


Upregulation of *REG IV* gene in human intestinal epithelial cells by lipopolysaccharide via downregulation of microRNA-24

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Abstract

The pathophysiology of inflammatory bowel diseases (IBD) reflects a balance between mucosal injury and reparative mechanisms. Some regenerating gene (*Reg*) family members (*REG I α* , *REG I β* and *REG IV*) are expressed in Crohn's disease (CD) and ulcerative colitis (UC) and involved as proliferative mucosal factors in IBD. We revealed that *REG I α* and *REG I β* were induced in cell culture system by IL-6/IL-22. Although *REG IV* was upregulated in IBD biopsy samples, the upregulation of *REG IV* was not at all induced in cell culture by autoimmune-related cytokines such as IL-6, IL-22 and TNF α . Here, we analysed *REG IV* expression in LS-174T and HT-29 human intestinal epithelial cells by real-time RT-PCR and ELISA. *REG IV* expression was induced by lipopolysaccharide (LPS). However, LPS did not activate *REG IV* promoter activity. As the LPS-induced upregulation of *REG IV* was considered to be regulated post-transcriptionally, we searched targeted microRNA (miR), which revealed that *REG IV* mRNA has a potential target sequence for miR-24. We measured the miR-24 level of LPS-treated cells and found that the level was significantly lower. The LPS-induced increase of *REG IV* mRNA was abolished by the introduction of miR-24 mimic but not by non-specific control RNA.

KEYWORDS

inflammatory bowel diseases, LPS, microRNA-24, *REG IV*

1 | INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC), the two primary forms of idiopathic human inflammatory bowel disease (IBD), are both characterized by chronic, destructive intestinal inflammation of unknown cause(s). Despite advances over the past decade in our understanding of the cellular and molecular mechanisms underlying chronic inflammation, the precise aetiopathogenic factors in IBD remain undefined.

Regenerating gene (*REG*) family proteins are structurally similar proteins belonging to the calcium-dependent (C-type) lectin

superfamily.¹ In humans, five *REG* family genes (i.e. *REG I α* , *REG I β* , *REG*-related sequence [*RS*] [pseudogene], hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein [*HIP/PAP*] [islet neogenesis associated protein: *INGAP*] and *REG III*) are tandemly ordered on chromosome 2p12, while *REG IV* is located on chromosome 1q12-q21.¹ The first *Reg* was discovered in regenerating pancreatic islets from 90% pancreatectomized rats receiving poly(ADP-ribose) polymerase (PARP) inhibitor, nicotinamide.^{2,3} *Reg* proteins have since been found in other physiological and pathophysiological processes. Their basic biological effects seem to be induction of cellular proliferation.^{1,4} *Reg* family proteins have been also suggested to be

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involved in cellular proliferation in gastrointestinal cells.^{5,6} Elsewhere in the gastrointestinal system, the proteins are found during tissue injury.¹ They are also overexpressed in gastric and colorectal cancers and in colorectal cancer cell lines.^{1,7,8}

Concerning IBD, overexpression of *REG Iα* and *REG Iβ* mRNA in resected colonic tissue from CD and UC was reported.⁹ We also showed the overexpression of *HIP/PAP* and *REG III* in IBD.¹⁰ Overexpression of *REG Iα* mRNA and protein in UC, particularly in dysplasia or cancer, and a possible role for *REG Iα* as a marker of UC-associated neoplasia were also reported. Recently, van Beelen Granlund et al. analysed four *REG* family genes (*REG Iα*, *REG Iβ*, *HIP/PAP* and *REG IV*) in five functional human *REG* family members and found the genes were overexpressed in IBD samples.¹¹

We recently examined all five *REG* family genes in IBD and found overexpression of *REG Iα*, *REG Iβ*, and *REG IV* mRNAs in CD and of *REG IV* mRNA in UC. Reporter gene assays and small inhibitory RNA (siRNA)-mediated knockdown experiments indicated that the overexpression of *REG Iα* and *REG Iβ* mRNA was mediated through several transcription factors, including myeloid zinc finger 1, related transcriptional enhancer factor-1/TEA domain transcription factor 4, and signal transducer and activator of transcription 3 in *REG Iα*; helicase-like transcription factor/forkhead box protein N2 in *REG Iβ*.^{12,13} However, induction mechanism of *REG IV* in IBD has been unclear. In the present study, we revealed *REG IV* was induced by lipopolysaccharide (LPS) and that the induction was abolished by the introduction of siRNA for LPS receptors (receptor for advanced glycation endproducts [RAGE] and Toll-like receptor [TLR] 4) and microRNA (miR)-24 mimic.

2 | MATERIALS AND METHODS

2.1 | Cell culture

LS-174T and HT-29 human intestinal epithelial cells were grown in RPMI 1640 (Nacalai Tesque) supplemented with 10% foetal bovine serum (Sigma), 100U/ml penicillin and 100µg/ml streptomycin (FUJIFILM Wako Pure Chemical Co.) as described in previous literature.^{12,13} LPS was purchased from FUJIFILM Wako. Advanced glycation endproducts (AGE)-bovine serum albumin (BSA) was purchased from Calbiochem®, Merck KGaA, high mobility group box (HMGB)1 was from Bio-Techne, and S100 Ca²⁺-binding protein B (S100B) was from Medical & Biological Laboratories Co., Ltd. Cells were treated with 1 µg/ml LPS, 150 µg/ml AGE-BSA, 1 µg/ml HMGB1 or 100 ng/ml S100B for 24h, as described in the literature.^{14,15}

2.2 | Measurement of viable cell numbers by tetrazolium salt cleavage

LS-174T and HT-29 cells (2×10^4 cells/100µl in 96-well plate) were incubated at 37°C overnight, and the siRNA against *REG IV* and scrambled RNA (control) were introduced and incubated at 37°C for

24h in the presence of 0–30 µg/ml LPS. The viable cell numbers were determined by a Cell Counting kit-8 (Dojindo Laboratories) according to the manufacturer's instructions. Briefly, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt) solution was added to cells in 96-well plates, and the cells were incubated at 37°C for 30 min. The optical density of each well was read at 450nm (reference wave length at 650nm) using a Sunrise™ microplate reader (Tecan), as described.^{14,16–18}

2.3 | Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated using a RNeasy Protect Cell Mini kit (Qiagen) from LS-174T and HT-29 human intestinal epithelial cells, and cDNA was synthesized from total RNA as a template using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) as described in previous studies.^{12–19} The cDNA was subjected to PCR with the following primers: *β-actin* (NM_001101) sense primer, 5'-GCGAGAAGATGACCCAGA-3' and antisense primer, 5'-CAGAGGCGTACAGGGATA-3'; *REG Iα* (NM_002909) sense primer, 5'-AGGAGAGTGGCACTGATGACTT-3' and antisense primer 5'-TAGGAGACCAGGGACCCACTG-3'; *REG Iβ* (NM_006507) sense primer, 5'-GCTGATCTCCTCCCTGATGTTT-3' and antisense primer, 5'-GGCAGCTGATTCCGGGGATTA-3'; *REG III* (AB161037) sense primer, 5'-GAATATTCTCCCCAACTG-3' and antisense primer, 5'-GAGAAAAGCCTGAAATGAAG-3'; *HIP/PAP* (NM_138937) sense primer, 5'-AGAGAATATTCGCTTAATTCC-3' and antisense primer, 5'-AATGAAGAGACTGAAATGACA-3'; *REG IV* (AY007243) sense primer, 5'-ATCCTGGTCTGGCAAGTC-3' and antisense primer, 5'-CGTTGCTGCTCCAAGTTA-3', and *RAGE* (NM_001136) sense primer, 5'-TGGAACCGTAACCCTGACCT-3' and antisense primer, 5'-CGATGATGCTGATGCTGACA-3'. All the PCR primers were synthesized by Nihon Gene Research Laboratories (NGRL). Real-time PCR was performed using KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems) and Thermal Cycler Dice Real Time System (Takara Bio Inc.) as described in the literature.^{12–18} PCR was performed with an initial step of 3 min at 95°C followed by 40cycles of 3 s at 95°C and 20s at 60°C for *β-actin*, *REG III*, *HIP/PAP* and *RAGE*, and 40cycles of 3 s at 95°C and 20s at 64°C for *REG Iα*, *REG Iβ* and *REG IV*. Target cDNAs were cloned into pBluescript SK(-) plasmid (Stratagene), and sequential 10-fold dilutions from $10^2 \sim 10^7$ copies/µl were prepared. The serial dilutions were run to verify the specificity and to test the sensitivity of the SYBR Green-based real-time RT-PCR. Target mRNA value was normalized to that of *β-actin* mRNA, which was used to account for differences in the efficiency of reverse transcription between samples.

2.4 | Measurement of *REG IV* in culture medium by enzyme-linked immunosorbent assay (ELISA)

Human LS-174T and HT-29 intestinal epithelial cells were exposed to 1 µg/ml LPS for 24h, culture medium was

collected, and the concentration of REG IV was measured by using Human REG IV ELISA kit (Cloud-Clone) according to the instructions of the supplier.

2.5 | Construction of reporter plasmid and luciferase assay

Reporter plasmid was prepared by inserting the promoter fragments of human *REG IV* (-1053~+22) upstream of a firefly luciferase reporter gene in the pGL3-Basic vector (Promega). The reporter plasmid was transfected into human LS-174T and HT-29 intestinal epithelial cells using Lipofectamine® 3000 (Invitrogen), as described in the literature,^{12,13} and the cells were stimulated by LPS (1 µg/ml) for 24h. The cells were harvested, and cell extracts were prepared in extraction buffer (0.1 M potassium phosphate, pH 7.8/0.2% Triton X-100; Life Technologies). To monitor transfection efficiency, pCMV•SPORT-βgal plasmid (Life Technologies) was co-transfected in all experiments at a 1:10 dilution. Luciferase activity was measured using a PicaGene luciferase assay system (Toyo-ink) and was normalized by the β-galactosidase activity^{12-14,17-19} using Beta-Glo® Assay System (Promega).

2.6 | MiRNA extraction, reverse transcription and real-time quantitative PCR

Total RNA including microRNA (miRNA) was isolated from LS-174T and HT-29 intestinal epithelial cells using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. An equal amount of DNase-treated RNA was Poly-A tailed using a Mir-X™ miRNA first strand synthesis kit (Clontech Laboratories, Inc.) according to the manufacturer's protocol. The condition for PCR was 95°C for 10 s, followed by 45 cycles of amplification (95°C, 5 s, 60°C, 20s). U6 small nuclear RNA was used as an endogenous control for miRNA as described in previous studies.^{13,17} The cDNA was subjected to PCR with the following primers: miR-24 sense primer, 5'-TGGCTCAGTTCAGCAGGAACA-3' and antisense primer, 5'-GTGCAGGGTCCGAGGT-3'; U6 sense primer, 5'-CTCGCTTCGGCAGCACA-3' and antisense primer, 5'-AACGCTTCACGAATTTGCGT-3'.

2.7 | MiR-24 mimic transfection

MiR-24 mimic (5'-UGGCUCAGUUCAGCAGGAACAGtt-3', 5'-CUGUCCUGCUGAACUGAGCCAtt-3') and non-specific control RNA (miR-24 mimic NC) (5'-UUCUCCGAACGUGUCACGUtt-3', 5'-ACGUGACACGUUCGGAGAAtt-3') were synthesized by NGRL and introduced into human LS-174T and HT-29 intestinal epithelial cells using Lipofectamine® RNAiMAX (Invitrogen)^{19,26} just before

LPS stimulation, and the mRNA levels of *REG IV* were measured by real-time RT-PCR, as described in previous studies.^{12-14,17}

2.8 | RNA interference (RNAi)

Small interfering RNAs (siRNAs) directed against human *REG IV*, *RAGE* and *TLR4* mRNAs were synthesized by NGRL. The sense sequences of siRNA for human *REG IV*, *RAGE* and *TLR4* were 5'-CAUGCUCUGGAAGCCAUCtt-3', 5'-AUCUACA AUUCUGGCUUCtt-3' and 5'-GAGCCGUCUGGUGU AUCUUU-3', respectively. The Silencer Select® scrambled siRNA was purchased from Thermo Fisher Scientific (Waltham, MA) and used as the control. LS-174T and HT-29 cells were transfected with 1 pmol and 5 pmol each of siRNA in a 96-well and 24-well culture dish (4×10^5 cells/ml) using the Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen), as previously described.^{12-15,17,18}

2.9 | Data analysis

Results are expressed as mean ± SE. Statistical significance was determined by Student's *t*-test using the GRAPHPAD PRISM software (GRAPH-PAD Software).

3 | RESULTS

3.1 | LPS induced *REG IV* mRNA

We added 1 µg/ml LPS in the culture medium of LS-174T and HT-29 human intestinal epithelial cells and cultured for 24h. After the stimulation, we prepared RNA from the cells and *REG IV* expression was measured by real-time RT-PCR. As shown in [Figures 1 and 2](#), *REG IV* mRNA was significantly increased in response to the LPS addition in both LS-174 and HT-29 human intestinal epithelial cells but not increased mRNAs of the other members of human *REG* family (*REG Iα*, *REG Iβ*, *REG III* and *HIP/PAP*) and *RAGE*.

3.2 | *REG IV* acts as a growth factor in human intestinal epithelial cells

In order to confirm *REG IV* acts a growth factor in intestinal epithelial cells, we introduced siREG IV RNA into LS-174 and HT-29 cells and added LPS (0–30 µg/ml) into the medium. As shown in [Figure 3](#), cell proliferation was significantly inhibited by LPS (0.5–30 µg/ml) in siRAGE IV-introduced cells ('+' in [Figure 3](#)). In contrast, inhibitory effects on cell proliferation by LPS (0.5–30 µg/ml) were not shown in scrambled RNA introduced cells ('-' in [Figure 3](#)), suggesting that *REG IV* expression in intestinal epithelial cells in response to LPS

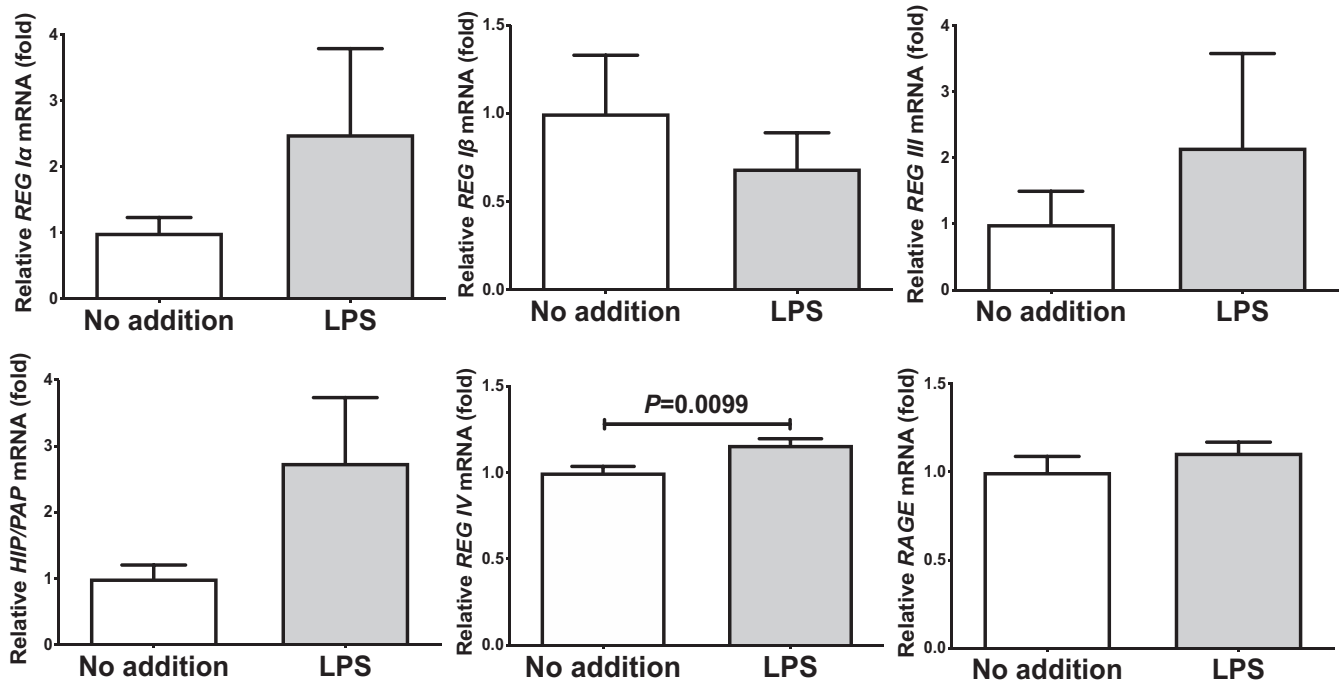


FIGURE 1 mRNA levels of REG family genes (REG I α , REG I β , REG III, HIP/PAP and REG IV) and RAGE in LS-174T human intestinal epithelial cells stimulated by LPS (1 μ g/ml) for 24 h. The mRNAs were measured by real-time RT-PCR using β -actin as an endogenous control. Data are expressed as mean \pm SE for each group ($n = 4$). The statistical analyses were performed using Student's *t*-test

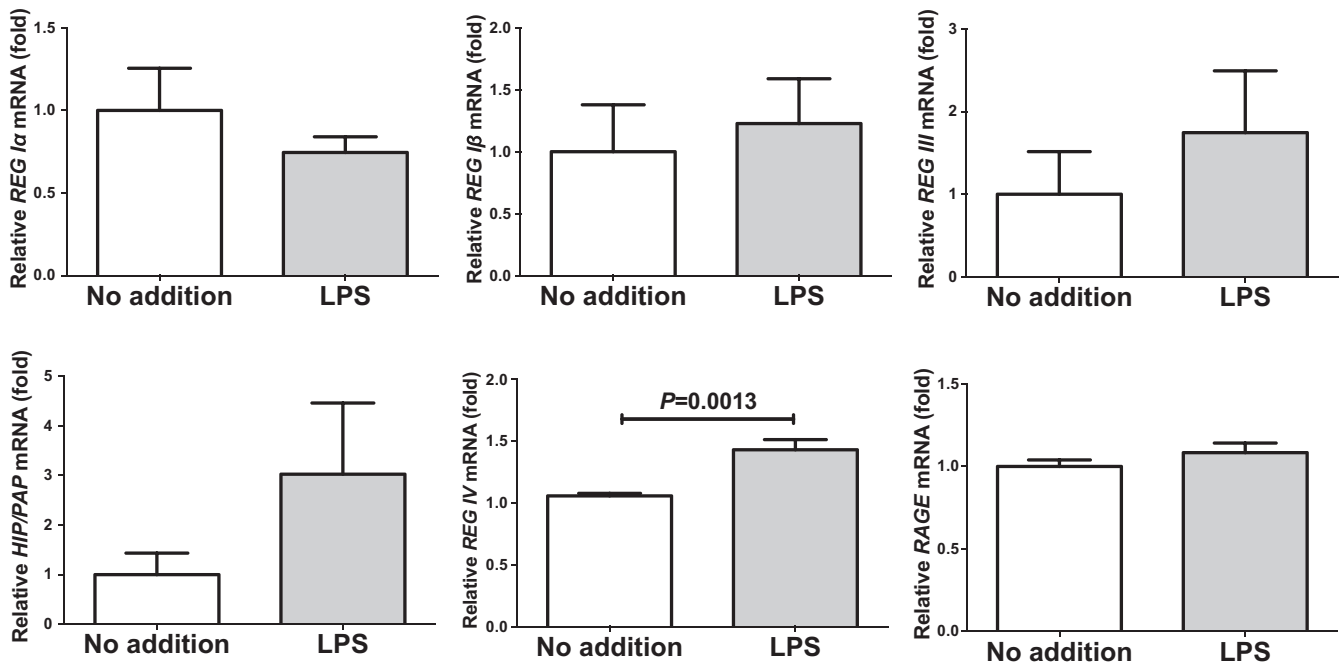


FIGURE 2 mRNA levels of REG family genes (REG I α , REG I β , REG III, HIP/PAP and REG IV) and RAGE in HT-29 human intestinal epithelial cells stimulated by LPS (1 μ g/ml) for 24 h. The mRNAs were measured by real-time RT-PCR using β -actin as an endogenous control. Data are expressed as mean \pm SE for each group ($n = 4$). The statistical analyses were performed using Student's *t*-test

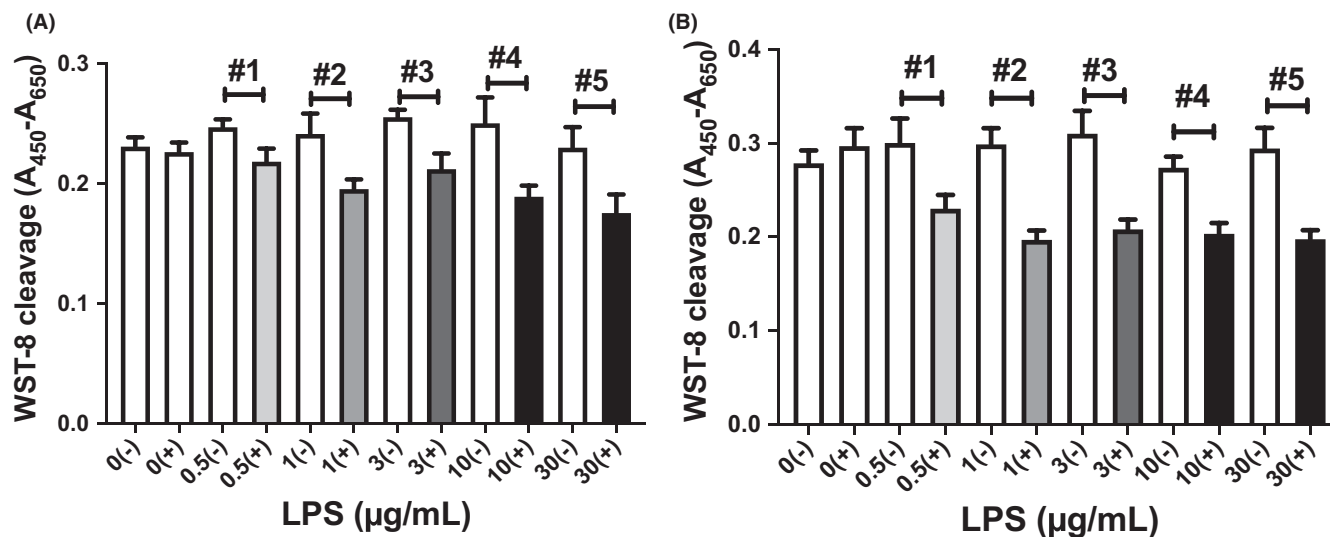


FIGURE 3 Effects of siRNA transfection on cell proliferation in (A) LS-174T and (B) HT-29 human intestinal epithelial cells stimulated by LPS. After transfection of siRNA against *REG IV* into LS-174T and HT-29 cells, LPS (0–30 µg/ml) was added. Cellular proliferation was measured by WST-8 assay. Data are expressed as mean \pm SE for each group ($n = 4-8$). 0, 0.5, 1, 3, 10 and 30 mean LPS concentrations (µg/ml). (-): Scrambled RNA, (+): siREG IV RNA, #1: $p = 0.0490$, #2: $p = 0.0267$, #3: $p = 0.0288$, #4: $p = 0.0212$, #5: $p = 0.0325$ in LS-174T cells (A). #1: $p = 0.0342$, #2: $p = 0.0002$, #3: $p = 0.0018$, #4: $p = 0.0008$, #5: $p = 0.0013$ in HT-29 cells (B). The statistical analyses were performed using Student's *t*-test

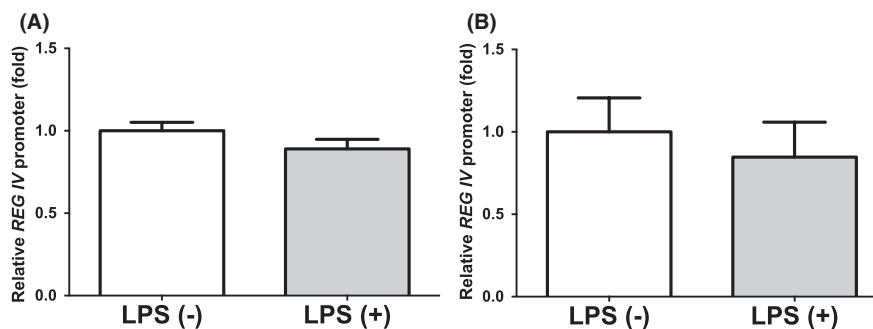


FIGURE 4 Luciferase reporter assays of promoter activities of *REG IV* in (A) LS-174T and (B) HT-29 human intestinal epithelial cells. Reporter plasmid prepared by inserting the promoter fragments of *REG IV* (–1053 ~ +22) upstream of a firefly luciferase reporter gene in pGL3-Basic vector was transfected into LS-174T and HT-29 human intestinal epithelial cells. After cells were stimulated by LPS (1 µg/ml) for 24h, the cells were lysed, and the promoter activities of *REG IV* were measured using β -galactosidase as a transfection efficiency control. All data are represented as the mean \pm SE of the samples ($n = 4$). The statistical analyses were performed using Student's *t*-test

stimulation and that *REG IV* works as a growth factor for intestinal epithelial cells in the presence of LPS.

3.3 | The promoter activities of *REG IV* were not increased by LPS

In order to determine whether the LPS-induced increases in *REG IV* mRNAs were caused by activation of transcription of *REG IV* gene, a 1075bp fragment containing 1053bp of the *REG IV* promoter was fused to the luciferase gene of pGL3-Basic vector and transfected it into LS-174T and HT-29 human intestinal epithelial cells. After LPS stimulation, we measured promoter activities and found that *REG IV* promoter activity was not significantly

increased by LPS in LS-174T nor HT-29 human intestinal epithelial cells (Figure 4). These results strongly suggested that the gene expression of *REG IV* in response to LPS stimulation was not regulated by transcription.

3.4 | LPS reduced microRNA-24 in human intestinal epithelial cells

We considered a possible explanation that the LPS-induced upregulation of *REG IV* (Figures 1 and 2) was controlled post-transcriptionally. Therefore, we searched targeted miRNA (miR) using the [MicroRNA.org](http://www.microrna.org/microrna/home.do) program (<http://www.microrna.org/microrna/home.do>), which revealed that *REG IV* mRNA has a potential

target sequence for miR-24. We then measured the miR-24 levels of LPS-treated cells by real-time RT-PCR and found that the miR-24 levels in LPS-treated cells were significantly lower than that of untreated cells (Figure 5: 0.02782 ± 0.006653 fold, $p = 0.0368$ in LS-174T cells; 0.1400 ± 0.06179 fold, $p = 0.0490$ in HT-29 cells).

3.5 | REG IV mRNA levels were regulated by microRNA-24 in human intestinal cells

In order to investigate whether *REG IV* expression in LPS-treated cells is regulated by miR-24, miR-24 mimic and non-specific control RNA (miR-24 mimic NC) were introduced into LS-174T and HT-29 human intestinal epithelial cells with LPS exposure, and the mRNA levels of *REG IV* and *REG IV* protein levels in cell culture medium were measured by real-time RT-PCR and ELISA, respectively. As shown in Figure 6, we found that the LPS-induced increases in *REG IV* mRNA and *REG IV* protein in culture medium were abolished by the introduction of miR-24 mimic but not by miR-24 mimic NC. These findings indicate that LPS down-regulates the miR-24 level in human intestinal epithelial cells (Figure 5) and that the levels of *REG IV* mRNA and *REG IV* protein are increased via the miR-24 mediated mechanism.

3.6 | LPS induced REG IV expression via RAGE and TLR4

LPS has been reported to act as a RAGE ligand²⁰ and RAGE ligands, such as AGE and HMGB1 induced *REG IV* gene expression in human LS-174T cells (data not shown). It is well known that RAGE was up-regulated in some cells such as adipocytes by RAGE ligands such as AGE and HMGB1¹⁵ and we found significant upregulation of RAGE mRNA in response to AGE and HMGB1 in LS-174T cells (data not shown). As RAGE and TLR4 are considered as receptors for LPS to transduce LPS signal into cells,²⁰⁻²² we investigated which signaling pathway is used in the LPS-induced *REG IV* expression in human intestinal epithelial cells using siRNA introductions for RAGE and TLR4. The LPS-induced upregulations of *REG IV* mRNA and *REG IV* protein in culture medium were clearly abolished in both LS-174T and HT-29 human intestinal epithelial cells by the introduction of siRNAs both for RAGE and TLR4 (Figure 7).

4 | DISCUSSION

Although overexpression of *REG IV* has been reported in IBD,^{11,23-25} the mechanisms of *REG IV* induction have been elusive.^{12,13} In cancer cells, overexpression of *REG IV* gene was also reported in colorectal carcinoma,²⁶⁻³⁰ gastric cancer,³¹⁻³⁴ gallbladder carcinoma,^{35,36} prostate cancer,^{37,38} pancreatic cancer,³⁹⁻⁴¹ glioma⁴² and ovarian carcinoma.⁴³⁻⁴⁵ Despite lots of reports described above, the mechanisms of *REG IV* induction in cancer cells and in inflammatory cells/tissues have also been elusive.

In this study, we demonstrated that LPS induced increases of *REG IV* mRNA and protein in human intestinal epithelial cells. We further studied the mechanisms by which LPS upregulates the gene expression of *REG IV* and found that the LPS-signal was transduced via RAGE and TLR4 receptors and that the *REG IV* mRNA level in human intestinal epithelial cells was post-transcriptionally regulated via miR-24-regulated mechanism.

Originally, *Reg* was isolated as a gene specifically expressed in regenerating rat islets induced by 90% pancreatectomy with PARP inhibitor administrations.^{2,3} The *Reg* and *Reg*-related genes were isolated and were revealed to constitute a multigene family, the *Reg* gene family. Based on the primary structures of the *Reg* proteins, the members of the family are grouped into four subclasses: types I, II, III and IV.¹ In humans, five *REG* family genes, that is *REG I α* , *REG I β* , *RS*, *HIP/PAP* and *REG III* are tandemly ordered on chromosome 2p12, while *REG IV* locates on chromosome 1.¹ In the mouse genome, all *Reg* family genes except for *Reg IV* (i.e. *Reg I*, *Reg II*, *Reg III α* , *Reg III β* , *Reg III γ* and *Reg III δ*) were mapped to a contiguous 75 kbp region of chromosome 6C,⁴⁶ while *Reg IV* was mapped on chromosome 3.⁴⁷ Type I (and type II) *Reg* proteins are expressed in regenerating islets and are involved in β -cell regeneration.^{1,3,4,19,48} *Reg* family proteins have been suggested to be involved in cellular proliferation in gastrointestinal cells, hepatic cells, cardiovascular cells and neuronal cells.¹ *Reg* protein was also shown to mediate gastrointestinal epithelial cell proliferation in rats.^{6,49} Yonemura et al.⁸ showed that the expression of the *REG I α* gene is closely related to the infiltrating property of gastric carcinoma and may be a prognostic indicator of differentiated adenocarcinomas of the stomach. These observations suggest that the *Reg* gene family is involved in cell proliferation in a variety of cell types, including gastrointestinal cells. Concerning human *REG* family gene expression in IBD, upregulations of *REG I α* ,^{12,13,24,50} *REG I β* ,^{12,13,24} *REG III*,¹⁰ *HIP/PAP*^{10,24,51} and *REG IV*^{11-13,24} were reported.

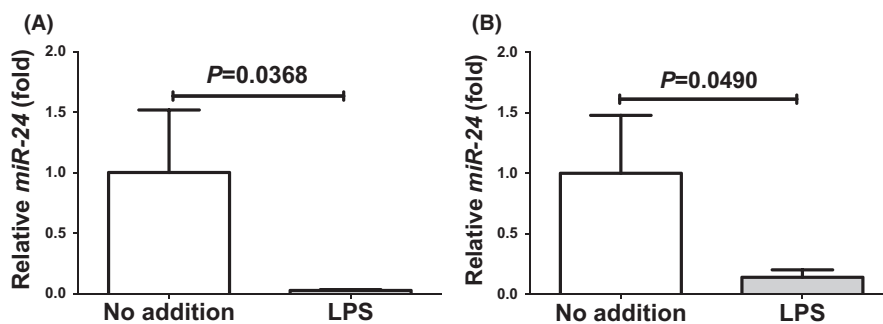


FIGURE 5 MicroRNA-24 levels in (A) LS-174T and (B) HT-29 human intestinal epithelial cells stimulated by LPS (1 μ g/ml) for 24 h. The miRNAs were measured by real-time RT-PCR using U6 RNA as an endogenous control. Data are expressed as mean \pm SE for each group ($n = 4$). The statistical analyses were performed using Student's *t*-test

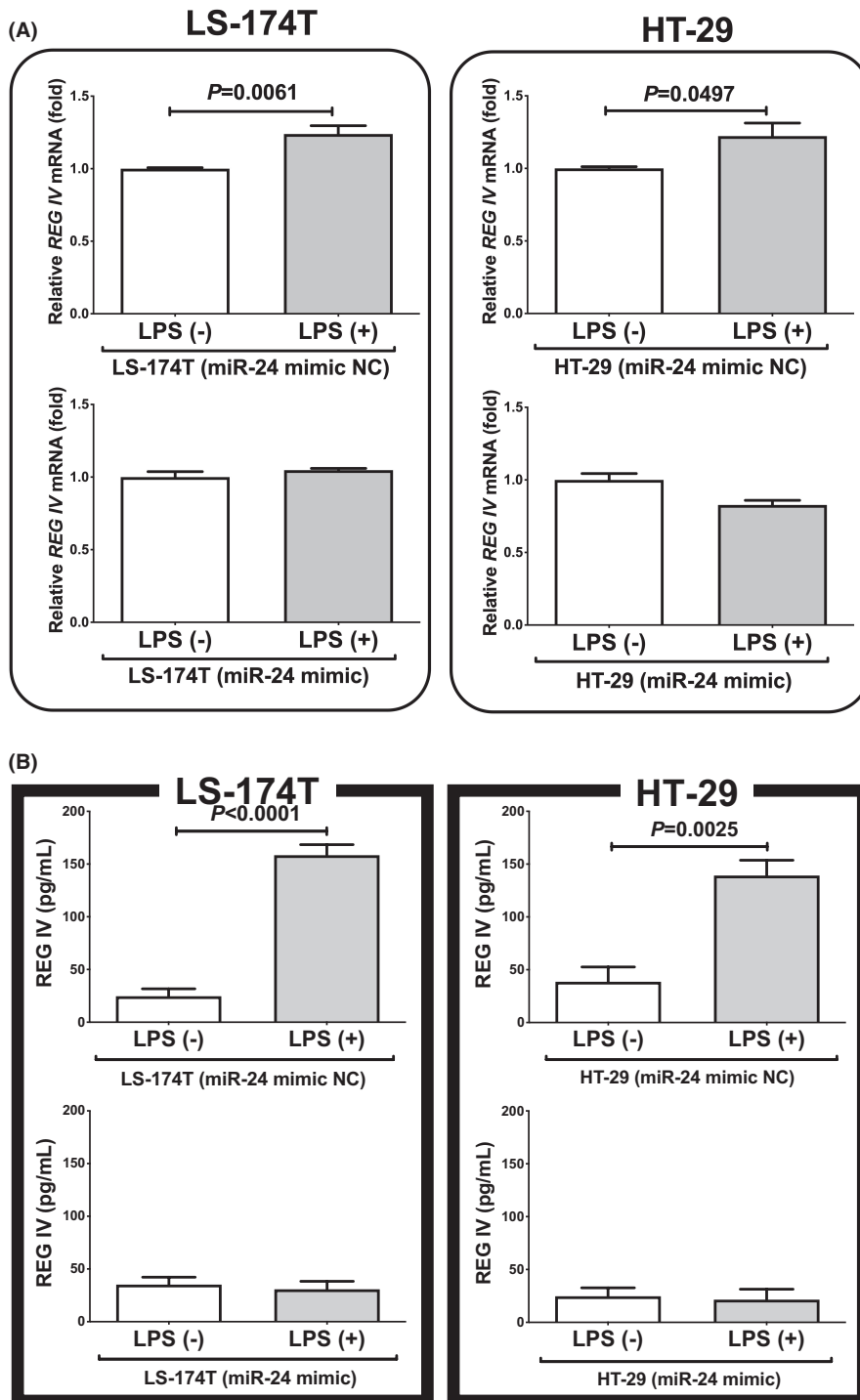


FIGURE 6 Effects of miR-24 mimic transfection on *REG IV* expression. The miR-24 mimic (5'-UGGCUCAGU UCAGCAGGAACAGtt-3' and 5'-CUGUU CCUGCUGAACUGAGCCAtt-3') and non-specific control RNA (miR-24 mimic NC) (5'-UUCUCCGACGUGUCACGUtt-3' and 5'-ACGUGACACGUUCGGAGAAtt-3') were synthesized by NGRL and introduced into LS-174T and HT-29 human intestinal epithelial cells using Lipofectamine® RNAiMAX just before LPS addition, and the mRNA levels of *REG IV* (A) were measured by real-time RT-PCR using β -actin as an endogenous control. The levels of *REG IV* in the cell culture medium (B) were measured by ELISA. Data were expressed as mean \pm SE for each group ($n = 4$). The statistical analyses were performed using Student's *t*-test

The mechanisms of upregulation in *REG I α* and *REG I β* in IBD were clarified,¹² and some upregulation mechanisms of mouse *Reg III* genes⁵² and human *REG III α* (*HIP/PAP*) gene⁵³ were also reported.

Upregulation of *REG IV* gene in IBD has been reported previously.^{11,23-25} However, the mechanism of *REG IV* expression in the colon is still controversial: caudal-type homeobox transcription factor 2 (*CDX2*) and GATA DNA-binding protein 6 (*GATA6*) involvements were reported. In our previous study, we found that *REG IV* mRNA was significantly upregulated in UC and CD samples and downregulated by the addition of TNF α in cultured colon cells.¹² We therefore

introduced siRNAs for *CDX2*, *GATA6* and suppressors of the cytokine signalling 3 (*SOCS3*) into LS-174T human intestinal epithelial cells and evaluated the effect of the siRNAs on the TNF α -induced suppression of *REG IV*. The introduction of *GATA6* siRNA, but not *CDX2* or *SOCS3* siRNA, significantly attenuated the TNF α -induced *REG IV* suppression, indicating that *GATA6* is essential for *REG IV* expression and TNF α -induced *REG IV* suppression in colon epithelial cells.¹³ Recently, miRs were reported to regulate some mRNA levels via degradation of target mRNAs.⁵⁴ MiR-24 was reported to reduce *REG IV* mRNA,⁵⁵ and miR-143 and miR-363 were reported to inhibit

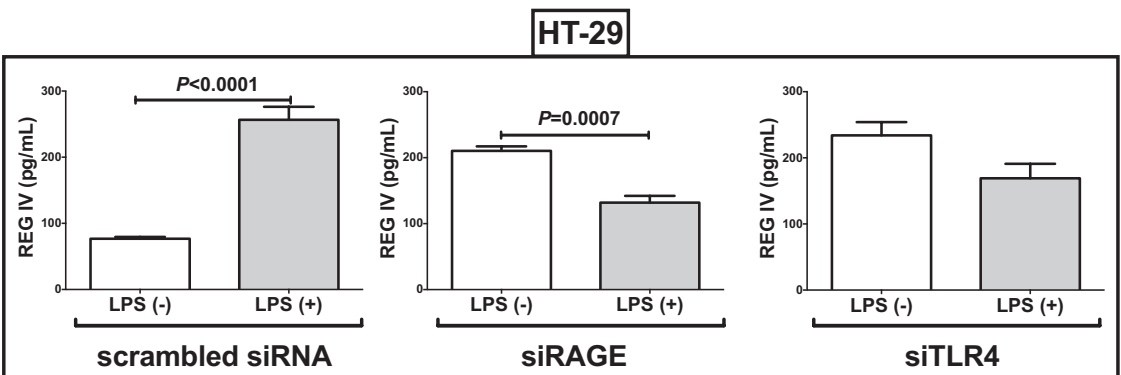
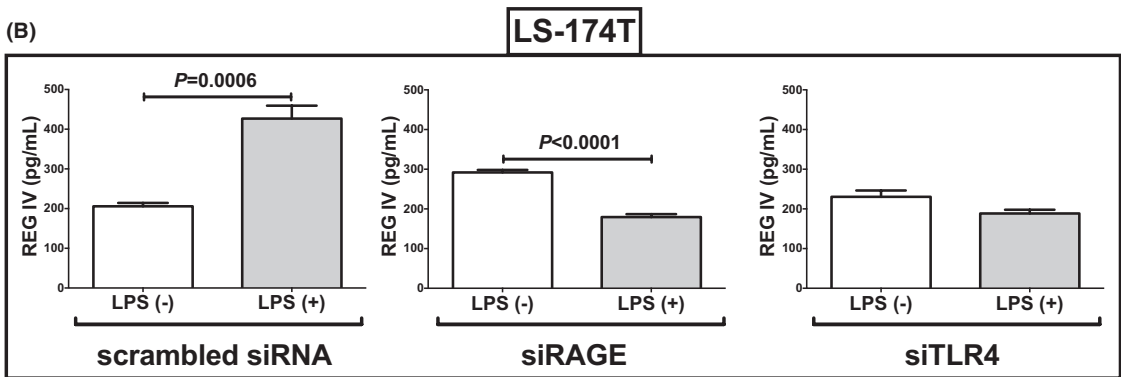
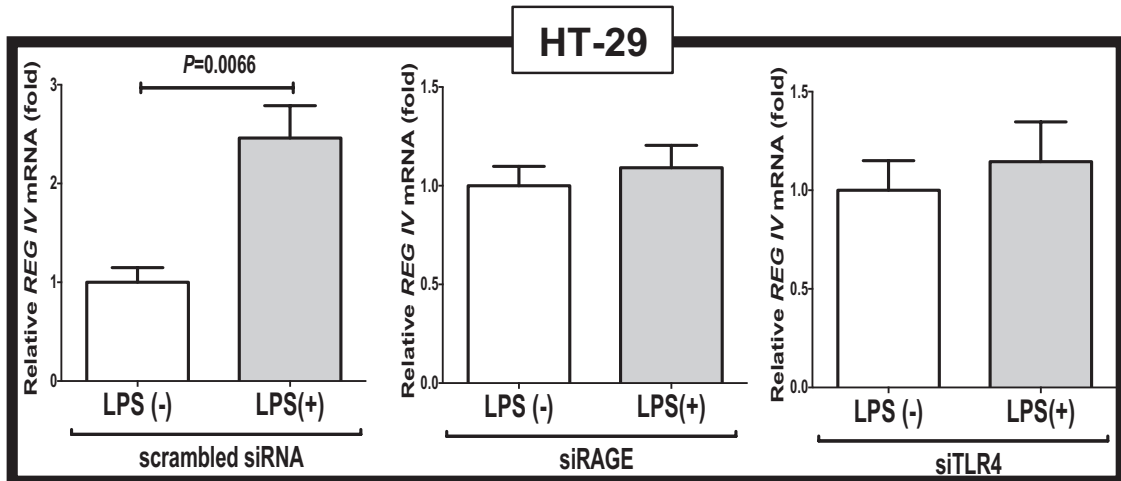
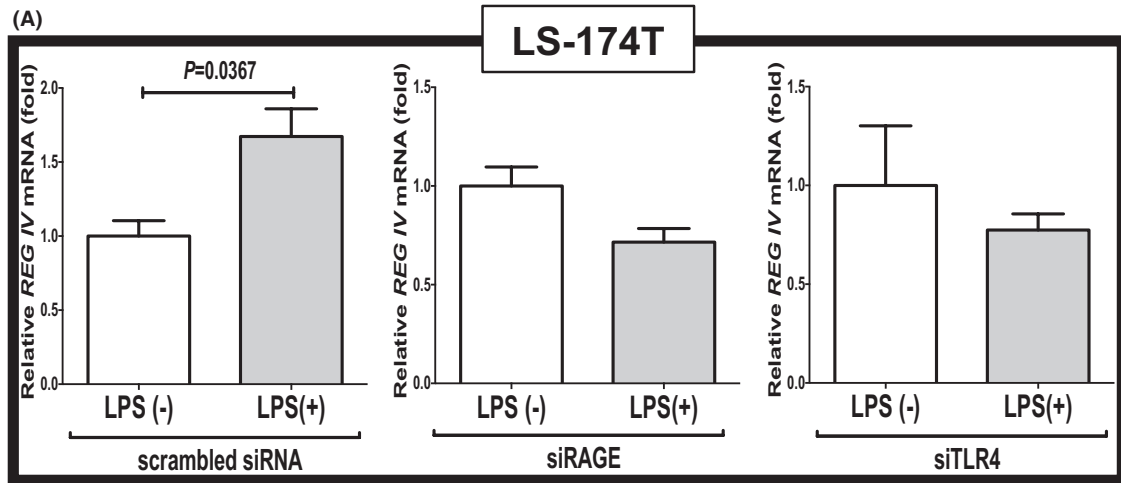


FIGURE 7 Effects of siRNAs directed against *RAGE* and *TLR4* on the LPS-induced gene expression of REG IV. After introduction of siRNAs for *RAGE* and *TLR4* into LS-174 T and HT-29 human intestinal epithelial cells, the cells were incubated with 1 μ g/ml LPS for 24 h. (A) The levels of REG IV mRNA were measured via real-time RT-PCR using β -actin as an endogenous control. (B) The levels of REG IV in the cell culture medium were measured via ELISA. Data are expressed as mean \pm SE for each group ($n = 4$). The statistical analyses were performed using Student's *t*-test

cell proliferation via downregulation of *GATA6* mRNA.^{56,57} We found that miR-363, but not miR-24 or -143, was significantly increased by TNF α stimulation and that the introduction of miR-363 mimic abolished the TNF α -induced REG IV downregulation.¹³

Although REG IV has been reported as a novel target for the enrichment of intestinal epithelial stem cells and mucosal healing,⁵⁸ the mechanism of upregulation of REG IV in human intestinal biopsies from IBD patients has still been unrevealed. The main reasons why the mechanism of REG IV upregulation in human intestinal epithelial cells has been unrevealed may be that we have been unable to establish an in vitro model of REG IV induction in human intestinal epithelial cells. In this aspect, *Reg IV* expression was not analysed in bacterial reconstitution model of mouse colitis¹⁰ although the model would be a good model to see *Reg IV* expression in colitis. Sun et al.⁵⁹ reported that *Reg IV* expression was predominant from the duodenum to the distal colon and that the expression level peaked in the colon in untreated and indomethacin-induced colitis mice. In the present study, we first found that LPS induced REG IV expression in human intestinal epithelial cells in vitro. The findings enable us to investigate how REG IV upregulation occurs not only in the LPS-stimulated cells but also LPS-stimulated animal models.

In this study, we showed that REG IV expression was induced by LPS in human intestinal epithelial cells via downregulation of miR-24. The LPS signal was mediated by RAGE/TLR4 receptors in intestinal epithelial cells. In IBD conditions, damaged intestinal cells were replenished by proliferation of intestinal cells. REG IV could work to proliferate intestinal cells to replenish intestinal mucosa as it is a growth factor for intestinal epithelial cells.

AUTHOR CONTRIBUTIONS

Shin Takasawa: Conceptualization (lead); data curation (equal); formal analysis (equal); methodology (equal); writing – original draft (lead); writing – review and editing (equal). **Chikatsugu Tsuchida:** Methodology (equal); writing – review and editing (equal). **Sumiyo Sakuramoto-Tsuchida:** Investigation (equal); methodology (equal); writing – review and editing (equal). **Tomoko Uchiyama:** Investigation (equal); methodology (equal); writing – review and editing (equal). **Mai Makino:** Investigation (equal); methodology (equal); writing – review and editing (equal). **Akiyo Yamauchi:** Investigation (equal); methodology (equal); writing – review and editing (equal). **Asako Itaya-Hironaka:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST

The authors confirm that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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