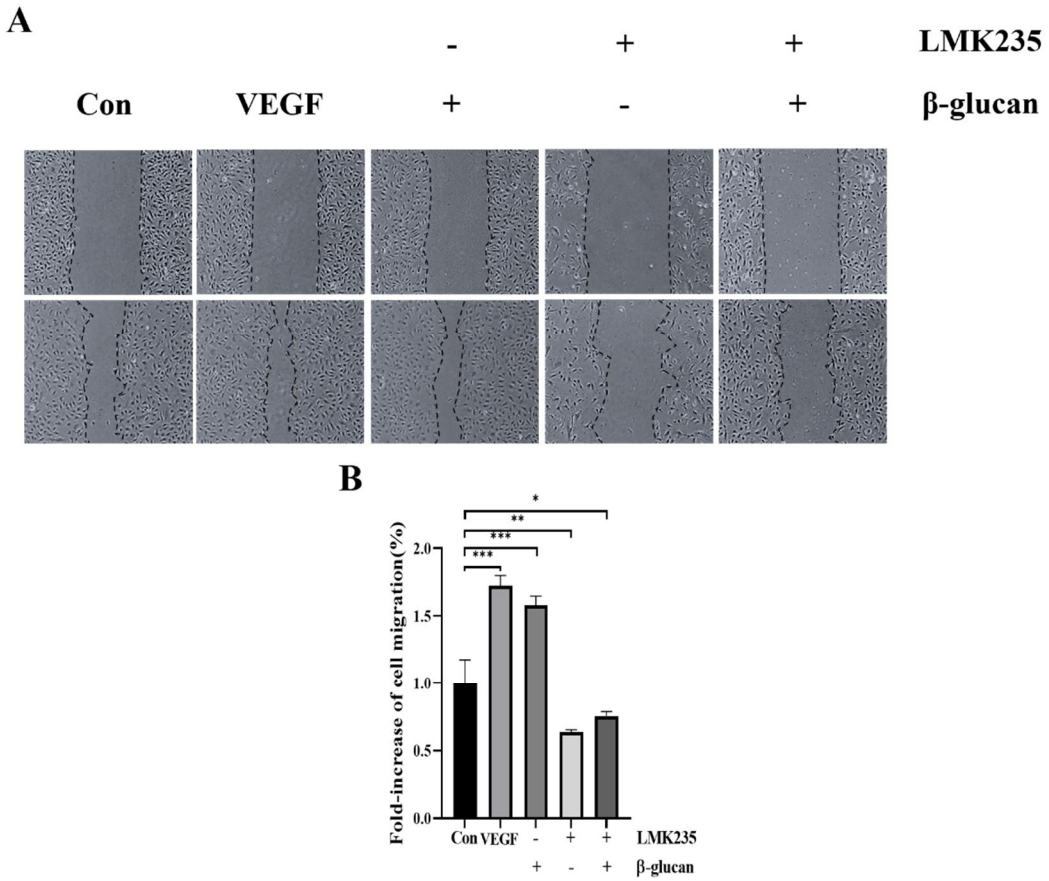
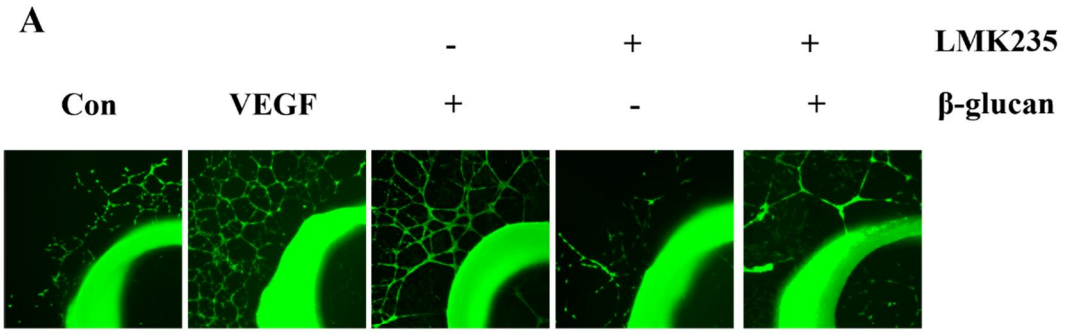

A
-
+
+
LMK235
Con
VEGF
+
-
+
β-glucan
B
*
**
***
***
Fold-increase of cell migration(%)
2.0
1.5
1.0
0.5
0.0
Con VEGF - + + LMK235
+ - + β-glucan
C
-
+
+
LMK235
Con
VEGF
+
-
+
β-glucan
D
*
***
***
Fold increase of the number of sprouts (%)
2.0
1.5
1.0
0.5
0.0
Con VEGF - + + LMK235
+ - + β-glucan

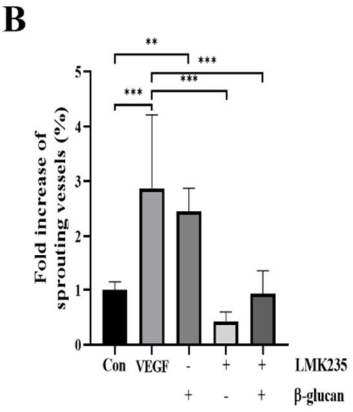
Figure.7. Functional analysis *in vitro* of HDAC5 in β-glucan-induced angiogenesis.

β-glucan (1μg/ml) and LMK235 (1μM). In scratched wound healing assay, LMK235 was treated for 30 minutes before β-glucan was treated. And then, HUVECs were incubated for 10 hours. (B) Quantitative analysis was performed by measuring the width of the cell-free region promptly after scratch and after 10 hours, respectively. (C) In the tube formation assay, LMK235 was treated for 30 minutes before β-glucan was treated. And then, HUVECs were incubated for 6 hours. (D) Quantitative analysis was done by counting the number of bridges extending from the tube after 6 hours. The presentative visuals and quantitative statistics are provided (n=3). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

8. Importance of HDAC5 in β-glucan-induced aortic ring angiogenesis.

Through the results of the *in vitro* angiogenesis assay, we confirmed whether similar results could be obtained *ex vivo*. So, to ensure the role of HDAC5 in inducing angiogenesis by β-glucan in vessels, we performed *ex vivo* rat aortic ring assay. Our data show that microvessels are stretched by β-glucan in the rat aortic ring assay. Furthermore, β-glucan stimulated the sprouting of microvessels in the aortic ring of Rat. But LMK235 repressed sprouting of microvessels, and when LMK235 and β-glucan were co-treated, sprouting was increased compared to the LMK235 treated (Figure 8. A and B). These data imply that HDAC5 is involved in the regulation of β-glucan-induced *ex vivo* angiogenesis.


A
- + + LMK235
Con VEGF + - + β-glucan


B
Fold increase of sprouting vessels (%)
0 1 2 3 4 5
** *** *** ***
Con VEGF - + + LMK235
+ - + β-glucan

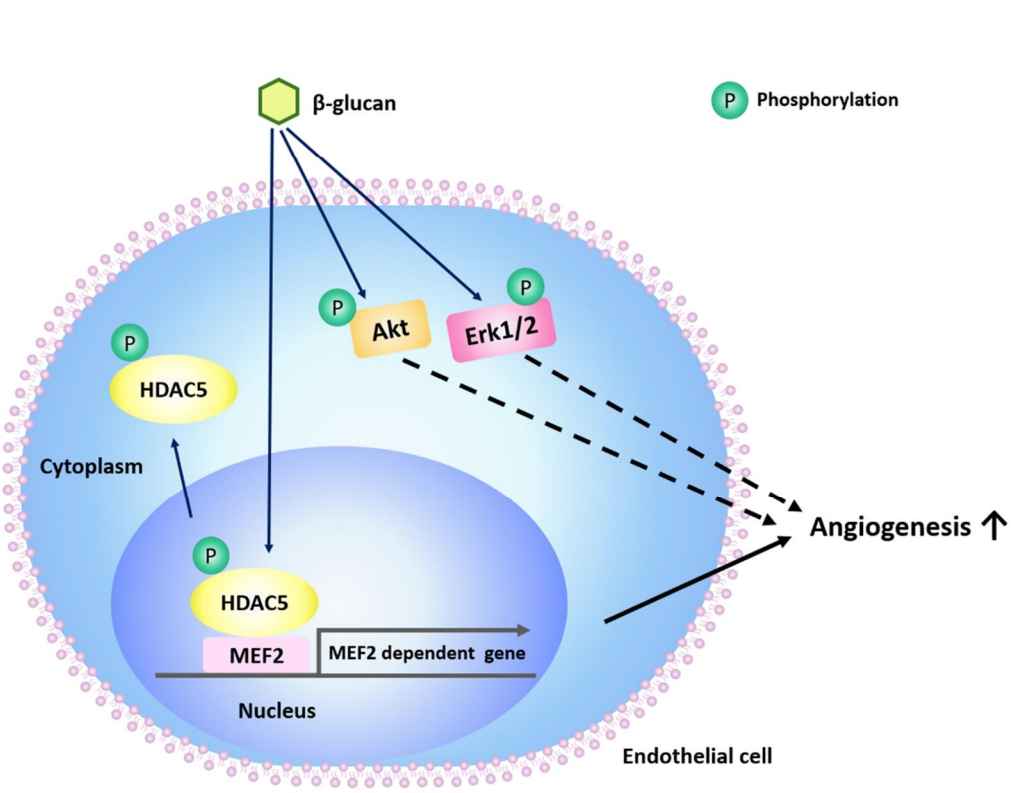

C
β-glucan
P Phosphorylation
P Akt
P Erk1/2
P HDAC5
Cytoplasm
P HDAC5
MEF2
MEF2 dependent gene
Nucleus
Angiogenesis ↑
Endothelial cell

Figure. 8. HDAC5 in β-glucan-induced aortic ring angiogenesis *ex vivo*.

(A) The aortic rings were isolated from Rat and exposed to VEGF (20ng/ml), β-glucan (1μg/ml), and LMK235 (1μM) as indicated. (B) And then, the aortic ring assay for *ex vivo* angiogenesis was performed. Representative images were shown (n=6). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. (C) Schema for potential role and pathways of HDAC5 for β-glucan-induced angiogenesis.

Discussion

The main finding in this study is that β-glucan of *S. cerevisiae* induces angiogenesis through HDAC5 in HUVEC cells. In endothelial cells, β-glucan causes phosphorylation of HDAC5 and nuclear export. And phosphorylated HDAC5 is involved in MEF2-dependent gene expression, endothelial cell migration, and tube formation. Our findings demonstrate that β-glucan-induced HDAC5 is important in endothelial cell angiogenesis.

β-glucan and mannoprotein make up the majority of the yeast cell wall (37). β-glucan, a glucose polymer linked by a 1→3 linear glycosidic chain core, has a various lengths and branching structures (5). Mannoprotein is known to stimulate angiogenesis in endothelial cells via the Erk/Akt/eNOS signaling pathway (9). The structural complexity of β-glucan determines their functionalities (5). β-glucan has a variety of biological activities, including alleviating cardiovascular disease, dyslipidemia, and obesity (8). Previous studies have shown that barley-derived β-glucan induces angiogenesis under oxidative microenvironment (10). However, the relationship between β-glucan derived from yeast and angiogenesis is unclear. This study revealed that yeast-derived β-glucan induces angiogenesis in endothelial cells. Furthermore, We showed that β-glucan is involved in endothelial cell migration and tube formation.

Angiogenesis is a process in which blood vessels are formed by stimulation of various factors such as VEGF, PIGF, FGF, and Ang-1 to existing primary vessels (38). Previous studies suggest that Akt and Erk1/2 induce angiogenesis in endothelial cells in response to stimulation of PIGF and VEGF (29). In particular, binding of VEGF to its corresponding receptor activates the RAS and PI3K pathways (11, 30). β-glucan can also phosphorylate Akt and Erk1/2 in endothelial cells, like how yeast-derived mannoprotein phosphorylates Akt and Erk1/2 to induce angiogenesis (9). So, further studies are needed to elucidate the Akt and Erk1/2

signaling pathways involved in β-glucan induction.

Besides Akt and Erk1/2, HDAC5 is well known as a major factor in VEGF-induced angiogenesis (20, 21). Class a HDACs (HDACs 4, 5, 7, and 9) are involved in cardiac hypertrophy, neurological disease, and development and differentiation of skeletal muscle (23, 28). Although HDAC5 is well known as a repressor of angiogenesis (19), it has been found to promote angiogenesis by being involved in VEGF signaling and gene expression (20, 21). However, the role of HDAC5 induced by β-glucan stimulation is not yet clear. Our study showed that β-glucan activates phosphorylation of the serine 259 residue of HDAC5, suggesting that β-glucan is involved in angiogenesis through HDAC5. But further investigations into how β-glucan phosphorylates HDAC5 is required.

Phosphorylation can modulate HDACs activity in a variety of ways, resulting in reactions that are particular to the enzyme and cellular microenvironment (28). In HDAC5, the nuclear localization signal (NLS) sequence at the N-terminus and the nuclear export signal (NES) sequence at the C-terminus control nuclear import and export, respectively (39, 40). We observed that when β-glucan stimulated endothelial cells, β-glucan induced the translocation of HDAC5 from the nucleus to the cytoplasm. This is consistent with previous reports that the nuclear export of HDAC5 requires phosphorylation of Ser259 and Ser498 (40).

Our study shows that β-glucan accumulates translocated HDAC5 in the cytoplasm in endothelial cells and increases the transcriptional activity of MEF2. Previous studies have reported that class HDACs repress the MEF2 binding site by forming a complex with MEF2 in gene regulatory elements (41). In particular, previous studies suggest that the inhibitory effect on MEF2 transcriptional activation is released before phosphorylated HDAC5 translocated into the cytoplasm (21). Inhibition of phosphorylation of HDAC5 serine 259 residues suppressed transcriptional activation of MEF2. In this study, we present that β-glucan mediates phosphorylation of HDAC5 and nuclear export.

MEF2 plays a pivotal role in the myogenesis and morphogenesis of skeletal and cardiac muscle, as well as the development of the central nervous system (22). MEF2-dependent gene have been reported as KLF2, EGR2, and NR4A2 (Nurr1) (25, 32, 35). In particular, previous studies that KLF2 enhances the angiogenic process by promoting differentiation from endothelial progenitor cells to mature endothelial cells (34). In addition, there are studies that NR4A2 is expressed in endothelial cells in response to VEGF, leading to angiogenesis (36). Indeed, we verified that β-glucan prompted the expression of KLF2, EGR2 and NR4A2 genes associated with angiogenesis. In this case, inhibition of HDAC5 controls the expression of these genes, but β-glucan induces the expression of these genes through phosphorylation of HDAC5. These findings suggest that HDAC5 is a transcriptional repressor for genes such as KLF2, EGR2, and NR4A2 that are implicated in angiogenesis.

Furthermore, based on these findings, we confirmed the angiogenesis process through in vitro and ex vivo experiments such as scratched wound assay, tube formation assay, and aortic ring assay. β-glucan-mediated endothelial cell migration and tube formation were inhibited by HDAC5 inhibitor. This suggests that β-glucan-induced HDAC5 is significant for angiogenesis.

Together, our study verified that β-glucan advances phosphorylation of HDAC5 and nuclear export in endothelial cells. In addition, our results proposed the importance of HDAC5 in β-glucan-induced KLF2, EGR2, and NR4A2 expression and endothelial cell migration, tube formation, and aortic ring assay. Consequently, this study elucidates the importance of HDAC5 in β-glucan induction in endothelial cells, suggesting that β-glucan is crucial in angiogenesis. Hence, β-glucan could aid developing new strategies in therapeutic angiogenesis such as cardiovascular disease and diabetes.

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국문요약

베타 글루칸은 박테리아, 곰팡이 및 효모의 세포벽 구성요소에서 높은 비율을 차지하는 다당류이다. 베타 글루칸의 생물학 및 생리학적 효과는 항종양, 항당뇨, 콜레스테롤 수치의 저하 및 면역 강화으로 보고되고 있다. 그러나 효모 유래의 베타 글루칸의 임상 및 생리학적 중요성에 대한 구체적인 연구는 거의 없어 혈관신생성에 대한 효모 유래 베타 글루칸의 역할은 밝혀지지 않았다. 따라서 본 연구는 인간 탯줄 정맥 내피 세포에서 베타 글루칸과 히스톤 탈아세틸화효소5 (HDAC5)의 상관관계를 보여 혈관신생성의 활성에 대한 베타 글루칸의 역할을 밝혔다.

HDAC5이 베타 글루칸의 자극으로 인산화 되어 핵에서 세포질로 방출됨을 면역 형광법을 통해 확인하였다. 본 연구에서는 베타 글루칸에 자극된 HDAC5가 근세포 증강자 인자2 (MEF2)의 전사 활성화를 매개한다는 것을 밝혔다. 그 결과 MEF2에 의존적이며 혈관신생성에 관여하는 KLF2, EGR2, NR4A2 유전자의 발현이 증가한다는 것을 확인할 수 있었다. 그리하여 *in vitro* 및 *ex vivo* 혈관신생성 분석으로, 세포의 이동과 튜브 형성 및 대동맥 링 분석을 통해 베타 글루칸의 혈관신생성의 활성을 확인하였다. 특히, HDAC5 억제제에 의한 HDAC5의 억제는 MEF2의 전사 활성화와 *in vitro* 및 *ex vivo*의 혈관신생성을 억제하였다. 그러나 베타 글루칸이 사용되면 억제되었던 MEF2의 전사 활성화가 회복되는 것을 보여주었다. 이는 HDAC5가 혈관신생성에 필수적이며 베타 글루칸이 혈관신생성의 활성을 유도한다는 것을 암시한다.

결론적으로, 본 연구에서는 베타 글루칸은 HDAC5를 통해 혈관신생성의 활성을 유도한다는 것을 입증한다. 또한 베타 글루칸이 혈관신생성을 조절하는 새로운 치료제로 잠재적인 가치를 가지고 있음을 시사한다.

중심단어: 베타 글루칸; 인간 탯줄 정맥 내피 세포; 히스톤 탈아세틸화효소5 (HDAC5); 근세포 증강자 인자2 (MEF2); 혈관신생성