CRITICAL REVIEW

R-spondin-mediated WNT signaling potentiation in mammary and breast cancer development

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Abstract

The mammary gland is a secretory organ, which develops as a network of growing epithelial ducts composed of luminal and basal cells that invade the surrounding adipose tissue through a series of developmental cycles. Mammary stem cells (MaSCs) maintain an accurate tissue homeostasis, and their proliferation and cell fate determination are regulated by multiple hormones and local factors. The WNT pathway plays a critical role in controlling the enormous tissue expansion and remodeling during mammary gland development through the maintenance and differentiation of MaSCs, and its deregulation has been implicated in breast cancer (BC) initiation and progression. The R-spondins (RSPOs) are four secreted proteins that strongly enhance target cell sensitivity to WNT ligands. Moreover, leucine-rich repeat-containing G-protein-coupled receptors (LGRs) 4–6 are considered obligate high-affinity receptors for RSPOs and have been described as stem cell markers. Importantly, elevated RSPO expression has been recently identified in several tumor types from patients, including BC, and it has been reported that they play a significant role in mammary tumor progression in experimental models. In this review, exploring our present knowledge, we summarize the role of the RSPO-LGR axis as a WNT-enhancing signaling cascade in the MaSC compartment and during the normal and neoplastic mammary gland development. In addition, we include an updated expression profile of the RSPOs and their action mediators at the cell membrane, the LGRs, and the ubiquitin-ligases ZNRF3/RNF43, in different BC subtypes. Finally and based on these data, we discuss the significance of tumor-associated alterations of these proteins and their potential use as molecular targets for detection and treatment of BC.

KEYWORDS

breast cancer, LGR, mammary gland, RSPO, WNT

Abbreviations: BC, breast cancer; BCSCs, breast cancer stem cells; BR, basic amino acid-rich; EMT, epithelial-to-mesenchymal transition; HSPGs, heparan sulfate proteoglycans; MaSCs, mammary stem cells; TEBs, terminal end buds; TSR, thrombospondin type I repeat.

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1 | WNT PATHWAY OVERVIEW

WNTs represent a numerous family of secreted lipid-modified, cysteine-rich glycoproteins which share a high degree of sequence homology. Activation of WNT

signaling pathways has been described over the past decades as cell-biological programs critically required for several processes including embryonic development, regeneration, and cellular differentiation. Among pathological conditions, these pathways play a clear role in all steps of carcinogenesis, from tumor initiation to cancer growth and dissemination (all this information is well-reviewed in References 1 and 2). Since WNTs are hydrophobic as a result of posttranslational lipid modifications,³ they are unlikely to diffuse freely. Thus, once in the extracellular matrix (ECM), WNTs are most commonly restricted to autocrine and juxtacrine signaling. However, with the assistance of carrier structures like WNT-binding chaperones, heparan sulfate proteoglycans (HSPGs), lipoproteins, exosomes, and cytonemes, and even in a cell-bound manner through cell divisions, it has been suggested that WNTs can act as short- and relative long-range paracrine signaling molecules influencing the behavior of target cells in a concentration gradient-dependent manner.^{4–7} Even though there have been several reports trying to elucidate this WNT extracellular transport (well-reviewed in References 8 and 9), how exactly WNT gradients are formed and how short- and relative long-range WNT spreading is achieved in tissue homeostasis and development is still poorly understood.

At the cell membrane, WNT ligands mostly initiate signal transduction through binding to a member of the frizzled (FZD) transmembrane receptor family, but atypical receptors have been described, including the receptor tyrosine kinase (RYK)-like orphan receptor 1 or 2 (ROR1/2) and the RYK.¹⁰ The large family of WNT factors has long been grouped into two main categories, “canonical” and “noncanonical” ligands, as the downstream signaling pathways they activate. However, the intracellular response to each individual WNT is markedly context-specific, since several reports described the ability of distinct WNTs to activate both canonical and noncanonical WNT signaling pathways.¹⁰ Thus, the intracellular output might be the result of a combination between WNTs and a given receptor/co-receptor at the cell membrane, rather than the nature of each individual WNT. Since these cascades have been extensively explained in numerous reviews,^{1,11} they will only be described briefly herein.

Canonical WNT pathway, often referred to as WNT/ β -catenin signaling, is essential to several developmental processes by regulation of cell proliferation, differentiation, and migration, while its deregulation underlies a wide range of pathologies including cancer. After binding to FZD and a member of the low density lipoprotein-related protein 5 or 6 (LRP5/6) family, WNT ligands are able to activate the canonical WNT signaling

pathway by inducing the release of β -catenin from a cytosolic, multiprotein destruction complex (Figure 1, upper panel). Therefore, β -catenin accumulates and translocates into the nucleus where it acts as a co-activator of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors, promoting the transcription of a large number of target genes (reviewed in Reference 1).

Several noncanonical WNT pathways, that do not involve β -catenin, have been described (Figure 1, lower panel): as the WNT/planar cell polarity (PCP) pathway, in which FZD-bound WNTs establish a PCP and regulate polarized cell migration through modulation of the cytoskeletal factors, RhoA and JUN kinase (JNK); the WNT/ Ca^{2+} pathway in which WNTs, through FZD binding, can trigger Ca^{2+} fluxes; the WNT-ROR2 binding that triggers an intracellular cascade to inhibit WNT/ β -catenin pathway by JNK activation; and the WNT-RYK binding that activate Src proteins (all four reviewed in Reference 10).

1.1 | WNT pathway potentiators: The R-spondins

R-spondins (roof plate-specific spondins; RSPOs) are a family of four secreted glycoproteins (RSPO1–4) that have highly conserved domain structures with two amino-terminal cysteine-rich furin-like repeats (FU1 and FU2) and a thrombospondin type I repeat (TSR) domain toward the basic amino acid-rich (BR) carboxyl terminal domain^{12,13}. RSPOs markedly differ in the length of the BR carboxyl domain, being RSPO3 the largest, followed by RSPO1.¹⁴ Once secreted, the RSPOs can bind to the leucine-rich repeat-containing G-protein-coupled receptors 4, 5, or 6 (LGR4–6)¹⁵ through their FU2 domain and/or to the extracellular domains of two membrane-bound E3 ubiquitin ligases, Zinc and RING finger 3/RING finger 43 (ZNRK3/RNF43), through the FU1 domain.¹⁶ Once secreted, in culture, RSPOs have been either poorly detected or nearly undetected in the conditioned medium of cells that express them.^{12,17} As it was observed that adding soluble heparin or sodium chlorate, an inhibitor for sulfation, strongly enhanced free RSPO levels, it was concluded that these proteins remain bound to the cell surface and/or ECM components, like HSPGs.¹⁷ Moreover, this study demonstrated that deletions of the BR or both the TSR and BR domains turned the RSPOs easily detected as soluble proteins.¹⁷ More precisely, it has been shown that positively charged amino acids enriched in the BR domain, as well as the TSR domain itself, promoted RSPO cell surface retention by interacting with different types of Syndecan,^{18,19} one of the major membrane-bound HSPGs (see Figure 2).

FIGURE 1 Canonical WNT pathway (upper panel). **WNT OFF.** Without WNT ligands, cytosolic β -catenin is retained in a multiprotein destruction complex composed by Axin, APC, GSK-3, and CK1 α , which leads to β -catenin phosphorylation, ubiquitination, and consequent degradation via proteasome. In the nucleus, TCF/LEF is in an inactive state and, therefore, the transcription of target genes is OFF (left). **WNT ON.** After WNT binds to its receptor FZD, and co-receptor LRP5/6, β -catenin is released from the destruction complex, since FZD-bound DVL recruits the multiprotein complex contributing to its destabilization. This prevents phosphorylation and degradation of β -catenin, resulting in its accumulation and translocation into the nucleus where it acts as a co-activator of TCF/LEF, promoting the transcription of target genes (right). Noncanonical WNT pathways (lower panel). FZD-bound WNTs act in combination with different co-receptors activating β -catenin-independent mechanisms. The WNT/Ca²⁺ pathway (left) promotes the activation of phospholipase C (PLC) activity, leading to intracellular Ca²⁺ fluxes and downstream modulations that promote cytoskeletal rearrangements and/or transcriptional responses, as the NFAT nuclear activity. In the WNT/PCP pathway (right), DVL binds to Rho by de-inhibition of DAAM1. Rac1 and Rho together trigger ROCK and JNK activation. This leads to JUN transcription factor activation and/or rearrangements of the cytoskeleton. DVL, dishevelled; DAAM1, dishevelled-associated activator of morphogenesis 1; FZD, frizzled; JNK, JUN kinase; LRP5, lipoprotein-related protein 5; NFAT, nuclear factor of activated T cell; ROCK, rho-associated protein kinase; TCF/LEF, T-cell factor/lymphoid enhancer factor

Therefore, it may be expected that RSPOs exert their activities in an autocrine or short-range paracrine manner. However, it remains to be elucidated whether other HSPGs or extracellular components, and even the markedly different BR length among RSPOs, are involved in

determining their location and spreading in different mammalian tissues.

LGR proteins are evolutionarily-conserved seven-transmembrane receptors that belong to the G protein-coupled receptor superfamily.²⁰ They contain a large

FIGURE 2 WNT signaling amplification by RSPOs. Upon binding of WNT ligands to their receptors and co-receptors, target gene expression and/or rearrangements of the cytoskeleton are activated. Without RSPOs, a negative feedback mechanism maintains WNT signaling regulated due to the activity of transmembrane ubiquitin ligases, ZNRF3/RNF43, identified as negative modulators of the WNT signaling. These related ligases decrease the availability of FZD promoting membrane clearance through endocytosis and ubiquitin-mediated degradation.¹ With RSPOs, WNT signaling is potentiated by three distinct mechanisms: RSPOs bind to LGR4/5/6 and ZNRF3/RNF43 triggering the auto-ubiquitination of ZNRF3/RNF43, increasing the availability of FZD receptors.² RSPO2 and RSPO3 potentiate WNT signaling by interacting with ZNRF3/RNF43 and membrane-bound HSPGs, as Syndecans, in an LGR-independent manner.³ The RSPO-LGR4 complex enhances WNT pathways through the recruitment of IQGAP1 in a ZNRF3/RNF43-independent mechanism.⁴ HSPG, heparan sulfate proteoglycan; IQGAP1, IQ motif containing GTPase-activating protein 1; LGR, leucine-rich repeat-containing G-protein-coupled receptor; RSPO, R-spondin; ZNRF3/RNF43, zinc and RING finger 3/RING finger 43

extracellular region (ectodomain) with multiple imperfect copies of a leucine-rich repeat protein interaction domain.²⁰ Structural equivalences among LGR 4, 5, and 6 have been pointed due to sequence alignment of their ectodomains.²¹ LGR4-6 were considered orphan receptors until 2011, when it was demonstrated that the RSPOs were their high-affinity ligands.¹⁵

Transmembrane E3 ubiquitin ligases ZNRF3 and RNF43 negatively regulate the WNT pathways by promoting endocytosis and turn-over of FZD and LRP6 in the absence of RSPOs^{22,23} (Figure 2). However, RSPO-to-LGR binding induces auto-ubiquitination and membrane clearance of ZNRF3/RNF43, promoting FZD accumulation at the cell surface and enhancing the activation of canonical and noncanonical WNT signaling pathways^{22–24} (Figure 2).

Additional mechanisms that explain RSPO potentiating ability have been reported. Upon RSPO stimulation, LGR4/5 binds to LRP5/6 and FZD enhancing canonical WNT signaling.^{25,26} Besides, it has been demonstrated that on top of RNF43/ZNRF3 clearance, the RSPO-LGR4 complex is also able to potentiate WNT pathways through the recruitment of the intracellular scaffold IQ

motif containing GTPase-activating protein 1 (IQGAP1), in a ZNRF3-independent manner.²⁷ Moreover, three recent independent studies have reported that RSPO2 and RSPO3 are able to enhance WNT signaling by interacting with ZNRF3/RNF43, without the requirement of LGR binding, but involving the interaction with membrane-bound HSPGs by the TSR/BR domains^{28–30} (Figure 2). Considering these data and the fact that HSPGs are expressed ubiquitously on the surface of most cells³¹ whereas LGRs are specifically expressed in some stem cell niches,²⁰ it could be suggested that an RSPO-to-LGR binding might be relevant when a high level of a WNT signaling pathway must be activated in a specific cell compartment, as in the stem cells of various tissues, while an HSPG-mediated RSPO signaling may allow a less specific WNT pathway activation during the development of different tissues. Therefore, given the fact that several RSPO receptors have been identified in various experimental models, further studies are required to clarify how RSPO proteins may utilize different receptors or a combination of different receptors depending on each specific context to amplify target cell responses to WNT ligands.

Functionally, and based on their ability to potentiate WNT pathway activation, the RSPOs play critical roles in embryonic development and organogenesis as well as in the self-renewal and maintenance of stem cells in several tissues (reviewed in References 14 and 32). Particularly, they have been implicated in sex determination, vasculature formation, and in the development and growth of lungs, hairs, nails, limbs, intestine crypts, and muscles (reviewed in References 14 and 32). Coincidentally, the LGRs have been described as stem cell markers and WNT-regulated target genes in different tissues, playing a relevant role in embryonic development and adult tissue homeostasis.^{20,33,34}

Under pathological conditions, elevated RSPO expression due to genomic rearrangements or transcriptional activation has been identified in mouse and human tumors,^{35,36} suggesting that these proteins may be functionally relevant for tumor development. Acting through canonical or noncanonical WNT pathway activation, the RSPOs have been implicated in the progression of several cancer types including colorectal, ovarian, lung, bladder, and breast cancer (BC).^{36–38} Here, we focus on the role of RSPO-LGR signaling axis in the normal and neoplastic mammary gland development.

2 | MAMMARY GLAND DEVELOPMENT AND BC: OVERVIEW

The mammary gland is an epithelium formed by an inner luminal sheet surrounded by an outer layer of myo-epithelial or basal cells.³⁹ This tissue is an exocrine gland of ectodermal origin, whose development starts during embryogenesis and continues during puberty. Since the fundamental aspects of mammary tissue organization and development are well conserved between rodents and humans, mice and rats have been commonly used as experimental models to analyze developmental processes and oncogenic pathways.⁴⁰ Mammary gland formation in mouse embryos starts on the ectoderm at mid gestation, around 10 days after mating has occurred (E10). In 2 days (by E12, approximately), five (three thoracic and two inguinal) mammary placodes develop into mammary buds at each flank. In males, the mesenchyme around these structures expresses the androgen receptor that leads to the destruction of the mammary anlage in many mouse strains. In female embryos, the buds remain quiescent until E15.5–E16.5, when mammary epithelial cells start to divide and the buds sprout into the developing mammary fat pads located within the dermis. Then, a process of ductal branching begins, giving rise to the rudimentary ductal tree observed at birth.⁴⁰

Fetal human and mouse mammary development were reported to be quite parallel.⁴¹ Nevertheless, the human process is more complex involving 10 phases associated with increasing embryonic length.⁴² Besides, in the human fetal gland, most epithelial cells are positive for both luminal and basal markers (CK19 and CK14, respectively) and only near delivery basal and luminal signatures can be distinguished as either C14⁺ or CK19⁺ cells; however, only CK14⁺ cells have been observed in embryonic mouse mammary glands. Another difference at birth is that in female human newborns various minor ductal trees converge at each nipple, while in mice, a single ductal network corresponds to each gland.⁴¹

Before puberty, mammary epithelium grows according to the body size. At the beginning of puberty (approximately 5 weeks after birth in mice and 8–12 years of age in humans), the release of ovarian hormones promotes ductal development and branching of the gland. In addition, in both species, there is a renewed growth of mammary stroma (fatty and fibrous tissue) at this time. During this phase, at the distal ends of the mammary ducts, bulbous structures composed by multiple layers of cuboidal epithelial cells called terminal end buds (TEBs) can be observed. They constitute the invading fronts of the ducts, which extend and branch by bifurcation until reaching the limits of the fat pad at 8–12 weeks in mice and 18–24 years in humans whereupon the TEBs regress.^{41,43} Then, in the mature nulliparous female, ductal trees with budding structures (mouse alveolar buds or human terminal duct lobular units, TDLUs), each of which correspond to a group of acini from a single terminal bud, can be observed. One significant difference between mammary glands of puberal humans and mice is that in the first, lobules develop at this stage, while in the second these structures only appear if pregnancy occurs. In both species there is an extensive ductal branch expansion from the mouse alveolar buds or human TDLUs at the initiation of pregnancy. Finally, just before delivery, mammary differentiation reaches its maximum with the development of alveoli that produce lipids and protein for postpartum milk secretion.⁴¹

Twenty years ago, using serial transplantation of retrovirally tagged mammary cells, it was demonstrated that certain individual cells were able to completely reconstitute normal mammary tissue upon transplantation into de-epithelialized (cleared) mammary fat pads of host syngeneic mice.⁴⁴ This study provided the basis of our current understanding of the mammary gland cellular hierarchy, based on the existence of multipotent mammary stem cells (MaSCs) that can reconstitute the whole gland upon transplantation into cleared mammary fat

pads.⁴⁵ However, lineage-tracing studies demonstrated that the mammary gland embryonically develops from bipotent MaSCs that are later replaced by unipotent progenitors, which show their enormous potential during puberty and in the successive pregnancies.³⁹ These unipotent progenitors produce two functional-differentiated subtypes: the hormone-sensing or secretory luminal cells, and the basal myoepithelial contractile cells.⁴⁶ Interestingly, the multipotent stem cell activity of these progenitors is revealed in the transplantation assays. Physiologically, this capacity may be very important for recovery from local lesions or diseases, such as mastitis.⁴⁶ In addition, it has also been shown that localized stem cells originate clonal regions within the epithelial tree and that there are progenitor cell populations restricted to lobular or ductal development (reviewed in Reference 46).

BC-initiating cells or BC stem cells (BCSCs) are subpopulations of breast tumor cells enriched for clonogenic activity, which play critical roles in tumor initiation, progression, therapy resistance, and cancer recurrence.⁴⁷ It has been determined that human BC cells that were positive for the antigen CD44 and had no or low expression of CD24 were enriched for tumorigenic activity in xenografts grown in immuno-compromised mice.⁴⁷ Therefore, CD44⁺/CD24^{lo/-} have been considered as a BCSC-enriched population. MaSCs and BCSCs share some fundamental features such as self-renewal capability and multilineage potency. However, the first are not necessarily the origin of the second, since more differentiated cells can acquire mutations resulting in dedifferentiation, gain of self-renewal capabilities and epithelial-to-mesenchymal transition (EMT) (reviewed in Reference 46). In spite of that, it is often proposed that tumors arise from transformed MaSCs that acquire heterogeneity through clonal evolution, especially because they are long-lived and therefore more likely to accumulate mutations (reviewed in Reference 46). The concept that a mammary neoplasia is commonly originated from MaSCs suggests that determinant factors for cell fate decisions during normal development, such as WNT pathway regulators, are likely to be crucial in defining tumor phenotype and therapy response.

Five intrinsic molecular subtypes of BC have been described.⁴⁸ They are known as Luminal A, Luminal B, HER2-enriched, Basal-like, and Claudin-low. When analyzed in combination with clinical data (tumor grade, hormone receptor status, and HER2 status), they display relevant differences in their incidence, risk factors, prognosis, and treatment sensitivity.⁴⁸ Luminal tumor cells resemble those lining the normal mammary ducts. Most Luminal A and B tumors express estrogen receptors (ER⁺). However, Luminal A, the most common subtype (30–70% of BCs), tend to display a negative HER2 status

(HER2⁻), that is, they do not show amplification/overexpression of the *HER2* gene, whereas Luminal B can be either HER2 positive or negative. Luminal BCs normally respond to hormone therapy and their prognosis has a favorable outlook, particularly the Luminal A subtype, when treated early.⁴⁸ The HER2-enriched subtype represents 5–15% of BCs. Although not all tumors expressing HER2 belong to this group, most of them are HER2⁺ as well as estrogen and progesterone receptor negative (ER⁻PR⁻). Tumors within this subtype tend to grow fast and be invasive; however, specific anti-HER2 therapeutic approaches have improved their treatment.⁴⁸ The Basal-like category is the most distinct among all the molecular subtypes and it is defined by a robust cluster of genes expressed by epithelial cells in the basal or outer layer of the adult mammary gland. Most of the Basal-like tumors are triple-negative (ER⁻PR⁻HER2⁻) and have a highly aggressive behavior. Thus, they have less specific therapeutic options and the worst prognosis among all BC subtypes.⁴⁹ Finally, the Claudin-low tumors, which have been more recently characterized, show low to none expression of luminal markers, high enrichment for EMT markers, and cancer stem cell-like features. Therefore, it has been hypothesized that the most primitive MaSCs may be the cellular origin for this subtype.⁵⁰ Clinically, most Claudin-low tumors are triple-negative with a poor prognosis and a high frequency of metaplastic differentiation, and their therapeutic options are similar to those available for the Basal-like subtype. However, it has been reported that the Claudin-low signature is enriched in residual tumor tissue remaining after either endocrine therapy or chemotherapy,⁵⁰ indicating that finding specific targets for its treatment would have an important impact in preventing BC relapse.

3 | WNT PATHWAY INVOLVEMENT IN MAMMARY GLAND DEVELOPMENT AND NEOPLASIA

WNT signaling has been implicated in determining the specification and morphogenesis of mammary cell populations during almost all stages of embryonic and postnatal mammary gland development (well-reviewed in References 51 and 52).

3.1 | Canonical WNT pathway in the mammary gland

During mouse embryogenesis, the canonical WNT pathway determines the cell fate of those composing the

mammary lines and placodes.⁵³ Inhibition of distinct WNT-related factors resulted in placodal impairments, while hyperactivation of WNT/ β -catenin signaling led to their accelerated induction.^{54,55} During postnatal mammary development, WNT/ β -catenin signaling plays a critical role in controlling the enormous tissue expansion and remodeling (i.e., branching morphogenesis, alveolar bud formation, and early lobulo-alveolar development during pregnancy) through the maintenance and differentiation of MaSCs within the basal mammary epithelium.^{55,56}

Several studies have demonstrated mammary tumor development upon canonical WNT pathway hyperactivation using different mouse models. Since WNT/ β -catenin activation regulates MaSC dynamics, it is expected that its pro-oncogenic capacity would reside in a deregulated, altered stem/progenitor cell expansion. In fact, overexpression of WNT1 or activated Δ N89 β -catenin led to mammary hyperplasias that displayed stem/progenitor markers in vivo and enhanced colony-forming ability and bipotency in culture.^{54,57} Moreover, canonical WNT signaling hyperactivation in BC was involved in BCSC survival, bulk-tumor expansion, and enhanced invasion. High cytoplasmic and nuclear accumulation of β -catenin has been detected in approximately 60% of human BCs,⁵⁸ particularly in the Basal subtype,^{59,60} associated with an increase in the CD44⁺ CD24⁻ stem-cell-like population.⁵⁹ WNT/ β -catenin activation was also observed in mouse-derived basal-like tumors that exhibited characteristics of this human BC subtype.⁶¹ Likewise, canonical WNT signaling blockage decreased the migratory potential and growth of Basal-like BC cells in culture and in several mouse xenograft models of triple-negative BC.^{62,63} However, canonical WNT signaling activation in BC is not commonly induced by β -catenin mutations. Therefore, studying new factors involved in its modulation may lead to new therapeutic strategies to control BC progression.

3.2 | Noncanonical WNT pathway in the mammary gland

Although WNT/ β -catenin signaling has been extensively studied and is clearly implicated in mammary gland development, it is known that noncanonical WNT pathways also modulate mammary stem/progenitor biology. WNT5A and WNT5B represent two noncanonical WNT ligands that play a role within the mammary epithelium, and whose expression is restricted to mature luminal epithelial cells.⁶⁴ Particularly, WNT5A inhibited

branching morphogenesis⁶⁵ as a result of WNT5A/ROR2 interaction at the cell membrane.⁶⁶ However, the same WNT ligand was able to promote mammary epithelial progenitor growth through RYK interaction,⁶⁶ suggesting that WNT5A may increase MaSC capacity and proliferation or inhibit mammary morphogenesis in a noncanonical RYK-specific manner. In addition, WNT5B has been described as a negative regulator of mammary stem/progenitor function by inhibition of the WNT/ β -catenin signaling and activation of the non-canonical Ca^{2+} /nuclear factor of activated T cell (NFAT) cascade.⁶⁷ Besides, overexpression of WNT5B resulted in strongly decreased mammary branching morphogenesis and impaired epithelial outgrowth.⁶⁷

WNT/ β -catenin signaling has also been more frequently implicated in mammary tumorigenesis. However, some studies have shown that the noncanonical WNT cascades have relevant implications on BC clinical outcome.⁶⁸ In mice, *WNT5a*-knockdown inhibited both canonical and noncanonical WNT pathways, leading to suppressed invasion and sphere formation of basal-like mammary tumor cells.⁶⁹ In addition, it was determined that the alternative WNT receptor ROR2 is expressed in different BC subtypes, including Luminal, Basal-like, and Claudin-low, whereas canonical WNT signaling is preferentially restricted to the basal-like subtype.⁶¹ Interestingly, ROR2 expression was negatively correlated with WNT/ β -catenin-dependent pathway activity in mouse tumors as well as in human breast tumor samples, establishing an inverse correlation between WNT/ β -catenin-dependent and WNT/ β -catenin-independent pathways.⁶¹ In the TP53-null model, *Ror2* depletion inhibited tumor growth and cell adhesion, increasing WNT/ β -catenin activation and cell migration.⁶¹ In human basal-like BC cells, silencing ROR1—also involved in noncanonical WNT pathway activation—impaired EMT, cell migration, invasion, and metastatic potential.⁷⁰ Besides, high ROR1 expression was tightly correlated with poor metastasis-free survival of BC patients.⁷⁰

Taken together, these studies show the complexity of the WNT pathway activity in the mammary gland. However, a deeper understanding of the specific WNT signaling function depending on the existing repertoire of WNT ligands, receptors, modulators, and downstream signaling components in normal human breast development and among the different BC subtypes is still missing. Moreover, deciphering the specific molecular mechanisms altered as a result of a WNT signaling deregulation will also provide valuable information to select the patient subsets that may benefit from the development of therapeutic drugs targeting this pathway.

4 | RSPO/LGR AXIS IN MAMMARY GLAND AND BC

4.1 | RSPOs in normal mammary gland development

It is well-documented that the RSPOs play critical roles in embryonic development and organogenesis as well as in the self-renewal and maintenance of stem cells in several adult tissues.^{14,32} Regarding the mammary gland, it was reported that *Rspo1*^(-/-) mice were unable to feed their pups,⁷¹ and that transplants of their glands into cleared fat pads of wild-type mice showed impaired side-branching in virgin females and reduced alveolar formation with a persistence of virgin markers during pregnancy.^{72,73} Importantly, it has been shown that RSPO1 expression is induced in ER⁻PR⁻ mammary cells of wild-type mice during pregnancy or upon treatment with estrogen, progesterone, or both hormones.⁷⁴ These results indicated that sex hormones would cause the secretion of a mediator that, in turn, would trigger RSPO1 synthesis in ER⁻PR⁻ mammary cells. In addition, it was shown that the maintenance of the MaSC compartment in vivo requires the presence of both RSPO1 and WNT4, which is expressed by ER⁺PR⁺ cells, to fully activate the WNT/ β -catenin signaling in the progenitor population.⁷⁴ Then, it was demonstrated that progesterone indirectly stimulates RSPO1 expression in the mammary gland through induction of the receptor activator of nuclear factor kappa B ligand in PR⁺ cells.⁷⁵ More recently, amphiregulin was identified as a relevant factor, secreted by ER⁺ cells, also able to upregulate RSPO1 in luminal ER⁻ cells in response to estrogen stimulation and in an epidermal growth factor receptor-dependent manner.⁷⁶

To the best of our knowledge, there are no reports indicating RSPO2 or RSPO4 contribution in the normal mammary gland development. Regarding RSPO3, we have shown that it is expressed in the basal stem cell-enriched compartment of adult virgin mouse mammary glands, whereas it is absent from committed mature luminal cells.³⁸ Besides, RSPO3 was detected in the mammary stroma and associated with the ECM, where it could exert a significant effect on the epithelial population.³⁸ In addition, in silico analysis revealed that the committed alveolar luminal cell population of mid-pregnant animals expresses lower levels of *Rspo3* than alveolar luminal stem/progenitor mammary cells of virgin mice.³⁸ In culture, RSPO3 impaired lactogenic differentiation in luminal-like, functionally normal mammary cells, whereas it promoted the acquisition of fibroblast-like, mesenchymal morphology in mammary epithelial cells through canonical WNT pathway activation, increasing the expression levels of mesenchymal markers

as fibronectin and vimentin.³⁸ These results suggest that RSPO3 could play a relevant role in the normal mammary gland biology, promoting the maintenance of an uncommitted basal-like mammary epithelial phenotype and inducing EMT-like features, which may be the underlying mechanisms for transforming BC cells that overexpress RSPO3.

4.2 | LGR4-6 in normal mammary gland development

LGR4 is expressed in the mammary basal compartment of virgin female mice. Particularly, Wang et al. demonstrated that *Lgr4* promoter was active in most TEBs, as well as in mammary duct basal cells of 6-week-old virgin female mice.³⁴ They also showed that LGR4 is required for mammary gland development, since *Lgr4*^{-/-} mice displayed delayed ductal development, decreased side-branching and a severely diminished MaSCs repopulation capacity.³⁴ Besides, mammospheres from *Lgr4*^{-/-} cells had decreased activation of the canonical WNT pathway and diminished expression of its target genes as *Sox2*, which represents one of the key transcription factors in cell reprogramming.³⁴ Interestingly, overexpression of this protein restored mammary regenerative potential of *Lgr4*^{-/-} MaSCs. These authors also observed that organoid branching from those cells was impaired. Nevertheless, WNT3a, but not RSPO1, treatment rescued MaSCs from this deficit.³⁴ In the human breast, LGR4 expression was observed in mammary ducts, but not in myoepithelial cells, suggesting that LGR4 might be involved in human mammary development.⁷⁷

LGR5 expression pattern varies during mouse mammary gland development, since it appears confined to the luminal layer until day 12 after birth, but afterward its expression switches to the basal compartment.⁷⁸ Furthermore, it was shown that basal LGR5⁺ cells are able to efficiently reconstitute mammary glands in vivo upon serial transplantations.³³ Using a three-dimensional (3D) imaging strategy and a stochastic multicolor Cre reporter, it has been observed that both luminal and basal mammary cell patches exhibited LGR5 expression along the ductal tree and contributed to alveoli formation, establishing the bipotential capacity of LGR5⁺ mammary cells.⁷⁹ Nevertheless, it has to be noticed that transgenic and knock-in Cre drivers used to perform lineage-tracing experiments are commonly heterogeneously expressed, compromising the interpretation of the obtained data. In fact, more recently, a rigorous assessment of these studies has shown that only unipotent progenitors would be involved in postnatal development and adult tissue remodeling of the mouse mammary gland.⁸⁰

Interestingly, Zhang et al. found that mammary organoids derived from murine LGR5⁺ single cells were sustainable during prolonged passaging using a growth factor cocktail containing EGF, WNT3a, and RSPO1, and consisted of at least four distinct cell populations self-organized into ductal-like structures.⁸¹ Through gene expression profiling of mouse LGR5⁺ basal epithelial cells, a new marker of MaSC subpopulations, Tetraspanin8 (Tspan8), has been proposed.⁸² Interestingly, LGR5⁺Tspan8^{hi} mouse mammary cell subset showed a remarkably similar expression profile to human Claudin-low tumors.⁸² Nevertheless, it has been indicated that LGR5 expression would not be, by itself, a reliable marker of the mammary progenitor population since protein C receptor (PROCR) expressing cells, even without detectable LGR5 expression (LGR5[−]PROCR⁺ mammary cells) showed regenerative capacity, whereas LGR5⁺PROCR[−] cells were unable to form colonies in vitro and had lower repopulating frequency than the average basal cells.⁸³

LGR6 has been reported as a relevant marker of unipotent progenitor cells in the luminal and basal compartments of the mouse mammary gland.⁸⁴ Lineage-tracing analysis showed that LGR6⁺ cells expand clonally during puberty and, with the onset of pregnancy or upon hormone stimulation, they regain proliferative potency contributing to the development of functional alveoli.⁸⁴

Taking together, these studies show a presumably distinct expression pattern and role of each LGR during different stages throughout the mouse mammary gland development. However, whether these reported differences depend on the availability of specific locally secreted RSPOs, or even a potentially overlapping function among LGR4, LGR5, and LGR6 in maintaining the mammary gland homeostasis, remain to be elucidated. Moreover, the precise upstream mechanism regulating LGR4-6 expression in this tissue is unexplored.

4.3 | RSPO/LGR signaling potentiation in mammary tumor initiation and maintenance

4.3.1 | RSPOs in mouse mammary tumors and human BC

Rspo2 and *Rspo3* sequences were first identified because of their proximity to common MMTV integration sites in mammary tumors induced by this retrovirus in inbred mice.^{85–87} In addition, overexpression of these genes was involved in mammary tumor initiation and progression.^{85–87} More recently, we have shown that RSPO3 induces mesenchymal-like features and migration capacity in nontumorigenic

mouse mammary cells.³⁸ Besides, we found that canonical WNT pathway activation and in vivo tumorigenicity of basal mouse mammary cancer cells depend on RSPO3 endogenous expression.³⁸ Regarding the regulation of RSPO3 expression, it was reported that the runt-related transcription factor 1 (RUNX1) can bind to the *Rspo3* promoter region, upregulating its levels in mammary tumor cells.⁸⁸ In the case of RSPO1 induction in BC, a synergy between MYC and the long noncoding RNA PVT1, has been proposed.⁸⁹ In addition, Yoldi et al.⁹⁰ reported that RANK signaling induces RSPO1 expression in MMTV-PyMT tumor cells, as previously reported in the normal mouse mammary gland.⁷⁵

In human BC, it has been reported that *RSPO2* as well as *RSPO4* are overexpressed in triple-negative and metaplastic tumors.⁹¹ In addition, even though RSPO3 expression was detected in both Luminal and Basal BC, the highest levels of expression also corresponded to the last subtype.³⁸ However, RSPO3 overexpression was even more pronounced in Claudin-low BC compared with any other category, if this subtype is considered separately.³⁸ Here, an updated analysis of the The Cancer Genome Atlas (TCGA)-BC RNA-Seq dataset composed by 1,097 primary breast carcinomas samples obtained from the UCSC Xena Browser (<https://xenabrowser.net>), performed as described in Tocci et al.,³⁸ is displayed (Figure 3a). This approach rendered similar results to what previously reported,^{38,91} indicating that RSPO3 and RSPO4 expression prevail in Basal-like tumors, while RSPO1 is particularly overexpressed in the Luminal A subtype. It would be interesting to determine whether RSPO1 overrepresentation in ER⁺PR⁺ BC might be related to the previously reported indirect-dependence of this gene expression on progesterone circulating levels.⁷⁴ Unfortunately, we were not able to include RSPO2 in Figure 3a because its expression levels in the analyzed samples were too low to draw any conclusion. As an overview, Figure 4 shows a graphical abstract depicting the expression pattern and activity of RSPO1 and RSPO3 in the normal adult mammary gland and among BC subtypes.

4.3.2 | LGR4-6 and ZNRF3/RNF43 in mammary tumors

Differently from the well-documented role of LGRs in MaSCs activity and normal mammary gland development, little is known about their role in BCSCs, BC subtypes, and their progression. However, it has been reported that LGR4-6 is overexpressed in the Basal-like subtype and that LGR levels correlate with RSPO, EMT, and stem cell marker expression in human BC.^{38,91} In addition, LGR4 down-regulation reduced cell migration,

FIGURE 3 RSPOs, LGRs, and ZNRF3/RNF43 expression levels obtained from a TCGA BC RNA-Seq dataset (1,097 primary breast carcinomas samples) from the UCSC Xena Browser (<https://xenabrowser.net>). LGR, leucine-rich repeat-containing G-protein-coupled receptor; RSPO, R-spondin; ZNRF3/RNF43, zinc and RING finger 3/RING finger 43

FIGURE 4 RSP01 and RSP03 expression patterns in the normal and neoplastic mammary gland. In the mature normal mammary gland, RSP01 and RSP03 participate in the maintenance of MaSCs. The committed luminal ER-cells and the basal compartment express RSP01 and RSP03, respectively. Overexpression of RSPOs and potentiation of the WNT pathway is observed in breast tumors. RSP01 expression is higher in the Luminal A subtype, while RSP03 predominates in the Basal BC subtype. BC, breast cancer; ER, estrogen receptor; MaSC, Mammary stem cell; RSPO, R-spondin

tumor xenograft, and lung metastasis growth of human BC cells and impaired *MMTV-WNT1* CSC survival upon transplantation in mice.⁹² Regarding LGR5, it has been reported that it promotes BC progression and the maintenance of stem-like cells through canonical WNT pathway activation.⁹³ Besides, analysis of women BC samples revealed LGR5 expression associated with triple-negative BCs, tumor size, and metastasis, as well as with lower relapse-free survival rates.⁹⁴ Finally, patients with high LGR6 expression showed a significantly lower disease-free survival and it has been demonstrated that Lgr6⁺ are potent luminal tumor initiating cells involved in mouse mammary cancer maintenance and growth.⁸⁴ However, this gene is mainly overrepresented in the Basal-like subtype of human BC (Figure 3b, Reference 38). Further studies to obtain a complete understanding on how LGR expression is regulated and how these proteins exert their biological effects under homeostatic conditions and in BC cells remain to be performed. Moreover, dissecting unique or redundant activities of each receptor on cells co-expressing LGRs will be of major relevance for the potential development of BC therapeutic approaches.

Although RSPOs are LGR ligands, it has been reported—and already mentioned herein—that RSPO2 and RSPO3 are capable of potentiating WNT signaling by binding to the membrane ubiquitin ligases ZNRF3/RNF43, without interacting with LGRs.^{28–30} Interestingly, our current data-mining analysis reveals that *RNF43* is overexpressed in all but the Basal subtype (Figure 3C). Therefore, it could be proposed that RSPOs may play a relevant role in promoting the development of those BC subtypes showing low LGR levels by directly binding to the RNF43 ubiquitin-ligase protein.

5 | RSPOS AS MOLECULAR TARGETS FOR BC TREATMENT: NEW PERSPECTIVES

The preeminent RSPO expression in BC undoubtedly supports their putative relevance as therapeutic targets for this cancer type. It has been reported that specific monoclonal antibodies against RSPOs significantly reduced tumor growth of different human cancer types in immuno-deficient mice.³⁶ Then, OncoMed Pharmaceuticals produced an anti-RSPO3 blocking monoclonal antibody, named Rosmantuzumab (OMP-131R10), which proved to exert antineoplastic effects on tumor cells in culture and in xenotransplanted mice.⁹⁵ However, a Phase1a/b clinical trial for treatment of solid tumors using Rosmantuzumab as adjuvant therapy did not supply compelling proof of clinical benefit for patients receiving humanized anti-RSPO3 (ClinicalTrials.gov

Identifier: NCT02482441). As described by the authors, during antibody generation and assessment, they only tested the ability of anti-RSPO antibodies to block RSPO interaction with LGRs.³⁶ Therefore, considering the fact that the RSPOs are able to interact with other membrane and extracellular components, as ZNRF3/RNF43 and HSPGs, to modulate WNT signaling activation in an LGR-independent manner, a polyvalent therapeutic strategy might be more efficient in reducing RSPO enhancing activity in cancer patients. However, it is conceivable that the efficacy of these putative polyvalent therapies would also depend on the repertoire of WNT ligands, membrane proteins, and mediators of the canonical and non-canonical WNT pathways expressed by tumor cells in each specific BC subtype.

6 | CONCLUDING REMARKS

WNT pathways play a critical role during mammary gland development through the maintenance and differentiation of MaSCs, and its deregulation has been implicated in BC progression. RSPOs strongly enhance target cell sensitivity to WNT ligands in several tissues through their interaction with LGRs and the E3 ubiquitin-ligases at the cell membrane. RSPO and LGR expressions have been identified during normal mammary gland development, suggesting that this RSPO-LGR axis might play a significant role as a WNT-enhancing signaling cascade in this secretory organ. Under pathological conditions, elevated RSPO and LGR levels have been detected in several cancer models, including BC, indicating that these proteins may be functionally relevant for breast tumorigenesis. However, more studies regarding their precise mechanisms of action, in the diverse scenarios they have been found, are required to determine the proper approach to tackle their impact on BC progression.

Traditional therapies against cancer, chemo and radiotherapy, have several limitations that lead to treatment failure and cancer recurrence. These limitations are due to systemic and local toxicity, since the agents are not selective enough, and to drug resistance, a property of the CSC population. Thus, eradicating CSCs, by targeting specific cell surface markers and modulating signaling pathways involved in CSC maintenance, becomes essential for cancer treatment by inducing a long-lasting clinical response to prevent tumor relapse. Since the available data indicate that RSPOs and LGRs are relevant for both CSCs and normal adult stem cells of different organs such as the mammary gland, they might be proposed as CSCs markers and molecular targets in cancer therapies. Particularly, for BC patients, therapeutic strategies that may alter the normal MaSC

compartment would not represent a clinical disadvantage, due to the nonvital character of this organ. However, since RSPO-LGR axis plays a relevant role in regulating the WNT pathway, critically required in tissue homeostasis, anti-RSPOs and anti-LGRs therapeutic strategies should be pursued hand in hand with specific delivery systems to avoid stem cell depletion in essential organs. Taking into account that a main goal of BC treatment is to prevent the development of distant metastasis, which may require systemic approaches, determining specific features of the RSPO-LGR axis in BC cells is crucial to generate valid therapeutic strategies.

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