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Clinical value of R-spondins in triple-negative and metaplastic breast cancers

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Background: RSPO ligands, activators of the Wnt/β-catenin pathway, are overexpressed in different cancers. The objective of this study was to investigate the role of RSPOs in breast cancer (BC).

Methods: Expression of *RSPO* and markers of various cancer pathways were measured in breast tumours and cell lines by qRT–PCR. The effect of *RSPO* on the Wnt/β-catenin pathway activity was determined by luciferase assay, western blotting, and qRT–PCR. The effect of *RSPO2* inhibition on proliferation was determined by using *RSPO2* siRNAs. The effect of IWR-1, an inhibitor of the Wnt/β-catenin pathway, was examined on the growth of an *RSPO2*-positive patient-derived xenograft (PDX) model of metaplastic triple-negative BC.

Results: We detected *RSPO2* and *RSPO4* overexpression levels in BC, particularly in triple-negative BC (TNBC), metaplastic BC, and triple-negative cell lines. Various mechanisms could account for this overexpression: presence of fusion transcripts involving *RSPO*, and amplification or hypomethylation of *RSPO* genes. Patients with *RSPO2*-overexpressing tumours have a poorer metastasis-free survival ($P = 3.6 \times 10^{-4}$). *RSPO2* and *RSPO4* stimulate Wnt/β-catenin pathway activity. Inhibition of *RSPO* expression in a TN cell line inhibits cell growth, and IWR-1 significantly inhibits the growth of an *RSPO2*-overexpressing PDX.

Conclusions: *RSPO* overexpression could therefore be a new prognostic biomarker and therapeutic target for TNBC.

Breast cancer (BC) is the leading cause of death by cancer in women (Jemal *et al*, 2007). Fifteen percent of primary BC are triple-negative BC (TNBC) with lack of expression of oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Morris *et al*, 2007). The majority of TNBC are invasive ductal carcinomas, but metaplastic BC (MBC) constitutes a rare subtype (<1%). MBC is characterised morphologically by the differentiation of neoplastic epithelium into squamous cells and/or mesenchymal cells. MBC displays a fairly aggressive clinical behaviour, and unlike other forms of TNBC, these tumours do not appear to respond to conventional chemotherapy regimens (Hennessy *et al*, 2006). Globally, women with TNBC or MBC have a poor prognosis, particularly due to the absence of targeted therapies (Aydiner *et al*, 2015).

The abnormally high activity of the canonical Wnt/β-catenin signalling pathway has a key role in the development of many

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cancers, such as colorectal cancer (CRC) and BC, particularly TNBC and MBC (Hayes *et al*, 2008; MacDonald *et al*, 2009; Bilir *et al*, 2013; Cai *et al*, 2013; Maubant *et al*, 2015). In CRC, Wnt/β-catenin pathway activation is predominantly (90%) due to mutations of *APC* and *CTNNB1* genes (coding for β-catenin) (Bienz and Clevers, 2000; Behrens and Lustig, 2004; Seshagiri *et al*, 2012). However, anomalies of *APC* and *CTNNB1* genes are rare in breast carcinogenesis (Cancer Genome Atlas Network, 2012). Identifying and understanding the mechanisms responsible for activation of the Wnt/β-catenin signal in BC are essential for the development of new biomarkers and therapeutic targets for this type of cancer, particularly TNBC.

The family of RSPO secreted proteins comprises four members (RSPO1–4). RSPOs are physiologically involved in embryogenesis and are ligands for the leucine-rich repeat containing G protein-coupled receptors (LGRs) 4–6. These proteins synergise with Wnt ligands to activate the Wnt/β-catenin pathway by inducing stabilisation of FZD and LRP 5/6 (Jin and Yoon, 2012). Recently, a new mechanism of activation of the Wnt/β-catenin pathway has been identified in 10% of CRC: overexpression of *RSPO2* and *RSPO3* due to gene fusions resulting from deletions of about 150 kb in the chromosome region 8q23 and chromosome inversions in the region 6q22.3 (involving *RSPO2* and *RSPO3*, respectively). These gene fusions lead to the formation of transcripts involving exon 1 of *EIF3E* and exon 2 of *RSPO2* (*EIF3E* (exon1)-*RSPO2* (exon2)) and transcripts involving either exon 1 or exon 7 of *PTPRK* and exon 2 of *RSPO3* (*PTPRK* (exon1 or exon7)–*RSPO3* (exon2)), resulting from overexpressed functional *RSPO2* and *RSPO3* proteins, respectively (Seshagiri *et al*, 2012; Shinmura *et al*, 2014). Expression of these *RSPO* fusion transcripts is mutually exclusive with *APC* and *CTNNB1* mutations and could therefore explain activation of the Wnt/β-catenin pathway in the 10% of CRC that do not harbor any *APC* or *CTNNB1* mutation.

RSPO2 and *RSPO3* genes were identified as Int, a common integration site of mouse mammary tumour virus (Callahan *et al*, 2012), and *RSPO2* overexpression in mouse mammary epithelial cells induces tumour growth (Klauzinska *et al*, 2012). These results suggest that overexpression of *RSPO*s could have a crucial role in breast carcinogenesis.

In the present study, we investigated the potential involvement of *RSPO* in breast carcinogenesis.

MATERIALS AND METHODS

Patients and samples. Samples from 446 unilateral invasive primary breast tumours excised from women, managed at Institut Curie (Saint-Cloud, France) between 1978 and 2008, and described in detail elsewhere (Meseure *et al*, 2016) were analysed. Standard prognostic factors of this tumour set are presented in Supplementary Data (Supplementary Table S1). With a median follow-up of 8.9 years (range: 6 months to 29 years), 171 patients developed metastasis. Ten specimens of adjacent normal breast tissue from BC patients or normal breast tissue from women undergoing cosmetic breast surgery were used as sources of normal RNA. An additional set of 28 MBCs collected by Institut Curie was analysed by qRT–PCR: 16 were diagnosed as MBC with mesenchymal elements, 4 as MBC with squamous metaplasia, and 8 as spindle cell carcinoma (Weigelt *et al*, 2015).

Cell lines. Thirty-two RNA samples from breast cell lines obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), including 16 triple-negative tumour cell lines, were analysed by qRT–PCR (Supplementary Table S2). The HBCc-15 cell line was established from the HBCx-15 TNBC patient tumour-derived xenograft (PDX).

MDA-MB-468, MDA-MB-231, and HEK293 cells were purchased from ATCC. Fibroblast L cells producing Wnt-3a and L cells stably transfected with pGKneo plasmids were a generous gift from Dr Shinji Takada.

Real-time qRT–PCR. Total RNA extraction and RT–PCR have been described previously (Bieche *et al*, 2001). qRT–PCR results, expressed as *N*-fold differences in *RSPO* and *LGR* (or genes of interest involved in various cancer pathways) gene expression relative to the *TBP* gene and called N_{target}, were determined as $N_{target} = 2^{\Delta Ctsample}$, where the ΔCt value of the sample was determined by subtracting the average Ct value of the target gene from the average Ct value of the *TBP* gene. N_{target} values of breast tumour samples were subsequently normalised so that the median N_{target} value for 10 normal breast tissues was equal to 1. Primers for fusion genes involving *RSPO2* and *RSPO3* described in the literature (Seshagiri *et al*, 2012) and for *TBP*, *RSPO1*, *RSPO4*, and *LGR4–6* genes were chosen with the assistance of the Oligo 6.0 software (National Biosciences, Plymouth, MN, USA) (Supplementary Table S3 and additional information on request; Bieche *et al*, 2001).

For the gene expression study in the breast tumour series, the 7 investigated genes (*RSPO1–4* and *LGR4–6*) were differentially expressed in the 10 normal breast tissues analysed. The median Ct of normal breast tissues was equal to 30.5 for *RSPO1*, 27.8 for *RSPO3*, 28.6 for *LGR4*, and 31.4 for *LGR6*. mRNA levels of these four genes in breast tumours are expressed relative to the endogenous RNA control *TBP*, normalised on the basis of normal breast tissue expression (median N_{target} for normal breast tissues = 1). Values $\geqslant 3$ were considered to represent overexpression. For the other 3 genes (*RSPO2*, *RSPO4* and *LGR5)*, absent ($Ct > 35$) or low level ($32 < Ct < 35$) expression was observed in the 10 normal breast tissues analysed. Consequently, mRNA levels in breast tissue samples were normalised to obtain a 'basal mRNA level' (smallest amount of mRNA quantifiable ($Ct = 35$)) equal to 1. Values $\geqslant 5$ were considered to represent high expression in breast tumour samples (Bieche *et al*, 1999; Bieche *et al*, 2003).

For the gene expression study in breast cell lines and PDX, mRNA levels were normalised to obtain a 'basal mRNA level' (smallest amount of mRNA quantifiable ($Ct = 35$)) equal to 1. Values $\geqslant 5$ were considered to represent high expression in breast tumour samples.

For the TCGA analysis, mRNA expression (RNA sequencing), methylation, and copy number alteration data were downloaded (http://www.cbioportal.org/) for TCGA Breast Cancer (provisional cohort, $n = 1105$; Cerami *et al*, 2012; Gao *et al*, 2013).

Western blotting. The methods are described in detail elsewhere (Lallemand *et al*, 2001). In this study, we used the following antibodies: anti-GAPDH (sc-20357, Santa Cruz Biotechnology, Dallas, TX, USA) used as an internal control, polyclonal rabbit anti-phospho LRP6 (ser 1490, Cell Signaling Technology, Danvers, MA, USA), monoclonal rabbit anti-LRP6 (C5c7, Cell Signaling Technology), polyclonal rabbit active β-catenin (05665, Millipore, Billerica, MA, USA), and anti-β-catenin (sc-7199, Santa Cruz Biotechnology).

Conditioned medium. Conditioned medium containing Wnt3a (Wnt3a-CM) was prepared as previously described (Shibamoto *et al*, 1998). To prepare *RSPO2*- or *RSPO4*-conditioned medium, 2×10^6 HEK293 cells were seeded in a 94-mm-diameter dishes. Twenty-four hours later, cells were transfected with the empty vector (PS100001, Origene, Rockville, MD, USA), *myc*-tagged *RSPO2* expression vector (RC224177, Origene), or *myc*-tagged *RSPO4* expression vector (RC224295, Origene). To study the effect of *RSPO2/4* and Wnt3a on the Wnt/β-catenin pathway activity, cells were cultured overnight in medium containing 50% RSPO medium and/or 30% Wnt3a.

The presence of myc-tagged RSPO in conditioned medium was verified by western blotting (Supplementary Figure S1).

Luciferase assays. The methods have been described in detail elsewhere (Lallemand *et al*, 2001). Firefly luciferase activity was normalised to Renilla luciferase activity and expressed as mean ± s.d. of triplicates from a representative experiment. Results are shown as fold induction of luciferase activity compared with control cells transfected with empty vectors alone.

Immunohistochemistry (IHC). Paraffin sections of HBCx-15 (xenograft with *RSPO2* overexpression) and HBCx-3 (xenograft without expression of RSPO) TNBC PDX were prepared as previously described (Landemaine *et al*, 2008). Briefly, PDX blocks were deparaffinised, treated with 3% H_2O_2, and incubated without (negative control) or with goat polyclonal anti-*RSPO2* antibody (Santa Cruz Biotechnology). Staining signals were revealed with the Leica bond biosystem (Leica Biosystems Newcastle Ltd, Newcastle-upon-Tyne, UK). Slides were counterstained with Mayer's haematoxylin.

SiRNA inhibition. For siRNA inhibition and real-time monitoring of cell proliferation studies, BT549 cells were seeded at a density of 5000 cells per well in 96-well E-Plates (ACEA Biosciences, San Diego, CA, USA). The *in vitro* growth curve was characterised by using the xCELLigence system (Roche Inc., Bale, Swiss). Cell were transfected 24 h later with validated human *RSPO2* siRNAs (reference SI04274760 and SI04303957) or negative control siRNA 1 (Qiagen, Hilden, Germany) at a final concentration of 30 nM in the presence of HiPerFect Transfection Reagent (Qiagen). All experiments were performed in quadruplicate, and normalised cell index values were calculated according to the manufacturer's instructions.

In vivo assay in PDX. The HBCx-60 TNBC PDX was directly established from a metaplastic TNBC and was routinely passaged by subcutaneous engraftment into the interscapular fat pad in Crl:NU(Ico)-Foxn1nu nude mice (Charles River Laboratories, Wilmington, MA, USA), with protocol and animal housing in accordance with national regulations and international guidelines (Marangoni *et al*, 2007). Female 8-week-old mice with 60–200 mm^3 tumours were randomly assigned to the control or treated groups. Mice (at least 7 per group) were treated with 100 μl of 200 μM of IWR-1 (IO161, Sigma, St Louis, MO, USA) diluted in PBS by subcutaneous injection, 4 times per week, as previously described (Rognoni *et al*, 2014). Tumour growth was evaluated measuring two perpendicular tumour diameters with a caliper twice a week. Individual tumour volumes were calculated: $V = a \times b^2/2$, where a is the largest diameter and b is the smallest diameter. For each tumour, volumes were expressed in relation to the initial volume as relative tumour volume (RTV).

Statistical analysis. Relationships between *RSPO* or *LGR* expression and clinical, histological, and laboratory parameters were estimated with Chi-square and Mann–Whitney tests. Metastasis-free survival (MFS) was determined as the interval between diagnosis and detection of the first metastasis. Survival distributions were estimated with the Kaplan–Meier method, and the significance of differences between survival rates was ascertained with the log-rank test. The Cox proportional hazards regression model was used to assess prognostic significance and the results are expressed as hazard ratios and 95% confidence intervals. Hierarchical clustering was performed with the GeneANOVA software (Didier *et al*, 2002).

RESULTS

Expression of RSPOs and LGRs in BC tumours, TNBC subtype, and BC cell lines. Pending the development of a reliable RSPO antibody (we tested three different commercial antibodies), the best way to study *RSPO* expression consists of mRNA assay. The difficulty of detecting the RSPO proteins by western blotting is a well-known problem in the literature (Seshagiri *et al*, 2012; Chartier *et al*, 2016). We first examined *RSPO* mRNA expression in a series of 446 breast tumours, including 68 TNBC. Overexpression of at least one *RSPO* gene was observed in 11.6% of BC and 55.8% of TNBC, mainly involving the *RSPO4* gene (9.2% in the total population of BC and 42.6% in TNBC) and *RSPO2* gene (2.7% in the total population of BC and 17.6% in TNBC; Table 1). In contrast, *RSPO1* and *RSPO3* were rarely overexpressed in BC (only 1.1% and 0.2%, respectively; Table 1). None of the 446 tumours overexpressed all *RSPO* at the same time. Interestingly, four TNBCs of our cohort were MBCs and all overexpressed at least one *RSPO* (one sample overexpressed *RSPO2*, two samples overexpressed *RSPO4* and one overexpressed *RSPO2* and *RSPO4*. To validate the very high prevalence of *RSPO* overexpression in MBC, we examined *RSPO* overexpression in a second cohort of 28 MBC. This study confirmed that *RSPO2* was more often overexpressed in this specific subtype than in TNBC (46% *vs* 17.6%; Table 1).

Among the 32 BC cell lines, 9 (28%) overexpressed at least one gene of the *RSPO* family with a majority of TNBC cell lines (7 out of 16: 43.7%; Table 1 and Supplementary Table S2) as observed for the tumour sample cohort.

We also verified the expression of the *RSPO* receptors, *LGR4*, *LGR5* and *LGR6*, in our series of 446 breast tumours and found that the majority of tumours expressed *LGR4* (median Ct of 27.43) and, to a lesser extent, *LGR5* and *LGR6* (median Ct of 36.4 and 31.92, respectively; data not shown). Distributions of *RSPO* receptor expression are detailed in Table 1 and Supplementary Table S2 for breast tumours and cell lines, respectively.

Mechanisms underlying RSPO overexpression in BC. It has been observed that *RSPO2* and *RSPO3* overexpression in CRC is at least partly due to the presence of fusion transcripts (*EIF3E* (exon1)–*RSPO2* (exon2), *PTPRK* (exon1)–*RSPO3* (exon2), and *PTPRK* (exon7)–*RSPO3* (exon2); Seshagiri *et al*, 2012). In order to explain cases of *RSPO2* and *RSPO3* overexpression in BC cells,

Table 1. Overexpression of RSPO and LGR in BC tumours and BC cell lines

Overexpression	RSPO1, *n* (%)	RSPO2, *n* (%)	RSPO3, *n* (%)	RSPO4, *n* (%)	All[a] RSPO, *n* (%)	LGR4, *n* (%)	LGR5, *n* (%)	LGR6, *n* (%)	All[a] LGR, *n* (%)
Patient tumour samples									
Whole population (*n* = 446)	5 (1.1%)	12 (2.7%)	1 (0.2%)	41 (9.2%)	52 (11.6%)	3 (0.6%)	16 (3.6%)	4 (0.9%)	23 (5.1%)
TNBC subgroup (*n* = 68)	3 (4.4%)	12 (17.6%)	1 (1.5%)	29 (42.6%)	38 (55.8%)	1 (1.5%)	10 (14.7%)	3 (4.4%)	14 (20.6%)
Metaplastic cohort (*n* = 28)	0 (0%)	13 (46%)	0 (0%)	12 (42.8%)	18 (64%)	0 (0%)	9 (32.1%)	0 (0%)	9 (32.1%)
Cancer cell lines									
Whole population (*n* = 32)	2 (6.2%)	2 (6.2%)	4 (12.5%)	4 (12.5%)	9 (28%)	4 (12.5%)	2 (6.2%)	9 (28%)	14 (43.8%)
TNBC subgroup (*n* = 16)	2 (12.5%)	2 (12.5%)	4 (25%)	2 (12.5%)	7 (43.7%)	1 (6.3%)	2 (12.5%)	8 (50%)	10 (62.5%)

Abbreviations: LGR = leucine-rich repeat containing G protein-coupled receptor; TNBC = triple-negtive breast cancer.
[a]Overexpression of at least one RSPO gene.

we therefore first looked for the presence for these fusion transcripts by qRT–PCR using specific primers (Supplementary Table S3). We identified fusion transcripts involving *RSPO2* only in two TNBC cell lines: HBCc-15 and BT549, and in HBCx-15 TNBC PDX from which the HBCc-15 cell line was established (Figure 1A). The two different fusion transcripts *EIF3E* (exon1)–*RSPO2* (exon2) and *EIF3E* (exon1)–*RSPO2* (exon3) were found in both the HBCx-15 PDX and the derived HBCc-15 cell line (Figure 1A, left panel), and fusion transcript *EIF3E* (exon1)–*RSPO2* (exon3) was found in the BT549 cell line (Figure 1A, right panel). The HBCc-15 and BT549 cell lines and the HBCx-15 PDX expressed the highest levels of *RSPO2* transcripts (Supplementary Table S2 and data not shown).

We confirmed RSPO2 expression by IHC in the HBCx-15 TNBC PDX, strongly suggesting that the fusion transcripts detected in this PDX are functional (Figure 1B).

We did not detect any fusion transcript involving *RSPO2* or *RSPO3* in our series of 446 breast tumours.

The presence of fusion transcripts therefore cannot explain the majority of the *RSPO* overexpression observed in BC. On the basis of TCGA data (http://www.cbioportal.org/, breast invasive carcinoma, TCGA provisional), another mechanism can be proposed, such as amplification or hypomethylation of *RSPO* genes, as, in the TCGA population, *RSPO2* expression was correlated with *RSPO2* gene amplification ($P = 8 \times 10^{-5}$ for the total population and $P = 3 \times 10^{-3}$ for the TN subtype) and *RSPO4* expression was significantly correlated with *RSPO4* gene hypomethylation ($P = 1.7 \times 10^{-6}$ for the total population and $P = 8.2 \times 10^{-4}$ for the TN subtype; Supplementary Figure S2).

Relationship between RSPO and LGR gene mRNA levels and clinical parameters. *RSPO2* and *RSPO4* overexpression levels were associated with high SBR histological grade ($P = 5.5 \times 10^{-3}$ and $P = 2.4 \times 10^{-3}$, respectively), negative hormonal receptor status ($P < 10^{-7}$ for ER and $P = 4.6 \times 10^{-5}$ for PR and $P < 10^{-7}$ for ER and $P = 2.6 \times 10^{-7}$ for PR, respectively), elevated *Ki67* mRNA ($P = 2.7 \times 10^{-2}$ and $P = 1.7 \times 10^{-7}$, respectively), and TNBC subtype ($P < 10^{-7}$ and $P = 2.1 \times 10^{-7}$, respectively) (Supplementary Tables S4 and S5). Results for LGR5 are detailed in Supplementary Table S6. These correlation tests cannot be performed for tumours overexpressing *RSPO1*, *RSPO3* and *LGR4* and *LGR6* owing to the small number of overexpressing samples.

These findings indicate that *RSPO2* and *RSPO4* overexpression levels are associated with aggressive characteristics of BC.

Relationship between RSPO gene mRNA level and MFS. A log-rank test was used to identify relationships between MFS and *RSPO2* and *RSPO4* mRNA levels. Patients with BC (Figure 2A) or TNBC (Figure 2B) overexpressing *RSPO2* had significantly poorer MFS ($P = 3.6 \times 10^{-4}$ and $P = 4.4 \times 10^{-2}$, respectively). The prognostic significance of the parameters identified in univariate analysis (including histopathological grade, lymph node status, macroscopic tumour size, PR status; Supplementary Table S1) and *RSPO2* expression status persisted (except for histopathological grade and PR status) in Cox multivariate regression analysis of MFS (Supplementary Table S7). These associations were not observed for *RSPO4* (data not shown).

LGR5 overexpression was not associated with MFS in the BC population or the TNBC subpopulation (data not shown). Survival

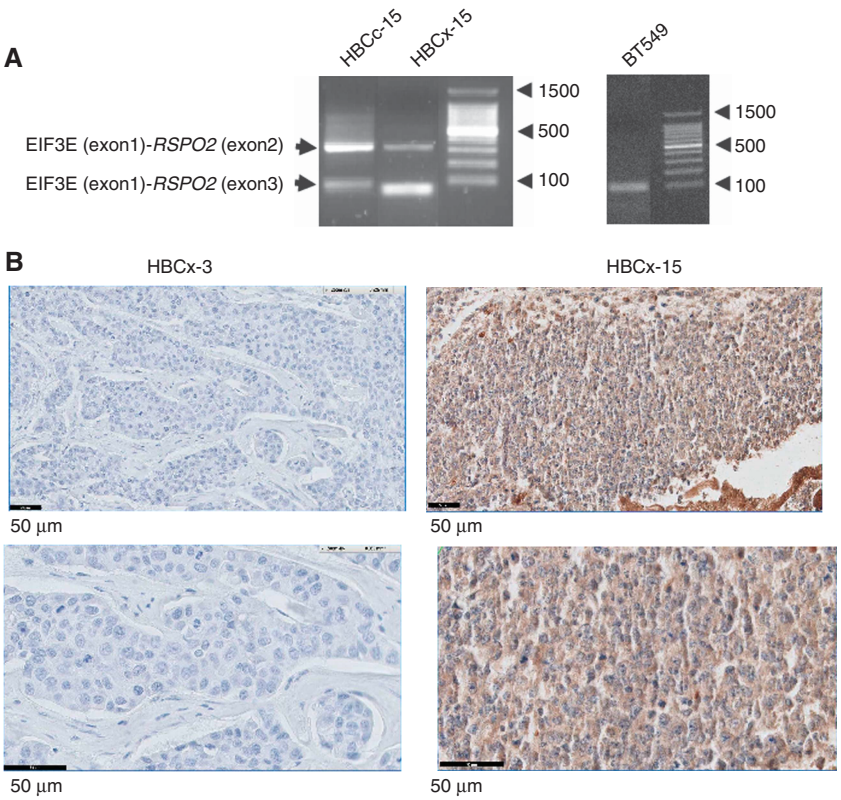

Figure 1. Presence of functional RSPO fusion transcripts in BC cells. (**A**) Detection of EIF3E (exon1)-*RSPO2* (exon2) and EIF3E (exon1)-*RSPO2* (exon3) fusion transcripts (351 pb and 88 pb, respectively, see Supplementary Table S3) in HBCx-15 TNBC PDX and its derived HBCc-15 cell line and BT549 cell line. (**B**) Detection of *RSPO2* protein in HBCx-15 TNBC PDX and HBCx-3 (negative control, TNBC PDX, which does not express *RSPO2*). HBCx-3 TNBC PDX (left panel) and HBCx-15 TNBC PDX (right panel) blocks were prepared and immunostained with anti-*RSPO2* antibody. Magnification × 20 (top panels) and × 40 (bottom panels).

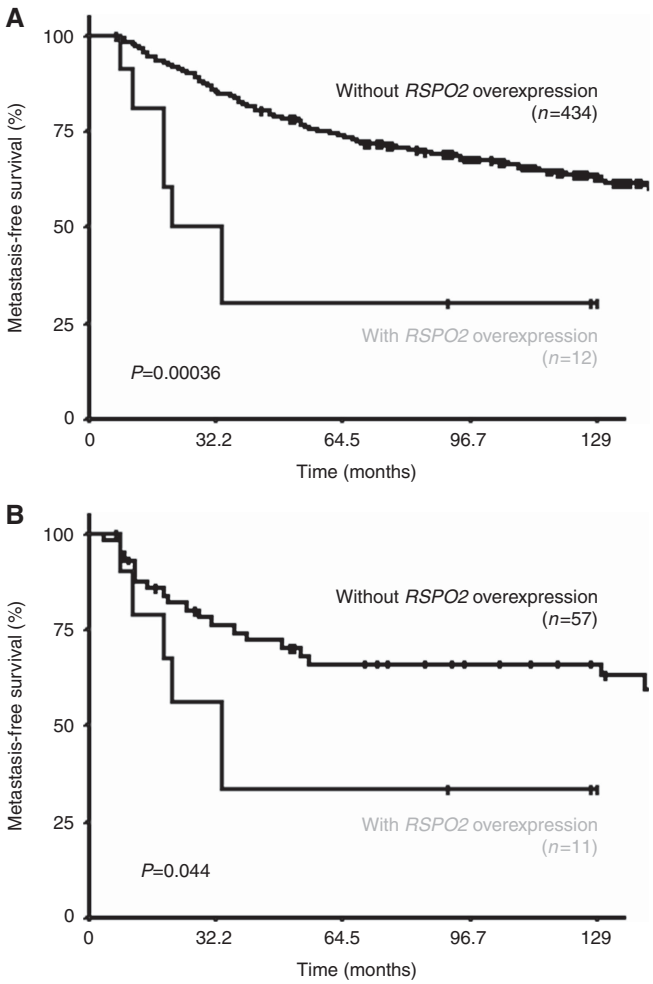

Figure 2. Relationship between *RSPO2* gene mRNA level and MFS in BC. (**A**) MFS curves for patients with *RSPO2* overexpression in the total population. (**B**) MFS curves for patients with *RSPO2* overexpression in patients with TNBC.

analysis cannot be performed for patients with tumours overexpressing *RSPO1*, *RSPO3*, *LGR4*, or *LGR6* because of the very small sample sizes.

Altogether, our results raise the hypothesis that *RSPO* overexpression, particularly *RSPO2* overexpression, may have an important role in the development of TNBC and MBC.

Correlation between expression of RSPO and LGR genes and genes involved in various cancer pathways. The TNBC subgroup is characterised by EMT, stem cell traits, and a high level of Wnt/β-catenin pathway activity (Sarrio *et al*, 2008; King *et al*, 2012). In order to further investigate our hypothesis, we therefore studied the correlations between the levels of expression of *RSPO* and their *LGR* receptors and EMT markers (*CDH1*, *VIM*, *ZEB1*, *ZEB2*, *SNAIL*, *SNAIL2*, *TWIST1*), stem cell markers (*ALDHA1A1*, *ALDHA1A3*, *CD133*), and Wnt/β-catenin activity (*AXIN2*, *DKK1*, *TCF4*, *LEF1*, *MMP7*) in TNBC. By hierarchical clustering of the samples, the 7 *RSPO/LGR* gene expression signature dichotomised the 68 TNBC analysed into two subgroups: a high *RSPO/LGR* expression group ($n=44$) and a low *RSPO/LGR* expression group ($n=24$). Mann–Whitney test demonstrated positive correlations between *RSPO/LGR* expression levels and EMT markers ($P=4.5\times10^{-2}$ for *VIM* and $P=4.7\times10^{-3}$ for *TWIST1*), stem cell markers ($P=4.5\times10^{-3}$ for *CD133* and $P=1.6\times10^{-2}$ for *ALDHA1A1*), and Wnt/β-catenin pathway activity ($P=4.8\times10^{-2}$ for *AXIN2* and $P=2.4\times10^{-2}$ for *MMP7*) (Table 2). *RSPO* overexpression may therefore have a role in TNBC carcinogenesis via activation of various pathways, such as the Wnt/β-catenin pathway.

Table 2. Relationships between *RSPO/LGR* gene expression and gene expression levels of different pathways of carcinogenesis

Genes	TNBC with high level of RSPO-LGR mRNA expression ($n=44$)	TNBC with low level of RSPO-LGR mRNA expression ($n=24$)	P^a
Stem cell pathway			
CD133	1.1 (0.02–7.9)	0.2 (0.006–5.8)	0.0045
ALDHA1A3	0.6 (0.1–11)	0.4 (0.08–14.2)	0.37
ALDHA1A1	0.1 (0.02–0.7)	0.09 (0.1–0.6)	0.016
EMT pathway			
CDH1	0.6 (0.01–3)	0.7 (0.047–5.4)	0.13
VIM	0.4 (0.15–2.1)	0.3 (0.02–0.8)	0.045
ZEB1	0.3 (0–1.2)	0.3 (0–0.8)	0.77
ZEB2	0.4 (0–1.9)	0.3 (0–0.9)	0.2
SNAIL	1.7 (0–8.2)	1.2 (0–13.1)	0.54
SNAIL2	0.6 (0–11.4)	0.5 (0–3.8)	0.054
TWIST1	0.4 (0.05–3.8)	0.2 (0.04–1)	0.0047
WNT/β-catenin pathway			
AXIN2	0.35 (0.07–3.6)	0.2 (0.04–1.3)	0.048
DKK1	5.5 (0–786)	8.8 (2.6–358)	0.97
TCF4	0.8 (0–3.6)	0.6 (0–3)	0.082
LEF1	0.3 (0–1.8)	0.2 (0.02–0.6)	0.32
MMP7	3.5 (0–58)	0.8 (0.02–19)	0.024

Abbreviations: EMT = epithelial–mesenchymal transition; LGR = leucine-rich repeat containing G protein-coupled receptor; TNBC = triple-negtive breast cancer.
[a]Mann–Whitney test.

RSPO2 and RSPO4 stimulate the Wnt/β-catenin pathway in human TNBC cell lines. To determine whether *RSPO2* and/or *RSPO4* overexpression (the two *RSPO* genes most frequently overexpressed in BC) may stimulate the activity of the Wnt/β-catenin pathway in BC cells, we assessed the effect of RSPO2-, RSPO4-, and Wnt3a-conditioned media on the expression of active β-catenin and phosphorylated-LRP6 (phospho-LRP6), two well-known markers of Wnt/β-catenin activity, by western blotting in the MDA-MB-468 TNBC cell line, which does not express any RSPO. *RSPO2* and *RSPO4* strongly enhanced the effect of Wnt3a on phospho-LRP6 levels and, to a lesser extent, active β-catenin (Figure 3A). Similar results were obtained with a second TNBC breast cell line, MDA-MB-231 (Supplementary Figure S3).

We then performed luciferase assays using TOPflash containing six TCF-binding elements as reported plasmid and found that RSPO2 and RSPO4 stimulated Wnt/β-catenin activity and potentiated the effect of Wnt3a on this signalling pathway in MDA-MB-468 cells (Figure 3B).

We also found that RSPO2 and RSPO4 strongly enhanced the positive effect of Wnt3a on the levels of two Wnt/β-catenin pathway target genes, *AXIN2* and *DKK1*, in the MDA-MB-468 cell line (Figure 3C).

Altogether, our results strongly suggest that *RSPO* overexpression, particularly *RSPO2* overexpression observed in BC, enhances breast carcinogenesis by activating the Wnt/β-catenin pathway.

Inhibition of RSPO2 expression inhibits proliferation of the BT549 TN breast cell line. Our previous findings suggested that inhibition of the biological function of *RSPO2* in BC cells would inhibit their proliferation. To examine this hypothesis, we first determined the effect of inhibition of *RSPO2* expression by using the siRNA approach on proliferation of the BT549 TN breast cell line, selected for its high level of *RSPO2* expression. As expected, effective inhibition of endogenous *RSPO2* expression by two different *RSPO2*-specific siRNA resulted in statistically significant

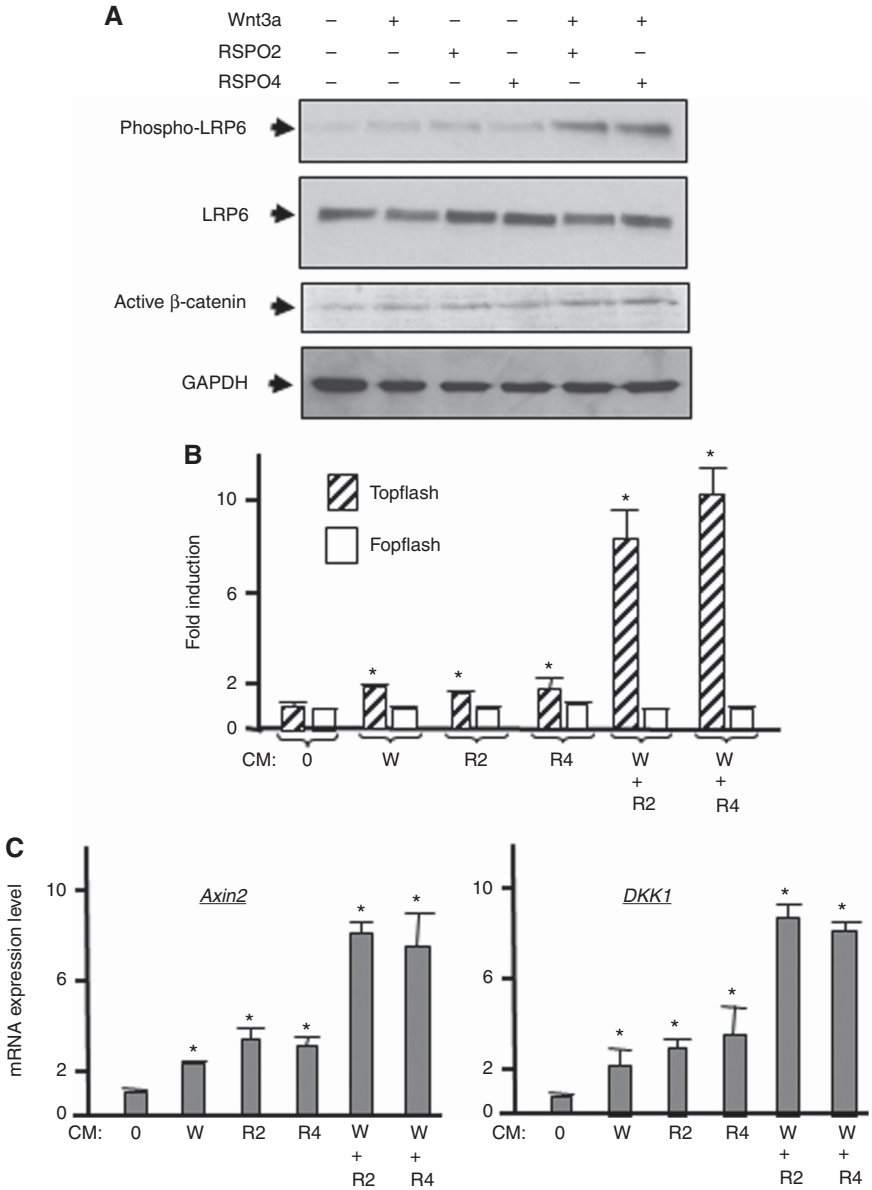

Figure 3. *RSPO2* and *RSPO4* stimulate Wnt/*β*-catenin pathway activity in MDA-MB-468 cells. (**A**) Effect of *RSPO2*, *RSPO4* and/or Wnt3a on the expression of phosphorylated LRP (phospho-LRP) and active *β*-catenin. (**B**) Effect of *RSPO2*, *RSPO4*, and/or Wnt3a on Wnt/*β*-catenin transcriptional activity. (**C**) Effect of *RSPO2*, *RSPO4*, and/or Wnt3a on the expression of Axin2 and DKK1. CM: conditioning medium, W: Wnt3a, R2: *RSPO2*, R4: *RSPO4*, *: indicates $P \leqslant 0.05$ *vs* control (CM: 0).

inhibition of BT549 cell proliferation ($P < 0.001$, Mann–Whitney test; Figure 4).

The Wnt/β-catenin inhibitor, IWR-1, inhibits the growth of a human TNBC PDX overexpressing *RSPO2*. We then tested the effect of the Wnt/*β*-catenin pathway inhibitor, IWR-1, on the growth of a human TNBC PDX (HBCx-60) overexpressing *RSPO2* (Ct = 27.94). This PDX displays a metaplastic phenotype. Treatment by IWR-1 resulted in significant tumour growth inhibition and this effect persisted after 2 weeks (Figure 4C). Optimal tumour growth inhibition (TGI) of treated tumours *vs* controls was calculated as the ratio of the mean RTV in the treated group to the mean RTV in the control group at the same time. Treatment by IWR-1 resulted in a TGI of 23%. Untreated xenografts rapidly reached the ethical size limit within 12 days and mice had to be killed.

Inhibition of Wnt/*β*-catenin pathway activity in this *RSPO2*-overexpressing breast tumour therefore induces tumour growth inhibition.

DISCUSSION

This study of a series of breast tumours showed, for the first time, that the *RSPO* gene family is overexpressed in 11.6% of tumours, mainly in TNBC (55.8%) and MBC (64%). This overexpression mainly concerns *RSPO2* (2.7% in BC, 17.6% in TNBC, and 46.4% in MBC) and *RSPO4* (9.2% in BC, 42.6% in TNBC, and 42.8% in MBC). These observations led us to assume that overexpression of certain *RSPO*s could confer an advantage for the development of the breast tumour.

Various studies have shown that *RSPO2* and *RSPO3* overexpression is due to the presence of gene fusions involving *RSPO*, *EIF3E*, and *PTPRK* genes in CRC and in malignant peripheral nerve sheath tumours (Seshagiri *et al*, 2012; Watson *et al*, 2013; Shinmura *et al*, 2014). Other studies have indicated that other mechanisms may also be involved in *RSPO* overexpression in cancers (Robinson *et al*, 2015; Gong *et al*, 2014). In our study, only

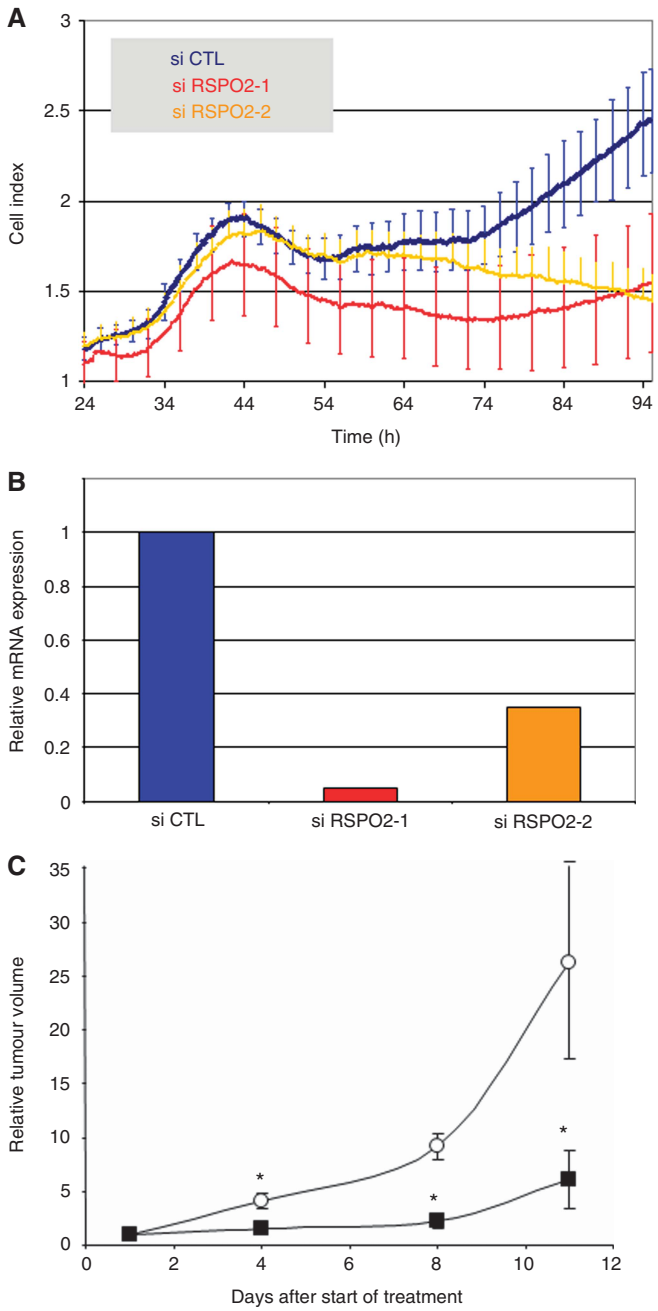

Figure 4. Effect of inhibition of RSPO2. (**A**) Inhibition of proliferation of BT549 cells by two siRNA of *RSPO2*. (**B**) Inhibition of mRNA expression determined by qRT–PCR: *RSPO2* expression was decreased by siRSPO2-1 and siRSPO2-2 to 5% and 35%, respectively, *vs* CTL (control). *$P<0{,}0001$, Mann–Whitney test. (**C**) The Wnt/β-catenin inhibitor IWR-1 inhibits the growth of HBCx-60 *RSPO2*-overexpressing human TNBC PDX; ■ = IWR-1, ○ = vehicle alone. Tumour growth was evaluated by plotting the mean RTV ± s.d. per group over time after first treatment. *$P<0.05$, unpaired *t*-test.

one case of *RSPO2* overexpression in BC (in the HBCx-15 TNBC PDX and the derived HBCc-15 cell line) can be explained by the presence of fusion transcripts involving *EIF3E* but not *PTPRK*. The small number of fusion transcripts indicates that other mechanisms are also involved in *RSPO* overexpression in BC. *RSPO2* is located on 8q23 near the *c-MYC* gene (8q24). *c-MYC* amplification is observed in many different cancers, particularly in 16% of cases of BC (TCGA, Cancer Genome Atlas Network, 2012). We observed a correlation between *RSPO2* expression and amplification of this region in BC (TCGA data, http://www.cbioportal.org/), suggesting that *RSPO2* overexpression in BC could be at least partly due to co-amplification with *MYC*. In this context, it is noteworthy that *MYC* and *PVT1* synergise to increase the level of *RSPO1* in BC (Sarver *et al*, 2016). *RSPO* overexpression could also be due to hypomethylation. Although carcinogenesis involves various pathways of gene alterations driven by DNA methylation, several studies have implicated activation of gene expression via hypomethylation. Global genomic hypomethylation is found in many types of cancer, particularly in BC, and is associated with high metastatic risk and death (Cheishvili *et al*, 2015). We observed a correlation between *RSPO4* expression and *RSPO4* hypomethylation in BC (TCGA data, http://www.cbioportal.org/). This mechanism could therefore be responsible for *RSPO4* overexpression in BC.

Patients with *RSPO2*-overexpressing TNBC have significantly poorer MFS than patients with TNBC not overexpressing this *RSPO*. It is not the case for *RSPO4*. Futhermore, the inhibition of *RSPO2* expression in the BT549 TN cell line inhibits cell growth. These findings strongly suggest that *RSPO* overexpression, particularly *RSPO2* overexpression, has an important role in the development of TNBC. These results suggest that overexpression of *RSPO2* has specific effects on the development of breast tumours. The hypothesis that *RSPO2* has specific function is supported by the fact that deficiency for RSPO2 in mice is postnatally lethal, indicating that this RSPO protein has functions that are non-redundant from the other RSPO members (Nam *et al*, 2007).

The role of *RSPO* in breast carcinogenesis is supported by the fact that *RSPO2* and *RSPO3* are overexpressed by mouse mammary tumour virus proviral insertions in mouse mammary tumours and that *RSPO2* overexpression in mammary cell lines stimulates tumour growth (Callahan *et al*, 2012; Klauzinska *et al*, 2012). *RSPO* overexpression has also been found in CRC, pancreatic ductal adenocarcinoma, lung adenocarcinomas, prostate cancer, and malignant peripheral nerve sheath tumours, suggesting a crucial role of *RSPO* in the development of various cancers (Seshagiri *et al*, 2012; Watson *et al*, 2013; Gong *et al*, 2014; Shinmura *et al*, 2014; Ilmer *et al*, 2015; Robinson *et al*, 2015).

Many experimental arguments and observations strongly suggest that the abnormally high activity of the Wnt/β-catenin pathway has a crucial role in the development of BC, specifically in TNBC, by stimulating EMT and stem cell growth (Lin *et al*, 2000; Li *et al*, 2003; Lindvall *et al*, 2006; Yook *et al*, 2006; Lehmann *et al*, 2011; Xu *et al*, 2012; Dey *et al*, 2013). Furthermore, constitutive activation of the Wnt/β-catenin pathway induces mammary metaplastic carcinomas in mice (Teuliere *et al*, 2005). However, the molecular mechanisms responsible for activation of the Wnt/β-catenin signalling pathway in BC have not been elucidated (Howe and Brown, 2004). Several reports suggest that hyperactivity of the Wnt/β-catenin pathway in this cancer could be due to aberrant expression levels of *LRP6/5*, *FZD7*, Wnt ligands, and Wnt inhibitors (King *et al*, 2012). The *APC* and *CTNNB1* mutations inducing β-catenin stabilisation observed in various type of cancer, such as CRC, are rare in BC. Our data raise the interesting hypothesis that *RSPO2/4* overexpression could be one of the mechanisms responsible for activation of the Wnt/β-catenin pathway, thereby activating breast carcinogenesis. First, we found that the *RSPO* gene family is mainly overexpressed in TNBC. Second, a positive correlation was observed between *RSPO/LGR* expression and EMT markers, stem cell markers, and Wnt/β-catenin pathway activity in TNBC. Third, we demonstrated that *RSPO2* and *RSPO4* stimulate the activity of this signalling pathway in two different TN breast cell lines. Fourth, inhibition of the growth of the HBCx-60 *RSPO2*-overexpressing metaplastic TNBC PDX by IWR-1 strongly suggests that the positive effect of *RSPO2* on breast tumour

growth is dependent on its ability to stimulate the activity of the Wnt/β-catenin pathway. Our hypothesis that *RSPO* overexpression could stimulate breast carcinogenesis by inducing Wnt/β-catenin pathway activity is strongly supported by recent studies showing that inhibition of RSPO by specific antibodies attenuates β-catenin signalling and tumorigenesis in multiple cancer types (ovarian cancer for *RSPO1*, colon and pancreatic cancer for *RSPO2*, non-small lung cancer, colorectal and ovarian cancer for *RSPO3*; Chartier *et al*, 2016).

CONCLUSION

Despite considerable progress in cancer research, the mortality rates of TNBC and MBC have remained unchanged over the past decade primarily due to the failure to identify specific targets. Responses to systemic chemotherapy, particularly for MBC, are suboptimal compared with patients with standard invasive ductal carcinoma and limited data are available concerning the optimal treatment modalities (Aydiner *et al*, 2015). Our study supports the identification of *RSPO* overexpression as a new therapeutic target and an attractive predictive biomarker for the development of therapies targeting the Wnt/β-catenin signalling pathway, particularly in TNBC and the metaplastic subtype.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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