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Review article

R-spondins: Multi-mode WNT signaling regulators in adult stem cells

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ABSTRACT

R-spondins (RSPOs) are secreted cysteine-rich glycoproteins that belong to a superfamily of thrombospondin type 1 repeat-containing proteins. RSPOs together with WNT proteins potentiate canonical WNT/β-catenin signaling activity. Over the last several years, the understanding of the regulatory mechanisms and functional roles of RSPOs in many biological contexts has increased. Particularly, because a leucine-rich repeat containing G protein-coupled receptor 5 (LGR5), a stem cell marker originally identified as a marker for intestinal stem cells, and two closely related proteins, LGR4 and LGR6, were identified as cognate receptors for RSPOs, significant research progress has been made in understanding the functional roles of RSPO/LGR signaling in stem cell biology. Given the crucial roles of canonical WNT signaling in self-renewal and differentiation of various types of stem cells, examination of RSPO function and underlying mechanism in these stem cells has provided new insight into the regulatory roles of WNT signaling in stem cell behavior. In this review, we summarize and discuss recent advances in the understanding of the signaling mechanism and roles of RSPOs in different stem cell contexts.

1. R-spondins and their signaling mechanisms

1.1. R-spondins and their receptors

R-spondins (RSPOs), roof-plate of neural tube-specific spondin-domain containing proteins, consist of four family members in mammals (Jin and Yoon, 2012; Kamata et al., 2004). *Rspo* genes were found in the genomes of all deuterostomes including chordates, hemichordates, and echinodermata, but no orthologs have been identified in invertebrates such as *Drosophila* and *Caenorhabditis* (de Lau et al., 2012; Kim et al., 2006; Yoon and Lee, 2012). The RSPO protein members (RSPO1, RSPO2, RPSO3, and RSPO4), encoded by four individual genes in mammals, are highly cysteine-rich glycoproteins that are 234–272 amino acids in length (Alowolodu et al., 2016; Jin and Yoon, 2012). The RSPO proteins share unique structural characteristics: a signal peptide at the N-terminus, two adjacent furin-like cysteine-rich domains (FU-CRD1 and 2) followed by a single thrombospondin type I repeat (TSR) domain, and a positively charged basic amino acid (BR) domain with a varying length at the C-terminus (Jin and Yoon, 2012; Kamata et al., 2004; Kim et al., 2006) (Fig. 1A). These domains exhibit high amino acid sequence homology (for instance, 40–60% identical amino acid sequence within FU-CRDs) (Jin and Yoon, 2012).

Interactions between different RSPO receptors and specific domains of RSPO are relatively well established by a collection of biochemical and structural analyses. The FU-CRD1 (amino acid residues 34–85 in human RSPO1) binds to two specific plasma membrane-associated E3 ubiquitin ligases, ZNRF3 (zinc and ring finger 3) and RNF43 (ring finger protein 43), which specifically regulate the degradation of Frizzled (FZD) receptors (Hao et al., 2012). Two amino acid residues, Arginine (R) 66 and Glutamine (Q) 71, within FU-CRD1 in human RSPO1 were

Abbreviations: APC, adenomatous polyposis coli; ASCL2, achaete-scute family bHLH transcription factor 2; AXIN, axis inhibition protein; BR, Basic region; CECs, corneal endothelial cells; CK1, casein kinase 1; CRC, colorectal cancer; ERK, extracellular signal-regulated kinase; FAH, Fumarylacetoacetate hydrolase; FU-CRD, furin-like cysteine-rich domain; FZD, Frizzled; GSCs, gastric stem cells; GSK-3β, glycogen synthase kinase 3 beta; HFSCs, hair follicle stem cells; HGF, hepatic growth factor; HSPGs, heparan sulfate proteoglycans; IQGAP, IQ motif containing GTPase activating protein; ISCs, intestinal stem cells; LGR4/5/6, leucine-rich repeat containing G protein-coupled receptor 4/5/6; LRP5/6, low density lipoprotein receptor-related protein 5/6; MaSCs, mammary gland stem cells; MEK, mitogen-activated protein kinase; MMTV, mouse mammary tumor virus; PAX7, paired box 7; PDGFRα, platelet derived growth factor receptor alpha polypeptide; RANK, receptor activator of nuclear factor kappa B; RNF43, ring finger protein 43; ROBO1, Roundabout 1; RSPO1/2/3/4, R-spondin1/2/3/4; SCA1, stem cell antigen 1; SkMSCs, skeletal muscle stem cells; SLIT2, slit guidance ligand 2; SOX, sex determining region Y box; TCF, T-cell factor; TSR, thrombospondin type I repeat domain; WNT, wingless-type MMTV integration site family; ZNRF3, zinc and ring finger 3

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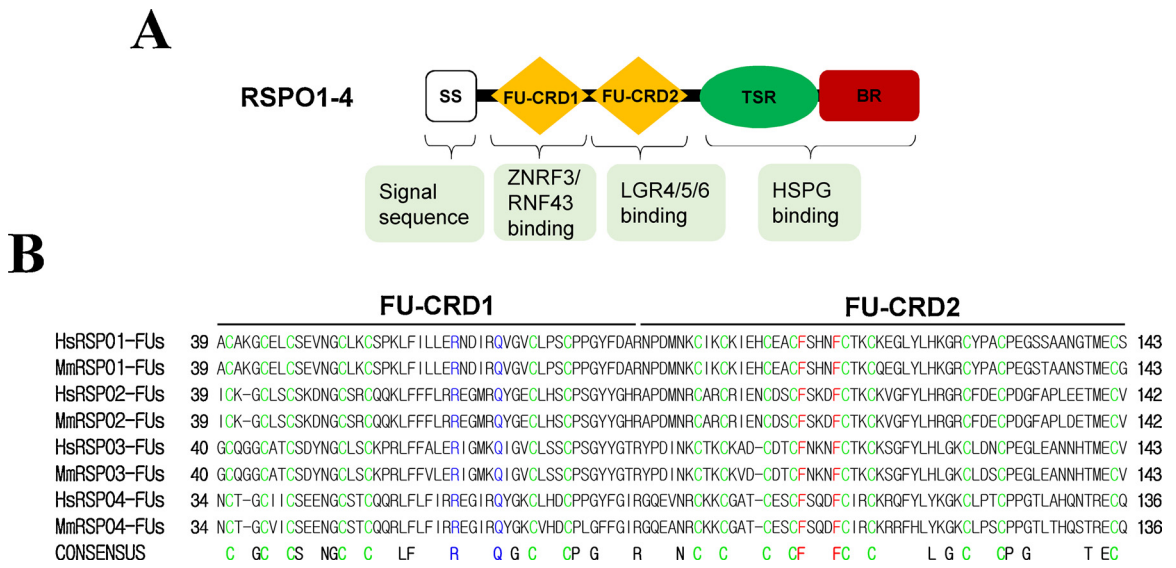

Fig. 1. The R-spondin (RSPO) protein family. (A) A schematic structure of the RSPO protein family illustrates specific domains of the RSPO proteins and their known functions. (B) Alignment of amino acid sequences of the FU-CRD of the RSPO protein family in human (Hs) and mouse (Mm). The conserved cysteines are marked in green. Blue and red letters indicate the key amino acids critical for RNF43/ZNRF3 and LGR4/5/6 binding, respectively.

identified as key amino acids in the direct binding to ZNRF3/RNF43 by X-ray crystallography. These two amino acids are conserved in other members of the RSPO family in humans and mice (Fig. 1B), suggesting other RSPOs also bind to ZNRF3/RNF43 (Table 1).

The FU-CRD2 (a.a. 91–135) directly interacts with the subfamily of leucine-rich repeat containing G protein-coupled receptors, LGR4, 5, and 6. (Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011; Peng et al., 2013). The LGR4 subfamily belongs to the type B class of the LGR family characterized by the presence of 16–18 leucine-rich repeats within the ectodomain (Hsu et al., 2000, 1998; McDonald et al., 1998). Within the FU-CRD2 domain, two phenylalanine (F) residues at positions 106 and 110 of human RSPO1, which are also conserved in other RSPOs (Fig. 1B), were found to be essential for LGR4/5/6 binding (Xie et al., 2013).

Finally, it is demonstrated that the TSR domain of RSPO (residues 147–207 in human RSPO1) binds to heparin (Nam et al., 2006), suggesting that the TSR domain can bind to heparan sulfate proteoglycans (HSPGs). More recently, HSPGs including glypicans and syndecans were identified as RSPO receptors (Lebensohn and Rohatgi, 2018; Ohkawara et al., 2011). In addition, several previous studies showed that RSPOs bind the canonical WNT coreceptor, LRP5/6 (Wei et al., 2007; Yoon and Lee, 2012), or Kremen (Binnerts et al., 2007). However, these results have not been further confirmed by the followed studies and remain elusive.

1.2. Mechanisms of RSPO-mediated canonical WNT signaling potentiation

The functional hallmark of RSPO proteins is their regulatory roles in the canonical WNT signaling pathway. *Rspo2* was isolated as a gene encoding for a positive activator of WNT signaling in *Xenopus* (Kazanskaya et al., 2004). Subsequent studies confirmed the link between RSPO and WNT signaling (Binnerts et al., 2007; Kim et al., 2008, 2006; Nam et al., 2006; Wei et al., 2007), as RSPOs can potentiate canonical WNT signaling activity induced by WNT proteins. While future studies are still required to determine the detailed molecular mechanisms of this potentiation, recent studies have increased the understanding of RSPO's regulatory mechanisms (Chen et al., 2013; Glinka et al., 2011; Hao et al., 2012; Lebensohn and Rohatgi, 2018; Park et al., 2018; Peng et al., 2013; Szenker-Ravi et al., 2018; Warner et al., 2015; Zebisch et al., 2013). In particular, identification and functional studies of the RSPO receptors mentioned above have revealed numerous aspects of the molecular mechanism of WNT signaling potentiation by RSPOs. Increasing evidence strongly suggests that the mechanisms by which RSPOs potentiate WNT signaling are complex and occur in a cellular context-dependent manner.

1.2.1. LGR4/5/6-dependent potentiation

In a non-activated state in the absence of WNTs and RSPOs, ZNRF3 and RNF43 induce the internalization and degradation of FZD and, perhaps, LRP5/6 receptors *via* the ubiquitin-dependent protein degradation pathway (Fig. 2A) (Hao et al., 2012). Simultaneously, β-catenin complexed with AXIN, APC, and GSK3β is phosphorylated and degraded by the proteasome. Canonical WNT signaling activity is maintained at a basal level.

In the presence of RSPO and WNT, RSPO binds to ZNFR3/RNF43 and LGR4/5/6 simultaneously through FU-CRD1 and FU-CRD2, respectively (Fig. 2B). Introduction of missense mutations at R66 and Q71 in FU-CRD1, direct binding sites for ZNFR3/RNF43, significantly reduced the capacity of RSPO to potentiate WNT signaling (Xie et al., 2013). Similarly, RSPO1 carrying missense mutations at F106 and F110 in FU-CRD2, specific binding sites for LGR4/5/6, also failed to potentiate WNT signaling (Xie et al., 2013). Therefore, RSPO binding to both ZNRF3/RNF43 and LGR4/5/6 is a key mechanism in WNT signaling potentiation. Formation of the ZNRF3/RNF43-RSPO-LGR4/5/6 tertiary complex inhibits the ubiquitin E3 ligase activities of ZNFR3/RNF43, resulting in accumulation of the FZD receptors on the plasma membrane. Cells with the increased FZD receptors on the plasma membrane respond to the existing WNT ligands in a more sensitized manner, resulting in a potentiated WNT signaling response.

1.2.2. LGR4/5/6-independent potentiation

The above model has been challenged by recent findings implyimg the occurrence of LGR4/5/6-independent WNT potentiation by RSPOs (Fig. 2C). First, in a human cell line lacking all three *LGR4* family genes, RSPO2 and RSPO3, but not RSPO1 and RSPO4, potentiated canonical WNT signaling (Lebensohn and Rohatgi, 2018). Mutant RSPO3 that is unable to bind the LGR4/5/6 receptors still generated high WNT potentiation activity, suggesting that LGR4/5/6 receptors are dispensable for RSPO3-mediated potentiation. However, considering the high similarity of protein sequences and structure in FU-CRD1 of RSPO1/4 and RSPO2/3, the molecular basis of this distinction is currently unknown. It is possible that other amino acid residues in FU-CRD1 only

Table 1
Summary of the roles of RSPOs in various adult stem cells.

Protein	Stem cell type	RSPO Source	Function	References
RSPO1	Intestinal stem cells (ISCs)	Unknown type of intestinal stromal cell	Supports ISCs expansion Potentiates intestinal regeneration in cooperation with SLIT2	Greicius et al. (2018), Kabiri et al. (2014), Yan et al. (2017) and Zhou et al. (2013)
	Gastric stem cells (GSCs)	Unknown	Supports growth of GSC organoids	Sigal et al. (2017)
	Mammary gland stem cells (MaSCs)	Sca1 $^{-ve}$ luminal cells	Maintains MaSCs expansion and self-renewal	Jarde et al. (2016), Chadi et al. (2009), Cai et al. (2014) and Dall et al. (2017)
	Skeletal muscle stem cells (MuSCs)	Skeletal muscle stem cells	Controls muscle differentiation and muscle cell fusion	Lacour et al. (2017)
	Hair follicle stem cells (HFSCs)	hair follicle dermal papilla	Induces the fate determination factors	Li et al. (2016)
	Corneal endothelial cells (CECs)	Unknown	Accelerates CECs proliferation and inhibit mesenchymal transformation	Hirata-Tominaga et al. (2013) and Okumura et al. (2014)
	Liver	Unknown	Promotes LGR5 $^{+ve}$ stem cell expansion and the ability to form organoid	Azuma et al. (2007); Huch et al. (2013) and Lin et al. (2017)
	Gall bladder	Unknown	Promotes LGR5 $^{+ve}$ stem cell expansion and the ability to form organoid	Lugli et al. (2016) and Huch et al. (2013)
	Pancreas	Unknown	Promotes LGR5 $^{+ve}$ stem cell expansion and the ability to form organoid	Huch et al. (2013)
RSPO2	Intestine stem cells (ISCs)	Unknown	Supports the growth of ISC organoids *in vitro* Stimulates the growth of intestinal crypts *in vivo* in mice	Park et al. (2018)
	Hair follicle stem cells (HFSCs)	Unknown	Represses the entry to catagen and prolongs anagen	Smith et al. (2016)
RSPO3	Intestinal stem cells (ISCs)	PDGFRα $^{+ve}$ pericryptal myofibroblasts stromal cells	Promotes self-renewal of ISCs	Greicius et al. (2018)
	Gastric stem cells (GSCs)	Gastric myofibroblasts	Supports growth of organoids Induces hyperproliferation of GSCs and gland hyperplasia	Sigal et al. (2017)

specific to RSPO1/4 or RSPO2/3 may determine this distinction. Furthermore, RSPO2/3 may mediate this potentiation by interacting with heparan sulfate proteoglycans (HSPGs) rather than binding to LGR4/5/6, because the removal of heparin-binding TSR and BR domains from RSPO3 resulted in the loss of potentiation activity (Lebensohn and Rohatgi, 2018). A previous study in *Xenopus* also demonstrated that RSPO3 binds to HSPGs including syndecan4 to modulate the non-canonical WNT pathway (Ohkawara et al., 2011). Whether these HSPGs also engage in potentiation of canonical WNT signaling requires further investigation.

Second challenging evidence was obtained from the study analyzing the phenotypes of mice lacking *Lgr4/Lgr5/Lgr6* (Szenker-Ravi et al., 2018). Strikingly, these mice rarely developed the defects observed in either *Rspo2* or *Rspo3* null mutant mice. For instance, hindlimb defects and lung hypoplasia detected in *Rspo2* gene knockout mice were not observed in *Lgr4/Lgr5/Lgr6* triple knockout mice, strongly suggesting that RSOP2 functions independently of LGR4/LGR5/LGR6 receptors in hindlimb and lung development. Furthermore, all RSPO proteins effectively potentiated WNT3A activity in the expression of a WNT signaling target gene, *Axin2*, and a *SuperTopFlash* WNT signaling reporter in fibroblasts derived from *Lgr4/Lgr5/Lgr6* triple knockout mice. Inhibiting *Znrf3* expression by RNA interference in the *Lgr4/Lgr5/Lgr6* triple knockout cells potentiated *Axin2* expression by WNT3A, mimicking the WNT signaling potentiation by RSPOs. Therefore, even though LGR4/5/6 were not required for RSPO2-induced potentiation, inhibition of ZNRF3 function by RSPO2 binding is essential for WNT signaling potentiation. Is an RSPO2-ZNRF3 complex alone sufficient enough to induce WNT signaling potentiation? Although the cooperative role of ZNRF3/RNF43 and HSPGs was not directly evaluated in these two studies. It is plausible that RSPOs can mediate the inhibition of internalization and degradation of the FZD receptor through the ZNFR3/RNF43 and HSPG complex (Fig. 2C).

1.2.3. ZNRF3/RNF43-independent potentiation

In addition to the above two mechanisms, a distinct ZNRF3/RNF43-independent mechanism of WNT signaling potentiation by RSPO was also reported (Fig. 2D) (Carmon et al., 2014). When *ZNRF3* expression was knocked down in HEK293 T cells by RNA interference, increased basal WNT signaling activity was observed, which is consistent with the negative role of ZNRF3/RNF43 in WNT signaling regulation. Surprisingly, RSPO was still able to potentiate WNT signaling activation in cells lacking ZNRF3 upon LGR4 overexpression. RSPO1 carrying the Q71 A mutation which fails to bind ZNRF3 can effectively potentiate WNT signaling in the presence of overexpressed LGR4. In this ZNRF3/RNF43-independent WNT potentiation, the scaffold IQGAP proteins that are known to modulate diverse intracellular signaling mediators such as MEK and ERK appear to play key roles. *IQGAP1* knockdown in HEK293 T cells significantly reduced WNT signaling potentiation by RSPO1. Furthermore, it is demonstrated that IQGAP1 directly binds to the cytoplasmic domain of the LGR4 receptor *via* the Ras GAP-related domain. It is further shown that RSPO stimulation enhances the IQGAP1 and Dishevelled interaction and recruits MEK1/2 and ERKs to the WNT signalosome, resulting in a phosphorylation of LRP6.

1.2.4. Inhibition of WNT signaling by RSPOs

RSPO2 or RSPO3 overexpression *via* recurrent gene fusion is closely associated with human colorectal cancer (CRC) lacking *APC* mutations (Seshagiri et al., 2012), displaying a typical WNT/β-catenin signaling potentiation by RSPOs in these cancers. Interestingly, it is demonstrated that RSPO2 functions as a tumor suppressor in some human CRCs in which RSPO2 expression is down-regulated (Wu et al., 2014). It appears that RSPO-LGR5 axis may inhibit WNT/β-catenin signaling. RSPO2 overexpression suppressed the proliferation and tumorigenicity of CRC cells by inhibiting WNT/β-catenin signaling (Wu et al., 2014). These activities of RSPO2 depend on LGR5. In opposite to LGR4/5/6-dependent mechanism (Fig. 2B), RSPO binding to both LGR5 and ZNRF3 can

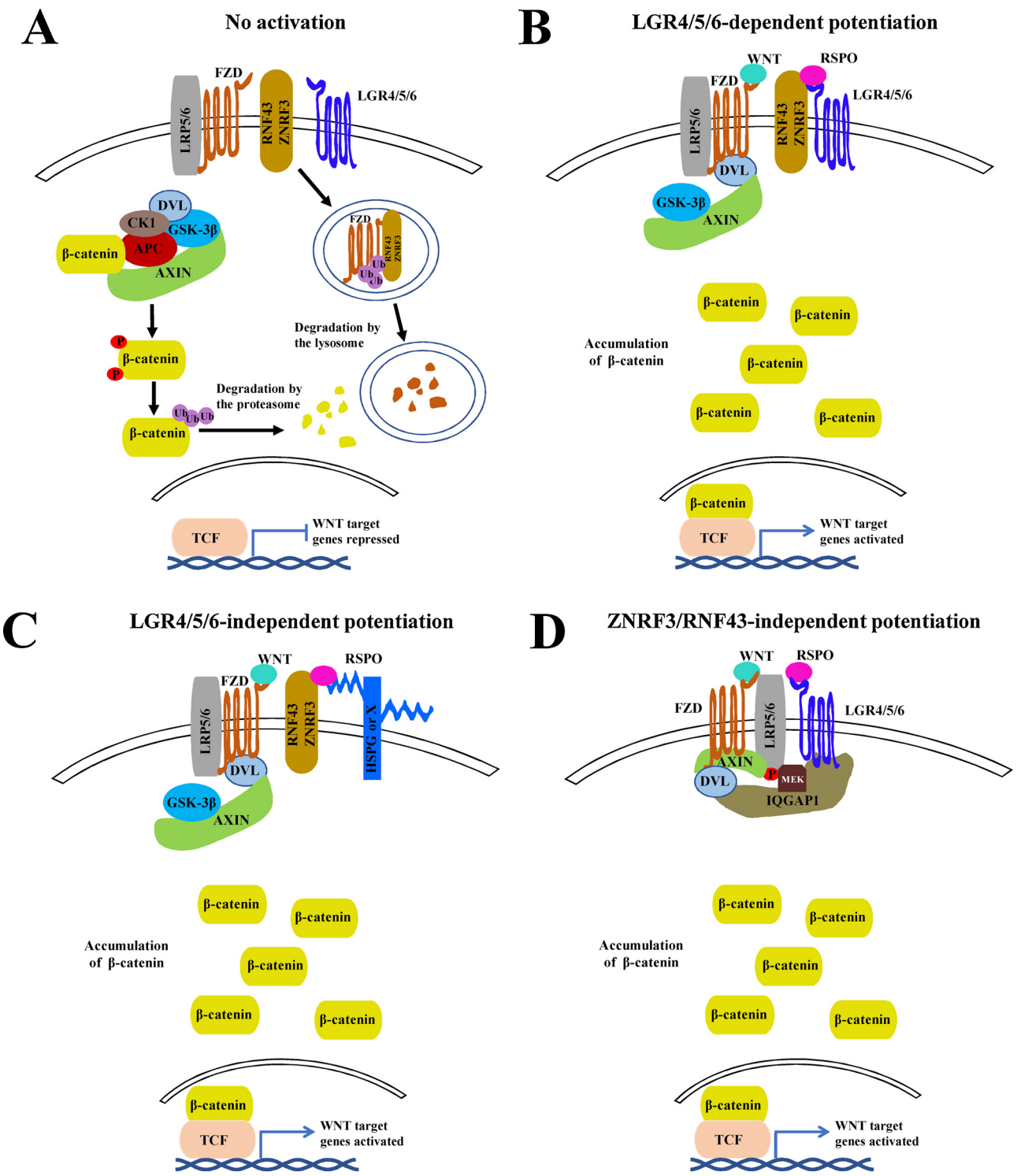

Fig. 2. Potentiation mechanisms of WNT signaling by RSPO. (A) In the absence of WNT ligand, the degradation complex consisting of APC, AXIN, GSK-3β, and CK1, phosphorylates β-catenin. Ubiquitination of phosphorylated β-catenin occurs followed by its degradation by the proteasome. Independently, ZNRF3/RNF43 ubiquitinate the Frizzled (FZD) receptors resulting in lysosome-dependent degradation of the FZD receptors. (B) LGR4/5/6-dependent potentiation of WNT signaling by RSPO. RSPO simultaneously binds to both LGR4/5/6 and ZNRF3/RNF43, leading to the clearance of ZNRF3/RNF43 from the plasma membrane. The increased level of the FZD receptors on the plasma membrane produce a higher WNT signaling activity. (C) LGR4/5/6-independent potentiation of WNT signaling by RSPO. RSPO binds to HSPG and ZNRF3/RNF43 simultaneously and potentiates WNT signaling. (D) ZNRF3/RNF43-independent potentiation of WNT signaling by RSPO. IQGAP1 directly binds to the cytoplasmic domain of the LGR receptor *via* the ras GAP-related domain. Upon RSPO stimulation, IQGAP1 and Dishevelled interaction is enhanced and MEK1/2 and ERKs are recruited to the WNT signalosome to generate active WNT/β-catenin signaling.

stabilize ZNRF3 to suppress WNT/β-catenin signaling. Notably, LGR5 was previously shown to be a downstream target gene of WNT/β-catenin signaling and was suggested as a negative feedback inhibitor of WNT/β-catenin signaling (Bottomly et al., 2010; Carmon et al., 2012). This unexpected tumor suppressor role of RSPO2 raises a major question of how RSPO2 exhibits opposite functions utilizing the same

molecular components.

In another CRC study, RSPO2 showed inhibitory activity towards CRC cell metastasis (Dong et al., 2017). Interestingly, this inhibitory action of RSPO2 did not occur through the WNT/β-catenin signaling pathway but rather through non-canonical WNT signaling. RSPO2 antagonized WNT5A-driven non-canonical WNT signaling activation of tumor cell migration by blocking the binding of WNT5A to the FZD7 receptor. Because RSPOs were previously shown to not bind FZD receptors (Nam et al., 2006; Wei et al., 2007), the mechanism of the antagonistic action of RSPO2 requires future investigation.

2. RSPOs: emerging regulators in adult stem cells

The initial link between RSPO and stem cell biology originated from an experimental observation that mice in which human RSPO1 gene was knocked into an immunoglobin ƙ locus resulting in continuous secretion of the RSPO1 protein into circulatory system by B cells developed intestinal epithelium hyperplasia (Kim et al., 2005). Systemic administration of recombinant RSPO1 protein also induced a similar phenotype in mice (Kim et al., 2005). RSPO1 significantly increases the proliferation of crypt epithelial cells, without affecting the number of goblet and paneth cells, suggesting that RSPO1 acts as a mitogen for intestinal stem cells with negligible effects on the maturation and migration of differentiated cells along the crypt-villus axis. Subsequently, RSPO proteins were found to be ligands for the stem cell markers LGR5 and LGR6, further emphasizing the importance of RSPO in regulating LGR5/6^{+ve} stem cells in various tissues including intestinal stem cells (ISCs).

2.1. Intestinal stem cells (ISCs)

The adult intestine has a high capacity for self-renewal and regeneration because it contains a stem cell population known as ISCs, which is in the bottom of crypts and expresses the RSPO receptor LGR5. RSPO ligands, particularly RSPO1, were shown to effectively support the *in vitro* expansion of LGR5^{+ve} ISCs in organoid culture (Greicius et al., 2018; Kabiri et al., 2014; Ootani et al., 2009; Sato et al., 2009; Yan et al., 2017). While the cell type that produces RSPO1 in the intestinal crypts has not been identified, RSPO3 is secreted from adjacent PDGFRα^{+ve} pericryptal myofibroblasts (Greicius et al., 2018; Kabiri et al., 2014). Considering the LGR5 receptor expression in the ISCs, it is speculated that the RSPO-LGR5 axis is crucial for the self-renewal of ISCs. However, an intestinal epithelium-specific *Lgr5* deletion in mice did not result in any overt intestinal phenotypes, indicating that *Lgr5* itself is functionally dispensable for ISCs (Mustata et al., 2013). Rather, gene deletion of a closely related family member, *Lgr4*, decreased ISC proliferation and induced crypt loss (Mustata et al., 2013). Moreover, ISCs from *Lgr4* null mice displayed impaired *ex vivo* organoid formation (Mustata et al., 2013). Therefore, it is likely that the expansion of ISCs is regulated by the RSPO-LGR4 axis.

Interestingly, a more recent study demonstrated that recombinant RSPO2 lacking the ability to bind LGR4 and LGR5 (likely LGR6 too) was able to support the growth of ISC organoids *in vitro* and stimulated the growth of intestinal crypts *in vivo* in mice (Park et al., 2018), suggesting that RSPO2 binding to LGR4/5/6 is not required for ISC growth. Although RSPO1 possesses a similar WNT potentiating activity like RSPO2 and RSPO3, RSPO1 binding to LGR4/5/6 is essential for WNT signaling potentiation (Lebensohn and Rohatgi, 2018). Whether the LGR4/5/6 receptors are actively involved in RSPO1-dependent, but not RSPO2/3-dependent regulation in ISCs remains to be determined.

Furthermore, intestine-specific deletion of the WNT signaling inhibitory RSPO receptor genes, *Rnf43* and *Znrf3*, in mice resulted in the expansion of crypt size associated with hyperproliferation and expansion of cycling ISCs, eventually leading to the formation of intestinal adenomas (Koo et al., 2012). These collective results strongly suggest a mechanistic model for ISCs in which RSPOs bind to ZNRF3/RNF43 to abolish their WNT signaling inhibitory activity, thereby potentiating WNT signaling activity (Fig. 2B and C).

Recently, the relative contributions of the extracellular WNT and RSPO ligands to homeostatic WNT signaling in the ISC niche were determined (Yan et al., 2017). In this

study, they showed that WNT proteins confer basal competency to ISCs by maintaining the expression of RSPO receptor genes (*Lgr5*, *Znrf3*, and *Rnf43*) which enables RSPO ligands to actively drive and specify the extent of ISC expansion. However, in the absence of both RSPO and WNT ligands, LGR5^{+ve} ISCs undergo a default differentiation program. This suggests the possible individual roles of WNT and RSPO in the priming and self-renewal of ISCs, respectively. However, it is unclear whether WNT and RSPO regulate priming and self-renewal independently or if each step requires a different strength of WNT signaling induced by WNT alone and WNT/RSPO cooperation.

How does RSPO-induced WNT signaling potentiation regulate ISC expansion at the molecular level? A transcription factor gene, *Ascl2*, was identified as an RSPO/WNT signaling target in ISCs (Schuijers et al., 2015). *Ascl2* overexpression enhances the self-renewal capacity of ISCs, whereas *Ascl2*$^{-/-}$ ISCs display increased sensitivity to WNT withdrawal in *in vitro* organoid culture. Because *Ascl2* expression is also regulated by a direct auto-activation loop, the ASCL2 transcription factor regulates gene transcription cooperatively with the β-catenin/TCF complex and functions as an on-off switch to maintain the stem cell state.

An additional molecular link for RSPO signaling in ISCs was revealed in a study of the regeneration of damaged intestinal epithelium (Zhou et al., 2013). *Slit2* and its receptor gene *Robo1* are expressed in ISCs. Partial deletion of *Robo1* decreased ISCs and resulted in villus hypotrophy, whereas *Slit2* overexpression increased ISCs and induced villus hypertrophy. Interestingly, administered RSPO1 acted cooperatively with the ROBO/SLIT signaling pathway during regeneration of the intestinal epithelium after administration of anti-tumor chemotherapeutics. The molecular nature of this cooperation has not been determined. Additionally, whether this cooperation occurs during normal intestinal epithelium homeostasis requires further investigation.

2.2. Gastric stem cells (GSCs)

LGR5^{+ve} cells are found at the base of mature pyloric glands in the adult mouse stomach (Barker et al., 2010). Using *in vivo* lineage tracing, these cells were shown to have a self-renewal capacity and are multipotent stem cells responsible for the long-term renewal of the gastric epithelium. Furthermore, single LGR5^{+ve} GSC efficiently generated long-lived organoids resembling the mature pyloric epithelium *in vitro* (Barker et al., 2010).

A recent study revealed a link between RSPO and GSCs and antral gland homeostasis (Sigal et al., 2017). It was previously shown that the gastric pathogen *Helicobacter pylori* penetrates deep into the antral glands and increases stem cell turnover manifested as gastric hyperplasia (Sigal et al., 2015). In this recent study, *Axin2* expression representing active WNT signaling was detected in two distinct stem cell populations within the antral glands: AXIN2^{+ve};LGR5^{+ve} and AXIN2^{+ve};LGR5^{-ve} cells. AXIN2^{+ve};LGR5^{+ve} cells represent long-lived stem cells as previously noted (Sigal et al., 2017). When LGR5^{+ve} cells are depleted, AXIN2^{+ve};LGR5^{-ve} cells are highly proliferative and can repopulate the entire antral glands, including the base. Specific *Rspo3* expression was detected in gastric myofibroblasts proximal to the stem cell compartment (Sigal et al., 2017). Along with several WNT ligands expressed in the proximity of the stem cell compartment, RSPO3 likely induces *Axin2* expression and potentiates WNT signaling in the GSCs. When antral gastric glands are co-cultured with primary myofibroblasts or cultured in the presence of recombinant RSPO1 or RSPO3, organoid growth is effectively supported (Sigal et al., 2017). Furthermore, administration of exogenous RSPO expands and accelerates the proliferation of AXIN2^{+ve} cells, but not LGR5^{+ve} cells, *in vivo* (Sigal et al.,

2017). Interestingly, *H. pylori* infection increases stromal *Rspo3* expression and expands the AXIN2^{+ve} cell pool, resulting in the hyperproliferation of gastric stem cells and gland hyperplasia. Therefore, RSPO3 controls LGR5^{-ve} GSCs likely *via* an LGR5-independent mechanism, although the possibility that RSPO3 transmits the signal depending on LGR4 or LGR6 cannot be completely ruled out.

2.3. Mammary gland stem cells (MaSCs)

LGR5^{+ve} cells are identified as epithelial stem cells in the mammary glands and these cells contribute to basal and luminal epithelial lineages in the postnatal mammary gland (Barker et al., 2013; de Visser et al., 2012). Similar to ISCs, *Lgr5* is functionally dispensable for both fetal and adult MaSC activities (Trejo et al., 2017). However, *Lgr4*$^{-/-}$ mice lack the capacity for MaSC repopulation, and mammospheres derived from *Lgr4*$^{-/-}$ MaSCs showed a decrease in the WNT signaling response (Wang et al., 2013), strongly suggesting that RSPO-LGR4 signaling axis is critical for MaSC function.

Rspo2 and *Rspo3* are potential oncogenes that induce mammary hyperplasia according to MMTV-based tumorigenesis screening in mice (Lowther et al., 2005; Theodorou et al., 2007). However, their expression in the normal mammary glands in mice has not been determined. In contrast, *Rspo1* is expressed in the Sca1^{-ve} luminal cells in the mouse mammary glands which are hormone receptor-negative, whereas numerous *Wnt* genes including *Wnt4*, *Wnt5A*, and *Wnt7b* are expressed in Sca1^{+ve} luminal cells (Cai et al., 2014; Dall et al., 2017), implicating a convincing possibility of RSPO1/WNT cooperation. In addition, upregulated *Rspo1* expression during pregnancy and estrogen and progesterone hormone treatment (Cai et al., 2014) and high *RSPO1* expression in human breast tissue (Haakensen et al., 2011) further suggest RSPO1 as a major RSPO in the mammary gland tissue.

Consistent with gene expression pattern, serial colony formation of MaSCs is significantly increased in the presence of WNT4 and RSPO1 conditioned medium. *Rspo1* knockdown in transplanted MaSCs results in alveologenesis defects during pregnancy and a decreased number of stem cells (Cai et al., 2014; Chadi et al., 2009). In contrast, MaSCs with *Wnt4* knockdown generated normal mammary outgrowth in virgins and during pregnancy. However, *Wnt4* and *Rspo1* double knockdown in MaSCs caused a severe failure to generate mammary outgrowths in transplantation assays (Brisken et al., 2000; Cai et al., 2014). Additionally, it is shown that RSPO1 in cooperation with Neuregulin1 maintains and expands mammary organoids efficiently for several months in culture (Jarde et al., 2016). These data suggest that RSPO1 along with WNT4 promote the self-renewal of MaSCs in an autocrine manner by amplifying WNT/β-catenin signaling.

Rspo1 expression appears to be at the downstream of RANK signaling. *Rank* null mice exhibited defects in the proliferation of hormone receptor-negative luminal and basal cells (Joshi et al., 2015). *Rspo1* expression is abolished in these luminal cells in *Rank* mutant mice. Administration of RSPO1 to these mutant mice rescued several key defects caused by *Rank* gene ablation, convincingly indicating that RSPO1 is a key mediator downstream of RANK signaling in regulating the WNT response and MaSC expansion (Joshi et al., 2015).

2.4. Skeletal muscle stem cells (SkMSCs)

Adult skeletal muscle shows a remarkable plastic and regenerative capacity in response to diverse stimuli, including stretching, exercise, injury, and electrical stimulation. Regeneration of the adult skeletal muscle relies on a pool of resident SkMSCs (also known as satellite cells) which are located between the basal lamina and myofibers. Quiescent SkMSCs expressing the paired box transcription factor PAX7 (Seale and Rudnicki, 2000) are activated and proliferate to give rise to myogenic precursor cells that can terminally differentiate into myofibers. Both canonical and non-canonical WNT signaling is crucial for SkMSCs during regeneration. A previous study implicated the canonical WNT1-induced β-catenin pathway in regulating muscle cell differentiation through the activation and recruitment of SkMSC-like reserve myoblasts for fusion with myotubes *in vitro* (Rochat et al., 2004). In contrast, another study showed that WNT/β-catenin signaling promotes SkMSCs proliferation in the first stages of their activation on isolated myofibers *in vitro* (Otto et al., 2008). *In vivo* injection of recombinant WNT3A to the regenerating adult muscle controls myogenic lineage progression by limiting pro-proliferative NOTCH signaling and thus promoting differentiation during muscle regeneration (Brack et al., 2008). Inhibition of NOTCH signaling by WNT3A is mediated by inhibition of GSK-3β. In contrast, WNT7A, *via* non-canonical WNT signaling, promotes the proliferation of SkMSCs through the planar cell polarity pathway effector VANGL2 (Le Grand et al., 2009). Moreover, WNT7A enhances myocyte fusion during regeneration, resulting in hypertrophy in regenerated myofibers by activating the AKT/mammalian target of rapamycin pathway (von Maltzahn et al., 2011).

Previously, *Rpso1/2/3* were shown to be expressed in proliferating SkMSCs and *Rpso1/3* were found to be up-regulated during their differentiation (Han et al., 2011). Furthermore, all *Rspo* genes are transiently expressed during muscle regeneration, reaching a peak at approximately 2–4 days of regeneration when SkMSCs are actively proliferated and initiate differentiation to form new myofibers (Yoon, unpublished), suggesting a specific role for RSPOs in WNT signaling during SkMSC proliferation and differentiation. Recently, it was reported that RSPO1 is expressed in quiescent SkMSCs and shows increased expression in activated SkMSCs (Lacour et al., 2017). Interestingly, *Rspo1* transcription was strongly up-regulated in primary myoblasts overexpressing *Pax7* (Soleimani et al., 2012) and PAX7 was shown to bind the *Rspo1* promoter, suggesting that *Rspo1* is a downstream target gene for PAX7 (Lacour et al., 2017).

Rspo1 ablation in mice caused a delay in myogenic differentiation during regeneration (Lacour et al., 2017). While the numbers of PAX7^{+ve} SkMSCs and progenitor cells were similar at day 4 of regeneration of injured tibialis anterior muscle, the number of Myogenin^{+ve} myocytes was decreased. Later in regeneration at 7 days after injury, there was no difference in newly formed myofibers in both wild-type and *Rspo1* null mice. However, the number of Myogenin^{+ve} cells inside myofibers was increased in *Rspo1* mutant muscle, suggesting a compensatory enhancement of fusion. Furthermore, compared to control muscles, fully regenerated *Rspo1* mutant muscles were larger, heavier and composed of larger myofibers containing a larger number of myonuclei. Loss of *Rspo1* may shift the WNT signaling balance from canonical WNT to non-canonical WNT signaling, as regenerating muscle phenotypes of *Rspo1* null mutants showed some similarities to those of WNT7A-overexpression mice. Three additional *Rspo* members are expressed during muscle regeneration. Whether there is a unique or compensatory role for each RSPO member requires further investigation.

2.5. Hair follicle stem cells (HFSCs)

The mammalian epidermis exhibits regenerative capacity within its three compartments: the hair follicle (HF), sebaceous gland, and interfollicular epidermis. HFs present in most areas of the skin undergo many hundreds of regeneration cycles (catagen (degeneration), teleogen (resting), and anagen (growth)) during our lifetime. While LGR5^{+ve} cells reside exclusively in the lower bulge and hair germ of telogen HFs (Barker et al., 2008), LGR6 marks the most primitive epidermal stem cells that generate the hair follicle and sebaceous gland (Snippert et al., 2010). Transplanted LGR5^{+ve} cells generated all skin components, including functional HFs, sebaceous glands, and interfollicular epidermis (Barker et al., 2008), whereas LGR6^{+ve} cells were involved long-term wound repair, including the formation of new HFs (Snippert et al., 2010).

All *Rspo* genes are highly expressed in the HF dermal papilla; particularly, *Rspo1* expression is noticeably upregulated before anagen

entry (Li et al., 2016). Intradermal injection of recombinant RSPO1 protein in mid-telogen causes early entry into the anagen cycle. RSPO1 activates WNT/β-catenin signaling in cultured bulge stem cells *in vitro* and induces the fate determination factors of HFs such as *Sox7* and *Sox21* without affecting cell proliferation (Li et al., 2016). *Rspo2* expression in mouse skin is higher in the anagen than in the catagen or telogen cycles. RSPO2^{+ve} cells are detected in the epidermis, matrix, and inner root sheath. Intradermal injection of RSPO2 induces the LGR5^{+ve} stem cell population in mice. RSPO2 injection represses entry into catagen and prolongs anagen, resulting in longer and thicker hair (Smith et al., 2016). These two studies suggested that RSPO1 and RSPO2, although they can amplify WNT signaling, play different roles in regulating the proliferation and differentiation of HF stem cells.

2.6. Other adult stem cells

LGR5^{+ve} cells, representing a potential stem cell population, have been identified in many additional normal or injured adult tissues (Curcio et al., 2015; Hirata-Tominaga et al., 2013; Huch et al., 2013; Lugli et al., 2016; Vidal et al., 2016). Studies of RSPO signaling in these cells have mainly focused on whether RSPO supports LGR5^{+ve} stem cells in culture, as these stem cells are difficult to grow in *in vitro* culture while maintaining their stem cell capacity. As demonstrated in the stem cells mentioned above, RSPOs have emerged as key regulatory factors of the growth of these stem cells.

LGR5 is uniquely expressed in limbal epithelial crypts and the peripheral region of human corneal endothelial cells (CECs). These LGR5^{+ve} cells show stem/progenitor cell characteristics (Curcio et al., 2015; Hirata-Tominaga et al., 2013). *RSPO1/2/3* are expressed in the peripheral region of human CECs (Hirata-Tominaga et al., 2013). In *in vitro* culture, RSPO1 accelerated CEC proliferation and inhibited mesenchymal transformation by activating the WNT/β-catenin pathway, suggesting a key role for RSPO1 in maintaining corneal endothelium homeostasis (Hirata-Tominaga et al., 2013; Okumura et al., 2014).

In the liver, LGR5^{+ve} cells appear upon injury (Huch et al., 2013). In RSPO1-containing culture, a single LGR5^{+ve} cell isolated from the damaged mouse liver was clonally expanded into an organoid that differentiated into functional hepatocytes upon transplantation into fumarylacetoacetate hydrolase *(Fah)*$^{-/-}$ mutant mice, a model for Tyrosinemia type I liver disease (Azuma et al., 2007; Huch et al., 2013). Deficiency of *Fah* resulted in liver failure unless the mice were administered with 2-(2-nitro-4-trifluoro-methylbenzyol)-1,3-cyclohexanedione. In a mouse liver fibrosis model, administration of RSPO1 in combination with hepatic growth factor (HGF) increased the number of LGR5^{+ve} liver stem cells and improved liver function by alleviating fibrosis (Lin et al., 2017). Consistent with the results from mouse study, LGR5^{+ve} liver stem cells identified in human liver fibrosis tissues formed organoids and their expansion was promoted by HGF/RSPO1 treatment (Lin et al., 2017).

Additionally, LGR5^{+ve} stem cells isolated from the gall bladder can be propagated as organoids in the presence of RSPO1 and the bone morphogenetic protein signaling inhibitor NOGGIN for more than a year (Lugli et al., 2016). Ductal fragments isolated from the mouse pancreas initiated *Lgr5* expression in RSPO1-containing culture and developed into budding cyst-like organoids that expanded for up to 40 weeks (Huch et al., 2013). A damage-induced pancreatic LGR5^{+} cell also exhibited the ability to form organoids in the presence of RSPO1 (Huch et al., 2013). In the adrenal gland, *Rspo3* deletion led to impaired organ growth and loss of sonic hedgehog signaling, suggesting that RSPO3 plays an important role in the replenishment of lost cells and maintains the properties of the zona glomerulosa (Vidal et al., 2016).

In the developing kidney, LGR5^{+ve} cells in nascent nephrons are detected and generate part of the Henle loop and distal convoluted tubule (Barker et al., 2012). In the cochlea of the inner ear, *Lgr5* is expressed in a subset of supporting cells (Chai et al., 2012; Shi et al., 2012). Lineage tracing experiments showed that these *Lgr5*-expressing cells differentiate into hair cells *in vivo*.

The hilum region of the ovarian surface epithelium also contains LGR5^{+ve} progenitor cells that regenerate the entire ovarian surface epithelium. (Flesken-Nikitin et al., 2013). No studies have demonstrated how RSPO signaling regulates the self-renewal and differentiation of these stem cells.

3. Conclusion

Understanding the signaling pathways with important roles in stem cell function and homeostasis is important for applying stem cells in regenerative medicine. WNT signaling was demonstrated to play an important role in regulating stem cell homeostasis by promoting the self-renewal of stem cells and controlling differentiation in many organs. RSPOs, which are WNT signaling regulators, play a critical role in regulating adult stem cells in various organs. As the diverse molecular mechanisms of RSPO signaling continue to be revealed, studies are needed to investigate how different RSPOs adopt a specific signaling mechanism in different stem cell contexts. A better understanding of RSPO signaling in different stem cell types may lead to developments in stem cell-based regenerative medicine.

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