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SHORT COMMUNICATION

Monitoring mRNA Expression Patterns in Macrophages in Response to Two Different Strains of Probiotics

Sang-Pil Choi[1,†], Si-Won Park[1,†], Seok-Jin Kang[1], Seul Ki Lim[2], Min-Sung Kwon[2], Hak-Jong Choi[2,*], and Taehoon Chun[1,*]

[1]Department of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul 02841, Korea
[2]Technology Innovation Research Division, World Institute of Kimchi, Gwangju 61755, Korea

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***Corresponding author** :
Hak-Jong Choi
Technology Innovation Research Division, World Institute of Kimchi, Gwangju 61755, Korea
Tel: +82-62-610-1729
Fax: +82-62-610-1850
E-mail: hjchoi@wikim.re.kr

Taehoon Chun
Department of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul 02841, Korea
Tel: +82-2-3290-3069
Fax: +82-2-3290-3507
E-mail: tchun@korea.ac.kr

***ORCID**
Sang-Pil Choi
https://orcid.org/0000-0001-8797-8332
Si-Won Park
https://orcid.org/0000-0002-1597-9683
Seok-Jin Kang
https://orcid.org/0000-0002-9701-8690
Seul Ki Lim
https://orcid.org/0000-0001-7371-2402
Min-Sung Kwon
https://orcid.org/0000-0001-8673-0255
Hak-Jong Choi
https://orcid.org/0000-0003-1185-0919
Taehoon Chun
https://orcid.org/0000-0002-5940-8620

† These authors contributed equally to this work.

Abstract As an initial study to elucidate the molecular mechanism of how probiotics modulate macrophage activity, we monitored mRNA expression patterns in peritoneal macrophages (PMs) treated with two different strains of probiotics. After treatment with either *Weissella cibaria* WIKIM28 or *Latilactobacillus sakei* WIKIM50, total RNAs from PMs were isolated and subjected into gene chip analyses. As controls, mRNAs from vehicle (phosphate-buffered saline, PBS)-treated PMs were also subjected to gene chip analysis. Compared to vehicle (PBS)-treated PMs, WIKIM28-treated and WIKIM50-treated PMs exhibited a total of 889 and 432 differentially expressed genes with expression differences of at least 4 folds, respectively. Compared to WIKIM28-treated PMs, WIKIM50-treated PMs showed 25 up-regulated genes and 21 down-regulated genes with expression differences of more than 2 folds. Interestingly, mRNA transcripts of M2 macrophage polarization marker such as *anxa1*, *mafb*, and *sepp1* were increased in WIKIM50-treated PMs comparing to those in WIKIM28-treated PMs. Reversely, mRNA transcripts of M1 macrophage polarization marker such as *hdac9*, *ptgs2*, and *socs3* were decreased in WIKIM50-treated PMs comparing to those in WIKIM28-treated PMs. In agreement with these observations, mRNA expression levels of tumor necrosis factor-α and interleukin-1α were significantly reduced in WIKIM50-treated macrophages compared to those in WIKIM28-treated macrophages. These results may indicate that probiotics can be classified as two different types depending on their ability to convert macrophages into M1 or M2 polarization.

Keywords differentially expressed gene, macrophage, probiotics

Introduction

Colonization of selective probiotics in gastrointestinal tract might lead to immune modulation within gut microenvironment by communications with mucosal immune cells (Belkaid and Hand, 2014). Furthermore, the crosstalk between probiotics and

immune cells could affect the status of various physiological conditions of humans (Kumari et al., 2021). According to this, several connections between gut and specific organs such as gut-brain, gut-liver, and gut-lung axes have been proposed to explain the functional importance of selective probiotics (Dumas et al., 2018; Powell et al., 2017; Tripathi et al., 2018).

Probiotics can modulate immune responses by mainly interacting with pattern recognition receptors (PRRs) expressed on professional antigen presenting cells (pAPCs) or epithelial cells (Fukata and Arditi, 2013). After interacting with PRRs, specific receptor-mediated signal transduction pathway can turn on and change the phenotype of pAPCs (Fukata and Arditi, 2013). Besides dendritic cells, macrophages are a major population of pAPCs that can modulate inflammatory responses by either triggering inflammation or suppressing inflammation for tissue repair (Lawrence and Natoli, 2011). Therefore, two types of macrophages, inflammatory macrophages (M1 macrophages) and anti-inflammatory macrophages (M2 macrophages), have been proposed depending on their functions (Lawrence and Natoli, 2011). Recently, many observations have demonstrated that M1 or M2 macrophage skewing conditions might affect several pathologic conditions in human diseases (Shapouri-Moghaddam et al., 2018).

Among probiotics, *Weissella cibaria* is considered as a good starter of Kimchi fermentation because it has many beneficial effects including anti-microbial activities, antioxidant activities and immunomodulatory effects (Lim et al., 2017; Yu et al., 2018; Zhu et al., 2022). *Latilactobacillus sakei* is also used for the food fermentation or preservation. Especially, *L. sakei* is known for a commercial meat starter of meat fermentation (Leroy et al., 2006). Therefore, *W. cibaria* and *L. sakei* are good resources for food industry.

In the present study, we profiled mRNA expression patterns in macrophages after treatment with two different strains of probiotics isolated from Kimchi, *W. cibaria* WIKIM28 and *L. sakei* WIKIM50 (Lim et al., 2017). Results of this study might provide valuable data to understand the molecular mechanisms of macrophage polarization triggered by different types of probiotics.

Materials and Methods

Probiotics

Isolation and species identification of *W. cibaria* WIKIM28 from Gat Kimchi have been described previously (Lim et al., 2017). WIKIM50 was isolated from Baechu Kimchi and identified as *L. sakei* based on 16S rRNA gene sequencing according to a previously established method (Lim et al., 2017). WIKIM28 and WIKIM50 were sub-cultured more than 10 times before treating peritoneal macrophages (PMs) for RNA-Seq data analyses.

Stimulation of peritoneal macrophages (PMs) by probiotics

PMs were isolated according to a previously established method (Pineda-Torra et al., 2015). Briefly, 3% thioglycolate (Becton Dickinson, Sparks, MD, USA) in phosphate-buffered saline (PBS) was intraperitoneally injected into C57BL/6 mice (Orient Bio, Seongnam, Korea) at 1 mL per mouse. On day 3, thioglycolate-injected mice were sacrificed to harvest peritoneal lavages. Cell pellets were then acquired after centrifuging peritoneal lavages at 400×g for 5 min at 4℃ and resuspended in DMEM/F-12 (Thermo Fisher Scientific, Waltham, MA, USA) medium supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin (DMEM/F-12-10). After resuspension, cells were plated into tissue culture plates and incubated in a humidified 5% CO_2 atmosphere at 37℃. After overnight culture, adherent cells were harvested and counted. The purity of PMs was validated by flow cytometry analyses using anti-mouse CD11b (eBioscience, San Diego,

CA, USA) and anti-mouse F4/80 (BioLegend, San Diego, CA, USA) antibodies. More than 90% of cells were $CD11b^{+}F4/80^{+}$ cells (data not shown). After isolation, PMs (2×10^{6} cell/well) were seeded into a 12-well plate in 1 mL of DMEM/F-12-10. Heat killed WIKIM28 or WIKIM50 (2×10^{7} CFU/mL) was co-cultured with PMs in a humidified 5% CO_2 atmosphere at 37℃ for 24 hours. As controls, vehicle (PBS) was used to treat PMs without incubation with probiotics.

Gene chip analyses

Total RNAs were isolated from vehicle (PBS)-, WIKIM28-, or WIKIM50-treated PMs using TRIzol (Thermo Fisher Scientific) according to the manufacturer's protocol. The quality and quantity of each RNA sample were measured using an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Gene chip analyses were then performed using Agilent SurePrint G3 Mouse gene expression V2 microarrays (8×60K) according to the manufacturer's protocol (Agilent Technology, V 6.5, 2010). Briefly, total RNAs from each sample were linearly amplified and labeled with Cy3-dCTP. These labeled cRNAs were then purified with an RNAeasy mini kit (Qiagen, Hilden, Germany). The concentration and specific activity of the labeled cRNAs (pmol Cy3/μg cRNA) were measured with an ND-1000 spectrophotometer. For hybridization of microarray, each labeled cRNA was fragmented by adding 5 μL of 10×blocking agent and 1 μL of 25×fragmentation buffer and then heated at 60℃ for 30 min. After heating, 25 μL of 2×hybridization buffer was added to dilute the labeled cRNA. Subsequently, 40 μL of hybridization solution was loaded into the gasket slide and assembled to microarray slides (Agilent Technologies). After assembling, microarray slides were incubated at 65℃ in an Agilent hybridization oven (Agilent Technologies) for 17 hours. After incubation, microarray slides were washed at room temperature using washing buffer provided by manufacturer (Agilent Technologies). The hybridized array was then immediately scanned with an Agilent Microarray Scanner D (Agilent Technologies).

Raw data were extracted using a Feature Extraction Software (v11.0.1.1) provided by Agilent (Agilent Technologies). Comparative analysis between test sample and control sample was carried out using paired t-test. False discovery rate was controlled by adjusting p-value using Benjamini-Hochberg algorithm (Benjamini and Hochberg, 1995). For a differentially expressed genes (DEG) set, hierarchical cluster analysis was performed using complete linkage and Euclidean distance as a measure of similarity.

Transcriptional analysis by quantitative reverse-transcriptase real-time PCR (qRT-PCR)

To confirm gene chip analyses, qRT-PCR were conducted as described previously (Choi et al., 2018). Briefly, total RNAs from vehicle (PBS)-, WIKIM28-, or WIKIM50-treated PMs were isolated for synthesizing cDNAs using a First-Strand cDNA Synthesis Kit with oligo-dT primers (SuperScript RT; Thermo Fisher Scientific). Subsequently, quantitative real-time PCR was performed using one μg of each total RNA with QGreen™ 2X qPCR Master Mix (GenDEPOT, Katy, TX, USA) on a Bio-Rad CFX96 Real-Time Detection System (Bio-Rad, Hercules, CA, USA). Expression of each mRNA transcript was compared to the expression level of mouse *Gapdh* to obtain relative gene expression. Primer sequences for mouse *gapdh* were 5′-ATGGTGAAGGTCGGTGTGAA-3′ (sense) and 5′-GGTCGTTGATGGCAACAATCTC-3′ (anti-sense), resulting in a 100 bp product (Kang et al., 2022). PCR primers of M1 or M2 marker genes used in this study are listed in Table 1.

Statistical analyses

Statistical significance of independent variable values was evaluated by Student's t-test. Significant differences at a confidence level of 95%, 99%, 99.9% are labeled on each graph and indicated by asterisks '*', '**', and '***', respectively.

Table 1. Primer sets of M1 or M2 macrophage signature genes analyzed by quantitative real-time reverse transcriptase-PCR (qRT-PCR)

Gene name	Classification	Primer sequence (5′→ 3′)	*T*m (℃)	Product size	Accession number
anxa1	M2 macrophage marker	F: AGCTCTGGATCTGGAACTGA	55.4	103	NM_010730.2
		R: TTCGTACAGCTTCTCGGCAA	55.4		
mafb	M2 macrophage marker	F: GCGAGCAACTACCAGCAGAT	57.4	125	NM_010658
		R: GCACTACGGAAGCCGTCGAA	59.5		
sepp1	M2 macrophage marker	F: GAGCATCTTGGCAGCAGTAA	55.4	140	NM_001042614
		R: GTGGTGTCTCAGCTCTCTAA	55.4		
hdac9	M1 macrophage marker	F: ATCAGCTCAGTGGACGTGAA	55.4	103	NM_024124
		R: GATCCACCACAGGCATCATC	57.4		
ptgs2	M1 macrophage marker	F: ATCAGGTCATTGGTGGAGAGG	58.2	137	NM_011198
		R: CATCAGACCAGGCACCAGA	57.3		
socs3	M1 macrophage marker	F: CCTCAAGACCTTCAGCTCCAA	58.2	82	NM_007707
		R: CCAGTAGAATCCGCTCTCCT	57.4		

Results and Discussion

Differentially expressed genes (DEGs) in peritoneal macrophages (PMs) treated with either WIKIM28 or WIKIM50

Compared with vehicle (PBS)-treated PMs, PMs treated with WIKIM28 and WIKIM50 showed a total of 889 and 432 DEGs with expression differences of at least 4 folds, respectively. Among them, 652 from WIKIM28-treated cells and 375 genes from WIKIM50-treated cells were up-regulated compared to those in vehicle (PBS)-treated PMs. Contrarily, 237 genes from WIKIM28-treated cells and 57 genes from WIKIM50-treated cells were down-regulated compared to those in vehicle (PBS)-treated cells.

DEGs with at least 2-fold expression difference in WIKIM50-treated cells compared to those in WIKIM28-treated cells are listed in Table 2. Among them, 25 genes were up-regulated, and 21 genes were down-regulated in WIKIM50-treated cells compared to those in WIKIM28-treated cells. Interestingly, several M2 macrophage marker genes such as *anxa1*, *mafb*, and *sepp1* were up-regulated in WIKIM50-treated cells, whereas several M1 macrophage marker genes such as *hdac9*, *ptgs2*, and *socs3* were down-regulated in WIKIM50-treated cells compared with those in WIKIM28-treated cells (Arnold et al., 2014; Barrett et al., 2015; Kim, 2017; Liu et al., 2021; Lu et al., 2017; Moraes et al., 2017). These results indicate that WIKIM28-treated macrophages are more likely to be polarized into M1 phenotype whereas WIKIM50-treated macrophages might exhibit M2 phenotype. Supporting this idea, mRNA transcripts of pro-inflammatory cytokines such as tumor necrosis factor-α and IL-1α were significantly decreased in WIKIM50-treated macrophages compared to those in WIKIM28-treated macrophages (Table 2).

Besides genes involved in macrophage polarization, genes encoding several cell surface proteins including PRRs, chemokine receptors, cytokine receptors were also identified as DEGs (Table 2). However, there was no clear physiological relevance in these DEGs.

Validation of gene expression patterns by quantitative reverse-transcriptase real-time PCR (qRT-PCR)

To validate gene chip data, mRNA expression levels of DEGs related to macrophage polarization were monitored by qRT-PCR (Fig. 1). Results of qRT-PCR analyses clearly showed that M2 macrophage marker genes such as *anxa1*, *mafb*, and

Table 2. DEGs between WIKIM28-treated macrophages and WIKIM50-treated macrophages[1)]

Gene symbol	Gene description	Fold increase	Accession number
Up-regulated genes in WIKIM50-treated macrophages compared to those in WIKIM28			
cd55	CD55 antigen	4.04	NM_010016
card11	Caspase recruitment domain family, member 11	3.97	NM_175362
rgs2	Regulator of G-protein signaling 2	3.64	NM_009061
cxcr3	Chemokine (C-X-C motif) receptor 3	3.35	NM_009910
mafb	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)	2.99	NM_010658
tlr12	Toll-like receptor 12	2.79	NM_205823
nlrp1b	NLR family, pyrin domain containing 1B	2.78	NM_001040696
mrc1	Mannose receptor, C type 1	2.74	NM_008625
nfatc2	Nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 2	2.63	NM_001136073
cd93	CD93 antigen	2.62	NM_010740
il6ra	Interleukin 6 receptor, alpha	2.51	NM_010559
thbs1	Thrombospondin 1	2.47	NM_011580
st6gal1	Beta galactoside alpha 2,6 sialyltransferase 1	2.45	NM_145933
pparg	Peroxisome proliferator activated receptor gamma	2.36	NM_011146
ccl6	Chemokine (C-C motif) ligand 6	2.34	NM_009139
c3ar1	Complement component 3a receptor 1	2.33	NM_009779
sepp1	Selenoprotein P, plasma, 1	2.22	NM_001042614
anxa1	Annexin A1	2.12	NM_010730
ccr5	Chemokine (C-C motif) receptor 5	2.12	NM_009917
masp1	Mannan-binding lectin serine peptidase 1	2.07	XM_006521828
iqgap3	IQ motif containing GTPase activating protein 3	2.04	NM_001033484
cd300lb	CD300 antigen like family member B	2.02	NM_199221
alox5	Arachidonate 5-lipoxygenase	2.01	NM_009662
celf2	CUGBP, Elav-like family member 2	2.01	NM_010160
clec9a	C-type lectin domain family 9, member a	2.00	NM_001205363
Down-regulated genes in WIKIM50-treated macrophages compared to those in WIKIM28			
cxcl2	Chemokine (C-X-C motif) ligand 2	–22.80	NM_009140
cxcl1	Chemokine (C-X-C motif) ligand 1	–17.74	NM_008176
ptgs2	Prostaglandin-endoperoxide synthase 2	–11.32	NM_011198
cav1	Caveolin 1, caveolae protein	–9.92	NM_001243064
cxcl5	Chemokine (C-X-C motif) ligand 5	–5.33	NM_009141
tnf	Tumor necrosis factor	–5.25	NM_013693
il1a	Interleukin 1 alpha	–4.74	NM_010554
hdac9	Histone deacetylase 9	–4.64	NM_024124
dnmt3l	DNA (cytosine-5-)-methyltransferase 3-like	–4.51	NM_019448
nlrp1a	NLR family, pyrin domain containing 1A	–3.35	NM_001004142
maff	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	–3.30	NM_010755
cxcl10	Chemokine (C-X-C motif) ligand 10	–3.23	NM_021274

Table 2. DEGs between WIKIM28-treated macrophages and WIKIM50-treated macrophages[1)] (Continued)

Gene symbol	Gene description	Fold increase	Accession number
il20rb	Interleukin 20 receptor beta	–3.21	NM_001033543
ccl3	Chemokine (C-C motif) ligand 3	–3.10	NM_011337
cd40	CD40 antigen	–3.00	NM_011611
ccl7	Chemokine (C-C motif) ligand 7	–2.96	NM_013654
il10	Interleukin 10	–2.94	NM_010548
socs1	Suppressor of cytokine signaling 1	–2.80	NM_009896
socs3	Suppressor of cytokine signaling 3	–2.72	NM_007707
rgs16	Regulator of G-protein signaling 16	–2.57	NM_011267
cd38	CD38 antigen	–2.40	NM_007646

[1)] DEGs with at least 2 folds.
DEGs, differentially expressed genes.

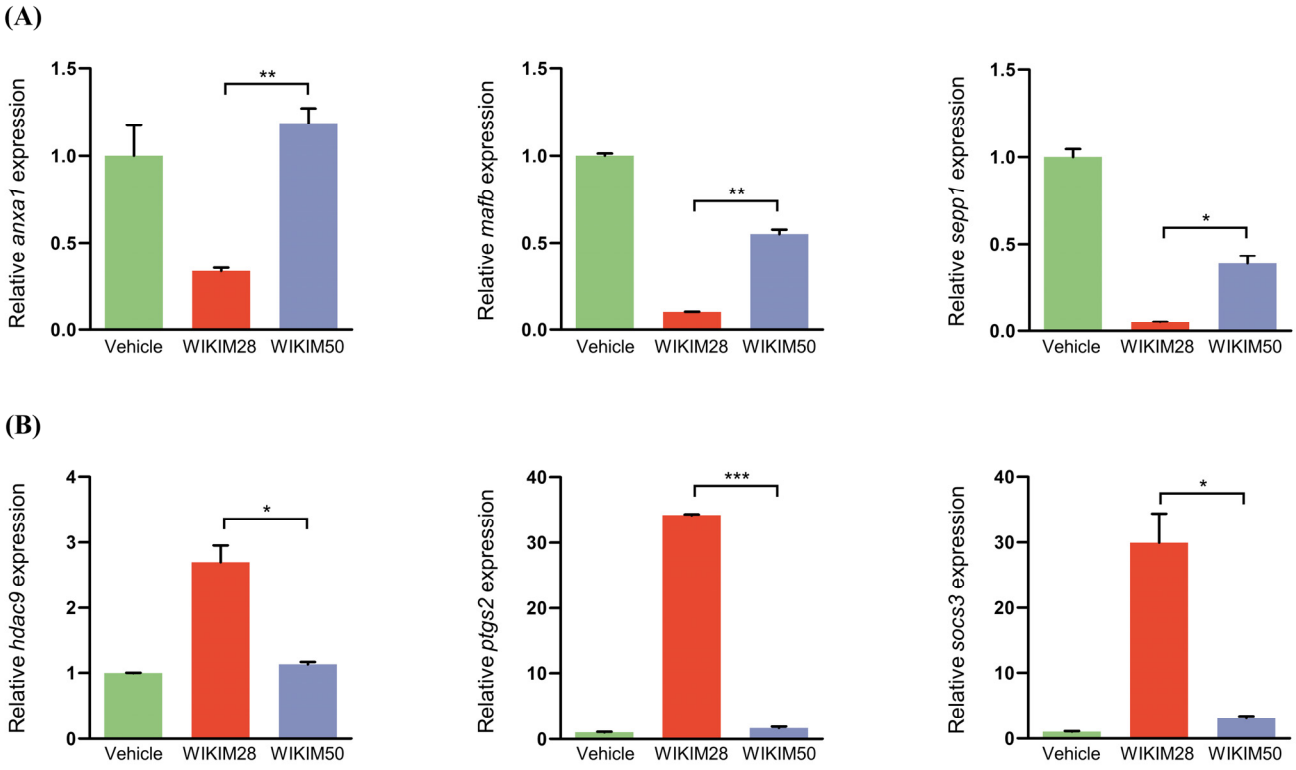

Fig. 1. WIKIM50-treated macrophages increase mRNA transcripts of M2 signature genes and decrease mRNA transcripts of M1 signature genes compared to WIKIM28-treated macrophages. Peritoneal macrophages (PMs; 2×10^6 cell/well) were co-cultured with heat killed WIKIM28 or WIKIM50 (2×10^7 CFU/mL) for 24 hours. Subsequently, quantitative reverse-transcriptase real-time PCRs (qRT-PCRs) were performed using total RNA from each sample as a template to determine relative mRNA expression levels of M1 or M2 macrophage signature genes. (A) Selected up-regulated genes in WIKIM50-treated PMs compared to those in WIKIM28. (B) Selected down-regulated genes in WIKIM50-treated PMs compared to those in WIKIM28. Specific gene primers of qRT-PCRs are listed in Table 1. The relative quantitation of each gene expression was normalized to expression level of mouse *gapdh*. All results are shown as means±SEs. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ (n=3). Vehicle, phosphate-buffered saline (PBS)-treated macrophages; WIKIM28, WIKIM28-treated macrophages; WIKIM50, WIKIM50-treated macrophages.

sepp1 were significantly decreased in WIKIM28-treated cells, whereas M1 macrophage marker genes such as *hdac9*, *ptgs2*, and *socs3* were significantly increased in WIKIM28-treated PMs compared with those in WIKIM50-treated PMs.

Similar to our study, previous reports have demonstrated modulation of macrophage polarization by several different probiotics (Wang et al., 2020). Therefore, consumption of particular probiotics which shifts macrophage into M1 or M2 phenotype might help alleviate specific pathologic conditions of human diseases. For example, oral uptake of several different strains of probiotics can ameliorate colitis or hepatic steatosis by inducing M2 macrophage polarization in a mouse model (Jang et al., 2014; Sohn et al., 2015). However, not many studies have attempted to dissect the molecular mechanism of how certain strains can induce macrophage polarization. Here, we provide evidence indicating that signature genes of macrophage polarization are changed depending on probiotic strains. These results might be useful for finding the molecular clue of macrophage polarization caused by probiotics. Further studies will be required to define molecular signaling pathways involved in WIKIM28- or WIKIM50-mediated macrophage polarizations.

Conclusion

In conclusion, our gene chip data suggest that macrophage polarization might be modulated by treatment with different probiotic strains. Therefore, oral uptake of specific strains of probiotics might change human physiology by shifting macrophage phenotypes into either M1 or M2 polarity.

Conflicts of Interest

The authors declare no potential conflicts of interest.

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Author Contributions

Conceptualization: Choi HJ, Chun T. Data curation: Choi SP, Park SW. Formal analysis: Choi SP, Park SW. Methodology: Choi SP, Park SW, Kang SJ, Lim SK, Kwon MS. Software: Kang SJ. Validation: Choi SP, Park SW. Investigation: Choi HJ, Chun T. Writing - original draft: Choi SP, Park SW. Writing - review & editing: Choi SP, Park SW, Kang SJ, Lim SK, Kwon MS, Choi HJ, Chun T.

Ethics Approval

A protocol of animal study was approved by the Institutional Animal Care and Use Committee (IACUC) of Korea University (protocol numbers: KUIACUC-2015-0430).

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