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

Supplementary Figures

Supplementary Figure S1

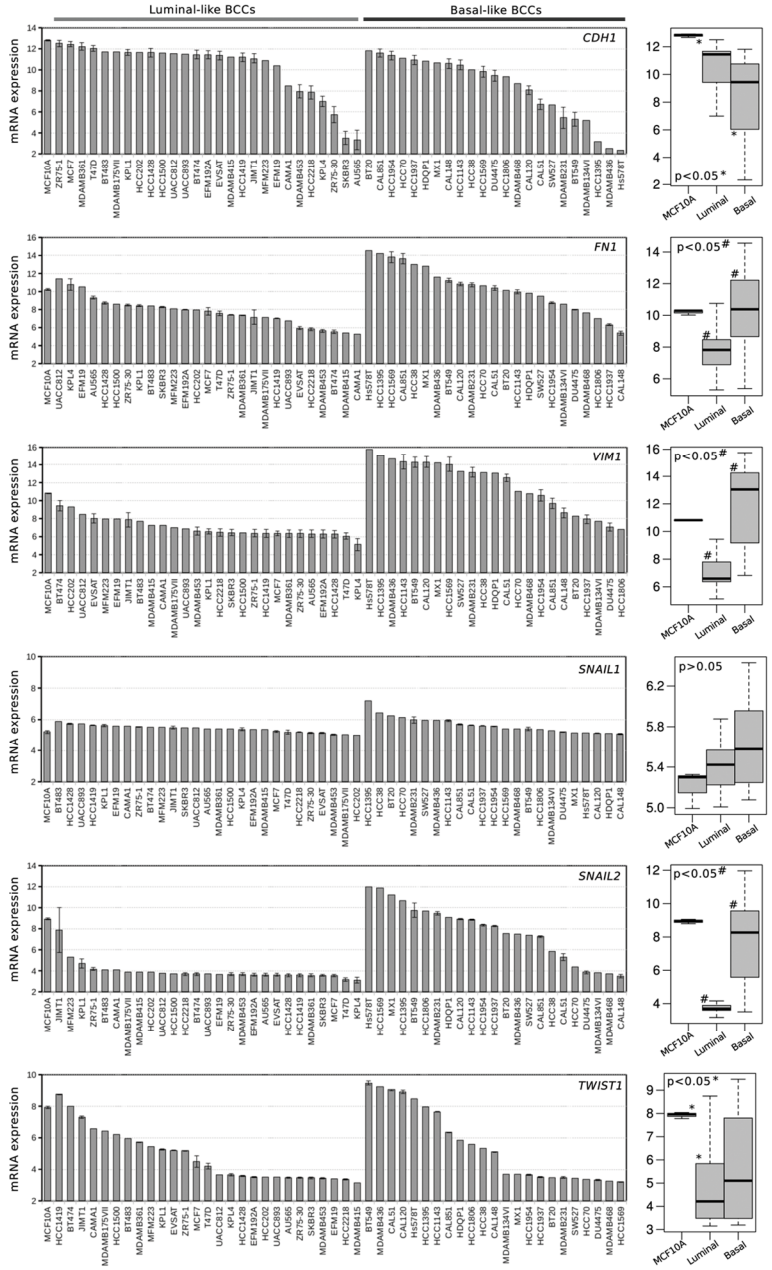


Figure S1. EMT and CSC gene expression in human mammary cancer cell lines. *CDH1*, *FN1*, *VIM*, *SNAI1*, *SNAI2*, and *TWIST* mRNA expression profile and box plots based on a compiled *in silico* dataset of 50 human breast cancer cell lines (BCCLs): 27 luminal-like and 23 basal-like BCCLs, and the non-tumorigenic MCF-10A cell line, obtained from GSE10843, GSE12777, and GSE41445 datasets showing statistically significant differences between groups.

Supplementary Figure S2

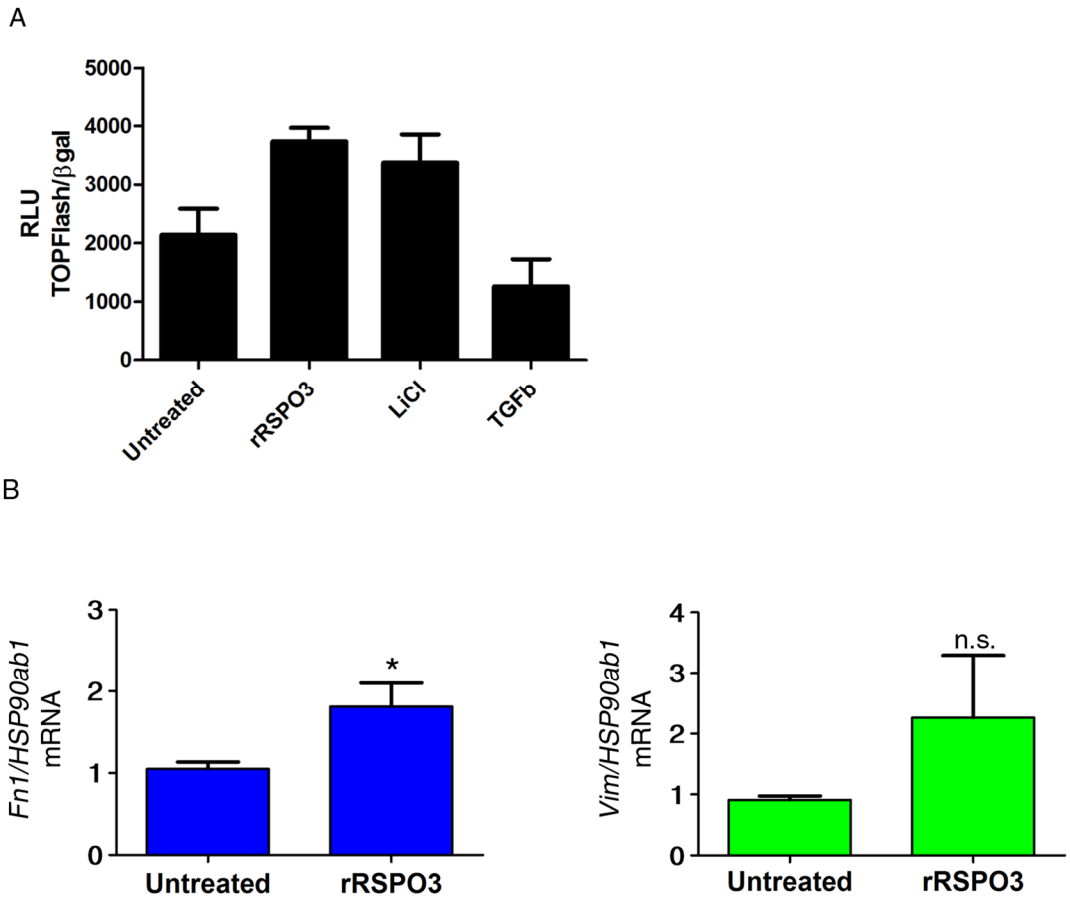


Figure S2. RSPO3 induces canonical WNT pathway activation and promotes mesenchymal/basal phenotype in mouse mammary epithelial cells. (A) NMuMG cells were transiently transfected with Luciferase β-catenin reporter and β-galactosidase vectors, and treated with rRSPO3 protein (60 ng/ml), TGF-β (2 ng/ml) or LiCl (20 mM) during 72 h. LiCl was used as a canonical Wnt pathway inductor. Error bars represent the s.d. (n=2). (B) RT-qPCR analysis of mesenchymal (vimentin and fibronectin) markers in luminal-like SCp2 cells treated or not with rRSPO3 protein. Gene expression data were normalized to *HSP90ab1* mRNA and are shown as fold change (mean +/- s.e.m.) relative to untreated cells. Student´s t-test, *: P<0.05; n.s. not significant; n=3.

Supplementary Figure S3

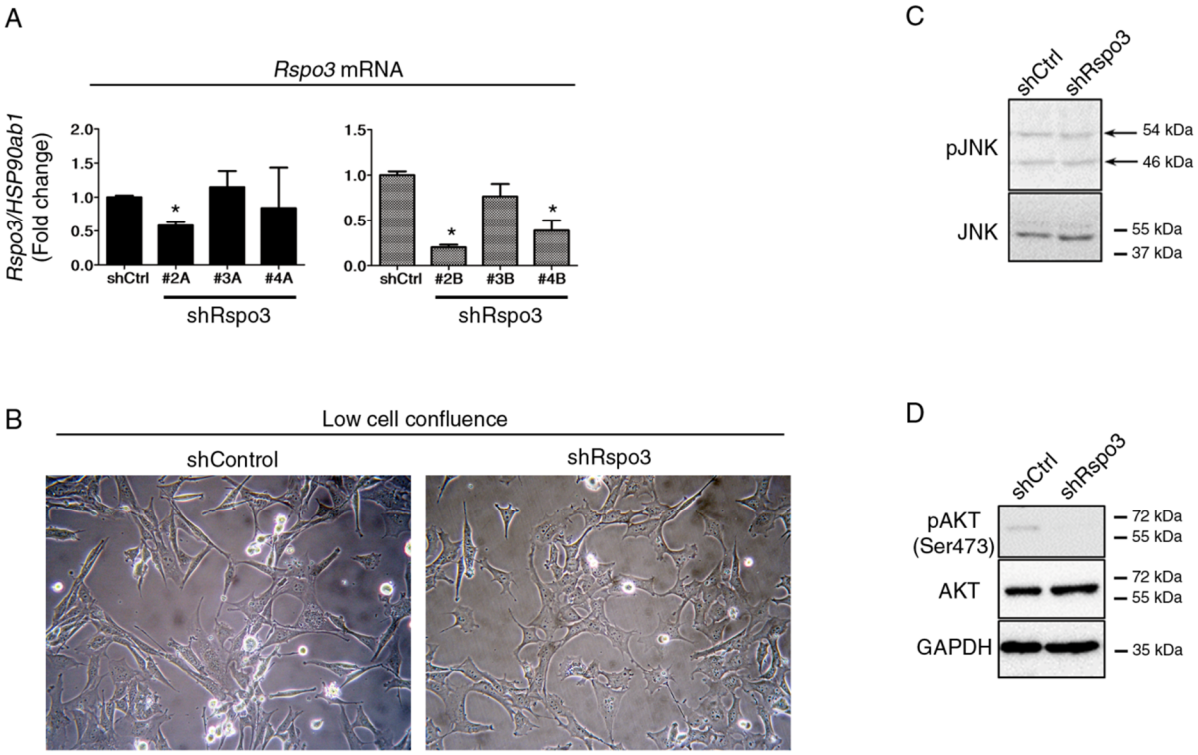


Figure S3. *Rspo3* down-regulation alters basal phenotype in tumor SCg6 mammary cells. (A) RT-qPCR analysis of *Rspo3* levels in SCg6 cells after stable transfection with a Scrambled shRNA (shControl) or with four different shRNA sequences against *Rspo3* (shRspo3). #2-4, A and B, represent different shRspo3 isolated clones obtained after stable transfection. mRNA expression data were *HSP90ab1*-normalized and represent fold change (mean +/- s.e.m.) compared to shControl cells. *P<0.05 indicates statistically significant differences vs shControl; Dunnett´s test following one-way ANOVA. (B) Representative images of shControl and shRspo3 cells at low confluence. (C) Representative WB analysis of total JNK and its phosphorylation levels, as a non-canonical *Wnt* pathway activation marker, in SCg6 stably transfected cells. (D) Representative WB of AKT phosphorylation levels, as a measure of PI3K/ATK pathway activation in shControl and shRspo3 cells. GAPDH was used as loading control.

Supplementary Figure S4

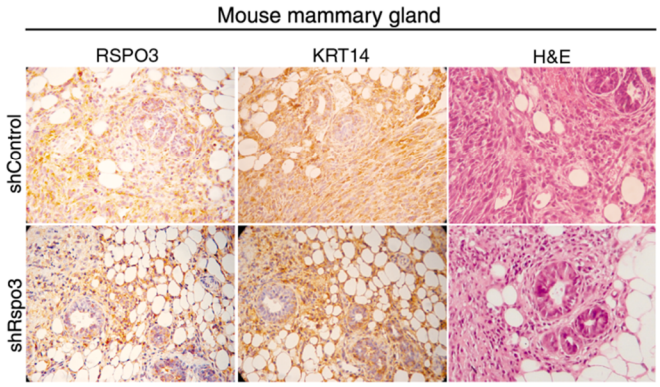
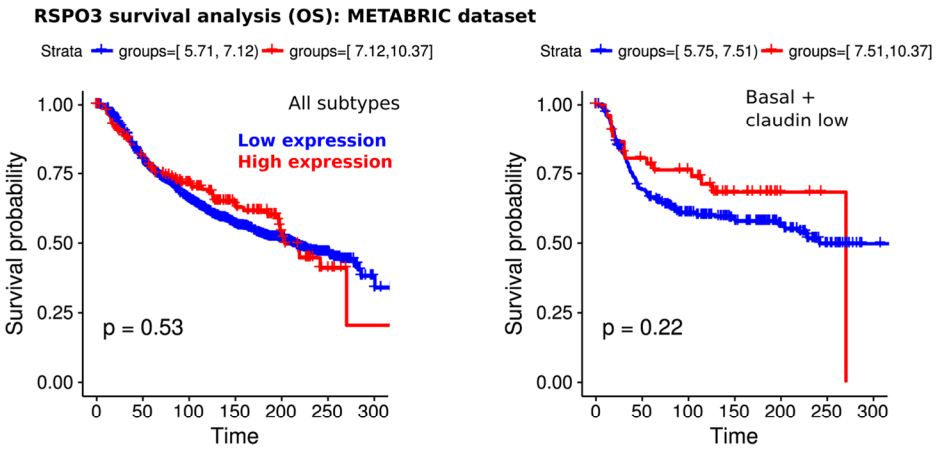


Figure S4. *Rspo3* down-regulation reduces SCg6 tumorigenicity *in vivo*. Representative micrographs of RSPO3 and KRT14 IHC, as well as H&E staining of intra-mammary implants of shRspo3 (clone 4B) and shControl cells 20 days post-inoculation. Original magnification: 400x.

Supplementary Figure S5

A



B

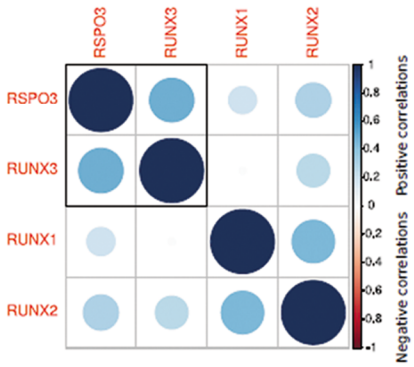


Figure S5. (A) Prognostic value analysis of human *RSPO3* expression among the two independent dataset employed in our study (TCGA-BRCA RNA-Seq and the METABRIC cohorts) using univariate (Kaplan–Meier survival curves and log-rank test) and multivariate method (Cox proportional-hazard model). Briefly, breast cancer patients were clustered into low or high *RSPO3* expression levels based on the median values of the normalized profiles. (B) *RSPO3* and *RUNX1-3* correlation matrix using the TCGA-Breast cancer RNA-Seq dataset obtained from UCSC Xena Browser.

Supplementary Figure S6

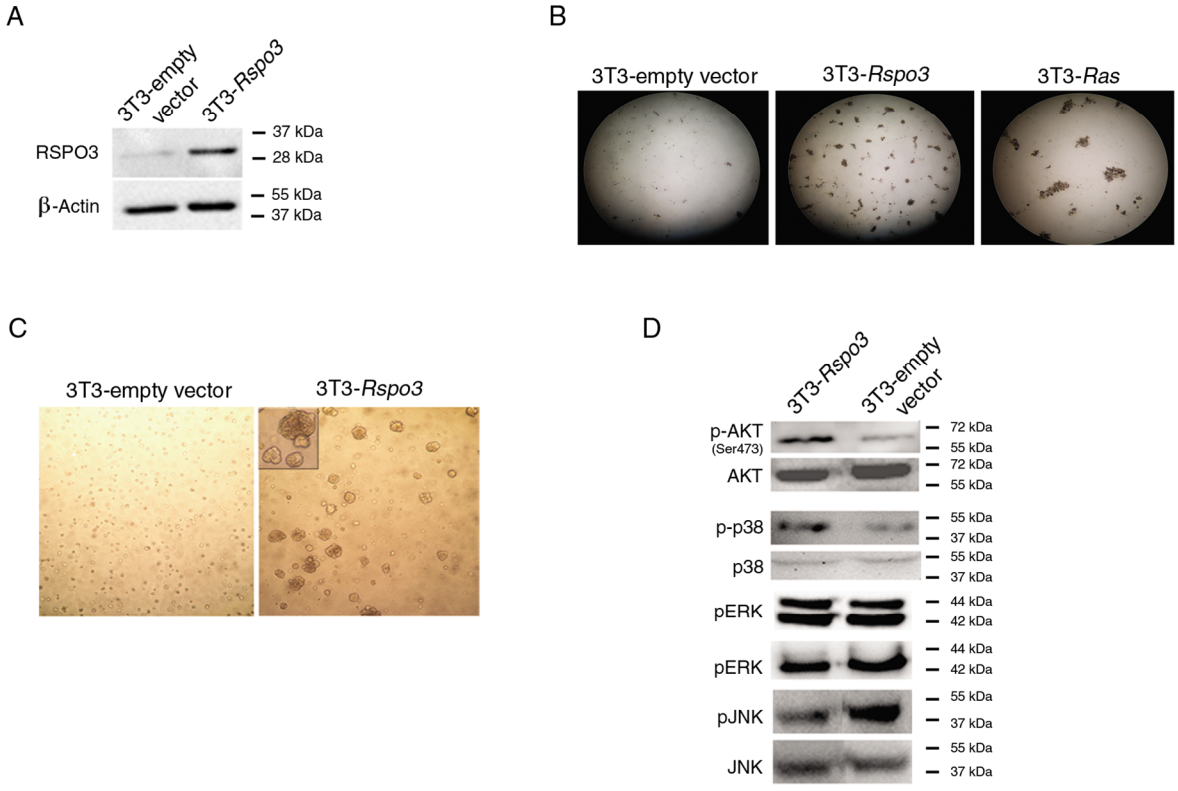


Figure S6. *Rspo3* overexpression induces anchorage-independent growth and suppresses cell contact inhibition of 3T3 cells through modulation of AKT signaling pathway. Full length *Rspo3* cDNA (870bp) was cloned in a pGEM-T vector and subcloned in the expression vector pCEFL under the EF1 promoter. Normal mouse epithelial fibroblasts (NIH-3T3 cell line) were transfected with this construct or the empty vector and selected by G418 antibiotic. (A) Representative WB analysis of RSPO3 expression in NIH-3T3 cells stably expressing either empty vector (3T3-Vector) or *Rspo3* cDNA (3T3-*Rspo3*). β-actin was used as a loading control. (B) Representative images of foci formation assay. NIH-3T3-vector or 3T3-*Rspo3* cells were cultured on top of a lawn of WT NIH-3T3 cells attached to plastic dishes. NIH-3T3-Ras cell line, overexpressing *Ras* cDNA, was used as a positive control of foci formation. (C) Representative images of soft-agar growth assay. NIH-3T3-vector or 3T3-*Rspo3* cells were cultured on agar-coated dishes. (D) Representative WB analysis of NIH-3T3-vector or 3T3-*Rspo3* total cell lysates showing expression levels of phosphorylated proteins involved in certain cell survival signaling pathways.

Supplementary Figure S7

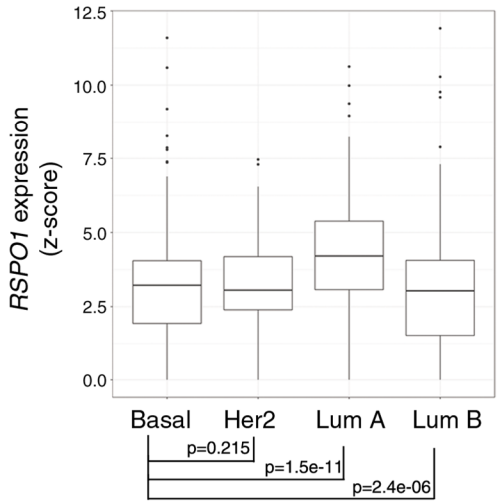


Figure S7. Comparative box plot analysis of *RSPO1* mRNA expression profile according to intrinsic subtypes (luminal A, luminal B, Her2, and basal-like) among primary breast carcinomas, by Pairwise comparisons using Tukey and Kramer test and the two independent dataset employed in our study (TCGA-BRCA RNA-Seq and METABRIC cohorts).