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β-Glucan from *Saccharomyces cerevisiae* induces SBD-1 production in ovine ruminal epithelial cells via the Dectin-1–Syk–NF-κB signaling pathway

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ABSTRACT

The ruminal mucosal epithelium can secrete defensins, which play a key role in innate and adaptive immunity and are considered potential replacements for antibiotics. Of these, sheep β-defensin-1 (SBD-1) is one of the most potent molecules produced by ovine ruminal epithelial cells (ORECs). β-glucan, safe and effective immune activators, can stimulate innate and adaptive immune responses. Here we examined whether β-glucan from *Saccharomyces cerevisiae* can induce SBD-1 expression in ORECs, as well as the underlying mechanism. First, ORECs were cultured, and quantitative real-time PCR (qPCR) and enzyme-linked immunosorbent assay (ELISA) were used to study the effects of different β-glucan concentrations. Then western blotting, immunohistochemistry, and immunocytofluorescence were performed to investigate the regulatory mechanism of β-glucan-induced SBD-1 upregulation. We show that β-glucan can induce the release of SBD-1 from ORECs; the highest SBD-1 mRNA and protein expression was achieved after treatment with 10 μg/mL at 2 and 4 h. Moreover, β-glucan-induced SBD-1 production was mediated by the activation of dendritic-cell-associated C-type lectin 1 (Dectin-1) receptors, Syk, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). These findings highlight the immunomodulatory effects of β-glucan on ORECs.

1. Introduction

In recent years, antibiotics have been used in animal husbandry to prevent animal diseases and promote growth [1]. However, because of the ever-increasing negative impact of antibiotic resistance and antibiotic residues [2], the search for alternative feed additives has intensified. Strategies to improve disease resistance via immune modulation, not only increase health and production performance, but also reduce the dependence on antibiotics in animal husbandry [3–6].

Antimicrobial peptides (AMPs) are important members of the innate immune system and play a key role in resistance to infection by external pathogens [7,8]. Defensins belong to a family of small cysteine peptides with amphiphilic and cationic properties that are thought to form part of the primitive and effective host defense system, and which are widely distributed in vertebrates [9]. Based on different structural characteristics, defensins are divided into three main classes including α-, β-, and θ-defensins [10]. β-defensins are cationic antimicrobial peptides consisting of 38–42 amino acids and are present in nearly all vertebrates [11]. They play a key role in innate and adaptive immunity and can kill a variety of pathogens including bacteria, fungi, viruses and parasites. Additionally, β-defensins are chemotactic attractants of immune cells that are involved in immune regulation [12]. Because of these pleiotropic functions, β-defensins have been actively explored for a class of antibiotic-resistant microorganisms and as new therapeutic agents against certain inflammatory diseases [13].

Defensins can be synthesized by chemical methods [14,15]. However, due to the high cost of chemical peptide synthesis, induction of defensin expression through dietary regulation might be a more efficient alternative method. Previous studies have demonstrated that probiotics such as *Ganoderma lucidum* and lipopolysaccharide can induce defensin expression in humans, rats, or hens, and enhance disease resistance [16–18]. Therefore, choosing a safe and effective method to

Abbreviations: SBD-1, sheep β-defensin-1; ORECs, ovine ruminal epithelial cells; qPCR, quantitative real-time PCR; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; Dectin-1, Dendritic-cell-associated C-type lectin 1; Syk, receptor tyrosine kinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; AMPs, antimicrobial peptides; PBS, phosphate-buffered saline; DMEM, Dulbecco's Modified Eagle's Medium; BSA, bovine serum albumin; IECs, intestinal epithelial cells; SD, standard deviation; ANOVA, analysis of variance; HCECs, human corneal epithelial cells; OOECs, ovine oviduct epithelial cells

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induce defensin expression is thought to be a good way to control and prevent disease. β-glucan, a type of polysaccharide that is widely present in plants, algae, bacteria, yeast and mushrooms, has well-known biological activities such as immunity-enhancing, anti-tumor, antibacterial, anti-viral, and wound-healing activities, in addition to stimulating innate and adaptive immune responses [19,20]. β-glucans are generally considered to be potent immunological activators [21] that can counteract the immunosuppressed state of fish, human, scallops, chickens, and mice by promoting non-specific immune responses, antibody production, or inflammatory responses [22–26]. Moreover, they are biocompatible, biodegradable, and safe for the environment and animal health [27,28].

Although β-glucan has been shown to induce the expression of defensins in rainbow trout, *Argyrosomus regius*, and broiler chickens [26,29,30], their effects on the expression of SBD-1 in ORECs and associated mechanisms remain poorly understood. Recently, one studies showed that Dectin-1, a C-type lectin receptor, uniquely recognizes β-glucan expressed on the surface of most fungi and plays a nonredundant role in host defense against a number of pathogens [31–33]. Many of these receptors are coupled to the Syk kinase, which allows these receptors to signal via CARD9, leading to NF-κB activation, which in turn contributes to the induction of innate immunity [34,35]. Further, recent reports have shown that β-glucan can induce defensin production via Dectin-1 and Syk in human intestinal epithelial cells (IECs) [36]. We therefore tested the effect of β-glucan on the expression of SBD-1 in ORECs and investigated the involvement of the Dectin-1-Syk-NF-κB signaling pathway in the β-glucan-induced expression of SBD-1.

2. Materials and methods

2.1. Reagents

β-glucan (Cat. NO. 346210), laminarin (Cat. NO. L9634), and the NF-κB inhibitor PDTC (Cat. No. P8765) were purchased from Sigma Chem. Co. (Munich, Germany). The Syk kinase inhibitor R406 (Cat. NO. inh-r406) was purchased from InvivoGen (Toulouse, France). The Lipofectamine™ 3000 Transfection Reagent (Cat. NO. L3000008) and Opti-MEM I medium (Cat. No. 31985–062) were purchased from Invitrogen (Shanghai, China). The anti-Dectin-1 antibody (Cat. No. ab140039), anti-Syk antibody (Cat. No. ab155187), anti-rabbit IgG H&L (Alexa Fluor® 680) secondary antibody (Cat. No. Ab175772) were obtained from Abcam (Cambridge, UK). Anti-p-p65 (Cat. No. C3033), anti-p65 (Cat. No. C4764), anti-IκBa (Cat. No. C4814), anti-p-IκBa (Cat. No. C2859), and anti-p-Syk (Tyr525/526) (Cat. No. C87C1) antibodies were obtained from Cell Signaling Technologies (Massachusetts, USA). The anti-β-actin antibody (Cat. No. AA128), HRP-conjugated anti-rabbit secondary (Cat. No. A0208), and HRP-conjugated anti-mouse secondary (Cat. No. A0216) antibodies were obtained from Beyotime Institute of Biotechnology (Shanghai, China). The UltraSensitive™S-P Sensitive Kit (Rat/Rabbit) (Cat. No. kit9710) was obtained from Fuzhou Maixin Biotech (Fujian, China). All of the other chemicals that were used were of analytical grade and obtained from commercial sources.

2.2. Sample collection

In this study, adult Mongolian sheep ($n = 10$) at 7–12 months of age were euthanized under the approval of the Animal Ethics Committee of the Inner Mongolia Agriculture University (License NO. SYXK, Inner Mongolia, 2016-0017). After euthanizing the animals, rumen tissues were harvested (20-cm^2), flushed with ice-cold physiological saline, placed in ice-cold phosphate buffered saline (PBS; Sigma-Aldrich) supplemented with 5% penicillin/streptomycin (Sigma), and transported to a biosafety cabinet for processing.

2.3. Primary OREC culture

The culture methods and conditions for ORECs were as described by our previous research [37]. The cells were cultured at 37 °C with 5% CO_2 in a cell incubator for subsequent testing.

2.4. Dilution of β-glucan

The β-glucan utilized was the glucan from *Saccharomyces cerevisiae*, with a purity of ≥95%. Sterilized deionized water was used as the vehicle for β-glucan dilution. β-glucan was diluted with PBS to a concentration of 10 mg/mL. It was then added to OREC cultures at concentrations of 5, 10, 20, 50, and 100 μg/mL.

2.5. Induction tests

After cells reached 80–90% confluence in the culture flask, they were seeded into black 6-well flat bottom plates (5×10^5 cells per well) in cell culture medium that did not contain fungizone and other antibiotics, and were incubated at 37 °C and 5% CO_2 overnight. Following starvation, the cells were randomly divided into two groups, namely the β-glucan-treated group and the control group. Treatment groups were exposed to a range of β-glucan concentrations (5, 10, 20, 50, and 100 μg/mL) at 37 °C and 5% CO_2 for 8 h. Control group cells were cultured in DMEM/F12 medium without β-glucan. Then, ORECs were stimulated with the optimal concentration of β-glucan (10 μg/mL) that induced the highest expression of SBD-1 for 2, 4, 8, 12, and 24 h; medium alone was used as control group.

2.6. Viability assays

ORECs were seeded in each well of 96-well plates at a density of 2×10^4 cells per well. They were then treated with β-glucan at 5, 10, 20, 50, and 100 μg/mL for 8 h. ORECs were also stimulated with the optimal concentration of β-glucan (10 μg/mL) for 2, 4, 8, 12, and 24 h; *medium* alone was used as a control. Then, ORECs were washed with PBS five times and the supernatant (100 μL) was replaced with DMEM/F12, and 10 μL MTT (Sigma-Aldrich) was added to each well, which was incubated for 4 h at 37 °C. Then, the absorbance was detected at 540 nm using a Hybrid microplate reader (BioTek Inc., Winooski, VT, USA). The assay was performed in triplicate.

2.7. Transfection of ORECs with siRNA

All siRNAs targeting Dectin-1 and Syk were synthesized by Sangon Biotech. Co., Ltd. (Shanghai, China). Nonspecific siRNA with random nucleotides was used in each experiment as a negative control (Table 1).

Table 1
Sequences of short interfering RNAs.

siRNA	Sequences
Dectin-1 siRNA1	GCUUUCAACUGGGCUCUAATT
	UUAGAGCCCAGUUGAAAGCTT
Dectin-1 siRNA2	CCUGAGUACCUUGGGUAUUTT
	AAUACCCAAGGUACUCAGGTT
Dectin-1 siRNA3	GGAUCCAUGUGUCAGACAUTT
	AUGUCUGACACAUGGAUCCTT
Syk siRNA#1	GCGGGUGGAAUAAUCUCAATT
	UUGAGAUUAUUCCACCCGCTT
Syk siRNA#2	CCUACAGGGUCAAGCUCUUTT
	AAGAGCUUGACCCUGUAGGTT
Syk siRNA#3	GCAGUGAAGUUUCUGCUAUTT
	AUAGCAGAAACUUCACUGCTT
Nonsilencing siRNA	UUCUCCGAACGUGUCACGUTT
	ACGUGACACGUUCGGAGAATT

Transfection of Dectin-1, Syk-specific and control siRNA was performed according to the manufacturer's instructions. Briefly, after Dectin-1 or Syk siRNA was incubated with Lipofectamine™ 3000 Transfection reagent for 15 min at room temperature, the mixture was added to each well of the 12-well culture plates. These plates were gently mixed by rocking back and forth. Transfected cells were incubated for 24 h before being stimulated with β-glucan.

2.8. Inhibition tests

Laminarin utilized was the soluble β-glucan from *Laminaria digitata*, with a purity of 98%, commonly used as a Dectin-1 antagonist. ORECs pretreated with laminarin (10, 50, 100, 200, and 500 μg/mL) for 30 min and then stimulated with 10 μg/mL β-glucan were considered the treatment groups. Cells treated only with laminarin, without β-glucan, served as negative controls. Cells treated only with β-glucan but not with laminarin were used as positive controls and cells that did not receive any treatment comprised the control group.

The Syk pathways were blocked using the specific inhibitor R406. ORECs were pretreated with 1 and 5 μM R406 for 30 min and then stimulated with 10 μg/mL β-glucan, which comprised the treatment groups. Cells treated with only R406, without β-glucan, served as negative controls. Cells treated only with β-glucan were used as the positive control group and cells that did not receive any treatment served as a control group.

NF-κB pathways were blocked using the specific inhibitor PDTC (10 μM). Cells were treated with the PDTC for 30 min and then stimulated with 10 μg/mL β-glucan (used as the treatment group). Cells treated only with PDTC, but not β-glucan, served as the negative controls. Cells treated only with β-glucan were used as a positive control and untreated cells also comprised a control group.

2.9. Isolation of RNA, reverse transcription and qPCR

Total RNA was extracted using the AxyPrep™ Multisource Total RNA Miniprep kit (Axygen Scientific, Inc. USA), according to the manufacturer's instructions. It was reverse transcribed into single-stranded cDNA using the PrimerScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Japan). qPCR thermal cycling was performed as follows: 95 °C for 30 s; 45 cycles of 95 °C for 5 s and 60 °C for 34 s. Each reaction was followed by a 46-step melt-curve analysis (95 °C for 5 s, 60 °C for 30 s, and 95 °C for 15 s). The relative abundance of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to the mean expression of *β-actin*. Each experiment was repeated at least three times. Primers are shown in Table 2.

2.10. Polymerase chain reaction (PCR)

For PCR amplification of *Dectin-1*and *β-actin*, cDNA (1 μL) were coamplified in a reaction mixture with a final volume of 50 μL and containing 25 μL Premix Taq (TaKaRa Taq™ Version 2.0 plus dye), 22 μL RNase-free dH_2O, and 20 μM of primers for *Dectin-1* and *β-actin* (Table 2). The reaction conditions were as follows: 5 min at 94 °C; 35 amplification cycles consisting of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s, and a final extension at 72 °C for 7 min. The PCR products were resolved via 1% agarose gel electrophoresis and visualized through ethidium bromide staining.

Table 2
Primer sequences for qPCR.

Gene	GenBank accession	Fragment size (bp)	Primer pair sequences(5′-3′)
β-actin	U39357	208	F: GTCACCAACTGGGACGACA R: AGGCGTACAGGGACAGCA
SBD-1	U75250	133	F:GCTCTTCTTCGTGGTCCTGT R: ACAGGTGCCAATCTGTCTCA
Dectin-1	AM167930.1	163	F:AGCGCTTGTCCCCCTAACT R:ACACTCGCCTTGATATAAACTCCA
Syk	XM_004004064.3	139	F:GGAGGAGGCGGAAGACTACCTG R:CCTCTCGATGGTGTAGTGATGTGC
NF-κB	XM_012159302.1	107	F:AGCACAAGAAGGCAGCACAA R:CCATCAGCAGCAGCAGACA

2.11. ELISA

ORECs were seeded in 6-well plates at a density of 5×10^5 cells per well; the culture supernatants from ORECs were collected and SBD-1 levels in culture media were measured using commercially available ELISA kits (Wuhan Xinqidi Biological Technology, China) according to the supplier's protocol.

2.12. Immunohistochemistry

Rumen tissues were fixed in 4% paraformaldehyde for 48 h, embedded in paraffin, and sectioned to a thickness of 5 μm. After dewaxing and rehydration, antigen retrieval treatment was performed by microwave heating in 0.01 M citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched by incubating the sections in reagent A from the UltraSensitive™S-P Sensitive Kit (Rat/Rabbit) for 10 min at room temperature. After rinsing with PBS, the sections were blocked with reagent B for 1 h at 37 °C and immunostained with anti-Dectin-1 (1:100) and anti-Syk (1:200) antibodies overnight at 4 °C. After washing with PBS for 6 min, the sections were incubated for 10 min with secondary antibody reagent C, and this was followed by further washing with PBS. Then, the sections were incubated for 10 min with reagent D according to the manufacturer's instructions. After washing with PBS for 20 min, the sections were incubated with DAB (DAB-0031, Fuzhou Maixin Biotech, China) solution for 3 min to detect immunostaining and then counterstained hematoxylin (CTS-1097, Fuzhou Maixin Biotech, China). An isotype-matched control antibody was used at equivalent concentrations. All images within each experiment were taken under the same conditions.

2.13. Immunofluorescence

Cells were seeded on cover slips in 12-well plates at 1×10^5 cells/well. The next day, cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.5% triton X-100 for 20 min, and blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature. The cells were then incubated with anti-Syk (1:200) antibody at 4 °C overnight, followed by staining with corresponding secondary antibodies for 2 h at room temperature, and counterstaining with DAPI (Beyotime Institute of Biotechnology). Cells were observed and photographed using a confocal microscope (LSM 800, Zeiss, Oberkochen, Germany). All images within each experiment were taken under the same conditions.

2.14. Western blot analysis

Confluent cells were exposed to β-glucan and total protein of ORECs were extracted with Western and IP cell lysates (Beyotime) according to the manufacturer's instructions. Protein concentrations were quantified using the BCA protein assay reagent (Cat. No. 23250, Thermo, USA), separated by 8–10% SDS-polyacrylamide gel electrophoresis, and then electro-transferred to PVDF membranes (Cat. No. ISEQ00010, Millipore, USA). After transfer, PVDF membranes were blocked for 2 h in 5% BSA in tris-buffered saline with 0.1% tween-20, and incubated overnight at 4 °C with primary antibodies against Dectin-1 (1:750), Syk (1:750), p-Syk (1:750), p65 (1:750), p-p65 (1:500), IκBα (1:750), p-IκBα (1:750), and β-actin (1:1000). Subsequently, PVDF membranes were incubated with the appropriate secondary antibody. Finally, the

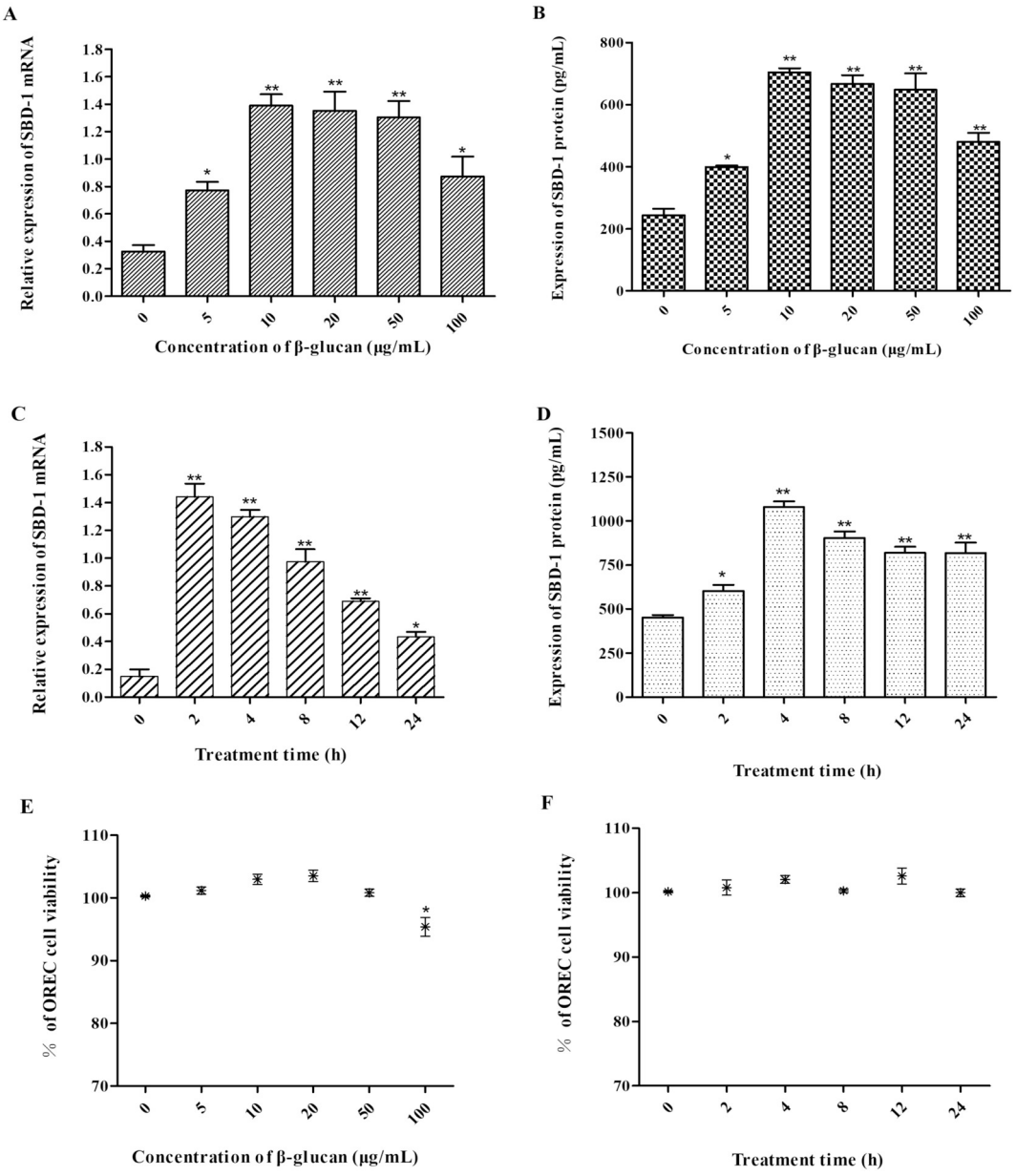

Fig. 1. β-glucan induces SBD-1 mRNA and protein expression. (A–B). SBD-1 mRNA and protein levels after treatment with varying doses (5, 10, 20, 50, 100 μg/mL) of β-glucan for 8 h based on qPCR and ELISA. (C–D). SBD-1 mRNA and protein levels were determined following treatment with β-glucan (10 μg/mL) for 0–24 h by qPCR and ELISA. (E–F) OREC cell activity. The % loss in viability of ORECs following β-glucan treatment was determined using the MTT leakage assay. Data are mean ± SD ($n = 3$). *P < 0.05; **P < 0.01 compared to the control group.

membranes were visualized using a Signal Chemiluminescent Detection System (Chem Studio, GER). The optical density of each band was analyzed by using Image-Pro Plus 6.0 software.

2.15. Statistical analysis

All data were plotted using GraphPad Prism5 software and reported as mean ± standard deviation (SD) of at least three experiments. Comparisons among multiple groups were made by performing an analysis of variance (ANOVA). The independent *t*-test was performed when only two groups were involved. Data analysis was carried out with SPSS version 20.0 (SPSS Institute Inc., USA); statistical significance was set at P < 0.05.

3. Results

3.1. β-Glucan induces SBD-1 mRNA and protein expression

We first measured SBD-1 mRNA and protein levels after treatment with various concentrations (5, 10, 20, 50, 100 μg/mL) of β-glucan for 8 h by qPCR and ELISA. As shown in Fig. 1A and B, treatment of ORECs with 10 μg/mL β-glucan significantly increased the expression of SBD-1 (P < 0.01) compared to that in the control group. Meanwhile, the expression of SBD-1 was determined following β-glucan treatment (10 μg/mL) for 0–24 h. The highest concentration of SBD-1 mRNA and protein was noted at 2 and 4 h post-treatment (P < 0.01; Fig. 1C, D). Next, to determine the possible toxicity of β-glucan, ORECs were treated with β-glucan (5, 10, 20, 50, 100 μg/mL) for 8 h. The survival rate of ORECs following β-glucan treatment was determined by performing MTT assays. It was observed that a β-glucan concentration of

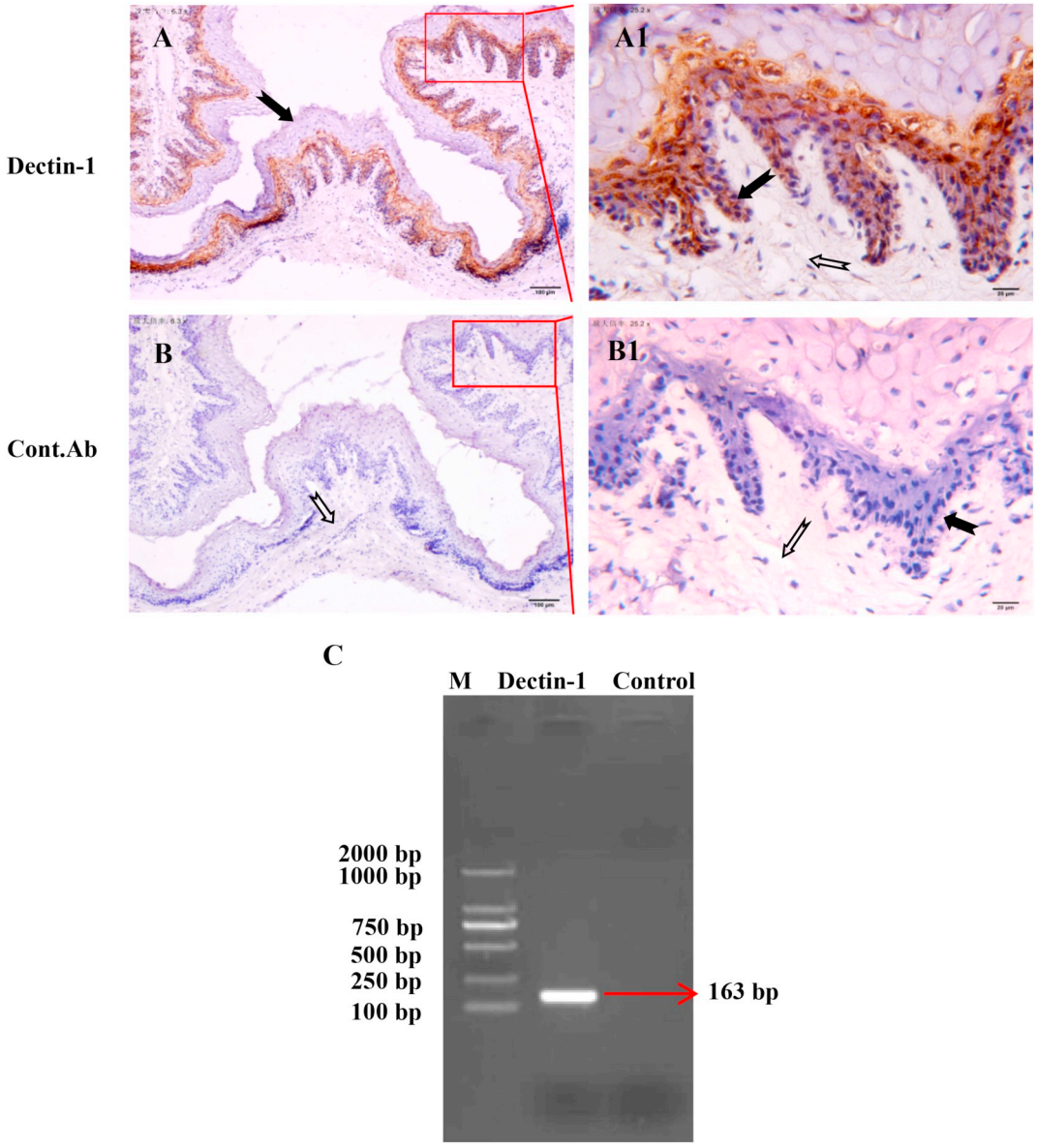

Fig. 2. Dectin-1 is expressed in ovine ruminal mucosa tissue and ORECs. (A) Dectin-1 expression in ovine ruminal mucosa tissue was assessed by immunohistochemistry. Formalin-fixed paraffin embedded sections of mucosa from ovine rumen were stained with an anti-Dectin-1 Ab (brown). (B) Staining with isotype-matched control Ab served as a negative control. Filled arrowheads indicate epithelium and open arrowheads indicate lamina propria. (C) The electrophoresis results of Dectin-1 PCR. M: D2000 DNA marker; Control: negative control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

10 μg/mL was not toxic ($P > 0.05$), whereas 100 μg/mL resulted in significant cell death in ORECs ($P < 0.05$; Fig. 1E). Next, ORECs were stimulated with 10 μg/mL β-glucan for 0–24 h, and MTT assays showed that the cells were viable when exposed to 10 μg/mL β-glucan for 0–24 h ($P > 0.05$; Fig. 1F).

3.2. Dectin-1 is expressed in ORECs

We then assessed whether Dectin-1 is expressed on ORECs; for this, paraffin-embedded sections generated from ruminal mucosa tissues were stained with anti-Dectin-1 and control antibodies. The results showed that Dectin-1 was mainly distributed in the stratum basale and stratum spinosum of the mucosal epithelium (Fig. 2A, B). To further understand the expression of Dectin-1 in ORECs, a PCR product of approximately 163 bp PCR was generated and visualized by electrophoresis using a 1% agarose gel (Fig. 2C). Then, the PCR products were purified and sent to BGI (Beijing) for sequencing. The sequences were analyzed using the BLAST utility from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nig.gov/). BLAST results showed that the sequence had 99% homology with mRNA of ovine Dectin-1 (GenBank: AM167930.1) from GenBank (data not shown).

Next, we attempted to knockdown Dectin-1 expression using Dectin-1 siRNA to further determine whether it is expressed in ORECs. Results shown in Fig. 3A indicate that three different Dectin-1 siRNAs (1, 2, and 3) resulted in decreased expression of Dectin-1 mRNA, and the effect of Dectin-1 siRNA3 was most pronounced. At the same time, MTT results showed that Dectin-1 siRNA (1, 2, and 3) and nonspecific siRNA had no toxic effect on the activity of ORECs ($P > 0.05$; Fig. 3B). The effectiveness of Dectin-1 siRNA3 knockdown was then confirmed by western blotting as shown in Fig. 3C, where Dectin-1 protein levels were reduced compared to control levels ($P < 0.01$). Taken together, these finding show that ORECs express Dectin-1.

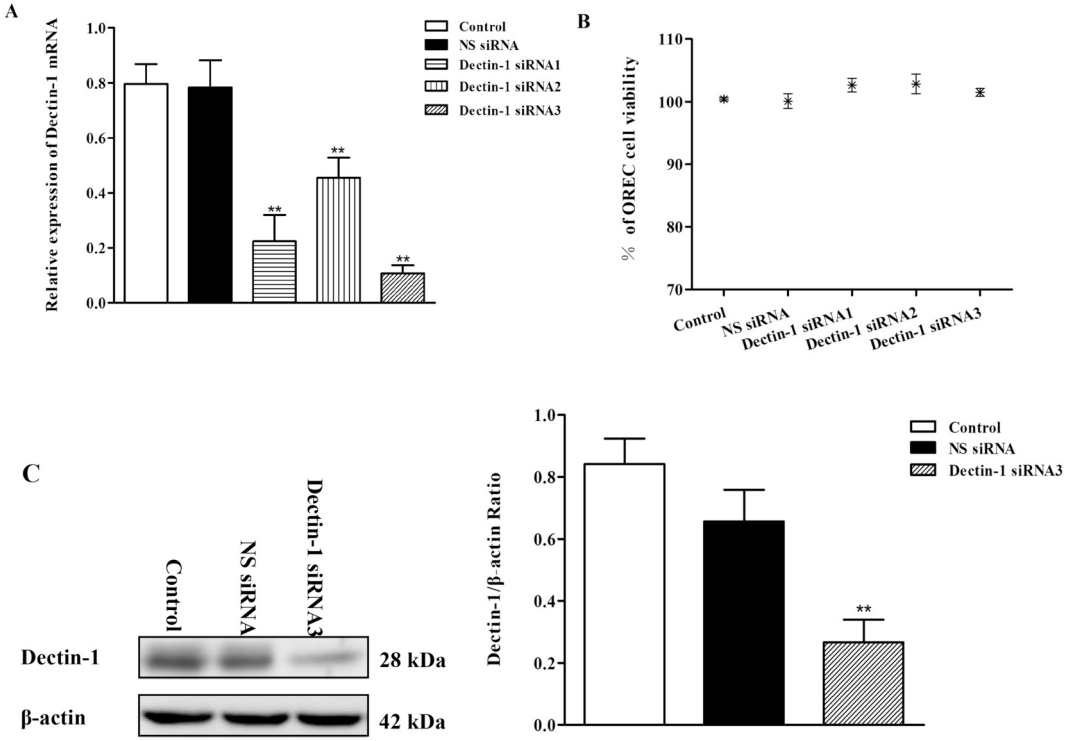

Fig. 3. Dectin-1 is expressed in ORECs. (A) qPCR were used to determine Dectin-1 knockdown with a series of siRNAs. NS, nonspecific siRNA. (B) OREC cell activity. The % loss in viability of ORECs was measured using the MTT leakage assay for cells treated with Dectin-1 siRNA1, 2, and 3 and NS siRNA. (C) Western blot analysis of cell lysates from ORECs revealed signals specific to Dectin-1 (28 kDa). Blots were reacted with an anti-Dectin-1 Ab, and with β-actin antibodies as loading controls. Western blotting also showed a marked decrease in Dectin-1 expression following 24 h of treatment with Dectin-1 siRNA3 compared to that with NS siRNA. Data are mean ± SD ($n = 3$). *P < 0.05, **P < 0.01 compared to the control group.

3.3. β-Glucan induces the expression of SBD-1 via Dectin-1

To determine whether the β-glucan-induced expression of SBD-1 is Dectin-1-dependent, ORECs were transfected with nonspecific siRNA and Dectin-1 siRNA3, or pre-treated with laminarin (10, 50, 100, 200, 500 μg/mL), and then stimulated with 10 μg/mL β-glucan. The effect of Dectin-1 knockdown on SBD-1 expression is shown in Fig. 4A and B. Transfection of ORECs with Dectin-1 siRNA3 suppressed the β-glucan-induced increase in SBD-1 mRNA and protein expression, whereas nonspecific siRNA did not significantly interfere with the ability of β-glucan to stimulate SBD-1 expression (P > 0.05). The effect of laminarin on SBD-1 expression is shown in Fig. 4C and D. Preincubation of ORECs with 100 μg/mL laminarin resulted in an extremely significant decrease in the β-glucan-induced expression of SBD-1, compared to that in the positive control group (P < 0.01). Further, SBD-1 expression was lowest when ORECs were stimulated with 500 μg/mL laminarin. No significant difference was observed between the negative control group and the control group (P > 0.05), indicating that the decrease in SBD-1 expression was not due to cytotoxic effects of the inhibitor. These results indicated that the induction of SBD-1 expression on ORECs by β-glucan is mediated by Dectin-1.

3.4. Syk is expressed in ORECs

We next tested whether Syk, the cell signaling mediator of Dectin-1 activation, is expressed in ORECs. Immunohistochemistry results showed that Syk was mainly distributed in the ruminal mucosal epithelium, whereas the lamina propria and submucosa were not stained (Fig. 5A, B). Furthermore, immunofluorescence results also showed that Syk was expressed in ORECs (Fig. 5C, D).

3.5. β-Glucan-induced SBD-1 expression is Syk-dependent

To test whether Syk is involved in the expression of SBD-1 in β-glucan-induced ORECs, we first examined whether β-glucan could activate the phosphorylation of Syk in ORECs. Western blotting (Fig. 6A) results showed that p-Syk was induced by β-glucan in ORECs, and that activation of these kinases was apparent within 15 min of treatment (P < 0.01 vs. control), which provides direct evidence that Syk signaling is involved in β-glucan-induced OREC activation.

Next, we attempted to knockdown Syk expression by siRNA to determine whether it is involved in the expression of SBD-1 in ORECs induced by β-glucan. As depicted in Fig. 6B, ORECs were treated with Syk siRNA (#1, #2, and #3) or nonspecific siRNA. All three siRNAs significantly reduced the expression of Syk, and the effect of Syk siRNA#2 was the most obvious. Meanwhile, we also found that Syk siRNAs and non-specific siRNA were not toxic to ORECs based on MTT assays (P > 0.05; Fig. 6C). Furthermore, the effectiveness of Syk siRNA#2 was evaluated by western blotting. As shown in Fig. 6D, Syk protein expression was decreased after treatment with Syk siRNA#2 as compared to control group (P < 0.01). Therefore, Syk siRNA#2 was used for subsequent experiments.

To determine whether β-glucan activation involves Syk signaling, ORECs were treated with nonspecific siRNA and syk siRNA#2 or pre-treated with 1 and 5 μM of the Syk inhibitor R406, and then stimulated with 10 μg/mL β-glucan. As shown in Fig. 7A and B, silencing Syk significantly reduced β-glucan-induced SBD-1 expression at mRNA and protein levels compared to that in β-glucan-treated controls (P < 0.01). Furthermore, a significant decrease in SBD-1 expression was observed when ORECs were incubated with 1 or 5 μM R406 prior to β-glucan treatment (P < 0.05). With respect to viability, no significant differences were observed between the negative control group and the control group (P > 0.05; Fig. 7C, D), indicating that the inhibitor had

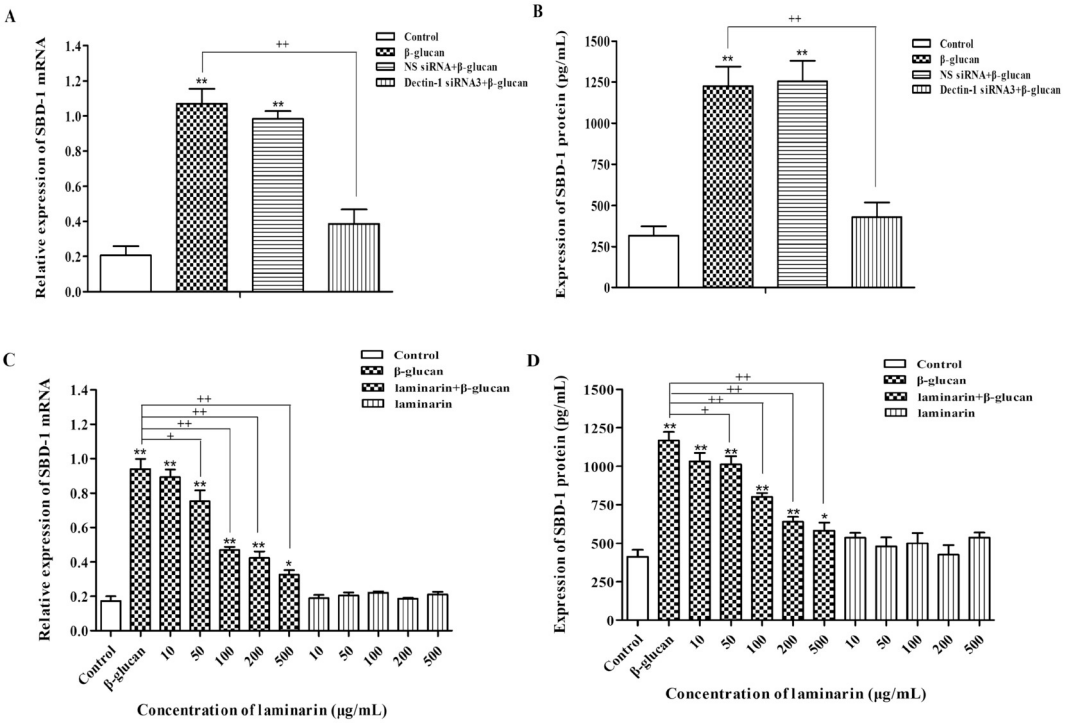

Fig. 4. β-glucan induces the expression of SBD-1 via Dectin-1. (A-B) Transfection of ORECs with Dectin-1 siRNA3 suppressed the β-glucan-induced increase in SBD-1 secretion. NS, nonspecific siRNA. (C-D) Dectin-1 inhibition by laminarin reduced β-glucan-induced SBD-1 secretion in ORECs. Data are mean ± SD (n = 3). *P < 0.05; **P < 0.01 compared to the control group. +P < 0.05, ++P < 0.01 compared to the β-glucan-induced group.

no toxic effect on the cells. Taken together, these results suggest that β-glucan-induced SBD-1 expression in ORECs is mediated by Syk.

3.6. Role of NF-κB in β-glucan-induced SBD-1 expression

Western blot was performed to investigate the possibility that β-glucan activates NF-κB signaling pathways in ORECs. Results showed that NF-κB was activated after treatment with 10 μg/mL β-glucan. Further, the apparent activation of p-IκBα and p-p65 occurred at 15 min (P < 0.01; Fig. 8A).

To further elucidate the function of NF-κB signaling in β-glucan-induced SBD-1 expression, qPCR and ELISA were performed to investigate the effects of the NF-κB inhibitor PDTC on SBD-1 mRNA expression induced by β-glucan. According to qPCR and ELISA results, the β-glucan-induced mRNA and protein expression of SBD-1 was dramatically reduced in ORECs pretreated with PDTC, as compared to that in the group treated with β-glucan alone (P < 0.01), and the reduction was not due to the cytotoxic effects of the inhibitors (P > 0.05; Fig. 8B, C). These results indicate that β-glucan can induce the upregulation of SBD-1 via the NF-κB pathway.

3.7. Activation of NF-κB signaling is mediated by Syk

In the aforementioned experiments, we demonstrated that β-glucan-induced SBD-1 expression is mediated by Dectin-1–Syk, and also found that the upregulation of SBD-1 by β-glucan requires the activation of NF-κB. However, it was unknown whether this activation is related to Syk. To investigate this, cells were divided into three groups as follows: control, β-glucan-treated, and Syk siRNA#2 combined with β-glucan. The results showed that NF-κB mRNA expression and the phosphorylation of p65 were decreased in cells treated with Syk siRNA#2 combined with β-glucan, compared to those in cells treated with β-glucan alone (P < 0.01). However, the expression of these markers was enhanced in cells treated with both Syk siRNA#2 and β-glucan compared to that in cells of the control group (P < 0.05; Fig. 9A, B). This indicated that β-glucan induces SBD-1 expression via the Syk-mediated NF-κB pathway in ORECs.

4. Discussion

It has recently been recognized that mucosal epithelial cells play a vital role in the initiation and regulation of immune responses. Innate immunity, the first line of defense against microorganisms, and defensins play an important role in the immune response [38]. Recently, studies have shown that β-glucan that is derived from the yeast cell walls of species such as *Saccharomyces cerevisiae* have immunomodulatory activities and can upregulate the expression of β-defensins in rainbow trout IECs [29]. However, the regulatory effect of β-glucan on SBD-1 expression in ORECs and the underlying mechanisms remain poorly understood. This study found that SBD-1 is abundantly expressed in ORECs in a concentration and time-dependent manner, and that levels of SBD-1 mRNA and protein were highest when ORECs were separately stimulated for 2 and 4 h with a concentration of 10 μg/mL. The expression of β-defensin (defb) in the *Argyrosomus regius* kidney was recently found to be significantly upregulated after the injection of LPS and β-glucan for 24 h, whereas defb expression was upregulated in kidney and intestinal cells at 4 and 24 h after stimulation with LPS and β-glucan [30]. Co-culture of 1×10^8 particles/mL Zymosan A from *Saccharomyces cerevisiae* with scallop *Argopecten purpuratus* hemocytes for 48 h was found to result in phagocytic activity and the production of reactive oxygen intermediates, as well as the upregulation of big defensin antimicrobial peptide transcription [25]. From the above studies, we speculated that the inductive effect of β-glucan depends on the dose, timing, and mode of administration. Too low of a dose or a short stimulation had no obvious effects, whereas high doses or long stimulation times had an inhibitory effect on cells or tissues.

Based on the aforementioned results, we sought to understand the mechanism of β-glucan-induced SBD-1 expression in ORECs. Previous studies showed that Dectin-1, as the specific receptor of fungal β-glucan, mediates a variety of fungal innate immune responses, and

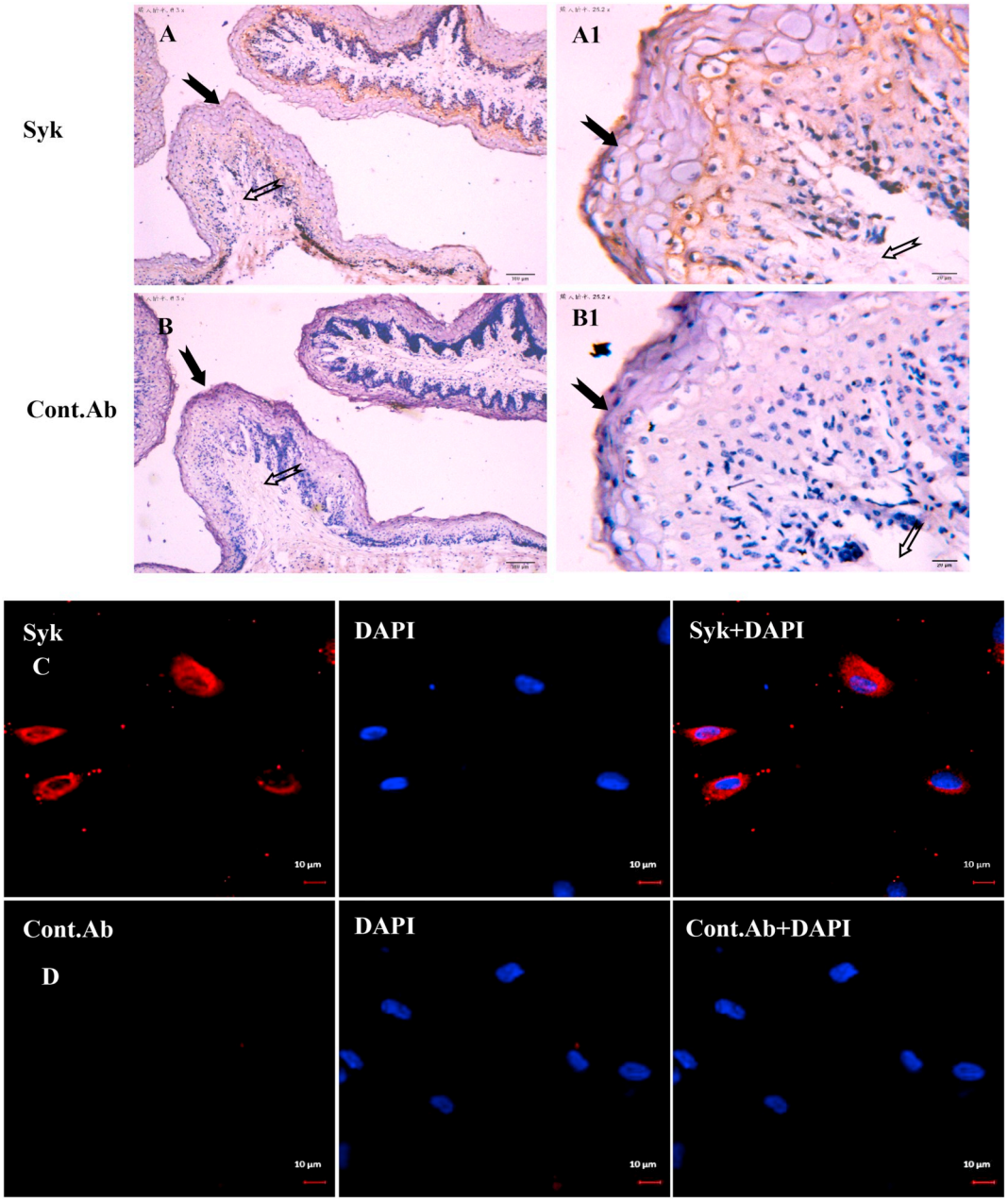

Fig. 5. Syk is expressed in ovine ruminal mucosa tissue and ORECs. (A–B) Syk expression in the ovine ruminal mucosa was assessed by immunohistochemistry. (A, A1) Formalin-fixed paraffin-embedded sections of mucosa from ovine ruminal mucosa were stained with an anti-Syk Ab. (B, B1) Staining with isotype-matched control Ab. Filled arrowheads indicate the epithelium and open arrowheads indicate the lamina propria. (C) ORECs were stained with an anti-Syk Ab (red) and DAPI (blue). (D) Staining with isotype control Ab. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

triggers signal transduction [39–41]. Dectin-1 is now known to be expressed by dendritic cells, macrophages, monocytes, neutrophils, and T cells [42]. Recently, it was reported that Dectin-1 is expressed by human corneal epithelial cells (HCECs), bronchial epithelial cells, and IECs [43–45]. Furthermore, Dectin-1 has been identified in ovine spleen, mesenteric lymph nodes, prescapular lymph nodes, lungs, kidney, skin, and bladder [46]. However, little information is available about the expression of Dectin-1 in the rumen of sheep. Here, we show for the first time, cell-surface expression of Dectin-1 on ruminal mucosal tissue and ORECs by immunohistochemistry and western blotting, which will provide the basis for future studies on the mechanism through which β-glucan induces SBD-1 expression in ORECs.

Recent studies have reported that β-glucan induces the upregulation of human β-defensin-2 and cathelicidin LL37 in HCECs via the Dectin-1 signaling pathway [47]. Our study also identified marked expression of SBD-1 in response to β-glucan in ORECs, as this effect was decreased after using the Dectin-1 siRNA3 and antagonist laminarin, similar to that reported by Iliev et al. [48], indicating that Dectin-1 is necessary for β-glucan-induced SBD-1 expression. Our results indicate that β-glucan might induce the expression of SBD-1 through the surface membrane receptor Dectin-1 of ORECs, thereby functioning in an immunoregulatory role. However, in vitro cell culture models do not fully mimic the complex in vivo microenvironment. Therefore, future research must include in vivo animal models to test whether β-glucan can exert their immunostimulatory effect in the rumen of sheep, and whether Dectin-1 plays a role in immunomodulation in vivo.

In immunocytes, the canonical Dectin-1 signaling pathway involves the intracellular mediator Syk, which becomes phosphorylated upon

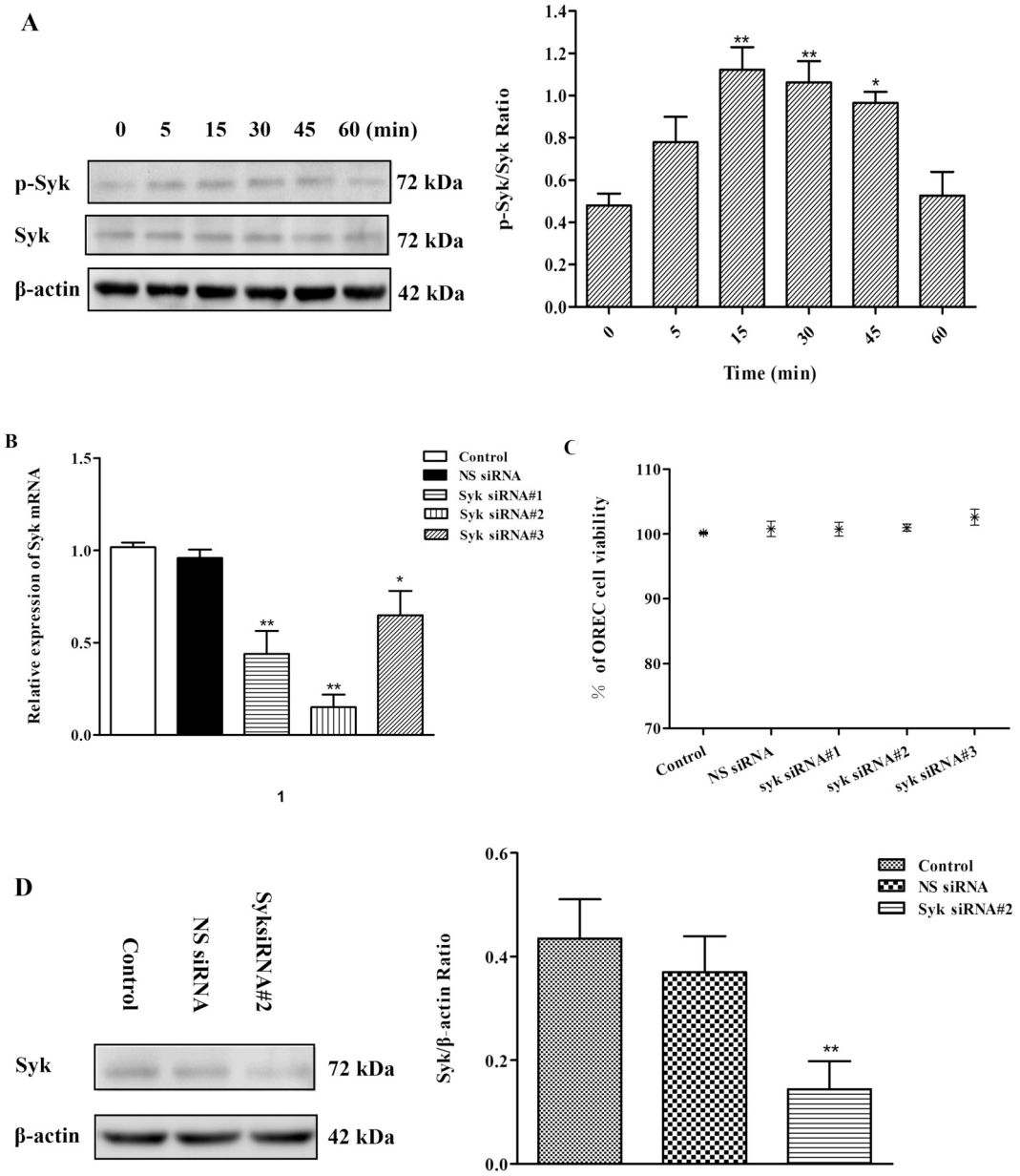

Fig. 6. β-glucan can activate the phosphorylation of Syk in ORECs. (A) Expression of Syk and p-Syk was determined by western blotting at 0, 5, 15, 30, 45, and 60 min after incubation with β-glucans (10 μg/mL). (B) qPCR were used to determine Syk knockdown with a series of siRNAs. (C) OREC cell activity. The % loss in viability of ORECs was measured using the MTT leakage assay for Syk siRNA#1, #2, and #3 and NS siRNA. (D) Western blotting also showed a decrease in Syk protein expression compared to that with NS siRNA with β-actin as the loading control. Data are mean ± SD (n = 3). *P < 0.05; **P < 0.01 compared to the control group.

Dectin-1 activation [49]. In the present study, we demonstrated that Dectin-1 is a membrane receptor involved in the β-glucan-induced upregulation of SBD-1, and further studied the role of its downstream signal adaptor Syk. Here we identified Syk expression in ovine ruminal mucosa tissues and ORECs by immunohistochemistry and immunofluorescence; further, the localization of Syk in ORECs was consistent with that in human IECs, airway epithelial cells, and B cells [36,50,51]. This is the first report of Syk expression in ORECs. According to Kolar et al. [47] β-glucan-induced antimicrobial peptide expression in HCECs involves Syk. Based on these studies, we found that the expression of SBD-1 on β-glucan-induced ORECs is also Syk-dependent because the Syk siRNA#2 and specific inhibitor R406 abrogated most of this secretion, which is consistent with the results of Kolar et al. [47] This finding implies that Syk participates in β-glucan-induced SBD-1 expression.

NF-κB plays a central role in innate and adaptive immune responses [52,53]. Over the past few years, it has been described that Dectin-1 receptors can mediate NF-κB activation, leading to the expression of pro-inflammatory cytokines, chemokines, and antimicrobial peptides, which are important effectors or mediators of innate and adaptive immune responses [36,54–56]. Canonical NF-κB activation relies on the degradation of IκBα, which is phosphorylated by active IKK [57]. In this study, 10 μg/mL of β-glucan treatment significantly increased the activity of p-IκBα and p-p65 in ORECs, and the activation of these kinases was apparent after 15 min. Furthermore, pretreatment of ORECs with the specific NF-κB inhibitor PDTC drastically decreased β-glucan-induced SBD-1 expression. Therefore, it is reasonable to speculate that the upregulation of SBD-1 by β-glucan might be a programmed process that requires the activation of NF-κB. Similar to our results, Wen et al. [58] showed that 17β-estradiol in ovine oviduct epithelial cells (OOECs)

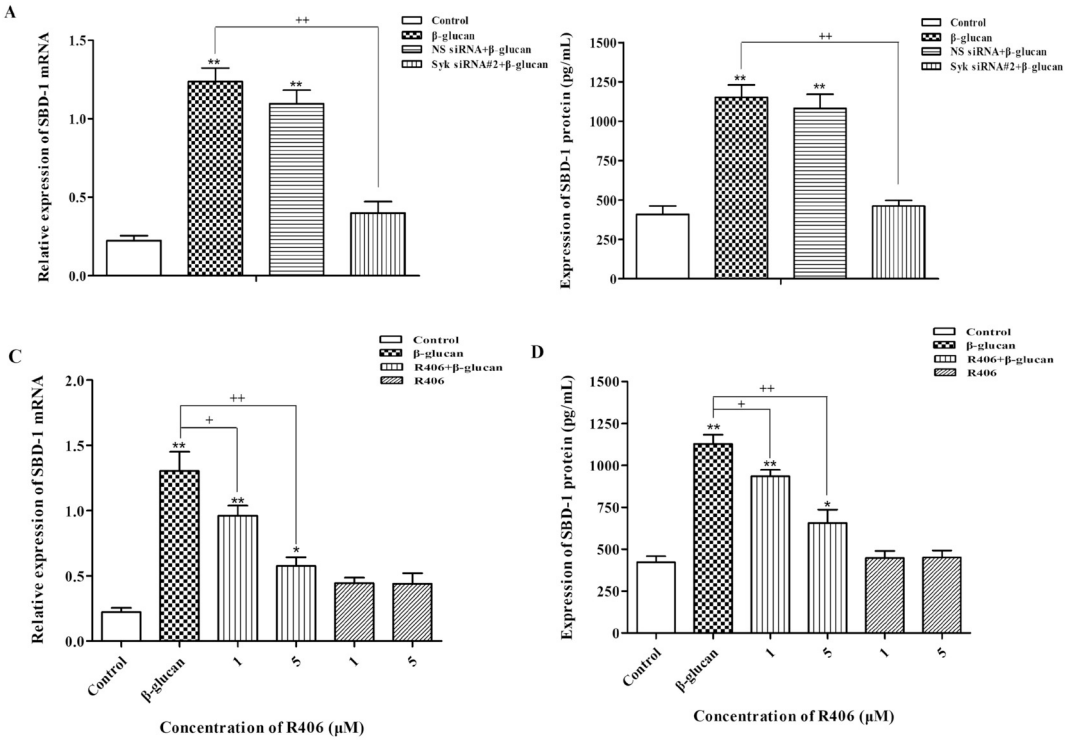

Fig. 7. β-glucan induces SBD-1 expression in a Syk-dependent manner. (A–B) Transfection of ORECs with Syk siRNA#2 suppressed the β-glucan-induced increase in SBD-1 secretion. NS, nonspecific siRNA. (C–D) Syk blockade by R406 reduces β-glucan-induced SBD-1 secretion in ORECs. Data are mean ± SD (n = 3). *P < 0.05; **P < 0.01 compared to the control group. +P < 0.05, ++P < 0.01 compared to the β-glucan-induced group.

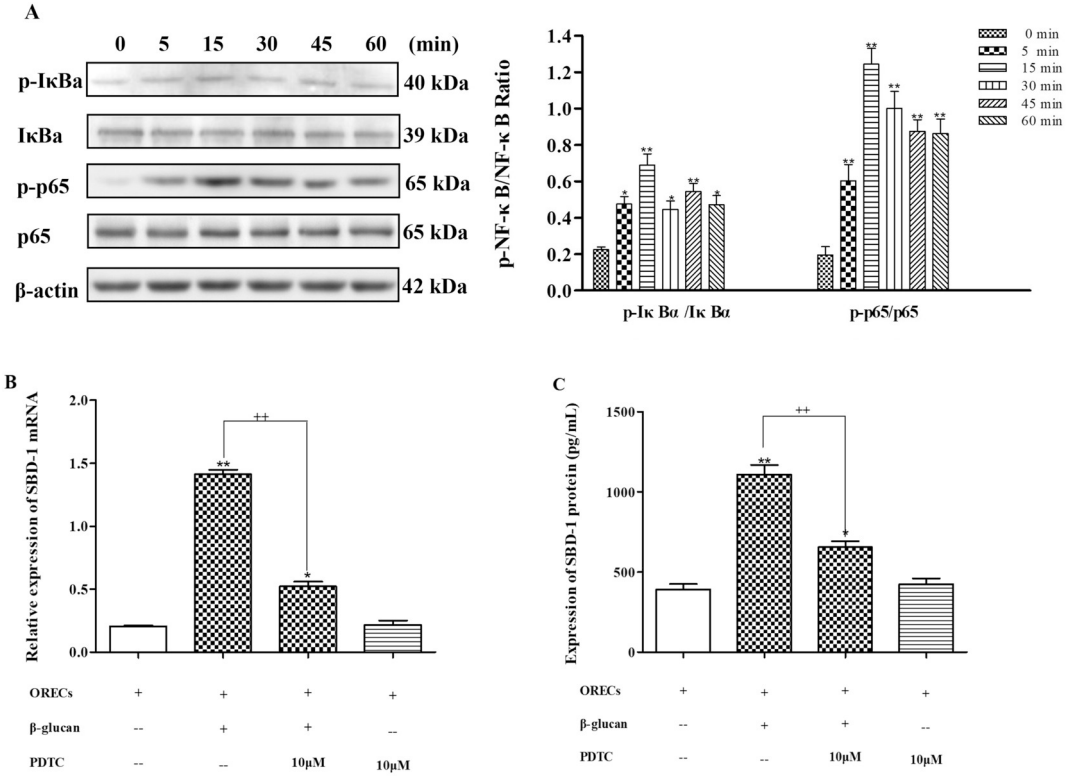

Fig. 8. Role of NF-κB in β-glucan-induced SBD-1 expression. (A) ORECs were treated with β-glucan (10 μg/mL) and harvested at the indicated time points (0, 5, 15, 30, 45, 60 min). Whole-cell lysates were prepared and used for western blot analysis with p-IκBα, IκBα, p-p65, and p65 antibodies. Densitometric analysis of the optical density (OD) of different p-IκBa and p-p65 bands relative to the OD of IκBa and p65 bands was also performed (right). All experiments were repeated at least three times. *P < 0.05, **P < 0.01 compared to the control group. (B—C) Effect of the NF-kB inhibitor on β-glucan-induced SBD-1 expression. Data are mean ± SD (n = 3). *P < 0.05, **P < 0.01 compared to the control group, +P < 0.05, ++P < 0.01 compared to the β-glucan-induced group.

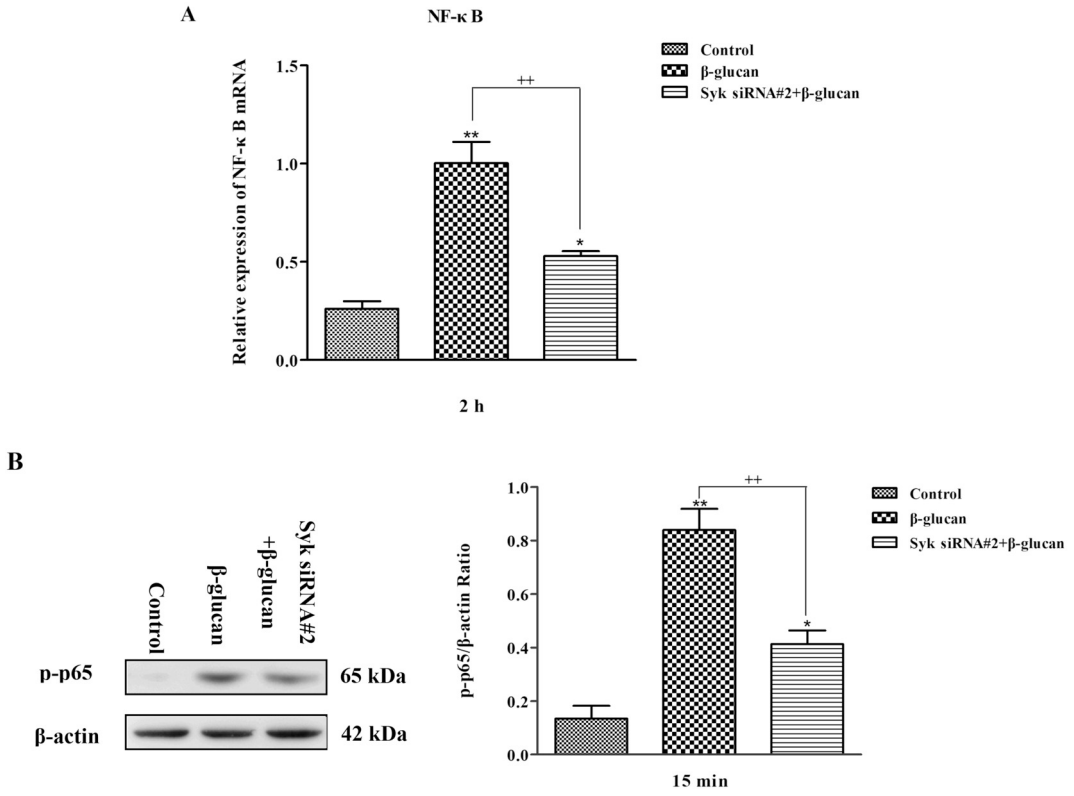

Fig. 9. Activation of NF-κB in β-glucan-induced ORECs is mediated by Syk. (A) Relative abundance of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method, relative to β-actin. (B) Protein amounts are represented by the value shown in gray for the p-p65/β-actin ratio. Statistical analyses were performed using ImageJ and SPSS 20.0 software. Data are mean ± SD (n = 3). *P < 0.05, **P < 0.01 compared to the control group; +P < 0.05, ++P < 0.01 compared to the β-glucan-induced group.

induced SBD-1 expression via NF-κB. In contrast, Li et al. [59] reported that lipopolysaccharide induced SBD-1 expression in OOECs, which was found to be mainly mediated by p38 MAPK. Therefore, the induction of SBD-1 release appears to be dictated by different signaling molecules upon diverse stimuli in different tissues. In addition, we also found that β-glucan significantly increased NF-κB mRNA expression and the phosphorylation of p65; moreover, the Syk siRNA#2 reversed NF-κB activation in ORECs. From this, we hypothesized that NF-κB activation is mediated by the Dectin-1 signal transduction protein Syk. However, determining whether other signal transduction proteins are involved requires further studies.

5. Conclusion

In summary, β-glucan can increase the expression of SBD-1 in ORECs and induce SBD-1 expression via Dectin-1 signaling pathways. These pathways involve Syk and NF-κB activation. We believe that these observations might provide a new explanation to understand how fungal probiotics exert their effects in animals in vivo, and will provide practical guidance for the improved development and utilization of fungal probiotics.

Ethics approval and consent to participate

All animal treatments and procedures used in the present study were approved by the Institutional Animal Care and Use Committee of the IMAU (License NO. SYXK, Inner Mongolia, 2016–0017) with adherence to IMAU guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceived and designed the experiments: MZ, YF. Performed the experiments: MZ, XJ. Analyzed and discussed the data: MZ, XJ. Wrote the paper: MZ, XJ. All authors read and approved the final manuscript.

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