DNA AND CELL BIOLOGY
Volume 40, Number 1, 2021

Pp. 70–79
DOI: 10.1089/dna.2020.5585

R-Spondins 2 and 3 Are Overexpressed in a Subset of Human Colon and Breast Cancers

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Wnt signaling is activated in many cancer types, yet targeting the canonical Wnt pathway has been challenging for cancer therapy. The pathway might be effectively targeted at many levels depending on the mechanism by which it has become hyperactive. Recently, mouse genetic screens have found that R-spondins (RSPOs) act as oncogenes. Evidence includes recurrent genomic rearrangements that led to increased *RSPO2* or *RSPO3* expression in human colorectal adenocarcinomas, exclusive of *APC* mutations. RSPOs modulate Wnt signaling to promote epithelial cell proliferation and survival. These secreted proteins modulate Wnt signaling by binding to G-coupled receptors LGR4/5/6, ultimately inhibiting frizzled membrane clearance by RNF43 and ZNRF3. They also exert their function independent of leucine-rich repeat-containing, G protein-coupled receptors (LGRs) by binding to ZNRF3 and RNF43. This results in increased β-catenin concentration that, after translocation to the nucleus, acts as a transcriptional coactivator of genes necessary for proliferation and cell survival. In this article, we aimed to identify the role of *RSPOs* in colon and breast cancers by using *in silico* and *in vitro* studies. We found that expression of *RSPO2* and *RSPO3* at high levels characterized a subset of colorectal cancers (CRCs). *RSPO2* expression was found to characterize a subset of triple-negative breast cancers. In both instances, increased expression of *RSPOs* was associated with an activated Wnt signaling gene expression profile. Furthermore, knockdown of *RSPO2* decreased Wnt signaling and proliferation in human breast cancer cells. Our findings show and confirm that *RSPO2* and *RSPO3* expression is upregulated in a subset of colorectal adenocarcinomas and breast cancers and that both are attractive druggable oncoprotein targets against such cancers. We also describe novel fusion transcripts that occur in CRC.

Keywords: breast cancer, colon cancer, RSPO2, oncogenic expression

Introduction

Activated canonical Wnt signaling is a feature of many types of cancer and has, subsequently, generated significant interest in targeting the Wnt pathway therapeutically. Multiple strategies for targeting the Wnt pathway have been developed, such as porcupine and tankyrase inhibitors with limited success in clinical trials (Anastas and Moon, 2013). Because Wnt signaling is integral for regulation of normal cell development and homeostasis, it is difficult to avoid off-target effects of systemic therapies aimed at reducing Wnt signaling (Barker and Clevers, 2006; Kahn *et al.*, 2014). Therefore, an ideal target would be a cancer-specific activator of Wnt signaling.

In cancer, multiple genetic events are known to activate Wnt signaling. For example, in colorectal cancer (CRC), the predominant mechanism of Wnt pathway activation is an inactivating mutation or deletion of *APC*, a core negative regulator of *CTNNB1*. Activating mutations in *CTNNB1*, loss of function mutations in *AXIN1/2*, and other pathway components are alternative routes to Wnt activation in CRC (Fodde *et al.*, 2001). In breast cancer, activation of Wnt signaling has been correlated with the basal subtype, but the events leading to Wnt activation in breast cancer are incompletely understood (Khramtsov *et al.*, 2010). Silencing of *SFRP1*, a secreted negative regulator of the Wnt pathway, is one proposed mechanism that promotes Wnt signaling in breast cancer (Ugolini *et al.*, 2001).

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R-spondins (RSPOs) have been described as secreted factors that modulate Wnt signaling. They are thought to modulate Wnt signaling by binding to LGR4/5/6 (de Lau *et al.*, 2012). They have also been found to exert their function independently of LGRs, by binding directly to RNF43 and ZNRF3 (Szenker-Ravi *et al.*, 2018). RSPOs were proposed as human oncogenes in 4–10% CRC cases that showed *RSPO2* and *RSPO3* overexpression, due to recurrent genomic rearrangements, in the absence of *APC* inactivating mutations (Seshagiri *et al.*, 2012; Shinmura *et al.*, 2014). Studies in mice also support the hypothesis that RSPOs are oncogenic and modulate Wnt signaling. Insertional mutagenesis screens performed in *Apc* wild-type mice identified *Rspo2* as a common insertion sites in gastrointestinal tract colorectal-like tumors (Lowther *et al.*, 2005; Theodorou *et al.*, 2007; Starr *et al.*, 2009; Takeda *et al.*, 2015). Moreover, murine mammary tumor virus screens identified *Rspo2* and *Rspo3* as breast tissue oncogenes (Lowther *et al.*, 2005; Theodorou *et al.*, 2007).

Interestingly, similar screens using *Apc* mutant mice did not identify RSPOs and the same exclusivity between *RSPO2/3* activation and *APC* mutation was also found in human CRC, suggesting that activation of RSPOs can substitute for loss of *APC* to drive Wnt signaling (March *et al.*, 2011; Starr *et al.*, 2011; Seshagiri *et al.*, 2012). Functional studies in mice demonstrated that targeted overexpression of *Rspo1* in the intestines promotes hyperplasia, whereas overexpression of *Rspo2* in mammary epithelium promotes breast cancer (Kim *et al.*, 2005; Klauzinska *et al.*, 2012). Other studies have also shown that *RSPO2* overexpression in its native form and in a fusion transcript activates Wnt signaling and induces colorectal tumors (Han *et al.*, 2017).

One mechanism proposed for sustained Wnt signaling through RSPO/LGR interaction is through inhibition of a negative feedback loop. Activation of Wnt signaling results in transcription of *ZNRF3*, an E3 ubiquitin ligase, that translocates to the cell membrane, ubiquitinates the frizzled (FZD) receptor, and dampens Wnt signaling in a canonical negative feedback loop. Recent studies demonstrated that RSPO1 and LGR4 together can cause membrane clearance of ZNRF3, breaking the negative feedback loop, resulting in enhanced Wnt signaling (Hao *et al.*, 2012; Koo *et al.*, 2012). Surprisingly, Wu *et al.* (2014) demonstrated the opposite effect when analyzing RSPO2 and LGR5 in certain CRC cell lines. They found that RSPO2 and LGR5 stabilize ZNRF3 at the surface, resulting in diminished Wnt signaling. Supporting their hypothesis that RSPO2 acts as a tumor suppressor, they found that the majority of CRC patients have down-regulated *RSPO2* through promoter methylation. These findings indicate the RSPOs have context-dependent pleiotropic effects. A clear analysis of expression data on *RSPO2* and *RSPO3* levels and canonical Wnt signaling-induced gene expression has not been reported for human cancer.

In this study, we focused on the role of RSPO2 and RSPO3 in two major epithelial cancers: colon and breast. We present evidence that *RSPO2* and *RSPO3* may function as oncogenes in a relatively small subset of these cancers based on analysis of expression levels, presence of *RSPO* fusion genes, and *in vitro* functional studies.

Methods

Acquisition of RNA-seq and somatic mutation data from The Cancer Genome Atlas

RNA-seq and somatic mutation data were extracted from The Broad Institute GDAC Firehose. These data were generated by The Cancer Genome Atlas (TCGA) Research Network. RNA-seq normalized counts (RSEM) were obtained for a set of 69 genes of interest related to RSPOs, Wnt signaling, and tissue-specific markers of differentiation and stemness, for 1884 samples comprising 41 normal colon, 434 CRCs, 111 normal breasts, and 1048 breast carcinomas. Somatic mutation data were obtained for *APC*, *CTNNB1*, *RSPO1*, *RSPO2*, *RSPO3*, and *RSPO4* from 266 CRCs and 768 breast carcinomas.

Primary breast tissue RNA isolation, cDNA synthesis, and quantitative reverse transcriptase polymerase chain reaction

Normal/tumor matched pairs were obtained from the University of Minnesota Tissue Procurement facility. In brief, cDNA synthesis and qPCR were performed as described (Burns *et al.*, 2013). Tissue RNA was from 100 mg flash-frozen tissue disrupted by a 2-h water bath sonication in 1 mL of Qiazol Lysis Reagent (RNeasy, Qiagen). Cell RNA was made using Qiashredder (RNeasy, Qiagen). qPCR was performed on a Roche Lightcycler 480 instrument. *RSPO2* and *RSPO3* primer sets were designed using the ProbeFinder version 2.48 for the Human Universal ProbeLibrary (UPL) from Roche Applied Science. The housekeeping gene *TBP* was used for normalization. Primer and probe sequences are listed in Supplementary Table S2.

Tissue culture reagents and cell lines

BT549, MCF10A, and MCF7 cells were obtained from the American Type Culture Collection (ATCC). BT549 and MCF7 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum and 0.023 IU/mL bovine insulin. MCF10A cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% horse serum, 20 ng/mL epidermal growth factor (EGF), 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 μg/mL insulin, and 1% penicillin/streptomycin (Debnath *et al.*, 2003). All cells were grown on tissue culture-treated plates under standard conditions of 37°C and 5% CO_2.

In vitro *gene knockdown and overexpression and proliferation assays*

For *RSPO2* knockdown experiments, plasmids encoding lentiviral shRNAmirs against *RSPO2* or a nonsilencing control shRNAmir were purchased from OpenBiosystems. Lentiviral particles were produced in 293T cells using the Trans-Lentiviral Packaging Kit (Thermo Scientific). For overexpression experiments, lentiviral expression vectors were cloned with *RSPO2* or dsRed regulated by a CAGGs promoter and followed by an IRES-GFP to monitor transduction efficiency. Lentiviral particles were produced in 293T cells by cotransfection with helper plasmids. For both knockdown and overexpression experiments, viral

supernatant was collected after 24 h of virus production, cleared, and applied to transduce experimental cells with 12 μg/mL polybrene overnight. Transduced cells were selected with 1 μg/mL puromycin. Knockdown efficiency and overexpression levels were assayed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

Quantitative reverse transcriptase polymerase chain reaction

RNA was isolated from cell lines using the Trizol method (Thermo Scientific). RNA samples were treated with DNase to remove contaminating genomic DNA (Turbo DNA-free Kit, Ambion). Complementary DNA was synthesized from 1 μg template RNA per sample using random hexamer primers (SuperScript III First-Strand Synthesis System, Invitrogen). qRT-PCRs were conducted with FastStart Universal SYBR Green Master mix (Roche), using 0.5 μL of cDNA template per 25 μL reaction. Primer sequences for qRT-PCRs are listed in Supplementary Table S2. Data were analyzed by normalization to *ACTB* using the following equation: relative expression = ((2^(CT_ACTB))/(2^(CT_GOI))).

Results

RSPO2 and RSPO3 are upregulated in fusion-transcript-positive colon cancer

To determine the frequency of *RSPO* overexpression and gene rearrangement, we analyzed RNA-seq data from 434 CRCs and 41 normal colon samples obtained through TCGA. We focused on *RSPO2* and *RSPO3* as previous data by Seshagiri and colleagues suggested that *RSPO2* and *RSPO3* are CRC drivers. Also, RSPO2 and RSPO3 have been shown to be the more potent Wnt modulators of the four RSPOs (Moad and Pioszak, 2013). In this cohort, *RSPO2* and *RSPO3* expression was decreased in the majority of CRCs compared with normal colon. Specifically, 422 of 434 (97.2%) CRCs have more than fourfold decreased *RSPO2* expression, and 250 of 434 (57.6%) have more than fourfold decreased *RSPO3* expression (Fig. 1A). This concurs with previous observations indicating *RSPO2* and *RSPO3* expression levels are suppressed in the majority of CRCs (Kazanskaya *et al.*, 2004; Seshagiri *et al.*, 2012; Shinmura *et al.*, 2014; Wu *et al.*, 2014).

Notably, a small subset of CRCs expressed high levels of *RSPO2/3*. ''RSPO-high'' tumors were defined as having greater than fourfold elevated mRNA levels compared with normal colon. In this cohort, two CRCs (0.5%) were RSPO2-high tumors and six CRCs (1.4%) were RSPO3-high tumors (Fig. 1A). Compared with the corresponding RSPO-low tumors, RSPO-high tumors expressed 231-fold higher *RSPO2* levels and 59-fold higher *RSPO3* levels.

Next, we sought to determine whether *RSPO*-high samples expressed *RSPO* gene fusions. Tumors that had paired-end RNA-seq data available (three of eight) were analyzed with DeFuse (McPherson *et al.*, 2011). All three expressed *PTPRK-RSPO3* fusion transcripts similar to prior studies (Storm *et al.*, 2016) (Fig. 1A). An additional novel *FLJ31306-RSPO2* fusion was found in a sample with elevated *RSPO2* expression less than the cutoff for RSPO2-high designation. We used Trinity (Grabherr *et al.*, 2011) to locate RSPO fusion transcripts in samples with single read RNA-seq data, and to confirm the *FLJ31306-RSPO2* fusion. This method identified one additional *PTPRK-RSPO3* fusion in an RSPO3-high sample, and a novel *CASC19-RSPO2* fusion in an RSPO2-high sample (Fig. 1B and Supplementary Fig. S1). The *CASC19-RSPO2* and *FLJ31306-RSPO2* fusions contained the native start codon in *RSPO2* exon 2 and were predicted to overexpress the complete native coding sequence, similar to the previously described *EIF3E-RSPO2* fusion (Seshagiri *et al.*, 2012). Examination of *RSPO2* mRNA levels on a per exon basis revealed a pattern of exon imbalance, further suggesting that the high levels of *RSPO2* mRNA in these samples consisted of fusion transcripts, as did the *RSPO3* fusion (Supplementary Fig. S1 and Supplementary Table S1). These data show that *RSPO2* can be activated by fusion with genes other than *EIF3E*.

RSPO2/3-high human CRCs have activated Wnt signaling and wild-type APC

To determine the relationship between *RSPO* overexpression and Wnt signaling, we next examined expression of a panel of Wnt-regulated genes (*AXIN2*, *TCF7*, *LEF1*) and *LGR5*, a Wnt target gene that is also the receptor for RSPOs. In the TCGA set of CRC samples, expression of three of four Wnt target genes was increased in RSPO-high tumors compared with normal colon (Fig. 1C). Consistent with prior reports, RSPO-high status was mutually exclusive with *APC* mutations (Seshagiri *et al.*, 2012; Shinmura *et al.*, 2014). Specifically, among RSPO-high CRCs with available somatic mutation data, four of four (100%) retained wild-type *APC*. These data are consistent with a model wherein RSPOs are an alternative route to activation of Wnt signaling.

It has been proposed that in CRC with an *RSPO* gene fusion causing overexpression, LGR5 must be downregulated to prevent Wnt inhibition through a repressive RSPO/LGR5 axis (Wu *et al.*, 2014). In contrast, we found that *LGR5* mRNA was downregulated in only one of eight RSPO-high CRCs, and was upregulated in the other seven samples, consistent with other Wnt-responsive genes (Fig. 1C). These data suggest that *LGR5* inactivation is not a requirement for Wnt activation in RSPO-high tumors. Because Wnt activation can promote tumorigenesis in other tissues, we next examined expression of *RSPO2* and *RSPO3* in primary human breast cancer, using TCGA data sets and independently analyzed primary samples.

RSPO2 is upregulated in human breast cancer and associated with basal/HER2 subtypes and active Wnt signaling

To analyze *RSPO* expression in breast cancer, we obtained pairs of matched normal and tumor tissues from 41 patients. *RSPO2* and *RSPO3* mRNA expression levels were quantified by qRT-PCR. Six tumors (14.6%) expressed *RSPO2* mRNA levels more than fourfold higher than their matched normal controls and were defined as ''RSPO2-high'' (range: 6.7 to 28.2-fold elevation) (Fig. 2A). RSPO2-high breast tumors did not express *EIF3E-RSPO2* fusion genes by RT-PCR. In the TCGA data set, we compared 1048 breast tumors with 111 normal breast tissues. *RSPO2* mRNA was expressed at a very low level in normal breast samples (normalized counts <1 in 100 of 111 samples, 90.1%). The majority of breast tumors also maintained low *RSPO2*

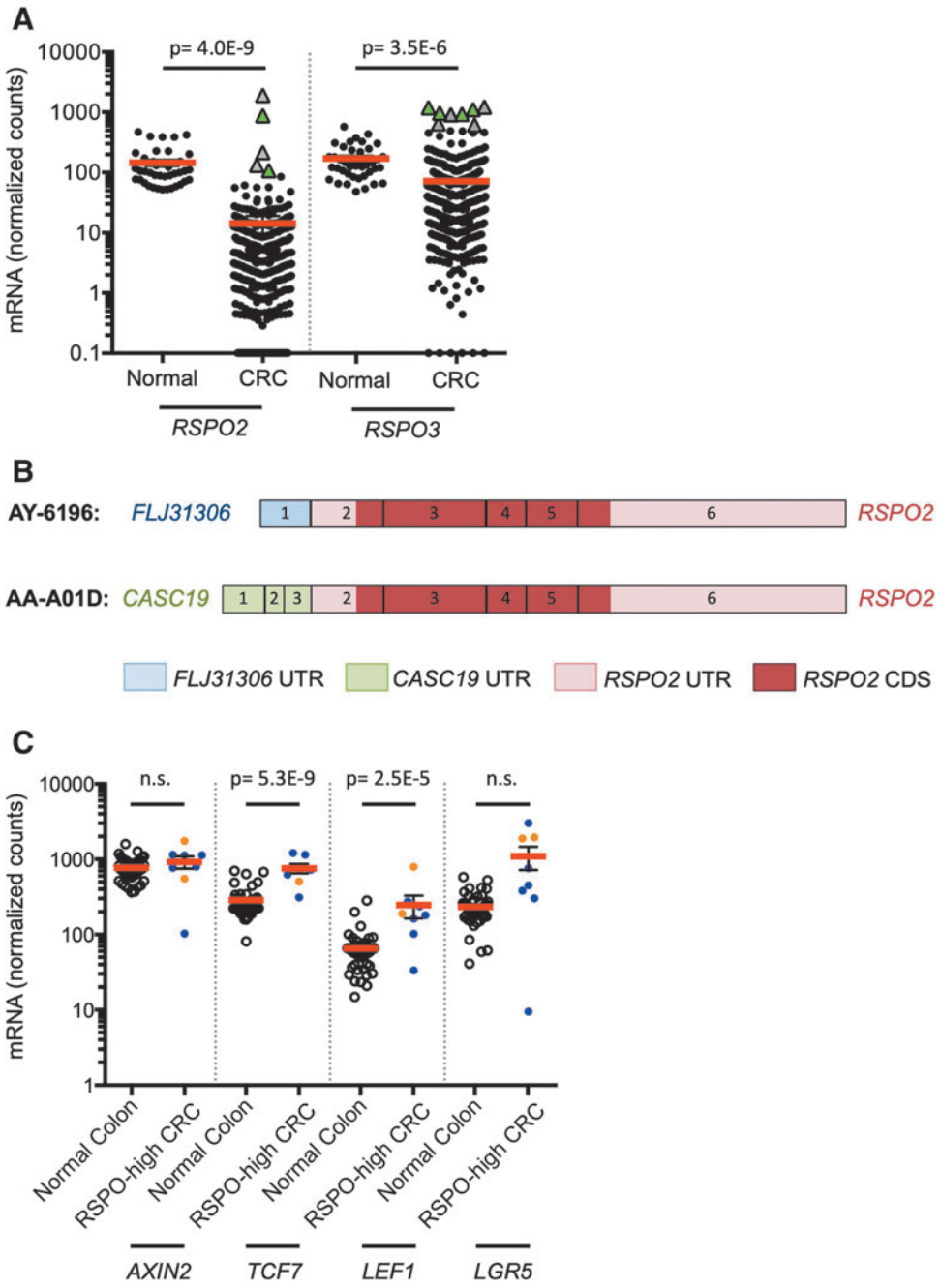

FIG. 1. *RSPO2* and *RSPO3* are highly expressed in rare colorectal tumors and associated with expression of RSPO fusion transcripts and activated Wnt signaling. **(A)** *RSPO2* and *RSPO3* mRNA levels in 41 normal colon tissues and 434 CRC tissues from TCGA RNA-Seq. RSPO fusion status is indicated by symbol shape and color: *black circle* = not assessed, *green triangle* = RSPO fusion detected, *gray triangle* = no RSPO2 or RSPO3 fusion detected, *red bar* = mean. **(B)** mRNA structure of novel *RSPO2* gene fusions: *FLJ31306-RSPO2* and *CASC19-RSPO2*. Numbers identify exons in the reference transcript. **(C)** mRNA expression of Wnt target genes (*AXIN2*, *TCF7*, and *LEF1*) in 41 normal colon tissues and 8 ''RSPO-high'' CRC tissues with R-spondin expression elevated more than fourfold compared with normal colon. Symbol colors: *orange* = RSPO2-high tumor, *blue* = RSPO3-high tumor, *red bar* = mean. CDS, coding sequence; CRC, colorectal cancer; TCGA, The Cancer Genome Atlas; UTR, untranslated region.

expression (normalized counts <1 in 842 of 1048 samples, 80.3%). However, 122 of 1048 (10.6%) of breast tumors had >4-fold increased *RSPO2* expression compared with the normal average (range: 4.0 to 300.7-fold elevated) (Fig. 2B). Similar to our observations in CRC, *RSPO3* levels were decreased in the majority of breast tumors, both in the set of 41 matched tumor/normal samples and in the TCGA data set (Fig. 3A and B). In the TCGA data set, 881 of 1048 (83.5%) of breast tumors were RSPO3-low, whereas rare tumors had elevated *RSPO3* levels (3 of 1048, 0.3%, Fig. 3B). DeFuse analysis of select RSPO-high breast tumors in the TCGA set did not identify expression of *RSPO* fusion transcripts (Table 1).

To determine the clinical relevance of *RSPO2* overexpression, we next analyzed PAM50 molecular subtype information for 521 breast tumors in the TCGA data set. We also looked into *RSPO3* levels in matched human breast cancer samples (Fig. 3A) and RNAseq data in TCGA (Fig. 3B). Interestingly *RSPO3* expression in human tumors is lower than in normal adjacent tumor. RSPO2-high status was significantly associated with basal ($p = 2.07E\text{-}5$) and HER2 ($p = 0.0259$) subtypes, and anticorrelated with luminal A ($p = 0.0052$) and luminal B ($p = 0.0093$) subtypes (Fig. 2C). Significantly, although basal-type tumors account for only 19% of tumors overall, 41% of RSPO2-high breast tumors were basal-type tumors (Fig. 2C).

Although active Wnt signaling, as measured by presence of nuclear CTNNB1, is associated with basal subtype tumors (Khramtsov *et al.*, 2010; Geyer *et al.*, 2011), expression of

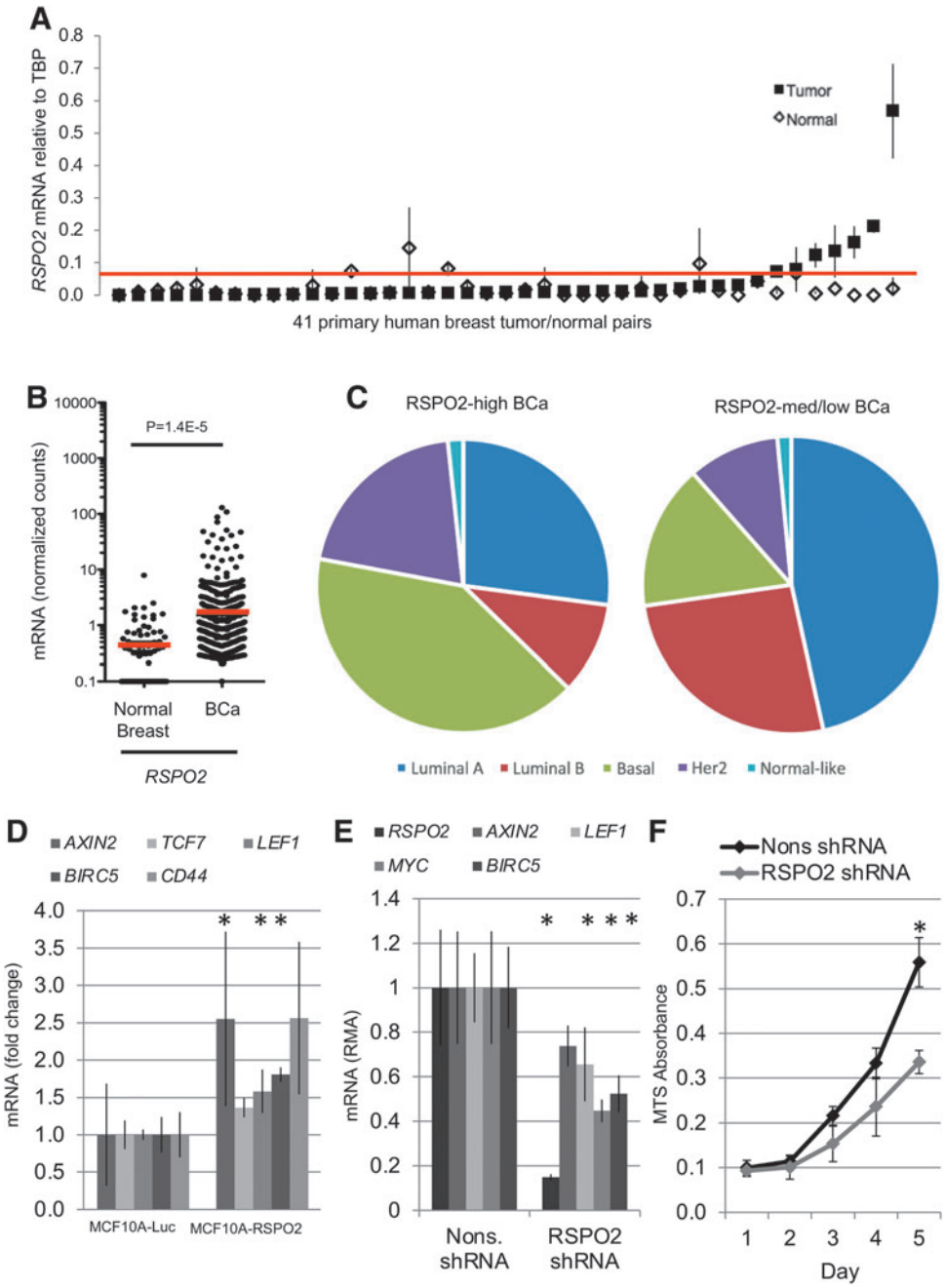

FIG. 2. *RSPO2* is highly expressed in a subset of breast tumors and associated with the basal and HER2 subtypes. **(A)** *RSPO2* mRNA levels in tumor (*black squares*) and matched normal breast tissue (*open diamond*) from 41 breast cancer patients. *RSPO2* mRNA levels were measured using qRT-PCR and normalized to *TBP* mRNA levels. The *red* line indicates mRNA expression level fourfold higher than the normal sample average. **(B)** *RSPO2* mRNA levels in 1048 breast cancer and 111 normal breast tissue samples from TCGA RNA-Seq. *Red* and *black bars* indicate mean and standard error. **(C)** Molecular subtype distribution of 59 RSPO2-high breast tumors compared with 463 tumors with medium or low *RSPO2* expression. Elevated expression of *RSPO2* is correlated with basal ($p = 2.07\text{E-}5$) and HER2 ($p = 0.0259$) subtypes and negatively correlated with luminal A ($p = 0.0052$) and luminal B ($p = 0.0093$) subtypes. **(D)** Wnt target gene mRNA levels in MCF10A cells transduced with lentivirus expressing RSPO2 or luciferase (Luc) control. mRNA levels were measured by qRT-PCR and normalized using ACTB levels. **(E)** Knockdown of *RSPO2* in BT549 cells decreases expression of Wnt/beta-catenin target genes. BT-549 cells were transduced with lentivirus encoding shRNA to RSPO2 or a nonsilencing (Nons) control. Knockdown efficiency and expression of Wnt target genes were quantified by qRT-PCR and normalized with beta-actin to the nonsilencing control. **(F)** Knockdown of *RSPO2* expression decreases proliferation of BT-549 cells. Proliferation was measured by MTS absorbance for three biological replicates per sample. *Denotes statistical significance. qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

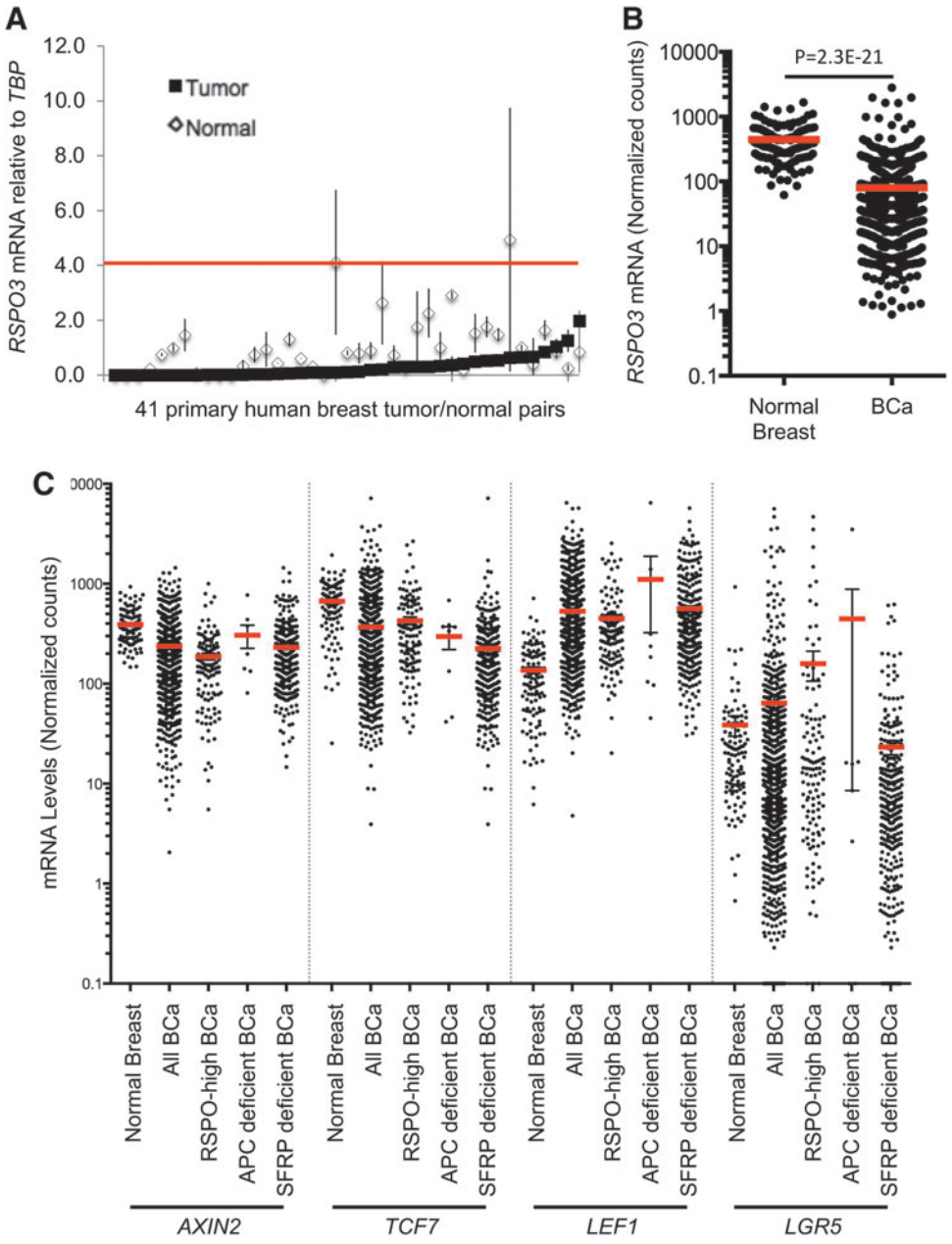

FIG. 3. Breast cancers have decreased expression of *RSPO3* and mixed expression of Wnt target genes. **(A)** *RSPO3* mRNA levels in tumor (*black squares*) and matched normal breast tissue (*open diamond*) from 41 breast cancer patients. *RSPO3* mRNA levels were measured using qRT-PCR and normalized to *TBP* mRNA levels. The *red* line indicates mRNA expression level fourfold higher than the normal sample average. **(B)** *RSPO3* mRNA levels in 1048 breast cancer and 111 normal breast tissue samples from TCGA RNA-Seq. *Red* and *black bars* indicate mean and standard error. **(C)** mRNA expression of Wnt target genes (AXIN2, TCF7, LEF1, and LGR5) in 111 normal breast tissues, 1048 BCa, 124 RSPO2/3-high BCa, 8 *APC*-deficient BCa, and 328 SFRP-deficient BCa. Samples were considered *APC* deficient that had mutations in *APC* that were nonsense, frameshift, or predicted functionally significant point mutations. Samples were considered SFRP deficient that had >100-fold decreased mRNA level.

Wnt target genes in RSPO-high breast tumors was not consistently elevated compared with normal tissue (Fig. 3C and Supplementary Fig. S3). Specifically, although expression levels of the Wnt target genes *LEF1* and *LGR5* were significantly elevated in RSPO-high tumors, those of *AXIN2* and *TCF7* were significantly downregulated (Fig. 3C). Examination of Wnt target gene expression in breast tumors with *APC* loss of function mutations or reduced expression of *SFRP1* revealed a similar pattern, suggesting that Wnt signaling is equivalently activated in these subsets (Fig. 3C).

RSPO2 regulates Wnt signaling and proliferation in breast cancer cells

To determine the functional significance of elevated *RSPO2* expression in breast cancer, we overexpressed *RSPO2* in a nontransformed basal-type breast epithelial cell line (MCF10A) (Coussy *et al.*, 2017), and we knocked down *RSPO2* in BT-549, a RSPO2-high basal-type breast cancer cell line. MCF10A cells overexpressing *RSPO2* showed transcriptional upregulation of Wnt target genes (Fig. 2D). Wnt target genes were selected based on data gathered and published by the Nusse laboratory's Wnt Homepage. BT-549 cells express extremely high levels of *RSPO2* compared with 58 breast cancer cell lines profiled in the Cancer Cell Line Encyclopedia (Fig. 3A). Knockdown of *RSPO2* in BT-549 cells resulted in decreased expression of Wnt target genes, as well as reduced cell proliferation (Fig. 2E, 2F, and Supplementary Fig. S2). These results suggest that RSPO2-high breast cancers may require *RSPO2* expression for activation of Wnt signaling and enhanced growth.

Discussion

The role of RSPOs in regulating the Wnt pathway in cancer is complex, as evidence suggests that RSPOs can function as oncogenes or tumor suppressors (Seshagiri *et al.*,

Table 1. Summary of Fusion Gene Analyses

Sample	*RNAseq library type*	*RSPO2 mRNA (normalized counts)*	*RSPO3 mRNA (normalized counts)*	*RSPO fusion gene detected*	*DeFuse result*	*Rearrangement*	*DeFuse breakpoints*	*Probability*	*Trinity result*	*Rearrangement*	*Trinity breakpoints (hg19)*
Colon tumor samples											
TCGA-AA-3664	Single reads	7.485	1188.6228	PTPRK-RSPO3	N/A	—	—	—	PTPRK-RSPO3 detected	Inversion	Chr6:128520259; Chr6:127148648
TCGA-DM-A1HB	Paired reads	0.806	1107.7977	PTPRK-RSPO3	PTPRK-RSPO3 detected	Inversion	Chr6:127469793–128505577	0.394251576	N/A	—	—
TCGA-CA-5255	Paired reads	2.7747	973.9179	PTPRK-RSPO3	PTPRK-RSPO3 detected	Inversion	Chr6:127469818–128840274	0.2784538275	N/A	—	—
TCGA-D5–6539	Paired reads	2.3259	931.7536	PTPRK-RSPO3	PTPRK-RSPO3 detected	Inversion	Chr6:127469793–128841404	0.5087362341	N/A	—	—
TCGA-AA-3695	Single reads	12.6316	910.5263	nd	N/A	—	—	—	No RSPO fusions detected	—	—
TCGA-CM-6167	Paired reads	214.6264	642.8193	nd	No RSPO fusions detected	—	—	—	N/A	—	—
TCGA-AZ-4323	Paired reads	131.9784	618.7429	nd	No RSPO fusions detected	—	—	—	N/A	—	—
TCGA-AA-3842	Single reads	4.2796	489.301	N/A	N/A	—	—	—	No RSPO fusions detected	—	—
TCGA-G4–6302	Paired reads	22.3339	486.8788	nd	No RSPO fusions detected	—	—	—	N/A	—	—
TCGA-A6–2672	Single reads	1.2739	463.6943	N/A	N/A	—	—	—	No RSPO fusions detected	—	—
TCGA-A6–6651	Paired reads	8.7949	455.6591	nd	No RSPO fusions detected	—	—	—	N/A	—	—
TCGA-AA-3518	Single reads	3.7594	453.6341	N/A	N/A	—	—	—	No RSPO fusions detected	—	—
TCGA-D5–6534	Paired reads	7.6674	399.404	nd	No RSPO fusions detected	—	—	—	N/A	—	—
TCGA-AY-6196	Paired reads	107.443	243.0972	FLJ31306-RSPO2	RSPO2 fusion detected	Interchromosomal	Chr 14: 58772830; Chr8:109115184	0.7214394704	FLJ31306-RSPO2 detected	Interchromosomal	Chr 14:58764673; Chr8:109095035
TCGA-AA-A01D	Single reads	874.4663	74.2955	CASC19-RSPO2	N/A	—	—	—	CASC19-RSPO2 detected	—	Chr 8:128200030; Chr 8:109095035
TCGA-AA-3520	Single reads	1907.0022	64.5514	N/A	N/A	—	—	—	No RSPO fusions detected	—	—
Colon tumor (avg)	—	14.2	72.3	—	—	—	—	—	—	—	—
Colon normal (avg)	—	146.2	171.8	—	—	—	—	—	—	—	—
Breast tumor samples											
TCGA-D8-A1X7	Paired reads	2.5	2792.0	nd	No RSPO fusions detected	—	—	—	N/A	—	—
TCGA-C8-A1HJ	Paired reads	1.1	1977.9	nd	No RSPO fusions detected	—	—	—	N/A	—	—
TCGA-AC-A2QH	Paired reads	108.8	30.5	nd	No RSPO fusions detected	—	—	—	N/A	—	—
TCGA-E9-A1RB	Paired reads	51.0	15.1	nd	No RSPO fusions detected	—	—	—	N/A	—	—
Breast tumor (avg)	—	1.5	80.0	—	—	—	—	—	—	—	—
Breast normal (avg)	—	0.4	443.3	—	—	—	—	—	—	—	—

N/A, not analyzed; nd, not detected; TCGA, The Cancer Genome Atlas.

2012; Wu *et al.*, 2014). To clarify the role of RSPOs, specifically RSPO2 and RSPO3, we analyzed gene expression, the presence of fusion transcripts, and functional effects of RSPO2 and RSPO3 in colon and breast cancers. We found that, in a minority of cases of both cancers, *RSPO2* or *RSPO3* are significantly upregulated and may play a functional role enhancing Wnt signaling and promoting tumor development in these cancers.

Colon cancer

In a majority of colon cancers, *RSPO2* expression is suppressed due to promoter methylation. Wu *et al.* (2014) demonstrated that RSPO2 along with LGR5 causes stabilization of ZNRF3, an E3 ubiquitin ligase that negatively regulates Wnt signaling. They proposed that loss of RSPO2 in CRC patients would lead to increased Wnt signaling through destabilization of ZNRF3. In a small subset of CRC patients, however, *RSPO2* or *RSPO3* is highly overexpressed due to gene fusions, such as *PTPRK-RSPO3* and *EIF3E-RSPO2*, which result in changes in promoter regulation (Seshagiri *et al.*, 2012; Shinmura *et al.*, 2014). We extended this finding by analyzing TCGA RNA-Seq data for gene fusions. We found gene fusions in 66% of *RSPO3*-high cancers and 50% of *RSPO2*-high cancers. In addition to previously identified fusions, we detected two novel *RSPO2* gene fusions that also contained the native start codon of *RSPO2*, similar to the *EIF3E-RSPO2* fusion.

Moreover, we identified *CASC19-RSPO2* and *FLJ31306-RSPO2* fusion genes to be enriched in a subset of CRCs. These new data suggest that there are many circumvented pathways that result in the overexpression of *RSPO2*, resulting in positive Wnt signaling. Such events may be as a result of loss of border elements, such as CCCTC-binding factor (CTCF) resulting in aberrant chromatin confirmation, resulting in fusion transcript generation, enhancer hijacking, and/or transcription run-off. Analysis of exon-level expression confirmed that high-expression levels in these cancers were likely due to transcription of the fusion transcript. In the report by Wu *et al.*, it was suggested that elevated levels of RSPO2 would only modulate Wnt signaling if LGR5 levels were downregulated. In the TCGA data set, however, we found that *LGR5* was upregulated in 87% (7/8) of RSPO2/3 high cancers, indicating loss of LGR5 is not necessary for RSPO-induced Wnt activation. These findings support a model in which *RSPO2* or *RSPO3* is upregulated in a subset of *APC* wild-type CRCs due to gene fusions or other events, correlating with activation of Wnt signaling.

Breast cancer

Similar to colon cancer, a majority of breast cancers have constitutively low levels of *RSPO2* and *RSPO3* expression. However, we found that a small subset has elevated *RSPO2* levels and this subset is enriched for the basal subtype of breast cancer. We manipulated levels of *RSPO2* by overexpression in an RSPO2-low breast epithelial cell line (MCF10A), which resulted in elevated levels of Wnt target genes. Decreasing levels of *RSPO2* using shRNA in an RSPO2-high cell line (BT-549) caused a decrease in expression of Wnt target genes and a decrease in proliferation. These functional studies indicate that RSPO2 can function as an enhancer of Wnt signaling in breast cancer. The mechanism of upregulation of *RSPO2* in breast cancer is unclear, as we did not detect fusion transcripts, nor did we detect a positive feedback loop through Wnt3a treatment, as proposed by Kazanskaya *et al.*, (2004). Moreover, others have found that increasing *RSPO2* expression in breast tumors results in decreased metastasis-free survival (Coussy *et al.*, 2017) (Supplementary Fig. S4). Further studies will be required to determine the mechanisms of RSPO upregulation and characterize Wnt signaling role and function in breast cancer to find sensible drug target.

We have found and confirmed that RSPOs are overexpressed in a small subset of human cancers of the colon and breast. This occurs exclusive of *APC* mutations and other Wnt pathway component alterations. Many genetic mechanisms are involved in the increased expression of RSPOs. Genomic rearrangements that result in fusion transcript expression, copy number variations, and epigenetic alterations that control proper promoter function have been identified. It is important then to further study RSPOs as druggable targets and biomarkers of human cancers, with a new focus on breast cancer (Chartier *et al.*, 2016; Storm *et al.*, 2016; Coussy *et al.*, 2017). Alternative or combination therapy with Wnt pathway inhibitors, such as porcupine and/or tankyrase inhibitors, is a strategy that could halt growth of RSPO-dependent colon and breast cancers (Watson *et al.*, 2013).

Authors' Contributions

C.B.C. and G.L.V.-R. designed, performed experiments, and prepared this article; M.B.B. performed experiments; S.K.R., J.E.A., and N.A.T. performed bioinformatics; T.K.S. and D.A.L. served as mentors, conceptualized, supervised, designed, and prepared this article.

Acknowledgments

The results published here are, in part, based upon data generated by the TCGA Research Network. This study utilized computing resources at the University of Minnesota Supercomputing Institute. The University of Minnesota Genomics Center provided Sanger sequencing and primer synthesis services.

Disclosure Statement

D.A.L. is the cofounder and co-owner of NeoClone Biotechnologies, Inc., Discovery Genomics, Inc., (recently acquired by Immusoft, Inc.), and B-MoGen Biotechnologies, Inc. He consults for Surrogen, Inc., and Genentech, Inc. is funding some of his research. The business of all these companies is unrelated to the contents of this article. Other authors have no conflict of interest to disclose.

Funding Information

This study was supported by grants to C.B.C. (T32 GM008244, F30 CA171547), G.L.V.-R. (T32 GM008244, T32 HL007741), D.A.L. (R01 CA134759, R01 CA113636), T.K.S. (5R00CA151672-03, P30-CA77598, Mezin-Koats Colon Cancer Research Fund, and ACS PF-06-282-01-MGO).

Supplementary Material

Supplementary Table S1
Supplementary Table S2
Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4

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Received for publication July 1, 2020; received in revised form October 9, 2020; accepted October 13, 2020.