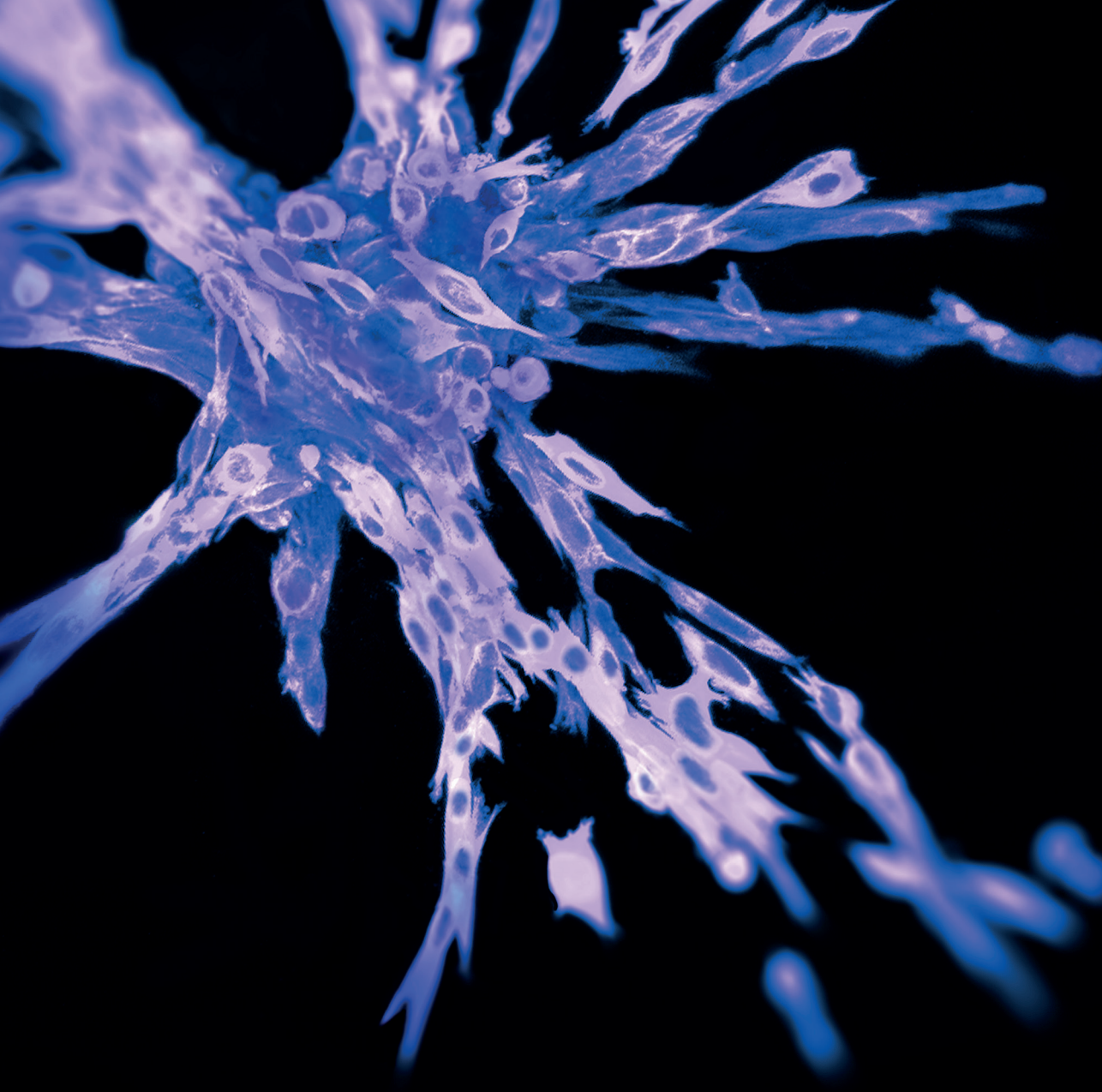


Insights in the contributions of the RSPO-LGR pathway to breast cancer

Eline Johanna ter Steege

Insights in the contributions of the RSPO-LGR pathway to breast cancer

Eline Johanna ter Steege

ISBN: 978-94-93278-61-5
Cover: Fenrir design
Layout and printing: Off Page, Amsterdam

Insights in the contributions of the RSPO-LGR pathway to breast cancer

Inzichten in de rol van de RSPO-LGR signaleringsroute in borstkanker

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof.dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

dinsdag 19 december 2023 des middags te 12.15 uur

door

Eline Johanna ter Steege
geboren op 8 augustus 1994
te Oldenzaal

Promotoren

Prof. dr. P.W.B. Derksen
Prof. dr. P.J. van Diest

Copromotor

Dr. E.R.M. Bakker

Beoordelingscommissie

Prof. dr. E. Danen
Prof. dr. S.C. Linn
Prof. dr. M.M. Maurice
Prof. dr. K. de Visser
Prof. dr. E. van der Wall (voorzitter)

Voor mijn ouders

Table of Contents

1 INTRODUCTION

The RSPO-LGR pathway

R-Spondins are a family of four secreted proteins (RSPO1-4) well known to agonize the canonical Wnt pathway, a crucial signaling route that exerts essential roles in developmental processes, homeostasis and stem cell niches of various tissues[1]. Canonical Wnt signaling is specified by the binding of extracellular Wnt ligands to Frizzled (FZD) receptors, leading to stabilization and nuclear localization of downstream effector β-catenin. Subsequently, β-catenin binds to T-cell factor (TCF)/Lef transcription factors, resulting in transcription of target genes. This signaling route is negatively regulated by ubiquitin ligases ZRNF3 and RNF43 that stimulate the degradation of FZD receptors, resulting in reduced membrane receptor availability and consecutive downstream signaling activity[2,3]. Alternatively, Wnt signals can activate non-canonical Wnt signaling cascades that do not transduce Wnt signals through cytoplasmic stabilization of β-catenin, but instruct signaling molecules acting upon cell migration and the cytoskeleton[4].

Wnt signaling is regulated at different levels by various natural inhibitors including Dickkopf (Dkk) proteins, Wnt Inhibitory Factor-1 (WIF-1) and secreted Frizzled-Related Proteins (sFRPs) that mostly influence signaling activity by modulating ligand-receptor interactions[1,5]. RSPOs additionally impact Wnt signaling by potentiating the pathway through neutralization of the negative regulation of ZNRF3/RNF43. By binding to leucine-rich repeat-containing G-protein-coupled receptors (LGR) 4-6, RSPOs induce membrane clearance of ubiquitin ligases ZNRF3/RNF43, resulting in enhanced Wnt receptor availability at the cell membrane and increased Wnt pathway activity[6–9]. In line with the crucial role of Wnt signaling in embryonic development, RSPOs have additionally been described to have pivotal, distinct roles in several developmental processes throughout the body such as ovarian, limb and placental development[10–14]. Furthermore, the identification as LGR ligands additionally unraveled roles for RSPOs in stem cell regulation and homeostasis, as LGRs are typically expressed on the membranes of stem-and progenitor cells[6,7,15–20]. Most we know about functioning of RSPOs in the stem cell niche comes from studies in the intestine. Here, RSPO3 is produced by stromal cells that lie in proximity to the intestinal stem cell niche, influencing stem cell dynamics by activating canonical Wnt signaling[15,21–23]. Since RSPOs and LGRs are expressed in multiple tissues, the stem cell influencing functions of RSPOs extend beyond the intestinal tract, occurring in various organs. Coherent with this instructive role for RSPOs in stem cell regulation, aberrant activation of RSPOs has been associated with the development of various cancer types and subsequently RSPOs have been proposed as interesting potential novel clinical therapeutic targets[24].

Mammary gland development

The mammary gland is a highly specialized organ that develops through distinct stages. Mammary gland organogenesis starts during embryonic development when the rudimental ductal tree is formed, followed by extensive ductal elongation of the epithelial tree during puberty. The epithelial tree holds a bilayered architecture consisting of an outer layer of

basal myoepithelial cells and an inner layer of luminal cells. Together these cells constitute a branched ductal system that endures multiple extensive remodeling events during menstrual cycles and pregnancy that are instructed by hormonal signals.

Embryonic mammary gland development

In the mouse, mammary gland development starts at embryonic day 10.5 (E10.5) when milk lines develop from the anterior to the posterior limb buds as a consequence of the enlargement of the single layered ectoderm. At E11.5, ectodermal cells migrate and cluster along the milk lines forming multilayered placodes in the regions of the future mammary glands. These placodes increase in size and embed into the underlying mesenchyme during E12 and E13, forming mammary epithelial buds. Mesenchymal cells enclosing the mammary epithelial buds condensate and differentiate to form the mammary mesenchyme. Continuing at day E15.5, epithelial cells in the mammary bud proliferate and elongate into the presumptive mammary fat pad followed by ductal branching, resulting in formation of the rudimentary ductal tree at E18.5 (Figure 1)[25,26].

Embryonic development of the mammary gland requires mutual cross-talk between instructive signals that originate from the epithelium and the underlying mesenchyme[25,26]. Among these are Wnt signals, and corresponding Wnt signaling activity is observed in both epithelial and mesenchymal cells of the milk line, placodes and developing ducts until E15.5[27]. During embryonic mammary gland development, *Wnt10b* is expressed along the milk lines and required for milk line specification[28]. Canonical Wnt ligands *Wnt6* and *Wnt10a* are additionally expressed by the ectoderm flanking the milk line while non-canonical Wnt ligands *Wnt11* and *Wnt5* are expressed in the underlying mesenchyme[27,28]. Following milk line formation, canonical Wnt signals stimulate the process of placode

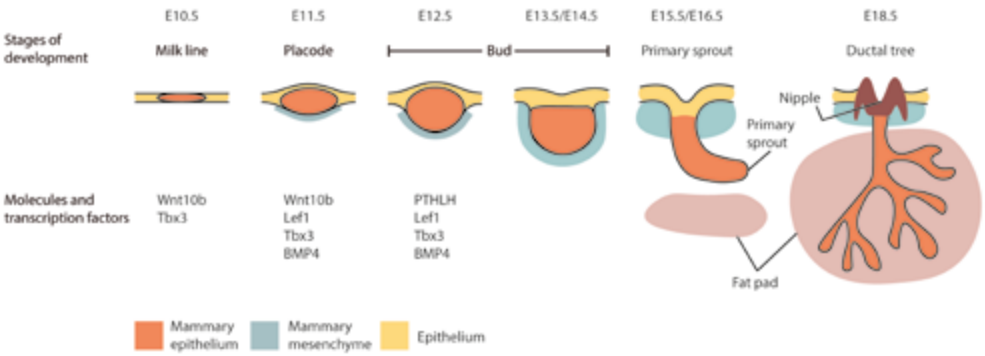


Figure 1. Embryonic mammary gland development in the mouse. Mammary gland development starts at E10.5 when the mammary milk lines are formed. At E11.5 ectodermal cells cluster at the positions of the future mammary glands forming the placodes. In following days, these placodes will grow and develop into mammary buds that embed into the underlying mesenchyme. From E15.5 onwards, the mammary buds start proliferating into the underlying mesenchyme towards the mammary fat pad. At E18.5, a rudimental ductal tree in the mammary fat pad has formed. Figure adapted from [26].

development and additionally increase placode size[27]. This was demonstrated by a study that overexpressed Wnt inhibitor Dkk1 in the ectoderm of transgenic mice that resulted in a complete blockage of placode formation[27]. At this stage of development, *Lef1* is additionally expressed in the mammary placodes and required for their formation since deletion of *Lef1* in mice leads to abolished placode formation and the absence of epithelial Wnt signaling activity[29,30]. *Lef1* and *Wnt10b* expression in the mammary placodes is induced by T-box protein 3 (Tbx3) that regulates positioning of the mammary placodes[31–33]. Through an interaction with bone morphogenetic protein 4 (Bmp4), Tbx3 localizes the expression of *Lef1*, thereby coordinating the dorso-ventral patterning of the mammary placodes [33].

During the next stage of mammary gland development, the mammary mesenchyme is formed followed by ductal branching and formation of the nipple. These processes are highly regulated by the parathyroid hormone-like hormone (PTHLH) that is produced by the epithelium and acts on its receptor expressed in the surrounding mesenchyme[34–37]. PTHLH stimulates ductal outgrowth and nipple formation by modulating BMP and Wnt signaling[38,39]. At this point, *Lef1* expression is downregulated in the epithelium and epidermis, but increasingly expressed in the mesenchyme[39]. In contrast, in mammary glands lacking PTHLH, *Lef1* expression is retained in the epithelial cells surrounding the presumptive nipple but absent in the mesenchyme[39]. Additionally, mesenchymal cells lose their mammary specific keratin expression and become squamous upon *Pthlh* deletion, indicating that PTHLH determines mammary cell fate, potentially by modulating Wnt signaling[39].

Postnatal mammary gland development

By the end of embryonic development a rudimental ductal tree is formed that remains quiescent until puberty when extensive ductal branching is driven by instructive signals from hormonal cues and growth factors including growth hormone (GH), Insulin-like growth factor-1 (IGF1) and estrogen[25,40–43] (Figure 2). Elongation of the ductal tree is essentially driven by the proliferation of epithelial cap cells that represent the outer layer of terminal end buds (TEBs), the ends of growing ducts. Proliferation of epithelial cap cells drives penetration into the fat pad after which they differentiate into the outer basal layer of the bilayered ductal structure. The inner epithelial body cells of the TEBs will form the eventual luminal layer of the ducts. Secondary branches will sprout from the primary ducts, filling up the mammary fat pad and forming the mature mammary gland[25]. In the mature, virgin mammary gland, short tertiary branches are formed upon stimulation by progesterone during each estrus cycle. Alveologenesis is induced in the presence of pregnancy hormone prolactin. During alveologenesis, alveolar buds are formed constituting milk-producing secretory cells in late pregnancy. Progesterone and prolactin are responsible for the extensive side-branching and differentiation of alveoli, but also estrogen is required for growth and maintenance of alveolar cells[43–48]. After lactation, hormone stimulation will regress, and the mammary gland architecture will again drastically change and involute back into its original adult state. Also in the postnatal mammary

gland, several canonical and non-canonical Wnt signals contribute to its development during puberty and pregnancy. Canonical Wnt protein Wnt4 demonstrated to be essential during side branching in early pregnancy and also endogenous *Rspo1* expression is significantly upregulated during pregnancy in the mouse mammary gland[49–52]. Coherently, depletion of *Rspo1* results in defects in side branching and subsequent alveolar formation upon pregnancy[51–53]. Loss of the RSPO receptor *Lgr4* moreover results in delayed ductal development and decreased side-branching in the mammary glands of mice[54]. Regarding non-canonical Wnt signaling, Wnt5a is particularly enriched in proliferating TEBs and required for proper ductal elongation and branching morphogenesis[55,56].

Mammary gland stem cell hierarchy

The exceptional dynamic character of the mammary gland is fueled by divers stem- and progenitor cells that reside in the adult mammary gland[57]. The exact stem cell hierarchy of the mammary gland is complex and not completely delineated. Multiple studies have focused on identifying specific mammary stem cell (MaSC) populations and although various cell populations potentially hold stem cell properties, the identity of MaSCs remains controversial[58–60]. Initial studies were limited to mouse mammary tissues and identified rare basal cell populations that presented with repopulation capacity when transplanted in cleared mammary fat pads of recipient female mice[58–60]. These findings

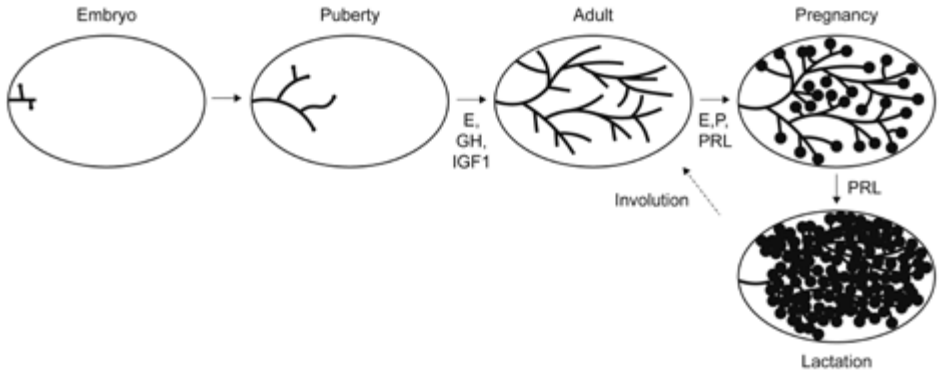


Figure 2. Postnatal mammary gland development in the mouse. After embryonic development, the mammary gland contains a rudimental ductal tree that remains quiescent until puberty. Under the influence of estrogen (E), Growth hormone (GH) and Insulin-like growth factor 1 (IGF1) ductal morphogenesis occurs, expanding the ductal tree to fill the mammary fat pad and form the adult mammary gland. During each estrus cycle short tertiary branches form and regress upon progesterone (P) stimulation. Under the influence of prolactin (PRL), the mammary gland undergoes another round of heavy remodeling and stimulates the proliferation and differentiation of alveolar cells into alveolar units in coherence with progesterone. Estrogen is at this stage of development required for growth and maintenance of alveolar cells. Prolactin remains expressed driving the lactational state of the mammary gland until weaning signals diminish and the mammary gland ductal tree involutes back into its resting adult state.

suggested that basal, multipotent MaSC reside at the top of the cellular hierarchy, giving rise to all lineages of the mammary gland. Subsequent studies, however, have reported a variety of multi- and unipotent stem and progenitor cells in the mammary gland[61–68] (Figure 3). These include short- and long-lived progenitors that are restricted to either the basal or the luminal lineage, quiescent stem cells and cycling stem cells. Short and long-lived basal and luminal progenitors have been identified that contribute to mammary gland homeostasis and postnatal ductal elongation during puberty[57]. In pregnancy, luminal progenitors are reported to give rise to mature alveolar cells, but also multipotent stem cells contribute to alveologenesis [63–65,69–72].

In diverse tissues, it has been well established that stem cell control is highly influenced by Wnt and RSPO signals[5,73]. Also in the mammary gland, canonical Wnt and RSPO signals are implicated in stem cell control, their expression being regulated by steroid hormone progesterone[51,74,75]. In the mouse mammary gland, *Rspo1* is identified as a key paracrine mediator of progesterone signaling, translating upstream hormonal signals to expansion of the mammary stem cell pool[51,74]. Progesterone acts on PR^+ luminal cells, inducing the expression of Receptor Activator of Nuclear factor Kappa-β Ligand (*Rankl*) and *Wnt4*[51,74–76]. Rankl in turn stimulates the expression of *Rspo1* by ER^-/PR^- luminal cells that in conjunction with Wnt4 induces canonical Wnt signaling, thereby enabling stem cell maintenance and cellular expansion (Figure 4)[51,74]. In the human breast, *RSPO3* is expressed by $ALDH^+$ cells that are proposed to hold stem/progenitor cell characteristics[77–79]. Different Wnt responsive mammary stem/progenitor cells are found present during embryonic and postnatal development of mice, giving rise to the basal and luminal lineages of the epithelial network[19,20,64,67,80]. $LGR6^+$ cells were recently described to present a rare subset of unipotent progenitors in the mouse mammary gland that expand during pubertal development, but diminish in adulthood[20]. Upon pregnancy or hormonal stimulation, adult $LGR6^+$ cells regain their proliferative capacity, contributing to alveolar development[20]. These $LGR6^+$ cells were additionally proposed to function as tumor initiating cells of luminal mammary tumors[20]. $LGR5^+$ cells have also been proposed as mammary stem cells, though that remains rather controversial[19,63,67,80]. While some studies demonstrate that $LGR5^+$ cells contribute to both cell lineages of the mammary gland, alternative studies describe a more restricted cell fate for $LGR5^+$ cells and their progeny, being committed to the myoepithelial lineage in the pubertal mammary gland[19,63,67,80]. Furthermore, it was described that $LGR5^+$ cells change fate during mammary gland development[19]. Whereas $LGR5^+$ cells and their progeny are restricted to the luminal compartment directly after birth, 12 days later they are found committed to the myoepithelial lineage of the mammary gland[19]. Similar findings were observed in a population of $Axin2^+$ cells that demonstrated to be restricted to the luminal cell lineage during embryonic development, but were found to contribute to the basal cell lineage after birth, indicating a potential change in Wnt signaling during this stage of mammary gland development[64]. Although above described studies acknowledge the presence of a hierarchical stem cell structure in the mammary

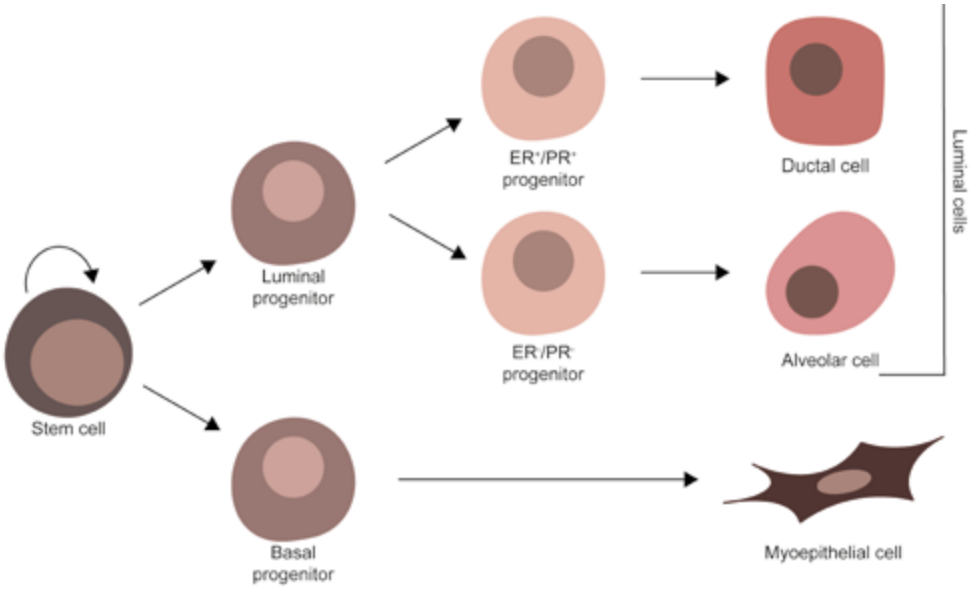


Figure 3. Hypothetical model of mammary gland stem cell hierarchy. The mammary gland stem cell lineage is proposed to consist of multiple co-existing stem and progenitor cells that together give rise to the designated differentiated cells of the bilayered epithelium. At the top of the hierarchy resides a multipotent stem cell that is capable of self-renewal and gives rise to the committed progenitors for the luminal and basal epithelial lineages. Figure adapted from [57].

gland, the exact built-up, regulation and position of Wnt responsive cells in this hierarchy remain to be delineated.

Breast cancer subtypes

Breast cancer is the most commonly occurring cancer in women worldwide[81]. Although breast cancer mortality has greatly reduced in the last decade due to early detection and increased therapeutic strategies, it is still the leading cause of cancer deaths among women[81]. In line with the complex mammary gland stem cell hierarchy, breast cancer is an exceptionally heterogeneous disease, consisting of a wide spectrum of subtypes.

Ductal versus lobular breast cancer

The majority of breast malignancies are carcinomas that account for 95% of all breast tumors. Carcinomas of the breast are broadly subdivided into invasive ductal carcinomas (IDC) and invasive lobular carcinomas (ILC), based on the histological features of the tumor. IDC is the most common type of breast cancer, while ILC accounts for 10-15% of all breast cancer cases[82]. IDC and ILC are biologically distinct entities, differing in histology, morphology, clinical features, and genetic profiles. IDC is a heterogeneous type of breast cancer that can be classified into several histological subtypes based on multiple clinic pathological

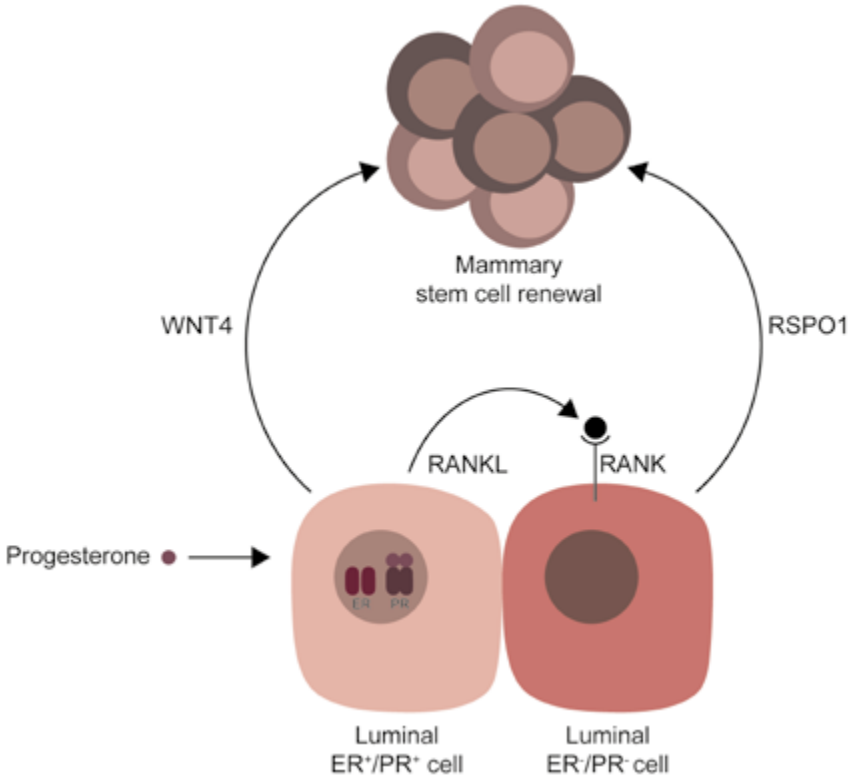


Figure 4. *Rspo1* expression and function in the mouse mammary gland. In the mouse mammary gland, progesterone stimulates the expression of *Rankl* and *Wnt4* from ER$^+$/PR$^+$ luminal cells. Paracrine Rankl signals stimulate neighboring luminal ER$^-$/PR$^-$ cells to produce and secrete RSPO1 that subsequently regulates mammary stem cell self-renewal in coherence with Wnt4.

features. IDC tumors commonly present as firm nodules that are relatively well detectable with mammography[83]. ILC is a luminal type breast cancer characterized by the expression of ER and caused by loss of the cell-to-cell adhesion molecule E-cadherin[84–86]. Due to E-cadherin loss, ILC represents with a specific growth pattern of small, round, non-cohesive cells that invade the stroma as single strand files[87,88]. This distinct growth pattern challenges the detection of ILC with mammography or ultrasound, resulting in potential false negative outcomes [88–90]. The differences in genetic and clinical features of IDC and ILC reflect in prognosis and treatment outcomes. Although 5-10 year overall and disease-free survival rates of IDC and ILC are largely similar, long-term prognosis for ILC patients are worse compared to IDC patients[87,91–93]. This might be explained by the presence of disseminated dormant cancer cells that remain quiescent for a long period of time prior to relapse[82,94]. Moreover, while both endocrine- and chemotherapy are commonly used to treat ILC and IDC, ILC patients appear less chemo sensitive compared to IDC patients and demonstrate to benefit more from endocrine therapy [82,95–97]. Now that studies have

identified major differences in molecular portraits of IDC and ILC, research must focus on improving treatment strategies for these distinct types of breast carcinoma to provide optimal treatment regimens for both IDC and ILC.

Molecular subtypes of breast cancer

Over the last decade, increasing efforts have been made to further characterize breast cancer based on molecular parameters and pathological markers to provide a better understanding of the high heterogeneous nature of breast cancer and be able to predict tumor progression and therapeutic strategies. Most regularly, breast cancer is characterized in 5 different intrinsic molecular subtypes based on gene expression profiles and the expression of ER, PR, and human epidermal growth factor receptor 2 (HER2). These subtypes involve Luminal A, Luminal B, HER2-positive, Basal-like and Claudin-low [98–101]. The Luminal A type associates with the best prognosis, followed by Luminal B breast cancer (Figure 5) [99]. Basal-like and Claudin-low subtypes present with the worst clinical outcomes[99,101]. Luminal breast cancers are characterized by expression of ER and PR and maintain a gene expression signature resembling those of luminal cells[100]. Luminal B type tumors present with higher levels of proliferation and cell cycle related genes compared to Luminal A type tumors and express lower levels of several luminal-related genes including PR[102,103]. Endocrine therapy interfering with ER is the primary systemic therapy for ER positive breast cancer[104]. Tamoxifen is effective in pre- and post-menopausal woman and competitively inhibits binding of estrogen to its receptor. Aromatase inhibitors are only effective in postmenopausal women and inhibit the conversion of androgen to estrogen, thereby decreasing circulating estrogen levels. Chemotherapy is not standardly used, but hormone receptor positive breast cancer cases may be treated with adjuvant chemotherapy based on gene expression profile, anatomic stage and tumor grade[104]. The HER2-positive subtype is characterized by high HER2 (ERBB2) expression that is caused by genomic amplification, and associates with high expression of proliferation-related genes[99,100]. Targeted therapeutic treatment for HER2-postive breast cancer patients can be achieved with functional targeting antibodies like Trastuzumab, which targets the extracellular domain of ERBB2 thereby inhibiting HER2-mediated signaling[104–106]. Trastuzumab is used in combination with chemotherapy and markedly improves disease-

Figure 5. Molecular subtypes of breast cancer. Breast cancer is broadly divided in 5 different molecular subtypes based on gene expression profiles. Luminal A and B express ER and PR and are associated with good short-term prognosis. The HER2 subtype is defined by HER2 amplification. Basal-like and Claudin-low tumors do not express ER, PR or HER2, have limited treatment options and are associated with a poor prognosis.

free survival and overall survival rates of HER2-positive breast cancer patients compared to single chemotherapy treatment[106]. Basal-like and Claudin-low subtypes lack expression of ER, PR and HER2, hence are labeled triple negative breast cancer (TNBC) and express genes typically associated with myoepithelial and basal cells[100]. Since current targeted treatment strategies are mainly focused on antagonizing ER and HER2, TNBC patients hold limited treatment options and chemotherapy is at present the standard systemic therapy for TNBC patients[104,107]. In addition, although classification of breast cancers into distinct subtypes can predict treatment response and recurrence risks, efficacy of current treatment regimens is challenged by tumor heterogeneity and treatment resistance[108–112]. Therefore, it is crucial to gain more understanding of the mechanisms that underlie breast cancer to identify novel therapeutic targets and improve treatment strategies for breast cancer patients.

Aberrant activation of the canonical Wnt signaling route is tightly associated with carcinogenesis in various organs, including breast cancer where the first link with Wnt signaling originated from a mouse study that identified *Wnt1* as a driver of mammary tumors[113]. In breast cancer patients, activation of the Wnt signaling route is particularly observed in patients with TNBC[114–123]. The mechanisms underlying increased Wnt pathway activity are not yet delineated as common Wnt pathway mutations such as *APC* and *CTNNB1* mutations are not generally observed in breast cancer patients[115,116,124]. Instead, alterations in antagonists and agonists of the Wnt pathway are frequently detected and suggeted to be to be the cause of increased Wnt/β-catenin pathway activity[125–129]. In this respect, RSPOs may additionally be proposed as potential candidates driving aberrant Wnt pathway activity in the breast. In colorectal cancer such a role for RSPOs has already been described. Here, gene fusions of *RSPO2* and *RSPO3* are observed in 4-10% of colorectal cancer patients and associated with increased expression of the corresponding RSPO protein[130,131]. Moreover, overexpression of *Rspo2* or *Rspo3* in the mouse intestine drives tumorigenesis accompanied by increased Wnt signaling and expansion of the proliferative stem cell compartment, confirming the oncogenic potential of RSPOs[132,133]. Recognizing the clinical relevance of RSPO signaling in colorectal cancer, several studies addressed the potential benefit of targeting RSPO signaling by using monoclonal antibodies that directly target RSPOs, or by indirect targeting using porcupine inhibitors (PORCNi) that impede the secretion of functionally active Wnt ligands[134–139]. Both strategies successfully decreased tumor growth and increased tumor differentiation in colorectal cancer patient derived xenograft (PDX) models with a gain in *RSPO*[134–139]. Responding to the promising clinical potential of RSPOs as novel therapeutic targets, several clinical studies started investigating the efficacy of PORCNi on colorectal cancer patients with genetic alterations in *RSPO2/3* either or not combined with other drugs[140–143]. Additionally, a clinical trial was started testing the safety and efficacy of anti-RSPO3 monoclonal antibody OMP131-R10 (Rosmantuzumab) on colorectal cancer patients with advanced solid tumors and metastatic cancer[144]. In the breast, indications that RSPOs might hold tumorigenic potential were provided by oncogenic mouse mammary tumor virus (MMTV) screens that identified

Rspo1, *Rspo2* and *Rspo3* as common integration sites[145–148]. Moreover, injection of breast cancer cell lines overexpressing *Rspo2* or *Rspo3* in the mammary glands of mice resulted in the formation of mammary tumors, further supporting a protumorigenic role for RSPOs in the mammary gland[147,149]. Regarding human breast cancer, patient data studies reported overexpression of *RSPO2*, *RSPO3* and *RSPO4* in breast tumors with a particular occurrence in TNBC[135,150,151]. Although these patient data studies only describe an associative role for RSPOs in breast cancer and do not provide direct evidence of RSPOs as oncogenes in the breast, these data together propose that RSPOs hold oncogenic capacity in the mammary gland. Moreover, the specific association with TNBC indicates a potential clinical benefit for RSPOs as alternative therapeutic targets in breast cancer and additionaly implies that RSPO-LGR signaling in breast cancer may occur independenty of upstream hormone signals. In agreement with the high need for novel therapeutic targets for breast cancer patients, it is therefore interesting to further investigate the role of RSPO-LGR signaling in breast cancer and potentially identify the RSPO-LGR pathway as a clinically relevant target for breast cancer.

Scope of the thesis

In this thesis, we will address the contributions of the RSPO-LGR pathway to breast cancer development and progression. In **chapter 2,** the current knowledge on the role of RSPOs in tumorigenesis of various organs is discussed. In **chapter 3**, we establish an oncogenic role for RSPO3 in the mammary gland. Here, we use a mouse model specifically overexpressing *Rspo3* in the mammary glands, resulting in the formation of invasive mammary tumors. We find that RSPO3-driven mammary tumors are molecularly distinct from WNT1-driven counterparts and greatly differ in histology and gene expression profiles, indicating that RSPO3 and WNT1 drive mammary tumorigenesis through distinct mechanisms. We further address this in **chapter 4**, where we study the activities through which RSPO3 drives breast cancer. Here, we demonstrate that RSPO3 drives the aberrant expansion of luminal progenitor cells. Moreover, we find that RSPO3 drives growth, proliferation, and invasion of breast cancer cells independently of Wnt signaling and demonstrate that treatment with a Wnt inhibitor does not interfere with these stimulatory effects of RSPO3. In **chapter 5**, we present a new mouse model for skin and mammary cancer by inactivating E-cadherin and P53 in LGR6^{+} cells. We find that inactivation of E-cadherin and P53 in LGR6^{+} cells results in the formation of invasive squamous cell carcinomas in the skin and a sporadic ductal, ER negative, mammary tumor. The novel insights gained on the contributions of RSPO3 and LGR6 to breast cancer are discussed in **chapter 6.**

1

References

1. Clevers, H. Wnt/β-Catenin Signaling in Development and Disease. *Cell* **127**, 469–480 (2006).
2. Hao, H. X. *et al.* ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner. *Nature* **485**, 195–202 (2012).
3. Koo, B. K. *et al.* Tumour suppressor RNF43 is a stem-cell E3 ligase that induces endocytosis of Wnt receptors. *Nature* **488**, 665–669 (2012).
4. van Amerongen, R. Alternative Wnt pathways and receptors. *Cold Spring Harb. Perspect. Biol.* **4**, 1–18 (2012).
5. Nusse, R. & Clevers, H. Wnt/β-Catenin Signaling, Disease, and Emerging Therapeutic Modalities. *Cell* **169**, 985–999 (2017).
6. Carmon, K. S., Gong, X., Lin, Q., Thomas, A. & Liu, Q. R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/β-catenin signaling. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 11452–11457 (2011).
7. De Lau, W. *et al.* Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. *Nature* **476**, 293–297 (2011).
8. Glinka, A. *et al.* LGR4 and LGR5 are R-spondin receptors mediating Wnt/β-catenin and Wnt/PCP signalling. *EMBO Rep.* **12**, 1055–1061 (2011).
9. Gong, X. *et al.* LGR6 is a high affinity receptor of R-Spondins and potentially functions as a tumor suppressor. *PLoS One* **7**, e37137 (2012).
10. Tomizuka, K. *et al.* R-spondin1 plays an essential role in ovarian development through positively regulating Wnt-4 signaling. *Hum. Mol. Genet.* **17**, 1278–1291 (2008).
11. Nam, J. S. *et al.* Mouse R-spondin2 is required for apical ectodermal ridge maintenance in the hindlimb. *Dev. Biol.* **311**, 124–135 (2007).
12. Aoki, M., Kiyonari, H., Nakamura, H. & Okamoto, H. R-spondin2 expression in the apical ectodermal ridge is essential for outgrowth and patterning in mouse limb development. *Dev. Growth Differ.* **50**, 85–95 (2008).
13. Aoki, M. *et al.* R-spondin3 is required for mouse placental development. *Dev. Biol.* **301**, 218–226 (2007).
14. Bell, S. M. *et al.* R-spondin 2 is required for normal laryngeal-tracheal, lung and limb morphogenesis. *Development* **135**, 1049–1058 (2008).
15. Yan, K. S. *et al.* Non-equivalence of Wnt and R-spondin ligands during Lgr5 + intestinal stem-cell self-renewal. *Nature* **545**, 238–242 (2017).
16. Sigal, M. *et al.* Stromal R-spondin orchestrates gastric epithelial stem cells and gland homeostasis. *Nature* **548**, 451–455 (2017).
17. Barker, N. *et al.* Lgr5+ve Stem Cells Drive Self-Renewal in the Stomach and Build Long-Lived Gastric Units In Vitro. *Cell Stem Cell* **6**, 25–36 (2010).
18. Barker, N. *et al.* Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* **449**, 1003–1007 (2007).
19. De Visser, K. E. *et al.* Developmental stage-specific contribution of LGR5+ cells to basal and luminal epithelial lineages in the postnatal mammary gland. *J. Pathol.* **228**, 300–309 (2012).
20. Blaas, L. *et al.* Lgr6 labels a rare population of mammary gland progenitor cells that are able to originate luminal mammary tumours. *Nat. Cell Biol.* **18**, 1346–1356 (2016).
21. Greicius, G. *et al.* PDGFRα+ pericryptal stromal cells are the critical source of Wnts and RSPO3 for murine intestinal stem cells in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **115**, E3173–E3181 (2018).
22. Kleeman, S. O. *et al.* Exploiting differential Wnt target gene expression to generate a molecular biomarker for colorectal cancer stratification. *Gut* **69**, 1092-1103 (2019)
23. Kang, E., Yousefi, M. & Gruenheid, S. R-spondins are expressed by

the intestinal stroma and are differentially regulated during Citrobacter rodentium- and dssinduced colitis in mice. *PLoS One* **11**, 1–13 (2016).

24. ter Steege, E. J. & Bakker, E. R. M. The role of R-spondin proteins in cancer biology. *Oncogene* **40**, 6469–6478 (2021).
25. Macias, H. & Hinck, L. Mammary gland development. *Wiley Interdiscip. Rev. Dev. Biol.* **1**, 533–557 (2012).
26. Robinson, G. W. Cooperation of signalling pathways in embryonic mammary gland development. *Nat. Rev. Genet.* **8**, 963–972 (2007).
27. Chu, E. Y. *et al.* Canonical WNT signaling promotes mammary placode development and is essential for initiation of mammary gland morphogenesis. *Development* **131**, 4819–4829 (2004).
28. Veltmaat, J. M., Van Veelen, W., Thiery, J. P. & Bellusci, S. Identification of the Mammary Line in Mouse by Wnt10b Expression. *Dev. Dyn.* **229**, 349–356 (2004).
29. Boras-Granic, K., Chang, H., Grosschedl, R. & Hamel, P. A. Lef1 is required for the transition of Wnt signaling from mesenchymal to epithelial cells in the mouse embryonic mammary gland. *Dev. Biol.* **295**, 219–231 (2006).
30. Van Genderen, C. *et al.* Development of several organs that require inductive epithelial- mesenchymal interactions is impaired in LEF-1-deficient mice. *Genes Dev.* **8**, 2691–2703 (1994).
31. Eblaghie, M. C. *et al.* Interactions between FGF and Wnt signals and Tbx3 gene expression in mammary gland initiation in mouse embryos. *J. Anat.* **205**, 1–13 (2004).
32. Davenport, T. G., Jerome-Majewska, L. A. & Papaioannou, V. E. Mammary gland, limb and yolk sac defects in mice lacking Tbx3, the gene mutated in human ulnar mammary syndrome. *Development* **130**, 2263–2273 (2003).
33. Cho, K. W. *et al.* Molecular interactions between Tbx3 and Bmp4 and a model for dorsoventral positioning of mammary gland development. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 16788–16793 (2006).
34. Wysolmerski, J. J. *et al.* Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone-related protein is essential for mammary gland development. *Development* **125**, 1285–1294 (1998).
35. Wysolmerski, J. J. *et al.* Absence of functional type 1 parathyroid hormone (PTH)/PTH-related protein receptors in humans is associated with abnormal breast development and tooth impaction. *J. Clin. Endocrinol. Metab.* **86**, 1788–1794 (2001).
36. Jobert, A. S. *et al.* Absence of functional receptors for parathyroid hormone and parathyroid hormone-related peptide in Blomstrand chondrodysplasia. *J. Clin. Invest.* **102**, 34–40 (1998).
37. Wysolmerski, J. J., McCaughern-Carucci, J. F., Daifotis, A. G., Broadus, A. E. & Philbrick, W. M. Overexpression of parathyroid hormone-related protein or parathyroid hormone in transgenic mice impairs branching morphogenesis during mammary gland development. *Development* **121**, 3539–3547 (1995).
38. Hens, J. R. *et al.* BMP4 and PTHrP interact to stimulate ductal outgrowth during embryonic mammary development and to inhibit hair follicle induction. *Development* **134**, 1221–1230 (2007).
39. Foley, J. *et al.* Parathyroid hormone-related protein maintains mammary epithelial fate and triggers nipple skin differentiation during embryonic breast development. *Development* **128**, 513–525 (2001).
40. Gallego, M. I. *et al.* Prolactin, growth hormone, and epidermal growth factor activate Stat5 in different compartments of mammary tissue and exert different and overlapping developmental effects. *Dev. Biol.* **229**, 163–175 (2001).
41. Mallepell, S., Krust, A., Chambon, P. & Brisken, C. Paracrine signaling through the epithelial estrogen receptor α is required for proliferation and morphogenesis in

the mammary gland. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 2196–2201 (2006).

42. Ruan, W. & Kleinberg, D. L. Insulin-like growth factor I is essential for terminal end bud formation and ductal morphogenesis during mammary development. *Endocrinology* **140**, 5075–5081 (1999).
43. Feng, Y., Manka, D., Wagner, K. U. & Khan, S. A. Estrogen receptor-α expression in the mammary epithelium is required for ductal and alveolar morphogenesis in mice. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 14718–14723 (2007).
44. Brisken, C. *et al.* Prolactin controls mammary gland development via direct and indirect mechanisms. *Dev. Biol.* **210**, 96–106 (1999).
45. Ormandy, C. J. *et al.* Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. *Genes Dev.* **11**, 167–178 (1997).
46. Lydon, J. P. *et al.* Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes Dev.* **9**, 2266–2278 (1995).
47. Horseman, N. D. *et al.* Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene. *EMBO J.* **16**, 6926–6935 (1997).
48. Brisken, C. *et al.* A paracrine role for the epithelial progesterone receptor in mammary gland development. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 5076–5081 (1998).
49. Brisken, C. *et al.* Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling. *Genes Dev.* **14**, 650–654 (2000).
50. Yu, Q. C., Verheyen, E. M. & Zeng, Y. A. Mammary development and breast cancer: A Wnt perspective. *Cancers (Basel).* **8**, 1–26 (2016).
51. Cai, C. *et al.* R-spondin1 is a novel hormone mediator for mammary stem cell self-renewal. *Genes Dev.* **28**, 2205–2218 (2014).
52. Chadi, S. *et al.* R-spondin1 is required for normal epithelial morphogenesis during mammary gland development. *Biochem. Biophys. Res. Commun.* **390**, 1040–1043 (2009).
53. Chadi, S. *et al.* Phenotypic and molecular alterations in the mammary tissue of R-spondin1 knock-out mice during pregnancy. *PLoS One* **11**, 1–15 (2016).
54. Wang, Y. *et al.* Lgr4 regulates mammary gland development and stem cell activity through the pluripotency transcription factor Sox2. *Stem Cells* **31**, 1921–1931 (2013).
55. Roarty, K. & Serra, R. Wnt5a is required for proper mammary gland development and TGF-β-mediated inhibition of ductal growth. *Development* **134**, 3929–3939 (2007).
56. Kouros-Mehr, H. & Werb, Z. Candidate regulators of mammary branching morphogenesis identified by genome-wide transcript analysis. *Dev. Dyn.* **235**, 3404–3412 (2006).
57. Fu, N. Y., Nolan, E., Lindeman, G. J. & Visvader, J. E. Stem cells and the differentiation hierarchy in mammary gland development. *Physiol. Rev.* **100**, 489–523 (2020).
58. Sleeman, K. E. *et al.* Dissociation of estrogen receptor expression and in vivo stem cell activity in the mammary gland. *J. Cell Biol.* **176**, 19–26 (2007).
59. Shackleton, M. *et al.* Generation of a functional mammary gland from a single stem cell. *Nature* **439**, 84–88 (2006).
60. Stingl, J. *et al.* Purification and unique properties of mammary epithelial stem cells. *Nature* **439**, 993–997 (2006).
61. Prater, M. D. *et al.* Mammary stem cells have myoepithelial cell properties. *Nat. Cell Biol.* **16**, 942–950 (2014).
62. Shehata, M. *et al.* Phenotypic and functional characterisation of the luminal cell hierarchy of the mammary gland. *Breast Cancer Res.* **14**, 1–19 (2012).
63. Van Keymeulen, A. *et al.* Distinct stem cells contribute to mammary gland development and maintenance. *Nature* **479**, 189–193 (2011).
64. Van Amerongen, R., Bowman, A. N. & Nusse, R. Developmental stage and

time dictate the fate of Wnt/β-catenin-responsive stem cells in the mammary gland. *Cell Stem Cell* **11**, 387–400 (2012).

65. Wuidart, A. *et al.* Quantitative lineage tracing strategies to resolve multipotency in tissue-specific stem cells. *Genes Dev.* **30**, 1261–1277 (2016).
66. Rios, A. C., Fu, N. Y., Lindeman, G. J. & Visvader, J. E. In situ identification of bipotent stem cells in the mammary gland. *Nature* **506**, 322–327 (2014).
67. Wang, D. *et al.* Identification of multipotent mammary stemcells by protein C receptor expression. *Nature* **517**, 81–84 (2015).
68. Chakrabarti, R. *et al.* Notch ligand Dll1 mediates cross-talk between mammary stem cells and the macrophageal niche. *Science* **360**, eaan4153 (2018).
69. Lafkas, D. *et al.* Notch3 marks clonogenic mammary luminal progenitor cells in vivo. *J. Cell Biol.* **203**, 47–56 (2013).
70. Rodilla, V. *et al.* Luminal Progenitors Restrict Their Lineage Potential during Mammary Gland Development. *PLoS Biol.* **13**, 1–24 (2015).
71. Kaanta, A. S., Virtanen, C., Selfors, L. M., Brugge, J. S. & Neel, B. G. Evidence for a multipotent mammary progenitor with pregnancy-specific activity. *Breast Cancer Res.* **15**, R65 (2013).
72. Fu, N. Y. *et al.* Identification of quiescent and spatially restricted mammary stem cells that are hormone responsive. *Nat. Cell Biol.* **19**, 164–176 (2017).
73. Raslan, A. A. & Yoon, J. K. R-spondins: Multi-mode WNT signaling regulators in adult stem cells. *Int. J. Biochem. Cell Biol.* **106**, 26–34 (2019).
74. Joshi, P. A. *et al.* RANK Signaling Amplifies WNT-Responsive Mammary Progenitors through R-SPONDIN1. *Stem Cell Reports* **5**, 31–44 (2015).
75. Joshi, P. A. *et al.* Progesterone induces adult mammary stem cell expansion. *Nature* **465**, 803–807 (2010).
76. Asselin-Labat, M. L. *et al.* Control of mammary stem cell function by steroid hormone signalling. *Nature* **465**, 798–802 (2010).
77. Lim, E. *et al.* Transcriptome analyses of mouse and human mammary cell subpopulations reveal multiple conserved genes and pathways. *Breast Cancer Res.* **12**, R21 (2010).
78. Ginestier, C. *et al.* ALDH1 Is a Marker of Normal and Malignant Human Mammary Stem Cells and a Predictor of Poor Clinical Outcome. *Cell Stem Cell* **1**, 555–567 (2007).
79. Colacino, J. A. *et al.* Heterogeneity of Human Breast Stem and Progenitor Cells as Revealed by Transcriptional Profiling. *Stem Cell Reports* **10**, 1596–1609 (2018).
80. Plaks, V. *et al.* Lgr5-Expressing Cells Are Sufficient And Necessary for Postnatal Mammary Gland Organogenesis. *Cell Rep.* **3**, 70–78 (2013).
81. Sung, H. *et al.* Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA. Cancer J. Clin.* **71**, 209–249 (2021).
82. Pramod, N. *et al.* Comprehensive Review of Molecular Mechanisms and Clinical Features of Invasive Lobular Cancer. *Oncologist* **26**, e943–e953 (2021).
83. Makki, J. Diversity of Breast Carcinoma : Histological Subtypes and Clinical Relevance. *Clin. Med. Insights Pathol.* **8**, 23–31 (2015).
84. Berx, G. *et al.* E-cadherin is a tumour/invasion suppressor gene mutated in human lobular breast cancers. *EMBO J.* **14**, 6107–6115 (1995).
85. Derksen, P. W. B. *et al.* Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. *Cancer Cell* **10**, 437–449 (2006).
86. Moll, R., Mitze, M., Frixen, U. H. & Birchmeiert, W. Differential Loss of E-Cadherin Expression in Infiltrating

Ductal and Lobular Breast Carcinomas. *Am. J. Pathol.* **143**, 1731–1742 (1993).

87. Arpino, G., Bardou, V. J., Clark, G. M. & Elledge, R. M. Infiltrating lobular carcinoma of the breast: Tumor characteristics and clinical outcome. *Breast Cancer Res.* **6**, 7–11 (2004).
88. Reed, M. E. M. C., Kutasovic, J. R., Lakhani, S. R. & Simpson, P. T. Invasive lobular carcinoma of the breast: Morphology, biomarkers and 'omics. *Breast Cancer Res.* **17**, 1–11 (2015).
89. Porter, A. J., Evans, E. B., Foxcroft, L. M., Simpson, P. T. & Lakhani, S. R. Mammographic and ultrasound features of invasive lobular carcinoma of the breast. *J. Med. Imaging Radiat. Oncol.* **58**, 1–10 (2014).
90. Berg, W. A. *et al.* Diagnostic accuracy of mammography, clinical examination, US, and MR imaging in preoperative assessment of breast cancer. *Radiology* **233**, 830–849 (2004).
91. Adachi, Y. *et al.* Comparison of clinical outcomes between luminal invasive ductal carcinoma and luminal invasive lobular carcinoma. *BMC Cancer* **16**, 1–9 (2016).
92. Chen, Z. *et al.* Invasive lobular carcinoma of the breast: A special histological type compared with invasive ductal carcinoma. *PLoS One* **12**, 1–17 (2017).
93. Pestalozzi, B. C. *et al.* Distinct clinical and prognostic features of infiltrating lobular carcinoma of the breast: Combined results of 15 International Breast Cancer Study Group clinical trials. *J. Clin. Oncol.* **26**, 3006–3014 (2008).
94. Du, T. *et al.* Invasive lobular and ductal breast carcinoma differ in immune response, protein translation efficiency and metabolism. *Sci. Rep.* **8**, 1–11 (2018).
95. Mathieu, M. C. *et al.* The poor responsiveness of infiltrating lobular breast carcinomas to neoadjuvant chemotherapy can be explained by their biological profile. *Eur. J. Cancer* **40**, 342–351 (2004).
96. Barroso-Sousa, R. & Metzger-Filho, O. Differences between invasive lobular and invasive ductal carcinoma of the breast: Results and therapeutic implications. *Ther. Adv. Med. Oncol.* **8**, 261–266 (2016).
97. Van Baelen, K. *et al.* Current and future diagnostic and treatment strategies for patients with invasive lobular breast cancer. *Ann. Oncol.* **33**, 769–785 (2022).
98. Herschkowitz, J. I. *et al.* Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. *Genome Biol.* **8**, 1–17 (2007).
99. Sørlie, T. *et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 10869–10874 (2001).
100. Perou, C. M. *et al.* Molecular portraits of human breast tumours. letters to nature 748. *Nature* **533**, 747–752 (2000).
101. Prat, A. *et al.* Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Res.* **12**, R68 (2010).
102. Prat, A. *et al.* Prognostic significance of progesterone receptor-positive tumor cells within immunohistochemically defined luminal a breast cancer. *J. Clin. Oncol.* **31**, 203–209 (2013).
103. The Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature* **490**, 61–70 (2012).
104. Waks, A. G. & Winer, E. P. Breast Cancer Treatment: A Review. *JAMA - J. Am. Med. Assoc.* **321**, 288–300 (2019).
105. Vu, T. & Claret, F. X. Trastuzumab: Updated mechanisms of action and resistance in breast cancer. *Front. Oncol.* **2**, 62 (2012).
106. Figueroa-Magalhães, M. C., Jelovac, D., Connolly, R. M. & Wolff, A. C. Treatment of HER2-positive breast cancer. *Breast* **23**, 128–136 (2014).

107. Bianchini, G., De Angelis, C., Licata, L. & Gianni, L. Treatment landscape of triple-negative breast cancer — expanded options, evolving needs. *Nat. Rev. Clin. Oncol.* **19**, 91–113 (2022).

108. Turashvili, G. & Brogi, E. Tumor heterogeneity in breast cancer. *Front. Med.* **4**, 227 (2017).

109. Lüönd, F., Tiede, S. & Christofori, G. Breast cancer as an example of tumour heterogeneity and tumour cell plasticity during malignant progression. *Br. J. Cancer* **125**, 164–175 (2021).

110. Hanker, A. B., Sudhan, D. R. & Arteaga, C. L. Overcoming Endocrine Resistance in Breast Cancer. *Cancer Cell* **37**, 496–513 (2020).

111. Swain, S. M., Shastry, M. & Hamilton, E. Targeting HER2-positive breast cancer: advances and future directions. *Nat. Rev. Drug Discov.* **22**, 101-126 (2022) doi:10.1038/s41573-022-00579-0.

112. Bai, X., Ni, J., Beretov, J., Graham, P. & Li, Y. Triple-negative breast cancer therapeutic resistance: Where is the Achilles' heel? *Cancer Lett.* **497**, 100–111 (2021).

113. Tsukamoto, A. S., Grosschedl, R., Guzman, R. C., Parslow, T. & Varmus, H. E. Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. *Cell* **55**, 619–625 (1988).

114. Khramtsov, A. I. *et al.* Wnt/β-catenin pathway activation is enriched in basal-like breast cancers and predicts poor outcome. *Am. J. Pathol.* **176**, 2911–2920 (2010).

115. Lin, S. Y. *et al.* β-catenin, a novel prognostic marker for breast cancer: Its roles in cyclin D1 expression and cancer progression. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 4262–4266 (2000).

116. Geyer, F. C. *et al.* B-Catenin pathway activation in breast cancer is associated with triple-negative phenotype but not with CTNNB1 mutation. *Mod. Pathol.* **24**, 209–231 (2011).

117. He, Y. *et al.* Expression and significance of Wnt signaling components and their target genes in breast carcinoma. *Mol. Med. Rep.* **9**, 137–143 (2014).

118. Jonsson, M., Borg, Å., Nilbert, M. & Andersson, T. Involvement of adenomatous polyposis coli (APC)/β-catenin signalling in human breast cancer. *Eur. J. Cancer* **36**, 242–248 (2000).

119. López-Knowles, E. *et al.* Cytoplasmic localization of â-catenin is a marker of poor outcome in breast cancer patients. *Cancer Epidemiol. Biomarkers Prev.* **19**, 301–309 (2010).

120. Prasad, C. P., Gupta, S. D., Rath, G. & Ralhan, R. Wnt signaling pathway in invasive ductal carcinoma of the breast: Relationship between β-catenin, disheveled and cyclin D1 expression. *Oncology* **73**, 112–117 (2008).

121. Ozaki, S. *et al.* Alterations and correlations of the components in the Wnt signaling pathway and its target genes in breast cancer. *Oncol. Rep.* **14**, 1437–43 (2005).

122. Karayiannakis, A. J. *et al.* Expression patterns of β-catenin in in situ and invasive breast cancer. *Eur. J. Surg. Oncol.* **27**, 31–36 (2001).

123. Li, S., Li, S., Sun, Y. & Li, L. The expression of β-catenin in different subtypes of breast cancer and its clinical significance. *Tumor Biol.* **35**, 7693–7698 (2014).

124. Incassati, A., Chandramouli, A., Eelkema, R. & Cowin, P. Key signaling nodes in mammary gland development and cancer: β-catenin. *Breast Cancer Res.* **12**, 1–14 (2010).

125. Ai, L. *et al.* Inactivation of Wnt inhibitory factor-1 (WIF1) expression by epigenetic silencing is a common event in breast cancer. *Carcinogenesis* **27**, 1341–1348 (2006).

126. Suzuki, H. *et al.* Frequent epigenetic inactivation of Wnt antagonist genes in breast cancer. *Br. J. Cancer* **98**, 1147–1156 (2008).

127. Veeck, J. *et al.* Epigenetic inactivation of the secreted frizzled-related protein-5 (SFRP5) gene in human breast cancer is associated with unfavorable prognosis. *Carcinogenesis* **29**, 991–998 (2008).

128. Veeck, J. *et al.* Prognostic relevance of Wnt-inhibitory factor-1 (WIF1) and Dickkopf-3 (DKK3) promoter methylation in human breast cancer. *BMC Cancer* **9**, 1–13 (2009).

129. van Schie, E. H. & van Amerongen, R. Aberrant WNT/CTNNB1 Signaling as a Therapeutic Target in Human Breast Cancer: Weighing the Evidence. *Front. Cell Dev. Biol.* **8**, 1–14 (2020).

130. Seshagiri, S. *et al.* Recurrent R-spondin fusions in colon cancer. *Nature* **488**, 660–664 (2012).

131. Sekine, S. *et al.* Frequent PTPRK-RSPO3 fusions and RNF43 mutations in colorectal traditional serrated adenoma. *J. Pathol.* **239**, 133–138 (2016).

132. Hilkens, J. *et al.* RSPO3 expands intestinal stem cell and niche compartments and drives tumorigenesis. *Gut* **66**, 1095–1105 (2017).

133. Han, T. *et al.* R-Spondin chromosome rearrangements drive Wnt-dependent tumour initiation and maintenance in the intestine. *Nat. Commun.* **8**, 1–12 (2017).

134. Storm, E. E. *et al.* Targeting PTPRK-RSPO3 colon tumours promotes differentiation and loss of stem-cell function. *Nature* **529**, 97–100 (2016).

135. Chartier, C. *et al.* Therapeutic targeting of tumor-derived r-spondin attenuates b-catenin signaling and tumorigenesis in multiple cancer types. *Cancer Res.* **76**, 713–723 (2016).

136. Fischer, M. M. *et al.* RSPO3 antagonism inhibits growth and tumorigenicity in colorectal tumors harboring common Wnt pathway mutations. *Sci. Rep.* **7**, 1–9 (2017).

137. Li, C. *et al.* Identification of RSPO2 Fusion Mutations and Target Therapy Using a Porcupine Inhibitor. *Sci. Rep.* **8**, 1–9 (2018).

138. Madan, B. *et al.* Wnt addiction of genetically defined cancers reversed by PORCN inhibition. *Oncogene* **35**, 2197–2207 (2016).

139. Picco, G. *et al.* Loss of AXIN1 drives acquired resistance to WNT pathway blockade in colorectal cancer cells carrying RSPO 3 fusions . *EMBO Mol. Med.* **9**, 293–303 (2017).

140. A Study to Evaluate the Safety and Tolerability of ETC-1922159 as a Single Agent and in Combination With Pembrolizumab in Advanced Solid Tumours. Available from: https://clinicaltrials.gov/ct2/show/NCT02521844

141. Study of WNT974 in Combination With LGX818 and Cetuximab in Patients With BRAF-mutant Metastatic Colorectal Cancer (mCRC) and Wnt Pathway Mutations. Available from: https://clinicaltrials.gov/ct2/show/NCT02278133

142. A study of LGK974 in Patients With Malignancies Dependent on Wnt Ligands. Available from: https://clinicaltrials.gov/ct2/show/NCT01351103

143. A study to Assess Efficacy of RXC004 +/- Nivolumab in Ring Finger Protein 43 (RNF43) or R-spondin (RSPO) Aberrated, Metastatic, Microsatellite Stable, Colorectal Cancer After Progression on Standard of Care (SOC). Available from: https://clinicaltrials.gov/ct2/show/NCT04907539

144. A Phase 1a/b Dose Escalation Study of the Safety, Pharmacokinetics, and Pharmacodynamics of OMP-131R10. Available from: https://clinicaltrials.gov/ct2/show/NCT02482441

145. Callahan, R. & Smith, G. H. Common integration sites for MMTV in viral induced mouse mammary tumors. *J. Mammary Gland Biol. Neoplasia* **13**, 309–321 (2008).

146. Klijn, C. *et al.* Analysis of Tumor Heterogeneity and Cancer Gene Networks Using Deep Sequencing of MMTV-Induced Mouse Mammary Tumors. *PLoS One* **8**, 1–10 (2013).

147. Theodorou, V. *et al.* MMTV insertional mutagenesis identifies genes, gene families and pathways involved in mammary cancer. *Nat. Genet.* **39**, 759–769 (2007).

148. Gattelli, A., Zimberlin, M. N., Meiss, R. P., Castilla, L. H. & Kordon, E. C. Selection

of Early-Occurring Mutations Dictates Hormone-Independent Progression in Mouse Mammary Tumor Lines. *J. Virol.* **80**, 11409–11415 (2006).

149. Klauzinska, M. *et al.* Rspo2/Int7 Regulates Invasiveness and Tumorigenic Properties of Mammary Epithelial Cells. *J. Cell Physiol.* **227**, 1960–71 (2012).

150. Coussy, F. *et al.* Clinical value of R-spondins in triple-negative and metaplastic breast cancers. *Br. J. Cancer* **116**, 1595–1603 (2017).

151. Tocci, J. M. *et al.* R-Spondin3 is associated with basal-progenitor behavior in normal and tumor mammary cells. *Cancer Res.* **78**, 4497–4511 (2018).

THE ROLE OF R-SPONDIN PROTEINS IN CANCER BIOLOGY

2

Eline J. ter Steege[1] and Elvira R.M. Bakker[1]

[1] Department of Pathology, University Medical Center Utrecht, The Netherlands

Published in Oncogene (2021)
doi: 10.1038/s41388-021-02059-y

Abstract

R-spondin (RSPO) proteins constitute a family of four secreted glycoproteins (RSPO1-4) that have appeared as multipotent signaling ligands. The best-known molecular function of RSPOs lie within their capacity to agonize the Wnt/β-catenin signaling pathway. As RSPOs act upon cognate receptors LGR4/5/6 that are typically expressed by stem cells and progenitor cells, RSPO proteins importantly potentiate Wnt/β-catenin signaling especially within these proliferative stem cell compartments. Since multiple organs express LGR4/5/6 receptors and RSPO ligands within their stem cell niches, RSPOs can exert an influential role in stem cell regulation throughout the body. Inherently, over the last decade a multitude of reports implicated the deregulation of RSPOs in cancer development. Firstly, *RSPO2* and *RSPO3* gene fusions with concomitant enhanced expression have been identified in colon cancer patients, and proposed as an alternative driver of Wnt/β-catenin hyperactivation that earmarks cancer in the colorectal tract. Moreover, the causal oncogenic capacity of RSPO3 overactivation has been demonstrated in the mouse intestine. As a paradigm organ in this field, most of current knowledge about RSPOs in cancer is derived from studies in the intestinal tract. However, *RSPO* gene fusions as well as enhanced *RSPO* expression have been reported in multiple additional cancer types, affecting different organs that involve divergent stem cell hierarchies. Importantly, the emerging oncogenic role of RSPO and its potential clinical utility as a therapeutic target have been recognized and investigated in preclinical and clinical settings. This review provides a survey of current knowledge on the role of RSPOs in cancer biology, addressing the different organs implicated, and of efforts made to explore intervention opportunities in cancer cases with RSPO overrepresentation, including the potential utilization of RSPO as novel therapeutic target itself.

Introduction

The R-spondin (RSPO) family is represented by four genes *RSPO1, 2, 3,* and *4*, encoding like-named secreted signaling proteins. Homologues of RSPOs are present amongst vertebrates and typically contain a thrombospondin type I repeat (TSR) domain, explaining their historical names as *Human Protein With ThromboSpondin type I Repeat (hPWTSR)* and *Cysteine-rich single thrombospondin type I repeat containing protein* (*Cristin)*[1,2]*. RSPO3* was the first member to be identified in a human fetal brain cDNA library in 2002, followed by the identification of mouse *Rspo1* in 2004[1,3]. As *Rspo1* expression was observed in the roof plate of the neural tube during mouse development, it was named Roof plate specific–Spondin (R-spondin). Subsequently, *RSPO2* and *RSPO4* were identified[4,5]. Genetic mouse and human studies have revealed divergent and pivotal roles for the four RSPO members during development. Mutations in *RSPO1* are linked with female-to-male XX sex reversal and *Rspo1* knockout in mice revealed an important role in ovarian development[6,7]. *Rspo2* is involved in limb and respiratory tract development as well as craniofacial patterning and morphogenesis[8–11]. *Rspo3* is essential for angiogenesis, vasculogenesis and placental development whereas genetic mutations in *RSPO4* were detected in people with anonychia, characterized by the absence of finger and toe nails[12–17].

In 2004, *Xenopus* studies first described what is now the best-known molecular activity of R-spondin proteins: potentiation of the Wnt/β-catenin pathway, a crucial signaling pathway that regulates multiple fundamental processes including proliferation, stem cell control, tissue homeostasis and regeneration[5,18]. Because of this fundamental role, the activity of the Wnt/β-catenin signaling pathway, in other words the downstream transcriptional activity of effector protein β-catenin, requires tight regulation which is executed at multiple levels. The central restraint of the pathway is provided by the intracellular APC containing destruction complex, which induces β-catenin degradation and as such inhibits the pathway (Figure 1A). The pathway is activated upon binding of extracellular Wnt ligands to LRP5/6 and Frizzled (FZD) membrane receptors, leading to dissociation of β-catenin from the degradation complex, stabilization and nuclear translocation of β-catenin and subsequent transcriptional regulation of target genes in the nucleus (Figure 1B). Another level of negative regulation is provided by ZNRF3 and RNF43, ubiquitin ligases that promote the degradation of LRP5/6 and FZD receptors, thereby reducing membranous Wnt receptor availability and subsequent downstream β-catenin signaling capacity[19,20]. It is this latter ZNRF3/RNF43-mediated negative feedback loop that RSPO proteins interfere with, providing an additional level of canonical Wnt pathway regulation. All four RSPOs hold a conserved domain pattern composed of an N-terminal signal peptide, 2 cysteine rich furin like (FU1-FU2) domains, a thrombospondin (TSP) domain and a basic amino acid rich (BR) C-terminal domain. The FU1, FU2 and TSP domains enable RSPO proteins to bind ZNRF3/RNF43, Leucine-rich repeat-containing G-protein coupled receptors (LGR) 4-6 and heparin sulfate proteoglycans (HSPGs) respectively[2,21–28]. Through interaction with ZNRF3/RNF43 and LGRs, RSPOs induce membrane clearance of ubiquitin ligases ZNRF3/

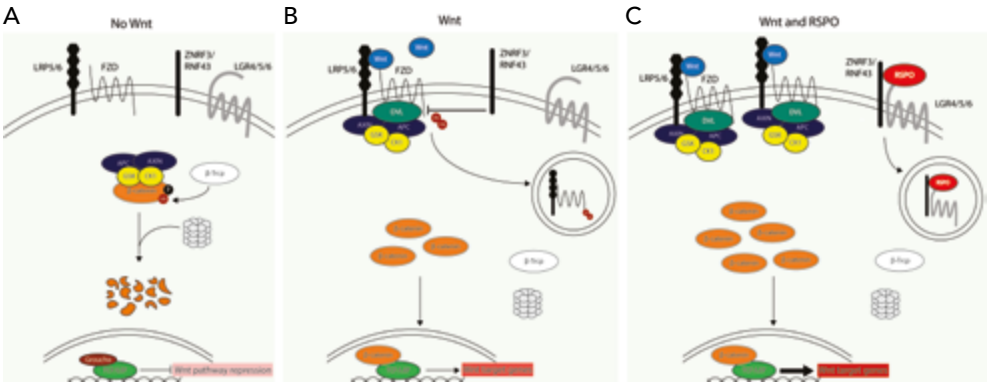


Figure 1. The canonical Wnt pathway and the potentiating effect of RSPO. A. In the absence of canonical Wnt ligands the central destruction complex induces β-catenin degradation, restraining the transcription of Wnt target genes. B. Canonical Wnt ligands induce dissociation of β-catenin from the degradation complex, leading to β-catenin accumulation, nuclear translocation and transcription of Wnt target genes. Ubiquitin ligases ZNRF3/RNF43 negatively regulate the Wnt pathway by internalizing and degrading membrane receptors LRP5/6 and FZD, thereby reducing Wnt receptor availability at the membrane. C. RSPOs potentiate the canonical Wnt pathway by clearing negative regulators ZNRF3/RNF43 from the membrane, thereby increasing membranous Wnt receptor availability and potentiation of Wnt ligand-mediated pathway activation.

RNF43, leading to enhanced Wnt receptor availability at the cell membrane and thereby potentiating Wnt ligand-mediated activation of the Wnt/β-catenin pathway (Figure 1C)[19].

Despite the high homology among the four RSPOs, differences exist in their ability to bind LGRs and ZNRF3/RNF43 and to potentiate the Wnt/β-catenin pathway, where RSPO2 and RSPO3 show highest ZNRF3/RNF43 binding affinity and activity[21,29]. Moreover, RSPO2 and RSPO3 can potentiate the Wnt/β-catenin pathway independently of LGR binding[27,30]. This activity requires the binding of RSPOs to HSPGs with the TSP and BR domains in addition to binding to ZNRF3/RNF43 with the FU1 domain[27,31]. Another study reported that RSPOs are also able to potentiate the Wnt/β-catenin pathway independent of ZNRF3/RNF43, where interaction of RSPO with LGR4 increases the affinity of scaffold protein IQGAP1 to bind DVL, resulting in LRP6 phosphorylation and potentiation of the Wnt/β-catenin pathway[32]. In addition to canonical Wnt signaling, RSPOs have also been implicated in non-canonical Wnt signaling in *Xenopus* embyros, where RSPO3 can modulate non-canonical Wnt/PCP signaling by binding to HSPG syndecan-4 and LGR4,5 to regulate gastrulation and head cartilage morphogenesis[28,33]. Moreover, a recent *Xenopus* study described that RSPO3 exerts antagonistic effects on the BMP signaling route additionally, through binding of ZNRF3 and BMP receptor 1A[34]. Thus, since their discovery multiple signaling activities have been attributed to RSPOs, especially in the regulation of canonical Wnt/β-catenin pathway but also beyond. In that perspective, more lessons will expectedly be learned considering the molecular activities of RSPOs.

The discovery that RSPOs represent ligands of the LGR4/5/6 receptors raised special interest, since these receptors are typically expressed by progenitor cells and stem cells, and as such the agonistic activity that RSPOs exert on Wnt/β-catenin signaling importantly influences the proliferative stem cell compartment[35–38]. As LGR5 was identified as a marker of stem cells in the intestine followed by the recognition of RSPOs representing LGR ligand, most knowledge on RSPO-LGR signaling currently exists in the field of the intestinal tract[33,35,39,40]. However, RSPOs and LGR4/5/6 receptors are present in multiple organs and therefore RSPOs can influence stem cell regulation throughout the body. In accordance with this broad stem cell regulatory role, deregulated RSPO activity has increasingly been implicated in cancer development lately and *RSPO* alterations have been reported to occur in multiple cancer types as reviewed below (Table 1).

Gastrointestinal tract

Intestine

The intestinal epithelium displays an exceptionally rapid turnover that is controlled by tightly balanced molecular signaling in conjunction with unique cellular build-up of the crypt-villus architecture. Canonical Wnt/β-catenin signaling plays a central role in fueling proliferation and self-renewal in the crypt region, from where most progeny cells migrate towards the villus region whilst differentiating. The intestinal stem cell niche housed in the crypt region holds a refined composition of cycling stem cells, being protected and instructed by neighboring Paneth cells, quiescent stem cells and transient amplifying cells. Importantly, the cycling stem cells that fuel the continuous epithelial renewal express the RSPO receptor LGR5, whilst LGR4 is expressed more broadly on cycling stem cells, transient amplifying cells and Paneth cells[35,41]. RSPO3 ligand is produced by stromal cells that lie in close proximity to the crypt stem cells both in mouse intestine and human colon[42–44]. Within this crypt environment, paracrine regulation of Wnt/β-catenin signaling by RSPO and Wnt ligands provide instructive signals to the intestinal stem cell niche, albeit in distinct manners[45]. Whereas Wnt ligands agonize canonical Wnt/β-catenin signaling, they are incapable of inducing renewal of LGR5$^+$ stem cells on their own[45]. Instead, Wnt ligands induce RSPO receptor expression, thereby optimizing conditions for RSPO ligands to exert their effects. RSPO ligands in their turn induce the self-renewal and expansion of stem cells, as such dictating the size of the intestinal stem cell pool[45]. In case of intestinal injury, stromal RSPO3 expression is elevated and is demonstrated to be indispensable for epithelial regeneration by inducing Wnt/β-catenin signaling in differentiated cells, probably through the LGR4 receptor[46]. Of note, LGR5$^+$ stem cells are dispensable in these epithelial regeneration processes, indicating that RSPO3 ligand is essential and dominantly instructive in epithelial repair in the gut[46–48]. Taken together, in the non-transformed intestine, RSPO3 is produced in the pericryptal stroma and plays a fundamental role in controlling stem cell numbers and epithelial recovery through activation of the Wnt/β-catenin pathway.

Table 1. *RSPO* alterations reported among cancer types.

Organ	*RSPO* alteration	Reference
Intestine	**Gene fusions**	
	EIF3E-RSPO2	49, 50, 51
	PIEZO1-RSPO2	51
	NRIP1-RSPO2	52
	PRR15L-RSPO2	53
	PTPRK-RSPO3	49, 50, 51, 52
	Overexpression	
	RSPO2	49, 50
	RSPO3	43, 49, 50
Stomach	**Gene fusions**	
	EMC2-RSPO2	67
	HNF4G-RSPO2	67
	Overexpression	
	RSPO3	64
Breast	**Overexpression**	
	RSPO2	113
	RSPO3	113, 115, 116
	RSPO4	113, 116
Ovary	**Overexpression**	
	RSPO1	116, 122
	RSPO3	116
Prostate	**Gene fusions**	
	GRHL2-RSPO2	131
	Overexpression	
	RSPO3	130
	Downregulation	
	RSPO3	132
Liver	**Gene fusions**	
	SINE-RSPO2	137
	Overexpression	
	RSPO2	136, 137, 138
Lungs	**Gene fusions**	
	EIF3E-RSPO2	141
	PTPRK-RSPO3	141
	Overexpression	
	RSPO3	116, 140
Pancreas	**Overexpression**	
	RSPO2	116, 142
Bladder	**Overexpression**	
	RSPO3	143

In line with the central role of Wnt/β-catenin signaling in intestinal stem cell maintenance, hyperactivation of this pathway is the hallmark feature of colorectal cancer (CRC). In the majority of CRC patients, this hyperactivation is caused by either inactivating *APC* mutations or activating mutations in the β-catenin gene *CTNNB1*, both resulting in constitutive activation of the Wnt/β-catenin pathway, independent of Wnt ligand binding. Importantly, in 2012 it was found that 4-10% of CRC patients harbor gene fusions of the *RSPO2* and *RSPO3* genes with *EIF3E* and *PTPRK* respectively, co-occurring with enhanced expression of the considerate *RSPO* gene[49,50]. These *RSPO2* and *RSPO3* gene rearrangements were found mutually exclusive with other Wnt pathway mutations, though co-occurring with either *KRAS* or *BRAF* mutations, suggestively serving as an alternative mechanism to achieve hyperactivation of the Wnt/β-catenin pathway and to hold oncogenic capacity[49,50]. Following up on the initial discovery of *RSPO* gene fusions in CRC patients, other studies identified additional gene fusions of *RSPO2* with *PIEZO1*, *NRIPI* and *PRR15L* and moreover, reported *RSPO* gene fusions to typically occur in traditional serrated adenoma (TSA) rather than conventional colon tumors[51–54]. Additionally, another CRC patient subpopulation has been described that harbors high *RSPO3* expression levels but seem to lack *RSPO* gene fusions or alternative Wnt pathway mutations[43]. Instead, in these tumors the elevated levels of RSPO3 are produced by the stromal compartment, and in line, most of these cases were of the CMS4 mesenchymal subtype[43]. These data suggest that enhanced *RSPO3* expression by stromal cells can substitute for epithelial *RSPO* mutations in driving CRC. As RSPOs are secreted ligands, these findings support the plausibility that especially the cells that receive the RSPO signals, rather than the producing cells, determine the oncogenic response, therefore being most interesting in understanding the biology of RSPO-driven cancer. For CRC, the typical occurrence of *RSPO* gene fusions in TSA might be informative in this regard, and it has been proposed that this might point towards a different, TSA-like evolutionary trajectory for RSPO-mutant tumor development, distinct from conventional CRC[55]. However, details on the potential cell of origin and mutation selection along the tumorigenic cascade within RSPO-driven cancer remain to be unraveled.

Formal evidence for the causal oncogenic capacity of *Rspo3* was provided by a mouse study where conditional *Rspo3* overexpression consistently induced abundant intestinal tumor formation, demonstrating that augmentation of *Rspo3* levels is causative in driving tumorigenesis[56]. RSPO3-driven tumors showed major expansion of crypt cells including $LGR5^+$ stem cells, quiescent stem cells, Paneth cells and $LGR4^+$ cells with modestly increased Wnt/β-catenin signaling[56]. Thus, enhanced *Rspo3* levels induced a magnification of the proliferative, self-renewing crypt compartment. Adding up to the oncogenic capacity of *Rspo3* overexpression, another mouse study showed that also the transgenic expression of either *EIF3E-RSPO2* or *PTPRK-RSPO3* gene fusion causally drives the formation of intestinal tumors, which comparably show expansion of proliferative cells and ectopic Paneth cells[57]. Inversely, targeted anti-RSPO3 treatment in a *PTPRK-RSPO3* xenograft CRC model was shown to induce tumor differentiation whilst reducing growth, stem cell marker

expression and canonical Wnt pathway activity[58]. Thus, these mouse studies demonstrated that RSPO gain of function, either through overexpression or genetic rearrangement, causally drives intestinal tumorigenesis, wherein deregulation of the proliferative stem cell compartment was shown to be involved. Notably, despite this and the occurrence of *EIF3E-RSPO2* fusions and enhanced *RSPO2* expression in CRC patients, some controversy exists considering the role of RSPO2 in CRC. Hence, RSPO2 has also been attributed tumor suppressive activities in CRC in some reports[59,60].

In summary, during the last decade, studies in the intestinal tract have revealed that a subset of CRC patients harbors a gain in RSPO, which can act as oncogenic driver through fueling aberrant expansion of the crypt stem cell compartment. Currently, most of our knowledge on RSPOs in cancer is derived from studies in the intestine, and for this organ, aberrant RSPO activation is recognized as oncogenic driver.

Stomach

As in the intestine, Wnt signaling plays a crucial role in regulating epithelial turnover in the stomach and aberrant activation of the Wnt/β-catenin pathway is an established driver of gastric cancer[61–63]. In the homeostatic stomach, Wnt ligands and RSPO3 are expressed in the stroma neighboring the gland base that constitutes the gastric stem cell compartment[63,64]. The stem cell compartment of mouse gastric antrum glands is composed of $Lgr5^{+}/Axin2^{+}$ cells at the base and more apical $Lgr5^{-}/Axin2^{+}$ cells[63]. Both these stem cell populations are capable of repopulating the gastric gland, giving rise to progenitor and differentiated cell types[37,63–65]. The $Lgr5^{-}/Axin2^{+}$ cells appear to be the main driver of homeostatic epithelial turnover, repopulating the glands in 7 days, whereas $Lgr5^{+}/Axin2^{+}$ show relatively less proliferation and a gland turnover time of 10-14 days[63,64]. In the stomach, RSPO3 is produced by myofibroblasts neighboring the stem cell compartment and plays a crucial role in regulating stem cell dynamics[64]. Interestingly, RSPO3 induces $Lgr5^{+}/Axin2^{+}$ stem cells to differentiate into secretory cells with antimicrobial activity, protecting the stem cell compartment against bacterial colonization[65]. In contrast, RSPO3 acts upon $Lgr5^{-}/Axin2^{+}$ cells by promoting their proliferation and expansion, probably through Lgr4 that is expressed on these cells[64]. Infection with *Helicobacter pylori (H. pylori)* enhances stromal *Rspo3* expression and leads to expansion of proliferative $Axin2^{+}$ stem cells and hyperplasia[64]. Importantly, *H. pylori* infection represents the main risk factor for the development of gastric cancer. Enhanced proliferation of gastric stem cells driven by RSPO3 upon *H.pylori* infection might contribute to this increased risk for cancer development[63,66]. Despite interesting links have been revealed among stem cell (de)regulation, RSPO3 and *H.pylori* infection in the stomach, more research is needed to further assess their possible interplay in gastric carcinogenesis. With regard to genetic alterations that might underlie RSPO deregulation in gastric cancer patients, current knowledge is relatively limited. Two cases of *RSPO2* gene fusions have been reported in gastric cancer patient-derived xenograft (PDX) material by one group[67].

Steroid hormone regulated organs

Breast

The mammary gland represents another organ where both RSPO and Wnt/β-catenin signaling have been implicated in stem cell regulation during homeostasis and carcinogenesis[68–74]. Although at first glance this involvement might seem comparable to the benchmark situation in the intestinal tract, it is important to realize that the mammary gland is a totally different, uniquely organized epithelial structure that is primarily instructed by steroid hormones estrogen and progesterone. The bilayered mammary epithelium consists of outer basal cells and inner luminal cells, latter being further segregated into luminal progenitor cells and mature luminal cells that express the estrogen receptor (ER) and progesterone receptor (PR). Steroid hormones regulate the exceptionally dynamic remodeling events that occur during puberty, menstrual cycles, pregnancy, lactation and involution. These processes require tightly controlled self-renewal, and the mammary epithelium constitutes a complex and unique, yet incompletely clarified hierarchy of co-existing progenitor and stem cell populations[38,71,74–83]. Mammary stem cells (MaSC) with repopulating capacity were firstly described to be part of the basal population[84,85] . More recent studies report stem cells both in basal and luminal populations, and Wnt/β-catenin signaling has been implicated in the regulation of MaSCs[68,71,72,74,78–83]. In human breast, RSPO3 is expressed in ALDH+ cells, a cell population that has been proposed to represent (cancer) stem cells and luminal progenitor cells[86–88]. In the mouse mammary gland, RSPO1 has emerged as a key regulator of MaSCs, leading to defects in side-branching and alveologenesis upon its depletion[70,73,89,90]. RSPO1 is produced by luminal progenitor cells, in proximity to mature luminal cells that produce Wnt4, which together cooperate in promoting the self-renewal of MaSCs[70,73]. Moreover, RSPO1 and Wnt4 are synchronously upregulated upon steroid hormone signals during pregnancy, leading to Wnt/β-catenin signaling potentiation and fueling the expansion of basal cells and luminal progenitor cells[70,73]. This collaborative RSPO1-Wnt4 action seems to represent the actual downstream executor of stem cell regulation, in response to upstream steroid hormone signals.

In line with the extensive stem cell hierarchy in the mammary gland, breast cancer is exceptionally heterogeneous, and uniquely classified based on the expression of the hormone receptors ER, PR and human epidermal growth factor receptor 2 (HER2). Triple negative breast cancer (TNBC) that lacks expression of these three receptors is the most aggressive subtype with poorest prognosis and most limited options for targeted treatment. Activation of the canonical Wnt pathway in breast cancer has been reported regularly, amongst multiple subtypes, though an association has been proposed especially with TNBC[91–100]. In striking contrast to CRC however, the majority of breast tumors lack mutations in *APC* or *CTNNB1*, obscuring the mutational cause of reported intracellular Wnt activation[68,93]. A possible explanation for this might lie in the different tissue-specific dosages of canonical Wnt signaling activation that support tumor growth, where tumor growth in the mammary epithelium favors a relatively weaker level of Wnt/β-catenin

activation compared to its intestinal counterpart[101–103]. Also, activation of the Wnt pathway might result from alterations in other pathway members[68,104–107]. In this regard, RSPOs might represent additional candidates, supported by the self-renewal promoting effects that RSPO exerts in the normal mammary gland. The first indications that RSPOs might potentially represent mammary oncogenes come from studies in which *Rspo1*, *Rspo2* and *Rspo3* were identified as common integration sites of the mouse mammary tumor virus (MMTV)[108–111]. This was further supported by experiments where injection of cell lines overexpressing *Rspo2* or *Rspo3* in the mouse mammary gland resulted in mammary tumor formation, and distant metastases in case of *Rspo2*[110,112]. With regard to RSPOs in breast cancer patients, some reports have suggested a protumorigenic role for overexpressed RSPOs, mostly based upon associative studies and *in vitro* data[113–115]. Overexpression of *RSPO2, RSPO3* and *RSPO4* have been reported in breast tumors, with a particular occurrence in TNBC and being associated with reduced patient survival in case of *RSPO2* upregulation[113,115,116]. Notably, *EIF3E-RSPO2* fusion transcripts known to occur in CRC were not found in a group of 446 breast tumors tested [113]. This directed approach for these fusions specifically does however not exclude the possibility that other *RSPO* gene fusions might occur in breast cancer. Notably, the two cell lines HBcc-15 and BT549 that are derived from breast cancer patients do have *EIF3E-RSPO2* gene fusions, and siRNA-mediated inhibition of *RSPO2* in BT549 cells was shown to reduce the proliferation of this TNBC cell line[113]. Together these data point towards a protumorigenic role for RSPOs in breast cancer, though further research is needed to better establish this.

Ovary

In ovarian development RSPO1 has appeared as a crucial player, regulating female sex determination and ovarian differentiation in cooperation with Wnt4[6,7,117-119]. RSPO1 and Wnt4 are expressed throughout ovarian development and influence cell proliferation and the entry of germ cells into meiosis by activating the Wnt/β-catenin signaling pathway[117,119,120]. In agreement with its essential role in ovarian development, the Wnt/β-catenin pathway has found to be frequently activated in ovarian cancer, being associated with epithelial-to-mesenchymal progression, chemotherapy resistance and poor prognosis[121]. Considering RSPOs in ovarian cancer, *in silico* analysis suggested relatively high *RSPO1* mRNA expression in ovarian cancer, and another study reported high expression of *RSPO1* and *RSPO3* in ovarian tumor xenograft material[116,122]. Also, SNPs in the *RSPO1* locus have been identified as risk factors for ovarian cancers of serous histology[122,123]. Moreover, a mouse study demonstrated that continuation of *Rspo1* expression after birth, normally downregulated in the ovaries at this stage, resulted in impaired ovulation and Wnt/β-catenin-mediated formation of granulosa cell tumors at the onset of puberty, suggesting that aberrant RSPO1 holds oncogenic potential in the ovaries[124].

Prostate

Wnt/β-catenin signaling is crucial during prostate development and both Wnt and RSPO ligands are expressed within the developing urogenital tract[125–128]. *In vitro* studies have indicated that RSPOs promote the growth and luminal differentiation in prostate organoid cultures[127,129]. In prostate cancer, aberrant regulation of RSPOs and Wnt/β-catenin pathway components have been described[130–133]. *APC* and *CTNNB1* mutations are regularly found in prostate cancer[131,133]. Moreover, *RSPO2* gene fusions associated with elevated *RSPO2* expression have been identified in prostate cancer patients, that were mutually exclusive with *APC* and *CTNNB1* mutations[131]. Unlike in CRC, these prostate cancer cases harbored fusions of *RSPO2* with *GRHL2* instead of *EIF3E*[131]. Also, *RSPO3* has been described as one of the genes being upregulated in prostate tumor stroma compared to healthy stroma[130]. In contrast, another group that studied gene expression data sets reported reduced levels of *RSPO3* in prostate tumors compared to healthy tissue, with further expression loss in metastatic disease and *RSPO3* loss correlating with an increased risk of relapse[132]. Thus, although *RSPO* fusions have been identified in prostate cancer patients and several reports have implicated RSPOs in prostate carcinogenesis, some controversy exists on the contribution of RSPOs to prostate cancer development.

Other organs

In addition to aforementioned cancers, RSPO activation has been implicated in tumorigenic processes in more tissues. In the liver, the RSPO-LGR pathway has been defined as a key regulator of zonation, size and regeneration[134,135]. Several reports have described *RSPO2* activation in liver cancer through distinct means[136–139]. Among these, *RSPO2* gene fusions have been identified, co-occurring with increased *RSPO2* expression levels, nuclear β-catenin localization and upregulation of Wnt target genes, resembling the situation of CRC cases with *RSPO2* gene fusions[137]. Several other studies reported subsets of hepatocellular carcinoma that harbor *RSPO2* copy number amplifications or enhanced *RSPO2* mRNA expression associated with Wnt/β-catenin activation[136,138,139]. Also, it has been shown that overexpression of *Rspo2* in a *Trp53* loss background caused tumor formation in the mouse liver[138]. In lung cancer, a protumorigenic role for RSPOs has been proposed as enhanced expression of RSPO ligands was observed in a subset of lung cancer cases, and enhanced *RSPO3* expression was associated with reduced patient survival[116,140]. These studies reported no underlying *RSPO* gene fusions, and it was proposed that enhanced *RSPO3* expression might have resulted from promoter demethylation and deficiency in tumor suppressor *KEAP1*[116,140]. Complementary *in vitro* and *in vivo* experiments suggested that RSPO3 promotes lung carcinogenesis through LGR4-IQGAP1 signaling[140]. Another group however did report *EIF3E-RSPO2* and *PTPRK-RSPO3* gene fusions in 1% and 2% of lung cancer patients respectively, being restricted to the squamous subtype of NSCLC[141]. Furthermore, enhanced *RSPO* expression and a tumor promoting role have also been described in pancreatic cancer and bladder cancer[116,142,143].

Therapeutic targeting of RSPO in cancer

Hyperactivation of the Wnt/β-catenin pathway has been linked to tumor development in multiple organs, and the underlying molecular alterations are divergent. In line, compelling efforts have been made to develop therapeutic agents that target the Wnt/β-catenin pathway at various levels, among which those intervening with FZD or LRP receptor activity or Wnt ligand maturation and secretion through porcupine inhibitors (PORCNi)[144]. Also the RSPO receptor LGR5 is subject of investigation as a candidate target for therapeutic intervention in cancer[145]. With regard to Wnt driven cancers, dichotomous distinction can be made between ligand-independent and ligand-dependent cases, including those with *APC* or *CTNNB1* mutations versus those with *RSPO* or *RNF43* mutations respectively[55].

The ligand-dependent cases hold relatively more opportunities for targeted intervention. Specifically, with the growing indications for *RSPO* gene fusions/upregulation and a concomitant oncogenic role in several cancer types, RSPOs have emerged as promising candidate targets for therapeutic intervention and inherently as potential biomarkers predicting therapy responsiveness. Accordingly, some first studies have been published exploring the possibilities to inhibit tumor growth through targeting RSPO activity. These intervention strategies either directly targeted the RSPO ligands themselves or rather interfered more indirectly with Wnt ligand activity through PORCNi (Figure 2). As the best described molecular activity of RSPOs lies within their capability to amplify the signal of canonical Wnt ligands, aberrant RSPO expression would expectedly sensitize tumors to Wnt ligand blockade using PORCNi. Hence, PORCNi block the secretion of functionally active Wnt ligands and in their absence, RSPO ligands are impaired to exert any potentiating effects (Figure 2B). Several preclinical studies have tested this using PORCNi in cancer cases with RSPO activation specifically[67,146,147]. Indeed, it was found consistently that PORCNi effectively reduced tumor growth whilst inducing tumor differentiation in PDX models of *RSPO*-fusion positive CRC and gastric cancers[67,146,147]. Currently, several clinical trials distinctively stratify cancer patients with genetic alterations in *RSPO2/3* as inclusion criteria to investigate the efficacy of PORNCi, either or not combined with other drugs[148–151]. As these trials specifically take into consideration the RSPO status of the considerate cancer patients, the drug efficacy data to be obtained by these trials will expectedly provide useful information for further decision making towards targeted intervention strategies in cancer patients with RSPO overactivation.

Direct targeting of RSPO proteins themselves with anti-RSPO antibodies represents another possible intervention approach (Figure 2C). Through this means, RSPO ligands are disabled to clear ZNRF3/RNF43 from the membrane, leading to Wnt receptor degradation and thereby to inhibited Wnt pathway activation. Of additive value, direct targeting of RSPOs might also interfere with potential oncogenic signaling activities beyond stimulating canonical Wnt signalling. Hence, RSPOs have been implicated in other signaling pathways, though possible oncogenic roles there are insufficiently clear yet. In cancer cases with RSPO overactivation, direct targeting of RSPOs themselves might be favorable, and several studies have addressed the efficacy of anti-RSPO antibodies[58,116,152]. Chartier *et*

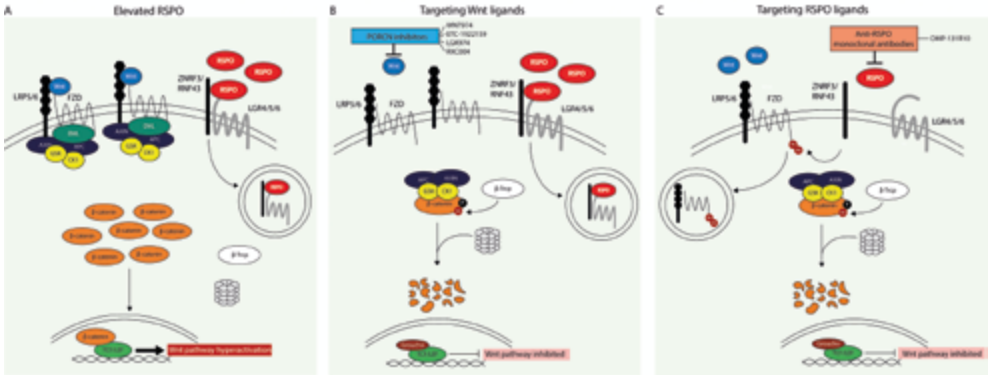


Figure 2. Schematic representation of therapeutic targeting opportunities in cancer cases with *RSPO* overactivation. A. Overexpression of RSPOs induces increased clearing of negative regulators ZNRF3/RNF43 from the membrane, thereby expanding membranous Wnt receptor availability and excessive activation of Wnt ligand-mediated pathway activation. B. PORCN inhibitors block the availability of functional Wnt ligands, allowing the destruction complex to form and degrade β-catenin, resulting in inhibition of the Wnt pathway. Indicated PORCNi are tested in clinical trials for solid cancers considering the RSPO status. C. Monoclonal anti-RSPO antibodies disable RSPOs to clear negative regulators ZNRF3/RNF43 from the membrane, causing ubiquitination and membrane clearance of Wnt receptors, as such inhibiting Wnt ligands to activate the pathway. Indicated antibody has been tested in a clinical trial for colorectal cancer.

al generated monoclonal antibodies against RSPO1-3, and showed that these inhibited tumor growth (both as single agent or in combination with chemotherapy) in multiple PDX cancer models with overexpression of the respective *RSPO*[116]. These included an ovarian tumor with *RSPO1*, pancreatic and colon tumors with *RSPO2*, and lung and CRC tumors with *RSPO3* overexpression. Despite efficacy in most of the models, a minority of *RSPO* expressing tumors were not responsive[116]. Another study by Storm *et al* used a CRC xenograft model specifically with a PTPRK-*RSPO3* gene fusion, and showed that anti-RSPO3 effectively reduced tumor growth and induced differentiation[58]. Both studies demonstrated that differentiation induced by anti-RSPO3 treatment was accompanied by downregulation of Wnt target and stem cell related genes[58,116]. Additionally, another study by Fisher *et al* tested anti-RSPO3 treatment on CRC PDX models harbouring *APC* mutations. Although these were not sensitive to anti-RSPO3 treatment only, the combination of anti-RSPO3 with paclitaxel synergistically reduced tumor growth in most cases, being accompanied by reduced nuclear β-catenin, proliferation and CSC frequency against enhanced differentiation[152]. In addition to these results within solid tumor models, a recent study also showed beneficial effects of anti-RSPO3 treatment in certain acute myeloid leukemia PDX models, where anti-RSPO3 treatment effectively inhibited leukemia stem cells without harming healthy stem cells[153].

Recognizing the promising clinical potential of RSPOs as novel therapeutic targets, a clinical trial has been set-up that tested the safety and efficacy of the neutralizing

monoclonal anti-RSPO3 antibody OMP131-R10 (Rosmantuzumab) in cancer patients with advanced solid tumors and metastatic CRC[154]. It was reported that OMP131-R10 was well-tolerated by patients, though serum bone markers appeared affected[155]. The trial was unfortunately halted in phase I as a consequence of insufficient evidence for clinical benefit[155]. However it seems that the inclusion criteria for this trial did not take into consideration the RSPO status. In that case, it is unknown whether any and how many patients were included in the trial that actually had a gain in *RSPO3* specifically. Therefore, and given the multitude of indications for the relevant oncogenic role of RSPOs, it remains valuable to further investigate the clinical potential of anti-RSPO monoclonal antibodies specifically in cancer patients that harbour *RSPO* alterations.

Taken together, in line with the growing indications for the clinically relevant oncogenic role of RSPOs, some first avenues have been instigated to explore how we can potentially interfere with RSPO overactivation in cancer. Clinical trials addressing the efficacy of indirect and direct RSPO targeting strategies through PORCNi and anti-RSPO3 antibodies respectively will hopefully provide more insight beneficial to the development of novel treatment strategies against RSPO driven cancer.

Conclusions and perspectives

RSPO ligands are powerful regulators of stem cell maintenance and tissue homeostasis. In accordance with this influential role, aberrant RSPO activation has increasingly been implicated in cancer development over the last decade. *RSPO* alterations, mostly represented by gene fusions or upregulation, have been reported to occur in patients of multiple cancer types. In addition, several studies have demonstrated that RSPO overactivation causally drives tumorigenesis in the mouse intestine, and provided indications that abnormal expansion of the stem cell compartment seems part of the mechanism. Most of our current knowledge on the molecular activities of RSPOs have been obtained by studies in the intestinal tract. Although these provide solid indications and relevant insight, only the first part of the puzzle seems uncovered, leaving many questions still unanswered. Among these, it remains unclear how the pathologic *RSPO* alterations are mechanistically achieved. Though specific breakpoints in the *RSPO* genes as well as specific fusion partner genes are involved in reported *RSPO* rearrangements, it is currently unknown how and under which conditions the *RSPO* fusions arise and are selected for along the tumorigenic cascade. A possible cell of origin for RSPO-driven cancer has not been reported yet, and its identification might be complicated by the fact that RSPOs are secreted proteins. Also, it is insufficiently clear what the specific molecular activities are that RSPO proteins instigate on receiving cells and that underpin carcinogenesis. Notably, for all these questions, the answers likely differ per organ.

Forthcoming, a better understanding on the molecular mechanisms of RSPOs with tissue-specific consideration is needed to provide well-founded directions for (pre)clinical studies. Current extensive indications for the oncogenic role of RSPOs however have

already instigated the exploration of potential therapeutic opportunities and RSPOs have been recognized as promising therapeutic targets. Preclinical studies demonstrated that PORCNi and anti-RSPO antibodies efficiently inhibited tumor growth in PDX models of cancer with RSPO activation. Moreover, therapeutic targeting through both PORCNi and anti-RSPO3 antibodies are evaluated in clinical trials and will expectedly provide valuable information for further development of novel targeted intervention strategies.

Acknowledgements

This work was financially supported by the Netherlands Organization for Scientific Research (NWO/ZonMW VENI 016.186.138) and the Dutch Cancer Society (KWF Young Investigator Grant 10957).

Author contributions

Review conceptualization by EB. EtS and EB co-wrote the manuscript. All authors have approved the final version.

Competing interests

The authors declare no competing interests.

References

1. Chen, J.-Z. *et al.* Cloning and identification of a cDNA that encodes a novel human protein with thrombospondin type I repeat domain, hPWTSR. *Mol. Biol. Rep.* **29**, 287–292 (2002).
2. Nam, J. S., Turcotte, T. J., Smith, P. F., Choi, S. & Jeong, K. Y. Mouse cristin/R-spondin family proteins are novel ligands for the frizzled 8 and LRP6 receptors and activate β-catenin-dependent gene expression. *J. Biol. Chem.* **281**, 13247–13257 (2006).
3. Kamata, T. *et al.* R-spondin, a novel gene with thrombospondin type 1 domain, was expressed in the dorsal neural tube and affected in Wnts mutants. *Biochim. Biophys. Acta - Gene Struct. Expr.* **1676**, 51–62 (2004).
4. Kim, K. A. *et al.* R-spondin proteins: A novel link to β-catenin activation. *Cell Cycle* **5**, 23–26 (2006).
5. Kazanskaya, O. *et al.* R-Spondin2 is a secreted activator of Wnt/β-catenin signaling and is required for Xenopus myogenesis. *Dev. Cell* **7**, 525–534 (2004).
6. Parma, P. *et al.* R-spondin1 is essential in sex determination, skin differentiation and malignancy. *Nat. Genet.* **38**, 1304–1309 (2006).
7. Tomizuka, K. *et al.* R-spondin1 plays an essential role in ovarian development through positively regulating Wnt-4 signaling. *Hum. Mol. Genet.* **17**, 1278–1291 (2008).
8. Nam, J. S. *et al.* Mouse R-spondin2 is required for apical ectodermal ridge maintenance in the hindlimb. *Dev. Biol.* **311**, 124–135 (2007).
9. Aoki, M., Kiyonari, H., Nakamura, H. & Okamoto, H. R-spondin2 expression in the apical ectodermal ridge is essential for outgrowth and patterning in mouse limb development. *Dev. Growth Differ.* **50**, 85–95 (2008).
10. Bell, S. M. *et al.* R-spondin 2 is required for normal laryngeal-tracheal, lung and limb morphogenesis. *Development* **135**, 1049–1058 (2008).
11. Yamada, W. *et al.* Craniofacial malformation in R-spondin2 knockout mice. *Biochem. Biophys. Res. Commun.* **381**, 453–458 (2009).
12. Aoki, M. *et al.* R-spondin3 is required for mouse placental development. *Dev. Biol.* **301**, 218–226 (2007).
13. Kazanskaya, O. *et al.* The Wnt signaling regulator R-spondin 3 promotes angioblast and vascular development. *Development* **135**, 3655–3664 (2008).
14. Blaydon, D. C. *et al.* The gene encoding R-spondin 4 (RSPO4), a secreted protein implicated in Wnt signaling, is mutated in inherited anonychia. *Nat. Genet.* **38**, 1245–1247 (2006).
15. Ishii, Y. *et al.* Mutations in R-spondin 4 (RSPO4) underlie inherited anonychia. *J. Invest. Dermatol.* **128**, 867–870 (2008).
16. Bergmann, C. *et al.* Mutations in the gene encoding the Wnt-signaling component R-spondin 4 (RSPO4) cause autosomal recessive anonychia. *Am. J. Hum. Genet.* **79**, 1105–1109 (2006).
17. Brüchle, N. O. *et al.* RSPO4 is the major gene in autosomal-recessive anonychia and mutations cluster in the furin-like cysteine-rich domains of the Wnt signaling ligand R-spondin 4. *J. Invest. Dermatol.* **128**, 791–796 (2008).
18. Clevers, H. Wnt/β-Catenin Signaling in Development and Disease. *Cell* **127**, 469–480 (2006).
19. Hao, H. X. *et al.* ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner. *Nature* **485**, 195–202 (2012).
20. Koo, B. K. *et al.* Tumour suppressor RNF43 is a stem-cell E3 ligase that induces endocytosis of Wnt receptors. *Nature* **488**, 665–669 (2012).
21. Zebisch, M. *et al.* Structural and molecular basis of ZNRF3/RNF43 transmembrane ubiquitin ligase inhibition by the Wnt agonist R-spondin. *Nat. Commun.* **4**, (2013).
22. Chen, P. *et al.* The structural basis of R-spondin recognition by LGR5 and RNF43. *Genes dev.* **27**, 1345–1350 (2013).

23. Xie, Y. *et al*. Interaction with both ZNRF3 and LGR4 is required for the signalling activity of R-spondin. *EMBO Rep*. **14**, 1120–1126 (2013).
24. Peng, W. C. *et al*. Structures of Wnt-antagonist ZNRF3 and its complex with R-spondin 1 and implications for signaling. *PLoS One* **8**, 1–10 (2013).
25. Wang, D. *et al.* Structural basis for R-spondin receptors. *Genes Dev.* **27**, 1339–1344 (2013).
26. Xu, K., Xu, Y., Rajashankar, K. R., Robev, D. & Nikolov, D. B. Crystal structures of Lgr4 and its complex with R-Spondin1. *Structure* **21**, 1683–1689 (2013).
27. Lebensohn, A. M. & Rohatgi, R. R-spondins can potentiate WNT signaling without LGRs. *Elife* **7**, 1–18 (2018).
28. Ohkawara, B., Glinka, A. & Niehrs, C. Rspo3 Binds Syndecan 4 and Induces Wnt/PCP Signaling via Clathrin-Mediated Endocytosis to Promote Morphogenesis. *Dev. Cell* **20**, 303–314 (2011).
29. Park, S. *et al*. Differential activities and mechanisms of the four r-spondins in potentiating wnt/-catenin signaling. *J. Biol. Chem*. **293**, 9759–9769 (2018).
30. Szenker-Ravi, E. *et al*. RSPO2 inhibition of RNF43 and ZNRF3 governs limb development independently of LGR4/5/6. *Nature* **557**, 564–569 (2018).
31. Dubey, R. *et al.* R-spondins engage heparan sulfate proteoglycans to potentiate WNT signaling. *Elife* **3**, 1–24 (2020).
32. Carmon, K. S., Gong, X., Yi, J., Thomas, A. & Liu, Q. RSPO-LGR4 functions via IQGAP1 to potentiate Wnt signaling. *Proc. Natl. Acad. Sci. U. S. A.* **111**, (2014).
33. Glinka, A. *et al*. LGR4 and LGR5 are R-spondin receptors mediating Wnt/β-catenin and Wnt/PCP signalling. *EMBO Rep*. **12**, 1055–1061 (2011).
34. Lee, H., Seidl, C., Sun, R., Glinka, A. & Niehrs, C. R-spondins are BMP receptor antagonists in Xenopus early embryonic development. *Nat. Commun.* **11**, 5570 (2020).
35. Barker, N. *et al*. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* **449**, 1003–1007 (2007).
36. Blaas, L. *et al.* Lgr6 labels a rare population of mammary gland progenitor cells that are able to originate luminal mammary tumours. *Nat. Cell Biol.* **18**, 1346–1356 (2016).
37. Barker, N. *et al*. Lgr5+ve Stem Cells Drive Self-Renewal in the Stomach and Build Long-Lived Gastric Units In Vitro. *Cell Stem Cell* **6**, 25–36 (2010).
38. De Visser, K. E. *et al.* Developmental stage-specific contribution of LGR5+ cells to basal and luminal epithelial lineages in the postnatal mammary gland. *J. Pathol.* **228**, 300–309 (2012).
39. Carmon, K. S., Gong, X., Lin, Q., Thomas, A. & Liu, Q. R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/β-catenin signaling. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 11452–11457 (2011).
40. De Lau, W. *et al.* Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. *Nature* **476**, 293–297 (2011).
41. Mustata, R. C. *et al*. Lgr4 is required for Paneth cell differentiation and maintenance of intestinal stem cells ex vivo. *EMBO Rep*. **12**, 558–564 (2011).
42. Greicius, G. *et al.* PDGFRα+ pericryptal stromal cells are the critical source of Wnts and RSPO3 for murine intestinal stem cells in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **115**, E3173–E3181 (2018).
43. Kleeman, S. O. *et al.* Exploiting differential Wnt target gene expression to generate a molecular biomarker for colorectal cancer stratification. *Gut* **69**, 1092-1103 (2020)
44. Kang, E., Yousefi, M. & Gruenheid, S. R-spondins are expressed by the intestinal stroma and are differentially regulated during Citrobacter rodentium- and dssinduced colitis in mice. *PLoS One* **11**, 1–13 (2016).
45. Yan, K. S. *et al*. Non-equivalence of Wnt and R-spondin ligands during Lgr5 + intestinal stem-cell self-renewal. *Nature* **545**, 238–242 (2017).

46. Harnack, C. *et al.* R-spondin 3 promotes stem cell recovery and epithelial regeneration in the colon. *Nat. Commun.* **10**, 4368 (2019).
47. Santos, A. J. M., Lo, Y. H., Mah, A. T. & Kuo, C. J. The Intestinal Stem Cell Niche: Homeostasis and Adaptations. *Trends in Cell Biology* **28**, 1062–1078 (2018).
48. Castillo-Azofeifa, D. *et al.* Atoh1 + secretory progenitors possess renewal capacity independent of Lgr5 + cells during colonic regeneration. *EMBO J.* **38**, 1–16 (2019).
49. Shinmura, K. *et al.* RSPO fusion transcripts in colorectal cancer in Japanese population. *Mol. Biol. Rep.* **41**, 5375–5384 (2014).
50. Seshagiri, S. *et al.* Recurrent R-spondin fusions in colon cancer. *Nature* **488**, 660–664 (2012).
51. Hashimoto, T. *et al.* EIF3E–RSPO2 and PIEZO1–RSPO2 fusions in colorectal traditional serrated adenoma. *Histopathology* **75**, 266–273 (2019).
52. Sekine, S. *et al.* Comprehensive characterization of RSPO fusions in colorectal traditional serrated adenomas. *Histopathology* **71**, 601–609 (2017).
53. Mizuguchi, Y. *et al.* Identification of a novel PRR15L-RSPO2 fusion transcript in a sigmoid colon cancer derived from superficially serrated adenoma. *Virchows Arch.* **475**, 659–663 (2019).
54. Sekine, S. *et al.* Frequent PTPRK-RSPO3 fusions and RNF43 mutations in colorectal traditional serrated adenoma. *J. Pathol.* **239**, 133–138 (2016).
55. Kleeman, S. O. & Leedham, S. J. Not all wnt activation is equal: Ligand-dependent versus ligand-independent wnt activation in colorectal cancer. *Cancers (Basel).* **12**, 1–16 (2020).
56. Hilkens, J. *et al.* RSPO3 expands intestinal stem cell and niche compartments and drives tumorigenesis. *Gut* **66**, 1095–1105 (2017).
57. Han, T. *et al.* R-Spondin chromosome rearrangements drive Wnt-dependent tumour initiation and maintenance in the intestine. *Nat. Commun.* **8**, 1–12 (2017).
58. Storm, E. E. *et al.* Targeting PTPRK-RSPO3 colon tumours promotes differentiation and loss of stem-cell function. *Nature* **529**, 97–100 (2016).
59. Wu, C. *et al.* RSPO2-LGR5 signaling has tumour-suppressive activity in colorectal cancer. *Nat. Commun.* **5**, 149 (2014).
60. Dong, X. *et al.* RSPO2 suppresses colorectal cancer metastasis by counteracting the Wnt5a/Fzd7-driven noncanonical Wnt pathway. *Cancer Lett.* **402**, 153–165 (2017).
61. Clements, W. M. *et al.* β-catenin mutation is a frequent cause of Wnt pathway activation in gastric cancer. *Cancer Res.* **62**, 3503–3506 (2002).
62. Koushyar, S., Powell, A. G., Vincan, E. & Phesse, T. J. Targeting wnt signaling for the treatment of gastric cancer. *Int. J. Mol. Sci.* **21**, 3927 (2020).
63. Fischer, A. S. & Sigal, M. The role of Wnt and R-spondin in the stomach during health and disease. *Biomedicines* **7**, 1–15 (2019).
64. Sigal, M. *et al.* Stromal R-spondin orchestrates gastric epithelial stem cells and gland homeostasis. *Nature* **548**, 451–455 (2017).
65. Sigal, M. *et al.* R-spondin-3 induces secretory, antimicrobial Lgr5+ cells in the stomach. *Nat. Cell Biol.* **21**, 812–823 (2019).
66. Tomasetti, C. & Vogelstein, B. Variation in cancer risk among tissues can be explained by the number of stem cell divisions. *Science* **347**, 78–81 (2015).
67. Li, C. *et al.* Identification of RSPO2 Fusion Mutations and Target Therapy Using a Porcupine Inhibitor. *Sci. Rep.* **8**, 1–9 (2018).
68. van Schie, E. H. & van Amerongen, R. Aberrant WNT/CTNNB1 Signaling as a Therapeutic Target in Human Breast Cancer: Weighing the Evidence. *Front. Cell Dev. Biol.* **8**, 1–14 (2020).
69. Alexander, C. M. The Wnt Signaling Landscape of Mammary Stem Cells and Breast Tumors. *Prog. Mol. Biol. Transl. Sci.* **153**, 271-298 (2018).

70. Cai, C. *et al.* R-spondin1 is a novel hormone mediator for mammary stem cell self-renewal. *Genes Dev.* **28**, 2205–2218 (2014).
71. Van Amerongen, R., Bowman, A. N. & Nusse, R. Developmental stage and time dictate the fate of Wnt/β-catenin-responsive stem cells in the mammary gland. *Cell Stem Cell* **11**, 387–400 (2012).
72. Zeng, Y. A. & Nusse, R. Wnt proteins are self-renewal factors for mammary stem cells and promote their long-term expansion in culture. *Cell Stem Cell* **6**, 568–577 (2010).
73. Joshi, P. A. *et al.* RANK Signaling Amplifies WNT-Responsive Mammary Progenitors through R-SPONDIN1. *Stem Cell Reports* **5**, 31–44 (2015).
74. Badders, N. M. *et al.* The Wnt receptor, Lrp5, is expressed by mouse mammary stem cells and is required to maintain the basal lineage. *PLoS One* **4**, e6594 (2009).
75. Visvader, J. E. & Stingl, J. Mammary stem cells and the differentiation hierarchy: Current status and perspectives. *Genes Dev.* **28**, 1143–1158 (2014).
76. Tharmapalan, P., Mahendralingam, M., Berman, H. K. & Khokha, R. Mammary stem cells and progenitors: targeting the roots of breast cancer for prevention. *EMBO J.* **38**, 1–19 (2019).
77. Plaks, V. *et al.* Lgr5-Expressing Cells Are Sufficient And Necessary for Postnatal Mammary Gland Organogenesis. *Cell Rep.* **3**, 70–78 (2013).
78. Rios, A. C., Fu, N. Y., Lindeman, G. J. & Visvader, J. E. In situ identification of bipotent stem cells in the mammary gland. *Nature* **506**, 322–327 (2014).
79. Wang, D. *et al.* Identification of multipotent mammary stemcells by protein C receptor expression. *Nature* **517**, 81–84 (2015).
80. Davis, F. M. *et al.* Single-cell lineage tracing in the mammary gland reveals stochastic clonal dispersion of stem/progenitor cell progeny. *Nat. Commun.* **7**, 13053(2016).
81. Wuidart, A. *et al.* Quantitative lineage tracing strategies to resolve multipotency in tissue-specific stem cells. *Genes Dev.* **30**, 1261–1277 (2016).
82. Van Keymeulen, A. *et al.* Distinct stem cells contribute to mammary gland development and maintenance. *Nature* **479**, 189–193 (2011).
83. Scheele, C. L. G. J. *et al.* Identity and dynamics of mammary stem cells during branching morphogenesis. *Nature* **542**, 313–317 (2017).
84. Stingl, J. *et al.* Purification and unique properties of mammary epithelial stem cells. *Nature* **439**, 993–997 (2006).
85. Shackleton, M. *et al.* Generation of a functional mammary gland from a single stem cell. *Nature* **439**, 84–88 (2006).
86. Lim, E. *et al.* Transcriptome analyses of mouse and human mammary cell subpopulations reveal multiple conserved genes and pathways. *Breast Cancer Res.* **12**, R21 (2010).
87. Colacino, J. A. *et al.* Heterogeneity of Human Breast Stem and Progenitor Cells as Revealed by Transcriptional Profiling. *Stem Cell Reports* **10**, 1596–1609 (2018).
88. Ginestier, C. *et al.* ALDH1 Is a Marker of Normal and Malignant Human Mammary Stem Cells and a Predictor of Poor Clinical Outcome. *Cell Stem Cell* **1**, 555–567 (2007).
89. Chadi, S. *et al.* R-spondin1 is required for normal epithelial morphogenesis during mammary gland development. *Biochem. Biophys. Res. Commun.* **390**, 1040–1043 (2009).
90. Chadi, S. *et al.* Phenotypic and molecular alterations in the mammary tissue of R-spondin1 knock-out mice during pregnancy. *PLoS One* **11**, 1–15 (2016).
91. Khramtsov, A. I. *et al.* Wnt/β-catenin pathway activation is enriched in basal-like breast cancers and predicts poor outcome. *Am. J. Pathol.* **176**, 2911–2920 (2010).
92. Lin, S. Y. *et al.* β-catenin, a novel prognostic marker for breast cancer: Its roles in cyclin D1 expression and cancer progression. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 4262–4266 (2000).

93. Geyer, F. C. *et al.* B-Catenin pathway activation in breast cancer is associated with triple-negative phenotype but not with CTNNB1 mutation. *Mod. Pathol.* **24**, 209–231 (2011).

94. He, Y. *et al.* Expression and significance of Wnt signaling components and their target genes in breast carcinoma. *Mol. Med. Rep.* **9**, 137–143 (2014).

95. Jonsson, M., Borg, Å., Nilbert, M. & Andersson, T. Involvement of adenomatous polyposis coli (APC)/β-catenin signalling in human breast cancer. *Eur. J. Cancer* **36**, 242–248 (2000).

96. López-Knowles, E. *et al.* Cytoplasmic localization of â-catenin is a marker of poor outcome in breast cancer patients. *Cancer Epidemiol. Biomarkers Prev.* **19**, 301–309 (2010).

97. Prasad, C. P., Gupta, S. D., Rath, G. & Ralhan, R. Wnt signaling pathway in invasive ductal carcinoma of the breast: Relationship between β-catenin, disheveled and cyclin D1 expression. *Oncology* **73**, 112–117 (2008).

98. Ozaki, S. *et al.* Alterations and correlations of the components in the Wnt signaling pathway and its target genes in breast cancer. *Oncol. Rep.* **14**, 1437–43 (2005).

99. Karayiannakis, A. J. *et al.* Expression patterns of β-catenin in in situ and invasive breast cancer. *Eur. J. Surg. Oncol.* **27**, 31–36 (2001).

100. Li, S., Li, S., Sun, Y. & Li, L. The expression of β-catenin in different subtypes of breast cancer and its clinical significance. *Tumor Biol.* **35**, 7693–7698 (2014).

101. Gaspar, C. & Fodde, R. APC dosage effects in tumorigenesis and stem cell differentiation. *Int. J. Dev. Biol.* **48**, 377–386 (2004).

102. Bakker, E. R. M. *et al.* β-Catenin signaling dosage dictates tissue-specific tumor predisposition in Apc-driven cancer. *Oncogene* **32**, 4579–4585 (2013).

103. Gaspar, C. *et al.* A targeted constitutive mutation in the Apc tumor suppressor gene underlies mammary but not intestinal tumorigenesis. *PLoS Genet.* **5**, e1000547 (2009).

104. Suzuki, H. *et al.* Frequent epigenetic inactivation of Wnt antagonist genes in breast cancer. *Br. J. Cancer* **98**, 1147–1156 (2008).

105. Veeck, J. *et al.* Prognostic relevance of Wnt-inhibitory factor-1 (WIF1) and Dickkopf-3 (DKK3) promoter methylation in human breast cancer. *BMC Cancer* **9**, 1–13 (2009).

106. Veeck, J. *et al.* Epigenetic inactivation of the secreted frizzled-related protein-5 (SFRP5) gene in human breast cancer is associated with unfavorable prognosis. *Carcinogenesis* **29**, 991–998 (2008).

107. Ai, L. *et al.* Inactivation of Wnt inhibitory factor-1 (WIF1) expression by epigenetic silencing is a common event in breast cancer. *Carcinogenesis* **27**, 1341–1348 (2006).

108. Callahan, R. *et al.* Genes affected by mouse mammary tumor virus (MMTV) proviral insertions in mouse mammary tumors are deregulated or mutated in primary human mammary tumors. *Oncotarget* **3**, 1320–1334 (2012).

109. Klijn, C. *et al.* Analysis of Tumor Heterogeneity and Cancer Gene Networks Using Deep Sequencing of MMTV-Induced Mouse Mammary Tumors. *PLoS One* **8**, 1–10 (2013).

110. Theodorou, V. *et al.* MMTV insertional mutagenesis identifies genes, gene families and pathways involved in mammary cancer. *Nat. Genet.* **39**, 759–769 (2007).

111. Gattelli, A., Zimberlin, M. N., Meiss, R. P., Castilla, L. H. & Kordon, E. C. Selection of Early-Occurring Mutations Dictates Hormone-Independent Progression in Mouse Mammary Tumor Lines. *J. Virol.* **80**, 11409–11415 (2006).

112. Klauzinska, M. *et al.* Rspo2/Int7 Regulates Invasiveness and Tumorigenic Properties of Mammary Epithelial Cells. *J. Cell Physiol.* **227**, 1960–71 (2012).

113. Coussy, F. *et al.* Clinical value of R-spondins in triple-negative and metaplastic breast cancers. *Br. J. Cancer* **116**, 1595–1603 (2017).

114. Tocci, J. M., Felcher, C. M., García Solá, M. E. & Kordon, E. C. R-spondin-mediated WNT signaling potentiation in mammary and breast cancer development. *IUBMB Life* **72**, 1546–1559 (2020).

115. Tocci, J. M. *et al*. R-Spondin3 is associated with basal-progenitor behavior in normal and tumor mammary cells. *Cancer Res.* **78**, 4497–4511 (2018).

116. Chartier, C. *et al*. Therapeutic targeting of tumor-derived r-spondin attenuates b-catenin signaling and tumorigenesis in multiple cancer types. *Cancer Res.* **76**, 713–723 (2016).

117. Chassot, A. *et al*. Activation of b -catenin signaling by Rspo1 controls differentiation of the mammalian ovary. *Hum. Mol. Genet.* **17**, 1264–1277 (2008).

118. Maatouk, D. M., Mork, L., Chassot, A., Chaboissier, M. & Capel, B. Disruption of mitotic arrest precedes precocious differentiation and transdifferentiation of pregranulosa cells in the perinatal Wnt4 mutant ovary. *Dev. Biol.* **383**, 295–306 (2013).

119. Chassot, A. A. *et al*. RSPO1/β-Catenin signaling pathway regulates oogonia differentiation and entry into meiosis in the mouse fetal ovary. *PLoS One* **6**, e25641 (2011).

120. Chassot, A. A. *et al*. WNT4 and RSPO1 together are required for cell proliferation in the early mouse gonad. *Dev.* **139**, 4461–4472 (2012).

121. Teeuwssen & Fodde. Wnt Signaling in Ovarian Cancer Stemness, EMT, and Therapy Resistance. *J. Clin. Med.* **8**, 1658 (2019).

122. Kuchenbaecker, K. B. *et al*. Identification of six new susceptibility loci for invasive epithelial ovarian cancer. *Nat. Genet.* **47**, 164–171 (2015).

123. Schindler, A. J., Watanabe, A. & Howell, S. B. LGR5 and LGR6 in stem cell biology and ovarian cancer. *Oncotarget* **9**, 1346–1355 (2018).

124. De Cian, M. C. *et al.* Amplification of R-spondin1 signaling induces granulosa cell fate defects and cancers in mouse adult ovary. *Oncogene* **36**, 208–218 (2017).

125. Francis, J. C., Thomsen, M. K., Taketo, M. M. & Swain, A. β-Catenin Is Required for Prostate Development and Cooperates with Pten Loss to Drive Invasive Carcinoma. *PLoS Genet.* **9**, e1003180 (2013).

126. Simons, B. W. *et al*. Wnt signaling though beta-catenin is required for prostate lineage specification. *Dev. Biol.* **371**, 246–255 (2012).

127. Luo, W. *et al*. Lgr4 is a key regulator of prostate development and prostate stem cell differentiation. *Stem Cells* **31**, 2492–2505 (2013).

128. Mehta, V. *et al*. Atlas of Wnt and R-spondin gene expression in the developing male mouse lower urogenital tract. *Dev. Dyn.* **240**, 2548–2560 (2011).

129. Karthaus, W. R. *et al*. Identification of multipotent luminal progenitor cells in human prostate organoid cultures. *Cell* **159**, 163–175 (2014).

130. Planche, A. *et al*. Identification of prognostic molecular features in the reactive stroma of human breast and prostate cancer. *PLoS One* **6**, e18640 (2011).

131. Robinson, D. *et al*. Integrative clinical genomics of advanced prostate cancer. *Cell* **161**, 1215–1228 (2015).

132. Mesci, A. *et al*. RSPO3 is a prognostic biomarker and mediator of invasiveness in prostate cancer. *J. Transl. Med.* **17**, 1–11 (2019).

133. Murillo-Garzón, V. & Kypta, R. WNT signalling in prostate cancer. *Nat. Rev. Urol.* **14**, 683–696 (2017).

134. Planas-Paz, L. *et al*. The RSPO-LGR4/5-ZNRF3/RNF43 module controls liver zonation and size. *Nat. Cell Biol.* **18**, 467–479 (2016).

135. Rocha, A. S. *et al*. The Angiocrine Factor Rspondin3 Is a Key Determinant of Liver Zonation. *Cell Rep.* **13**, 1757–1764 (2015).

136. Kan, Z. *et al*. Whole-genome sequencing identifies recurrent mutations in

hepatocellular carcinoma. *Genome Res.* **23**, 1422–1433 (2013).

137. Longerich, T. *et al.* RSPO2 gene rearrangement: A powerful driver of β-catenin activation in liver tumours. *Gut* **68**, 1287–1296 (2019).
138. Conboy, C. B. *et al.* R-spondin 2 Drives Liver Tumor Development in a Yes-Associated Protein-Dependent Manner. *Hepatol. Commun.* **3**, 1496–1509 (2019).
139. Ahn, S. M. *et al.* Genomic portrait of resectable hepatocellular carcinomas: Implications of RB1 and FGF19 aberrations for patient stratification. *Hepatology* **60**, 1972–1982 (2014).
140. Gong, X. *et al.* Aberrant RSPO3-LGR4 signaling in Keap1-deficient lung adenocarcinomas promotes tumor aggressiveness. *Oncogene* **34**, 4692–4701 (2015).
141. Karkera, J. *et al.* Identification of R-spondin fusions in NSCLC. [abstract]. *Proc. AACR-IASLC Jt. Conf. Mol. Origigins Lung cancer*.
142. Ilmer, M. *et al.* RSPO2 enhances canonical wnt signaling to confer stemness-associated traits to susceptible pancreatic cancer cells. *Cancer Res.* **75**, 1883–1896 (2015).
143. Chen, Z. *et al.* RSPO3 promotes the aggressiveness of bladder cancer via Wnt/β-catenin and Hedgehog signaling pathways. *Carcinogenesis* **40**, 360–369 (2019).
144. Blagodatski, A., Poteryaev, D. & Katanaev, V. L. Targeting the Wnt pathways for therapies. *Mol. Cell. Ther.* **2**, 28 (2014).
145. Morgan, R. G., Mortensson, E. & Williams, A. C. Targeting LGR5 in Colorectal Cancer: Therapeutic gold or too plastic? *Br. J. Cancer* **118**, 1410–1418 (2018).
146. Madan, B. *et al.* Wnt addiction of genetically defined cancers reversed by PORCN inhibition. *Oncogene* **35**, 2197–2207 (2016).
147. Picco, G. *et al.* Loss of AXIN1 drives acquired resistance to WNT pathway blockade in colorectal cancer cells carrying RSPO 3 fusions . *EMBO Mol. Med.* **9**, 293–303 (2017).
148. Study of WNT974 in Combination With LGX818 and Cetuximab in Patients With BRAF-mutant Metastatic Colorectal Cancer (mCRC) and Wnt Pathway Mutations. Available from: https://clinicaltrials.gov/ct2/show/NCT02278133
149. A Study to Evaluate the Safety and Tolerability of ETC-1922159 as a Single Agent and in Combination With Pembrolizumab in Advanced Solid Tumours. Available from: https://clinicaltrials.gov/ct2/show/NCT02521844
150. A study of LGK974 in Patients With Malignancies Dependent on Wnt Ligands. Available from: https://clinicaltrials.gov/ct2/show/NCT01351103
151. A study to Assess Efficacy of RXC004 +/- Nivolumab in Ring Finger Protein 43 (RNF43) or R-spondin (RSPO) Aberrated, Metastatic, Microsatellite Stable, Colorectal Cancer After Progression on Standard of Care (SOC). Available from: https://clinicaltrials.gov/ct2/show/NCT04907539
152. Fischer, M. M. *et al.* RSPO3 antagonism inhibits growth and tumorigenicity in colorectal tumors harboring common Wnt pathway mutations. *Sci. Rep.* **7**, 1–9 (2017).
153. Salik, B. *et al.* Targeting RSPO3-LGR4 Signaling for Leukemia Stem Cell Eradication in Acute Myeloid Leukemia. *Cancer Cell* **38**, 263-278 (2020).
154. A Phase 1a/b Dose Escalation Study of the Safety, Pharmacokinetics, and Pharmacodynamics of OMP-131R10. Available from: https://clinicaltrials.gov/ct2/show/NCT02482441
155. Bendell, J. *et al.* Initial results from a phase 1a / b study of OMP-131R10 , a first-in-class anti-RSPO3 antibody , in advanced solid tumors and previously treated metastatic colorectal cancer (CRC). *Eur. J. Cancer* **69**, S29–S30 (2016).

3

R-SPONDIN-3 IS AN ONCOGENIC DRIVER OF POORLY DIFFERENTIATED INVASIVE BREAST CANCER

Eline J. ter Steege[2], Mandy Boer[1], Nikki C. Timmer[1], Carola M.E. Ammerlaan[1], Ji-Ying Song[3], Patrick W.B. Derksen[2], John Hilkens[1*] and Elvira R.M. Bakker[1,2*]

[1] Department of Molecular Genetics, The Netherlands Cancer Institute, Amsterdam, The Netherlands
[2] Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands
[3] Department of Experimental Animal Pathology, The Netherlands Cancer Institute, Amsterdam, The Netherlands
* these authors contributed equally

Published in Journal of Pathology (2022)
doi: 10.1002/path.5999

Abstract

R-spondins (RSPOs) are influential signaling molecules that promote the Wnt/β-catenin pathway and self-renewal of stem cells. Currently, RSPOs are emerging as clinically relevant oncogenes, being linked to cancer development in multiple organs. Although this has instigated the rapid development and testing of therapeutic antibodies targeting RSPOs, functional evidence that RSPO causally drives cancer has focused primarily on the intestinal tract. Here, we assess the oncogenic capacity of RSPO in breast cancer, in a direct fashion by generating and characterizing a novel mouse model with conditional *Rspo3* expression in the mammary gland. Also, we address the prevalence of *RSPO* gene alterations in breast cancer patients. We found that a quarter of breast cancer patients harbor *RSPO2/RSPO3* copy number amplifications, associating with lack of steroid hormone receptor expression and reduced patient survival. Foremost, we demonstrate the causal oncogenic capacity of RSPO3 in the breast, as conditional *Rspo3* overexpression drives the development of mammary adenocarcinomas consistently in our novel *Rspo3* breast cancer model. RSPO3-driven mammary tumors typically show poor differentiation, areas of EMT and metastatic potential. Given reported interplay in the Wnt/β-catenin pathway, we comparatively analyzed *Rspo3*-driven mouse mammary tumors versus classical *Wnt1*-driven analogues. This revealed that *Rspo3*-driven tumors are distinct, as the poorly differentiated tumor morphology and metastatic potential were observed in RSPO3-driven tumorigenesis exclusively, being further substantiated by clearly differentiating gene expression profiles. Co-expression of *Rspo3* and *Wnt1* induced mammary tumors with a mixed phenotype, harboring morphological features characteristic of both transgenes. Conclusively, we report that a quarter of breast cancer patients harboring *RSPO2/RSPO3* copy number gains have worse prognosis, whilst providing *in vivo* evidence that RSPO3 causally drives poorly differentiated invasive breast cancer in mice. Herewith, we establish RSPO3 as a driver of breast cancer with clinical relevance, proposing RSPO3 as a novel candidate target for therapy in breast cancer.

Introduction

R-spondin proteins (RSPO1-4) are secreted ligands that have emerged as multipotent signaling molecules. Among their activities, potentiation of the Wnt/β-catenin pathway in cooperation with Wnt ligands has been established best. As RSPO ligands act through the LGR4, LGR5 and LGR6 transmembrane receptors typically expressed on stem- and progenitor cells, they play an influential role by potentiating Wnt/β-catenin signaling and proliferation in various stem cell compartments[1,2]. Despite the fact that RSPOs cooperate with Wnt ligands to drive canonical Wnt/β-catenin signaling, RSPO and Wnt ligands also exert distinct, non-interchangeable roles in the intestinal stem cell niche[3]. Here, RSPOs actively fuel self-renewal and expansion of stem cells, dictating the size of the stem cell pool, in contrast to Wnt ligands that are unable to induce stem cell self-renewal[3]. In line with the instrumental role of RSPOs in stem cell regulation, aberrant RSPO activation has been increasingly implicated in carcinogenesis over the last decade[4]. The oncogenic role of RSPOs have been especially recognized for the intestinal tract, as mouse studies have provided functional evidence that aberrant *Rspo* expression causally drives intestinal tumorigenesis, associated with aberrant expansion of the proliferative stem cell compartment[5,6]. Moreover, a gain in *RSPO2* or *RSPO3* levels is evident in a subpopulation of colorectal cancer patients, caused either by stromal overexpression or specific gene fusions, among which *EIF3E-RSPO2* and *PTPRK-RSPO3* occur mutually exclusive with classical *APC* and *CTNNB1* driver mutations[7-13]. These findings put forward RSPO2 and RSPO3 as novel, clinically relevant cancer drivers in the intestinal tract, which has accordingly been recognized by a clinical trial targeting RSPO3 in colorectal cancer[14].

As RSPOs have been implicated in many cancer types, the potential clinical utility extends beyond the intestinal tract, therefore urging further investigation. Among these other types is breast cancer, which represents a uniquely different cancer type, where the expression of steroid hormone receptors ER and PR rather play a crucial role in stratifying therapeutic treatment options, reflecting the instrumental role of hormonal regulation in the mammary epithelium. Hence, in the normal mammary gland, upstream steroid hormone signals are instructive in regulating mammary stem cell dynamics, and also Wnt/β-catenin signaling plays an important stem cell regulatory role herein[4,15-17]. Importantly, mouse studies have indicated that the dictating role of upstream steroid hormone signals is executed through a collaborative Rspo1-Wnt4 signaling axis that potentiates Wnt/β-catenin signaling and stimulates the self-renewal of mammary stem cells in the normal mammary gland[18,19]. Wnt/β-catenin signaling also contributes to mouse mammary cancer, initially found through the identification of *Wnt1* as a mammary oncogene[20]. Transgenic mouse studies have shown that hyperactivation of the Wnt/β-catenin pathway, e.g. in MMTV-*Wnt1* and MMTV-ΔN89-β-catenin mice, causes the development of mammary tumors[21,22]. In breast cancer patients especially of triple negative subtype, overactivation of the Wnt/β-catenin pathway has been frequently reported, however the responsible underlying mechanisms remain obscure as *APC* and *CTNNB1* mutations are rarely found[4,23-25]. Wnt pathway activation may be rather

achieved by alterations in alternative pathway members, as reported for Wnt antagonists[26-29]. Alterations in RSPOs might potentially present another explanation. Overexpression of *RSPO2*, *RSPO3* and *RSPO4* have been reported in breast cancer patients, in particular in triple-negative cases, where enhanced *RSPO2* expression associated significantly with reduced metastasis-free survival[30,31]. Underlying *RSPO* fusions were not detected in 446 breast tumors screened by Coussy et al[30]. However, the triple-negative breast cancer cell line BT549 to harbors the *EIF3E-RSPO2* gene fusion. From earlier MMTV insertional mutagenesis screens in mice, *Rspo1, Rspo2* and *Rspo3* had already been proposed as potential mammary oncogenes[32-35]. Despite that these data suggest a pro-tumorigenic role for RSPOs in breast cancer, functional *in vivo* evidence for their causal oncogenic capacity has remained limited. In this regard, we exploited a yet available and validated transgenic *Rspo3* mouse model to investigate RSPO3 as a paradigm. In current study, we demonstrate that a gain in RSPO3 causes the development of poorly differentiated invasive mammary tumors in mice, providing functional evidence for the causal oncogenic capacity of RSPO3 in driving breast cancer. Also, we show that mammary tumors driven by RSPO3 are morphologically and molecularly distinct from WNT1 driven tumors, presenting with higher metastatic potential. These findings suggest that RSPO3 potentially represents a novel candidate therapy target for breast cancer patients with a gain in RSPO3.

Materials and methods

In silico copy number analysis

Copy number analysis was performed using the METABRIC breast cancer patient dataset and Cbioportal.org.

Mouse strains and tumor study

We generated the *Rspo3*^{inv} mouse model on 129/Ola background previously (official 129P2-Gt(Rosa)26Sor$^{tm6(CAG-Rspo3)Nki}$/A (MGI:5697338, abbreviated to *Rspo3*^{inv}), of which a detailed description is provided in Ref. [5]. In the *Rspo3*^{inv} mouse line, the *Rspo3* coding sequence is present in antisense orientation between two sets of non-homologous *Lox* sites in a head to head orientation (Figure 2A and S1A). In current study, *Rspo3*^{inv} mice (129/Ola) were crossbred with MMTV-*Cre*;MMTV-*Wnt1* mice[22,36] (FVB), generating required cohorts on F1 hybrid background (maintaining all alleles heterozygous). Utilized cohorts comprised: single transgenic *Rspo3*^{inv} control females (no transgenic expression), double transgenic MMTV-*Cre*;*Rspo3*^{inv} (transgenic *Rspo3* expression) and MMTV-*Wnt1*;*Rspo3*^{inv} (transgenic *Wnt1* expression) females, and triple transgenic MMTV-*Cre*;*Rspo3*^{inv} ;MMTV-*Wnt1*females (transgenic *Rspo3/Wnt1* co-expression). Mice of all genotypes were forced bred and monitored for tumor development, up to a maximum age of 600 days. All animal experiments were performed with approval of the Animals Ethics Committee and according to Dutch legislation.

Histology and immunohistochemistry

Harvested tissues were fixed in formalin or EAF and paraffin-embedded, followed by haemotoxylin eosin (H&E) staining according to routine protocols. For the postmortem analysis of lung metastases, paraffin-embedded lungs were sectioned and H&E stained at five different levels throughout the lungs. Immunohistochemistry was performed using rabbit-anti-Cytokeratin-5 (Covance PRB-160P), rat-anti-Cytokeratin-8 (DSHB Troma-I), rabbit-anti-ERα (Santa Cruz sc-542) and rabbit-anti PR (Thermo Scientific RM-9102).

RNA isolation, cDNA synthesis and expression analysis of whole tissue

For RT-PCR analysis, RNA was isolated from mammary tissues using TissueLyser LT (Qiagen) and RNeasy Plus Mini Kit (Qiagen), and cDNA was generated using the Maxima First Strand cDNA Synthesis Kit (Fisher Scientific). RT-PCR was performed with MyTaq Red DNA Polymerase using the following primer sequences: Sense *Rspo3* F 5′ TGGGCAACGTGCTGGTTATT 3′, Sense *Rspo3* R 5′ CCTATCTGCTTCATGCCAATCC 3′, *Actb* F 5′ TGAGACCTTCAACACCCCAG 3′, *Actb* R 5′ GAGCCAGAGCAGTAATCTCC 3′. RNA sequencing of mouse mammary tumor tissues was performed using Illumina Hiseq2000 platforms (Illumina) as previously described[5]. Limma's Voom was used for normalization and normalized expression values were statistically analyzed using the Benjamini–Hochberg method in R. Gene ontology analysis was performed with Qiagen Ingenuity Pathway Analysis.

Results

RSPO2 and *RSPO3* copy number amplifications associate with poor breast cancer prognosis

We analyzed all four *RSPO* genes for the occurrence of copy number alterations in the METABRIC breast cancer dataset. Among the *RSPO* members, copy number amplifications of the *RSPO2* gene occurred most frequently, presenting in a profound 23% (503/2 173) of breast cancer patients. In addition, 1% harbored copy number amplifications of the *RSPO1* gene (26/2 173), 2% of the *RSPO3* (47/2 173) and 2% of the *RSPO4* gene (48/2 173). Importantly, breast cancer patients harboring *RSPO2* and *RSPO3* copy number amplifications both showed significantly reduced overall survival (Figure 1A). In line with these results, the presence of *RSPO2* and *RSPO3* copy number amplifications associated with higher histological tumor grade (Figure 1B) and lack of expression of steroid hormone receptors ER (Figure 1C) and PR (Figure 1D). Together these data indicate that a substantial quarter of breast cancer patients harbors *RSPO2* or *RSPO3* amplifications, presenting with reduced clinical outcome.

Conditional *Rspo3* expression drives mammary tumorigenesis

As patient data suggested a protumorigenic role for RSPO2 and RSPO3 in breast cancer, we aimed to determine the oncogenic potential of RSPO in breast cancer. For this purpose, we used the conditional *Rspo3*^{inv} mouse model which we generated and validated

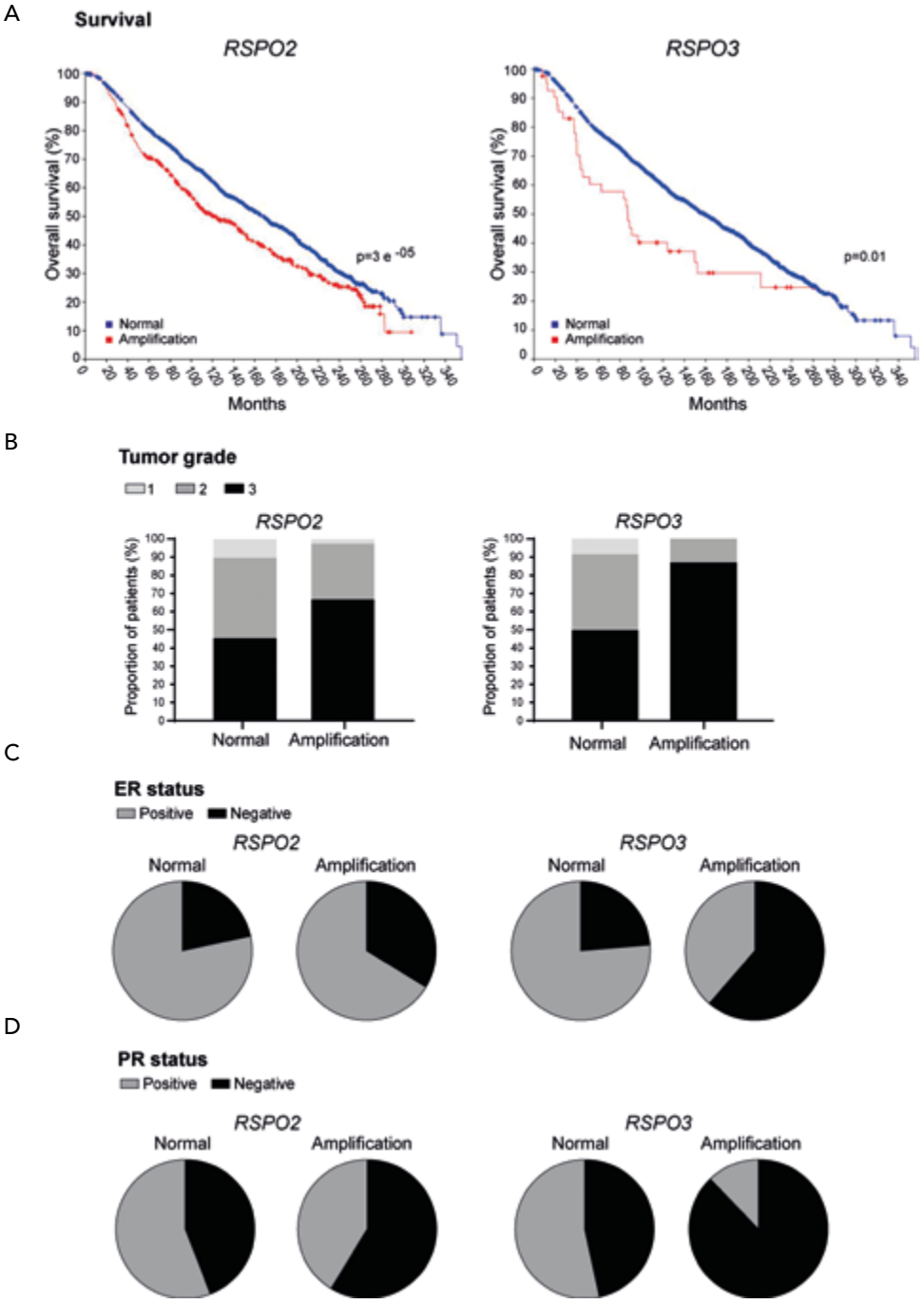


Figure 1. Analysis of *RSPO2* (left) and *RSPO3* (right) copy number amplifications in breast cancer patients. Copy number amplifications of both *RSPO2* and *RSPO3* associate with A. reduced overall survival significantly (LogRank test), B. enhanced histological tumor grade and lack of C. ER and D. PR expression.

previously[5]. Shortly, in this transgenic mouse model, the *Rspo3* coding sequence is placed in the inverse orientation between 2 sets of *Lox* sites, preventing transgene expression in this antisense configuration (Figure 2A and S1A). By providing directed Cre recombinase activity, the *Rspo3* transgene is inverted into sense orientation, leading to overexpression. To investigate the consequences of *Rspo3* overexpression in the mammary gland, this *Rspo3*^{inv} mouse model was combined with MMTV-*Cre* mice[36], providing abundant Cre expression throughout the mammary epithelium. Efficient conditional expression of transgenic *Rspo3* was confirmed in mammary gland tissues of double transgenic MMTV-*Cre*;*Rspo3*^{inv} mice, whereas single transgenic *Rspo3*^{inv} control mice did not express the *Rspo3* transgene (Figure S1B), demonstrating its correct regulation.

To assess the oncogenic capacity of RSPO3 in the mammary gland, we generated a cohort of MMTV-*Cre*;*Rspo3*^{inv} double transgenic females (n=43) and a corresponding control cohort of single transgenic *Rspo3*^{inv} females (n=42). Importantly, we observed that MMTV-*Cre*;*Rspo3*^{inv} female mice developed mammary tumors consistently, providing *in vivo* evidence for the causal oncogenic capacity of RSPO3 in the mammary gland. Accordingly, the tumor-free survival of MMTV-*Cre*;*Rspo3*^{inv} females was significantly reduced to a median of 343 days, compared to 600 days in the control cohort that lacks transgenic expression (Figure 2B). The mammary tumors that developed in MMTV-

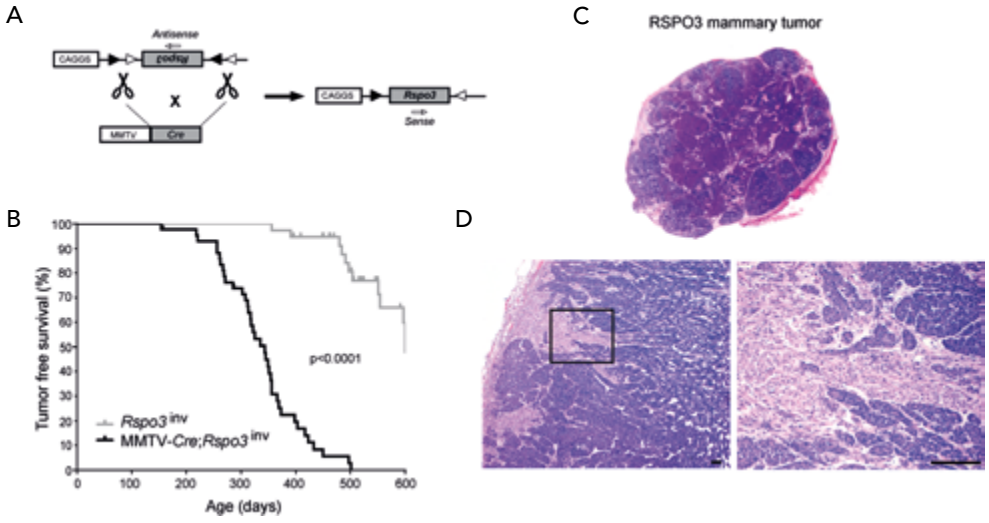


Figure 2. Conditional *Rspo3* mouse model of breast cancer. A. Schematic representation of the *Rspo3*^{inv} mouse model in which the *Rspo3* transgene is present in the antisense orientation between 2 pairs of *Lox* sites. Crossbreeding with MMTV-*Cre* allows Cre-mediated inversion of the *Rspo3* transgene into sense orientation in the mammary gland. Adapted from Ref. [5] with permission of BMJ Publishing Group Ltd. B. Survival curves showing significantly reduced tumor-free survival of double transgenic MMTV-*Cre*;*Rspo3*^{inv} mice (n=43) versus single transgenic *Rspo3*^{inv} control mice (n=42) (LogRank test). C. Scan of total and D. microscopic pictures (4x and 20x objective) of H&E stained mammary tumors of MMTV-*Cre*;*Rspo3*^{inv} female mice.

Cre;*Rspo3*^{inv} mice (i.e. RSPO3-driven tumors) macroscopically appeared as solid, compact structures, as confirmed microscopically by H&E staining (Figure 2C,D). RSPO3-driven mammary tumors typically presented as adenocarcinomas with mixed solid acinar and ductal arrangements, focal regions of squamous metaplasia and areas with epithelial-to-mesenchymal transition (EMT) (Figure 2D).

RSPO3-driven murine breast tumors are poorly differentiated and invasive

To further reveal the features of RSPO3-driven mammary tumors we performed immunohistochemical analyses. First, RSPO3-driven mammary tumors were largely negative for the steroid hormone receptors ERα and PR (Figure S2). We next analyzed expression of the established mammary epithelial markers cytokeratin-8 (K8) and cytokeratin-5 (K5), indicating the luminal and basal compartments respectively. In RSPO3-driven mammary tumors, K8 expression was observed throughout solid epithelial tumor structures but in a weak and patchy staining pattern (Figure 3A upper panel). Also, individual K8 positive spindle shaped cells were observed in EMT regions. K5 expression was found most abundantly in EMT areas, and to lesser extent in solid tumor structures (Figure 3A lower panel). Thus, the solid epithelial tumor structures harbored weak K8 expression whereas EMT regions showed K5 expression predominantly. Although RSPO3-driven mammary tumors contain both basal and luminal keratins, the relatively weak and disorganized expression patterns indicated poor differentiation.

To put this poorly differentiated RSPO3 tumor phenotype into further perspective, we comparatively analyzed WNT1-driven mammary tumors that developed in the co-bred MMTV-*Wnt1*;*Rspo3*^{inv} cohort (only *Wnt1* transgene expression given the lack of Cre). WNT1-driven mouse mammary tumors showed consistent and strong expression for both K8 and K5 in a bi-layered fashion, clearly segregating luminal and basal cell layers and indicating a distinctive degree of differentiation (Figure 3B). This further emphasized the relative poor differentiation of mammary tumors driven by RSPO3, together with the typical presence of EMT areas suggesting increased dissemination potential. Therefore we examined the lungs of mice bearing WNT1- or RSPO3-driven mammary tumors to determine distant metastasis potential. In line with histological features, lung metastases were found in 6 out of 21 (29%) mice bearing RSPO3-driven mammary tumors, mostly presenting in multitude, ranging up to 25 metastatic lesions per mouse (Figure 3C,D). In clear contrast, no lung metastases were found in mice with WNT1-driven mammary tumors (Figure 3C). Together these findings demonstrate that RSPO3-driven mammary tumors are poorly differentiated and metastatic.

RSPO3-driven mammary tumors are molecularly distinct from WNT1-driven tumors

The phenotypical difference between WNT1- versus RSPO3-driven tumors might seem striking, since Wnt1 is a classical canonical Wnt ligand driving Wnt/β-catenin signaling and R-spondins are well-known to potentiate this same Wnt/β-catenin route. To look further

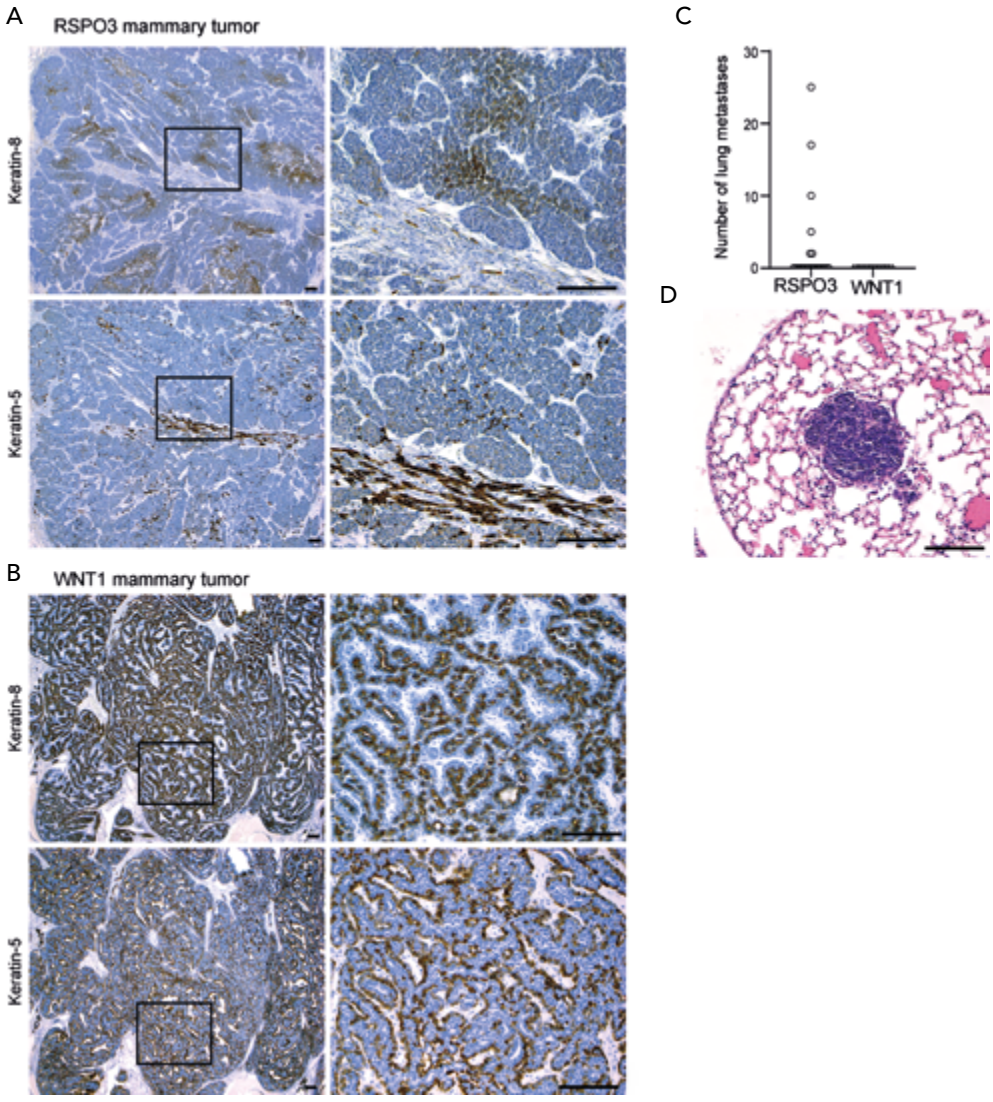
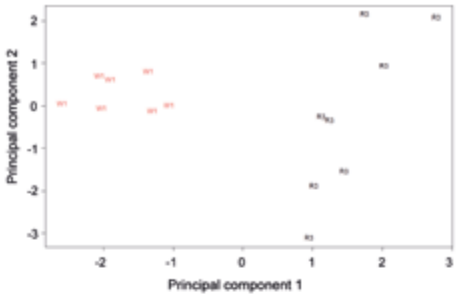


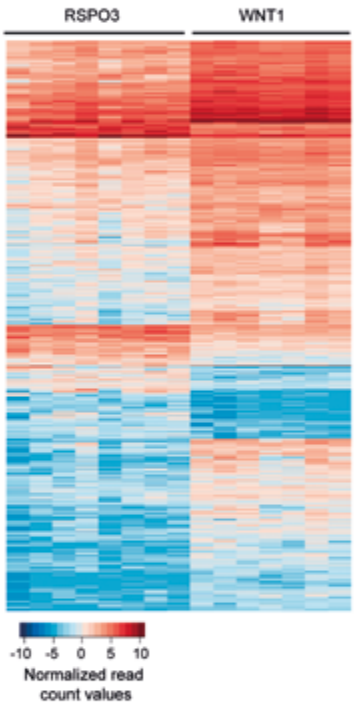
Figure 3. Immunohistochemical stainings for luminal marker K8 (upper) and basal marker K5 (lower) on mammary tumors developing in A. MMTV-*Cre*;*Rspo3*^{inv} female mice and B. MMTV-*Wnt1*; *Rspo3*^{inv} female mice. Left panels 4x objective, right 20x objective. C. Number of lung metastases observed per mouse of either MMTV-*Cre*;*Rspo3*^{inv} (n=21) or MMTV-*Wnt1* (n=10) model. D. Representative example of H&E stained lung metastasis in MMTV-*Cre*;*Rspo3*^{inv} mouse (20x objective).

into this, we assessed the molecular gene expression profiles of RSPO3- versus WNT1-driven mouse mammary tumors by performing RNAsequencing analyses on the respective mammary tumor tissues. Principal component analysis indicated separate clustering of RSPO3- versus WNT1-driven tumors, in line with their distinctive morphology (Figure 4A).

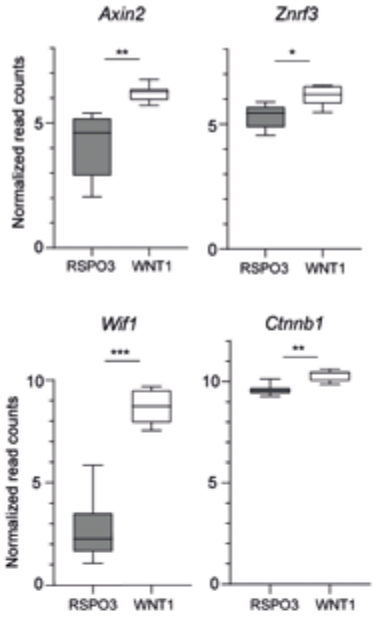
A



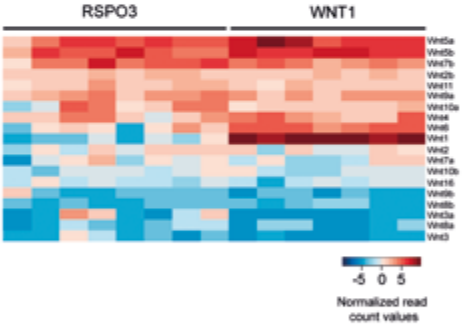
B



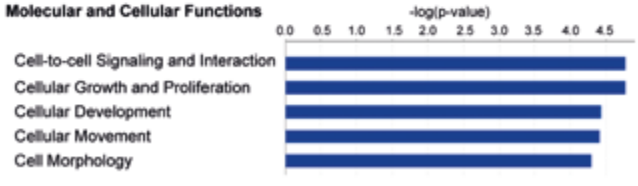
C



D



E



F

Gene	log FC
Mmp1a	4.7
Pax6	3.5
Gjb1	3.5
Il23a	3.4
Hbegf	2.8
Ret	2.6
Egf	2.4
Gata6	2.3
Tgfa	1.7

G

Signaling pathway	Gene ratio	Genes
cAMP mediated signaling	6/229	*Adcy8, Akap12, Chrm3, Dusp9, Gnao1, P2ry14*
Hepatic Fibrosis / Hepatic Stellate Cell Activation	5/186	*Col27a1, Col9a1, Egf, Mmp1, Tgfa*
eNOS Signaling	4/159	*Adcy8, Aqp2, Aqp5, Chrm3*

Gene expression analysis revealed that 881 genes were differentially expressed, of which 683 genes showed relative upregulation in WNT1 tumors compared to 198 genes being enhanced in RSPO3-driven tumors (Figure 4B, filtered $p<0.05$ and log fold change >1.5). Among these and in line with above findings, expression of steroid hormone receptors *Pgr* and *Esr1* were significantly reduced in RSPO3 mammary tumors compared to WNT1 tumors (Figure S3A). With regard to activation of the canonical Wnt pathway, we observed that RSPO3-driven breast tumors expressed Wnt/β-catenin target genes, however to a significantly lower extent than its WNT1-driven counterparts, as readout by expression of target genes *Axin2, Wif1, Znrf3* and *Ctnnb1* itself (Figure 4C). As R-spondins need Wnt ligands to potentiate the Wnt/β-catenin pathway, we inventoried the endogenous expression of Wnt ligands in the tumors and noticed the presence of a variety of Wnt ligands in both WNT1- and RSPO3-driven tumors (Figure 4D). Compared to RSPO3-driven tumors, WNT1-driven tumors showed significantly higher expression of *Wnt1* itself, but also of *Wnt6* and *Wnt5b*. Wnt ligands that were expressed in both tumor cohorts included *Wnt5a, Wnt5b* and *Wnt7b* foremost, in accordance with the reported expression of these ligands in mammary epithelium[18]. Moreover, *Wnt4* ligand was expressed too, which is a crucial cooperator of RSPO1 in Wnt/β-catenin activation and stem cell expansion in the mouse mammary gland[18,19]. This indicates that in RSPO3-driven tumors, endogenous Wnt ligands are available for possible cooperation with RSPO3. Also, broad expression of Wnt and RSPO receptors was confirmed (Figure S3B,C).

To obtain comprehensive insight in the molecular routes that are differentially activated in RSPO3-driven compared to WNT1-driven mammary tumors, we performed gene ontology analysis. In RSPO3-driven tumors, the most significantly enriched molecular and cellular functions were related to cellular signaling, growth, development, movement and morphology (Figure 4E). Genes that most often underlied high ranking of these functions were *Mmp1a, Pax6, Gjb1, Gata6, Ret* and signaling molecules *Egf, Hbegf, Il23a, Tgfa* (Figure 4F). Activated signaling pathways that were most unique to RSPO3 driven tumors were cAMP-mediated signaling, hepatic fibrosis/stellate cell activation and eNOS signaling, involving the upregulation of relatively small sets of genes (Figure 4G). In WNT1-driven tumors, molecular and cellular functions that were most significantly up

Figure 4. Gene expression analysis of RSPO3-driven (n=8) versus WNT1-driven (n=7) mouse mammary tumors. A. Principal component analysis indicating separate clustering of RSPO3 (R3) tumors from WNT1 (W1) tumors. B. Heat map illustrating normalized expression values of differentially expressed genes per sample (filtered $p<0.05$ and 2log fold change >1.5). C. Normalized expression counts of Wnt/β-catenin pathway genes in RSPO3-driven (grey boxes) versus WNT1-driven (white boxes) mammary tumors. Box plots show minimal, median and maximal expression values and adjusted P values * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (Benjamini–Hochberg method in R). D. Heat map illustrating normalized expression values of Wnt ligands. Gene ontology analysis showing E. most significantly enhanced molecular and cellular functions, F. associated upregulated genes and 2log fold change values and G. top 3 upregulated pathways in RSPO3-driven mammary tumors.

were related to cellular morphology, assembly, signaling, death and survival (Figure S4A). The top 3 pathways activated in WNT1 tumors were axonal guidance signaling, regulation of the epithelial mesenchymal transition in development pathway and human embryonic stem cell pluripotency (Figure S4B). Altogether, in line with the different tumor morphology of RSPO3- and WNT1-driven mammary tumors, gene expression analysis revealed that their molecular profiles are distinct as well.

RSPO3 and WNT1 co-expression drives mixed-phenotype mammary tumors

To fully facilitate and investigate a possible synergism between RSPO3 and WNT1 in the context of mammary tumorigenesis, we generated a cohort of compound MMTV-*Cre*;*Rspo3*^{inv};MMTV-*Wnt1* female mice that expressed both transgenic *Rspo3* and *Wnt1* in their mammary glands (n=31). Compared to *Rspo3*^{inv};MMTV-*Wnt1* mice that overexpress the *Wnt1* transgene only (n=49), there was no significant difference in tumor free survival (p=0.06), despite a slight trend towards reduced survival (Figure 5A). Histological analysis revealed that mammary tumors developing in mice with RSPO3/WNT1 co-expression showed a mixed phenotype, typically exhibiting characteristics of both RSPO3 and WNT1 driven tumors (Figure 5B). Grossly, the RSPO3/WNT1 mammary tumors showed a combination of compact solid areas as well as more dilated, cystic areas typically seen in RSPO3- and WNT1-driven mammary tumors respectively (Figure 5B left panel). In these RSPO3/WNT1 co-expression tumors, both keratin-8 and keratin-5 were expressed broadly, however the staining pattern was less organized compared to the bilayered staining pattern in WNT1-tumors, indicating reduced epithelial organization and differentiation (Figure 5B, 3C). Thus, RSPO3 co-expression with WNT1 affects tumor morphology, and accordingly, distant lung metastases were found in 3 out of 9 RSPO3/WNT1 mice (Figure 5C). Since no lung metastases were observed in mice with WNT1 overexpression only (Figure 3D), these findings indicate that RSPO3 contributes to WNT1-driven tumorigenesis by promoting malignant progression.

Discussion

RSPOs have gained attention as clinically relevant oncogenes lately, a novel character of RSPOs that has been established especially in the intestinal tract[4]. Considering RSPOs in breast cancer, data indicative for a protumorigenic role have been reported, though remained rather associative and indirect. Among these, *Rspo2* and *Rspo3* have been proposed as potential mammary oncogenes given their frequent activation in MMTV insertional mutagenesis screens in mice[32-35]. In breast cancer patients, *RSPO2, RSPO3* and *RSPO4* overexpression have been reported, being associated with hormone receptor negative tumor status and for *RSPO2* also with reduced patient survival[30,31]. Adding to this, we found that a profound quarter of breast cancer patients harbor *RSPO2* or *RSPO3* copy number amplifications, which associate with high tumor grade, ER and PR negative tumor status and reduced survival, indicating the clinical relevance of a gain in RSPO. Here, we

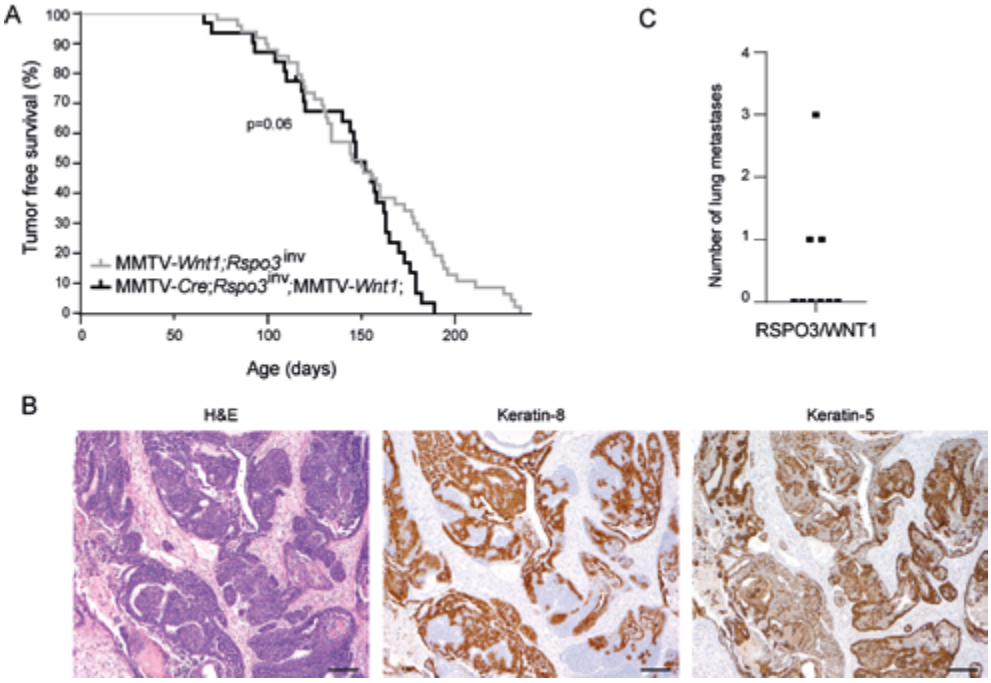


Figure 5. Combined transgenic *Rspo3* and *Wnt1* expression in the mammary gland. A. Tumor free survival curves of mice with expression of the *Wnt1* transgene exclusively (MMTV-*Wnt1*;*Rspo3*^{inv}, n=49) versus both *Wnt1* and *Rspo3* transgenes (MMTV-*Cre*;*Rspo3*^{inv};MMTV-*Wnt1*, n=31) (LogRank test). B. Representative RSPO3/WNT1 mammary tumor subjected to H&E, Keratin-8 and Keratin-5 staining (10x objective). C. Number of lung metastases observed per MMTV-*Cre*;*Rspo3*^{inv};MMTV-*Wnt1* mouse (n=9).

provide direct *in vivo* evidence that *Rspo3* acts as an oncogenic driver in the mammary gland, as *Rspo3* overexpression consistently caused the development of mammary tumors in mice. The RSPO3-driven mammary tumors typically appear as poorly differentiated adenocarcinomas with metastatic potential. These findings directly establish the oncogenic role of RSPO overactivation in the mammary gland, thus extending the clinical relevance of RSPOs among cancer types.

In colon cancer, *RSPO2* and *RSPO3* gene fusions have been proposed to potentiate Wnt/β-catenin signaling, providing a mutational alternative for classical *APC* and *CTNNB1* mutations[7]. In our previous study, we showed that *Rspo3* overexpression causes tumorigenesis in the mouse intestine, indeed accompanied by a modest increase in Wnt signaling[5]. Wnt pathway activation has been implicated in tumorigenesis in the breast too, although the underlying mutational causes remain incompletely understood[4,23]. Conditional *Wnt1* overexpression in the mouse mammary gland is well-known to induce robust mammary tumorigenesis[22]. Since RSPOs are most often envisioned as agonists of the canonical Wnt pathway, we studied our RSPO3 breast cancer mouse model in parallel to the WNT1-driven counterpart. Strikingly, we found that RSPO3-driven mammary tumors

appeared as completely different entities than those driven by WNT1. Whereas WNT1 was able to drive tumorigenesis faster compared to RSPO3, RSPO3 caused mammary tumors that were more malignant, showing poor differentiation, areas of EMT and distant metastases. These morphological differences were further substantiated upon RNA sequencing analysis, which revealed that RSPO3 versus WNT1-driven mammary tumors have very distinctive molecular profiles. Generally, many more upregulated genes were observed in WNT1-driven tumors (683) compared to RSPO3-driven tumors (198). Although Wnt/β-catenin target genes were expressed in RSPO3-driven tumors, levels were clearly less than in WNT1-driven tumors. Because RSPOs need Wnt ligands to be able to potentiate Wnt/β-catenin signaling, we examined the presence of endogenous Wnt ligands. We confirmed the expression of several Wnt ligands, including Wnt4, implying that Wnt ligands were available for possible synergy with RSPO3. Despite this, the relatively low expression of Wnt/β-catenin target genes in RSPO3-driven mammary tumors suggests that tumorigenesis driven by RSPO3 might be less reliant on Wnt/β-catenin pathway activation. Instead, or in parallel, RSPO3 might rely on alternative molecular routes and supportively, 198 genes were upregulated in RSPO3 driven mammary tumors. At the cellular level, we previously noticed that in the intestine, RSPO3-driven tumorigenesis was accompanied by a striking expansion of stem cell and niche compartments[5]. Additional studies reported likewise that in the intestine, RSPO3 activation is accompanied by tumorigenic growth and a proliferative stem cell phenotype[6,37]. This is in accordance with the reported ability of RSPOs to fuel self-renewal and expansion of stem cells in the intestine[3]. Thus, considering a possible mechanism through which RSPOs contribute to tumorigenesis, most of current knowledge is obtained from studies in the intestine and point towards RSPO mediated deregulation of the proliferative stem cell compartment. Although the mammary gland differs greatly from the intestine, also in the mammary gland, RSPO is known to play an essential role in stem cell regulation[18,19]. In the normal mouse mammary gland, RSPO1 has appeared as a key regulator of stem cells, acting together with Wnt4 to regulate the expansion of mammary progenitor cells[18,19]. Comparably to the setting in the intestine, RSPO3 overexpression might fuel tumorigenic growth through abnormal expansion of mammary progenitor cells. More research is required to further delineate the molecular and cellular activities through which RSPO3 fuels mammary tumorigenesis.

Conclusively, with this study we provide *in vivo* evidence for the causal oncogenic capacity of RSPO3 in the breast, extending its clinical relevance beyond the intestine. RSPO3-driven mouse mammary tumors are very distinct from WNT1-driven counterparts, and uniquely present with poor differentiation, malignant transformation and metastatic potential. Moreover, we found that a quarter of breast cancer patients harbors *RSPO2/RSPO3* copy number amplifications, associating with worse prognosis and lack of steroid hormone receptor expression, restricting therapeutic options. Targeting RSPO instead might potentially create a novel window of opportunity for alternative therapeutic intervention in steroid hormone receptor negative breast cancer patients and thereby provide significant clinical benefit. As therapeutic anti-RSPO antibodies already exist and

anti-RSPO3 has been demonstrated well-tolerated by patients in a clinical trial for colon cancer, realistic and relatively fast opportunities lay ahead to explore RSPO targeting in breast cancer patients.

Acknowledgements

The authors thank the NKI animal facility, animal pathology department (E Riem, L de Vrije and J van der Meer) and genomics core facility (I de Rink) for their service and technical assistance. This work was financially supported by the Netherlands Organization for Scientific Research (NWO/ZonMW VENI 016.186.138) and the Dutch Cancer Society (KWF Young Investigator Grant 10957).

Author contributions

ERMB and JH conceived, designed and supervised the project. Mouse studies: MB, NCT, CMEA, ERMB, JH, histology: J-YS, EJtS, ERMB, JH, PWBD. PWBD interpreted results and provided input. Gene expression analysis: ERMB, EJtS, JH. In silico analysis EJtS and ERMB. ERMB and EJtS wrote the manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

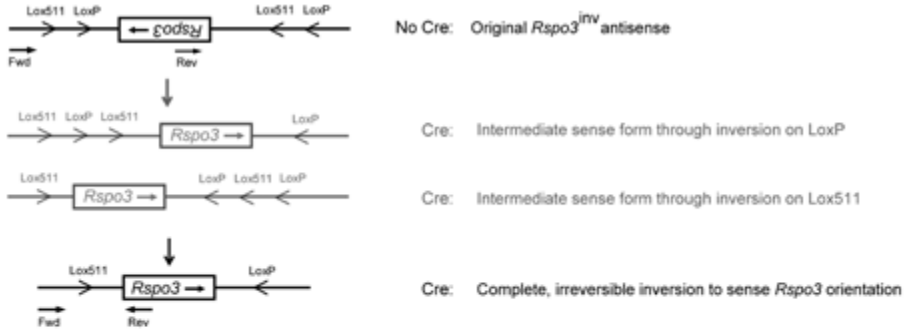
References

1. de Lau, W. *et al.* Lgr5 homologues associate with Wnt receptors andmediate R-spondin signalling. *Nature* **476**, 293-297 (2011).
2. de Lau, W.B., Snel, B. & Clevers, H.C. The R-spondin protein family. *Genome Biol.* **13**, 242 (2012).
3. Yan, K.S. *et al.* Non-equivalence of Wnt and R-spondin ligands during Lgr5(+) intestinal stem-cell self-renewal. *Nature* **545**, 238-242 (2017).
4. Ter Steege, E.J. & Bakker, E.R.M. The role of R-spondin proteins in cancer biology. *Oncogene* **40**, 6469-6478 (2021).
5. Hilkens, J. *et al.* RSPO3 expands intestinal stem cell and niche compartments and drives tumorigenesis. *Gut* **66**, 1095-1105 (2017).
6. Han, T. *et al.* R-Spondin chromosome rearrangements drive Wnt-dependent tumour initiation and maintenance in the intestine. *Nat. Commun.* **8**, 15945 (2017).
7. Seshagiri, S. *et al.* Recurrent R-spondin fusions in colon cancer. *Nature* **488**, 660-664 (2012).
8. Shinmura, K. *et al.* RSPO fusion transcripts in colorectal cancer in Japanese population. *Mol. Biol. Rep.* **41**, 5375-5384 (2014).
9. Kleeman, S.O. *et al.* Exploiting differential Wnt target gene expression to generate a molecular biomarker for colorectal cancer stratification. *Gut* **69**, 1092-1103 (2020).
10. Hashimoto, T. *et al.* EIF3E-RSPO2 and PIEZO1-RSPO2 fusions in colorectal traditional serrated adenoma. *Histopathology* **75**, 266-273 (2019).
11. Mizuguchi, Y. *et al.* Identification of a novel PRR15L-RSPO2 fusion transcript in a sigmoid colon cancer derived from superficially serrated adenoma. *Virchows. Arch.* **475**, 659-663 (2019).
12. Sekine, S. *et al.* Comprehensive characterization of RSPO fusions in colorectal traditional serrated adenomas. *Histopathology* **71**, 601-609 (2017).
13. Sekine, S. *et al.* Frequent PTPRK-RSPO3 fusions and RNF43 mutations in colorectal traditional serrated adenoma. *J. Pathol.* **239**, 133-138 (2016).
14. A Phase 1a/b Dose Escalation Study of the Safety, Pharmacokinetics, and Pharmacodynamics of OMP-131R10. Available from: https://clinicaltrials.gov/ct2/show/NCT02482441.
15. Alexander, C.M. Wnt signaling and mammary stem cells. *Vitam. Horm.* **116**, 21-50 (2021).
16. van Amerongen, R., Bowman, A.N. & Nusse, R. Developmental stage and time dictate the fate of Wnt/beta-catenin-responsive stem cells in the mammary gland. *Cell Stem Cell* **11**, 387-400 (2012).
17. Zeng, Y.A. & Nusse, R. Wnt proteins are self-renewal factors for mammary stem cells and promote their long-term expansion in culture. *Cell Stem Cell* **6**, 568-577 (2010).
18. Cai, C. *et al.* R-spondin1 is a novel hormone mediator for mammary stem cell self-renewal. *Genes Dev.* **28**, 2205-2218 (2014).
19. Joshi, P.A. *et al.* RANK Signaling Amplifies WNT-Responsive Mammary Progenitors through R-SPONDIN1. *Stem Cell Reports* **5**, 31-44 (2015).
20. Nusse, R. & Varmus, H.E. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* **31**, 99-109 (1982).
21. Imbert, A. *et al.* Delta N89 beta-catenin induces precocious development, differentiation, and neoplasia in mammary gland. *J. Cell Biol.* **153**, 555-568 (2001).
22. Tsukamoto, A.S. *et al.* Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. *Cell* **55**, 619-625 (1988).
23. Geyer, F.C. *et al.* beta-Catenin pathway activation in breast cancer is associated with triple-negative phenotype but not with CTNNB1 mutation. *Mod. Pathol.* **24**, 209-231 (2011).

24. Incassati, A. *et al.* Key signaling nodes in mammary gland development and cancer: beta-catenin. *Breast Cancer Res.* **12**, 213 (2010).
25. Lin, S.Y. *et al.* Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 4262-4266 (2000).
26. Ai, L. *et al.* Inactivation of Wnt inhibitory factor-1 (WIF1) expression by epigenetic silencing is a common event in breast cancer. *Carcinogenesis* **27**, 1341-1348 (2006).
27. Suzuki, H. *et al.* Frequent epigenetic inactivation of Wnt antagonist genes in breast cancer. *Br. J. Cancer* **98**, 1147-1156 (2008).
28. Veeck, J. *et al.* Epigenetic inactivation of the secreted frizzled-related protein-5 (SFRP5) gene in human breast cancer is associated with unfavorable prognosis. *Carcinogenesis* **29**, 991-998 (2008).
29. Veeck, J. *et al.* Prognostic relevance of Wnt-inhibitory factor-1 (WIF1) and Dickkopf-3 (DKK3) promoter methylation in human breast cancer. *BMC Cancer* **9**, 217 (2009).
30. Coussy, F. *et al.* Clinical value of R-spondins in triple-negative and metaplastic breast cancers. *Br. J. Cancer* **116**, 1595-1603 (2017).
31. Tocci, J.M. *et al.* R-spondin3 Is Associated with Basal-Progenitor Behavior in Normal and Tumor Mammary Cells. *Cancer Res.* **78**, 4497-4511 (2018).
32. Callahan, R. *et al.* Genes affected by mouse mammary tumor virus (MMTV) proviral insertions in mouse mammary tumors are deregulated or mutated in primary human mammary tumors. *Oncotarget* **3**, 1320-1334 (2012).
33. Gattelli, A. *et al.* Selection of early-occurring mutations dictates hormone-independent progression in mouse mammary tumor lines. *J. Virol.* **80**, 11409-11415 (2006).
34. Klijn, C. *et al.* Analysis of tumor heterogeneity and cancer gene networks using deep sequencing of MMTV-induced mouse mammary tumors. *PLoS One* **8**, e62113 (2013).
35. Theodorou, V. *et al.* MMTV insertional mutagenesis identifies genes, gene families and pathways involved in mammary cancer. *Nat. Genet.* **39**, 759-769 (2007).
36. Wagner, K.U. *et al.* Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice. *Transgenic Res.* **10**, 545-553 (2001).
37. Storm, E.E. *et al.* Targeting PTPRK-RSPO3 colon tumours promotes differentiation and loss of stem-cell function. *Nature* **529**, 97-100 (2016).

Supplementary Figures

A



B

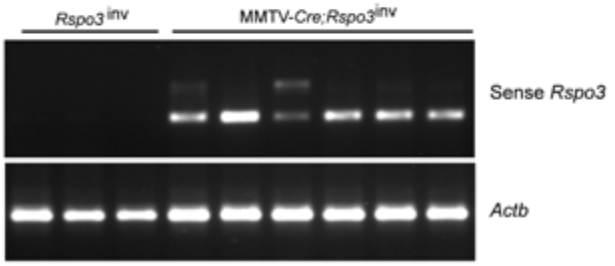


Figure S1. Regulation of *Rspo3* transgene expression in the *Rspo3*^{inv} mouse model. A. Simplified scheme of the original *Rspo3*^{inv} construct and resulting derivatives upon Cre activity. Cre mediates inversion of the *Rspo3* coding sequence using either the *LoxP* or *Lox511* sequences that are oppositely oriented, giving intermediate forms with sense *Rspo3* orientation that can revert back to antisense. Further Cre activity causes excision of the remaining sequences between homologous *Lox* sites that are now oriented in equivalent direction. This provides the final product with irreversible sense orientation of the *Rspo3* transgene. Figure adapted from ref 5 with permission of BMJ Publishing Group Ltd. Fwd and Rev arrows indicate the location and orientation of the primers used to detect mRNA expression of the *Rspo3* transgene. B. RT-PCR confirming sense-oriented *Rspo3* mRNA expression in mammary gland tissues of MMTV-*Cre*;*Rspo3*^{inv} but not *Rspo3*^{inv} mice. Upper bands represent the intermediate products and lower bands the final, locked product.

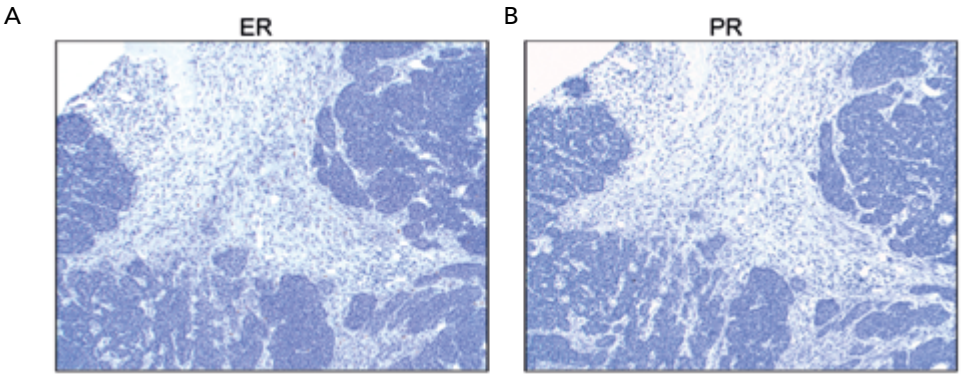


Figure S2. Steroid hormone receptor staining in an RSPO3-driven mouse mammary tumor. Representative immunohistochemical staining of an RSPO3-driven mouse mammary tumor for A. ERα and B. PR.

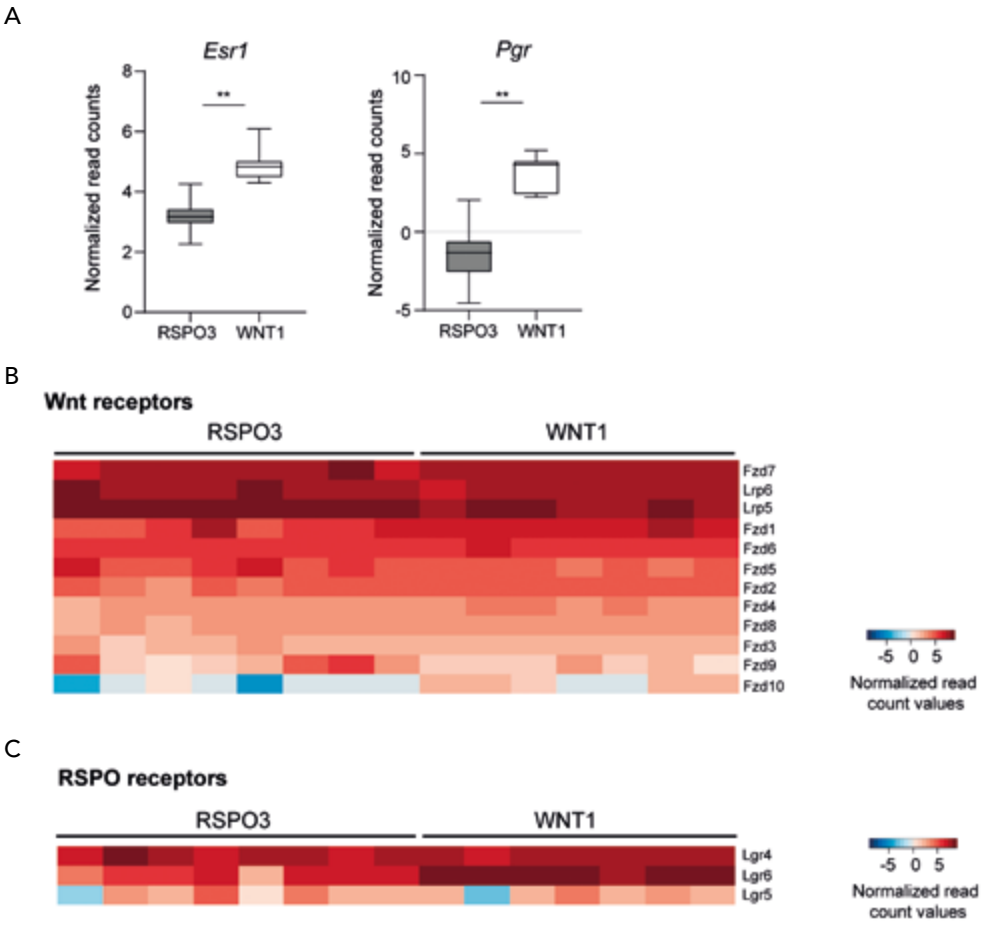
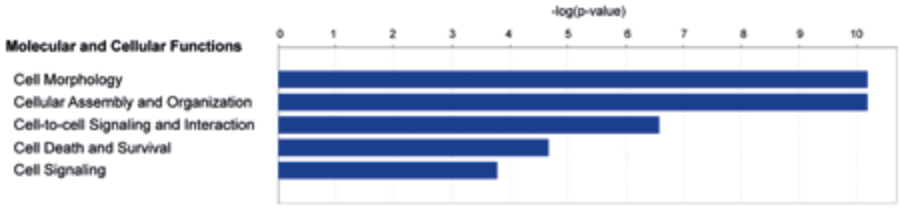


Figure S3. RNA expression analysis of receptors for steroid hormones, Wnt, and RSPO in RSPO3- and WNT1-driven mammary tumors. A. Normalized expression counts of estrogen- and progesterone receptors in RSPO3-driven (grey boxes) versus WNT1-driven (white boxes) mammary tumors. Box plots show minimal, median, and maximal values, and adjusted *P* values. $^{**}p < 0.01$ (Benjamini–Hochberg method in R). Heat maps illustrating normalized expression values of B. Wnt and C. RSPO receptors.

A



B

Signaling pathway	Gene ratio	Genes
Axonal Guidance Signaling	31/494	*Ablim1, Adam22, Adamts3, Adamts9, Adamts15, Adamts17, Adamts18, Bdnf, Bmp7, Bmp15, Dpysl5, Epha4, Fzd10, Gnai1, Gnaz, Lrrc4c, Myl9, Ntf3, Ntng1, Ntng2, Ntrk3, Pappa, Pik3c2g, Plce1, Ptch2, Robo1, Robo2, Sema6d, Wnt1, Wnt6, Wnt5b*
Regulation of the Epithelial Mesenchymal Transition in Development Pathway	10/84	*Axin2, Fzd10, Jag2, Ptch2, Pygo1, Snai2, Tcf7, Wnt1, Wnt6, Wnt5b*
Human Embryonic Stem Cell Pluripotency	12/135	*Bdnf, Bmp7, Bmp15, Fgfr2, Fzd10, Ntf3, Ntrk3, Pik3c2g, Tcf7, Wnt1, Wnt6, Wnt5b*

Figure S4. Gene ontology analysis showing A. the most significantly enhanced molecular and cellular functions and B. the top three upregulated pathways in WNT1-driven mammary tumors.

4

R-SPONDIN-3 PROMOTES PROLIFERATION AND INVASION OF BREAST CANCER CELLS INDEPENDENTLY OF WNT SIGNALING

Eline J. ter Steege[1], Loes W. Doornbos[1*], Peter D. Haughton[1*], Paul J. van Diest[1], John Hilkens[2], Patrick W. B. Derksen[1] and Elvira R. M. Bakker[1,2]

[1]Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands
[2]Department of Molecular Genetics, Netherlands Cancer Institute, Amsterdam, The Netherlands
* these authors contributed equally

Published in Cancer Letters (2023)
doi: 10.1016/j.canlet.2023.216301

Abstract

We recently identified R-spondin-3 (RSPO3) as a novel driver of breast cancer associating with reduced patient survival, expanding its clinical value as potential therapeutic target that had been recognized mostly for colorectal cancer so far. (Pre)clinical studies exploring RSPO3 targeting in colorectal cancer approach this indirectly with Wnt inhibitors, or directly with anti-RSPO3 antibodies. Here, we address the clinical relevance of RSPO3 in breast cancer and provide insight in the oncogenic activities of RSPO3. Utilizing the RSPO3 breast cancer mouse model, we show that RSPO3 drives the aberrant expansion of luminal progenitor cells expressing cancer stem cell marker CD61, inducing proliferative, poorly differentiated and invasive tumors. Complementary studies with tumor organoids and human breast cancer cell lines demonstrate that RSPO3 consistently promotes the proliferation and invasion of breast cancer cells. Importantly, RSPO3 exerts these oncogenic effects independently of Wnt signaling, rejecting the therapeutic value of Wnt inhibitors in RSPO3-driven breast cancer. Instead, direct RSPO3 targeting effectively inhibited RSPO3-driven growth of breast cancer cells. Conclusively, our data indicate that RSPO3 exerts unfavorable oncogenic effects in breast cancer, enhancing proliferation and malignancy in a Wnt-independent fashion, proposing RSPO3 itself as a valuable therapeutic target in breast cancer.

Introduction

The expression of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) are instrumental in breast cancer diagnosis and treatment, determining tumor subtype and intervention strategy respectively. Targeted therapies for breast cancer patients are largely directed against these receptors, of which the efficacy is challenged by tumor heterogeneity and therapy resistance. Moreover, triple negative breast cancers (TNBC) that lack expression of these three receptors are not susceptible for targeted treatments and hold relatively poor prognosis. To improve options for intervention strategies, it is crucial to obtain better insight into the molecular mechanisms that underlie breast cancer and to identify novel therapeutic targets.

In this perspective, R-spondin (RSPO) proteins, primarily known as agonists of the canonical Wnt/β-catenin pathway and regulators of stem cell niches, have emerged as clinically relevant oncogenes with apparent potential as therapeutic target[1]. Initially identified and recognized in the intestinal tract, pioneering (pre)clinical studies have mainly addressed the potential utility of targeting RSPO signaling in colorectal cancer[1–7]. These studies showed that both the direct targeting of RSPO ligands with monoclonal antibodies and the indirect targeting of Wnt signaling with porcupine inhibitors successfully reduced tumor growth and induced tumor differentiation in PDX models of colorectal cancer with a gain in *RSPO*[8–13]. Accordingly, in the mouse intestine, RSPO overexpression fueled tumor development, being accompanied by increased Wnt/β-catenin signaling and expansion of the proliferative stem cell compartment[14,15].

Interestingly, deregulation of RSPOs has also been linked to breast cancer, whereby overexpression of *RSPO2*, *RSPO3* and *RSPO4* have particularly been reported in patients with TNBC[9,16,17]. In agreement with these findings, we recently reported copy number amplifications of *RSPO2* and *RSPO3* in respectively 23% and 2% of breast cancer patients, which associated with lack of ER/PR expression, high tumor grade and reduced patient survival[18]. This association with receptor negative tumor status importantly indicates the potential clinical benefit that may be provided by RSPO as an alternative target in breast cancer. Additionally, we have recently reported that RSPO3 acts as a causal driver of breast cancer, as conditional overexpression of *Rspo3* in mouse mammary glands caused the consistent formation of poorly differentiated mammary tumors with metastatic potential[18]. We found that RSPO3-driven mammary tumors hold extensive differences in morphology and gene expression profiles compared to classical WNT1-driven mammary tumors, presenting as more malignant entities with relatively low Wnt activity[18]. In current study, we provide more insight in the activities through which RSPO3 drives breast cancer and the potential approach to target these activities, complementary using human breast cancer cell lines, an orthotopic transplantation model for human breast cancer, the RSPO3 breast cancer mouse model and tumor organoids models. We show that RSPO3 fuels increased proliferation and invasion of breast cancer cells, independent of Wnt signaling. Our data indicate that in breast cancer, RSPO3 functions as a Wnt independent and multi-

faceted oncogene, presenting itself as a promising therapeutic target, whereas indirect targeting through Wnt inhibiton lacks therapeutic value in this setting.

Materials and methods

Transgenic and orthotopic transplantation mouse studies

Rspo3^{inv} mice (more detailed description of the *Rspo3*^{inv} mouse model provided in Ref. [14]) were crossed with MMTV-*Cre* mice[19], generating double transgenic MMTV-*Cre*;*Rspo3*^{inv} mice. The MMTV-*Wnt1* mouse model was kept in parallel[20]. Transgenic alleles were maintained heterozygous.

For the orthotopic transplantation study, B6;129*Rag2*^{tm1Fwa}*Il2rg*^{tm1Rsky}/DwlHsd immunodeficient mice (Envigo) were injected with 1x10^6 MCF7 pInducer hRSPO3 Flag/HA cells in the fourth mammary fat pad on the right side of each mouse. 24 hours prior to fat pad transplantation, mice switched to β-estradiol containing water (4 μg/ml, Sigma). When tumors reached a volume of 50 mm^3, mice were randomized over two groups, either or not receiving doxycyline food (200 mg/kg, ssniff). Tumor volumes were monitored weekly and mice were sacrificed when tumors reached a volume of 1000 mm^3. All animal experiments were performed following Dutch legislation and with approval of the Animals Ethics Committee.

Immunohistochemistry

Isolated tissues were fixed in 4% formaldehyde for 24h and paraffin embedded. Hematoxylin eosin (HE) staining and immunohistochemistry (IHC) was performed according to routine protocols. For mouse tissues, the following antibodies were used: Ki67 (Abcam), p63 (D-9, Santa Cruz), Sox9 (Millipore), β-catenin (E247; Abcam), Cleaved Caspase3 (Asp175; Cell Signaling). IHC on transplanted human materials was performed on the Roche Ventana™ using K14 (SP53, Roche), K8 (B22.1, Roche), Ki67 (30-9, Roche), ERα (SP1, Roche) antibodies. Percentages of lung metastases were quantified by measuring the total surface area of all metastatic lesions and the total surface area of the lungs per mouse per section using Slide Score (Slide Score B.V.).

Flow cytometry

Isolated mouse tissues were kept in Hanks Balanced Salt solution (HBSS, Lonza) and minced using a Tissue Chopper (McIlwain). Tissue pieces were transferred to a digestion mix containing 3mg/ml Collagenase type 3 (Worthington), 0.35 mg/ml Hyaluronidase (Sigma) and 0.1mg/ml DNAse I (Stem cell technologies) in DMEM/F12 (Gibco) and left shaking at 37°C for 1h. After digestion, red blood cells were removed using Red Blood Cell Lysis Buffer (BioVision). Cells were then incubated for 20' at 37°C in a solution of 1x Trypsin (Sigma) with 0.1 mg/ml DNAse I. Trypsin was inactivated by incubating the cells for 5' at 37°C in a solution of DMEM/F12, 10% FCS (Bodinco) and 0.1 mg/ml DNAse I. Single cells were then collected in HBSS using a 70 μm cell strainer (Falcon) and 500,000

cells were stained per condition. Cells were incubated with the primary antibodies for 30' and with the secondary antibody streptavidin for 20' on ice in the dark. Antibodies used: CD49f-PerCP-Cy5.5 (GoH3, BioLegend), EpCAM (CD326)-BV510 (G8.8, BioLegend), CD61-PE-CY7 (2C9.G2 (HMβ3-1), BioLegend), TER-119-Biotin (TER-119, eBioscience), CD31 (PECAM-1)-Biotin (390, eBioscience), CD45-Biotin (30-F11, eBioscience) and Streptavidin-BV605 (BioLegend).

Generation of mouse tumor organoids

Isolated mammary tumors were collected in Advanced DMEM/F12 (Gibco) supplemented with 10 mM HEPES (Thermo Fischer), 1% Pen-Strep (Lonza) and 1% Ultra-Glutamine (Gibco) (AdDF+++). Tumors were minced into small pieces with a scalpel for approximately 50 times. Tumor pieces were placed in a Liberase digestion mix (0.1mg/ml, Sigma) and left shaking at 37°C for 1-1,5 hours. Cells were spun down, washed with AdDF+++ and treated with 0.1mg/ml DNAse I (Stem cell technologies) for 5' at RT. Following, differential centrifuging was performed to select for epithelial tumor components. Finally, the epithelial organoids were placed in a 50 µl Basement Membrane Extract (BME, R&D Systems) hanging drop and left to polymerize for 45' at 37°C before medium was added, being AdDF+++ supplemented with , 1x B27 (Thermo scientific), 1.25 mM N-Acetyl-L-cysteine (Sigma), 42.5 ng/ml FGF2 (Thermo Fischer) and 50 µg/ml Primocin (Invivogen).

Immunofluorescence

Cells and organoids grown in BME hanging drops were fixed with 4% PFA (Electron microscopy sciences) + 0.25% Glutaraldehyde (Sigma) to prevent degradation of the BME followed by treatment with 0.1% $NaBH_4$ to quench residual aldehyde groups after fixation. Organoids placed in a collagen-1 matrix were fixed with 4% PFA only. Samples were blocked in 0.3% Triton X-100 (Sigma) 5% normal goat serum (NGS, Sigma) in PBS (Sigma) for 1h at RT while rocking, followed by incubation with primary antibodies diluted in 0.3% Triton X-100, 1% BSA (Roche) in PBS for 24h at RT while rocking. Cells were washed 4 times 15' with PBS and incubated with secondary antibodies overnight at RT while rocking. Primary antibodies used: K14 (Poly19053, BioLegend), K8 (TROMA-I, Developmental Studies), GFP (D5.1, Cell Signaling), BrdU-Alexa Fluor® 647 (3D4, BD Biosciences). Secondary antibodies used: Anti-rat-Alexa-488 (Invitrogen), Anti-rabbit-Alexa-647 (Invitrogen). Immunofluorescence images were made using a Zeiss LSM880 microscope.

CellTiter-Glo assay

50,000 organoid-derived tumor cells were plated in a 50 µL BME hanging drop. Organoids were treated with DMSO control or 200 nM C59 (Cellagen Technology). At day 5, cell viability was measured using the CellTiter-Glo 3D cell viability assay (Promega) following manufacturers' protocol. Luminescence was measured using a spectrophotometer.

Invasion assay

RSPO3- and WNT1- driven tumor organoids were grown for 5 or 7 days in a BME hanging drop whilst treated with or without C59 (200 nM, Cellagen Technology). Next, cultures were incubated with 6 mg/ml Dispase (Gibco) for 20' at 37°C and reseeded in a Collagen-I matrix as previously described[21]. After 24h, collagen gels were fixed for 10' with 4% PFA. Images were made using an EVOS M500 microscope using phase contrast.

Quantitative real-time PCR

RNA was extracted from organoids using Trizol (Life Technologies) and cDNA was synthesized using a transcription kit (BioRad iScript cDNA Synthesis Kit) according to the manufacturers' instructions. qPCR was performed using Fast Start Universal SYBR Green (Roche) on a BioRad CFX96 Real-Time system. The following primers were used: *Axin-2* F- 5' GCTCCAGAAGATCACAAAG 3', *Axin-2* R-5' CTTCAGCATCCTCCTGTAT 3', *Rspo3* F- 5' AGATAGGAGTGTGTCTCTCTTCG 3', *Rspo3* R- 5' AGTATGATTGTTGGCTTCTAA CC 3'. *Actb* F- 5' AGACCTCTATGCCAACACAG 3', *Actb* R- 5' CACAGAGTACTTGCGCTCAG 3'

Cell lines and medium

Cell lines were obtained from ATCC. MCF10A cells were grown in DMEM/F12 (Gibco) supplemented with 5% Horse serum (Fischer Scientific), 1% Pen/Strep, 20 ng/ml EGF (Peprotech), 100 ng/ml Cholera Toxin (Sigma), 10µg/ml insulin (Sigma) and 0.5 mg/ml Hydrocortisone (Sigma). MCF7 and T47D cells were cultured in DMEM/F12 supplemented with 12% FCS, 1% Pen-Strep and 1% Ultra-glutamine.

Cloning and generation of stable cell lines

A pcDNA3.1 hRSPO3 Flag/HA vector (kind gift from the Clevers lab, Hubrecht institute, Utrecht, the Netherlands) was used as template to generate a hRSPO3 Flag/HA PCR product flanked by *BAMHI* and *ECORI* restriction sites. The resulting hRSPO3 Flag/HA PCR product was cloned into pEntry vector using GATEWAY cloning (Thermo Fisher Scientific). Subsequently, a LR reaction was performed, transferring the hRSPO3 Flag/HA sequence into the pInducer20 vector[22], generating pInducer hRSPO3 Flag/HA (pInd-hRSPO3). Wnt signaling reporter constructs used: 7TGP (7x Tcf-eGFP-Puro resistance cassette, #24305, Addgene) and 7TFP (7x Tcf-luciferase-Puro resistance cassette, #24308, Addgene). Stable cell lines were generated by lentiviral transduction followed by selection with appropriate antibiotic selection markers.

Immunoblotting

Cells were lysed in sample buffer, separated by SDS-PAGE and blotted. The following antibodies were used for immunoblotting: Flag (M2, Sigma) and GAPDH (Millipore). Detection was performed using IRDy680 goat anti-mouse antibody (Li-Cor) and the Amersham Typhoon Biomolecular Imager.

Growth and proliferation assays

MCF7 pInd-hRSPO3, T47D pInd-hRSPO3 and MCF10A pInd-hRSPO3 cells were treated with or without 1 mg/ml doxycyline 24h prior to the start of the experiment. 5,000 cells were plated in each 50 µl BME hanging drop. After BME polymerization, culture medium without or with doxycycline (1 mg/ml), Rosmantuzumab/OMP-131R10 (100 µg/ml, Proteogenix) or C59 (200 nM, Cellagen Technology) was added to the wells and medium was refreshed every other day. After 8 (MCF10A) and 10 (MCF7 and T47D) days, pictures were made of the 3D structures with an EVOS M500 microscope using phase contrast and cell surface area was measured using OrganoSeg[23].

For the BrdU incorporation assay, 10 µM BrdU (BD Biosciences) was added to the medium at day 5 (MCF10A) or day 8 (MCF7 and T47D). After 4h of incubation, cells were fixed 10' with 4% PFA containing 0.25% glutaraldehyde. Fixed cells were treated with 2M HCl for 90' at RT followed by a 10' 0.1% $NaBH_4$ incubation step. Cells were then processed for immunofluorescence. The percentage of $BrdU^+$ cells was quantified using FIJI, counting the number of $BrdU^+$ and total number of nuclei (using DAPI) per 3D structure.

Luciferase assay

50,000 cells (MCF7 pInd-hRSPO3;TFP &T47D pInd-hRSPO3;TFP) or 20.000 cells (MCF10A pInd-hRSPO3;TFP) cells were plated per well in a 24-well plate. After 24h the culture medium was replaced by experimental medium containing either 1 mg/ml doxycycline (Sigma), 25% Wnt3a conditioned medium or a combination. 48h after stimulation luciferase activity was measured using the luciferase assay system (Promega) following manufacturers' protocol. A Berthold technologies Centro XS^3 LB 960 bioluminescencemeter was used for readout.

Statistics

Statistics were performed using two-sided Student's t-test in Graphpad Prism. Error bars indicate standard deviation (SD). Non-significant is indicated as ns, significance as *$p < 0.05$, **$p < 0.01$, ***$p<0.001$ and ****$p < 0.0001$.

Results

RSPO3 fuels aberrant expansion of luminal progenitor cells during mammary tumorigenesis

To investigate the activities of RSPO3 in breast cancer, we used the conditional *Rspo3*^{inv} breast cancer mouse model that we recently published[14,18]. We previously characterized RSPO3-driven mammary tumors as poorly differentiated, and weak Keratin-5 (K5) and Keratin-8 (K8) staining patterns suggested that the solid tumor masses might largely consist of poorly differentiated luminal cells[18]. Staining for the luminal progenitor marker Sox9 demonstrated that the majority of the RSPO3-driven tumors is indeed Sox9-positive

(Figure 1A)[24], while basal cell marker p63 presented only in a minor subset of cells (Figure S1A), together indicating the predominance of the luminal cell compartment in RSPO3 driven mammary carcinomas. Proliferation marker Ki67 revealed overt proliferative activity, especially in cells located along the edges of solid tumor masses (Figure 1B). In contrast, apoptotic cells were rare, as indicated by low cleaved caspase-3 expression (Figure S1B). β-catenin was expressed moderately in the RSPO3-driven tumors, being restricted to the cell membranes (Figure 1C).

To further specify the cellular composition of RSPO3-driven breast carcinomas, we performed extensive flow cytometric analyses of RSPO3-driven tumors, neighboring mammary glands (MG) with RSPO3 overexpression and control mammary glands from mice lacking RSPO3 overexpression. Segregating CD49f^{hi}EpCAM^{low} basal cells from CD49f^{low}EpCAM^{hi} luminal cells, we observed an increase in the relative proportion of luminal cells in the mammary glands of MMTV-*Cre*;*Rspo3* mice compared to control glands (Figure 1D,E). Moreover, RSPO3-driven mammary tumors displayed an even greater expansion of the luminal cell population, displaying an approximate 4-fold increased luminal-to-basal ratio (Figure 1D,E). Following this confirmation of luminal expansion, we analyzed CD61, a marker for luminal progenitor cells that has been reported as cancer stem cell marker[24–28]. Segregating the luminal population into CD61^{-} mature and CD61^{+} progenitor luminal cells, we observed that in the non-neoplastic mammary gland, RSPO3 overexpression induced a significant increase in CD61^{+} luminal progenitor cells (Ctrl MG: 0.6%, RSPO3 MG: 12.3%) which was further increased in RSPO3-driven mammary tumors (52.9%) (Figure 1F,G). These data demonstrate that RSPO3-driven breast tumors are highly enriched in CD61^{+} luminal progenitor cells, a feature that is unique to RSPO3 driven tumors, as this was not observed in WNT1-driven mammary tumors (Figure S1C-D)[29]. Together, these data show that during mammary tumor development, RSPO3 drives the aberrant expansion of luminal progenitor cells marked by cancer stem cell marker CD61, distinctively from WNT1.

RSPO3-tumor organoids grow independently of the Wnt signaling pathway

To functionally investigate RSPO3 driven tumorigenesis *in vitro*, we generated mammary tumor organoids from MMTV-*Cre*;*Rspo3* and comparatively from MMTV-*Wnt1* mice (Figure 2A). Increased *Rspo3* mRNA levels validated *Rspo3* overexpression in the RSPO3 tumor-derived organoids (Figure S2A). Characterization of the RSPO3 tumor organoids with immunofluorescence revealed unevenly distributed expression of luminal marker K8 and basal marker K14 throughout the organoid structures, as well as hybrid cells expressing both keratins (Figure 2B upper panel). This disorganized phenotype was unique to RSPO3 tumor organoids, as WNT1 tumor organoids displayed a distinct polarized keratin expression pattern indicating an outer layer of basal cells and an inner layer of luminal cells (Figure 2B lower panel).

To investigate whether the growth of RSPO3-driven tumor organoids depends on Wnt pathway activity, we treated RSPO3- and WNT1 tumor organoids with porcupine inhibitor

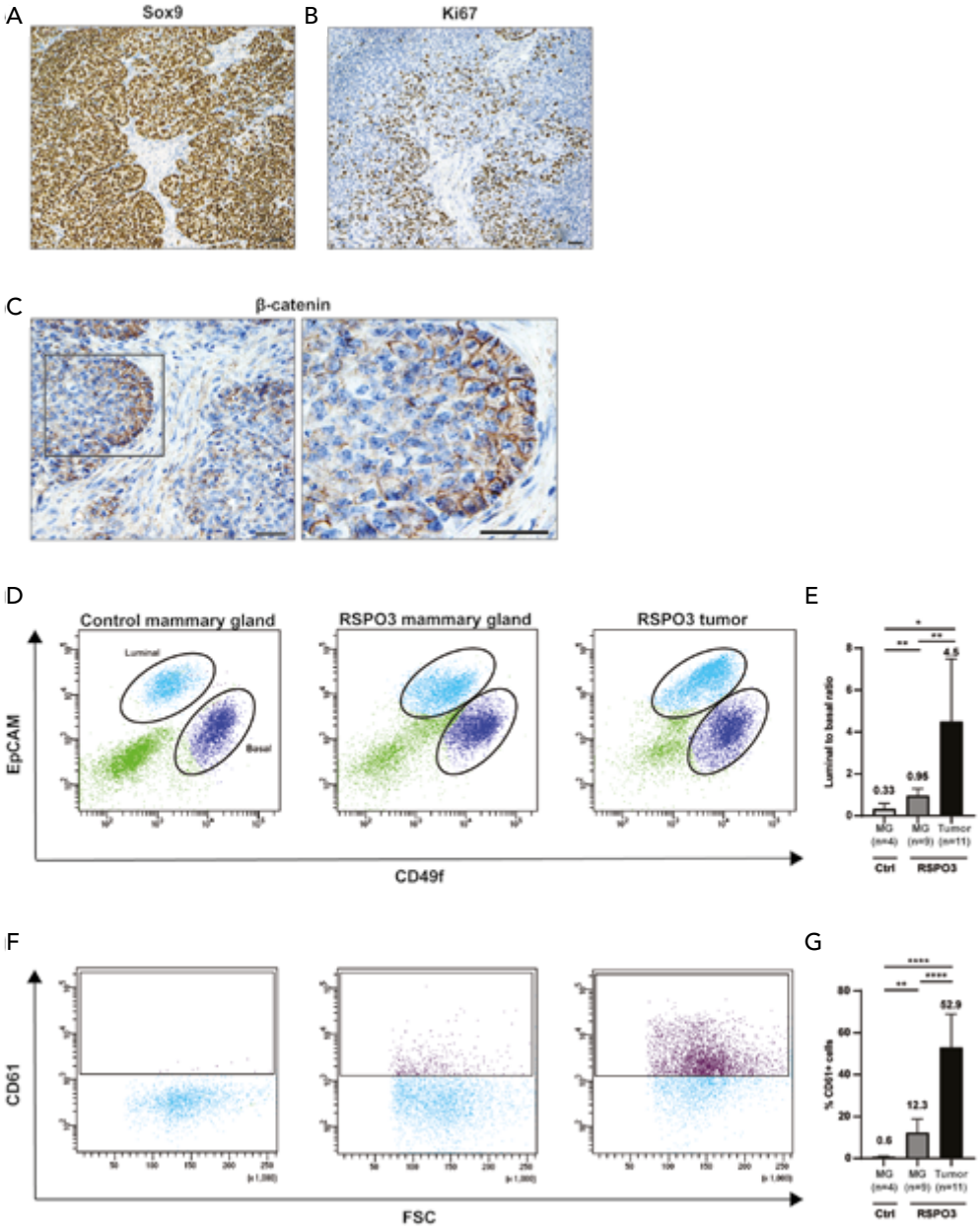


Figure 1. RSPO3-driven mammary tumors are enriched in luminal progenitor cells. Immunohistochemical stainings for A. Sox9, B. Ki67 (20x objective) and C. β-catenin (40x objective) on mammary tumors of MMTV-*Cre;Rspo3*^{inv} mice. D-G: Flow cytometric analyses of mammary glands (MG) of single transgenic control (Ctrl) mice and mammary glands and tumors of MMTV-*Cre;Rspo3*^{inv} (RSPO3) mice. D. FACS plots of mammary epithelial cells segregating luminal and basal populations and E. average luminal to basal ratios. F. FACS plots of the luminal cell population further specifying luminal progenitor cells based upon CD61 expression and G. percentages of CD61^{+} cells.

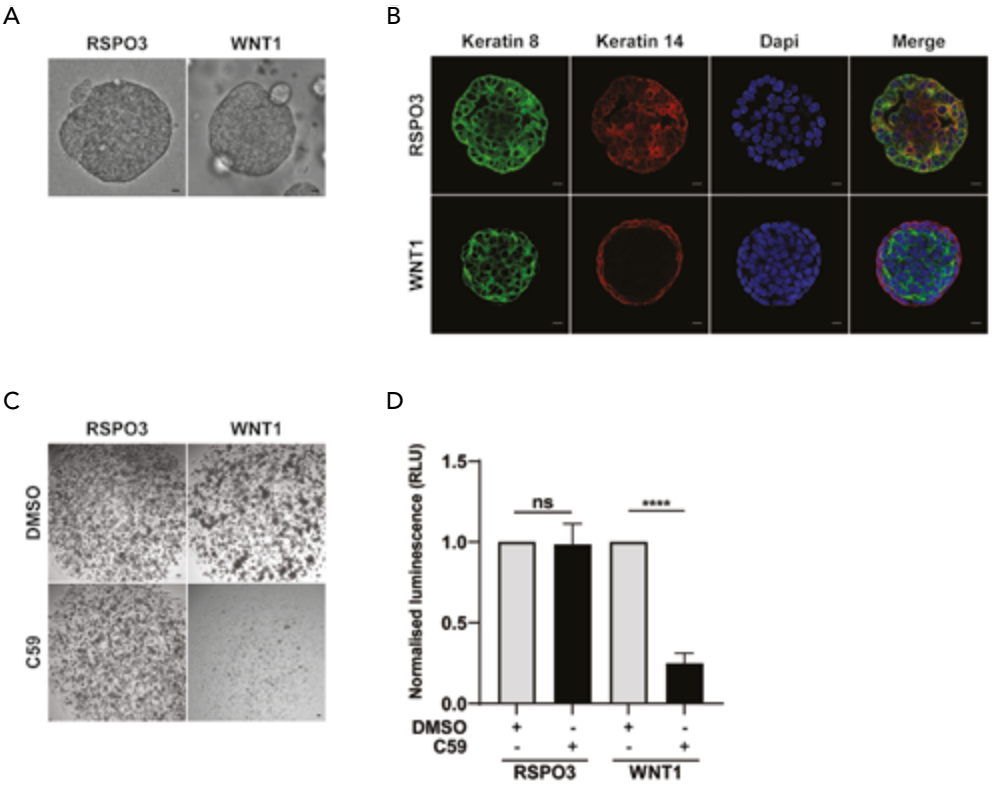


Figure 2. RSPO3 tumor organoids are disorganized and grow independently of Wnt signaling. A. Representative brightfield images of RSPO3- and WNT1-driven mammary tumor organoids. Scale bars: 10 µm B. Immunofluorescent staining of K8 and K14 on RSPO3 and WNT1- driven tumor organoids. Scale bars: 10 µm. C. Brightfield images of RSPO3- and WNT1- driven tumor organoids treated with C59 for 5 days. Scale bars: 100 µm. D. Normalized luminescence values of RSPO3- and WNT1- driven tumor organoids treated with C59.

(PORCNi) C59 to inhibit both canonical and non-canonical Wnt signals. Interestingly, C59 had no effect on the growth of RSPO3 tumor organoids, indicating that RSPO3 driven mammary tumor cells proliferate independent of Wnt signals (Figure 2C,D). Expectedly, the growth of WNT1 tumor organoids was drastically inhibited by C59 (Figure 2C,D). Together these data demonstrate that RSPO3 mammary tumor-derived organoids recapitulate the disorganized *in vivo* tumor phenotype and grow independent of the Wnt signaling route.

RSPO3 potentiates proliferation of human breast cancer cells independently of Wnt signaling

To extrapolate above findings to human cells, we generated model systems of human breast and breast cancer, in which RSPO3 (tagged by Flag-HA) expression can be induced

by doxycycline (Figure 3A). We used the non-malignant breast epithelial cell line MCF10A, and the luminal breast cancer cell lines MCF7 and T47D, in which correct regulation of inducible RSPO3 overexpression upon doxycycline was confirmed by Western blot (Figure 3B). Upon culturing these cell lines in 3D BME matrices, we consistently found that in all three models, RSPO3 overexpression induced a significant increase in growth (Figure 3C,D). To synchronously visualize canonical Wnt pathway activation, we used a reporter construct containing 7 TCF sites with GFP[30]. In all three models we noticed that despite the growth stimulatory phenotype caused by RSPO3, RSPO3 expression did not induce TCF reporter expression by itself, but only when combined with Wnt3a ligand stimulation (Figure S3 A-C). These findings show that RSPO3 promotes the growth of non-malignant breast and breast cancer cell lines independent of canonical Wnt signaling. An alternative Wnt/β-catenin reporter with luciferase as readout confirmed these results (Figure S3 D-F)[30].

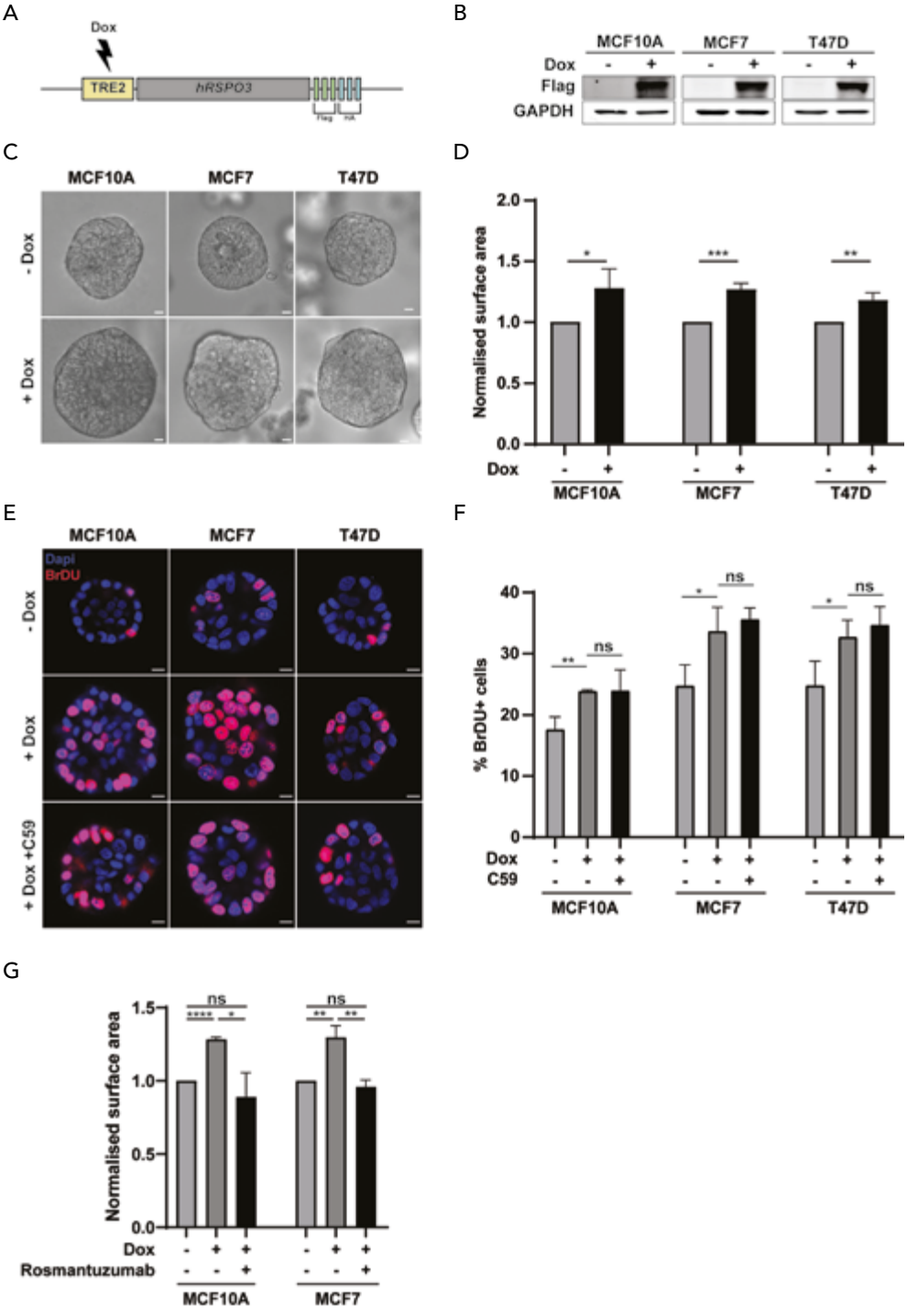
To affirm the growth regulatory effect of RSPO3 to proliferation, BrdU incorporation was subsequently assessed. In accordance with the above results, all three cell lines show an increase in the percentage of $BrdU^+$ cells upon RSPO3 overexpression, demonstrating that RSPO3 promotes proliferation of human breast (cancer) cells (Figure 3E,F). Moreover, C59-mediated inhibition of Wnt signaling did not affect the proliferation stimulatory effect of RSPO3 in all three cell lines (Figure 3E,F), confirming that RSPO3 drives growth and proliferation of human breast cancer cells in a Wnt-independent fashion.

To test whether direct targeting of RSPO3 is a more effective strategy to inhibit growth, we executed the 3D growth experiment in the presence of the therapeutic anti-RSPO3 antibody Rosmantuzumab (OMP-131R10) that was previously tested in a clinical trial with colorectal cancer patients[31]. Importantly, treatment with Rosmantuzumab successfully inhibited the growth stimulatory effect of RSPO3 in both MCF10A and MCF7 cell lines (Figure 3G), exemplifying the specificity of the obtained results.

RSPO3-tumor organoids are highly invasive, independently of Wnt signaling

As previously reported, RSPO3 overexpression induces mammary carcinomas that typically harbor EMT features and metastasize to the lungs[18]. To investigate the invasive potential *in vitro*, organoids derived from RSPO3-driven mammary tumors were placed in a Collagen-I dense matrix. RSPO3 tumor organoids were highly invasive, rapidly forming protrusions within 24 hours (Figure 4A). In contrast, WNT1 tumor organoids failed to invade in a collagen matrix, even after 5 days, further confirming that the invasive phenotype is a feature specific for RSPO3 driven mammary tumors. Invasive RSPO3 tumor organoids retained a disorganized K8 and K14 expression pattern, with the invading strands of RSPO3 tumor organoids containing a mixture of $K8^+$, $K14^+$ and double positive cells (Figure 4B). WNT1 tumor organoids maintained segregation of an outer layer of basal $K14^+$ cells and an inner layer of $K8^+$ luminal cells as in regular matrix (Figure 4B).

To assess whether RSPO3-driven invasion depends on Wnt signaling, we performed the Collagen-I invasion assays in the presence of C59. Although C59 treatment effectively


A
Dox
TRE2
hRSPO3
Flag
HA
B
MCF10A
MCF7
T47D
Dox
Flag
GAPDH
C
MCF10A
MCF7
T47D
- Dox
+ Dox
D
Normalised surface area
Dox
MCF10A
MCF7
T47D
E
MCF10A
MCF7
T47D
Dapi
BrDU
- Dox
+ Dox
+ Dox +C59
F
% BrDU+ cells
Dox
C59
MCF10A
MCF7
T47D
ns
G
Normalised surface area
Dox
Rosmantuzumab
MCF10A
MCF7
ns

inhibited the Wnt pathway (Figure S2B), the invading capability of RSPO3-driven tumor organoids was unaffected by C59 (Figure 4C,D). This indicates that in addition to growth, RSPO3 also drives invasion independently of the Wnt signaling route.

RSPO3 enhances distant metastasis of human breast cancer cells *in vivo*

To investigate the effects of RSPO3 on human breast cancer cells in a preclinical *in vivo* model, we orthotopically injected MCF7 cells with the inducible RSPO3 overexpression construct in the mammary fat pads of mice. When xenografts reached a volume of 50 mm^3, mice were randomized into two groups receiving either normal or doxycycline food to induce RSPO3 overexpression. Tumor growth was followed over time until a volume of 1000 mm^3 was reached (Figure 5A).

Induction of RSPO3 expression did not significantly affect the volume of primary mammary tumors (Figure S4A). This is in contrast to our *in vitro* findings, which consistently indicated that RSPO3 enhances proliferation and growth of breast cancer cells. Most likely, this discrepancy resulted from the use of β-estradiol in the *in vivo* setting, commonly used to sustain tumor growth. β-estradiol also promotes tumor cell growth, masking the growth-stimulatory effect of RSPO3, as mimicked *in vitro* (Figure S4B). Histological analysis of primary tumors developing in control and RSPO3 overexpression mice revealed high levels of K8, ER and Ki67 but no expression of K14, and no differences in expression levels of either staining between groups (Figure S4C).

Interestingly, histological analysis of the lungs revealed enhanced distant metastasis in mice with RSPO3 overexpressing breast cancer cells, reaching an average of 7.8% of the lung area being covered by metastatic lesions, compared to 2.6% without RSPO3 overexpression (Figure 5B,C). Corresponding to the primary tumors, metastatic lesions showed high levels of K8, ER and Ki67, no K14 and no differences in staining pattern between groups (Figure S4D). These data demonstrate that RSPO3 overexpression is sufficient to enhance the metastatic potential of human breast cancer cells *in vivo* and suggest that RSPO3 targeting may be beneficial for intervention with RSPO3 driven breast cancer.

Figure 3. RSPO3 drives proliferation of human breast cancer cell lines. A. Schematic representation of the inducible hRSPO3 Flag/HA construct introduced in breast cell lines. B. Western blot for Flag confirming overexpression of RSPO3 upon treatment with doxycycline for 48h in MCF10A, MCF7 and T47D cell lines. C. Representative brightfield images and D. surface area quantifications of MCF10A, MCF7 and T47D cell lines upon RSPO3 overexpression at day 8 (MCF10A) and day 10 (MCF7, T47D). Scale bars, 10 μm. E. Immunofluorescent staining and F. quantifications of BrdU incorporation in MCF10A, MCF7 and T47D cells upon RSPO3 overexpression and treatment with PORCNi C59. Scale bars, 10 μm. G. Surface area quantifications of MCF10A and MCF7 cell lines upon RSPO3 overexpression and Rosmantuzumab treatment at day 8 (MCF10A) and day 10 (MCF7). Scale bars, 10 μm.

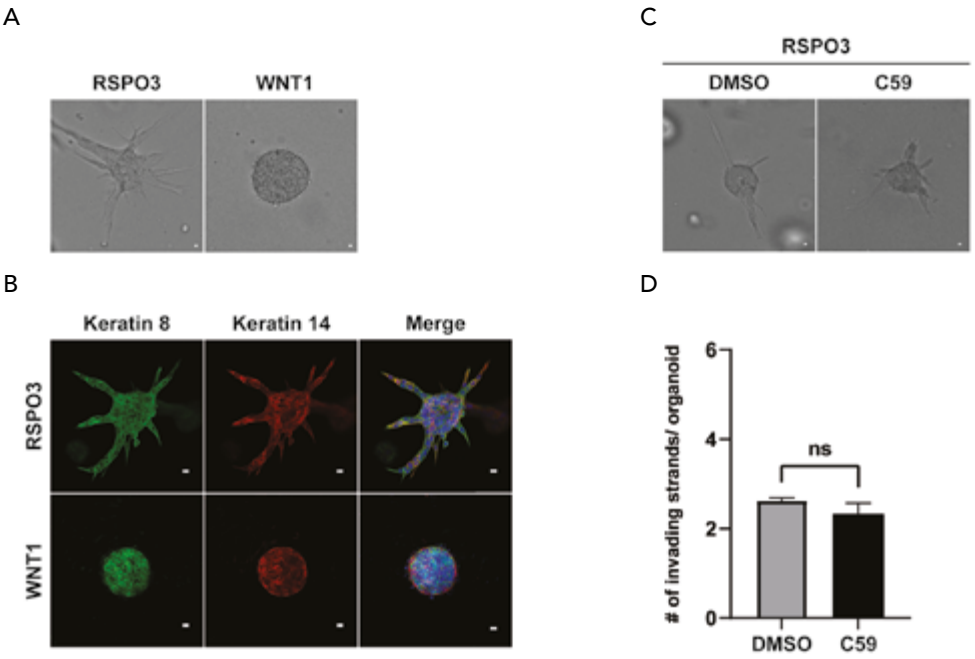


Figure 4. RSPO3 drives invasion independently of Wnt signaling. A. Representative brightfield images of RSPO3- and WNT-1 driven tumor organoids grown in a collagen matrix for 24h. B. Immunofluorescent staining for K8 and K14 on RSPO3- and WNT1- driven tumor organoids grown in a collagen matrix. Images are maximum intensity projections. C. Brightfield images and D. quantifications of the number of invading strands/organoid of RSPO3 driven tumor organoids in collagen upon C59 treatment. The number of invading strands were quantified 24h after collagen embedding in the presence of C59. Scale bars, 10 μm.

Discussion

Recently, we developed a mouse model for RSPO3-driven breast cancer, established RSPO3 as an oncogenic driver of breast cancer and demonstrated the consistent formation of poorly differentiated mammary tumors with high invading potential upon overexpression of *Rspo3* in the mouse mammary glands[18]. In human breast cancer, *RSPO* overexpression is particularly found in TNBC patients and accordingly we reported the presence of copy number alterations of *RSPO2* and *RSPO3* in respectively 23% and 2% of breast cancer patients that associated with ER and PR negative receptor status and high histological tumor grade[9,16–18]. Exploring the potential utility of RSPO3 as an alternative therapeutic target, we aimed to get more insight in the oncogenic activities through which RSPO3 contributes to breast cancer. Previously, we found that RSPO3-driven mammary tumors were poorly differentiated but had limited information on the cellular built-up[18]. Now, we show that RSPO3-driven mammary tumors are highly enriched in luminal progenitor cells marked by CD61. CD61 has been reported to mark luminal progenitor cells with highly enriched tumorigenic potential, as such being proposed as cancer stem cells[28]. Our data

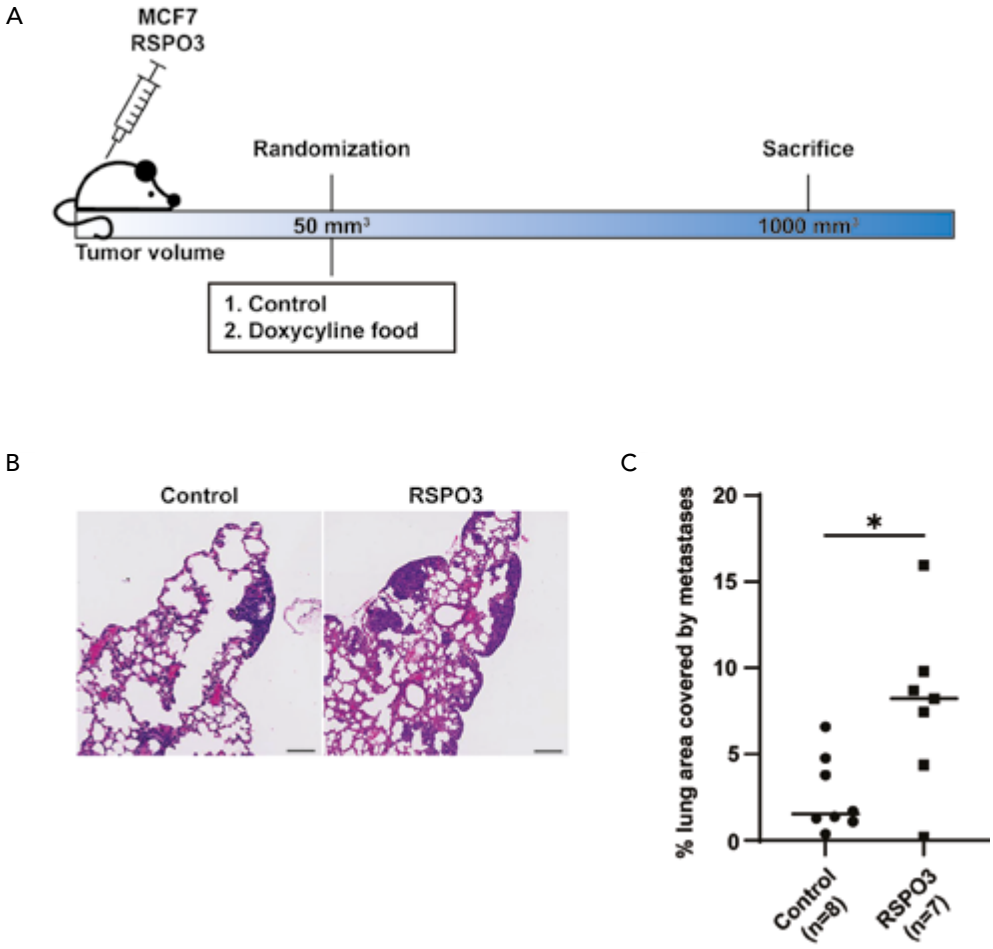


Figure 5. RSPO3 enhances metastasis of human breast cancer cells *in vivo*. A. Schematic overview of the experimental set-up with control or RSPO3 overexpressing breast cancer cells B. H&E stained sections of lungs with metastatic lesions. C. Quantifications of the percentages of lung area being covered by metastatic lesions. Scale bars, 200 µm.

show that RSPO3 drives the aberrant expansion of luminal progenitor cells, potentially cancer stem cells. This stem/progenitor cell expanding activity exerted by RSPO3 in mammary tumorigenesis appears to align with the findings in the intestine, where RSPO3-driven cancer is associated with expansion of crypt stem- and progenitor cells[8,14,15]. This oncogenic activity appears as a deregulated extrapolation of RSPOs normal function in stem cell regulation[32,33]. Despite the seemingly comparable stem cell expanding activity of RSPO3 in the intestine and mammary gland, these organs differ greatly in their stem cell hierarchy, dynamics and regulation, with the mammary epithelium being instructed majorly by steroid hormones. In the mouse mammary gland, steroid hormones induce expression of RSPO1 in luminal progenitor cells, that in turn promotes mammary stem

cell self-renewal in conjunction with WNT4[32,34]. That implies that RSPO1 acts downstream of steroid hormone signals and as such might stimulate stem cell expansion independent of these upstream hormone signals in case of RSPO upregulation. In alignment, RSPO3 overexpression thus induced ER and PR negative mammary tumors highly enriched in $CD61^+$ luminal progenitor cells, or cancer stem cells.

In addition to the progenitor expanding effect exerted by RSPO3, our *in vitro* studies with tumor organoids and human breast cancer cell lines demonstrate that RSPO3 consistently promotes the proliferation, invasion and metastasis of breast cancer cells. With the identification of these oncogenic effects, we show that RSPO3 is involved in breast cancer development and progression, importantly adding up to previous reports that presented *Rspo3* as a tumor initiator in the mammary gland and to the clinical relevance as a potential therapeutic target[18,35–38]. As such, during continuation of our research we focused at the effects of RSPO3 on proliferation, invasion and metastasis, thus at tumor development and progression phases rather than initiation.

We investigated the role of Wnt signaling in RSPO3-driven breast cancer. Hence, potentiation of the Wnt/β-catenin pathway is the best known signaling activity of RSPOs, and in the intestine, this seems to occur during RSPO-driven tumorigenesis and can be successfully targeted using porcupine inhibitors[11–15]. However, in line with their different stem cell hierarchies, cancer developing in the intestine and breast represent very different diseases. Where colorectal cancer classically holds high levels of Wnt/β-catenin activation through *APC* or *CTNNB1* mutations, breast cancer is associated with a relatively lower degree of Wnt/β-catenin activation and underlying mutations are not often found[39–43]. Moreover, we previously reported lower expression of Wnt target genes in RSPO3-driven tumors compared to WNT1-driven tumors, indicating that RSPO3-driven tumors may be less dependent on Wnt signaling activity[18]. Also, the morphology, invasive potential and gene expression profiles of RSPO3-driven mammary tumors differed greatly from that of WNT1 counterparts[18]. In this study, we demonstrate several effects exerted by RSPO3 during breast tumorigenesis and show that all of these were independent of Wnt signaling. No nuclear β-catenin translocation was observed in the RSPO3-driven mouse mammary tumors. With mouse organoid models, we showed that the growth of RSPO3 mammary tumor organoids is unaffected by treatment with PORCNi C59. Also, WNT1 derived organoids are non-invasive, whereas RSPO3 mammary tumor organoids were demonstrated to have high invasive capacity, which again could not be inhibited with C59. Then, with a novel panel of human breast cancer cell lines with inducible RSPO3 expression we showed that RSPO3 promotes growth and proliferation consistently *in vitro*. Utilizing reporters for canonical Wnt signaling, we showed that the growth stimulatory effect induced by RSPO3 did not coincide with reporter activity. Also, C59 was not able to inhibit the RSPO3-induced growth stimulatory effects. Together, these data consistently demonstrate that RSPO3 promotes proliferation and invasion of breast cancer cells in a Wnt-independent fashion. This further indicates that RSPO3 driven mammary tumorigenesis is not reliant on Wnt signaling, excluding both canonical and non-canonical

as PORCNi C59 acts through inhibiting the functionality of all WNT ligands. This implies that in breast cancer with a gain in *RSPO*, indirect targeting with porcupine inhibitors will most likely be ineffective, in contrast to earlier findings in the intestine[11–13]. Instead, our data indicated that direct targeting of RSPO3 using the humanized monoclonal RSPO3 antibody Rosmantuzumab was effective in inhibiting the growth stimulatory effect of RSPO3 both in MCF10A and MCF7 cell lines, providing rationale to further investigate the potential of RSPO3 targeting in breast cancer.

In conclusion, we demonstrate that RSPO3 promotes growth and metastasis of breast cancer cells. As these unfavorable tumorigenic activities are exerted by RSPO3 in a Wnt independent manner, breast cancer patients with a gain in RSPO will not benefit from indirect targeting with Wnt inhibitors, but importantly, will expectedly benefit from treatment directly targeting RSPO3. This study provides solid rationale for (pre)clinical follow-up investigation to further assess the utility of RSPO3 as a therapeutic target in breast cancer.

Acknowledgements

The authors thank the animal facilities of the Netherlands Cancer Institute (NKI) Amsterdam and the Gemeenschappelijk Dierenlaboratorium (GDL) in Utrecht. We are grateful for histology support from the NKI Animal Pathology Department and the UMC Utrecht Pathology Tissue Facility, for technical advice from the NKI Flow Cytometry Facility and technical assistance from Lotte Enserink. This work was financially supported by the Netherlands Organization for Scientific Research (NWO/ZonMW VENI 016.186.138) and the Dutch Cancer Society (KWF Young Investigator Grant 10957).

Author contributions

Eline ter Steege: Investigation, Methodology, Mouse studies, Analysis, Writing. Loes Doornbos: Investigation, Methodology, Mouse studies, Analysis. Peter Haughton: Investigation, Methodology, Analysis. Paul van Diest: Resources, Analysis. John Hilkens: Mouse studies, Resources. Patrick Derksen: Conceptualization, Methodology, Analysis, Resources. Elvira Bakker: Conceptualization, Funding, Supervision, Methodology, Investigation, Analysis, Mouse studies, Writing.

Competing interests

The authors declare no competing interests.

References

1. ter Steege, E. J. & Bakker, E. R. M. The role of R-spondin proteins in cancer biology. *Oncogene* **40**, 6469–6478 (2021).
2. Seshagiri, S. *et al.* Recurrent R-spondin fusions in colon cancer. *Nature* **488**, 660–664 (2012).
3. Shinmura, K. *et al.* RSPO fusion transcripts in colorectal cancer in Japanese population. *Mol. Biol. Rep.* **41**, 5375–5384 (2014).
4. Hashimoto, T. *et al.* EIF3E–RSPO2 and PIEZO1–RSPO2 fusions in colorectal traditional serrated adenoma. *Histopathology* **75**, 266–273 (2019).
5. Mizuguchi, Y. *et al.* Identification of a novel PRR15L-RSPO2 fusion transcript in a sigmoid colon cancer derived from superficially serrated adenoma. *Virchows Arch.* **475**, 659–663 (2019).
6. Sekine, S. *et al.* Comprehensive characterization of RSPO fusions in colorectal traditional serrated adenomas. *Histopathology* **71**, 601–609 (2017).
7. Sekine, S. *et al.* Frequent PTPRK-RSPO3 fusions and RNF43 mutations in colorectal traditional serrated adenoma. *J. Pathol.* **239**, 133–138 (2016).
8. Storm, E. E. *et al.* Targeting PTPRK-RSPO3 colon tumours promotes differentiation and loss of stem-cell function. *Nature* **529**, 97–100 (2016).
9. Chartier, C. *et al*. Therapeutic targeting of tumor-derived r-spondin attenuates b-catenin signaling and tumorigenesis in multiple cancer types. *Cancer Res.* **76**, 713–723 (2016).
10. Fischer, M. M. *et al.* RSPO3 antagonism inhibits growth and tumorigenicity in colorectal tumors harboring common Wnt pathway mutations. *Sci. Rep.* **7**, 1–9 (2017).
11. Li, C. *et al.* Identification of RSPO2 Fusion Mutations and Target Therapy Using a Porcupine Inhibitor. *Sci. Rep.* **8**, 1–9 (2018).
12. Madan, B. *et al.* Wnt addiction of genetically defined cancers reversed by PORCN inhibition. *Oncogene* **35**, 2197–2207 (2016).
13. Picco, G. *et al.* Loss of AXIN1 drives acquired resistance to WNT pathway blockade in colorectal cancer cells carrying RSPO 3 fusions . *EMBO Mol. Med.* **9**, 293–303 (2017).
14. Hilkens, J. *et al.* RSPO3 expands intestinal stem cell and niche compartments and drives tumorigenesis. *Gut* **66**, 1095–1105 (2017).
15. Han, T. *et al.* R-Spondin chromosome rearrangements drive Wnt-dependent tumour initiation and maintenance in the intestine. *Nat. Commun.* **8**, 1–12 (2017).
16. Coussy, F. *et al.* Clinical value of R-spondins in triple-negative and metaplastic breast cancers. *Br. J. Cancer* **116**, 1595–1603 (2017).
17. Tocci, J. M. *et al.* R-Spondin3 is associated with basal-progenitor behavior in normal and tumor mammary cells. *Cancer Res.* **78**, 4497–4511 (2018).
18. ter Steege, E. J. *et al.* R-spondin-3 is an oncogenic driver of poorly differentiate invasive breast cancer. *J. Pathol.* **258**, 289–299 (2022).
19. Wagner, K. U., Ward, T., Davis, B., Wiseman, R. & Hennighausen, L. Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice. *Transgenic Res.* **10**, 545–553 (2001).
20. Tsukamoto, A. S., Grosschedl, R., Guzman, R. C., Parslow, T. & Varmus, H. E. Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. *Cell* **55**, 619–625 (1988).
21. Koorman, T. *et al.* Spatial collagen stiffening promotes collective breast cancer cell invasion by reinforcing extracellular matrix alignment. *Oncogene* **41**, 2458–2469 (2022).

22. Meerbrey, K. L. *et al.* The pINDUCER lentiviral toolkit for inducible RNA interference in vitro and in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 3665–3670 (2011).
23. Borten, M. A., Bajikar, S. S., Sasaki, N., Clevers, H. & Janes, K. A. Automated brightfield morphometry of 3D organoid populations by OrganoSeg. *Sci. Rep.* **8**, 1–10 (2018).
24. Malhotra, G. K. *et al.* The role of Sox9 in mouse mammary gland development and maintenance of mammary stem and luminal progenitor cells. *BMC Dev. Biol.* **14**, 1–11 (2014).
25. Asselin-Labat, M. L. *et al.* Gata-3 is an essential regulator of mammary-gland morphogenesis and luminal-cell differentiation. *Nat. Cell Biol.* **9**, 201–209 (2007).
26. Domenici, G. *et al.* A Sox2–Sox9 signalling axis maintains human breast luminal progenitor and breast cancer stem cells. *Oncogene* **38**, 3151–3169 (2019).
27. Wenjun Guo, Zuzana Keckesova, Joana Liu Donaher, Tsukasa Shibue, Verena Tischler, Ferenc Reinhardt, Shalev Itzkovitz, Aurelia Noske, Ursina Zürrer-Härdi, George Bell, Wai Leong Tam, Sendurai A. Mani, Alexander van Oudenaarden, and R. W. Slug and Sox9 Cooperatively Determine the Mammary Stem Cell State. **27**, 417–428 (2009).
28. Vaillant, F. *et al.* The mammary progenitor marker CD61/β3 integrin identifies cancer stem cells in mouse models of mammary tumorigenesis. *Cancer Res.* **68**, 7711–7717 (2008).
29. Teissedre, B. *et al.* MMTV-Wnt1 and -ΔN89β-catenin induce canonical signaling in distinct progenitors and differentially activate hedgehog signaling within mammary tumors. *PLoS One* **4**, (2009).
30. Fuerer, C. & Nusse, R. Lentiviral vectors to probe and manipulate the Wnt signaling pathway. *PLoS One* **5**, (2010).
31. A Phase 1a/b Dose Escalation Study of the Safety, Pharmacokinetics, and Pharmacodynamics of OMP-131R10.
32. Cai, C. *et al.* R-spondin1 is a novel hormone mediator for mammary stem cell self-renewal. *Genes Dev.* **28**, 2205–2218 (2014).
33. Yan, K. S. *et al.* Non-equivalence of Wnt and R-spondin ligands during Lgr5 + intestinal stem-cell self-renewal. *Nature* **545**, 238–242 (2017).
34. Joshi, P. A. *et al.* RANK Signaling Amplifies WNT-Responsive Mammary Progenitors through R-SPONDIN1. *Stem Cell Reports* **5**, 31–44 (2015).
35. Theodorou, V. *et al.* MMTV insertional mutagenesis identifies genes, gene families and pathways involved in mammary cancer. *Nat. Genet.* **39**, 759–769 (2007).
36. Callahan, R. *et al.* Genes affected by mouse mammary tumor virus (MMTV) proviral insertions in mouse mammary tumors are deregulated or mutated in primary human mammary tumors. *Oncotarget* **3**, 1320–1334 (2012).
37. Klijn, C. *et al.* Analysis of Tumor Heterogeneity and Cancer Gene Networks Using Deep Sequencing of MMTV-Induced Mouse Mammary Tumors. *PLoS One* **8**, 1–10 (2013).
38. Gattelli, A., Zimberlin, M. N., Meiss, R. P., Castilla, L. H. & Kordon, E. C. Selection of Early-Occurring Mutations Dictates Hormone-Independent Progression in Mouse Mammary Tumor Lines. *J. Virol.* **80**, 11409–11415 (2006).
39. Gaspar, C. *et al.* A targeted constitutive mutation in the Apc tumor suppressor gene underlies mammary but not intestinal tumorigenesis. *PLoS Genet.* **5**, (2009).
40. Gaspar, C. & Fodde, R. APC dosage effects in tumorigenesis and stem cell differentiation. *Int. J. Dev. Biol.* **48**, 377–386 (2004).
41. Bakker, E. R. M. *et al.* β-Catenin signaling dosage dictates tissue-specific tumor predisposition in Apc-driven cancer. *Oncogene* **32**, 4579–4585 (2013).

42. van Schie, E. H. & van Amerongen, R. Aberrant WNT/CTNNB1 Signaling as a Therapeutic Target in Human Breast Cancer: Weighing the Evidence. *Front. Cell Dev. Biol.* **8**, 1–14 (2020).

43. Geyer, F. C. *et al.* β-Catenin pathway activation in breast cancer is associated with triple-negative phenotype but not with CTNNB1 mutation. *Mod. Pathol.* **24**, 209–231 (2011).

Supplementary Figures

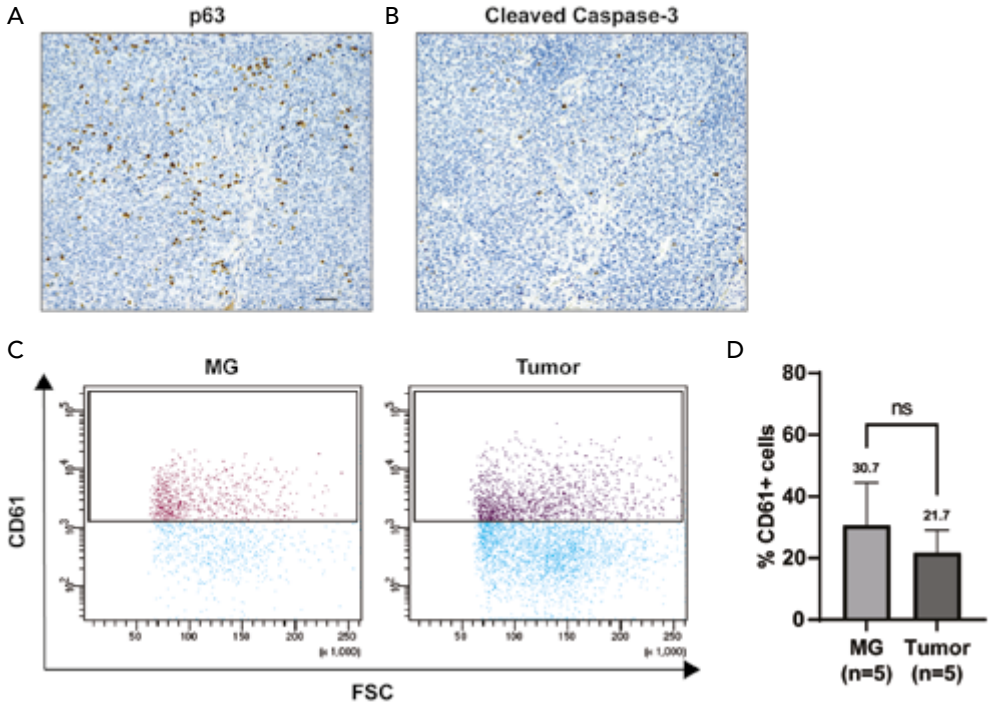


Figure S1. A-B: Immunohistochemical stainings for p63 (A) and Cleaved Caspase-3 (B)(20x objective). C-D: FACS plots (C) and quantifications (D) of the percentages of $CD61^+$ cells in mammary glands (MG) and mammary tumors of MMTV-*Wnt1* mice.

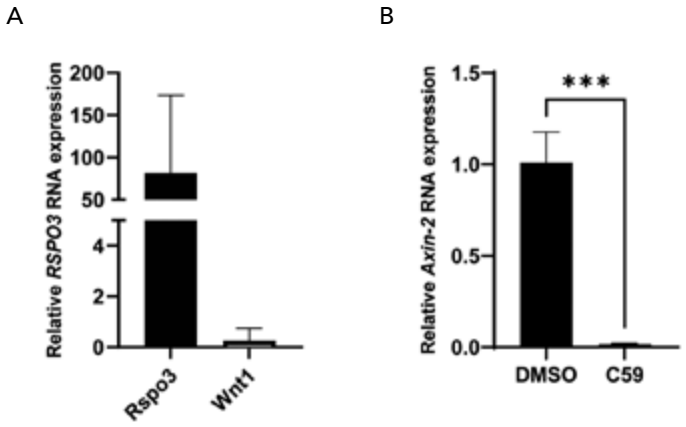
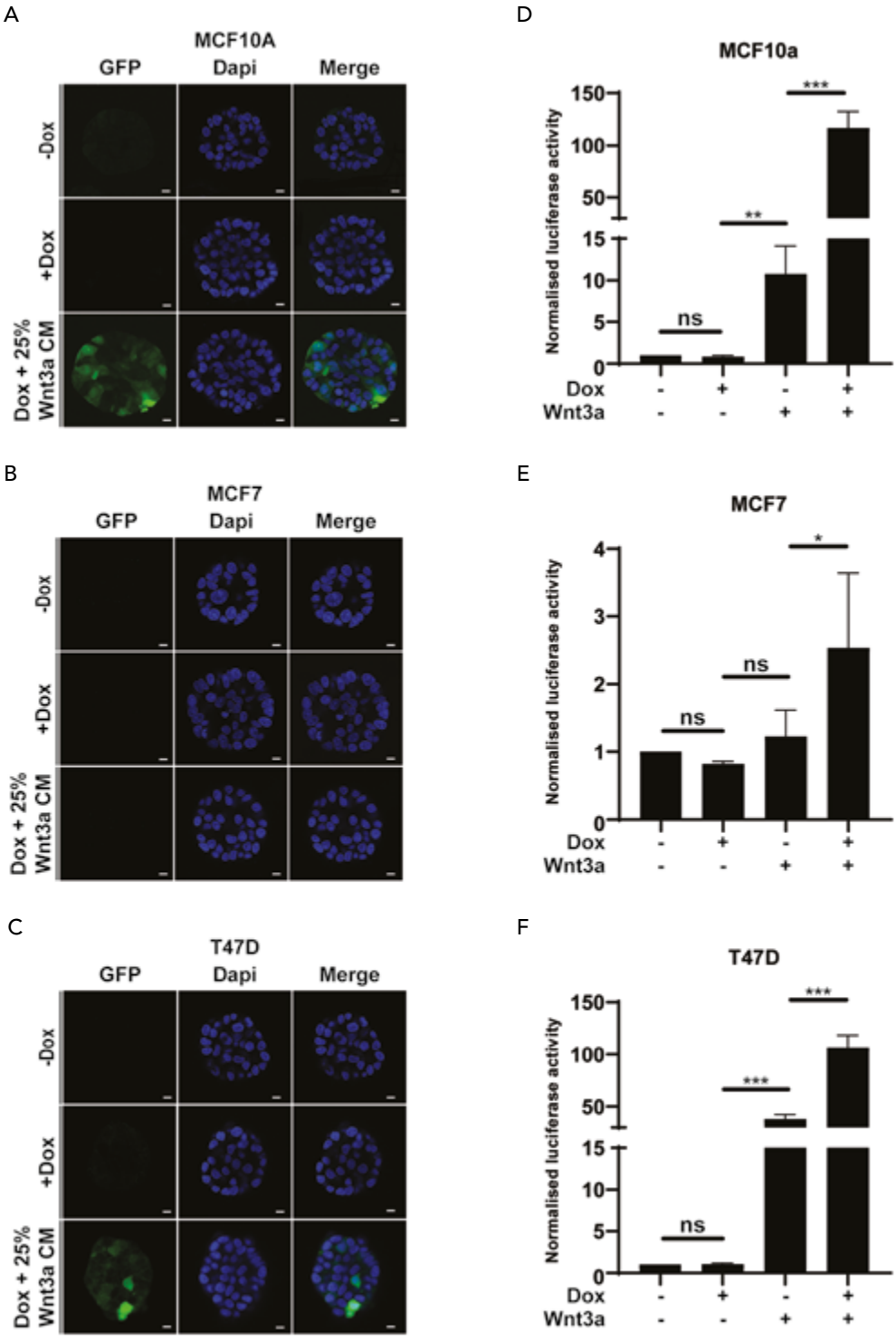


Figure S2. A. Relative *Rspo3* RNA expression in RSPO3- and WNT1- driven tumor organoids (n=3). B. Relative *Axin-2* RNA levels in control and C59 treated RSPO3- driven tumor organoids (n=3).

Figure S3. A-C: Immunofluorescence staining for GFP of MCF10A pInd-hRSPO3;TGP (A), MCF7 pInd-hRSPO3;TGP (B) and T47D pInd-hRSPO3;TGP (C) cells under control conditions (upper panels), upon RSPO3 overexpression (middle panels) and in combination with WNT3a conditioned medium (lower panels). D-F: Normalized luciferase activity measured in MCF10A pInd-hRSPO3;TFP (D), MCF7 pInd-hRSPO3;TFP (E) and T47D pInd-hRSPO3;TFP (F) cells upon RSPO3 overexpression, stimulation with WNT3A conditioned medium, or a combination. Scale bars, 10 μm.


A
MCF10A
GFP
Dapi
Merge
-Dox
+Dox
Dox + 25% Wnt3a CM
B
MCF7
GFP
Dapi
Merge
-Dox
+Dox
Dox + 25% Wnt3a CM
C
T47D
GFP
Dapi
Merge
-Dox
+Dox
Dox + 25% Wnt3a CM
D
MCF10a
Normalised luciferase activity
150
100
50
15
10
5
0
***
**
ns
Dox - + - +
Wnt3a - - + +
E
MCF7
Normalised luciferase activity
4
3
2
1
0
*
ns
ns
Dox - + - +
Wnt3a - - + +
F
T47D
Normalised luciferase activity
150
100
50
15
10
5
0
***
***
ns
Dox - + - +
Wnt3a - - + +

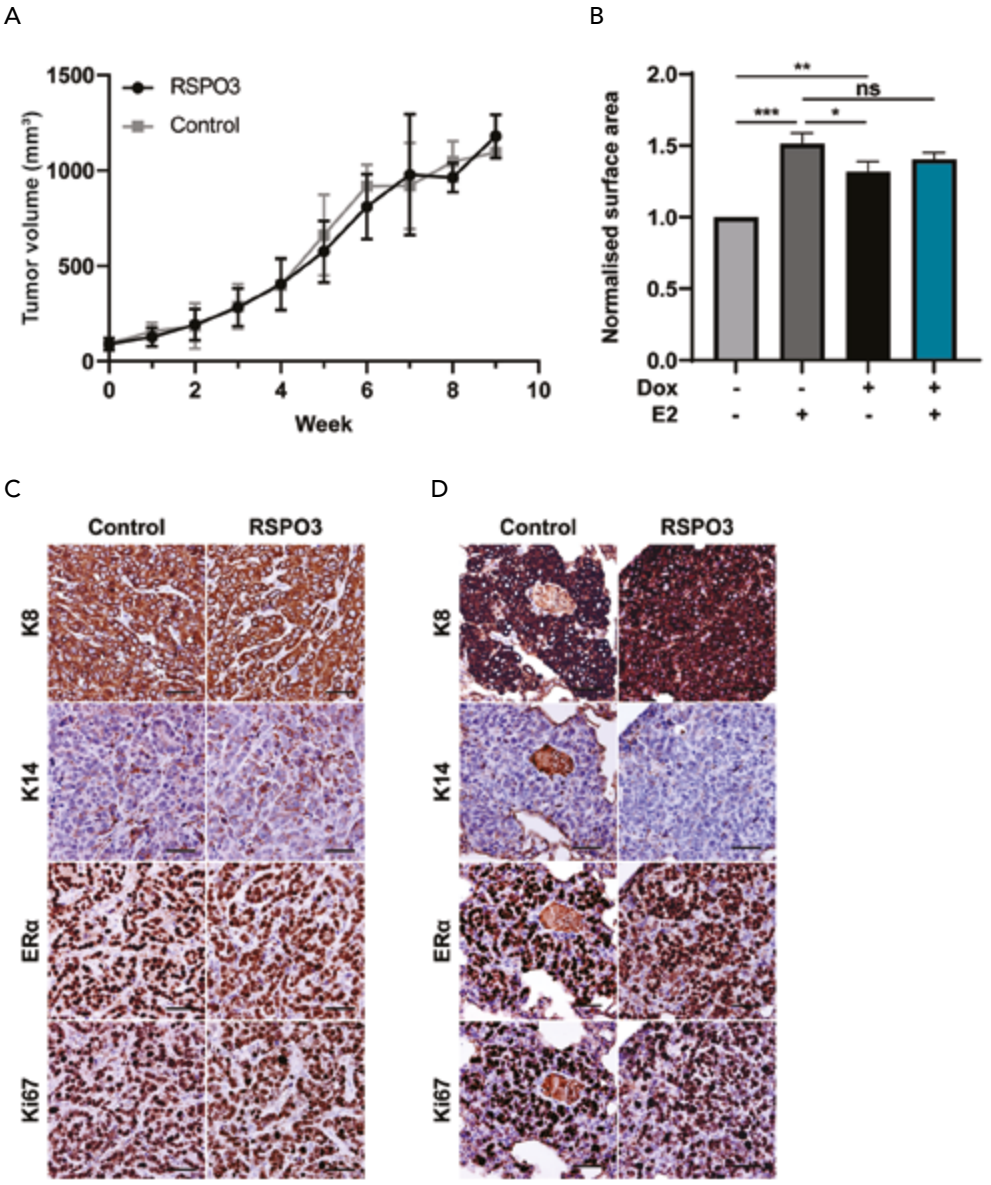


Figure S4. A. Primary tumor volumes measured weekly for control mice and mice with RSPO3 overexpression (week 0 = Randomization). B. Normalized surface area of MCF7 pInd-hRSPO3 cells grown in 3D measured upon RSPO3 overexpression, stimulation with β-estradiol (E2), or in combination. C-D: Immunohistochemical stainings for K8, K14, ERα and Ki67 of primary (C) and metastatic lesions in the lungs (D) of control mice and mice with RSPO3 overexpression. Scale bars: 500 μm.

5

LGR6-DEPENDENT CONDITIONAL INACTIVATION OF E-CADHERIN AND P53 LEADS TO INVASIVE SKIN AND MAMMARY CARCINOMAS IN MICE

Eline J. ter Steege*[1], Thijmen Sijnesael*[1], Lotte Enserink[1], Sjoerd Klarenbeek[2], Wisse E. Haakma[1], Elvira R.M. Bakker[1] and Patrick W.B. Derksen[1]

[1] The Department of Pathology, University Medical Center Utrecht, The Netherlands
[2] Experimental Animal Pathology, Netherlands Cancer Institute, Amsterdam, The Netherlands.
* these authors contributed equally

Published in Neoplasia (2023)
doi: 10.1016/j.neo.2022.100844

Abstract

Tissue-specific inactivation of E-cadherin combined with tumor suppressor loss leads to invasive and metastatic cancers in mice. While epidermal E-cadherin loss in mice induces squamous cell carcinomas, inactivation of E-cadherin in the mammary gland leads to invasive lobular carcinoma. To further explore the carcinogenic consequences of cell-cell adhesion loss in these compartments, we developed a new conditional mouse model inactivating E-cadherin (*Cdh1*) and p53 (*Trp53*) simultaneously in cells expressing the leucine-rich repeat-containing G-protein coupled receptor 6 (Lgr6), a putative epithelial stem cell marker in the skin and alveolar progenitor marker in the mammary gland.

Compound *Lgr6-CreERT2;Cdh1^{F};Trp53^{F}* female mice containing either heterozygous or homozygous *Cdh1^{F}* alleles were bred, and Lgr6-driven Cre expression was activated in pre-puberal mice using tamoxifen. We observed that 41% of the mice (16/39) developed mostly invasive squamous-type skin carcinomas, but also a non-lobular mammary tumor was formed. In contrast to previous K14cre or WAPcre E-cadherin and p53 compound models, no significant differences were detected in the tumor-free survival of *Lgr6-CreERT2* heterozygous *Cdh1$^{F/WT}$;Trp53$^{F/F}$ versus* homozygous *Cdh1$^{F/F}$;Trp53$^{F/F}$* mice (778 versus 754 days, p=0.5). One *Cdh1^{F}* homozygous mouse presented with lung metastasis that originated from a non-lobular and ERα negative invasive mammary gland carcinoma with squamous metaplasia. In total, 2/8 (25%) *Cdh1^{F}* heterozygous and 3/12 (25%) *Cdh1^{F}* homozygous mice developed metastases to lungs, liver, lymph nodes, or the gastro-intestinal tract.

In conclusion, we show that inducible and conditional Lgr6-driven inactivation of E-cadherin and p53 in mice causes squamous cell carcinomas of the skin in approximately 40% of the mice and an occasional ductal-type mammary carcinoma after long latency periods.

Introduction

E-cadherin is the central component of the adherens junction (AJ), a structure that is crucial for epithelial integrity by controlling cell-cell adhesion through homotypic extra-cellular interactions[1]. In line with its central function, loss of E-cadherin expression has been causally linked to tumor development and progression of several cancers such as hereditary diffuse gastric cancer[2,3], invasive lobular breast cancer (ILC) [4–6] and recently, plasmacytoid bladder cancer [7]. Loss of E-cadherin in lobular breast cancer has been studied extensively, showing that mutational inactivation leads to tumor progression through the acquisition of anoikis resistance, mostly through constitutive activation of growth factor receptor signaling and p120-catenin (p120) dependent actomyosin contraction[8–13].

Mammary gland epithelium consists of an outer myoepithelial layer and an inner layer of luminal cells that can be further subdivided in a ductal and an alveolar lineage. Despite this modest heterogeneity, multiple breast cancer subtypes can be distinguished based on histology, suggesting that not the progenitor cell type, but specific genetic lesions define the breast cancer histo-morphological type. Indeed, mammary gland-specific conditional inactivation of E-cadherin leads to the development of lobular-type tumors in mice when combined with loss of p53[5], PTEN[14], or activation of PI3K[15], regardless of whether a luminal whey acidic protein (WAP) Cre or myoepithelial cytokeratin 14 (K14) Cre driver is used. These models, however, do not express the estrogen receptor (ER), a common feature of human ILC[16]. In sum, these data may suggest that the genetic inactivation of E-cadherin drives the development of lobular breast cancer in the mouse mammary gland, and not the progenitor cell type [5,17].

Leucine-rich repeat-containing G-protein coupled receptor 6 (Lgr6) has been identified as a marker of stem cells of the lungs[18], alveolar taste buds[19] and skin[20,21], and associates with tumor development and progression in these organs[22,23]. In the mammary gland, Lgr6 marks progenitor cells that contributes to alveolar expansion during pregnancy[17]. Moreover, Lgr6^{POS} epithelial progenitor cells were reported to underpin the development of luminal ER^{POS} mammary carcinomas in mice upon inactivating *Brca1* and *Trp53* mutations in these cells[17].

Given the reported retention of ER expression in *Lgr6-CreERT2;Brca1^{F};Trp53^{F}*mice, we investigated the consequences of tamoxifen-induced inactivation of E-cadherin and p53 in Lgr6^{POS} cells. Concomitant loss of these key tumor suppressors upon systemic administration of tamoxifen induced the formation of mostly invasive squamous skin carcinomas with a long-term latency. We observe development of a non-lobular mammary tumor in 1 mouse that progressed towards metastatic disease.

Materials and methods

Generation of *Lgr6-EGFP-Ires-creERT2;Cdh1^{F};Trp53^{F}* female mice

Lgr6Cre;Cdh1^{F};Trp53^{F} mice were generated by crossing heterozygous female *Lgr6-EGFP-Ires-creERT2* (Lgr6Cre) mice[20] with male *Cdh1$^{F/F}$;Trp53$^{F/F}$* mice[5]. The resulting

heterozygous *Lgr6*Cre;*Cdh1*$^{F/wt}$;*Trp53*$^{F/wt}$ offspring was backcrossed onto homozygous *Cdh1*$^{F/F}$;*Trp53*$^{F/F}$ mice and intercrossed to produce female *Lgr6Cre;Cdh1*$^{F/wt}$;*Trp53*$^{F/F}$ (n=17) and *Lgr6Cre;Cdh1*$^{F/F}$;*Trp53*$^{F/F}$ (n=22) offspring. Eight-week-old female mice were injected with 100 µL intraperitoneal Tamoxifen (Sigma) (10 mg/mL dissolved in corn oil (Sigma)) three times with two-day intervals to activate Cre recombinase. Mice were monitored weekly and sacrificed when tumors reached a maximum tumor volume of 1,500 mm^3 (mammary tumors), or 1,000 mm^3 (skin tumors), when mice were moribund and displayed severe discomfort, or when mice reached an age of >800 days. Mice that presented multiple tumors were sacrificed when a cumulative tumor volume of 1,500 mm^3 was reached. All animal experiments were performed in accordance with local, national, and European guidelines under permit AVD115002015623 issued by The Netherlands Food and Consumer Product Safety Authority (NVWA) of the Ministry of Agriculture, Nature and Food.

Genotyping

DNA was isolated from ear punches with DirectPCR Lysis Reagent (Ear) buffer (Viagen) containing 4% Proteinase K, and incubated overnight at 56 °C. Proteinase K was inactivated the following day by heating the sample to 95°C. In post-experimental tissues, DNA was isolated using the Qiagen DNeasy blood and tissues kit (Qiagen). Detection of Cre, *Trp53*^{F}, *Trp53*$^\Delta$, *Cdh1*^{F}, and *Cdh1*$^\Delta$ was performed as previously described[5].

Histology and immunohistochemistry

Tissues were fixed in 4% formaldehyde for 24 hrs. and paraffin embedded. Immunohistochemistry (IHC) and hematoxylin eosin (HE) staining were performed on 4 µm thick tissue sections as described previously[5]. For IHC, antigen retrieval was accomplished by boiling for 20 min in a Tris-EDTA pH 9.0 buffer or by proteinase K incubation (10 ug/mL) at 37°C, followed by an overnight primary antibody incubation at 4°C. Sections were then incubated for 30' with secondary ab followed by incubation with liquid permanent Red (DAKO) when required. Hematoxylin was used as a counterstaining. Membranous E-cadherin staining intensity was scored as negative (0) or positive (1). All scoring was performed in a blinded fashion and was performed by at least two observers.

Antibodies

The following antibodies were used: mouse anti-E-cadherin (Clone 36; 1:200; BD Bioscience), rabbit anti-Keratin-14 (Poly19053; 1:10000; BioLegend), rat anti-Keratin 8 (TROMA-I; 1:100; Developmental Studies), rabbit anti-GFP (D5.1; 1:1000; Cell Signaling) and ERα (6F11; 1:100; Invitrogen). The following secondary antibodies were used: rabbit anti-rat HRP (1:100; DAKO), Brightvision anti rabbit-AP (Immunologic), Brightvision anti Mouse-AP (Immunologic).

Results

Inactivation of E-cadherin and p53 in Lgr6^{POS} cells induces tumor formation

To study the oncogenic effect of tumor suppressor inactivation in Lgr6^{POS} progenitor cells, we crossed *Lgr6-EGFP-Ires-creERT2* (Lgr6Cre) mice[20] with conditional E-cadherin and p53 knockout (*Cdh1^{F};Trp53^{F}*) mice[5]. Heterozygous E-cadherin *Lgr6Cre;Cdh1$^{F/wt}$;Trp53$^{F/F}$* and homozygous E-cadherin *Lgr6Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* mice (8-10 weeks old; n=39) were injected with tamoxifen to induce Cre recombinase-mediated inactivation of the conditional alleles in LGR6 expressing cells (Figure 1A). Both heterozygous *Lgr6Cre;Cdh1$^{F/wt}$;Trp53$^{F/F}$* and homozygous *Lgr6Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* mice developed tumors with a median latency of 778 and 732 days, respectively (Figure 1B,C). We observed tumor development in 8 out of 17 (47%) *Lgr6Cre;Cdh1$^{F/wt}$;Trp53$^{F/F}$* and 12 out of 22 (55%) *Lgr6Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* mice up to a period of 800 days, of which most were skin carcinomas (Table 1). Homozygous deletion of *Cdh1^{F}* did not accelerate development of cancer in *Lgr6Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* compared to heterozygous *Lgr6Cre;Cdh1$^{F/wt}$;Trp53$^{F/F}$* mice (p=0.5). The genetic status of *Cdh1* and *Trp53* was determined in all tumors that developed in the *Lgr6Cre;Cdh1$^{F/wt}$;Trp53$^{F/F}$* and *Lgr6Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* mice (Table S1). Homozygous loss of the conditional *Trp53* alleles was detected in all skin and mammary tumors, whereas the conditional *Cdh1* was retained in some tumors that developed in both *Lgr6Cre;Cdh1$^{F/wt}$;Trp53$^{F/F}$* and *Lgr6Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* mice. These findings suggest that, in contrast to previous studies using K14cre[5,16], homozygous loss of E-cadherin does not provide a selective advantage for Lgr6^{POS} cancer stem cells in the skin (Table S1). We also observed the development of lymphomas in 4/22 (18%) *Lgr6Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* mice, but only one lymphoma showed switching (deletion) of the conditional p53 alleles (Table 1 and S1). In contrast to the *Lgr6Cre;Cdh1$^{F/wt}$;Trp53$^{F/F}$* heterozygous mice, one homozygous *Lgr6Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* mouse developed a mammary tumor(1/22, 5%) (Table 1), suggesting that in this model, bi-allelic deletion of *Cdh1* may be detrimental to the induction of mammary tumor formation in Lgr6^{POS} cells. Altogether, our data show that concomitant loss of E-cadherin and p53 in Lgr6^{POS} cells in mice results in the modest formation of skin and an occasional mammary tumor.

Inactivation of E-cadherin and p53 in Lgr6 expressing cells induces skin squamous cell carcinoma

Inactivation of E-cadherin and p53 in Lgr6^{POS} cells induced skin tumor formation in 6/17 (35%) *Lgr6Cre;Cdh1$^{F/wt}$;Trp53$^{F/F}$* mice and 10/22 (45%) *Lgr6Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* mice. Skin tumors were predominantly diagnosed as squamous cell carcinomas (SCC) with either expansive or invasive growth patterns (Table 1) and (Figure 2). Although we observed more invasive carcinomas in the E-cadherin homozygous cohort, this difference was not statistically significant when comparing the development of expansive *versus* invasive carcinomas in *Lgr6Cre;Cdh1$^{F/wt}$;Trp53$^{F/F}$* and *Lgr6Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* mice (Table 1, p=0.11). SCCs were mostly formed in head and neck regions or the left and right flanks with no

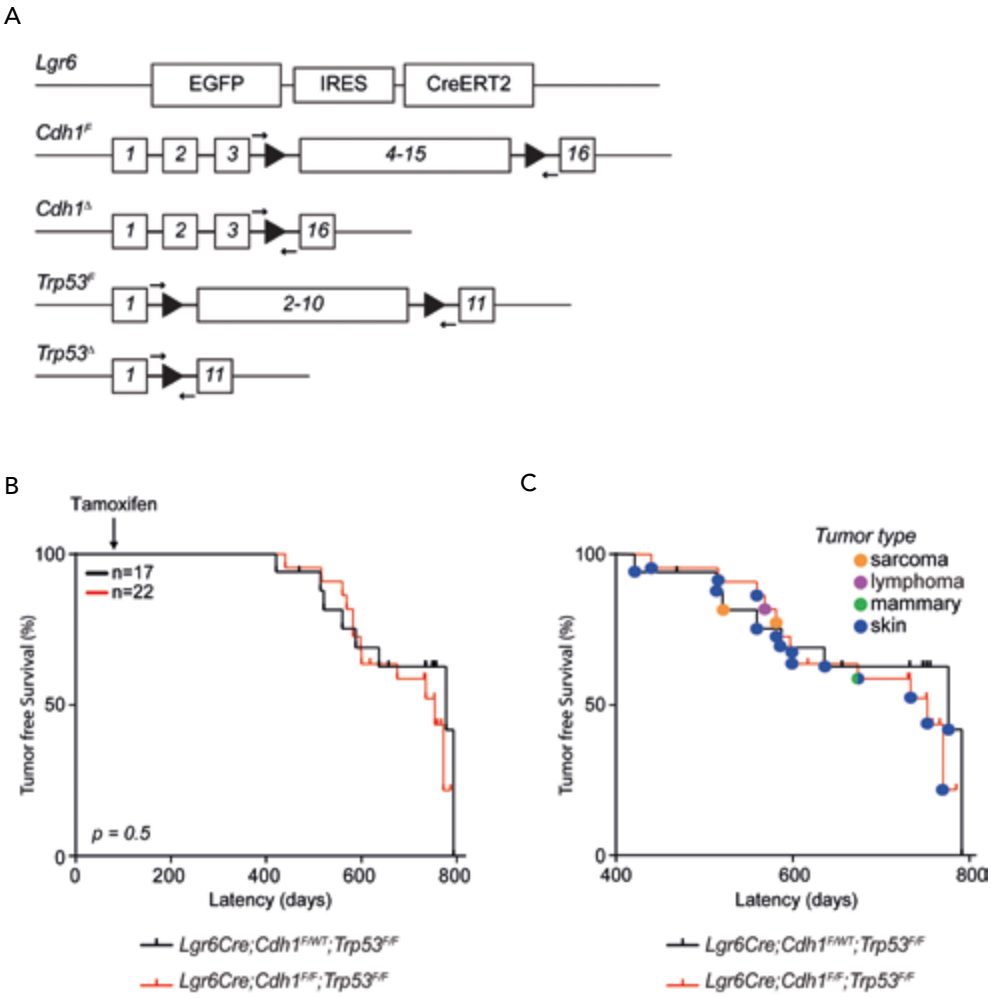


Figure 1. Conditional deletion of *Cdh1* and *Trp53* drives tumor development in mice. A: Schematic model of tamoxifen induced Cre dependent deletion of the conditional $Cdh1^{F}$ and $Trp53^{F}$ alleles in $Lgr6^{POS}$ cells. Eight to ten-week old mice were injected 3 times with tamoxifen to activate Cre in $Lgr6^{POS}$ cells, resulting in deletion of the conditional $Cdh1^{F}$ and $Trp53^{F}$ alleles. Arrows indicate the positions of the genotyping primers. B: Kaplan-Meier tumor free survival curves of $Lgr6Cre;Cdh1^{F/wt};Trp53^{F/F}$ versus $Lgr6Cre;Cdh1^{F/F};Trp53^{F/F}$ female mice (P = 0.5, log-rank test). Arrow indicates the time point of tamoxifen administration. C: Spectrum of tumors formed in $Lgr6Cre;Cdh1^{F/wt};Trp53^{F/F}$ and $Lgr6Cre;Cdh1^{F/F};Trp53^{F/F}$ mice. Tumor types for each individual mouse are visualized in colored bullets. Only tumors with switched $Trp53^{F}$ and/or $Cdh1^{F}$ (Δ) alleles are shown in (B) and (C). For tumor details see Table S1.

differences in tumor sites between both mouse cohorts (Table S1). One $Lgr6Cre;Cdh1^{F/wt};Trp53^{F/F}$ mouse diagnosed with invasive SCC presented with lung metastasis (Figure S1B). Additional IHC confirmed loss of E-cadherin protein expression in the tumors that

Table 1. Inactivation of E-cadherin and p53 in Lgr6^{POS} cells induces carcinoma of the skin and mammary gland.

	Lgr6Cre;Cdh1$^{F/wt}$;Trp53$^{F/F}$	*Lgr6Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$*	χ^2 *p*-value, df
Skin SCC			
Expansive	2/17 (12%)	1/22 (5%)	0.11, 1
Invasive	4/17 (24%)	9/22 (41%)	
Mammary gland			
Carcinoma	0/17 (0%)	1/22 (5%)	
Other			
Necrotizing dermatitis	1/17 (6%)	0/22 (0%)	
Histiocytic sarcoma	0/17 (0%)	1/22 (5%)	
Osteosarcoma	1/17 (6%)	0/22 (0%)	
Leukemic lymphoma	0/17 (0%)	1/22 (5%)	

SCC = Squamous cell carcinoma

developed in *Lgr6Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* mice, in contrast to the tumors that developed in Cdh1 heterozygous female mice (Figure 3A,B). Cytokeratin-14 (CK14) was heterogeneously expressed throughout all SCC samples (Figure 3C,D). Since the conditional *Cdh1* and *Trp53* alleles were deleted specifically in Lgr6-EGFP-Ires-CreERT2 cells, we determined the presence of Lgr6^{POS} cells in the SCC samples using the surrogate GFP marker (see Figure 1A). GFP expressing Lgr6^{POS} cells were detected in the non-neoplastic skin cells surrounding the tumor front, but not in the tumor cells (Figure S2A,B), suggesting that Lgr6 expression does not contribute to tumor maintenance or progression.

Mammary gland carcinoma development in *Lgr6Cre;Cdh1^{F};Trp53^{F}* mice

Because Lgr6^{POS} progenitor cells in the mouse mammary gland have been advocated as a tumor initiating cell[17], we investigated the consequences of *Cdh1* and *Trp53* loss in the *Lgr6Cre;Cdh1^{F};Trp53^{F}* model. In contrast to the frequent formation of skin tumors, we observed incidental mammary carcinoma development in one *Lgr6Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* female mouse (1/22, 5%) (Table 1). The mammary tumor was classified as mammary gland carcinoma with squamous metaplasia (Table 1 and Figure 4A). Additionally to the mammary carcinoma, this mouse also developed a SCC that was localized proximal to the tumor bearing mammary gland, as well as a metastatic lesion in the lungs (Figure S1A and Table S1). Histomorphological analysis indicated that the metastatic cancer cells originated from the mammary carcinoma, as metastatic lesions contained nest-like structures with characteristic nuclear atypia similar to the mammary carcinoma (Figure S1A, left and middle panels). In contrast, cells from the primary invasive skin tumor contained abundant cytoplasm and formed keratin pearls (Figure S1A, right panels), a feature that was also observed in a lung metastasis originating from a primary SCC (Figure S1B). As expected, we did not detect plasma membrane-localized E-cadherin expression in the mammary tumor

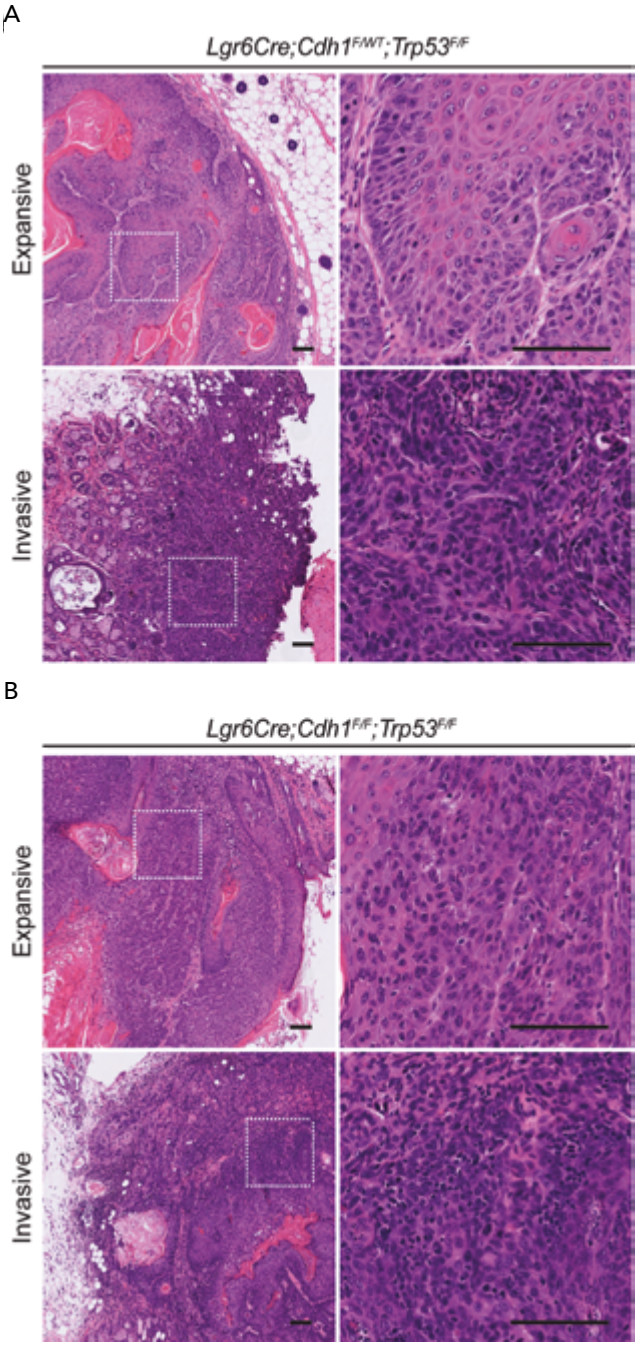


Figure 2. Conditional inactivation of E-cadherin and p53 in Lgr6^{POS} cells induces skin squamous cell carcinoma. A&B: H&E stained sections of skin squamous cell carcinomas (SCC) that developed in *Lgr6Cre;Cdh1$^{F/wt}$;Trp53$^{F/F}$* (A) or *Lgr6Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* (B) female mice with expansive and invasive phenotypes. Insets in the left panels depict the zoomed image in the right panels. Scale bars, 100 μm.

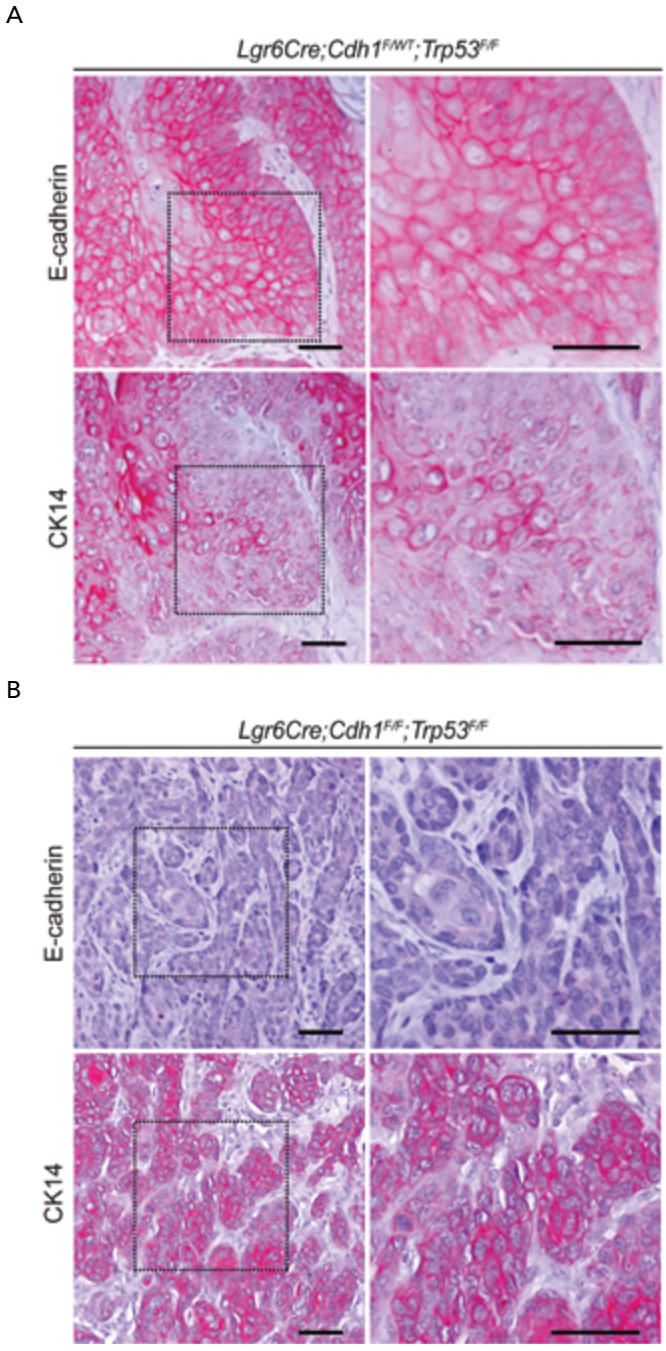


Figure 3. E-cadherin and CK14 expression in SCCs of *Lgr6Cre;Cdh1*$^{F/wt}$*;Trp53*$^{F/F}$ and *Lgr6Cre;Cdh1*$^{F/F}$*; Trp53*$^{F/F}$ mice. A&B: Immunohistochemical analysis on SCC that developed in *Lgr6Cre;Cdh1*$^{F/wt}$*; Trp53*$^{F/F}$ (A) or *Lgr6Cre;Cdh1*$^{F/F}$*;Trp53*$^{F/F}$ female mice (B). Shown are E-cadherin (top panels) and CK14 protein expression (bottom panels). Insets in the left panels depict the zoomed image in the right panels. Scale bars, 100 μm.

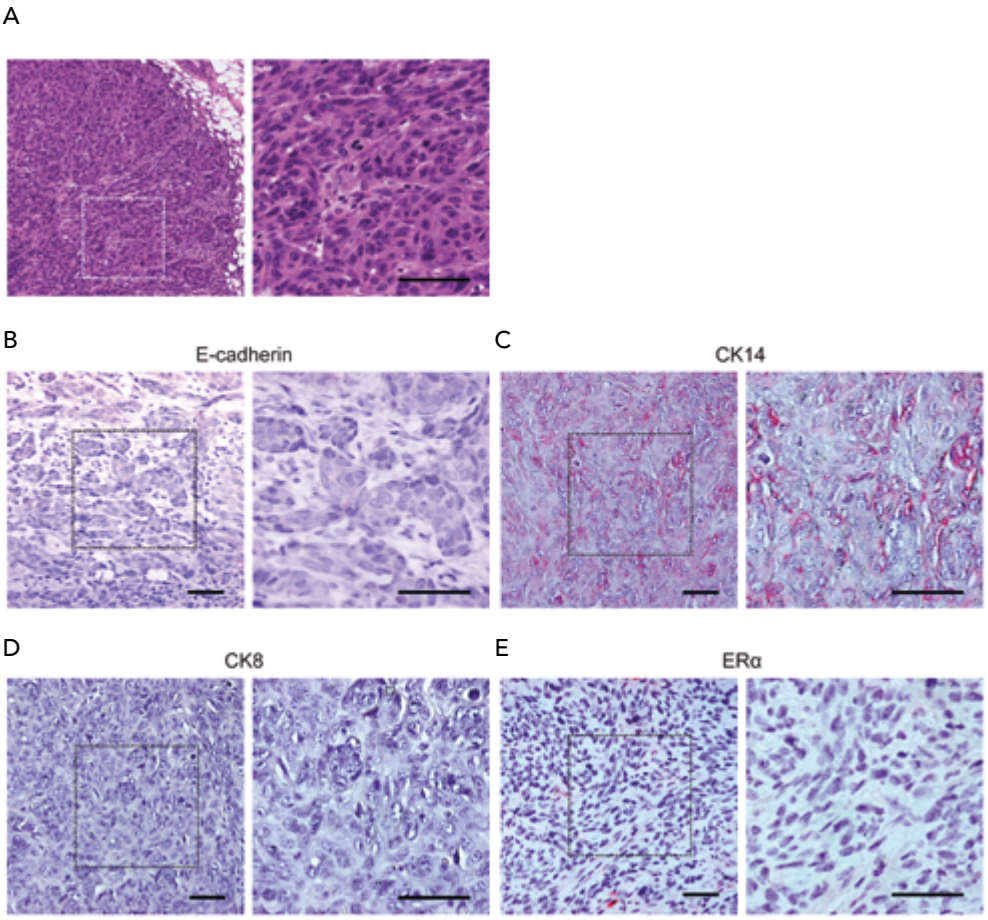


Figure 4. Homozygous deletion of *Cdh1* and *Trp53* in Lgr6^{POS} cells induces sporadic mammary carcinoma formation. A: H&E stained sections of an invasive mammary gland carcinoma that developed in a *Lgr6Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* female mouse. Insets in the left panel depicts the zoomed image in the right panel. Scale bars, 100 µm. B-E: Immunohistochemical analysis of the mammary carcinoma shown in (A), analyzed for protein expression of E-cadherin (B), CK14 (C), CK8 (D) and ERα (E). Insets in the left panels depict the zoomed image in the right panels. Scale bars, 100 µm.

that developed in the *Lgr6Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* female mouse. (Figure 4B). Basal CK14 expression was diffuse while luminal CK8 and ERα were not expressed in the mammary carcinoma (Figure 4C-E). In line with its expression pattern in the skin, we did not observe Lgr6^{POS} cells in the mammary tumors while we did find expression in the basal layer of healthy epithelium (Figure S2 C, D).

In summary, these data indicate that homozygous deletion of *Cdh1* and *Trp53* in Lgr6^{POS} cells induces sporadic formation of non-lobular mammary tumors with metastatic potential.

Discussion

E-cadherin is a cell-cell adhesion molecule that controls tissue homeostasis and epithelial integrity. In the mouse mammary gland, early conditional inactivation of E-cadherin and p53 results in the formation of ILC[5,16]. Unfortunately, mouse lobular tumors and the resulting metastatic disease in these models do not express estrogen receptor (ER), a common feature of human ILC[24]. We therefore developed a compound conditional mouse model to enable somatic inactivation of E-cadherin and p53 in a candidate ER^{POS} luminal progenitor cell type. For this, we used an Lgr6-dependent and inducible Cre recombinase mouse model[20], based on published data that conditional concomitant inactivation of *Brca1* and *Trp53* using Lgr6-Cre leads to ER^{POS} mammary carcinomas with a tumor-free latency period of approximately 1 year[17]. Unfortunately, while one mammary carcinoma developed in the *Lgr6Cre;Cdh1^{F};Trp53^{F}* female mice, we mainly observed the development of squamous skin tumors. Moreover, and in contrast to the published *Lgr6Cre;Brca^{F};Trp53^{F}* model[17], we observed an average tumor-free survival latency of 766 days. This was a surprising finding, given that both studies used the same Lgr6Cre mouse model and compound *Cdh1^{F};Trp53^{F}* mice that have a near identical genetic background as the *Brca^{F};Trp53^{F}*model. Furthermore, our experimental induction of Cre in mice using tamoxifen was based on the published materials of the aforementioned study[17]. The alternative oncogenic drivers or inactivated tumor suppressors in both mouse models can possibly explain the differences in latency time. Although both *Brca1* and *Cdh1* are strongly associated with breast cancer when mutated, it may be that conditional deletion of E-cadherin, even in the context of concomitant p53 inactivation, may not be tolerated in $Lgr6^{POS}$ mammary progenitor cells or provides a selective disadvantage in these cells. Additionally, although we confirmed loss of E-cadherin in our mammary tumor, this carcinoma did not express typical ILC characteristics. Notably, the mammary carcinoma did not express ERα, despite the finding that $Lgr6^{POS}$ cells can function as tumor initiating cells of luminal and ER^{POS} mammary tumors[17]. Given that dual E-cadherin and p53 loss leads to ILC in mice using either CK14 or WAP-dependent Cre drivers[5,16], we initially reasoned that the tumor phenotype is mainly guided by the genetic lesion, not the progenitor or cancer stem cell type. However, the lack of ILC development in our model may render an interplay between cell of origin and mutational load as a more likely hypothesis. Because we detected only one mammary tumor in a cohort of 39 mice, and given that all *WAPcre;Cdh1^{F};Trp53^{F}* female mice develop tumors of which roughly 50% are diagnosed as ILC[16], we consider it more probable that the absence of ILC development is due to the low propensity of $LGR6^{POS}$ mammary cells to develop tumors following E-cadherin loss.

Somatic inactivation of *Cdh1* and *Trp53* using Lgr6Cre predominantly resulted in the formation of invasive SCC in mice. Development of skin SCC in the *Lgr6cre;Cdh1^{F};Trp53^{F}* model is comparable with previous published results, where E-cadherin and p53 were stochastically inactivated using K14Cre[5]. Although both mouse models develop skin SCC, tumor-free survival latencies are considerably longer in the current Lgr6-driven mouse model, and only 41% of the mice develop tumors. Additionally, to skin tumors, we observed

sarcomas and lymphomas in both cohorts. Since these tumors did not have genetic deletion of the *Cdh1* alleles, it is likely to suggest they arose due to age. The relatively low penetration of tumor development in the current model may be due to either the variance in Cre driver activation, or because he skin hosts a more abundant presence of CK14^{POS} *versus* Lgr6^{POS} stem/progenitor cells. Alternatively, the dissimilar localization of CK14^{POS} and Lgr6^{POS} in the hair follicle may underpin the observed differences. While Lgr6^{POS} cells are strictly located to the interfollicular epidermis (IFE), the central isthmus and sebaceous gland, CK14^{POS} cells are located more broadly throughout the hair follicle [20]. Although our data clearly show that homozygous E-cadherin loss induces a more invasive phenotype, this did not lead to a significant difference in tumor development latency between *Lgr6Cre;Cdh1$^{F/wt}$;Trp53$^{F/F}$* and *Lgr6Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* mice. Of note, Lgr6^{POS} cells in the skin contribute to the epidermal lineage and can fully reconstitute hair follicles[20]. Given this essential homeostatic role of Lgr6 in the skin, we anticipate that simultaneous deletion of E-cadherin and p53 attenuates epidermal differentiation of Lgr6^{POS} cells and as such hinders tumor formation. While deletion of E-cadherin and p53 in Lgr6^{POS} cells specifically results in the formation of SCC, we observe that these carcinomas heterogeneously express CK14, but lack expression of Lgr6. The lack of Lgr6 expressing cells in the SCC samples may be a consequence of epidermal cell differentiation, where Lgr6 expressing stem/progenitor cells contribute to tumor onset but not to further progeny in current mutational model. This assumption is in line with data showing that loss of Lgr6 associates with increased proliferation and differentiation of the epidermal lineage[23].

In conclusion, we demonstrate that stochastic loss of E-cadherin and p53 in Lgr6^{POS} cells induces the modest formation of SCC and incidental ductal-type mammary carcinomas in mice. In contrast to previously reported K14cre and WAPcre drivers, our work shows that Lgr6-dependent loss of E-cadherin and p53 does not lead to the development of lobular cancer in the mouse mammary gland. These findings either confirm the existence of multiple different progenitor cell types that underpin the formation of different mammary cancer types or suggest that E-cadherin loss is not tolerated in an Lgr6-driven alveolar progenitor cell type. Notwithstanding these findings, our mouse model represents a valuable tool to study the oncogenic contributions of Lgr6^{POS} cells to the development of invasive skin carcinoma.

Acknowledgements

We would like to acknowledge the UMC Utrecht Pathology Tissue Facility for histology support. We are also grateful for the support of the Utrecht University animal facility, Gemeenschappelijk Dieren Laboratorium (GDL). This work was supported by grants from the Dutch Research Counsel-Talent Scheme VENI (NWO/ZonMW VENI 016.186.138), the Dutch Cancer Society (KWF Young Investigator Grant 10957, KWF-UU-2016-10456), and the European Union's Horizon 2020 FET Proactive program under the grant agreement No. 731957 (MECHANO-CONTROL). This publication is also partially based upon work

from COST action *LOBSTERPOT* (CA19138), supported by COST (European Cooperation in Science and Technology).

Author contributions

EJtS, TS, ERMB and PWBD designed the experiments. Mouse studies: LE. Histology: EJtS, LE, SK and PWBD. Immunohistochemistry: WH, EJtS, TS, and PWBD. ERMB interpreted results and provided input. EJtS, TS and PWBD wrote the manuscript. EJtS and TS contributed equally.

Competing interests

The authors declare no competing interests.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. Meng, W. & Takeichi, M. Adherens junction: molecular architecture and regulation. *Cold Spring Harb Perspect Biol*. **1**, a002899 (2009).
2. Guilford, P. *et al*. E-cadherin germline mutations in familial gastric cancer. *Nature* **392**, 402–405 (1998).
3. Shimada, S. *et al*. Synergistic tumour suppressor activity of E-cadherin and p53 in a conditional mouse model for metastatic diffuse-type gastric cancer. *Gut* **61**, 344-353 (2012).
4. Berx, G. *et al*. E-cadherin is a tumour/invasion suppressor gene mutated in human lobular breast cancers. *Embo J*. **14**, 6107–6115 (1995).
5. Derksen, P.W.B. *et al*. Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. *Cancer Cell* **10**, 437–449 (2006).
6. Moll, R., Mitze, M., Frixen, U.H. & Birchmeier, W. Differential loss of E-cadherin expression in infiltrating ductal and lobular breast carcinomas. *Am. J. Pathol*. **143**, 1731–1742 (1993).
7. Al-Ahmadie, H.A. *et al*. Frequent somatic CDH1 loss-of-function mutations in plasmacytoid variant bladder cancer. *Nat. Genet*. **48**, 356–358 (2016).
8. Bajrami, I. *et al*. E-Cadherin/ROS1 Inhibitor Synthetic Lethality in Breast Cancer. *Cancer Discov.* **8**, 498–515 (2018).
9. Hornsveld, M. *et al*. Restraining FOXO3-dependent transcriptional BMF activation underpins tumour growth and metastasis of E-cadherin-negative breast cancer. *Cell Death Differ.* **23**, 1483–1492 (2016).
10. Nagle, A.M. *et al*. Loss of E-cadherin enhances IGF1-IGF1R pathway activation and sensitizes breast cancers to anti-IGF1R/InsR inhibitors. *Clin. Cancer Res.* **24**, 5165-5177 (2018)
11. Schackmann, R.C.J. *et al*. Cytosolic p120-catenin regulates growth of metastatic lobular carcinoma through Rock1-mediated anoikis resistance. *J. Clin. Invest.* **121**, 3176–3188 (2011).
12. Teo, K. *et al*. E-cadherin loss induces targetable autocrine activation of growth factor signalling in lobular breast cancer. *Sci. Rep.* **8**, 15454 (2018).
13. Rätze, M.A.K. *et al*. Loss of E-cadherin leads to Id2-dependent inhibition of cell cycle progression in metastatic lobular breast cancer. *Oncogene* **41**, 2932-2944 (2022).
14. Boelens, M.C. *et al*. PTEN Loss in E-Cadherin-Deficient Mouse Mammary Epithelial Cells Rescues Apoptosis and Results in Development of Classical Invasive Lobular Carcinoma. *Cell reports* **16**, 2087–2101 (2016).
15. Annunziato, S. *et al*. Modeling invasive lobular breast carcinoma by CRISPR/Cas9-mediated somatic genome editing of the mammary gland. *Gene Dev.* **30**, 1470–1480 (2016).
16. Derksen, P.W.B. *et al*. Mammary-specific inactivation of E-cadherin and p53 impairs functional gland development and leads to pleomorphic invasive lobular carcinoma in mice. *Dis. Model Mech.* **4**, 347–358 (2011).
17. Blaas, L. *et al*. Lgr6 labels a rare population of mammary gland progenitor cells that are able to originate luminal mammary tumours. *Nat. Cell Biol.* **18**, 1346–1356 (2016).
18. Oeztuerk-Winder, F., Guinot, A., Ochalek, A. & Ventura, J.J. Regulation of human lung alveolar multipotent cells by a novel p38α MAPK/miR-17-92 axis. *Embo J.* **31**, 3431–3441 (2012).
19. Ren, W. *et al*. Single Lgr5- or Lgr6-expressing taste stem/progenitor cells generate taste bud cells ex vivo. *Proc. National Acad. Sci.* **111**, 16401–16406 (2014).
20. Snippert, H.J. *et al*. Lgr6 Marks Stem Cells in the Hair Follicle That Generate All Cell Lineages of the Skin. *Science* **327**, 1385–1389 (2010).

21. Füllgrabe, A. *et al.* Dynamics of Lgr6 + Progenitor Cells in the Hair Follicle, Sebaceous Gland, and Interfollicular Epidermis. *Stem Cell. Rep.* **5**, 843–855 (2015).

22. Guinot, A., Oeztuerk-Winder, F. & Ventura, J.J. miR-17-92/p38α Dysregulation Enhances Wnt Signaling and Selects Lgr6+ Cancer Stem-like Cells during Lung Adenocarcinoma Progression. *Cancer Res.* **76**, 4012–4022 (2016).

23. Huang, P.Y. *et al.* Lgr6 is a stem cell marker in mouse skin squamous carcinomas. *Nat. Genet.* **49**, 1624–1632 (2017).

24. Christgen, M. *et al.* Lobular Breast Cancer: Histomorphology and Different Concepts of a Special Spectrum of Tumors. *Cancers* **13**, 3695 (2021).

Supplementary Figures

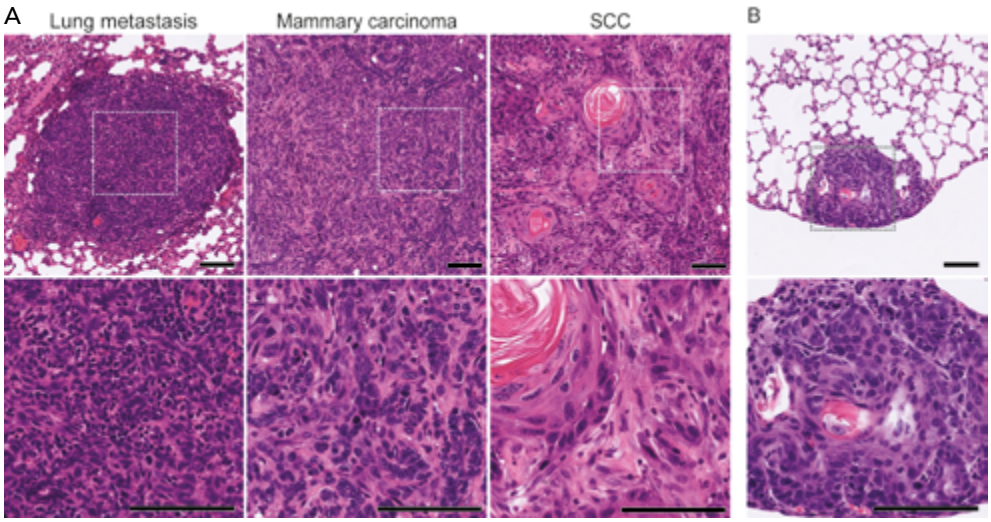


Figure S1. Lung metastasis in tumor bearing *Lgr6Cre;Cdh1*$^{F/wt}$*;Trp53*$^{F/F}$ and *Lgr6Cre;Cdh1*$^{F/F}$*;Trp53*$^{F/F}$ mice. A: H&E stained sections of the lung metastasis (left panel) that developed in a *Lgr6Cre;Cdh1*$^{F/F}$*; Trp53*$^{F/F}$ mouse with a mammary carcinoma (middle panel) and skin SCC (right panel). Histological analysis indicates a mammary origin. B: H&E stained section of the lung metastasis in a *Lgr6Cre;Cdh1*$^{F/wt}$*; Trp53*$^{F/F}$ female mouse that developed SCC. Insets in the upper panels depict the zoomed image in the lower panels. Scale bars, 100 μm.

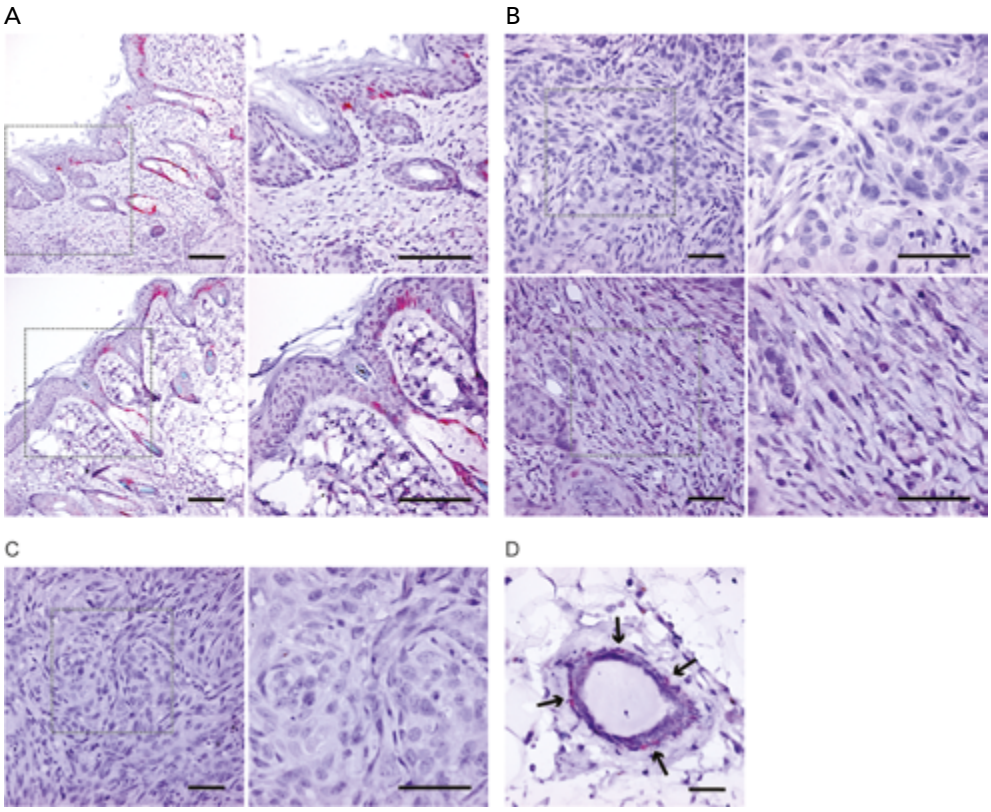


Figure S2. Lgr6 is not expressed in skin and mammary tumors of *Lgr6Cre;Cdh1^{F};Trp53^{F}* mice. A-D: Immunohistochemical sections from the skin (A), SCCs (B), mammary tumor (C) and mammary gland (D) of *Lgr6Cre;Cdh1^{F};Trp53^{F}* mice demonstrating Lgr6 expression in normal epithelium(A, D) but not in the tumors (B, C). Insets in the left panels depict the zoomed image in the right panels. Scale bars, 100 μm.

Table S1.Tumor phenotypes and genotypes in the *Lgr6Cre;Cdh1^{F};Trp53^{F}* mouse model

	Mouse #	Age (Days)	Tumor Pathology	Growth Pattern	Tumor site	Ecad
LGR6Cre;Cdh1$^{F/wt}$;Trp53$^{F/F}$			**Skin**			
	797944	421	SCC	Expansive	Nose	wt/F
	795795	588	SCC	Invasive	Flank (L)	wt/F
	796652	637	SCC	Invasive	Neck	wt/Δ
	797975	560	SCC	Expansive	Head	wt/F
	793513	778	SCC (2)	Invasive	Flank (L) & Neck	wt/Δ
	796654	793	SCC (2)	Invasive	Flank (L) & Nose	wt/Δ
			Other			
	795796	514	Necrotizing dermatitis	-		wt/Δ
	794304	521	Osteosarcoma	Invasive		wt/F
	793522	657	Harderian gland adenoma	Expansive		wt/F
LGR6Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$			**Skin**			
	797305	440	SCC	Invasive	Neck	Δ/Δ
	797304	516	SCC	Invasive	Flank (R)	Δ/Δ
	792529	600	SCC, exophytic	Expansive	Shoulder (R)	Δ/Δ
	795388	560	SCC	Invasive	Nose	Δ/Δ
	795455	582	SCC	Invasive	Hip (L)	Δ/Δ
	794847	598	SCC	Invasive	Shoulder (L)	Δ/Δ
	797277	675	SCC, Mammary gland carcinoma	Invasive	Flank (R), MG 3 (R)	F/F
	792528	754	SCC	Invasive	Chest	Δ/Δ
	794303	772	SCC	Invasive	Neck	F/F
	797964	735	SCC	Invasive	Neck	Δ/Δ
			Other			
	793532	598	Lymphoma	-		F/F
	797952	732	Lymphoma	Systemic		F/F
	797956	732	Lymphoma	Systemic		F/F
	795790	582	Histiocytic sarcoma	Invasive		F/F
	796649	569	Leukemic lymphoma	Systemic		F/F

P53	Metastatic site	Additional Pathology
Δ/Δ	No	Mammary gland: Mild periductular inflammation
Δ/Δ	Yes, Lungs	Uterus: Cystic endometrial hyperplasia. Ovary: Cystic
Δ/Δ	No	Skin: Epidermal hyperplasia. Uterus: Cystic endometrial hyperplasia, Ovary: Cystic
Δ/Δ	No	Mammary gland: Duct ectasia. Liver: Pancreatic lymph node. Lungs: Leukemia
Δ/Δ	No	Uterus: Cystic endometrial hyperplasia. Ovary: Cystic
Δ/Δ	No	Skin: Multifocal epidermal hyperplasia and dysplasia,
Δ/Δ	No	Tail: folliculitis, epidermal hyperplasia, ulceration, crusting
Δ/Δ	Yes, Liver	
F/F	No	Uterus: Cystic endometrial hyperplasia. Ovary: Cystic
Δ/Δ	No	Mammary gland: Mild perivascular inflammation, lymphocytic. Lungs: Focal alveolar epithelial hyperplasia
Δ/Δ	No	Mammmary gland: Mild perivascular and periductal inflammation
Δ/Δ	No	Mammary gland: Mild periductular inflammation, mild hyperplasia of ductular epithelium. Liver: Periportal myeloid immune cell clusters. Uterus: cystic endometrial hyperplasia.
Δ/Δ	No	Ovaries: Cystic. Lungs: Mild perivascular inflammation Uterus: Cystic endometrial hyperplasia. Ovary: Cystic
Δ/Δ	No	Kidney: Mild perivascular immune cell infiltrates. Uterus: Cystic endometrial hyperplasia Ovary: Cystic
Δ/Δ	No	Mammary gland: Perivascular inflammation, lymphocytic. Lungs: Mild perivascular and peribronchial lymphocytic infiltrates. Kidney: Perivascular infiltrates.
Δ/Δ	Yes, Lungs	Uterus: Mild cystic endometrial hyperplasia. Ovary: Cystic. Head: Perivascular lymphocytic infiltrates in parotid salivary gland
Δ/Δ	No	Mammary gland: Duct ectasia, periductal inflammation. Lungs: Perivascular inflammation
Δ/Δ	No	Uterus: Cystic endometrial hyperplasia. Ovary: Cystic. Lymphocytic clusters in many organs
Δ/Δ	No	Lungs: Suspected leukemia
F/F	No	Pancreas: Mild peritonitis and interstitial pancreatitis. Kideny: Renal lymph node; lymphoma
F/F	Systemic	Spleen: Lymphoma, focal osseous metaplasia. Uterus: Cystic endometrial hyperplasia. Ovary: Cystic
F/F	Systemic	Skin: Multifocal epidermal hyperplasia and dysplasia. Mammary gland: adenomyoepithelioma. Liver: Lymphoma, leukemia. Lungs: Lymphoma, leukemia. Uterus: Cystic endometrial hyperplasia. Ovary: Cystic.
Δ/Δ	Yes, Liver, lungs, peritoneum, uterus, lymph nodes	
Δ/Δ	Systemic	

6

GENERAL DISCUSSION

Breast cancer presents as a highly heterogeneous disease represented by multiple different subtypes that are mostly classified based on the expression of the estrogen receptor (ER), the progesterone receptor (PR) and the human epidermal growth factor receptor 2 (HER2). Targeted treatment options for breast cancer mainly focus on antagonizing these receptors, however the great heterogeneous nature of breast cancer and resistance toward current therapeutic treatments challenge their efficacy. Moreover, patients with Triple Negative Breast Cancer (TNBC) that lack the expression of the ER, PR and HER2 receptors remain with poor prognosis due to limited treatment options.

Therefore, further molecular insight is required to identify novel therapeutic targets to increase treatment options for breast cancer patients. In recent years, RSPOs have emerged as clinically relevant oncogenes and demonstrated to hold great potential as therapeutic targets. Although indications of a protumorigenic role for RSPOs in the mammary gland have been reported, direct evidence of RSPOs as mammary oncogenes remained absent. In this thesis, we used various *in vivo* and *in vitro* breast tumor models to address the contributions of RSPO3 and its receptor LGR6 to breast cancer development and progression. We identified RSPO3 as a causal oncogene driving poorly differentiated, invasive breast cancer, presenting itself as a promising therapeutic target for breast cancer.

RSPO3 is an oncogenic driver of invasive breast cancer

Aberrant expression of RSPOs has been linked to tumor development and progression in a multitude of organs as discussed in **chapter 2**. In the mammary gland, the identification of *Rspo1*, *Rspo2* and *Rspo3* as common integration sites of the mouse mammary tumor virus (MMTV) initially proposed a potential oncogenic role for RSPOs in breast cancer[1–4]. In human breast cancer patients, overexpression of *RSPO2*, *RSPO3* and *RSPO4* has furthermore been reported and particularly found associated with TNBC[5–7]. Correspondingly, we described the presence of *RSPO2* and *RSPO3* copy number amplifications in respectively 23% and 2% of breast cancer patients, associated with reduced overall survival and ER and PR negative hormone receptor status in **chapter 3**. Although these data indicate a potential tumorigenic role for RSPOs in the breast, they do not provide direct evidence of RSPOs as oncogenic drivers of breast cancer. Therefore, in **chapter 3**, we assessed the oncogenic potential of RSPO3 by using a mouse model specifically overexpressing *Rspo3* in the mammary glands. Overexpression of *Rspo3* resulted in the consistent formation of mammary tumors, establishing RSPO3 as an oncogenic driver of breast cancer. The RSPO3-driven mammary tumors presented with weak and disorganized expression patterns of basal and luminal markers, indicative of a poorly differentiated status. Further delineation of the cellular expansion upon RSPO3 overexpression in **chapter 4** revealed an aberrant enrichment of $CD61^+$ luminal progenitor cells in the RSPO3-driven tumors. CD61 was previously reported as a marker of luminal progenitor cells, but also identified as marker of cancer stem cells[8,9]. Together, these observations suggest that RSPO3 possibly fuels tumorigenesis by expanding mammary stem/progenitor cells. Such a role for RSPO3 was previously described

in the mouse intestine, where a major expansion of the proliferative stem cell niche was observed upon RSPO3-driven tumorigenesis[10–12]. This aberrant proliferation of stem cells is in line with the fundamental role of RSPO3 to regulate stem cell control in the intestinal stem cell niche[13]. Even though the stem/progenitor cell expanding activities of RSPO3 in the mammary gland seem comparable to those in the intestine, it must be noted that both organs maintain unique stem cell hierarchies that are completely differently regulated, the mammary gland stem cell niche being highly regulated by steroid hormones[14,15]. In the mouse mammary gland, progesterone induces expression of RSPO1 that in turn acts coherently with Wnt4 to promote mammary stem cell self- renewal[14,15]. RSPO3 could potentially act on the mammary gland stem cell pool in a similar fashion, driving the formation of mammary tumors through expansion of mammary stem/progenitor cells upon overexpression. The lack of ER/PR expression in RSPO3-driven mammary tumors, however, may suggest that RSPO3 does not require upstream hormonal signals to exert its tumorigenic effects.

Additional to the expansion of luminal progenitor cells, RSPO3-driven tumors displayed high invasive capability. RSPO3 tumor bearing mice regularly presented with lung metastases and *in vitro,* RSPO3- driven tumor organoids demonstrated high invasiveness in a collagen matrix. Furthermore, overexpression of RSPO3 in human breast cancer cells enhanced distant metastases *in vivo*, demonstrating that RSPO3 provides high invasive potential. These data coincide with the particular association of *RSPO* overexpression with TNBC, a highly invasive form of breast cancer[5–7].

In summary, we have identified RSPO3 as an oncogenic driver of poorly differentiated, invasive, hormone receptor negative mammary tumors, nominating RSPO3 as a clinically relevant candidate for targeted therapy in breast cancer.

RSPO3-driven mammary tumors are distinct from WNT1-driven mammary tumors

To put the characteristics of RSPO3-driven tumors into further perspective, we additionally analyzed mammary tumors formed upon WNT1 overexpression[16]. Interestingly, WNT1-driven mammary tumors demonstrated to differ majorly on histological level compared to their RSPO3 counterparts. WNT1- driven mammary tumors presented with a high degree of differentiation, maintaining distinct basal and luminal layers. In coherence with these data and in contrast to RSPO3-driven tumors, we did not observe enrichment of luminal progenitor cells in WNT1-driven tumors. Moreover, WNT1 tumor bearing mice harbored no lung metastasis and WNT1-driven tumor organoids did not demonstrate invasive characteristics *in vitro*. These striking differences between RSPO3- and WNT1- driven tumors proposed both models as completely different entities and insinuate that RSPO3 and WNT1 possibly drive tumorigenesis through divergent mechanisms. Non-equivalent interactions between RSPO and WNT ligands were formerly described in the intestinal stem cell niche that is highly regulated by instructive signals provided by these ligands[13].

Here, Wnt ligands are not able to induce stem cell self- renewal on their own but instead maintain RSPO receptor expression, creating optimal conditions for RSPOs to regulate stem cell self-renewal and expansion[13]. The significant morphological discrepancies observed between RSPO3- and WNT1-driven mammary tumors may propose that RSPO3 and WNT1 maintain comparable non-interchangeable interactions in the mammary gland such as described in the intestine. Alternatively, RSPO3 and WNT1 may act on different cell types. As described in **chapter 2**, Wnt and RSPO ligands modulate Wnt signaling by binding to distinct receptors. While Wnt ligands bind to Frizzled receptors, RSPOs can potentiate Wnt signaling by binding to LGRs. Frizzled receptors are in general widely expressed by a variety of cell types, but LGRs are mostly found expressed on the membranes of stem and progenitor cells and also in the mammary gland, stem/progenitor cell populations are described that express LGRs[17–23]. Therefore, it may be hypothesized that distinct cell populations in the mammary gland are responsive to WNT and RSPO ligands, hence resulting in the formation of unique tumors upon overexpression. In this case, distinct epithelial cell populations in the mammary gland potentially respond to WNT1 signaling, driving the formation of highly differentiated tumors, while RSPO3 expression mainly affects LGR expressing stem- and progenitor cells, resulting in expansion of this specific pool of cells and the formation of poorly differentiated tumors. Apart from acting on different cell types, RSPO3 and WNT1 may stimulate divergent signaling routes, as such driving the formation of distinct tumors.

RSPO3 drives breast cancer independently of Wnt signaling

Following the identification of RSPO3 as oncogene of breast cancer, we aimed to get more insight in the activities through which RSPO3 drives mammary tumorigenesis. Being identified as agonist of the Wnt/β-catenin signaling route, it is likely to assume that RSPO3 drives tumorigenesis through activation of this pathway. Analysis of the molecular gene expression profiles of RSPO3-driven mammary tumors and its WNT1-driven counterparts in **chapter 3**, however, demonstrated that RSPO3-driven tumors retained significant lower expression levels of Wnt-target genes compared to WNT1-driven tumors, suggesting that RSPO3 -driven tumors might be less dependent on Wnt/β-catenin signaling. We confirmed the expression of Wnt ligands, Wnt- and RSPO receptors in RSPO3- driven tumors, implying that Wnt ligands were available for possible synergy with RSPO3. In **chapter 4**, we further investigated the contributions of Wnt signaling activity in RSPO3-driven tumorigenesis. Here, we demonstrated that RSPO3 consistently promotes the proliferation and invasive potential of different breast cancer models. Interestingly, RSPO3 exerted these tumorigenic effects independently of the Wnt signaling route. Interference with both canonical and non-canonical Wnt signaling using PORCN inhibitor (PORCNi) C59 (Wnt-C59) did not affect growth of RSPO3- tumor derived organoids whilst growth of WNT1-driven tumor organoids was severely disturbed. Moreover, the invasive capability of RSPO3-driven tumor organoids was not affected upon PORCNi treatment, indicating that

RSPO3 promotes growth and invasion independently of the Wnt signaling route. By using several reporters for Wnt activity, we indeed demonstrated that solely RSPO3 was not able to activate the Wnt/β-catenin pathway in human breast (cancer) cell lines. Only in the presence of Wnt ligands, pathway activation was detected. Coherently, inhibition of Wnt signaling did not interfere with RSPO3 induced proliferation in human breast cancer cell lines, confirming that the growth stimulatory and invasive properties of RSPO3 are executed in a Wnt independent manner. These observations are in contrast with the situation in colorectal cancer, where overexpression of RSPO3 is associated with upregulation of Wnt pathway target genes[10,12,24,25]. This discrepancy between the colon and the breast may be explained by the tissue specific Wnt signaling activity required to support tumor growth. The mammary epithelium favors a relative weak level of Wnt signaling activation for tumor growth compared to the colon where hyper-activation of this pathway is a hallmark of colorectal cancer[26–28].

Together, our data demonstrates that RSPO3 drives proliferation and invasion of breast cancer cells independently of the Wnt pathway, indicating that RSPO3 exerts its effects through stimulation of alternative molecular mechanisms. Implications of RSPOs in signaling mechanisms other than the Wnt pathway are still largely undefined and currently RSPOs are described to play a role in one other signaling pathway alternative to the Wnt signaling route; the BMP pathway[29]. RSPO2 and RSPO3 are reported to antagonize this signaling route during early embryonic *Xenopus* development, independently of Wnt pathway activity. RNA sequencing of the RSPO3-driven mouse mammary tumors in **chapter 3** demonstrated that the most significantly enriched molecular and cellular functions were related to cellular signaling, growth and proliferation, development, and movement. In **chapter 4,** we confirmed the growth and proliferative stimulatory effects of RSPO3 *in vitro* and demonstrated that RSPO3 enhances metastasis formation *in vivo*, but efforts to pinpoint specific pathways activated by RSPO3 unfortunately remained inconclusive. Genes found upregulated in RSPO3- driven mouse mammary tumors included *Ret, Mmp1a, Pax9, Gjb1*, transcription factor *Gata6* and signaling molecules *Egf*, *Tgfa, Hbefg* and *Tgfa*. Using our various *in vitro* RSPO3 overexpressing breast cancer models, we were unable to confirm upregulation of these specific genes upon RSPO3 overexpression on protein or RNA level using western blot or qPCR (data not shown). These *in vitro* studies moreover did not lead to an indication that RSPO3 drives breast tumorigenesis through well-known oncogenic pathways such as the Akt and Mapk signaling routes and also the BMP pathway was not affected by RSPO3 overexpression (data not shown). Furthermore, RNA sequencing of RSPO3 overexpressing MCF7 and MCF10A human breast (cancer) cell lines only resulted in a limited number of differentially expressed genes that did not overlap between the two cell lines and did not correspond with the genes found upregulated in the RSPO3-driven tumors (data not shown). The absence of a clear noticeable effect of RSPO3 on molecular signaling could reflect that the effects of RSPO3 are time dependent and potentially occur at a time point prior to our detection. Furthermore, since we mostly studied the effects of RSPO3 on RNA level, it may be that RSPO3 primarily functions on protein level. Alternatively,

RSPO3 may stimulate a pathway that yet has to be discovered, potentially by signaling through receptors different from LGRs. As discussed in **chapter 2**, RSPOs can bind to various receptors, including Heparin Sulfate Proteoglycans (HSPGs)[30–32]. Interestingly, RNA sequencing data demonstrated expression of HSPGs Syndecan 1-4 in the RSPO3- driven mammary tumors and RSPO3 overexpressing MCF7 and MCF10A human breast (cancer) cell lines (data not shown). It may therefore be valuable to investigate a potential role for Syndecans as alternative receptors for RSPO3-driven mammary tumorigenesis, especially since high expression of Syndecan 1 is previously demonstrated to be associated with poor prognosis and reduced overall survival in breast cancer patients[33,34].

Taken together, we demonstrated that RSPO3 does not rely on Wnt signals to drive proliferation and invasion of breast cancer cells. As our efforts to depict distinct signaling mechanisms through which RSPO3 drives breast cancer remained ambiguous, further research is challenged to define the molecular mechanisms driven by RSPO3 in the mammary gland.

RSPO3 as a therapeutic target for breast cancer

The identification of *Rspo3* as an oncogene of poorly differentiated, invasive breast cancer as well as our observations that RSPO3 drives proliferation and invasion of breast cancer cells has emerged RSPO3 as an interesting potential clinical target for breast cancer. Indirect targeting of RSPOs using PORCNi demonstrated to be effective in different colorectal cancer models overexpressing *RSPO2/3,* resulting in inhibition of tumor growth, increased tumor differentiation and downregulation of stem cell and Wnt pathway related genes[35–37]. Interestingly, in our various RSPO3-driven breast cancer models, we did not observe efficacy of PORCNi C59 on growth, proliferation, and invasion, implying that in the case of breast cancer, patients harboring aberrant *RSPO3* expression may not benefit from PORCNi. Instead, breast cancer patients may rather benefit from direct targeting of RSPOs. Anti-RSPO treatment effectively reduced tumor growth of multiple PDX cancer models harboring increased *RSPO* expression as single agent or in combination with chemotherapeutics[7,12,38]. In PDX models of colorectal cancer, this reduced tumor growth was furthermore accompanied by increased differentiation and downregulation of stem cell and Wnt pathway related genes[7,12]. To determine the potential benefit of RSPO targeting in breast cancer, we addressed the efficacy of anti-RSPO3 monoclonal antibody Rosmantuzumab in our RSPO3 overexpressing tumor models. Rosmantuzumab indeed inhibited RSPO3-driven growth *in vitro*, further suggesting that breast cancer patients may benefit from specific RSPO targeting. A clinical trial using anti-RSPO3 monoclonal antibody Rosmantuzumab (OMP-131) was already started on patients with advanced solid tumors and metastatic colorectal cancer[39]. Although anti-RSPO3 was well-tolerated by patients, the trial was ended in phase-1 due to insufficient evidence for clinical benefit[40]. Unfortunately, this study did not discriminate between *RSPO* statuses of patients, which may be the cause of the unobserved clinical benefit of the monoclonal antibody.

Together, we have recognized a potential clinical benefit of RSPO3 targeted treatment, but not PORCNi treatment, for breast cancer patients harboring aberrant *RSPO3* expression. Along with the availability of anti-RSPO3 monoclonal antibodies, opportunities lie ahead to examine RSPO3 as a novel targeted therapeutic option for breast cancer patients with a gain in *RSPO3*.

Contributions of LGR6 to breast cancer development

In the mammary gland, LGR6 has been reported to mark a rare population of unipotent progenitor cells that contribute to alveolar expansion during pregnancy[18]. Additionally, LGR6$^+$ cells were proposed to be tumor-initiating cells as inactivation of tumor suppressors *Brca1* and *Trp53* in these particular cells resulted in the development of ER expressing luminal mammary tumors[18]. These specific features of LGR6$^+$ mammary cells provide a possible new tool to study ER$^+$ luminal breast cancers such as invasive lobular breast cancer (ILC) that currently lack good models. ILC is characterized by loss of E- cadherin and although many mouse models have already been developed for ILC, the tumors and the resulting metastatic disease formed in these mouse models did not express ER, thus lacking a common feature of human ILC[41–44]. In **chapter 5**, we therefore investigated the consequences of E-cadherin and p53 inactivation specifically in LGR6$^+$ cells and developed a *Lgr6-CreERT2;Cdh1^F;Trp53^F* mouse model. Unexpectedly, we did not observe consistent formation of mammary tumors as only one (1/39) mouse formed a mammary tumor with ductal characteristics that lacked ER expression. Several reasons could potentially explain the absence of mammary tumors in our model. First, it may be that conditional deletion of E-cadherin is not tolerated in LGR6$^+$ mammary cells. E-cadherin loss itself is not tolerated in mammary luminal cells and has to coincide with inactivation of a tumor suppressor such as p53 to confer resistance to E-cadherin loss induced apoptosis[41,44,45]. Although we combine E-cadherin loss with inactivation of p53, it is a possibility that LGR6$^+$ mammary cells cannot confer this resistance, hence are not able to drive tumorigenesis upon deletion of E-cadherin and p53. Secondly, the low abundance of LGR6$^+$ cells in the mammary gland may play a role in the absence of ILC formation. In previous ILC models, E-cadherin inactivation was driven by either luminal whey acidic protein (WAP) or myoepithelial Keratin-14 (K14) expressing cells that have a higher abundance in the mammary gland compared to LGR6$^+$ cells, rendering LGR6$^+$ cells with a too low propensity to drive ILC[18,41–44]. Third, mice were injected with Tamoxifen at 8 weeks old to induce E-cadherin and p53 loss. At this stage in mouse mammary development, LGR6 expression is almost exclusively restricted to the basal compartment with the number of LGR6$^+$ cells greatly reducing during adulthood[18]. It therefore may be that we inactivated E-cadherin in a basal subpopulation of LGR6$^+$ cells that rapidly decreases during adulthood and therefore were not able to induce mammary tumor development.

The fact that we did not observe lobular mammary tumors in our *Lgr6;Cdh1^F;Trp53^F* mouse model sheds new light on the role of LGR6$^+$ cells as tumor initiating cells of luminal breast cancer. The formation of ER$^+$ luminal tumors upon inactivation of *Brca1* and *Trp53*

in LGR6^{+} cells suggested that the cell of origin, rather than the genetic lesions determines tumor outcome. However, the lack of (lobular) mammary tumors in our model may rather suggest an interplay between cell of origin and mutational burden that eventually dictates tumor development.

Concluding remarks

We investigated the contributions of RSPO3 and LGR6 to breast cancer development. The work in this thesis casts new light upon the role of LGR6^{+} cells as tumor initiating cells of luminal breast cancer. We identified RSPO3 as a causal driver of poorly differentiated, invasive breast cancer and demonstrated that RSPO3 consistently promotes the proliferation and invasive potential of breast cancer cells. These oncogenic activities of RSPO3 did not depend on canonical and non-canonical Wnt signals, and accordingly, indirect targeting with Wnt inhibitors had no effect, implicating no clinical benefit for breast cancer patients with a gain in *RSPO*. Altogether, the oncogenic features of RSPO3 in the mammary gland described in this thesis nominate RSPO3 as a clinically relevant target for the treatment of breast cancer. Especially since anti-RSPO3 monoclonal antibodies presented with favorable outcomes on preclinical PDX models and additionally inhibited growth in our RSPO3 overexpressing tumor models, promising opportunities await to explore RSPO3 targeting in breast cancer patients with a gain in *RSPO3*.

References

1. Callahan, R. & Smith, G. H. Common integration sites for MMTV in viral induced mouse mammary tumors. *J. Mammary Gland Biol. Neoplasia* **13**, 309–321 (2008).
2. Theodorou, V. *et al.* MMTV insertional mutagenesis identifies genes, gene families and pathways involved in mammary cancer. *Nat. Genet.* **39**, 759–769 (2007).
3. Gattelli, A., Zimberlin, M. N., Meiss, R. P., Castilla, L. H. & Kordon, E. C. Selection of Early- Occurring Mutations Dictates Hormone-Independent Progression in Mouse Mammary Tumor Lines. *J. Virol.* **80**, 11409–11415 (2006).
4. Klijn, C. *et al.* Analysis of Tumor Heterogeneity and Cancer Gene Networks Using Deep Sequencing of MMTV-Induced Mouse Mammary Tumors. *PLoS One* **8**, 1–10 (2013).
5. Coussy, F. *et al.* Clinical value of R-spondins in triple-negative and metaplastic breast cancers.*Br. J. Cancer* **116**, 1595–1603 (2017).
6. Tocci, J. M. *et al.* R-Spondin3 is associated with basal-progenitor behavior in normal and tumor mammary cells. *Cancer Res.* **78**, 4497–4511 (2018).
7. Chartier, C. *et al.* Therapeutic targeting of tumor-derived r-spondin attenuates b-catenin signaling and tumorigenesis in multiple cancer types. *Cancer Res.* **76**, 713–723 (2016).
8. Asselin-Labat, M. L. *et al.* Gata-3 is an essential regulator of mammary-gland morphogenesis and luminal-cell differentiation. *Nat. Cell Biol.* **9**, 201–209 (2007).
9. Vaillant, F. *et al.* The mammary progenitor marker CD61/ 3 integrin identifies cancer stem cells in mouse models of mammary tumorigenesis. *Cancer Res.* **68**, 7711–7717 (2008).
10. Hilkens, J. *et al.* RSPO3 expands intestinal stem cell and niche compartments and drives tumorigenesis. *Gut* **66**, 1095–1105 (2017).
11. Han, T. *et al.* R-Spondin chromosome rearrangements drive Wnt-dependent tumour initiation and maintenance in the intestine. *Nat. Commun.* **8**, 1–12 (2017).
12. Storm, E. E. *et al.* Targeting PTPRK-RSPO3 colon tumours promotes differentiation and loss of stem-cell function. *Nature* **529**, 97–100 (2016).
13. Yan, K. S. *et al.* Non-equivalence of Wnt and R-spondin ligands during Lgr5 + intestinal stem- cell self-renewal. *Nature* **545**, 238–242 (2017).
14. Joshi, P. A. *et al.* RANK Signaling Amplifies WNT-Responsive Mammary Progenitors through R- SPONDIN1. *Stem Cell Reports* **5**, 31–44 (2015).
15. Cai, C. *et al.* R-spondin1 is a novel hormone mediator for mammary stem cell self-renewal.*Genes Dev.* **28**, 2205–2218 (2014).
16. Tsukamoto, A. S., Grosschedl, R., Guzman, R. C., Parslow, T. & Varmus, H. E. Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. *Cell* **55**, 619–625 (1988).
17. Wang, Y. *et al.* Lgr4 regulates mammary gland development and stem cell activity through the pluripotency transcription factor Sox2. *Stem Cells* **31**, 1921–1931 (2013).
18. Blaas, L. *et al.* Lgr6 labels a rare population of mammary gland progenitor cells that are able to originate luminal mammary tumours. *Nat. Cell Biol.* **18**, 1346–1356 (2016).
19. Plaks, V. *et al.* Lgr5-Expressing Cells Are Sufficient And Necessary for Postnatal Mammary Gland Organogenesis. *Cell Rep.* **3**, 70–78 (2013).
20. Van Keymeulen, A. *et al.* Distinct stem cells contribute to mammary gland development and maintenance. *Nature* **479**, 189–193 (2011).
21. Wang, D. *et al.* Identification of multipotent mammary stemcells

by protein C receptor expression. *Nature* **517**, 81–84 (2015).

22. De Visser, K. E. *et al.* Developmental stage-specific contribution of LGR5+ cells to basal and luminal epithelial lineages in the postnatal mammary gland. *J. Pathol.* **228**, 300–309 (2012).
23. Huang, H.-C. & Klein, P. S. The Frizzled family: receptors for multiple signal transduction pathways Gene organization and evolutionary history Characteristic structural features. *Genome Biol.* **5**, 1–7 (2004).
24. Seshagiri, S. *et al.* Recurrent R-spondin fusions in colon cancer. *Nature* **488**, 660–664 (2012).
25. Fischer, M. M. *et al.* RSPO3 antagonism inhibits growth and tumorigenicity in colorectal tumors harboring common Wnt pathway mutations. *Sci. Rep.* **7**, 1–9 (2017).
26. Gaspar, C. *et al.* A targeted constitutive mutation in the Apc tumor suppressor gene underlies mammary but not intestinal tumorigenesis. *PLoS Genet.* **5**, (2009).
27. Gaspar, C. & Fodde, R. APC dosage effects in tumorigenesis and stem cell differentiation. *Int. J. Dev. Biol.* **48**, 377–386 (2004).
28. Bakker, E. R. M. *et al.* -Catenin signaling dosage dictates tissue-specific tumor predisposition in Apc-driven cancer. *Oncogene* **32**, 4579–4585 (2013).
29. Lee, H., Seidl, C., Sun, R., Glinka, A. & Niehrs, C. R-spondins are BMP receptor antagonists in Xenopus early embryonic development. *Nat. Commun.* **11**, (2020).
30. Ohkawara, B., Glinka, A. & Niehrs, C. Rspo3 Binds Syndecan 4 and Induces Wnt/PCP Signaling via Clathrin-Mediated Endocytosis to Promote Morphogenesis. *Dev. Cell* **20**, 303–314 (2011).
31. Lebensohn, A. M. & Rohatgi, R. R-spondins can potentiate WNT signaling without LGRs. *Elife* **7**, 1–18 (2018).
32. Dubey, R. *et al.* R-spondins engage heparan sulfate proteoglycans to potentiate WNT signaling. *Elife* **3**, 1–24 (2020).
33. Barbareschi, M. *et al.* High syndecan-1 expression in breast carcinoma is related to an aggressive phenotype and to poorer prognosis. *Cancer* **98**, 474–483 (2003).
34. Leivonen, M., Lundin, J., Nordling, S., Von Boguslawski, K. & Haglund, C. Prognostic value of syndecan-1 expression in breast cancer. *Oncology* **67**, 11–18 (2004).
35. Li, C. *et al.* Identification of RSPO2 Fusion Mutations and Target Therapy Using a Porcupine Inhibitor. *Sci. Rep.* **8**, 1–9 (2018).
36. Picco, G. *et al.* Loss of AXIN1 drives acquired resistance to WNT pathway blockade in colorectal cancer cells carrying RSPO 3 fusions . *EMBO Mol. Med.* **9**, 293–303 (2017).
37. Madan, B. *et al.* Wnt addiction of genetically defined cancers reversed by PORCN inhibition. *Oncogene* **35**, 2197–2207 (2016).
38. Salik, B. *et al.* Targeting RSPO3-LGR4 Signaling for Leukemia Stem Cell Eradication in Acute Myeloid Leukemia. *Cancer Cell* **38**, 263-278.e6 (2020).
39. A Phase 1a/b Dose Escalation Study of the Safety, Pharmacokinetics, and Pharmacodynamics of OMP-131R10.
40. Bendell, J. *et al.* Initial results from a phase 1a / b study of OMP-131R10 , a first-in-class anti- RSPO3 antibody , in advanced solid tumors and previously treated metastatic colorectal cancer (CRC). *Eur. J. Cancer* **69**, S29–S30 (2016).
41. Derksen, P. W. B. *et al.* Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. *Cancer Cell* **10**, 437–449 (2006).
42. Boelens, M. C. *et al.* PTEN Loss in E-Cadherin-Deficient Mouse Mammary Epithelial Cells Rescues Apoptosis and Results in Development of Classical Invasive Lobular Carcinoma. *Cell Rep.* **16**, 2087–2101 (2016).
43. Annunziato, S. *et al.* Modeling invasive lobular breast carcinoma by CRISPR/Cas9-mediated somatic genome

editing of the mammary gland. *Genes Dev.* **30**, 1470–1480 (2016).

44. Derksen, P. W. B. *et al*. Mammary-specific inactivation of E-cadherin and p53 impairs functional gland development and leads to pleomorphic invasive lobular carcinoma in mice. *DMM Dis. Model. Mech.* **4**, 347–358 (2011).

45. Boussadia, O., Kutsch, S., Hierholzer, A., Delmas, V. & Kemler, R. E-cadherin is a survival factor for the lactating mouse mammary gland. *Mech. Dev.* **115**, 53–62 (2002).

NEDERLANDSE SAMENVATTING

CURRICULUM VITAE

LIST OF PUBLICATIONS

DANKWOORD

Nederlandse samenvatting

Borstkanker is de meest voorkomende kankersoort bij vrouwen, en ondanks dat verbetering van diagnostiek en behandelmethodes hebben geleid tot lagere sterftecijfers is borstkanker nog steeds de voornaamste overlijdensoorzaak door kanker onder vrouwen. De meest voorkomende vorm van borstkanker is invasief ductaal carcinoom (IDC) gevolgd door invasief lobulair carcinoom (ILC), welke ontstaan in respectievelijk de melkgangen versus de melk producerende lobuli. Borstkanker is enorm heterogeen en over de jaren is er veel geïnvesteerd in het verder karakteriseren van borstkanker. Over het algemeen wordt borstkanker onderverdeeld in 5 verschillende subtypes die vooral gebaseerd zijn op basis van de expressie van hormoonreceptoren oestrogeen receptor (ER), progesteron receptor (PR) en de humane epidermale groei factor receptor 2 (HER2). Deze subtypes zijn: Luminaal A (ER^+, PR^+, $HER2^-$), Luminaal B (ER^+, PR^+, $HER2^+$), HER2 verrijkt ($HER2^+$), Basal-like (ER^-, PR^-, $HER2^-$) en Claudin- low (ER^-, PR^-, $HER2^-$). De Basal-like en Claudin-low subtypes brengen geen ER, PR of HER2 tot expressie en worden daarom ook wel triple negatieve borstkanker genoemd. De huidige behandelingsopties voor borstkanker patiënten zijn voornamelijk gebaseerd op de expressie van de ER en HER2 receptoren, waardoor patiënten met triple negatieve borstkanker, zonder expressie van deze receptoren, gelimiteerde behandelingsopties en slechtere prognose hebben. Bovendien zijn tumor heterogeniteit en resistentie tegen therapieën ook factoren die de huidige behandelingsmethodes uitdagen en de overlevingskansen van borstkankerpatiënten negatief beïnvloeden. Hierom hebben we meer inzicht nodig om specifieke en klinisch relevante targets te ontdekken en nieuwe behandelingsmethodes te ontwikkelen.

In deze thesis bediscussiëren we de rol van R-spondin (RSPO) eiwitten, met name RSPO3, in borstkanker ontwikkeling en progressie. RSPOs zijn een familie van gesecreteerde signalerings eiwitten bestaande uit vier participanten: RSPO1-4. Door te binden aan receptoren van de leucine-rich repeat- containing G-protein-coupled receptor-familie (LGR4-6) kunnen RSPOs in combinatie met Wnt eiwitten de activiteit van de Wnt/β-catenin signaleringsroute intensiveren, welke cruciaal is voor correcte stamcel regulatie in het humane lichaam. LGRs komen vaak voor op stam- en voorloper cellen en zijn essentieel gebleken voor het goed functioneren van stamcel niches in meerdere organen. Daarbij is ook aangetoond dat RSPOs vooral belangrijke functies uitvoeren in het controleren van de stamcel zelf-vernieuwing. Coherent aan deze belangrijke rol voor RSPOs in stamcel regulatie is recentelijk steeds meer naar voren gekomen dat afwijkende RSPO expressie geassocieerd is met tumor ontwikkeling in verschillende organen zoals besproken in **hoofdstuk 2**. Hier bediscussiëren we de momentane kennis omtrent de rol van RSPOs in tumor ontwikkeling en progressie in verschillende organen van het humane lichaam zoals de darm, maag en borst. Tot op heden weten we het meeste over de rol van RSPOs in darmkanker, waar in ~10% van de darmkanker patiënten verhoogde expressie van *RSPO2* of *RSPO3* wordt gevonden als gevolg van een fusie met een ander gen (*EIF3E* or *PTPRK*, respectievelijk). Additioneel zijn er in de borst ook aanwijzingen dat RSPOs geassocieerd zijn

met tumor ontwikkeling. Zo hebben eerdere genetische studies in muizen *Rspo1*, *Rspo2* en *Rspo3* aan het licht gebracht als potentiële oncogenen in de borst. Ook hebben patiënten-studies verhoogde expressie van *RSPO2*, *RSPO3* en *RSPO4* beschreven in borstkanker patiënten en dan voornamelijk patiënten met triple negatieve borstkanker. Deze studies beschrijven echter vooral een associatieve, indirecte rol voor RSPOs in borstkanker. In **hoofdstuk 3** beschrijven we aanvullend dat 23% en 2% van de borstkankerpatiënten copynumber amplificaties hebben van *RSPO2* of *RSPO3*, respectievelijk, wat samenhangt met slechtere prognose van de betreffende patiënten. Vervolgens onderzoeken we de consequenties van toegenomen RSPO3 expressie in de borst door gebruik te maken van een innovatief, nieuw muismodel dat *Rspo3* verhoogd tot expressie brengt in specifiek de borstklieren. We tonen aan dat verhoogde RSPO3 expressie consistent leidt tot de ontwikkeling van slecht gedifferentieerde, invasieve borsttumoren, concluderend dat RSPO3 een causaal oncogen is in de borstklier. We vergelijken de RSPO3 gedreven borsttumoren in ons muismodel met borsttumoren die gevormd zijn in muizen die WNT1, een activator van de Wnt/β-catenin signaleringsroute, tot overexpressie brengen in de borstklieren. We vinden grote verschillen in de histologie, morfologie en genetische expressie profielen tussen de twee tumor modellen, implicerend dat RSPO3 en WNT1 tumoren induceren via verschillende mechanismen. Dit onderzoeken we verder in **hoofdstuk 4**, waar we dieper ingaan op de oncogene activiteiten van RSPO3 gedurende borsttumor ontwikkeling. We tonen aan dat RSPO3 gedreven borsttumoren sterk verrijkt zijn met luminale voorloper cellen, suggererend dat ook in de borst RSPO3 mogelijk invloed uitoefent op de stamcel niche. Ook beschrijven we dat RSPO3 groei, proliferatie en invasie van borstkankercellen induceert, onafhankelijk van de Wnt signaleringsroute. Verder demonstreren we in een preklinisch muismodel dat RSPO3 uitzaaiing van luminale, ER^+ borstkankercellen naar de longen verhoogt. In **hoofdstuk 5** onderzoeken we of we een nieuw muismodel voor ILC kunnen ontwikkelen door E-cadherine en P53 te inactiveren in $LGR6^+$ cellen die in de borst zijn gerapporteerd als tumor initiërende cellen van luminale, ER^+ borstkanker. Ondanks dat één muis een ER^-, invasieve ductale borsttumor vormde, observeerden we vooral de formatie van huidtumoren die waren gekarakteriseerd als plaveiselcelcarcinomen met expansieve of invasieve groei.

In zijn geheel draagt deze thesis bij aan een beter begrip over de rol van RSPO3 en LGR6 in borstkanker ontwikkeling en progressie. We hebben RSPO3 geïdentificeerd als causaal oncogen van slecht gedifferentieerde, invasieve borstkanker. Verder hebben we aangetoond dat ondanks dat RSPOs agonisten zijn van de Wnt/β-catenin signaleringsroute, RSPO3 onafhankelijk van deze signaleringsroute functioneert in borstkanker. Het identificeren van RSPO3 als oncogen van borstkanker opent mogelijk nieuwe deuren voor RSPO3 als een potentiele therapeutische target voor de behandeling van borstkanker. Aangezien klinische anti-RSPO3 monoclonale antilichamen al zijn ontwikkeld, zijn er veelbelovende kansen om in de borst RSPO3 te onderzoeken als potentiaal moleculair target. In tegenstelling tot RSPO3 is de rol van LGR6 in borstkanker minder duidelijk

aangezien we in ons muismodel geen constante formatie van borstkankers identificeerden. In **hoofdstuk 6** worden de resultaten van deze thesis verder bediscussieerd.

Curriculum Vitae

Eline ter Steege was born on the 8th of August 1994 in Oldenzaal, the Netherlands. She graduated from Twents Carmel College de Thij in 2012 after which she started the bachelor Biomedical Sciences at the University of Utrecht which she completed in 2015. Following she enrolled in the Biomedical Sciences research Master's program Cancer, Stem cells and Developmental Biology at the University of Utrecht. She performed her first internship in the lab of Dr. Tobias Dansen at the Center for Molecular Medicine, UMC Utrecht under the supervision of Dr. Sasha de Henau. Here, she studied the role of redox regulation in mitosis. For her second internship, Eline visited the lab of Prof. Dr. Sophie Dumont at the University of California, San Francisco for 10 months where she studied the contributions of microtubule occupancy and Spindly on silencing of the mammalian spindle assembly checkpoint. She graduated from the master's program in 2017. After a short intermezzo at Nutricia Research, she started as a PhD-student in the Lab of Dr. Elvira Bakker at the department of Pathology, UMC Utrecht. The results of her PhD work are presented in this thesis. In January 2023, Eline joined GenDx as a technical support specialist.

List of publications

Eline J. ter Steege, Loes W. Doornbos*, Peter D. Haughton*, Paul J. van Diest, John Hilkens, Patrick W.B. Derksen and Elvira R.M. Baker. R-spondin-3 promotes proliferation and invasion of breast cancer cells independently of Wnt signaling. *Cancer letters* 568:216301(2023)

Eline J. ter Steege*, Thijmen Sijnesael*, Lotte Enserink, Sjoerd Klarenbeek, Wisse E. Haakma, Elvira R.M. Bakker and Patrick W.B. Derksen. LGR6-dependent conditional inactivation of E-cadherin and p53 leads to invasive skin and mammary carcinomas in mice. *Neoplasia* 35: 100844 (2023)

Eline J. ter Steege, Mandy Boer, Nikki C. Timmer, Carola M.E. Ammerlaan, Ji-Ying Song, Patrick W.B. Derksen, John Hilkens and Elvira R.M. Bakker. R-spondin-3 is an oncogenic driver of poorly differentiated invasive breast cancer. *The Journal of Pathology* 258:289-299 (2022)

Eline J. ter Steege and Elvira R.M. Bakker. The role of R-spondin proteins in cancer biology. *Oncogene* 40:6469-6478 (2021)

Alexander J. Hale*, **Eline J. ter Steege*** and Jeroen den Hertog. Recent advances in understanding the role of protein-tyrosine phosphatases in development and disease. *Developmental Biology* 428:283-292 (2017).

*Authors contributed equally

Dankwoord

De afgelopen jaren waren interessant, uitdagend, maar ook vooral leerzaam. Ik ben iedereen die mij geholpen en gesteund heeft tijdens mijn PhD traject ontzettend dankbaar. Het tot stand komen van dit proefschrift zou onmogelijk zijn geweest zonder jullie. In het bijzonder zou ik graag de volgende mensen willen bedanken:

Als eerste natuurlijk **Elvira**. Samen zijn wij het Bakker lab gestart. Van muizenlijnen opzetten tot cellijnen maken, het was een hele ervaring. Ook al viel het soms even tegen, samen met ons kleine RSPO-team hebben we er iets moois van gemaakt. Ik wil je ontzettend bedanken dat je mij de kans hebt gegeven om mijn promotietraject in jouw groep te mogen doen. In de afgelopen jaren heb ik veel van je mogen leren, niet alleen op wetenschappelijk gebied, maar ook op andere gebieden en daar ben ik je dankbaar voor. Ik bewonder je kritische blik, doorzettingsvermogen en je talent voor het altijd bedenken van goede verjaardagscadeautjes. Ik benieuwd wat de toekomst je gaat brengen.

Patrick, graag wil ik jou ook bedanken voor de afgelopen jaren. Je dacht altijd mee met mijn projecten en nam de tijd om met mij hierover te brainstormen. Bedankt voor alle kennis die je met mij gedeeld hebt, je kritische blik en je feedback op alle stukken die in dit proefschrift staan. Ook bedankt voor alle leuke momenten in het Derksen lab zoals de bowling uitjes en Secret Santa. Ik genoot altijd erg van de gezonde competitie tijdens het bowlen en het eeuwige speculeren over wie nou wie z'n Secret Santa was.

Paul, Elsken, Sabine en andere leden van de BCRM, bedankt voor al jullie input en interessante discussies tijdens de Breast Cancer Research Meetings.

Mijn paranimfen, **Loes** en **Peter**. Lieve **Loes**, een half jaar na mijn start als PhD student kwam jij het lab versterken als analist. Gelijk bleek al dat wij het ontzettend goed konden vinden met elkaar. Gezamenlijke interesses zoals sport, legpuzzels maken, het opgroeien in een uithoek van het land en het drinken van een lekker biertje bood de fundatie voor een goede relatie. Ook toen de corona-pandemie uitbrak spraken we elkaar regelmatig en wisselden we legpuzzels uit zodat we ons niet hoefden te vervelen. Niet alleen op werkgebied, maar ook op persoonlijk vlak heb je me veel geholpen, waarvoor ik je ontzettend dankbaar ben. Ondanks dat we niet meer samen werken, zien we elkaar gelukkig nog steeds om een van onze favoriete hobby's uit te oefenen; Drankjes drinken op de terrassen van Utrecht. **Peter**, crazy Irish dude. When you joined the lab, we also realized quickly we could get along very well. Soon we started our daily joined bike rides home. Every day we would concur the rainbow road home, telling stories about life, how drunk you got on the weekend and I would always attempt to try to teach you Dutch. For a long time we joked about being authors on each other papers and now dreams finally came true, although I am still waiting for an invitation from you… ;)

I am super grateful to not just have been able to work with you two, but also to have gained two new friends.

Natuurlijk wil ik ook graag mijn andere labgenoten bedanken. Laat ik beginnen bij **Daan**, ofwel Dr. D., zoals ik jou graag noemde. 4,5 jaar lang hebben wij een kantoor(tje) gedeeld waarin vaak (veel te) lange gesprekken werden gevoerd. Soms serieus, soms minder serieus. Ik moet eerlijk zeggen dat ik soms je eindeloze verhalen zonder clou wel mis! Bedankt voor alle fijne gesprekken en het altijd goede advies dat je me hebt gegeven! **Thijs**, Dr. Koorman, m'n andere kantoorgenoot. Je stond altijd klaar om mij te helpen en mee te denken met mijn projecten. Bedankt voor al je wijze woorden, ik heb ze ter harte genomen en meegenomen naar mijn huidige werk. **Lotte**, jouw lach krijg ik niet meer uit mijn hoofd. Soms denk ik dat 'm hoor en dan moet ik gelijk glimlachen. Bedankt voor je aanstekelijke vrolijkheid, al je hulp met de muizen en je heerlijke baksels. **Thijmen** met een h, muizenbuddy, coauteur van een van mijn hoofdstukken. Bedankt voor je goede verhalen, enthousiasme en fijne samenwerking. Heel veel succes nog met de laatste loodjes! **Max**, Maximillian, toen ik mijn PhD begon, was jij de enige andere PhD student. Bedankt dat je me hebt geholpen bij de start. Ik waardeer je harde werk en je doorzettingsvermogen en wens je heel veel succes toe bij het afronden van je PhD. **Wisse**, bedankt voor al je hulp bij de kleuringen. Heel veel succes met je PhD in Amerika, ik hoop dat je een fantastische tijd hebt daar! **Sridevi**, thank you for sharing your knowledge on organoids with me and introducing me to the South-Indian kitchen. I wish you all the best of luck in the future.

My gratitude also goes to other colleagues from the second floor of the Stratenum; (former) members of the **Maurice, Janssen, Klumperman** and **Lens labs,** thank you for all the good conversations, Deutsche Donnertags and karaoke sessions that I had with you in either the 2D or 3D culture labs. Thank you for all the fun lunches, cookie times and the everlasting discussions on which radio station the radios in the labs had to be set to (p.s. still rooting for Q-Music). Each and anyone of you has helped me in some sort of way during my PhD, so thank you very much for that!

Mijn studenten **Mara** en **Elsemieke**. Voor jullie beiden verliep jullie stage iets anders dan jullie initieel bedacht hadden vanwege de pandemie, maar jullie hebben allebei ontzettend hard gewerkt om het tot een succesvol einde te brengen. Ontzettend bedankt voor al jullie hulp bij de projecten en ik wens jullie heel veel succes in jullie verdere carrière.

Een speciale plek ik dit dankwoord verdienen mijn lieve Twentse vriendinnetjes, **Sharon**, **Willemien**, **Merle**, **Florien**, **Marieke** en **Evelien**. Jullie weten allemaal dat het laten zien van mijn emotionele kant niet mijn sterkste punt is, dus ga er maar even goed voor zitten, hier komt een ode van liefde aan jullie: Meiden, wat is het een genot om jullie als vriendinnen te hebben! Ondanks dat jullie wellicht niet altijd begrepen waar ik nou precies mee bezig

was, hebben jullie altijd oprechte interesse getoond in mijn studie en mijn PhD traject. Als ik het allemaal even niet meer zag zitten stonden jullie voor mij klaar en als er dan een experiment eindelijk gelukt was of een paper gepubliceerd, waren jullie altijd nog enthousiaster dan dat ik zelf was. Mede door jullie is het gelukt om dit traject naar een succesvol einde te brengen en ik kan daarom alleen maar boffen met vriendinnen zoals jullie. In het speciaal wil ik graag nog even **Evelien** benoemen, mijn Utrecht buddy. Jij hebt het meeste met mij moeten dealen de afgelopen jaren en dus ook het meeste moeten verduren van iedereen. Bedankt dat je het met mij volhield, je bent een gigantische topper!

After tentamen kletsers, **Jelte**, **Suzanne**, **Saskia** en **Lisette**. We hebben elkaar ontmoet in het eerste jaar van de bachelor Biomedische wetenschappen. Toen spraken we altijd na een tentamen af om even bij te kletsen en het tentamen te bespreken. Nog steeds spreken we regelmatig af, alleen zijn nu de gespreksonderwerpen vaker gerelateerd aan eten, drinken en werk óf bedenken we welke stad we willen bezoeken op onze volgende stedentrip. Ik vind het altijd een feestje om met jullie op stap te gaan en ik hoop dan ook dat nog vele stedentrips zullen volgen. Bedankt voor al jullie steun, bemoedigende woorden en advies dat jullie me hebben gegeven de laatste jaren.

Erik, ook jij hebt veel voor mij betekend gedurende mijn PhD. Ik vond het erg leuk om jou als huisgenoot te hebben. Ik weet dat ik niet altijd een even gemakkelijke huisgenoot was, dus bedankt dat je het met mij hebt volgehouden! Onze gedeelde passie voor lekker eten en goede wijn leidde ertoe dat we er samen regelmatig op uit trokken om nieuwe restaurants in Utrecht te ontdekken. Bedankt voor alle gezellige avonden, etentjes en de vele glazen wijn die we samen hebben gedronken. Ik wens je al het geluk van de wereld toe samen met je lieve Nienke

VVU Dames 5&Heren 5, graag wil ik jullie ook bedanken voor alle support. Volleyballen is voor mij een ontzettend belangrijke uitlaatklep geweest de afgelopen jaren. Bedankt dat ik te laat mocht komen tijdens de trainingen/wedstrijden, omdat er toch nog even een experiment afgemaakt moest worden of omdat ik nog even het lab in was gedoken op zaterdagochtend (of omdat ik gewoon te laat was). Lieve **Dames 5**, bedankt dat jullie altijd naar mijn wetenschappelijke verhalen wilden luisteren, met mij meeleefden en elk mijlpaal met mij gevierd hebben. Bedankt voor alle leuke momenten in het veld, maar ook vooral buiten het veld. **Heren 5**, jullie mogen in dit dankwoord uiteraard ook niet ontbreken. Ondanks dat jullie me soms kopzorgen bezorgen, is het toch altijd weer een feestje met jullie. Ik wil ook jullie ontzettend bedanken voor jullie support en de leuke momenten de afgelopen jaren. Ook al zit training geven er niet meer in, we hebben altijd de zaterdagen nog.

Left over Bo gong gang, **Gaby**, **Hessel**, **Joep** en natuurlijk ook **Jelmer**. Deze club is ooit ontstaan in de studentenkamer op de derde verdieping van het Stratenum en still

going strong (ook al is de club wel een beetje gekrompen). Bedankt voor alle gezellige spelletjesavonden, de goede gesprekken en al het advies. Het was fijn om soms even met een groepje PhD studenten te sparren en ervaringen te delen.

Lieve leden van de BEC, **Bastiaan**, **Mark**, **Michiel**, **Rianne**, **Saskia** en **Suzanne**. Ook al is het al een tijdje geleden dat we de buitenlandse excursie hebben georganiseerd en zelf ook een buitenlandse excursie hebben gemaakt, zijn we nog steeds op de hoogte van elkaars levens. Ik vind het mooi om te zien dat we altijd voor elkaar klaar staan en elkaar steunen waar nodig. Bedankt voor al jullie support, ik kijk uit naar de volgende buitenlandse excursie die we zullen maken.

PhD committee, **Margit**, **Anton**, **Elvin, Sanne**, **Juri** and **Louk**, thank you for all the fun meetings we had while organizing the retreat. Sometimes it felt like we were more busy planning where and when to go for karaoke then organizing the retreat, but despite the pandemic we organized a successful online retreat!

Jurr van Hout van **Fenrir Design**, ook al zeg je elke keer dat het geen moeite was, toch heb je moeite gestoken in het ontwerpen van mijn cover welke echt fantastisch is geworden, mega bedankt!

Lieve papa en mama, lieve **Jan** en **Jenny**, bedankt dat jullie mij altijd onvoorwaardelijk hebben gesteund. Van mijn keuze om in Utrecht te gaan studeren tot mijn keuze om voor een jaar naar Amerika te gaan en vervolgens een PhD traject te beginnen. Jullie hebben mij geleerd om het uiterste uit mijzelf te halen en altijd door te zetten. Bedankt dat jullie altijd in mij hebben geloofd, zonder jullie was dit allemaal niet mogelijk geweest. Broertje, **Jan**, en **Marleen**, jullie zijn er ook altijd voor mij geweest gedurende dit proces. Door onze drukke schema's en de afstand tussen Denekamp en Utrecht was het niet altijd even makkelijk om elkaar te zien. Daarom was het des te leuker als we elkaar wel even konden zien om bij te kletsen en spelletjes te spelen met Lott en Nore.

Lieve **Victor**, als laatste wil ik jou graag bedanken voor al jouw support de afgelopen jaren. Jij was mijn baken van rust in tijden van stress. Met je wijze woorden wist je mij altijd weer met beide benen op de grond te krijgen. Je hield me in balans, motiveerde me op momenten waar nodig, maar remde me ook regelmatig af. Nu mijn PhD avontuur aan een eind is gekomen, is het tijd die van jou te starten en ik kan alleen maar hopen dat ik jou op dezelfde manier kan steunen als jij dat bij mij hebt gedaan. Ik ben trots op je.