N-glycosylation of R-spondin1 at Asn137 negatively regulates its secretion and Wnt/β-catenin signaling-enhancing activity

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Received March 10, 2015; Accepted March 18, 2016

DOI: 10.3892/ol.2016.4425

Abstract. *N*-glycosylation is a post-translational protein modification with a wide variety of functions. It has been predicted that R-spondin1 (RSPO1) is *N*-glycosylated, although this remains unknown. The present study identified that RSPO1 was *N*-glycosylated at Asn137, and that *N*-glycosylation of RSPO1 negatively influenced its secretion and enhancing effect on Wnt/β-catenin signaling. *In vitro* treatment with peptide-*N*-glycosidase F increased the electrophoretic mobility of RSPO1. Furthermore, treatment of wild-type (wt) RSPO1-overexpressing HT1080 cells with tunicamycin (TM), which inhibits *N*-glycosylation, resulted in a significant reduction in the molecular weight of RSPO1. However, TM treatment had no effect in the RSPO1 mutant whereby the Asn137 residue was replaced by Gln (N137Q). These results demonstrated for the first time that RSPO1 is *N*-glycosylated at Asn137. RSPO1 is a secreted protein that has Wnt/β-catenin signaling-enhancing activity and is expected to have therapeutic applications. The role of *N*-glycosylation in RSPO1 was evaluated by conducting comparative experiments with wt and N137Q RSPO1, which revealed that the N137Q mutant increased the secretion and Wnt/β-catenin signaling-enhancing effect of RSPO1, compared with wt RSPO1. These results suggest that *N*-glycosylation of RSPO1 has a negative influence on its secretion and Wnt/β-catenin signaling-enhancing effect.

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Abbreviations: RSPO, R-spondin; CK1, casein kinase 1; GSK3, glycogen synthase kinase 3; LRP6, low-density lipoprotein receptor-related protein 6; TCF/LEF, T-cell factor/lymphoid enhancer factor; LGR, leucine-rich repeat containing G protein-coupled receptor; ZNRF3, zinc and ring finger 3; RNF43, ring finger protein 43; ER, endoplasmic reticulum; DMEM, Dulbecco's modified Eagle's medium; PNGase F, peptide-*N*-glycosidase F; PBS, phosphate-buffered saline; TM, tunicamycin

Key words: glycosylation, heparin-binding protein, *N*-linked glycosylation, protein secretion, R-spondin1, Wnt signaling

Introduction

R-spondin1 (RSPO1) is a secreted protein that has Wnt signaling-enhancing effects and is essential in gender determination (1-3). Previous studies have demonstrated that RSPO1 exerts proliferative effects on intestinal stem cells, and is expected to have therapeutic applications by enhancing the host tolerance to aggressive chemoradiotherapy and ameliorating systemic graft-versus-host disease following allogeneic bone marrow transplantation (4-6).

Wnt/β-catenin signaling is part of the canonical Wnt signaling pathway and plays essential roles in the development and maintenance of adult tissues. However, its aberrant activation is involved in various types of human cancer (7-14). This signaling pathway regulates gene expression by controlling the stability of β-catenin, which is phosphorylated upon forming a complex with adenomatous polyposis coli, Axin, casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3) (15). The phosphorylation of β-catenin leads to its ubiquitylation and proteasomal degradation. Binding of Wnt to Frizzled and its coreceptor, low-density lipoprotein receptor-related protein 6 (LRP6), along with binding of Dishevelled, results in LRP6 phosphorylation, which in turn activates the binding of Axin, GSK3 and CK1 to Frizzled and LRP6, and inhibits the degradation of β-catenin (15). Stabilized β-catenin translocates to the nucleus, where it forms a complex with the transcription factor T-cell factor/lymphoid enhancer factor (TCF/LEF) and induces the expression of Wnt target genes, including c-Myc, cyclin D1 and matrix metalloproteinases (15). Accordingly, aberrant activation of Wnt signaling is a cause of cancer (15).

Previous experiments have demonstrated that RSPO1 enhances the activity of the Wnt/β-catenin signaling pathway (2). Secreted RSPO1 binds leucine-rich repeat containing G protein-coupled receptor (LGR)4 or LGR5, in addition to the cell-surface transmembrane E3 ubiquitin ligase zinc and ring finger 3 (ZNRF3) or its homolog, ring finger protein 43 (RNF43) (16,17). ZNRF3 and RNF43 act as negative regulators of Wnt/β-catenin signaling by decreasing the membrane levels of Frizzled and LRP6 (17,18). RSPO1 induces membrane clearance of ZNRF3/RNF43 in an LGR4/5-dependent manner and induces LRP6 phosphorylation, thus potentiating Wnt/β-catenin signaling (16,17,19).

Glycosylation is a common post-translational modification that is important for protein stability, folding, secretion and a wide variety of protein functions (20-24). There are four main

types of glycosylation: *N*-, *O*- and *C*-linked glycosylation, and glycosylphosphatidylinositol anchor (25). RSPO1 contains a consensus sequence for *N*-glycosylation (1), but it remains unknown whether RSPO1 is *N*-glycosylated. In *N*-glycosylation, an oligosaccharide chain is covalently linked to an Asn residue in the consensus sequence Asn-Xaa-Ser/Thr (where Xaa is any amino acid with the exception of Pro) of secreted or membrane-bound proteins (26,27). This glycosylation is caused by a continuous enzymatic reaction inside the lumen of the endoplasmic reticulum (ER) and Golgi apparatus (28,29). When a nascent glycoprotein enters the ER, a preformed *N*-linked sugar chain is attached to a particular Asn in the protein (28,29). This attached sugar chain is then processed via the ER and the Golgi apparatus, prior to the secretion of the *N*-glycosylated protein outside the cell (28,29). The present study demonstrated that RSPO1 is *N*-glycosylated at Asn137, and suggested that *N*-glycosylation of RSPO1 has a negative effect on its secretion levels and Wnt/β-catenin signaling-enhancing effects.

Materials and methods

Cell culture. The human fibrosarcoma cell line HT1080 was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) and the human embryonic kidney (HEK) cell line HEK293T was obtained from RIKEN BioResource Center (Tsukuba, Japan). HT1080 and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; catalogue no. 05919; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 5% (v/v) fetal bovine serum (Bovogen Biologicals Pty Ltd., Melbourne, Australia), 100 mg/l kanamycin (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin G (Sigma-Aldrich), 600 mg/l L-glutamine (Sigma-Aldrich) and 2.25 g/l $NaHCO_3$ (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Cells were incubated at 37˚C in a humidified incubator with 5% CO_2.

Plasmids construction. Human complementary (c)DNA coding for RSPO1 was amplified by polymerase chain reaction (PCR) from a cDNA library derived from human prostate cancer PC3 cells (which was kindly donated by Dr Nobuyuki Tanaka, Keio University School of Medicine, Tokyo, Japan), using KOD FX Neo (Toyobo Co., Ltd., Osaka, Japan), according to the manufacturer's protocol. The sequences of the primers used (which were synthesized by Thermo Fisher Scientific, Inc., Waltham, MA, USA) were as follows: Forward, 5'-TTT TCTCGAGATGCGGCTTGGGCTGTGTG-3' and reverse, 5'-TTTTGCGGCCGCCTAGGCAGGCCCTGCAG-3'. The reaction was conducted in a C1000™ thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the cycling conditions were as follows: 94˚C for 2 min, followed by 35 cycles at 94˚C for 15 sec, 63˚C for 30 sec and 68˚C for 1 min. To introduce C-terminal Myc-His_6 tags, polymerase chain reaction (PCR) was performed with primers possessing Myc and His_6 codons. The sequences of the tags were as follows: Myc, 5'-GAACAAAAACTCATCTCAGAAGAGGATCTG-3' and His_6, 5'-CATCATCACCATCACCAT-3'. Subsequently, the RSPO1-Myc-His_6 cDNA was subcloned into the pCI-neo vector (Promega Corporation, Madison, WI, USA). To create the mutant N137Q RSPO1, the Asn137 residue in wild-type (wt) RSPO1 was substituted with a Gln residue by inverse PCR in a C1000™ thermal cycler, using wt RSPO1 plasmids as a template. Inverse PCR was performed using KOD FX Neo according to the manufacturer's protocol. The sequences of the primers (Thermo Fisher Scientific, Inc., Waltham, MA, USA) used for the mutagenesis were as follows: Forward, 5'-GCT CCTCAGCTGCCCAGGGCACCATGGAGT-3' and reverse, 5'-ACTCCATGGTGCCCTGGGCAGCTGAGGAGC-3'. The cycling conditions used for inverse PCR were as follows: 94˚C for 2 min, followed by 20 cycles at 94˚C for 15 sec, 60˚C for 30 sec and 68˚C for 3.5 min.

Establishment of RSPO1-overexpressing cell line. Stable cell lines expressing wt or mutant RSPO1-Myc-His_6 were established by transfecting the pCI-neo vectors expressing wt or N137Q mutant RSPO1-Myc-His_6 into HT1080 cells and using 400 μg/ml G418 (Roche Applied Science, Penzberg, Germany) for the selection of RSPO1-Myc-His_6-clones. Those clones that expressed high levels of Myc-His_6-tagged wt RSPO1 and N137Q RSPO1 were named HT1080-RSPO1-MH and HT1080-RSPO1/N137Q-MH, respectively. Cells transfected with pCI-neo were called HT1080-neo and served as control.

Western blotting. Western blotting was performed using a slightly modified version of a previously described protocol (30-32). Cells were lysed in a lysis buffer [50 mM Tris (Sigma-Aldrich)-HCl (Kanto Chemical Co., Inc., Tokyo, Japan) (pH 7.5), 150 mM NaCl (Wako Pure Chemical Industries, Ltd.), 0.1% (w/v) sodium dodecyl sulfate (SDS; Wako Pure Chemical Industries, Ltd.), 1% (v/v) Triton X-100 (Wako Pure Chemical Industries, Ltd.), 1% (w/v) sodium deoxycholate (Wako Pure Chemical Industries, Ltd.) and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich)] and homogenized with sonication (20 kHz, 50 W, 10 sec, twice) in an ultrasonic homegenizer (UH-50; SMT, Fuji America Corporation, Vernon Hills, IL, USA). The cell lysates were centrifuged at 18,000 x g for 10 min in an MX-307 centrifuge (Tomy Digital Biology Co., Ltd., Tokyo, Japan), and the total amount of protein in each lysate was measured with the Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Sample buffer 6X [350 mM Tris-HCl (pH 6.8), 30% (w/v) glycerol (Kanto Chemical Co., Inc.), 0.012% (w/v) bromophenol blue (Kanto Chemical Co., Inc.), 6% (w/v) SDS and 30% (v/v) 2-mercaptoethanol (2-ME; Kanto Chemical Co., Inc.)] was added to each cell lysate, which was subsequently boiled at 98˚C for 3 min and electrophoresed on 12.5% SDS-polyacrylamide gels using a mini gel slab electrophoresis tank (catalogue no. NA-1010; Nihon Eido Co., Ltd., Tokyo, Japan) and a power supply (catalogue no. NC-1017; Nihon Eido Co., Ltd.) with current and time set at 23 mA and 1.5 h, respectively. Proteins were next transferred to polyvinylidene fluoride membranes (GE Healthcare Life Sciences, Chalfont, UK) using Trans-Blot SD semi-dry electrophoretic transfer cell (catalogue no. 1703940JA, Bio-Rad Laboratories, Inc.) and a power supply with voltage and time set at 12 V and 1 h, respectively. Membranes were blocked with Tris-buffered saline-Tween 20 [TBST; 20 mM Tris-HCl (pH 7.6), 137 mM NaCl and 0.1% (v/v) Tween 20 (Kanto Chemical Co., Inc.)] containing 5% Difco™ skim milk (catalogue no. 232100; BD Biosciences, Franklin Lakes, NJ, USA) for 30 min at

room temperature, and immunoblotted with rabbit polyclonal anti-c-Myc (dilution, 1:5,000 in TBST containing 5% Difco™ skim milk; catalogue no. C3956, Sigma-Aldrich, St. Louis, MO, USA) or mouse monoclonal anti-α-tubulin (dilution, 1:8,000 in TBST; catalogue no. T5168, Sigma-Aldrich) antibodies for 1 h at room temperature. Subsequently, membranes were incubated with TBST containing 5% Difco™ skim milk with secondary horseradish peroxidase (HRP)-conjugated sheep polyclonal anti-mouse immunoglobulin (Ig)G (dilution, 1:5,000; catalogue no. NA931V; GE Healthcare Life Sciences) or donkey polyclonal anti-rabbit IgG (dilution, 1:5,000; catalogue no. NA934V; GE Healthcare Life Sciences) antibodies for 1 h at room temperature. The membranes were washed six times with TBST for 5 min. Detection was performed with an enhanced chemiluminescence reagent (Immobilon Western Chemiluminescent HRP Substrate; EMD Millipore, Billerica, MA, USA) and ImageQuant LAS 4000 mini (GE Healthcare Life Sciences). Quantification of the protein bands was performed with ImageQuant™ TL version 8.1 software (GE Healthcare Life Sciences).

Peptide-N-glycosidase F (PNGase F) treatment of RSPO1. PNGase F treatment was performed as previously described, with a slight modification (22). Cells were lysed with sonication in 50 mM phosphate buffer (pH 7.5), which contained NaH_2PO_4 (Kanto Chemical Co., Inc.), Na_2HPO_4 (Kanto Chemical Co., Inc.), 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 1 mM PMSF and 50 mM 2-ME, and boiled for 5 min to inactivate endogenous enzymes. Next, 0.75% (v/v) Triton X-100 was added to the samples, which were subsequently incubated with 0.5 U PNGase F (Roche Applied Science) at 37˚C for 3 h. The samples were next electrophoresed and analyzed by western blotting as described above.

Semiquantitative reverse transcription (RT)-PCR. Semiquantitative RT-PCR was performed using a slightly modified version of a previously described protocol (22,24,33). Total RNA was extracted from cultured cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, and solutions containing 2 μg total RNA were subjected to RT reaction using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The cDNAs obtained were then used for PCR amplification with EmeraldAmp PCR Master Mix (Takara Bio, Inc., Otsu, Japan) in a C1000™ thermal cycler. The number of PCR cycles for each product was determined upon confirming the efficacy of the amplification and having defined the linear exponential phase of the amplification. The sequences of the primers (Thermo Fisher Scientific, Inc.), number of cycles and annealing temperatures used for semiquantitative RT-PCR were as follows: Transfected exogenous RSPO1, forward, 5'-CTCTGCTCTGAAGTCAACGG-3' and reverse, 5'-GTGATGGTGATGATGCAGATCCTCTTC TGAGATGAG-3', 25 cycles, 63˚C; and β-actin, forward, 5'-CTT CTACAATGAGCTGCGTG-3' and reverse, 5'-TCATGAG-GTAGTCAGTCAGG-3', 20 cycles, 58˚C. The PCR products were electrophoresed on agarose gels, which were prepared in 1X Tris/borate/ethylenediaminetetraacetic acid (EDTA) buffer [50 mM Tris, 48.5 mM boric acid (Wako Pure Chemical Industries, Ltd.) and 2 mM EDTA (Kanto Chemical Co., Inc., Tokyo, Japan)], supplemented with 0.5 μg/ml ethidium bromide (Sigma-Aldrich), and visualized with an ultraviolet illuminator (Desktop Gel Imager Scope21; Optima Inc., Tokyo, Japan).

Detection of secreted RSPO1. Cells were washed twice with phosphate-buffered saline [PBS; containing 137 mM NaCl, 2.7 mM KCl (Kanto Chemical Co., Inc.), 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4] and cultured in serum-free DMEM with 50 μg/ml heparin sodium salt (catalogue no. H3149; Sigma-Aldrich) for 24 h. Conditioned medium (CM) was then collected, and Ni-nitrilotriacetic acid (NTA) agarose (Qiagen GmbH, Hilden, Germany) was added to the CM, and the mixture was incubated for 2 h at 4˚C. Next, the Ni-NTA agarose was washed with washing buffer A [900 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 and 20 mM imidazole (USB Corporation, Cleveland, OH, USA)], and Ni-NTA-bound RSPO1 was subsequently eluted with an elution buffer (900 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 and 500 mM imidazole, pH 7.4). Upon concentration with Ni-NTA agarose, the total amount of protein in the CM was estimated from the total amount of protein in the cell lysates. Samples were next electrophoresed and analyzed by western blotting as mentioned above.

Immunofluorescence. Cells were grown on coverslips at 37˚C for 24 h. To observe the localization of the Golgi apparatus, the cells were washed twice with PBS containing 50 μg/ml heparin sodium salt, fixed with 4% paraformaldehyde (Wako Pure Chemical Industries, Ltd.) for 10 min and permeabilized with 0.1% Triton X-100 for 5 min. Upon blocking with PBS containing 2% bovine serum albumin (BSA; catalogue no. 12660; EMD Millipore), cells were incubated with rabbit polyclonal anti-Golgi reassembly-stacking protein of 65 kDa (GRASP65) antibody (dilution, 1:100; catalogue no. sc-30093; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and mouse monoclonal anti-c-Myc antibody (dilution, 1:100; catalogue no. sc-40; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Subsequently, cells were incubated for 1 h at room temperature with Alexa Fluor® 568-conjugated anti-rabbit IgG (catalogue no. A11036; Invitrogen; Thermo Fisher Scientific, Inc.) and Alexa Fluor® 488-conjugated anti-mouse IgG (catalogue no. A11029; Invitrogen; Thermo Fisher Scientific, Inc.) secondary antibodies diluted 1:500 in PBS containing 2% BSA. Following two washes with PBS, the cells were incubated with 2 μg/ml Hoechst 33258 (Polysciences, Inc., Warrington, PA, USA) for 10 min at room temperature to stain the nuclei. The cells were then washed with PBS and visualized under a fluorescence microscope (EVOS® FL Cell Imaging system; Life Technologies; Thermo Fisher Scientific, Inc.).

Purification of recombinant RSPO1 and luciferase activity assay. Cells were washed twice with PBS and cultured in serum-free DMEM for 24 h with 1% (v/v) Heparin Sepharose 6 Fast Flow (GE Healthcare Life Sciences). Following 24 h, Heparin Sepharose 6 beads were collected, washed twice with PBS and eluted with washing buffer A. The eluted solutions were concentrated with Ni-NTA agarose as aforementioned described, and buffer-exchanged into PBS using Vivaspin® 500 centrifugal filter units (Sartorius AG, Göttingen, Germany), according to the manufacture's

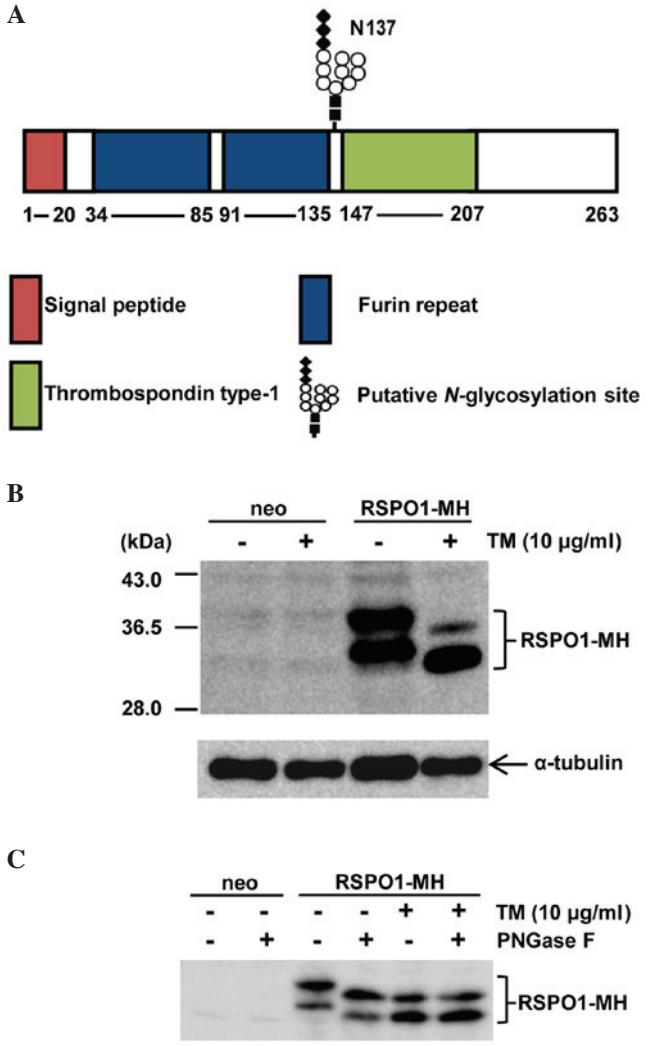

Figure 1. *N*-glycosylation of human RSPO1. (A) Schematic diagram of human RSPO1 protein, which consists of 263 amino acids. The location of a putative *N*-glycosylation site (Asn137) is indicated by a sugar chain. Protein domain architecture denotes signal peptide (red box), furin repeat (blue boxes) and thrombospondin type-1 (green box). (B) HT1080-neo and HT1080-RSPO1-MH cells were treated with or without 10 μg/ml TM for 24 h. Subsequently, cells were lysed, and aliquots of each cell lysate were electrophoresed and immunoblotted with anti-c-Myc or anti-α-tubulin antibodies. (C) Deglycosylation of RSPO1 by PNGase F *in vitro*. HT1080-neo and HT1080-RSPO1-MH cells were treated with or without 10 μg/ml TM for 24 h. Subsequently, cells were lysed and treated with or without PNGase F for 3 h. Samples were then electrophoresed and immunoblotted with anti-c-Myc antibody. RSPO1, R-spondin1; TM, tunicamycin; PNGase F, peptide-*N*-glycosidase F; neo, pCI-neo vector.

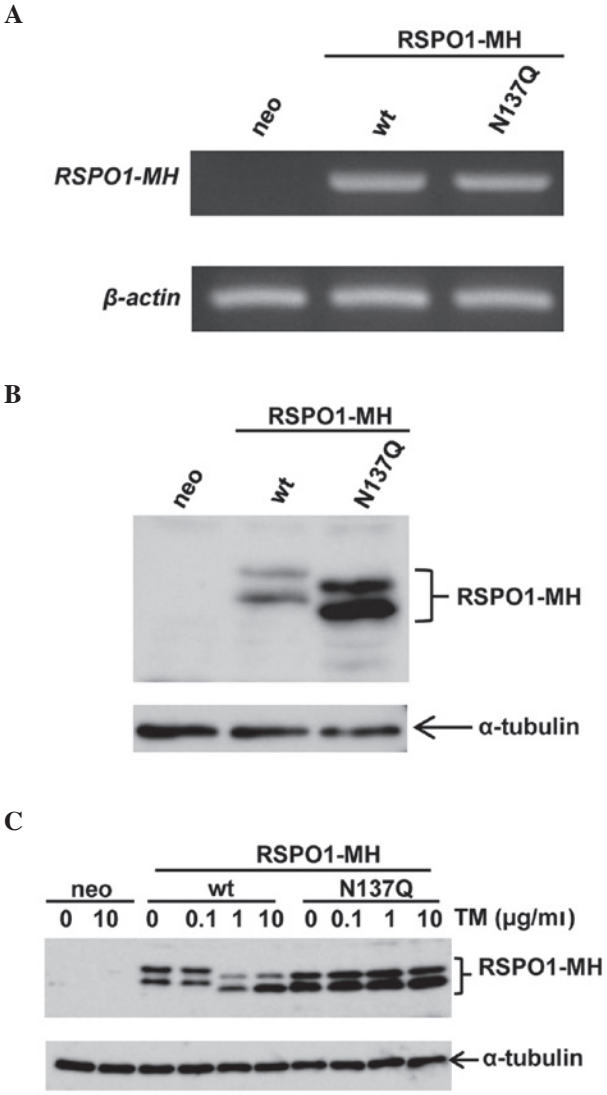

Figure 2. RSPO1 is *N*-glycosylated at Asn137. (A and B) Establishment of wt and *N*-glycosylation-defective RSPO1 mutant-expressing HT1080 cells. (A) Total RNA was isolated from each cell line, and semiquantitative reverse transcription-polymerase chain reaction analysis was performed (B) Cells were lysed, and aliquots of the cell lysates were electrophoresed and immunoblotted with anti-c-Myc or anti-α-tubulin antibodies. (C) Inhibition of *N*-glycosylation of RSPO1. HT1080-neo, HT1080-RSPO1-MH and HT1080-RSPO1/N137Q-MH cells were treated with the indicated concentrations of tunicamycin for 24 h. Subsequently, cells were lysed, and aliquots of the cell lysates were electrophoresed and immunoblotted with anti-c-Myc or anti-α-tubulin antibodies. RSPO1, R-spondin1; TM, tunicamycin; neo, pCI-neo vector; wt, wild-type.

protocol. Purified RSPO1 was electrophoresed and detected by western blotting as aforementioned described, and diluted RSPO1 samples were used for luciferase activity assay.

For the luciferase activity assay, HEK293T cells were plated into 24-well plates, and 24 h later, cells were transiently transfected with 400 ng canonical Wnt signaling reporter Super 8x TOPFlash plasmid (firefly luciferase; catalogue no. 12456; Addgene, Inc., Cambridge, MA, USA) or mutant reporter Super 8x FOPFlash plasmid (firefly luciferase; catalogue no. 12457; Addgene, Inc.) (34) and 20 ng phRL-TK vector (*Renilla* luciferase; Promega Corporation) in the presence of 10% (v/v) Wnt3A-CM produced from L Wnt3A cells (catalogue no. CRL-2647; American Type Culture Collection, Manassas, VA, USA), as previously described (10). Subsequently, cells were treated with equal amounts of wt or mutant RSPO1 protein or vehicle control (PBS). Following 24 h, cells were lysed, and firefly and *Renilla* luciferase activities were respectively measured by Luciferase Assay System (catalogue no. E1500; Promega Corporation) and *Renilla* Luciferase Assay System (catalogue no. E2810; Promega Corporation), according to the manufacturer's protocol, using Infinite® 200 PRO microplate reader (Tecan Group Ltd., Männedorf, Switzerland). TOPFlash and FOPFlash activities were normalized to *Renilla* luciferase activity.

Statistical analysis. Statistical analyses were performed using two-tailed Student's *t*-test. The results were presented as the

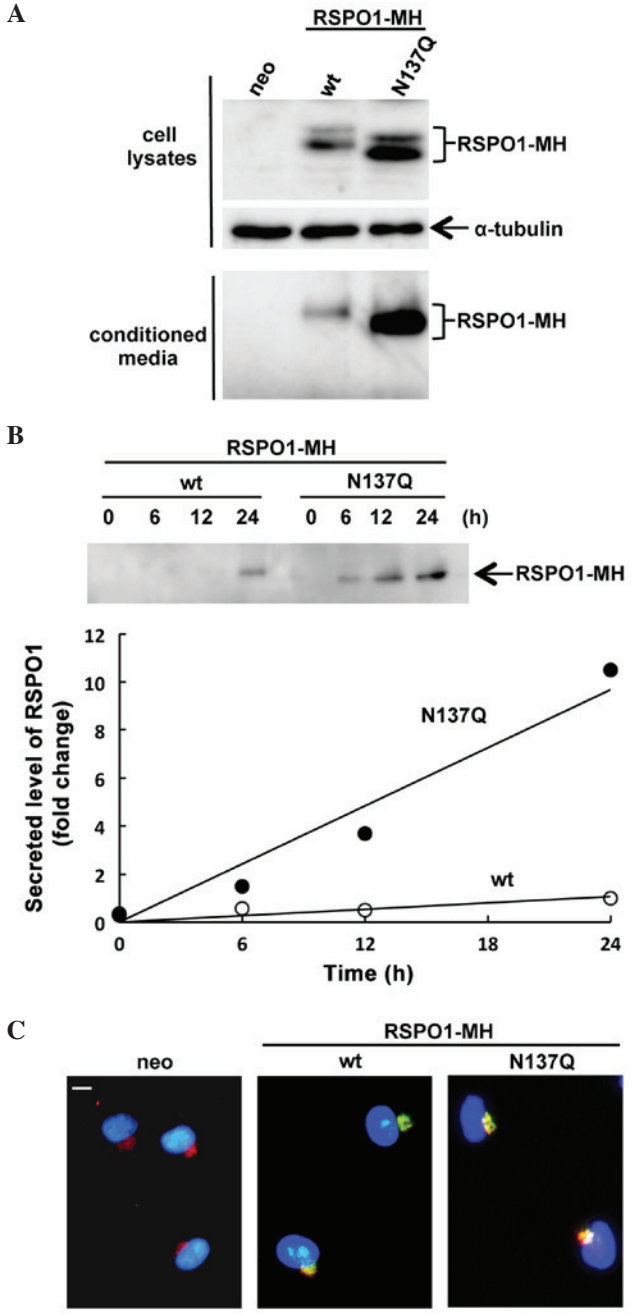

Figure 3. Effect of *N*-glycosylation on the secretion and trafficking of RSPO1. (A) Effect of *N*-glycosylation on the secretion of RSPO1. HT1080-neo, HT1080-RSPO1-MH and HT1080-RSPO1/N137Q-MH cells were cultured in serum-free DMEM for 24 h in the presence of 50 μg/ml heparin, and CM and cell lysates were collected. Samples from CM were concentrated with Ni-NTA agarose, and alongside aliquots of the cell lysates, were electrophoresed and immunoblotted with anti-c-Myc or anti-α-tubulin antibodies. (B) Effect of *N*-glycosylation on the kinetics of RSPO1 secretion. Each cell line was cultured in serum-free DMEM with 50 μg/ml heparin for the indicated time points, and CM were collected. Following concentration with Ni-NTA agarose, the samples were electrophoresed and immunoblotted with anti-c-Myc antibody. Protein bands were quantified using ImageQuant™ TL version 8.1 software to generate the graph. Secreted levels of RSPO1 are the levels of wt RSPO1 protein quantified by the software at 24 h, which is defined as 1. (C) Effect of *N*-glycosylation on the intracellular trafficking of RSPO1. HT1080-neo, HT1080-RSPO1-MH and HT1080-RSPO1/N137Q-MH cells were fixed prior to be stained with Hoechst 33258 (blue), anti-c-Myc antibody (green) and anti-Golgi reassembly-stacking protein of 65 kDa antibody (red), and observed by fluorescence microscopy. Areas of overlapping stains indicating co-localization were represented in yellow when the images were superimposed. Bar, 10 μm. Magnification, x400. RSPO1, R-spondin1; neo, pCI-neo vector; wt, wild-type; DMEM, Dulbecco's modified Eagle's medium; NTA, nitrilotriacetic acid; CM, conditioned media.

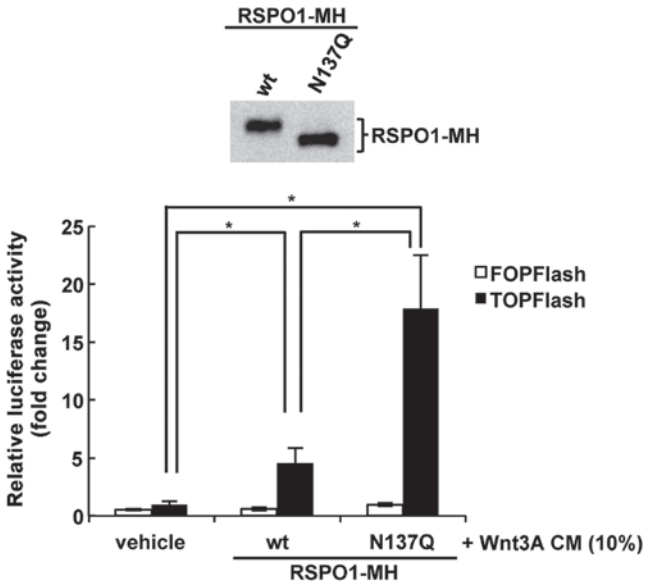

Figure 4. Effect of *N*-glycosylation on the Wnt signaling-enhancing effect of RSPO1. Wt and mutant RSPO1 proteins were purified from their corresponding CM and quantified by immunoblotting (inset). HEK293T cells were transiently transfected with TOPFlash or FOPFlash (firefly luciferase) and phRL-TK (*Renilla* luciferase) vectors, prior to be stimulated with equal amounts of recombinant wt or N137Q mutant RSPO1 in the presence of Wnt3A-CM. Following 24 h, luciferase activities were measured and normalized with *Renilla* luciferase activity. Data are the mean± standard deviation. *P<0.05. RSPO1, R-spondin1; wt, wild-type; CM, conditioned medium.

mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

RSPO1 is N-glycosylated. As human RSPO1 has one putative *N*-glycosylation site at Asn137 in its amino acid sequence (Fig. 1A), the present study tested whether RSPO1 was *N*-glycosylated or not. To examine the presence of *N*-glycosylation in RSPO1, RSPO1-overexpressing HT1080 cells were established (Fig. 1B). Treatment of HT1080-RSPO1-MH cells with tunicamycin (TM; catalogue no. T7765; Sigma-Aldrich), an inhibitor of *N*-glycosylation, increased the electrophoretic mobility of RSPO1-MH, suggesting that RSPO1 was *N*-glycosylated (Fig. 1B). Next, it was assessed whether the increment of RSPO1 electrophoretic mobility by TM was the result of inhibition of *N*-glycosylation. An *in vitro* deglycosylation assay with PNGase F and RSPO1-MH cell lysates also resulted in a significant reduction in the molecular weight of RSPO1-MH (Fig. 1C), which was identical to that of wt RSPO1-MH treated with TM (Fig. 1C). These results suggested that RSPO1 is *N*-glycosylated.

RSPO1 is N-glycosylated at Asn137. The present authors intended to identify the *N*-glycosylation site(s) within RSPO1. To confirm that RSPO1 is *N*-glycosylated at the putative *N*-glycosylation site Asn137 (Fig. 1A), an *N*-glycosylation-defective RSPO1 mutant-expressing HT1080 cell line was established (HT1080-RSPO1/N137Q-MH). Equal amounts of transfected exogenous RSPO1-MH messenger (m)RNA were confirmed to be present in the two stable cell

lines by semiquantitative RT-PCR (Fig. 2A). As expected, the N137Q mutant RSPO1 had an increased electrophoretic mobility, compared with wt RSPO1 (Fig. 2B), and was identical to that of wt RSPO1 treated with 10 μg/ml TM (Fig. 2C). No effects in the molecular weight of the protein were observed following TM treatment in cells overexpressing N137Q mutant RSPO1 (Fig. 2C). These results demonstrated that RSPO1 is *N*-glycosylated only at Asn137.

Effects of N-glycosylation of RSPO1 on its secretion. The present study attempted to clarify the role of *N*-glycosylation in the functions of RSPO1. Since the protein expression levels of N137Q mutant RSPO1 in the cells were higher than those of wt RSPO1 (Fig. 2B), the effect of *N*-glycosylation on the secretion of RSPO1 was examined by comparative experiments with wt and N137Q mutant RSPO1-overexpressing cells. Surprisingly, the levels of secreted RSPO1 were increased in N137Q mutant RSPO1-overexpressing cells, compared with wt RSPO1-overexpressing cells (Fig. 3A and B). This result suggested that *N*-glycosylation of RSPO1 negatively regulates its secretion. Next, the intracellular localization of wt and mutant RSPO1 was evaluated. Co-immunostaining demonstrated that both wt and N137Q mutant RSPO1 were colocalized with the Golgi apparatus marker GRASP65 (Fig. 3C), suggesting that there are no differences in the intracellular localization of wt and N137Q mutant RSPO1.

N-glycosylation of RSPO1 influences Wnt/β-catenin signaling. Since previous studies have demonstrated that RSPO1 enhances Wnt/β-catenin signaling activity synergistically with Wnt (2), the effect of *N*-glycosylation on the Wnt signaling-enhancing activity of RSPO1 was examined in the present study. Since the secreted levels of RSPO1 differed between wt and N137Q mutant RSPO1-overexpressing cells (Fig. 3A), these recombinant RSPO1 proteins were purified from the corresponding CM of each cell culture, and equal amounts of RSPO1 were prepared to treat HEK293T cells for Wnt/β-catenin signaling stimulation (Fig. 4). To determine the effects of *N*-glycosylation on the Wnt signaling-enhancing activity of RSPO1, the TCF/LEF reporter plasmid TOPFlash was used (34). As previously reported, wt RSPO1 enhanced TOPFlash activity synergistically with Wnt3A (10), and N137Q mutant RSPO1 significantly increased TOPFlash activity, compared with equivalent amounts of wt RSPO1 ($P<0.05$; Fig. 4). These results suggested that *N*-glycosylation of RSPO1 reduces its Wnt/β-catenin signaling-enhancing effect.

Discussion

RSPO1 is a secreted protein that exhibits Wnt signaling-enhancing effects (1,2). However, the role of *N*-glyscosylation in the function of RSPO1 remains obscure. Therefore, the present study investigated whether RSPO1 is *N*-glycosylated or not, and demonstrated that RSPO1 is *N*-glycosylated only at Asn137, and *N*-glycosylation of RSPO1 has a negative influence on its secretion and Wnt/β-catenin signaling-enhancing effects.

Recombinant RSPO1-MH from HT1080-RSPO1-MH cell lysates was detected as a double band of ~36.5 kDa (Fig. 1B). In contrast, the purified RSPO1-MH from CM was detected as a single band (Fig. 3A). Therefore, it was hypothesized that the difference in the molecular weight between RSPO1-MH in the cell lysate and that in the CM was due to *N*-glycosylation, since only *N*-glycosylated RSPO1 was expected to be secreted. Contrarily to the above hypothesis, the electrophoretic mobility of each of the bands corresponding to RSPO1-MH from cell lysates was increased following treatment with PNGase F and TM. This result suggested that all intracellular RSPO1 is *N*-glycosylated, and that the double band of wt RSPO1 present in the cells is not due to *N*-glycosylation. Therefore, further studies are required to clarify the mechanism and roles for each of these bands.

The present study also demonstrated that the secreted levels of RSPO1 were increased in non-*N*-glycosylated (N137Q mutant) RSPO1, compared with wt RSPO1. Previous experiments suggested that RSPO1 binds to LGR4/5 with high affinity in the extracellular region of the cell, and is then co-internalized with these receptors inside the cell (16). Thus, the alteration in the secreted levels of RSPO1 due to *N*-glycosylation that was observed in the present study may be explained by changes in the kinetics of secretion, internalization and/or stability of the protein in the extracellular region. However, the amount of RSPO1 present in the CM was not influenced by the internalization and stability of RSPO1 in the extracellular region (data not shown). Therefore, it was speculated that the increased secreted levels of N137Q mutant RSPO1 observed in the present study may be mainly due to an increase in the kinetics of secretion. Indeed, the intracellular localization of N137Q mutant RSPO1, which was present in the Golgi apparatus and ER, was similar to that of wt RSPO1. These results suggested that *N*-glycosylation of RSPO1 may regulate the transportation of RSPO1 from the Golgi apparatus to the extracellular region, rather than its transportation from the ER to the Golgi apparatus. Therefore, these results suggested that the kinetics of RSPO1 secretion may be regulated by *N*-glycosylation. Further studies are required for understanding the regulatory mechanisms of RSPO1 secretion by *N*-glycosylation.

Furthermore, although the N137Q mutant RSPO1 increased the levels of secretion of RSPO1, the amount of intracellular RSPO1 also increased in cells expressing this mutant. Based on these results and the fact that the mRNA levels of exogenous wt and N137Q mutant RSPO1 were equal, it may be hypothesized that *N*-glycosylation of RSPO1 regulates the kinetics of its secretion by influencing its intracellular stability. Several proteins have been reported to be stabilized by *N*-glycosylation (35-37), but there are limited studies reporting whether *N*-glycosylation causes protein destabilization (24). Thus, the present findings may extend the knowledge about the role of *N*-glycosylation in protein recognition and secretion.

The present study revealed that non-*N*-glycosylated RSPO1 increased the Wnt/β-catenin signaling activity significantly more than wt RSPO1 did. RSPO1 binds to heparin with high affinity, and it has been previously suggested that secreted RSPO1 is associated with heparan sulfate proteoglycans of the plasma membrane and extracellular matrix (2). Therefore, the present authors hypothesized that *N*-glycosylation of RSPO1 influences the association between RSPO1 and heparin, thus

affecting Wnt/β-catenin signaling activity. However, in the present study, *N*-glycosylation of RSPO1 had no effect on the association between RSPO1 and heparin (data not shown). Previous studies have proposed that the membrane proteins LGR4 and LGR5 recruit RSPO1 and induce the interaction with RSPO1 and ZNRF3/RNF43, which are antagonists of the Wnt/β-catenin signaling pathway (16,17,38). This interaction leads to membrane clearance of ZNRF3/RNF43 and consequently enhances the Wnt/β-catenin signaling activity (16-18,38). Therefore, *N*-glycosylation of RSPO1 is likely to affect the association between RSPO1 and LGR4/5, and/or between RSPO1 and ZNRF3/RNF43, thus regulating Wnt/β-catenin signaling activity.

It has been reported that aberrant *N*-glycosylation causes multiple diseases (39). In addition, dysregulated activation of Wnt/β-catenin signaling is involved a variety of human tumors (11,12,14), and overexpression of LGR4/5 has been reported in several types of cancer (40,41). Therefore the *N*-glycosylation status of RSPO1 may be a key regulator of cancer by aberrantly activating Wnt/β-catenin signaling. In contrast, RSPO1 has also been observed to exert proliferative effects on intestinal crypt and stem cells, and is therefore expected to have therapeutic applications (4-6). In this context, the non-*N*-glycosylated RSPO1 mutant, which increased Wnt/β-catenin signaling activity in the present study, may be more effective than the above proposed therapeutic applications of RSPO1.

In conclusion, the present study demonstrated that RSPO1 is *N*-glycosylated at Asn137, and the non-*N*-glycosylated N137Q mutant of RSPO1 exhibited increased secretion levels and Wnt/β-catenin signaling-enhancing effects. These results suggest that *N*-glycosylation of RSPO1 has a negative influence on the secretion and Wnt/β-catenin signaling-enhancing effects of RSPO1.

Acknowledgments

The authors would like to thank Professor Randall T. Moon (University of Washington, Seattle, WA, USA) for kindly donating Super 8x TOPFlash and Super 8x FOPFlash plasmids.

The present study was partly supported by grants from the programs Grants-in-Aid for Scientific Research (B) [Japan Society for the Promotion of Science (JSPS); grant no. 24310167) and JSPS Fellows (grant no. 254256). Mr. Yuki Niwa is a JSPS Research Fellow.

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