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Cellular signaling and biological functions of R-spondins

Jeong Kyo Yoon* and Jin-Seon Lee

Program in Stem Cell and Regenerative Medicine, Center for Molecular Medicine, Maine Medical Center Research Institute, 81 Research Drive, Scarborough, ME 04074, USA

Abstract

R-spondins (RSPOs) are a family of cysteine-rich secreted proteins containing a single thrombospondin type I repeat (TSR) domain. A vast amount of information regarding cellular signaling and biological functions of RSPOs has emerged over the last several years, especially with respect to their roles in the activation of the WNT signaling pathway. The identification of several classes of RSPO receptors may indicate that this family of proteins can affect several signaling cascades. Herein, we summarize the current understanding of RSPO signaling and its biological functions, and discuss its potential therapeutic implications to human diseases.

1. Introduction

R-spondins (RSPOs) are a family of four cysteine-rich secreted proteins containing a single thrombospondin type I repeat (TSR) domain. Over the last several years, significant progress has been made in RSPO research, especially with respect to their roles in the activation of the WNT signaling pathway. Recent identification of multiple receptors for the RSPO proteins suggests that this family of proteins can affect several signaling cascades by utilizing different receptors. In this review, we summarize what is currently known about RSPO signaling and biological functions of the RSPO proteins, and discuss their potentials for therapeutic applications to human diseases.

2. *Rspo* genes and their expression

The first member of the *Rspo* gene family to be discovered was *RSPO3*, originally named *PWTSR*, as encoding a novel human protein with the TSR domain [1]. Discovery of mouse and *Xenopus Rspo* genes followed. Mouse *Rspo1* (roof plate-specific spondin), named for its expression in the roof plate of the neural tube in mouse embryos, was identified from the screening of genes specifically expressed in the developing spinal cord [2]. Mouse *Rspo3*, initially named *Cristin1* (Cysteine-rich single thrombospondin type I repeat containing protein1), was independently discovered as a gene differentially expressed in mouse embryos null for the *pMesogenin* gene [3]. These embryos display defects in maturation of the presomitic mesoderm and somitogenesis. Using cell-based functional screening, *Xenopus Rspo* genes were identified as novel activators of WNT/ β -catenin signaling [4].

The *Rspo* gene family is evolutionary conserved and can be found in the genomic and transcript databases of all deuterostomes including the hemichordate, *Saccoglossus*

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*Corresponding author yoonje@mmc.org (J.K. Yoon).

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kowalevskii (acorn worm), the chordate, *Ciona intestinalis* (tunicate), and the echinoderm, *Stongylocentrotus purpuratus* (purple sea urchin) [5]. Four members of *Rspo* gene family are known to exist in most mammals [6] and birds, while three members (missing *Rspo4*) are present in *Xenopus* and zebrafish.

Expression studies of the *Rspo* genes have mainly been performed in mouse and *Xenopus* embryos [4, 7]. These genes are expressed in a broad range of embryonic tissues with different spatio-temporal specificities. In particular, *Rspos* are expressed in several signaling centers known for their critical roles during development. For instance, expression in the dorsal part of the neural tube (or roof plate), tail bud, apical ectodermal ridge (AER) of the limb, and developing brain suggests that *Rspos* play critical roles during development. Additionally, *RSPO* mRNA expression in human adult tissues indicates the broad and diverse roles of *RSPOs* in many different organs and cell types [2, 4].

3. Structure and secretion of the RSPO proteins

The human RSPO proteins range from 234 to 272 amino acids in length (Figure 1A). The amino acid sequences of the RSPO proteins are highly conserved, especially within vertebrate species. Four structurally distinct domains are recognized (Figure 1A): (i) a hydrophilic, putative signal peptide sequence for secretion, (ii) a cysteine-rich furin-like (CR) domain, (iii) a thrombospondin type I repeat (TSR) domain, and (iv) a basic amino acid-rich (BR) domain of variable length. Sequence similarities within the identified domains, especially CR and TSR, are significant and implicate conserved protein functions (Figure 1).

Consistent with the presence of a putative signal peptide sequence, secretion of the RSPO proteins into the extracellular milieu was verified [3, 4]. Moreover, subcellular localization of the RSPO proteins in the endoplasmic reticulum and the Golgi apparatus indicates that RSPOs are processed through the canonical secretory pathway [3]. It is notable that although the RSPO proteins are secreted from cells, the proteins are either poorly detected or nearly undetected in the conditioned medium, suggesting that the RSPO proteins are likely associated with the extracellular matrix (ECM) or cell surface or both [3, 4].

Heparan sulfate proteoglycans (HSPGs), including Syndecans and Glypicans, are located on the cell surface and in the ECM, and function as regulators of various signaling pathways including WNT, FGF, BMP, and SHH [8, 9]. Treatment of cells with soluble heparin or sodium chlorate, an inhibitor for sulfation, remarkably enhanced the free RSPO protein level detected in conditioned medium, and RSPO proteins strongly bound to immobilized heparin [3], suggesting that RSPOs can bind to HSPGs.

Domain deletion studies demonstrated that the RSPO protein lacking the BR domain or both the TSR and BR domains was easily detected as a soluble protein in conditioned medium [3, 4] and displayed significantly less affinity to heparin [3]. Positively charged amino acids enriched in the BR domain promote cell surface retention presumably by interacting with HSPGs [10]. Molecular modeling of the TSR domain of the RSPO4 protein showed that positively charged surface amino acids in the TSR domain may contribute to heparin binding in cooperation with those in the BR domain [11]. Recently, the TSR domain of RSPO3 was shown to bind Syndecans, confirming an interaction between the RSPO proteins and HSPGs [12]. Alternatively spliced isoforms of human RSPO1 lacking the TSR domain [13] and human RSPO4 lacking both the TSR and BR domains (the NCBI protein database) have been identified. Although the precise functions of these isoforms are not known, they may represent more diffusible forms of RSPOs.

The CR domain may be involved in the regulation of RSPO secretion. Within the CR domain of mouse RSPO2, substitutions of cysteine residues 78 and 113 with tyrosine and arginine, respectively led to reduced secretion of the mutant RSPO2 protein [14]. It remains to be determined how these mutations negatively influence the secretion of the mutant protein.

4. RSPO and WNT signaling

4.1. Overview of the WNT signaling pathway

The WNT signaling pathway is one of the key pathways regulating various biological processes in development and homeostasis. Aberrant regulation of WNT signaling often results in pathological conditions including cancer in humans [15, 16]. The WNT ligands activate two major intracellular pathways: known as the canonical (or β -catenin-dependent) [15, 16] and non-canonical (β -catenin-independent) pathways [17–19]. In the canonical WNT pathway, binding of the WNT ligands to their cell-surface receptors, Frizzled (FZD) and LDL-receptor related protein 5/6 (LRP5/6), results in ternary complex formation and induces cytoplasmic accumulation and subsequent nuclear translocation of the β -catenin protein. In the nucleus, β -catenin forms a complex with TCF/LEF transcription factors to regulate target gene transcription.

In the non-canonical WNT pathway, the WNT signal is transduced in a FZD-dependent but LRP5/6-independent manner, and activates two downstream signaling branches. WNT induces cytoskeletal changes via activation of the small GTPases RHOA and RAC1 in the regulation of cell polarity (called the WNT/PCP pathway). WNT also modulates cell adhesion and motility and transcription by NFAT via activation of the heterotrimeric G-proteins, calcium calmodulin-dependent kinase II, and protein kinase C in the WNT/ Ca^{++} pathway.

4.2. RSPO activation of canonical WNT signaling

Xenopus RSPOs were originally identified through the functional screening for canonical WNT signaling activators [4]. Several other studies, confirmed by various assays to measure canonical WNT signaling activation, have shown that RSPOs from different vertebrate species display the properties of the canonical WNT signaling activators [3, 20]. The CR domain of the RSPO proteins is primarily responsible for mediating the activation of the WNT/ β -catenin signaling pathway. Deletion of all or part of the CR domain from RSPO abolishes its ability to activate canonical WNT signaling [3, 4, 21]. The TSR and BR domains are proposed to regulate the strength of RSPO activity on canonical WNT signaling, because the RSPO protein lacking the TSR and BR domains activates canonical WNT signaling less effectively [21].

4.3. Multiple receptors for RSPO in canonical WNT signaling

While RSPO activation of canonical WNT signaling is well accepted, the cell surface receptors for RSPOs and the underlying molecular mechanisms by which RSPO activates canonical WNT signaling remain controversial due to conflicting results. Three studies determined that RSPOs are ligands for the LRP5/6 receptors [3, 14, 22]. Nam et al. (2006) showed by using both a cell-free binding assay and co-immunoprecipitation from cell lysates that the RSPO1 and RSPO3 proteins directly bound to the extracellular domain of the LRP6 receptor and more weakly to the extracellular domain of the FZD8 receptor. RSPO2 was shown to bind the LRP6 receptor in a co-immunoprecipitation assay [14]. The CR domain appears to bind directly to the extracellular domain of the LRP6 receptor [14]. Interestingly, substitution of glutamine 70 to arginine within the CR domain of RSPO2 caused diminished activation of canonical WNT signaling without affecting LRP6 binding

[14]. In another study, RSPO1 was also shown to bind the extracellular domain of the LRP6 receptor in both cell-free and cell surface binding assays [22]. This study determined that the *K_d* of RSPO1-LRP6 interaction was approximately 1.2 nM. The same study found that RSPO1 binding to the FZD receptor was negligible or very weak.

Consistent with these results, overexpression of the LRP6 receptor in cells further enhanced canonical WNT signaling activation by all RSPOs [3, 4, 22]. However, overexpression of the specific FZD receptors, FZD1, FZD5, and FZD8, intriguingly also enhanced RSPO1 activity in canonical WNT signaling; whereas overexpression of other FZD receptors did not [22]. Two WNT antagonists, DKK1 and sFRP1, showed differential inhibitory effects on RSPO2 activity [23]. While DKK1 effectively reversed RSPO2-induced WNT signaling activation, sFRP1 was unable to inhibit RSPO2 activity, suggesting that RSPOs activate canonical WNT signaling by a mechanism distinct from that of WNT. These studies concluded that the RSPO proteins are ligands for the LRP6 receptor. The mechanism by which the FZD receptors mediate RSPO action remains undetermined.

Several other studies, however, failed to detect interaction between the RSPO proteins and the LRP6 receptor, and identified new potential RSPO receptors [4, 12, 24–26]. Carmon et al. (2011) and de Lau et al. (2011) independently identified LGR4, and LGR5, two members of the leucine-rich repeat containing G protein-coupled receptor family, using a cell surface binding assay and by co-immunoprecipitation followed by mass spectrometry, respectively [25, 26]. Both studies failed to detect RSPO binding to the LRP6 receptor. The *K_d* of RSPO binding to the LGR5 family receptors broadly distributed within a nanomolar range, i.e., the *K_d* of RSPO1-LGR5 interaction was about 3.1 nM [25, 26].

Binnerts et al. (2007) identified Kremen1 (KRM1), a transmembrane protein known for negatively regulating the LRP6 receptor by DKK1-mediated endocytosis, as a specific receptor for RSPO1 [24]. Interestingly, in this study, soluble heparin was used in the binding assay to reduce background binding. Because RSPOs are heparin-binding proteins and soluble heparin may modify RSPO activity for canonical WNT signaling activation, it is possible that the RSPO protein complexed with soluble heparin may behave differently from the native protein. Further studies are required to ascertain whether or not this is the case. Independently, RSPO2 was shown to bind KRM1 in a co-immunoprecipitation assay [14].

The reason for the conflicting results is currently unclear. Different experimental approaches such as cell-free binding vs cell-based binding assays used in the different studies may be one of the reasons. Also, activity of co-receptors like HSPGs may potentially modify RSPO interactions with its cognate receptors. Clarification of these conflicting results and an integration of multiple receptor functions in RSPO signaling is a major remaining challenge.

4.4. Synergistic cooperation between RSPO and WNT

One intriguing and crucial aspect of RSPO action is its synergistic cooperation with WNT ligands in activation of canonical WNT signaling. Many studies confirm that co-treatment of cells with the RSPO and WNT proteins generates synergistic activation of canonical WNT signaling in many different cell types and biological contexts [4, 20]. Because there are significant overlaps of *Rspo* and *Wnt* gene expression domains in vivo, for instance in the tailbud, dorsal neural tube, and AER of the limb buds during mouse embryogenesis [4, 7], co-existence of RSPO and WNT proteins is expected to produce robust activation of canonical WNT signaling compared to that induced by the individual protein.

A model based on RSPOs and WNT1 binding [3] may explain this synergistic activation (Figure 2, Model I). The WNT1 and different RSPO proteins were co-immunoprecipitated when they were overexpressed in cultured cells, suggesting that they may form a complex

[3]. Because RSPOs exhibited weak or negligible binding affinity to the FZD receptors and the WNT proteins bound to the FZD receptor with a high affinity [27], one possible scenario is that the WNT-RSPO complex acts as a higher affinity ligand of the WNT signaling receptor complex than RSPO or WNT alone, and enhances the formation of FZD-LRP5/6 complex.

The LGR4/5/6 receptors appear to play a role in synergistic activation by RSPO and WNT (Figure 2, Model II) [25, 26]. In HEK293 cells with siRNA-mediated knockdown of *LGR4* and *LGR5*, RSPO1-induced activation of canonical WNT signaling was severely reduced, whereas activation by WNT3A was unaffected [25, 26]. Overexpression of the LGR4/5/6 receptors rescued the RSPO activity in these cells. Synergistic cooperation between RSPO1 and WNT3A in canonical WNT signaling activation was not observed in *LGR4/5*-knockdown cells [25, 26].

However, LGR5 overexpression in naive HEK293 cells produced an intriguing result. While WNT3A-induced LRP6 phosphorylation and β -catenin stabilization was enhanced in LGR5-overexpressing cells, RSPO1 treatment failed to generate any enhancement [25]. Furthermore, co-treatment of WNT3A and RSPO1 reduced the levels of cytoplasmic β -catenin and the phosphorylation of the LRP6 receptor in an RSPO1 dose-dependent manner. It appears that LGR5 overexpression enhanced the turnover of the phosphorylated LRP6, suggesting that LGR5 may play a role in RSPO/WNT-induced synergistic activation of WNT/ β -catenin signaling.

An inhibitory effect of RSPO1 on LRP6 receptor internalization induced by KRM1 and DKK1 was observed (Binnerts, Kim et al. 2007). This study proposed a model in which the RSPO protein acts as a competing ligand to DKK1 for KRM1, thereby accumulating the LRP6 receptor on the plasma membrane and keeping cells in a more sensitized state for activation by the WNT protein (Figure 2, Model III). Therefore, activation of WNT signaling by RSPO is an outcome of endogenous WNT protein activity in sensitized cells rather than a direct consequence of RSPO-induced signaling. This model also explains why the RSPO and WNT proteins activate β -catenin signaling in a synergistic manner. However, this model remains questionable because the precise mechanism of KRM1 function on canonical WNT signaling, especially LRP6 internalization, is still elusive [28]. LRP6 internalization occurs within minutes after WNT3A stimulation and is an essential step for β -catenin activation [29]. It needs to be resolved how these seemingly antagonistic events, the inhibition and induction of LRP6 internalization by RSPO and WNT3A, respectively, result in a synergistic cooperation in canonical WNT activation.

4.5. RSPO in non-canonical WNT signaling

An additional question regarding RSPO's role in WNT signaling is whether RSPO action is limited only to the canonical WNT pathway. Recently, Syndecans were determined to be novel receptors of RSPOs (Figure 2, Model IV) [12]. The RSPO2 and RSPO3 proteins bound Syndecans with high affinity (K_d , 0.88 nM) via their TSR domain in cell surface binding assays, whereas the RSPO1 and RSPO4 proteins did not. In *Xenopus* embryos, a functional connection of RSPO3 with WNT5A and Syndecan4 was determined by their regulatory functions in gastrulation and head cartilage morphogenesis, two processes regulated by the non-canonical WNT/PCP pathway. *Xenopus* RSPO3 activates WNT/PCP signaling in cooperation with WNT5A by promoting Syndecan4-mediated FZD7/WNT5A complex internalization. RSPO3-induced endocytosis of FZD7/WNT5A complex was attenuated by clathrin-specific inhibitors, demonstrating that RSPO3 is associated with clathrin-mediated endocytosis. Although this study was unable to clearly address how the RSPO protein imposes its activity to the FZD receptor without direct binding, it provided insight into a new role for RSPO in WNT signaling. It remains to be determined whether

Syndecans and other HSPGs are similarly involved in activation of non-canonical WNT signaling by RSPO in mammalian cell models. Additionally, there is little information on the regulatory roles of HSPGs in RSPO-induced activation of canonical WNT signaling. Future studies investigating this specific aspect of HSPG regulation are warranted.

4.6. Reciprocal regulation of *Rspo* and *Wnt* transcription

There is very limited information about how transcription of *Rspo* genes is regulated. Because *Rspos* and *Wnts* are expressed in an overlapping pattern or in the neighboring domains during mouse embryogenesis, it is speculated that they reciprocally regulate each other's expression. Indeed, several studies strongly indicate that this is likely the case. *Rspo1* transcript levels in the spinal cord of mouse embryos was diminished in *Wnt1*-knockout animals and was almost eliminated in *Wnt1/Wnt3a* double-knockout mice [2]. In mouse preosteoblastic MC3T3 cells, *Wnt11* overexpression significantly increased *Rspo2* mRNA level compared to that in control MC3T3 cells, and this effect was reversed by expression of a dominant-negative form of TCF4 [30]. Conversely, loss of the *Rspo2* gene led to reduced *Wnt3* expression in the AER [31] and the developing lung in mice [32]. Mice lacking the *Rspo1* gene also showed reduced *Wnt4* expression in the gonads [33]. Expression of the *Wnt4* gene in the mouse mammary gland temporally overlaps with *Rspo1* expression [34]. Thus, it is highly likely that transcription of both the *Rspo* and *Wnt* genes is regulated by each other's signaling activity, thereby establishing a positive feedback regulatory circuit in specific cell types.

5. Biological functions of RSPOs

Because of the pivotal and diverse roles of WNT signaling in cell proliferation, differentiation, migration, and death during embryogenesis and in the adult [16, 35], RSPO's ability to positively modulate canonical WNT signaling has raised significant interest and initiated investigations in a wide range of in vitro and in vivo biological model systems. Gene targeting approaches in mice and human genetic studies have begun to reveal the in vivo function of each *Rspo* gene. In vitro cell culture approaches are also uncovering roles for RSPOs in new biological and disease contexts.

5.1. In vivo functions of *RSPO/Rspo* genes identified from genetic studies

Recent genetic studies in humans and mice have begun to unravel the in vivo function of the *RSPO/Rspo* gene family. Genetic disorders linked to human *RSPO1* and *RSPO4* mutations have been reported. Mice carrying targeted mutant alleles of all *Rspo* genes, with the exception of *Rspo4*, have been generated and their phenotypes reported. Additionally morpholino-based gene ablation studies in *Xenopus* embryos also reveal *Rspo* gene function during amphibian embryogenesis. In this section, we summarize the phenotypes associated with *Rspo* gene inactivation and mutation.

5.1.1. *RSPO 1/Rspo1*—The in vivo function of *RSPO1/Rspo1* is revealed from both human and mouse genetic studies. Mutations in the human *RSPO1* gene were identified in individuals with female to male (XX) sex reversal or XX true hermaphroditism [13, 36]. Individuals from one family displaying XX sex reversal with palmoplantar hyperkeratosis and predisposition to squamous cell carcinoma of the skin were homozygous for a mutation with a single nucleotide insertion in exon 4, corresponding to codon 36 of the coding sequence immediately after the signal sequence. This mutation results in a stop codon due to a frameshift at the 10th amino acid downstream from the insertion site. Another individual from a different family in the same study was homozygous for a deletion that included exon 4. In another study, an individual with XX hermaphroditism and palmoplantar hyperkeratosis was homozygous for a point mutation that presumably disrupts the splicing between exon 5

and exon 6 of the *RSPO1* gene. This mutation results in a deletion of the second half of the CR domain of the RSPO1 protein. Because the CR domain is a key domain for RSPO-activation of the WNT/ β -catenin pathway, these *RSPO1* mutations cause severe disruption of RSPO1 protein activity to induce WNT/ β -catenin signaling. These mutations highlight the critical role of WNT/ β -catenin signaling in female sex determination in human.

The potential role of RSPO1 as a female sex determinant is further confirmed in gene-targeted mice lacking *Rspo1* [33, 37, 38]. *Rspo1* null XX mice displayed extremely poor fertility and had masculinized ovaries (ovotestis) with epididymis and vas deferens-like structures. The *Rspo1* null ovaries also showed fetal oocyte depletion, XY-specific vascularization, and steroidogenesis. *Wnt4* expression and active WNT/ β -catenin signaling was either absent or severely reduced in the ovaries of *Rspo1* null mice. Interestingly, unlike in humans, loss of the mouse *Rspo1* gene did not result in complete phenotypic male conversion in XX mice.

In most mammals, the sex-determining region Y (*SRY*) gene located on the Y chromosome is necessary for testis development during embryogenesis [39]. In contrast, ovary development is thought to be a passive process resulting from the absence of *SRY* expression in the female gonad. *RSPO1/Rspo1* genetic studies strongly indicate that RSPO1-induced WNT/ β -catenin signaling is an active regulatory cue to induce female differentiation over male differentiation. Indeed, a crucial role of WNT/ β -catenin signaling in ovary development was further confirmed by studies of conditional inactivation [40] and gain-of-function of *Ctnnb1* gene encoding the β -catenin protein in the gonads [41]. In addition, *Rspo1* showed a female-specific expression profile in vertebrates including chicken and turtle [42], implying a conserved role of *Rspo1* in the vertebrate female-sex determination pathway [43].

5.1.2. *RSPO2/Rspo2*—Although the human *RSPO2* gene locus was determined to be involved in genetic susceptibility to Dupuytren's disease, a benign fibromatosis, in a recent genome-wide association study [44], no specific *RSPO2* gene mutations were identified in Dupuytren's disease or other human diseases. However, multiple mouse lines bearing *Rspo2* mutant alleles have been generated by both transgenesis [45, 46] and gene targeting approaches [31, 47]. *Footless* mice are homozygous for an inserted transgene designed to study *H⁺K⁺-ATPase* gene promoter regulation, and showed limb defects with a missing distal region, especially in the hindlimb [45]. Positional cloning revealed that this transgene is inserted into the 5'-upstream region proximal to the *Rspo2* gene; therefore, *footless* is a hypomorphic allele of the *Rspo2* gene [46]. Mice carrying an *Rspo2* null mutant allele were generated by a gene targeting strategy [31, 47]. All reported *Rspo2* homozygous mutant mice died immediately after birth and displayed multiple defects including limb defects, craniofacial and laryngeal-tracheal malformation, and lung hypoplasia [31, 46]. Interestingly, female mice heterozygous for *Rspo2* mutation showed a partially penetrant, age-dependent sterility accompanied with development of polycystic ovaries [31, 45]. However, no study on ovarian development in *Rspo2* homozygous females has been reported.

Abnormal limb development manifested by a disruption of distal skeletal structures, especially in the hindlimbs was observed in *Rspo2* homozygous mutant mice [31, 46, 47]. Reciprocal FGF signaling between limb mesenchyme and overlaying epithelium (or AER at later stage) is crucial to controlling limb outgrowth and patterning [48]. Both mesenchyme-specific *Fgf10* and AER-specific *Fgf8* expression were not affected in *Rspo2* null mutant embryos younger than E10.5 [31]. However, expression of *Fgf10*, *Fgf8*, and *Fgf4*, another *Fgf* gene induced by FGF10 in the AER, were all significantly reduced in the limbs of *Rspo2* null embryos older than E10.5, indicating a delay or failed maturation of the AER [31, 46,

47]. Expression of *Axin2* and the *TopGAL* transgene (an in vivo WNT/ β -catenin signaling reporter) were significantly reduced in the AER of *Rspo2* null mutant embryos [31, 46, 47], indicating that WNT/ β -catenin signaling is responsible for mediating RSPO2 activity in the AER. The existence of genetic epistasis between the *Rspo2* and *Lrp6* genes confirmed that *Rspo2* function is associated with WNT/ β -catenin signaling [46].

Craniofacial defects including clefting of the secondary palate and abnormalities of the skeletal structures derived from branchial arch 1 (BA1) were observed in *Rspo2* null mice [49]. These defects appear to be mainly a direct consequence of improper patterning and morphogenesis of BA1 during embryogenesis. Gene expression of several signaling cytokines and transcription factors including *Dlx5/6*, *Msx1/2*, and *Hand1/2*, was significantly disrupted in BA1 mesenchyme of *Rspo2* null mice [49]. Additionally, laryngeal, tracheal and bronchial cartilage derived from BA2 to 6 was malformed, suggesting multiple roles for *Rspo2* in BA development [46]. Similar to the AER of *Rspo2* null mice, reduced expression of *Axin2* and *TopGAL* was detected within BA1, and genetic epistasis between *Rspo2* and *Lrp6* was also present in craniofacial development [49].

Lung hypoplasia and branching defects were also evident in *Rspo2* mutant mice [32, 46, 49]. The weight of fetal lungs isolated from *Rspo2* mutant mice was only 50% of wild type lungs. However, hypoplasia in the *Rspo2* mutant lung was not associated with differentiation defects of specific types of cells. In lung explant culture, growth and branching was severely retarded in *Rspo2* mutant explants compared to wild type explants [46]. The branching defect was rescued by culturing the *Rspo2* mutant explants in conditioned medium containing the RSPO2 protein. *Rspo2* mutant lungs showed reduced WNT/ β -catenin signaling and synergistic enhancement of hypoplasia in *Rspo2* and *Lrp6* compound mutant mice, indicating that *Rspo2* acts through the WNT/ β -catenin pathway during lung development [46].

In *Xenopus*, inhibition of *Rspo2* expression by injecting *Rspo2* morpholino anti-sense oligonucleotides into four-cell stage embryos impaired the expression of myogenic marker genes, *Myf5* and *MyoD*, and later muscle development [4]. Myogenic *Rspo2* function is mediated by the WNT/ β -catenin pathway, as *Myf5* expression inhibited by *Rspo2* morpholino injection was rescued by β -catenin expression. Recently, it was reported that limb-specific *Myf5* expression was significantly decreased in *Rspo2* null mutant mouse embryos, further supporting the role of *Rspo2* in myogenesis [23].

The *Rspo2* gene was also identified in a genome-wide association study to determine genetic variants for dog coat types [50]. Wire-haired breeds such as the Portuguese water dog are homozygous for the *Rspo2* gene mutation containing a 167 bp insertion in the 3'-untranslated region of *Rspo2* mRNA. This insertion does not cause any change in the *Rspo2* coding sequence, but affects *Rspo2* mRNA stability, thus resulting in an increase of steady-state *Rspo2* mRNA in the skin [50].

5.1.3. *Rspo3*—Targeted inactivation of the mouse *Rspo3* gene impaired normal placental development due to improper vascularization into the labyrinthine layer of the developing placenta [51, 52]. Additional vascular defects in the yolk sac and embryo were also detected in one of the *Rspo3* mutants [52]. In this *Rspo3* null mutant, PECAM staining showed that the primary capillary plexus in the yolk sac formed but failed to remodel, resulting in underdeveloped vasculature. The angiogenic function of *Rspo3* is mediated through WNT/ β -catenin signaling [52], because expression of the *Vegf* gene, a direct target of β -catenin signaling [53], was severely reduced in *Rspo3* null mutants. The placental defect in *Rspo3* null mice resulted in embryonic lethality around E10, which prevents the assessment of *Rspo3* function beyond this developmental stage. With an available conditional *Rspo3* gene

knockout allele [52], we anticipate that additional functions of the *Rspo3* gene during development and postnatal stages will be forthcoming.

In *Xenopus* embryos, inhibition of the *Rspo3* gene by injection of *Rspo3*-specific morpholinos resulted in three major developmental defects [12, 52]. The defects induced by *Rspo3* inhibition appear to be mediated by both the WNT/ β -catenin and WNT/PCP pathways.

First, embryos injected with *Rspo3*-specific morpholinos into all four blastomeres at the four-cell stage displayed ventral edema and vascular defects in tadpoles [52]. Inhibition of the *Rspo3* gene induced a disruption of the balance between endothelial and blood differentiation and promoted blood cell specification. *Msr* expression, which is associated with the lateral plate mesoderm where angioblasts are localized, was diminished, whereas expression of two blood cell markers, α -globin and *Scl*, was expanded in *Rspo3*-morpholino injected embryos and ventral marginal zone explants. Similar to *Rspo3* null mutant mice, *Vegf* expression was regulated by *Rspo3* in *Xenopus* embryos through the WNT/ β -catenin pathway.

In the second set of experiments, gastrulation defects were detected in embryos injected with *Rspo3*-specific morpholinos into two dorsal blastomeres at the four-cell stage [12]. Consistent with the gastrulation defects, inhibition of *Rspo3* in animal cap explants repressed activin-induced convergent extension, suggesting that *Rspo3* action during gastrulation is mediated by the WNT/PCP pathway. Interestingly, morpholino inhibition of the non-canonical WNT pathway-specific *Wnt5a* gene reversed the gastrulation defects caused by *Rspo3* overexpression [12].

Third, *Rspo3* morpholino injection into four animal blastomeres of eight-cell stage embryos, a manipulation where inhibition of *Rspo3* function is mainly limited to the neural crest population, caused malformation of the head cartilage in tadpoles [12]. Since a similar defect in head cartilage morphogenesis was induced by *Wnt5a* inhibition, these results suggest that *Rspo3* acts through the WNT/PCP pathway to regulate this process.

5.1.4. *RSPO4/Rspo4*—Anonychia/hyponychia is an autosomal recessive condition defined as total or partial absence of finger and toe nails [54]. Genome-wide mapping studies identified *RSPO4* gene mutations as being causative for anonychia [55–59]. Similar to *RSPO1* mutations, most mutations in the *RSPO4* gene were localized within the CR domain encoded by exons 2 and 3 [56, 58]. In consanguineous families with recessive inheritance of anonychia or hyponychia, the Gln65, Cys73, Cys95, Cys107, and Cys118 (amino acids highly conserved across all four RSPOs, Figure 1B) were mutated, thereby implying that the defects are linked to the inability to activate the canonical WNT pathway. During nail development, *Rspo4* is expressed in the primordia of the claw in mouse embryos [55, 56], and the expression of *RPSO4* transcript was detected in human primary fibroblasts but not in keratinocytes [60]. Therefore, *RSPO4*-mediated β -catenin activation at the dermal-epidermal boundary is likely to be critical for nail development. Furthermore, considering that the nail bed in patients with *RSPO4* mutations is fully formed, *RSPO4* is involved in advanced stages of nail development rather than in early stages [57]. Since the claws were also missing in *Rspo2* null mutant mice, it will be intriguing to investigate whether human *RSPO2* gene mutations are linked to *RSPO4*-independent anonychia/hyponychia [61].

5.2. Regulation of tissue-specific cellular processes by RSPOs

5.2.1. Bone formation—The WNT/ β -catenin signaling pathway plays a key role in bone development and metabolism, exemplified by several null and activating mutations of the

human WNT receptor gene, *LRP5*, linked to osteoporosis pseudoglioma and high bone mass syndromes, respectively [62, 63]. In osteoblast culture, activation of WNT/ β -catenin signaling promoted osteogenic differentiation. In mouse multipotential myogenic progenitor C2C12 cells and primary mouse osteoblast cells, RSPO1 induced expression of the osteoblast differentiation marker genes, osteocalcin and osteoprotegerin, and increased activity of alkaline phosphatase. Nuclear accumulation of β -catenin and activation of WNT/ β -catenin signaling were induced by RSPO1 treatment of these cells [64].

In MC3T3E1 pre-osteoblast cells primed by BMP2 protein, the non-canonical WNT11 ligand enhanced expression of osteoblast-associated genes and osteoblast maturation and mineralization [30]. Interestingly, these WNT11-induced effects were reversed by the expression of a dominant-negative TCF4 construct, suggesting that WNT11 activity contributes to the canonical WNT pathway. The *Rspo2* gene is identified as a potential target to mediate activation of WNT/ β -catenin signaling by WNT11. Similarly, *Rspo2* overexpression promoted osteogenic differentiation in MC3T3 cell pretreated with BMP2, but not in naive MC3T3E1 cells. However, in contrast to WNT11 and the expected actions of RSPO2 protein, osteogenic differentiation promoted by *Rspo2* was not reversed by dominant-negative TCF4, indicating the presence of an alternative RSPO2-mediated downstream signaling cascade that is separate from the canonical WNT pathway for promoting osteoblast maturation and mineralization in MC3T3E1 cells. Given that RSPO3 activates the non-canonical WNT pathway in a Syndecan-dependent manner in *Xenopus* embryos, it would be intriguing to determine whether RSPO2 transmits its activity through the non-canonical WNT pathway in MC3T3E1 cells. In addition, RSPO2-WNT11 signaling has no direct effect on osteoblast differentiation in C2C12 cells. It is possible that RSPO2 plays different roles in uncommitted (C2C12) and committed (MC3T3E1) osteoblastic cells.

5.2.2. Skeletal myogenesis—WNT signaling regulates skeletal muscle differentiation and development at different stages. During primary myogenesis occurring in somites, WNT ligands derived from the dorsal neural tube in combination with SHH from the notochord and the floor plate regulates the expression of *Myf5* [65, 66]. *Myf5* expression is reduced in *Wnt1/Wnt3a* double knockout mice [67]. *Rspo1* and *Rspo3* are also expressed in the dorsal neural tube of mouse embryos [4, 7]. RSPOs along with WNTs from the dorsal neural tube may account for the regulation of *Myf5* expression in the somites. In support of this, *Xenopus Rspo2* functions as a positive regulator of *Myf5* and *MyoD* through the WNT/ β -catenin pathway, as discussed above [4]. Furthermore, recent studies in our laboratory confirmed that *Myf5* expression was regulated by RSPOs in a WNT/ β -catenin pathway-dependent manner in C2C12 cells and primary satellite cells, whereas *MyoD* expression was unaffected [23]. Developing limbs of mouse embryos lacking the *Rspo2* gene also showed reduced *Myf5* expression, suggesting that RSPO signaling is a key regulatory signal for *Myf5* expression during primary myogenesis. We found that all *Rspo* genes except *Rspo4* were expressed in satellite cells, where they are likely to act as autocrine factors. In satellite cells, WNT/ β -catenin signaling is proposed to initiate myoblast differentiation through the inhibition of pro-proliferative Notch signaling through GSK-3 β [68]. Whether RSPOs indeed regulate this transition in satellite cells remains to be determined. In addition to *Myf5* regulation, RSPO treatment during myogenic differentiation of C2C12 cells generates enhanced myogenic differentiation and myotube fusion, suggesting additional roles of RSPOs in various steps of skeletal myogenesis [23].

5.2.3. Pancreatic beta-cell proliferation—WNT/ β -catenin signaling is required for islet beta-cell proliferation [69] and exendin4 induces beta cell proliferation via WNT/ β -catenin signaling [70]. The link of *Rspo1* to WNT/ β -catenin signaling in the beta cell was recently revealed [71]. Endogenous *Rspo1* transcripts in the MIN6 beta cell line were positively regulated by exendin4, and treatment of cells with recombinant RSPO1 protein

stimulated cell proliferation and insulin secretion. However, in vivo analysis using *Rspo1* null mice has failed to provide supporting evidence for roles of RSPO1 shown in the in vitro model, possibly due to compensation by other *Rspos* [72].

5.2.4. Intestinal stem cells—The WNT/ β -catenin pathway is important for maintaining the intestinal epithelium by regulating the self-renewal process occurring along the crypt-villus axis [73]. Canonical WNT ligands function as mitogens for crypt progenitor cells [74]. Consistent with the role of RSPOs as positive regulators of the WNT/ β -catenin pathway, a significant increase in proliferation of crypt epithelial cells, without affecting the number of goblet and paneth cells, was evident in the small intestine and colon of mice injected with the recombinant RSPO1 protein and in RSPO1 gain-of-function mice [20]. This study suggests that RSPO1 acts as a mitogen for intestinal epithelium with negligible effects on maturation and migration of differentiated cells along the crypt-villus axis [20]. This proliferative effect of RSPO1 is conserved in all four RSPO family members [5]. RSPO1 was further shown to effectively support the survival and proliferation of LGR5-positive intestinal stem cells in vitro [75, 76].

5.3. RSPO genes in cancer

Aberrant canonical WNT signaling, i.e., mostly hyperactivation, is strongly associated with many forms of human cancer. From three different genetic screenings, *Rspo* genes were linked to tumorigenesis in mice [77–80].

First, the *Rspo2* gene was identified as *Int7*, a common integration site 7 of mouse mammary tumor virus (MMTV) associated with mammary tumorigenesis in mice [79]. A single insertion of MMTV near the *Rspo2* locus (at 2.5 to 3.5 kb upstream region) resulted in the preneoplastic and hyperplastic outgrowth, the early events of mammary tumorigenesis. MMTV insertion caused an elevation of *Rspo2* mRNA expression in mammary tissues, indicating a gain-of-function mutation. Interestingly, co-insertion of MMTV into other loci such as *Wnt3a*, *Wnt10*, and *Fgf3/4* induced mammary tumors with or without metastasis, suggesting a functional synergy between *Int7* and these loci in mammary tumorigenesis. *Rspo2* and *Wnt1* co-overexpressing mouse mammary epithelial cells displayed strong invasive properties in three-dimensional culture and metastatic potential in vivo [81]. However, invasive phenotype is not β -catenin dependent, because DKK1 treatment or siRNA knockdown of β -catenin failed to reverse the phenotype [81].

Second, the *Rspo3* gene was identified based on its oncogenic potential from a similar MMTV insertional screening [77]. The oncogenic potential of the *Rspo3* gene was verified by *Rspo3* overexpression in a p53-deficient mammary epithelial cell line [77].

Third, transposon-based genetic screening identified that the *Rspo2* gene was altered in colorectal cancer in mice [80]. Consistent with this screening result, *RSPO2* expression was up-regulated in stable forkhead box Q1 (FOXQ1) expressing human colorectal cancer H1299 cell lines [82]. FOXQ1 is markedly overexpressed in colorectal cancer specimens compared to noncancerous samples, and also shows angiogenic and anti-apoptotic effects in various cancer cell lines [82]. Although the FOXQ1-RSPO2-mediated effect on colorectal cancer is undetermined, RSPO2 is likely one of the candidate FOXQ1 -downstream targets. It remains to be determined whether overexpression of *RSPO2* or other *RSPO* gene is correlated with breast and colorectal cancers in human.

In contrast to the oncogenic function in breast and colon cancers, *RSPO 1* is suggested as a potential tumor suppressor gene in both acute and chronic lymphocytic leukemia [83, 84]. CpG islands in the promoter of *RSPO 1* gene were hypermethylated in human leukemia cell lines and the leukemia patients, resulting in suppression of *RSPO1* gene expression.

Furthermore, humans carrying *RSPO1* null mutations are predisposed to the skin carcinoma [13]. The *RSPO4* gene also showed aberrant DNA methylation in esophageal squamous cells and in human esophageal squamous carcinoma specimens. The *RSPO4* methylation level was positively correlated with smoking duration of individuals [85]. Therefore, it is likely that *RSPOs* can function either as an oncogene or a tumor suppressor gene depending on the types of cancer.

6. Therapeutic implications

6.1. Chemotherapy-induced mucositis and inflammatory bowel diseases

Mitogenic activity of RSPO1 protein in intestinal stem cells may offer several therapeutic opportunities. First, when cancer patients undergo chemotherapy with anti-proliferative agents, mucositis is a major side effect [86]. Recombinant RSPO1 protein was tested in a mouse model of mucositis induced by the administration of the chemotherapeutic agent (5-fluorouracil, 5-FU). RSPO1 treatment reduced mucositis-associated symptoms such as diarrhea and weight loss, and protected the small intestine and colon from architectural damage by 5-FU. Second, there is therapeutic potential for RSPO1 treatment of inflammatory bowel diseases such as Crohn's disease in which the intestinal epithelial cells undergo continuous cell death due to the inflammatory response [20, 87]. Similar to mucositis, administration of recombinant RSPO1 protein in a mouse model of inflammatory bowel disease significantly reduced symptoms of the disease [87].

6.2. Joint disorders including arthritis

The therapeutic potential of RSPO proteins on joint disorders including arthritis was suggested in a recent study [88]. RSPO1-injected into *TNFTg* mice, a *TNF α* transgenic mouse model of arthritis, inhibited osteoclast development and bone resorption, as the osteoclasts and osteoclast precursors decreased in number. Expression of type I collagen (bone resorption marker) was reduced, whereas expression of osteoprotegerin (a potent inhibitor of bone resorption) was enhanced. Consequently, RSPO1 injection prevented bone erosion and improved cartilage integrity in the joints of *TNFTg* mice. Reduced proteoglycan loss and chondrocyte death was observed in RSPO1-injected mice with decreased expression level of VDIPEN, a neoepitope generated by matrix metalloproteinase-mediated proteoglycan damage, in the cartilage.

7. Conclusion

Our understanding of the activities of the RSPO proteins in WNT signaling and their biological functions has been significantly advanced in recent years as summarized above. However, further investigations are warranted to define RSPO signaling mechanisms and to determine additional biological functions. First, given the fact that a number of different cognate receptors have been identified in various experimental paradigms, it needs to be determined how these receptors integrate to mediate RSPO signaling. The specific RSPO protein may utilize different receptors or a combination of the different receptors depending on WNT protein availability in different tissues. Second, synergistic cooperation of RSPO and WNT in canonical WNT activation is evident, and determining the molecular mechanism by which this cooperation is regulated is essential to understand RSPO/WNT signaling. Third, additional conditional alleles of the *Rspo* genes in mice are necessary to shed more light on RSPO function in various biological contexts, especially postnatal development and disease conditions. Efforts to define *in vivo* functions associated with specific RSPO signaling activities, i.e., canonical or non-canonical WNT activation, are warranted. Finally, as we learn about the functions and signaling mechanisms of the RSPO

proteins in WNT signaling, it will be feasible to develop therapeutic applications based on RSPO signaling for human diseases where modulation of WNT signaling is crucial.

Highlights

- R-spondins are a family of cysteine-rich, thrombospondin type I repeat containing proteins.
- R-spondins activate both the WNT/ β -catenin and WNT/PCP pathways through multiple receptors.
- R-spondins are associated with human genetic diseases and also essential for various developmental processes in mice and frogs.

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Figure 1.

Conserved secondary structure of the human RSPO proteins. (A) Schematic structure of the human RSPO proteins. SP, signal peptide sequence; CR, cysteine-rich furin-like; TSR, thrombospondin type I repeat; BR, basic amino acid-rich. Percentiles indicate amino acid similarities with the recognized domains among the members. (B) Alignment of amino acid sequences of the CR domains of the human RSPO proteins. (C) Alignment of amino acid sequence of the TSR domains of the human RSPO proteins. ClustalW was used for alignment.

Figure 2.
Models for WNT signaling activation by RSPO.