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The R-spondin family of proteins: emerging regulators of WNT signaling

Yong-Ri Jin and Jeong Kyo Yoon*

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

Abstract

Recently, the R-spondin (RSPO) family of proteins has emerged as important regulators of WNT signaling. Considering the wide spectrum of WNT signaling functions in normal biological processes and disease conditions, there has been a significantly growing interest in understanding the functional roles of RSPOs in multiple biological processes and determining the molecular mechanisms by which RSPOs regulate the WNT signaling pathway. Recent advances in the RSPO research field revealed some of the in vivo functions of RSPOs and provided new information regarding the mechanistic roles of RSPO activity in regulation of WNT signaling. Herein, we review recent progress in RSPO research with an emphasis on signaling mechanisms and biological functions.

1. Introduction

Since *hPWTSR* (later renamed *hRSPO3*), the first member of the *Rspo* gene family, was identified in a high throughput sequencing study of human fetal brain cDNA library (Chen et al., 2002), other *Rspo* genes from different species were subsequently discovered (Kamata et al., 2004, Kazanskaya et al., 2004, Nam et al., 2006). The formal name of the *Rspo* (roof plate-specific spondin) gene was adopted from the mouse *Rspo1* gene that is expressed in the roof plate of the neural tube of developing embryos (Kamata et al., 2004). In mammals, the RSPO protein family consists of four members, namely RSPO1 through RSPO4. They share approximately 40–60% amino acid sequence identity and substantial structural homologies within human and mouse members (Kazanskaya et al., 2004, Nam et al., 2006, Kim et al., 2006). A major breakthrough in RSPO research occurred when *Rspo2* was identified by a functional screening study in *Xenopus* as a gene encoding a novel canonical WNT/ β -catenin signaling activator (Kazanskaya et al., 2004). Later studies further confirmed that the other RSPO proteins from different species have a similar capacity to activate WNT/ β -catenin signaling (Kim et al., 2006, Nam et al., 2006, Binnerts et al., 2007, Wei et al., 2007, Kim et al., 2008). Gene disruption studies in mice and genetic studies in humans began to uncover the in vivo functions of the *Rspo* genes in a wide array of developmental and physiological processes (Aoki et al., 2008, Aoki et al., 2007, Parma et al., 2006, Tomaselli et al., 2008, Blaydon et al., 2006, Tomizuka et al., 2008, Nam et al., 2007a, Chassot et al., 2008). During the last few years, there have been significant advances in identifying multiple RSPO receptors and understanding the regulatory function of RSPO

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*Correspond to Jeong Kyo Yoon at yoonje@mmc.org.

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in the WNT signaling pathway. In this review, we summarize recent findings in RSPO signaling and biological functions.

2. Structural and biochemical properties of the RSPO proteins

The human RSPO1-RSPO4 proteins range from 234 to 272 amino acids (aa) in length and feature the following: (i) a hydrophobic, putative signal peptide sequence at the N-terminus for secretion; (ii) two adjacent cysteine-rich furin-like (CR) domains (92–94 aa in length); (iii) a thrombospondin type I repeat (TSR) domain (55–57aa in length); and (iv) a basic amino acid-rich (BR) domain with varying length at the C-terminus (Figure 1). There are significant sequence similarities detected within the CR and TSR domains of the four hRSPO members, which are also apparent in the RSPO members of other mammalian species, thereby suggesting their conserved protein functions (Kazanskaya et al., 2004, Nam et al., 2006, Kim et al., 2008).

Consistent with the presence of signal sequences at the N-terminus, the RSPO proteins are secreted proteins (Nam et al., 2006, Kazanskaya et al., 2004). The subcellular localization of the RSPO proteins in the endoplasmic reticulum and the Golgi apparatus indicates that RSPOs are processed through the canonical secretory pathway (Nam et al., 2006). Generally, poor detection of the RSPO proteins in conditioned medium obtained from cell culture in which the RSPO proteins are ectopically expressed suggests that secretion of the RSPO proteins is tightly regulated or that the RSPO proteins can bind to the cell surface and extracellular matrix (Kazanskaya et al., 2004, Nam et al., 2006). Interestingly, substitutions of cysteine residues 78 and 113 with tyrosine and arginine within the CR domain of the mouse RSPO2 protein lead to diminished secretion of the mutant RSPO2 protein, suggesting that the CR domain is involved in the regulation of RSPO secretion (Li et al., 2009).

Because the TSR domain of the thrombospondin protein engages in binding to heparin sulfate proteoglycans (HSPGs) (Chen et al., 1996), it is predicted that HSPGs affect cell surface binding of RSPO. Indeed, the addition of soluble heparin (a sugar moiety similar to the one found on HSPGs) or sodium chlorate (an inhibitor of sulfatases mediating sulfation of HSPGs) to the medium during culture of cells overexpressing *Rspos* significantly enhanced the detection of secreted RSPO proteins in the conditioned medium (Nam et al., 2006), suggesting that secreted RSPOs are associated with HSPGs on the cell surface. Furthermore, deletion of the TSR domain from RSPOs resulted in an increase of soluble RSPO proteins in conditioned medium (Nam et al., 2006). Recently, the TSR domain of RSPO3 was found to bind syndecans and glypicans, thereby confirming a direct interaction between the RSPO proteins and HSPGs (Ohkawara et al., 2011). RSPO2 and RSPO3 proteins bind syndecans with high affinity via their TSR domain (e.g., a K_d of RSPO3 binding to syndecan4 is 0.88 nM). Interestingly, the RSPO1 and RSPO4 proteins do not show any significant interaction with syndecans despite their high amino acid sequence homology to that of RSPO2 and RSPO3 within the TSR domain (Ohkawara et al., 2011). Alternatively spliced isoforms of human RSPO1 lacking the TSR domain and human RSPO4 lacking both the TSR and BR domains have been identified from the NCBI protein database. Although the precise functions of these isoforms are unknown, they may exist as more diffusible forms of RSPOs.

3. Regulation of WNT signaling activation by RSPOs

3. 1. Overview of the WNT signaling pathway

WNT signaling regulates cell proliferation, cell survival, cell polarity, and cell fate determination during embryonic development and tissue homeostasis. Aberrant regulation of WNT signaling often results in pathological conditions including birth defects, cancer, and

other diseases in humans (Grigoryan et al., 2008, Clevers, 2006). The WNT ligands activate two major intracellular pathways known as the canonical (or β -catenin-dependent) and non-canonical (β -catenin-independent) pathways (He et al., 2004, Kohn and Moon, 2005, Angers and Moon, 2009). In the canonical WNT pathway, two cell membrane proteins, Frizzled (FZD) and LDL-receptor related protein 5/6 (LRP5/6), function together as receptors for the WNT ligands. In both humans and mice, the FZD receptor family has ten members that belong to the seven-pass transmembrane G-protein coupled receptor superfamily (Schulte and Bryja, 2007). The LRP5/6 receptors are single-pass transmembrane proteins with an extracellular portion containing four EGF-repeats (He et al., 2004). WNT ligand binding to both FZD and 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 that regulate target gene transcription (He et al., 2004, Angers and Moon, 2009).

In the non-canonical WNT pathway, the WNT signal is transduced via the FZD receptors independently from LRP5/6, activating two downstream signaling branches (Angers and Moon, 2009, Kohn and Moon, 2005). WNT induces cytoskeletal changes via activation of the small GTPases RHOA and RAC1 in the regulation of cell polarity, a pathway called the WNT/planar cell polarity (PCP) pathway. WNT also modulates cell adhesion and motility and transcription by nuclear factor of activated T-cells (NFAT) via activation of the heterotrimeric G-proteins, Ca^{++} /calmodulin-dependent kinase II, and protein kinase C cascade in the WNT/ Ca^{++} pathway.

Endocytosis of the LRP and FZD receptors through different plasma membrane compartments after WNT ligand binding is essential for WNT signaling and its specificity (Kikuchi et al., 2009). After stimulation with WNT3A, LRP6 is phosphorylated and internalized into a lipid raft-dependent caveolin-positive vesicular compartment, where it stabilizes β -catenin and activates downstream events (Bilic et al., 2007, Yamamoto et al., 2006). LRP6 internalization and LRP6 phosphorylation are not dependent upon each other, yet both are required for signal propagation (Yamamoto et al., 2008). In contrast, the FZD receptors (e.g., FZD5) undergo clathrin-dependent endocytosis upon WNT ligand stimulation (Yamamoto et al., 2006). Interestingly, with coexpression of the LRP6 and FZD5 receptors, the tertiary complex formed by WNT3A, LRP6, and FZD5 is internalized in a caveolin-dependent and clathrin-independent manner (Yamamoto et al., 2006). However, mice lacking both the *caveolin1* and *caveolin3* genes in which no caveolin expression was detected in most tissues did not show an overt disruption or modification of canonical WNT signaling (Park et al., 2002), thus the precise role of caveolin-mediated receptor endocytosis in canonical WNT signaling remains unclear.

3. 2. RSPO in canonical WNT/ β -catenin signaling

The functional connection of RSPOs to the WNT signaling pathway was established by the discovery of Rspo2 in screening for canonical WNT signaling activators in *Xenopus* (Kazanskaya et al., 2004). Subsequently, several studies have confirmed that RSPOs from different vertebrate species also possess properties of canonical WNT signaling activators (Kim et al., 2006, Nam et al., 2006, Binnerts et al., 2007, Wei et al., 2007, Kim et al., 2008). The CR domain of the RSPO proteins is primarily responsible for activation of WNT/ β -catenin signaling (Figure 1). Deletion of either one of two furin-like motifs within the CR domain of RSPO abolishes its ability to activate canonical WNT signaling (Kazanskaya et al., 2004, Nam et al., 2006, Kim et al., 2008). The Q70R mutation in the CR domains of RSPO2 dramatically reduced the WNT signaling activity of RSPO2 without affecting its secretion (Li et al., 2009). The TSR and BR domains are also presumed to regulate the strength of RSPO activity on canonical WNT signaling because the RSPO proteins lacking the TSR and BR domains activate canonical WNT signaling less effectively (Kim et al.,

2008). Interestingly, the RSPO proteins show strong synergistic or potentiating activity in canonical WNT signaling activated by WNT ligands (Kazanskaya et al., 2004, Nam et al., 2006, Binnerts et al., 2007, Wei et al., 2007, Kim et al., 2008). However, the precise molecular mechanism of this potentiation is not completely understood.

Although the RSPO proteins act as positive WNT signaling regulators and exhibit ligand properties, there are conflicting results regarding the cell surface receptors interacting with the RSPO proteins and the molecular mechanisms of RSPO action in WNT signaling. The requirement of the LRP5/6 receptors to transmit RSPO activation of canonical WNT signaling pathway is well established (Nam et al., 2006, Binnerts et al., 2007, Wei et al., 2007). However, whether or not the LRP5/6 receptors function as a genuine receptor for the RSPO protein still remains controversial. Several laboratories independently demonstrated that the RSPO proteins bind to the LRP6 receptor in cell-free binding and co-immunoprecipitation assays (Nam et al., 2006, Wei et al., 2007, Li et al., 2009). Human RSPO1 protein binds to the LRP6 receptor with a K_d of 1.2 nM and this interaction occurs between the CR domain of RSPO1 and multiple β -propeller repeat domains of LRP6 (Wei et al., 2007). In contrast, several laboratories reported conflicting results. These studies failed to detect effective binding of the RSPO proteins to LRP6 in cell surface binding, co-immunoprecipitation and mass spectrometric assays (Kazanskaya et al., 2004, Binnerts et al., 2007, Carmon et al., 2011, de Lau et al., 2011, Glinka et al., 2011). The reason for these conflicting data may be due to the different experimental conditions and approaches employed in different laboratories.

In several WNT-dependent stem cell compartments such as the small intestine, colon, stomach, and hair follicles, the leucine-rich repeat containing G protein-coupled receptor 5 (LGR5) was identified as a WNT signaling target and validated as a marker for stem cells (van der Flier and Clevers, 2009, Jaks et al., 2008, Sato et al., 2009). Interestingly, the RSPO1 protein showed a strong mitogenic activity on LGR5⁺ cells of the intestinal crypts and hair follicles (Sato et al., 2009, Jaks et al., 2008). Several subsequent studies conclusively determined that the RSPO proteins are the ligands for the LGR4/5/6 receptors (Carmon et al., 2011, de Lau et al., 2011, Glinka et al., 2011, Carmon et al., 2012, Ruffner et al., 2012). The direct interaction of RSPO to LGR4/5/6 was determined by cell surface binding and cell-free co-immunoprecipitation assays, a tandem affinity purification mass spectrometry, and surface plasmon resonance array-imaging approach (Carmon et al., 2011, de Lau et al., 2011, Glinka et al., 2011). The K_d s of binding between different RSPOs and LGRs are within nM ranges (e.g., hRSPO1 binding to LGR5 is 3.1 nM) (de Lau et al., 2011, Glinka et al., 2011). The first leucine-rich repeat domain at the N-terminus of the LGR5 receptor is required for RSPO1 binding (Glinka et al., 2011, de Lau et al., 2011). While the CR domain of the RSPO protein is involved in the interaction with LGR4 and LGR5 (Glinka et al., 2011, de Lau et al., 2011), the TSR domain of the RSPO proteins is not involved in binding to the LGR receptors (Glinka et al., 2011). Activation of WNT/ β -catenin signaling by RSPOs is severely compromised when the *LGR4* and *LGR5* gene expression is suppressed by siRNA-mediated gene knockdown approach (Carmon et al., 2011, de Lau et al., 2011, Glinka et al., 2011). However, under the same conditions, WNT3A-mediated β -catenin signaling activation did not seem to be significantly affected in these cells (Carmon et al., 2011, de Lau et al., 2011, Glinka et al., 2011). Intriguingly, the level of β -catenin in LGR5 overexpressing cells is higher immediately after RSPO stimulation, but declines faster than control cells (Carmon et al., 2011). Moreover, enhanced WNT/ β -catenin signaling is observed in the intestine of *Lgr5* mutant mice (Garcia et al., 2009). Considering that the *Lgr5* gene is a WNT/ β -catenin signaling target, it would be interesting to determine if LGR5 acts as a negative feedback inhibitor in the absence of RSPOs, even though it engages in activation of WNT/ β -catenin signaling by RSPO binding. In addition, unlike the other LGR family members (McDonald et al., 1998, Hsu et al., 1998), the LGR 4/5/6

receptors do not induce typical GPCR signaling activities (Carmon et al., 2011, de Lau et al., 2011, Glinka et al., 2011, Carmon et al., 2012, Gong et al., 2012).

It is generally thought that RSPOs do not directly bind to the FZD receptors (Wei et al., 2007). Because FZD are major receptor components in WNT/ β -catenin signaling, it will be important to determine how the FZD receptors are involved in RSPO-induced WNT/ β -catenin activation. Interestingly, RSPO1-stimulated WNT signaling is enhanced by the overexpression of the specific FZD members, such as FZD1, FZD4, FZD5, and FZD8, but not by some other FZD proteins (Wei et al., 2007). Furthermore, in co-immunoprecipitation/mass spectrometric analysis using the LGR4 or LGR5 receptor as bait, interactions between LGR4 and FZD5/FZD7, and LGR5 and FZD5/FZD6 were detected (de Lau et al., 2011), suggesting that the FZD receptors are likely a critical component of RSPO-induced WNT/ β -catenin signaling, although the precise molecular mechanism remains to be determined.

3.3. Potentiation of WNT/ β -catenin signaling by RSPOs and WNTs

Synergistic activation of WNT/ β -catenin signaling observed in cells treated with both RSPO and WNT ligands suggests that this synergism is likely a crucial regulatory mechanism governing the activation of WNT/ β -catenin signaling. Significant overlap or close proximity of *Wnt* and *Rspo* gene expression in mouse embryos suggest that WNT and RSPO proteins co-exist in many cellular contexts (Nam et al., 2007b, Kamata et al., 2004, Kazanskaya et al., 2004). Currently available data imply two models (Figure 2).

In the first model, RSPO and WNT together act on the LRP5/6 and LGR4/5/6 receptors directly and immediately activate WNT/ β -catenin signaling. Phosphorylation of serine residue at 1490 in the LRP6 receptor, which is the earliest molecular event occurring during activation of WNT/ β -catenin signaling, is detected within 30 minutes after RSPO stimulation (Wei et al., 2007, Carmon et al., 2011), and simultaneously, endocytosis of the ligand-receptor complex occurs (Carmon et al., 2012). It was previously demonstrated that the endocytosis of the FZD and LRP6 receptors is a critical step in WNT/ β -catenin signaling (Yamamoto et al., 2006, Kikuchi et al., 2009). In contrast to caveolin-dependent LRP6 endocytosis after WNT stimulation (Yamamoto et al., 2006), the endocytosis of LRP6 and LGR4/5 induced by WNT and RSPO co-treatment appears to be mediated by clathrin (Glinka et al., 2011, Carmon et al., 2012). However, whether endocytosis of the LRP6-LGR4 receptor complex is a critical step for WNT/ β -catenin signaling activation remains unknown. While blocking endocytosis of the LRP6 and LGR5 receptors induced by the WNT and RSPO proteins showed no effect on WNT/ β -catenin signaling activation in one study (Carmon et al., 2012), in another study, endocytosis of the receptor complex appeared to be critical for WNT signaling activation (Glinka et al., 2011).

In the second model, RSPOs act to prevent the degradation or removal of LRP6 and possibly FZD from the plasma membrane. In the presence of RSPO, more LRP6 and FZD receptors are present on the membrane, a condition allowing the WNT ligands to generate much stronger signals (Binnerts et al., 2007, Hao et al., 2012). Two proteins are proposed to be involved in this process. First, Binnerts et al. showed that RSPO binds to the Kremen family of transmembrane proteins that negatively regulates the LRP6 receptor by DKK1-mediated endocytosis and antagonizes the Kremen/DKK1-mediated LRP6 receptor downregulation on the plasma membrane (Binnerts et al., 2007). However, this model remains unsubstantiated because RSPO induces WNT/ β -catenin signaling activation in *Krm1/2* double mutant fibroblast cells as effectively as wild type fibroblasts (Ellwanger et al., 2008). Second, the cell-surface transmembrane E3 ubiquitin ligases, zinc and ring finger 3 (ZNRF3) and its homologue ring finger 43 (RNF43), emerged as negative feedback regulators of WNT signaling by promoting the turnover of the FZD and LRP6 receptors on the cell surface (Hao et al., 2012, Koo et al., 2012). Through its interactions with the extracellular domains of

ZNRF3 and LGR4, RSPO1 was demonstrated to play an antagonistic role against ZNRF3 by inducing the removal of the ZNRF3 protein (Hao et al., 2012). However, further study is required to delineate how these two seemingly different models of RSPO action can be integrated, and under what biological systems they function.

3.4. RSPO in the non-canonical WNT/PCP pathway

In addition to their regulatory role in WNT/ β -catenin signaling, RSPOs also regulate non-canonical WNT signaling (Ohkawara et al., 2011). During gastrulation and head cartilage morphogenesis in *Xenopus* embryos, two processes regulated by the non-canonical WNT/PCP pathway, *Xenopus* RSPO3 activates WNT/PCP signaling in cooperation with WNT5A in a syndecan4-dependent manner. RSPO3 promotes syndecan4-mediated FZD7/WNT5A complex internalization (Ohkawara et al., 2011). This RSPO3-induced endocytosis of FZD7/WNT5A complex is attenuated by clathrin-specific inhibitors, indicating that RSPO3 is associated with clathrin-dependent endocytosis (Ohkawara et al., 2011). Because direct binding between RSPO3 and FZD7 may not exist, it remains unclear how the RSPO protein imposes its activity on FZD7 without direct binding. It also remains to be determined whether syndecans and other HSPGs are similarly involved in the activation of non-canonical WNT signaling by RSPO in mammalian cell models. In addition, there is little information on the regulatory roles for HSPGs in RSPO-induced activation of canonical WNT signaling, even though the roles of HSPGs in the regulation of WNT signaling have been investigated.

4. In vivo functions of the RSPO genes

WNT signaling is broadly involved in regulating cell proliferation, differentiation, migration, survival and polarity during embryonic development and tissue homeostasis (Clevers, 2006, Grigoryan et al., 2008). Interest in RSPO's ability to function as canonical and non-canonical WNT signaling regulators has initiated a wide range of in vivo and in vitro investigations including human genetic studies and gene targeting approaches in mice (Table 1). These studies have begun to reveal the in vivo functions of the *Rspo* genes and also suggest the potential of the RSPO proteins and their signaling as novel therapeutic targets.

4.1. RSPO1

Recently, individuals with complete female-to-male sex reversal or XX true hermaphroditism from two families were identified as homozygous for RSPO1 gene mutations (Parma et al., 2006, Tomaselli et al., 2008). The identified mutations of the *RSPO1* gene resulted in either no functional protein production (Parma et al., 2006) or deletion of the second half of the CR domain of the RSPO1 protein (Tomaselli et al., 2008), thereby causing severe disruption of the RSPO1-dependent WNT signaling activation. Additionally, these individuals also displayed palmoplantar hyperkeratosis and predisposition to squamous cell carcinoma of the skin.

The function of mouse *Rspo1* gene was assessed by a gene-targeting strategy (Chassot et al., 2008, Tomizuka et al., 2008). Unlikely in humans, loss of the mouse *Rspo1* gene only caused masculinized ovaries (ovotestis) with epididymis and vas deferens-like structures rather than a complete phenotypic male conversion in XX mice. The *Rspo1* null ovaries also showed fetal oocyte depletion, XY-specific vascularization, and steroidogenesis relevant to extremely poor fertility of *Rspo1* null female mice. Molecular analyses demonstrated that the compromised WNT/ β -catenin signaling and an absence of female-specific activation of *Wnt4* might account for the XY-like vascularization and steroidogenesis. Germ cells of XX knockout embryos showed changes in cellular adhesions and a failure to enter XX specific

meiosis. Interestingly, loss of *Rspo1* and *Foxl2*, a transcription factor required for follicular formation, amplifies female-to-male sex reversal in XX mice (Auguste et al., 2011).

In mammals, the sex of the individual is determined by the male or female genetic program-directed testicular or ovarian organogenesis. While the Y chromosome-located *sex-determining region Y (SR Y)* gene is absolutely required for testis development, ovary development is regarded as a passive process due to the absence of *SR Y* expression in the female gonad (Liu et al., 2009). *RSPO1/Rspo1* genetic studies provided the first evidence that RSPO1-induced WNT/ β -catenin signaling positively regulates female differentiation by suppressing male differentiation in females. Consistent to this notion, RSPO1/ β -catenin signaling is demonstrated to be involved in meiosis in fetal germ cells and contributes to the cellular decision of germ cells to differentiate into oocyte or sperm (Chassot et al., 2011). In addition, *Rspo1* showed a female-specific expression profile in vertebrates including chicken and turtle (Smith et al., 2008), indicating a conserved role of *Rspo1* in the vertebrate female-sex determination pathway.

Additionally, some *Rspo1* null XX mice remain sub-fertile and are able to produce offspring; however, these mice are unable to feed their pups (Chadi et al., 2009). Transplantation of mammary tissues from *Rspo1*^{-/-} virgin mice into nude mice revealed that the lack of *Rspo1* expression resulted in the absence of duct side-branching development and subsequent alveolar formation, indicating that a local epithelial RSPO1 signal is required for normal development of the mammary gland.

4.2. RSPO2

Recently, recurrent *RSPO2* gene fusion events were identified in human colon tumors (Seshagiri et al., 2012). The fusion between the non-coding exon 1 of the *eukaryotic translation initiation factor 3 subunit E (EIF3E)* gene and exon2 of the *RSPO2* gene resulted in elevated expression of full-length RSPO2 protein driven by the *EIF3E* promoter. Additionally, a genome-wide association study initially suggested that the human *RSPO2* gene locus is linked to genetic susceptibility in Dupuytren's disease, a benign fibromatosis (Dolmans et al., 2011).

Four independent mouse lines bearing *Rspo2* mutant alleles have been generated by both transgene insertion and gene targeting approaches (Bell et al., 2003, Nam et al., 2007a, Yamada et al., 2009, Aoki et al., 2008). *Footless* mice are homozygous for an inserted transgene of the rat *H^tK^t-ATPase* gene promoter driving chloramphenicol acetyltransferase, and showed an asymmetric pattern of limb malformations (Bell et al., 2003). Positional cloning revealed that this transgene is inserted into the 5'-upstream region proximal to the *Rspo2* gene; *footless* is a strong hypomorphic allele of the *Rspo2* gene (Bell et al., 2008). *Footless* and *Rspo2* homozygous mutant mice (Nam et al., 2007a, Aoki et al., 2008, Yamada et al., 2009) die immediately after birth and display multiple defects including limb defects, craniofacial and laryngeal-tracheal malformation, lung hypoplasia, and kidney malformations (Nam et al., 2007a, Aoki et al., 2008, Bell et al., 2008, Yamada et al., 2009). Interestingly, female mice heterozygous for these *Rspo2* mutations showed partially penetrant, age-dependent sterility accompanied by development of polycystic ovaries (Bell et al., 2003, Nam et al., 2007a).

Disruption of distal skeletal structures, especially in the hindlimbs, occurs in *Rspo2* homozygous mutant and *footless* mice (Bell et al., 2003, Nam et al., 2007a). Reciprocal FGF signaling between limb mesenchyme and overlaying epithelium (or AER at later stages) is crucial to control limb outgrowth and patterning. *Fgf* gene expression in *Rspo2* mutant mice suggests that maturation of the AER is either delayed or fails. This defect results from compromised WNT/ β -catenin signaling as measured by reduced expression of the

transcriptional target *Axin2* and the in vivo WNT/ β -catenin signaling reporter *TopGAL* in the AER of *Rspo2* null mutant embryos (Yamada et al., 2009, Nam et al., 2007a). The existence of genetic epistasis between the *Rspo2* and *Lrp6* genes further confirmed that *Rspo2* function is associated with WNT/ β -catenin signaling (Bell et al., 2008). A redundant function of *Rspo2* and *Rspo3* during hind limb development was recently determined. Although limb mesenchyme-specific *Rspo3* gene ablation does not cause any noticeable hindlimb defect, hindlimb truncation defects detected in *Rspo3* and *footless* double mutant mice are more severe than those observed in *footless* mutant mice (Neufeld et al., 2012).

Craniofacial defects including cleft of the secondary palate with or without cleft lip and abnormalities of the skeletal structures derived from branchial arch 1 (BA1) occur in *Rspo2* null mice (Yamada et al., 2009, Jin et al., 2011). Gene expression of several signaling cytokines and transcription factors including *Fgf8*, *Endothelin1*, *Dlx5/6*, and *Msx1/2*, was significantly disrupted in BA1 mesenchyme of *Rspo2* null mice. Additionally, laryngeal, tracheal and bronchial cartilage derived from BA2 to BA6 was malformed (Bell et al., 2008), suggesting multiple roles for *Rspo2* in BA development. Similar to the AER of *Rspo2* null mice, reduced canonical WNT/ β -catenin signaling was detected within BA1, and genetic epistasis between *Rspo2* and *Lrp6* was also present in BA1-derived skeletal development (Jin et al., 2011).

Hypoplasia and branching defects within the lungs were also observed in *Rspo2* mutant mice (Bell et al., 2008, Yamada et al., 2009). The fetal lungs isolated from *Rspo2* mutant mice were approximately half the size of the lungs of wild type littermates but generally retained normal structure. The hypoplasia of the *Rspo2* mutant lung was not associated with differentiation defects of specific types of cells. *Rspo2* mutant lung explants showed severely impaired growth and branching in ex vivo culture, when compared to wild type explants (Bell et al., 2008). The branching defect was effectively rescued by the addition of RSPO2 conditional medium into the culture (Bell et al., 2008). In agreement with AER and BA1, *Rspo2* acts through the LRP6-mediated WNT/ β -catenin signaling during lung development as *Rspo2* mutant lungs showed reduced WNT/ β -catenin signaling and *Rspo2* and *Lrp6* compound mutant mice exhibited synergistic hypoplasia phenotype (Bell et al., 2008).

The *Rspo2* gene was also identified as a key genetic variant for dog coat types in a genome-wide association study (Cadieu et al., 2009). 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 (Parker et al., 2010). This insertion affects *Rspo2* mRNA stability without causing any change in RSPO2 protein coding sequence, thus leading to increased steady-state *Rspo2* mRNA in the skin.

In *Xenopus*, injection of *Rspo2* morpholino anti-sense oligonucleotides in four-cell stage embryos impaired the expression of the myogenic marker genes, *Myf5* and *MyoD*, and later muscle development. Inhibition of *Myf5* expression by *Rspo2* morpholino was reversed by ectopic β -catenin expression, confirming that *Rspo2* function is mediated by the WNT/ β -catenin pathway (Kazanskaya et al., 2004). In mice, loss of the *Rspo2* gene causes significant reduction of *Myf5* expression in developing limbs, suggesting the role of *Rspo2* in myogenesis is conserved in vertebrate species (Han et al., 2011).

4.3. RSPO3

Similar to the *RSPO2* gene, recurrent *RSPO3* gene fusions were identified in human colon tumors (Seshagiri et al., 2012). The coding exon 1 of the *Protein tyrosine phosphatase receptor type K (PTPRK)* gene was fused to the exon 2 of the *RSPO3* gene, resulting in expression of a fusion protein that exhibited a full capacity of RSPO3 activity in WNT

signaling. Interestingly, both RSPO2 and RSPO3 fusions occurred in an exclusive manner to *APC* mutations, indicating that these mutations represent a unique subclass of human colon tumors.

Rspo3 gene knockout mutants die around E10 due to angiogenesis defects in yolk sac and placenta (Kazanskaya et al., 2008, Aoki et al., 2007). Consistent with specific expression of *Rspo3* in the allantoic component of the labyrinth, the fetal blood vessels did not penetrate into the chorion in the placenta of *Rspo3* homozygous mutant, suggesting a critical role for *Rspo3* in the interaction between chorion and allantois in labyrinthine development (Aoki et al., 2007). PECAM staining and histological analysis also showed that the primary capillary plexus in the yolk sac of mutant embryos formed but failed to remodel, resulting in an underdeveloped vasculature (Aoki et al., 2007, Kazanskaya et al., 2008). A compromised VEGF expression, which is under the regulation by RSPO3-mediated WNT/ β -catenin signaling, is likely the major cause of the angiogenesis defect (Kazanskaya et al., 2008). The embryonic lethality around E10 prevents the assessment of *Rspo3* function in the embryo beyond this developmental stage. With available conditional *Rspo3* gene knockout alleles (Kazanskaya et al., 2008, Neufeld et al., 2012), additional functions of the *Rspo3* gene during development and postnatal stages will be uncovered. Most recently, it is reported that *Rspo3* gene function may be partly redundant to that of the *Rspo2* gene in hindlimb development as *Rspo2* and *Rspo3* double gene deletion in limb mesenchymal cells caused more severe hindlimb defects than those of *Rspo2* mutant mice (Neufeld et al., 2012).

In *Xenopus* embryos, inhibition of the *Rspo3* gene by *Rspo3*-specific morpholino injection resulted in three major developmental defects. The first defect caused by *Rspo3* inhibition is ventral edema and vascular defects in tadpoles and imbalance of endothelial and blood differentiation towards increased blood cell specification. These defects seem to be WNT/ β -catenin-dependent (Kazanskaya et al., 2008). The second and third defects caused by *Rspo3* inhibition are disruption of gastrulation and malformation of head cartilage (Ohkawara et al., 2011). Because the WNT/PCP pathway regulates both of these developmental processes, it is reasonable to speculate that *Rspo3* may play critical roles in these processes by regulating WNT/PCP signaling.

4.4. RSPO4

Anonychia/hyponychia is a condition defined by total or partial absence of fingernails and toenails without significant bone anomalies in human (Baran and Kechijian, 2001). The link of human *RSPO4* gene mutations to anonychia was discovered in several genome-wide mapping studies (Bergmann et al., 2006, Blaydon et al., 2006, Blaydon et al., 2007, Seitz et al., 2007, Ishii et al., 2008, Bruchle et al., 2008, Wasif and Ahmad, 2012). Individuals homozygous for these *RSPO4* mutations exhibit anonychia. Most identified mutations are clustered within the CR domain of the *RSPO4* protein (Blaydon et al., 2006, Ishii et al., 2008), resulting in production of *RSPO4* protein lacking an ability to activate canonical WNT signaling. *Rspo4* is expressed in the primordia of the claw during mouse nail development (Bergmann et al., 2006, Blaydon et al., 2006, Ishii et al., 2008). In addition, expression of *RPSO4* transcript was detected in human primary fibroblasts but not in keratinocytes (Blaydon et al., 2007). Therefore, *RSPO4* functions as a key regulator of nail development and potentially modulates WNT/ β -catenin signaling activation at the dermal–epidermal boundary. 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 (Seitz et al., 2007). Since the claws were also missing in *Rspo2* null mutant mice (Nam et al., 2007a, Bell et al., 2003), it remains to be investigated whether human *RSPO2* gene mutations are linked to *RSPO4*-independent anonychia/hyponychia (Nam et al., 2007a, Nakamura and Miyachi, 2008).

5. Therapeutics potentials of RSPOs

5.1. Skeletal diseases

WNT signaling plays a crucial role in normal bone development and remodeling. Aberrant WNT signaling, exemplified by null, hypo- and hypermorphic mutations in two WNT receptor genes, *LRP5* and *LRP6*, is strongly associated with bone diseases such as osteoporosis and osteoarthritis in humans (Kerkhof et al., 2008, van Meurs et al., 2008). Recent studies imply a therapeutic potential of RSPO and RSPO signaling in bone diseases. In mouse multipotential myogenic progenitor C2C12 cells and primary mouse osteoblast cells, RSPO1 treatment induces expression of the osteoblast differentiation marker genes, osteocalcin and osteoprotegerin, and increased alkaline phosphatase level through the WNT/ β -catenin signaling pathway (Lu et al., 2008). In MC3T3-E1 pre-osteoblast cells pretreated with BMP2, the non-canonical WNT11 ligand enhanced expression of osteoblast-associated genes and osteoblast maturation and mineralization (Friedman et al., 2009). In this study, the *Rspo2* gene was identified as a potential target to mediate WNT11 activity, and *Rspo2* overexpression promoted osteogenic differentiation in MC3T3-E1 cells pretreated with BMP2, but not in naive MC3T3-E1 cells. Interestingly, *Rspo2* activity to promote osteogenic differentiation was not WNT/ β -catenin-dependent. Considering the ability of RSPO to activate WNT/PCP signaling in *Xenopus* (Ohkawara et al., 2011), it is plausible that RSPO2 transmits its activity through the non-canonical WNT pathway in MC3T3-E1 cells.

It was found recently that elevated TGF- β 1 downregulated *RSPO2* expression, resulting in a reduction of WNT/ β -catenin signaling in primary cultured human osteoblasts from tibia plateaus of osteoarthritis patients (Abed et al., 2011). Importantly, while siRNA-mediated *RSPO2* gene knockdown decreased mineralization of normal osteoblasts, treatment with recombinant RSPO2 protein during bone differentiation enhanced mineralization of osteoblasts isolated from osteoarthritis patients (Abed et al., 2011).

Additional proof of the therapeutic potential of the RSPO1 protein on joint disorders was demonstrated. When the recombinant RSPO1 protein was locally injected into the joints of TNF α overexpressing transgenic mice (TNFtg), a mouse model of arthritis, it effectively inhibited osteoclast development and bone resorption (Kronke et al., 2010). 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 TNFtg mice with less damage in the cartilage manifested by decreased expression level of VDIPEN, a neoepitope generated by matrix metalloproteinase-mediated proteoglycan damage (Kronke et al., 2010). In addition, the *Pgia8* gene locus is associated with proteoglycan-induced arthritis in mice and rheumatoid arthritis in humans with a gender disparity (Kudryavtseva et al., 2011). The mouse *Rspo2* gene is one of four genes correlated with arthritis severity in *Pgia8* congenic mice, which illustrates the strong connection between *Rspo2* (and RSPO2 signaling) and inflammatory arthritis. These studies show the therapeutic potential of the recombinant RSPO protein in treatment of some skeletal diseases.

5.2. Inflammatory bowel disease and chemotherapy-induced mucositis

Human RSPO1 protein effectively supports survival and proliferation of LGR5-positive intestinal stem cells in vitro through activation of WNT/ β -catenin signaling (Sato et al., 2009). Ectopic expression of human *RSPO1* in the B cell lineage in mice causes severe abdominal distention by 8 weeks of age, which is characterized by a substantial increase in the diameter, length, and weight of the small intestine (Kim et al., 2005). Consistently, 3 days of administration of human RSPO1 protein into normal mice fully recapitulated the

intestinal phenotypes (Kim et al., 2005). This mitogenic activity of RSPO1 on intestinal stem cells may be useful to treat two specific diseases. First, RSPO1 may be clinically useful in the therapeutic treatment of inflammatory bowel disease because of its stimulating effect on crypt cell growth to accelerate mucosal regeneration. In both acute and chronic phases of colitis in mouse models, administration of the RSPO1 protein preserves mucosal integrity in both small and large bowels by stimulating crypt epithelial cell mitosis (Zhao et al., 2007). This results in the regeneration of both crypt and villus compartments in the mouse intestinal mucosa. Importantly, RSPO1 treatment suppresses intestinal inflammation, leukocyte infiltration, and overproduction of proinflammatory cytokines observed during colitis (Zhao et al., 2007). Second, RSPO1 can be used as a supplement to prevent architectural damage of the small intestine and colon arising from cytotoxicity of anti-cancer drug for cancer patients undergoing chemotherapy. Significant reverse of the intestinal epithelial cell damage caused by 5-FU was observed in a mouse model carrying a tumor (Kim et al., 2005).

5.3. Cancer

Aberrant canonical WNT signaling is strongly associated with many forms of human cancer (Clevers, 2006). The *Rspo2* and *Rspo3* genes are identified for their oncogenic potential in mouse mammary tumor virus associated with mammary tumorigenesis in mice (Theodorou et al., 2007, Klauzinska et al., 2012, Lowther et al., 2005). The oncogenic potential of the *Rspo3* gene was further verified by the enhanced tumor forming capability of *Rspo3* overexpressing p53-deficient mammary epithelial cells when they are subcutaneously injected into nude mice (Theodorou et al., 2007). Additionally, alteration of the *Rspo2* gene is linked to colorectal cancer in a transposon-based genetic screening in mice (Starr et al., 2009). Recent discovery of recurrent gene fusion of *RSPO2* and *RSPO3* in human colon tumors with no APC mutations illustrates the critical role of RSPOs and their activation of WNT signaling in some type of human colon cancer (Seshagiri et al., 2012). Antagonizing RSPO activity can be an effective therapeutic strategy against APC-negative colon cancers.

In contrast to oncogenic function of *Rspo2* and *Rspo3* in breast and colon cancers in mouse models, *RSPO1* is suggested as a potential tumor suppressor gene in both acute and chronic lymphocytic leukemia in humans (Kuang et al., 2008, Tong et al., 2010). Humans carrying RSPO1 null mutations are also predisposed to the skin carcinoma (Kuang et al., 2008, Tong et al., 2010, Parma et al., 2006). The RSPO4 gene also showed aberrant DNA methylation in esophageal squamous cells and in human esophageal squamous carcinoma specimens (Oka et al., 2009). Interestingly, recent studies suggest that LGR5 functions as a tumor suppressor, while increased expression of LGR5 in colon cancer and basal cell carcinoma may be potentially enriched in cancer stem cells (Tanese et al., 2008, McClanahan et al., 2006, Merlos-Suarez et al., 2011). In addition, the *LGR6* gene is hypermethylated in its promoter region in approximately 20% of colon cancer (Schuebel et al., 2007), thereby suggesting that its transcription is repressed. A loss-of-function *LGR6* mutation was also found in colon cancer (Gong et al., 2012). It is likely that RSPOs and their signaling can function as both oncogenic or tumor suppressive, depending on the types of cancer. Therefore, carefully designed inhibition or activation of RSPO signaling activity may be a promising anti-cancer strategy in various types of human cancers.

6. Conclusion

The importance of the RSPO family of proteins, which act as positive regulators of canonical and non-canonical WNT signaling pathways, has been established in several in vitro and in vivo studies in various animal models and human genetic studies. Although there has been a significant increase in our understanding of how RSPO regulates the WNT signaling pathway at the molecular level, many questions still remain unanswered. A

fundamental question is whether the RSPO proteins themselves act as activating ligands for WNT signaling or simply function as potentiators of the WNT ligands. Additionally, as noted above, integration of the different models of RSPO action is needed for comprehensive understanding of the molecular mechanism by which RSPO regulates WNT signaling, especially canonical WNT signaling. Furthermore, additional conditional alleles of the *Rspo* and *Lgr4/5/6* genes in mice are necessary to identify functions of RSPO signaling during postnatal development and disease conditions. Finally, our growing knowledge of the functions and signaling mechanisms of the RSPO proteins will facilitate development of therapeutic applications based on RSPO signaling for human diseases in which modulation of WNT signaling is critical.

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Abbreviations and Acronyms

| | |
|--------------|-------------------------------------------------------------------------------------------|
| BA | <u>b</u> ran <u>ch</u> ial <u>a</u> rch |
| BMP | <u>b</u> one <u>m</u> orphogenetic <u>p</u> rotein |
| BR | <u>b</u> asic amino acid- <u>r</u> ich |
| CR | <u>c</u> ysteine- <u>r</u> ich |
| DKK1 | <u>d</u> ick <u>k</u> opf homolog 1 |
| Dlx | <u>d</u> istal- <u>l</u> ess homeobox |
| EIF3E | <u>e</u> karyotic translation <u>i</u> nitiation <u>f</u> actor <u>3</u> subunit <u>E</u> |
| EGF | <u>e</u> pidermal <u>g</u> rowth <u>f</u> actor |
| FGF | <u>f</u> ibroblast <u>g</u> rowth <u>f</u> actor |
| 5-FU | 5- <u>f</u> luorou <u>r</u> acil |
| Foxl | <u>f</u> orkhead <u>b</u> ox <u>L</u> 2 |
| FZD | Frizzled |
| HSPGs | <u>h</u> eparan <u>s</u> ulfate <u>p</u> roteoglycans |
| KRM | kremen |
| LEF1 | <u>l</u> ymphoid <u>e</u> nhancer binding <u>f</u> actor 1 |
| LGR | <u>l</u> eu <u>c</u> ine-rich repeat containing <u>G</u> protein-coupled <u>r</u> eceptor |
| LRP | <u>L</u> ow density lipoprotein-receptor <u>r</u> elated <u>p</u> rotein |
| Msx | <u>m</u> sh-like homeobox |
| NFAT | <u>n</u> uclear <u>f</u> actor of <u>a</u> ctivated <u>T</u> -cells |
| PCP | <u>p</u> lanar <u>c</u> ell <u>p</u> olarity |
| PTPRK | protein tyrosine <u>p</u> hosphatase <u>r</u> eceptor type <u>K</u> |
| RAC1 | <u>R</u> as-related <u>C</u> 3 botulinum substrate 1 |
| RHOA | <u>R</u> as <u>h</u> omolog gene family, member <u>A</u> |
| RNF43 | <u>r</u> ing <u>f</u> inger protein43 |

| | |
|-------------------------------|--------------------------------------------------------------------------------|
| RSPO | roof plate-specific <u>s</u> pondin |
| hPWTSR | <u>h</u> uman protein <u>w</u> ith <u>t</u> hrombospondin type I repeat domain |
| WNT | <u>w</u> ingless-type MMTV <u>i</u> ntegration site family |
| SRY | <u>s</u> ex-determining region of chromosome <u>Y</u> |
| TCF | <u>T</u> cell specific HMG-box <u>f</u> actor |
| TNFα | <u>t</u> umor <u>n</u> ecrosis <u>f</u> actor <u>α</u> |
| TSR | <u>t</u> hrombospondin type I repeat |
| VEGF | <u>v</u> ascular <u>e</u> ndothelial growth <u>f</u> actor |
| ZNRF3 | <u>z</u> inc <u>a</u> nd <u>r</u> ing <u>f</u> inger3 |

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Highlights

- R-spondins are a family of cysteine-rich, thrombospondin type I repeat containing proteins that activate both the WNT/ β -catenin and WNT/PCP pathways.
- The LGR4/5/6 proteins act as receptors for R-spondins.
- R-spondins potentiate WNT ligand activity as either coactivators or inhibitors of WNT signaling receptor degradation.
- The *R-spondin* gene mutations are associated with human genetic diseases and cancer.
- The *R-spondin* genes are essential for various developmental processes in mice.

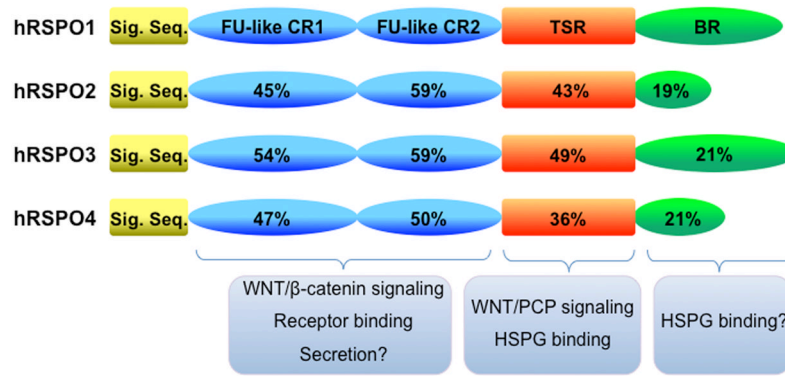


Figure 1. The human RSPO family of proteins

Schematic representation is shown for four human RSPO proteins. The RSPO1, 2, 3 and 4 proteins are 263, 243, 292 and 234 amino acids in length, respectively. Four different domains are illustrated: signal sequence, two cysteine-rich furin-like repeats (FU-like CR), a single thrombospondin type1 repeat (TSR) domain, and a basic amino-acid-rich (BR) domain with variable lengths. The percentages of identical amino acids within these domains show relative protein sequence conservation. The known and suggested functions of each domain are described.

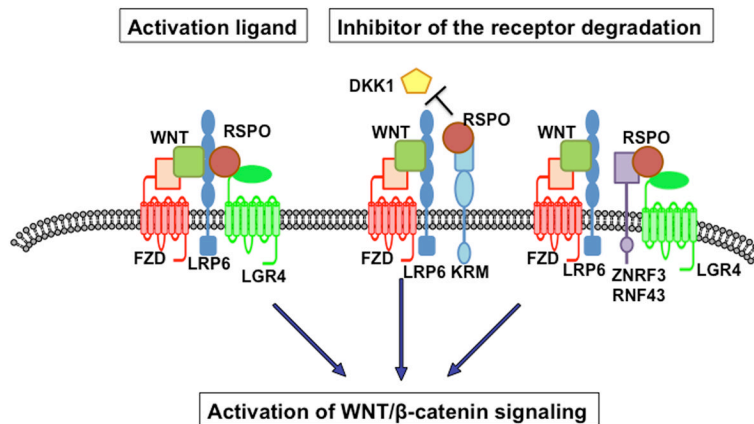


Figure 2. Models of RSPO-induced WNT/β-catenin signaling activation

In the left side, a model illustrates that RSPO acts as an activation ligand on LRP6 and LGR4 to form a multiple ligands-receptors-cluster with WNT and FZD. In a model presented in the middle and right side, RSPO inhibits degradation of the LRP6 and FZD receptors by antagonizing either DKK1-Kremen or ZNRF3.

Table 1
Loss-of-function (LOF) and gain-of-function (GOF) mutations of the *R-spondin* genes in mammals

| Gene | Species | Mutation type | Phenotypes | References |
|-------------------|---------|---------------|--------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------|
| <i>R-spondin1</i> | Human | LOF | Female to male (XX) sex conversion XX true hermaphroditism Palmoplantar hyperkeratosis Predisposition to skin squamous cell carcinoma | (Parma et al., 2006, Tomaselli et al., 2008) |
| | Mouse | LOF | Pseudohermaphroditism Masculinized ovaries Abnormal development of the mammary glands | (Auguste et al., 2011, Chadi et al., 2009, Chassot et al., 2011, Tomizuka et al., 2008) |
| | Human | GOF | APC-negative colon cancers | (Seshagiri et al., 2012) |
| <i>R-spondin2</i> | Mouse | LOF | Distal limb defects Craniofacial and laryngeal-tracheal malformation Lung hypoplasia Kidney defect Polycystic ovaries in heterozygous mice | (Aoki et al., 2008, Bell et al., 2003, Bell et al., 2008, Jin et al., 2011, Nam et al., 2007, Yamada et al., 2009) |
| | Dog | GOF | Dog coat type change | (Cadieu et al., 2009) |
| | Human | GOF | APC-negative colon cancers | (Seshagiri et al., 2012) |
| <i>R-spondin3</i> | Mouse | LOF | Impaired placental development Vascular defects in the yolk sac and embryo, Enhanced limb defect under <i>Rspo2</i> homozygous background | (Aoki et al., 2007, Kazanskaya et al., 2008, Neufeld et al., 2012) |
| | Human | LOF | Anonychia/hyponychia | (Bergmann et al., 2006, Blaydon et al., 2006, Bruchle et al., 2008, Ishii et al., 2008, Seitz et al., 2007) |