R-spondin3 is associated with basal-progenitor behavior in normal and tumor mammary cells

Supplementary Materials and Methods

Cell culture, transfections, and differentiation procedures

HC11 cells [53] were cultured in RPMI 1640 supplemented with 10% (v/v) FBS, 1% penicilin/streptomycin, and 5 µg/ml insulin (Sigma-Aldrich). For differentiation assay, confluent HC11 cells were cultured in 2% FBS medium with 5 µg/ml insulin for 72 h, followed by lactogenic stimuli addition of 5 µg/ml prolactin (Sigma-Aldrich) and 10^{-7} M dexamethasone (Sigma-Aldrich) during 72 h prior to harvesting. SCg6, SCp2 [54], and EpH4 [55] were grown in DMEM/F12 with 2% (v/v) FBS/penicilin/streptomycin and 5 µg/ml insulin. To induce differentiation, SCp2 and EpH4 cells were cultured in serum-free DMEM/F12 containing 5 µg/ml insulin, 1 µg/ml hydrocortisone (Sigma-Aldrich), and 5 µg/ml prolactin, with or without 1.5% v/v laminin-rich basement membrane (Matrigel; Corning) and 60 ng/ml recombinant RSPO3 protein (R&D-Systems) for 72 h. LM3 cells [56] were cultured in MEM (Gibco) supplemented with 5% (v/v) FBS and penicilin/streptomycin. NMuMG cells [57] were grown in RPMI 1640 supplemented with 10% (v/v) FBS and penicilin/streptomycin. All cells were cultured at 37°C with 5% CO_2. Medroxyprogesterone (MPA)-induced (ER+, PR+) C4-derived mammary tumor cells (L2: line2, HI: hormone independent; HD: hormone dependent; HIR: hormone independent, RU-486 resistant) [58] were not cultured in our laboratory. Instead, RNAs, used in figure 1E, were kindly provided by Dr. Claudia Lanari (IByME-CONICET, Argentina).

RT-qPCR

All cDNAs were synthesized with 200U MMLV reverse transcriptase (Promega); 25 ng/liter random primers or oligo-dT (Invitrogen); 2 g/liter RNAsin (Promega), and 1.5 mM dNTPs (Invitrogen). RT was performed at 40°C for 60 min followed by 15 min at 72°C. All RT-qPCR reactions were conducted in a volume of 25 µl containing 3-4 mM $MgCl_2$ (Invitrogen), 0.2 mM dNTPs, 0.75U Taq polymerase (Invitrogen), 1:30,000 SYBR Green (Roche), and specific oligonucleotides for each gene (see Primer Table below). Reactions were run for 40 cycles under the following conditions: 15 sec at 94°C, 20 sec at 60-68°C (according to primers), and 25 sec at 72°C. The amplification of unique products in each reaction was verified by melting curve and ethidium bromide (Sigma Aldrich)-stained agarose gel electrophoresis.

Primer Table. Primer sequences used in this study.

Gene	Primers	Length (bp)	Reference
Actinb1	**Fw:** 5'-CGGTTGGCCTTAGGGTTCAGGGGGG-3' **Rv:** 5'- GTGGGCCGCTCTAGGCAC-3'	245	[59]
Csn2	**Fw:** 5´-GATGCCCCTCCTTAACTCTGAA-3´ **Rv:** 5´-TTAGCAAGACTGGCAAGGCTG-3´	75	[60]
Cdh1	**Fw:** 5'- GCTTCAGTTCCGAGGTCTACAC-3' **Rv:** 5'- CTGTGATGGTGCCGTCTGTC-3'	162	Designed
Fn1	**Fw:** 5'- TACCAAGGTCAATCCACACCCC -3' **Rv:** 5'- CAGATGGCAAAAGAAAGCAGAGG -3'	366	[61]
Gapdh	**Fw:** 5'-AGAAGGTGGTGAAGCAGGCATC-3' **Rv:** 5'-CGAAGGTGGAAGAGTGGGAGTTG-3'	111	Designed
Hsp90ab1	**Fw:** 5'- CCAAAAAGCACCTGGAGATCA -3' **Rv:** 5'- TGTCGGCCTCAGCCTTCT -3'	72	[62]
Krt14	**Fw:** 5'- CCTCTGGCTCTCAGTCATCC -3' **Rv:** 5'- TGAGCAGCATGTAGCAGCTT -3'	144	[63]
Krt18	**Fw:** 5'- AAGGTGAAGCTTGAGGCAGA -3' **Rv:** 5'- CTGCACAGTTTGCATGGAGT -3'	111	[63]
Lgr4	**Fw:** 5'- AACAGTACCCAGTGAAGCCATT -3' **Rv:** 5'- GATGTTGTCATCCAGCCACAGA -3'	139	[64]
Rspo3	**Fw:** 5'- CCAAGTGGATATTACGGAACTCG -3' **Rv:** 5'- GCAACTGTCAAGGCACTTTCC -3'	124	Designed
Vim	**Fw:** 5'- TCCTGATTAAGACGGTTGAGAC -3' **Rv:** 5'- GCAGTAAAGGCACTTGAAAGC -3'	169	Designed

Protein analysis

Total proteins were extracted from cell culture in RIPA buffer (50 mM Tris-HCl; 150 mM NaCl; 1% Triton X-100; 0.25% sodium deoxycholate; 1 mM EDTA, pH 7.4) supplemented with protease cocktail set I (Calbiochem) and phosphatase inhibitors (1 mM NaF and 1 mM Na_2VO_4). The lysate was centrifuged at 13,000g and 4°C for 20 min and the pellet was discarded. Protein concentration was determined by Bradford´s method using BSA as a standard. For WB, cleared lysates were combined with SDS sample buffer (50 mM Tris, pH 6.8; 1% SDS; 0.1% bromophenol blue; 10% glycerol; 100 mM DTT), boiled for 5 min and electrophoresed for 3 h at 100 V in a 10-12%, SDS-polyacrylamide gel, transferred to a PVDF membrane (GE Healthcare Life Sciences) by electroblotting in transfer buffer (20% methanol; 0.19 M glycine; 0.025 M Tris-base; pH 8.3) at 300 mA for 1.5 h. Blots were blocked 1 h at room temperature in TBS (20 mM Tris-Cl, pH 7.5; 500 mM NaCl) containing low-fat 5% powdered milk and 0.1% Tween 20. Membranes were then incubated overnight at 4°C in blocking buffer (Tris-buffered saline containing 3% skim milk and 0.1% Tween 20) with primary antibodies. Membranes were then incubated 2 h at room temperature with the secondary peroxidase-labeled antibodies in blocking buffer. Immunoreactive protein bands were detected using the enhanced chemiluminescence system (ECL Plus

system; GE Healthcare Life Sciences) and the FujiFilm ImageReader LAS-1000 (Fuji Film). Densitometric analysis of protein levels was performed with ImageJ 1.34s software (Wayne Rasband, NIH, http://rsb.info.nih.gov/ij/). For band quantification, obtained images were converted to grayscale and equal areas encompassing each band were drawn. Then, integrated density in each rectangle was obtained and background noise was subtracted. Obtained values were normalized as indicated.

β-catenin transcriptional activity-Luciferase reporter assay

NMuMG, shControl and shRspo3 SCg6 cells were plated in 6-well plates at a density of 25×10^4 per well. The following day, cells were transfected with 1 μg/well of pGL3-OT, an improved version of TOP-Flash vector provided by Dr. Jorge Filmus (Sunnybrook Health Sciences Centre, Canada) which contains a firefly luciferase gene under the control of TCF/LEF binding sites, and with 1 μg/well of pCMVLacZ vector, which expresses high levels of β-galactosidase and was used as a reference (control) vector. Transfection was performed using Polyethylenimine (Polysciences) in serum-free medium according to manufacturer's instructions. Cell extracts were prepared 48 h or 72 h after transfection using Reporter Lysis Buffer (Promega). Luciferase activity was determined by Luciferase Reporter Assay system (Promega), and β-galactosidase activity was detected using Z Buffer (0.06M $Na_2HPO_4.7H_2O$; 0.04M $NaH_2PO_4.H_2O$; 0.01M KCl; 0.001M $MgSO_4$; 0.05M β-mercaptoethanol; pH 7.0) with 4 mg/ml ortho-Nitrophenyl-β-galactoside. Then, pGL3-OT activity was normalized to β-galactosidase activity and expressed relative to control conditions.

Immunofluorescence

Cells were grown on glass coverslips in 6-well plates (25×10^4 cells/well) for 24 h, fixed with 4% Paraformaldehyde/Sucrose (1:1) (Sigma-Aldrich) at room temperature for 30 min, and blocked with 3% (w/v) BSA (Sigma-Aldrich) for 2 h. Cells were subsequently incubated with the fluorescent F-actin probe Rhodamine Phalloidin (dilution 1:200; Sigma-Aldrich) at room temperature for 30 min or with the following primary antibodies: anti-β-catenin (dilution 1:200; BD Biosciences; #610154), anti-Vimentin (dilution 1:75; Abcam; 7783-500) or anti-Fibronectin (dilution 1:400; Sigma; F6140; clone FN-3E2) overnight at 4°C in PBS containing 0.1% BSA, followed by incubation with the fluorescently labeled secondary antibodies Alexa Fluor 555 goat anti–mouse IgG, Alexa Fluor 647 donkey anti–mouse IgG, and Alexa Fluor 647 donkey anti–rabbit IgG (dilution 1:400; Life Technologies) at room temperature for 2 h. Cells were stained with DAPI (dilution 1:2500; Roche) at room temperature for 10 min to visualize cell nuclei. Slides were covered with 80% glycerol/PBS mounting medium and examined under an Olympus FV100 confocal microscope. Images were analyzed with MacBiophotonics ImageJ.

Mouse mammary cell isolation

Lymph node-free mammary glands from five 12-week-old female virgin mice were minced and digested for 1 h at 37°C in serum-free PBS buffer containing 2 mg/ml Collagenase A (Roche) and 0.5 mg/ml Trypsin (Sigma-Aldrich) in a rotating shaker. Digested mammary suspension was later washed in 10% (v/v) FBS/PBS for enzyme inactivation. After spinning, the epithelial cell pellet was resuspended and washed in red blood cell lysis buffer (NH_4Cl/Tris pH 7.2) twice for 5 min. In order to obtained single-cell suspensions, the cell pellet was then incubated with 0.25% (w/v) Trypsin/EDTA (Gibco) and 1 μg/ml DNase I (Promega) in serum-free PBS buffer for 2 min at 37°C. After enzyme inactivation with 10% (v/v) FBS/PBS buffer, cell suspension was filtered through a 40-μm cell strainer (BD), centrifuged at 1400g for 5 min, and single-cell pellet was resuspended in 3 ml of 10% (v/v) FBS/PBS for cell counting.

In silico analysis

Comparative analysis of *RSPO3, CDH1*, *FN1*, *VIM*, *SNAI1*, *SNAI2* and *TWIST* expression performed in breast cancer cell lines was analyzed using inSilicoDb and inSilicoMerging R/Bioconductor packages [65]. Gene expression profiles were developed using the Affymetrix HG-U133 Plus2 platform (GPL570). Briefly, frozen RMA pre-processed expression profiles of these studies were downloaded from the InSilico database and merged using the COMBAT algorithm as batch removal method. Data integration and visualization of differentially expressed transcripts were done with R/Bioconductor and the MultiExperiment Viewer software (MeV v4.9).

Mouse *Rspo3* mRNA expression was analyzed among luminal cells isolated from the mammary glands of adult virgin female mice derived from the GSE47377 dataset. Briefly, *Rspo3* mRNA pre-processed data (1443187_at) was obtained from GEO2R resource and further analyzed with R/Bioconductor.

Comparative analysis of *RSPO1*, RSPO3, *LGR4-6*, and *RUNX1-3* mRNA expression profiles across primary human breast carcinomas was performed using TCGA Network, breast cancer project [66] available at UCSC Xena resource (http://xena.ucsc.edu/). Intrinsic subtype classification of 1097 primary breast carcinomas was performed using the 50-gene (PAM50) predictor bioclassifier R script [67]. ESTIMATE algorithm was used to infer the immune and stromal components from each sample [68]. Comparative analysis of *RSPO3*, *ER/PR/HER2* and EMT marker (*CDH1*, *VIM*, *SNAI2*, and *TWIST*) mRNA expression among 1985 breast cancer samples was done using the METABRIC dataset available at cBioPortal resource (http://www.cbioportal.org/).

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