RSPO2 Enhances Canonical Wnt Signaling to Confer Stemness-Associated Traits to Susceptible Pancreatic Cancer Cells

Matthias Ilmer1, Alejandro Recio Boiles1, Ivonne Regel2, Kenji Yokoi3, Christoph W. Michalski4, Ignacio I. Wistuba1, Jaime Rodriguez1, Eckhard Alt5, and Jody Vykoukal1

Abstract

Cancer stem cells (CSC) present a formidable clinical challenge by escaping therapeutic intervention and seeding tumors through processes that remain incompletely understood. Here, we describe small subpopulations of pancreatic cancer cells with high intrinsic Wnt activity (Wnthigh) that possess properties indicative of CSCs, including drug resistance and tumor-initiating capacity, whereas cell populations with negligible Wnt activity (Wntlow) preferentially express markers of differentiation. Spontaneous response to extrinsic Wnt signals induces signaling networks comprising ERK1/2 and epithelial–mesenchymal transition that subsequently confer cancer stemness traits to susceptible cells. Wnt enhancer R-Spondin 2 (RSPO2) seems to play a prominent upstream role in regulating this interplay. In this context, Wnthigh cells were more likely to give rise to Wnthigh progeny, tended to be more metastatic, and revealed higher levels of RSPO2 expression. Our studies reveal adaptive aspects of pancreatic cancer stemness arising from driver populations of CSCs that misappropriate functional and responsive elements of archetypical self-renewal pathways. Blocking such stemness-promoting pathways in conjunction with established chemotherapy could provide means to disrupt dynamic CSC process and present novel therapeutic targets and strategies. Cancer Res. 75(9): 1883–96. ©2015 AACR.

Introduction

Adaptive phenotypic and functional diversity is characteristic of tumor survival. Apart from differences between tumor types, solid cancers also display conspicuous heterogeneities with (often dynamic) distinct biologic properties within the same tumor (1, 2). The concept of cancer stem cells (CSC) has been formulated to account for various levels of differentiation, with the least differentiated and tumor-propagating CSC at the apex of the hierarchy that must be eradicated to achieve durable cure (3). Pancreatic ductal adenocarcinoma (PDAC) is reported to belong to the CSC-organized set of solid tumors and, indeed, is encountered clinically as an aggressive tumor type associated with drug resistance, formation of remote metastasis, and an overall dismal prognosis despite increased research efforts in recent years (4, 5).

It is known that cancer stemness is not predefined, but rather a fluent condition that can be modified in susceptible cells by contextual signals. For example, cancer stem–like traits such as epithelial–mesenchymal transition (EMT), a well-conserved and critical process in embryonic development (6), can be reactivated in adult tissue cells, especially cancer cells (7). Various factors can provide these signals, e.g., members of self-renewal pathways such as TGFβ, Hedgehog, or Wnt (8). Canonical Wnt signaling has been widely investigated because driver mutations in core elements of the pathway, particularly in APC and β-catenin, were found in colorectal and liver cancers (9, 10). In “classical” canonical Wnt signaling, the transcriptional regulator β-catenin remains under strict control of the destruction complex consisting of APC, AXIN2, and GSK3β. Upon binding of Wnt ligands to LRPS/6 and Frizzled coreceptors, β-catenin escapes its degradation, translocates to the nucleus, and acts as transcription factor at Tcf/Lef-binding sites (11). Recently, it has been shown that Wnt activation can be markedly enhanced by simultaneous ligand–receptor binding of R-Spondins to LGR4, 5, or 6 (12).

Remarkably, even in cancers with activating β-catenin mutations, heterogeneous Wnt signaling activities can be encountered, and this so-called “β-catenin paradox” phenomenon might be indicative of cancer stemness trait regulation (13). Most PDACs do not harbor driver mutations in the canonical Wnt signaling pathway, and thus its involvement in regulating CSC behavior in PDAC is less clear. However, very recent evidence suggests that Wnt signaling might be necessary for pancreatic carcinogenesis and that this activation is most likely conveyed by ligand–receptor interactions (14). Moreover, monoclonal antibody inhibition of Wnt signaling was shown to improve antitumor treatment in an orthotopic mouse model that suggests that...
Materials and Methods

Cell culture

Cell lines were obtained from the ATCC or established at the University of Texas MD Anderson Cancer Center and kindly provided by Dr. Dominic Fan (L3.6pl) or Dr. Thiru Anumugam (MDA-Patx1 (Patx1), ref. 16). Cells were grown as described before at 37°C in MEM-α medium supplemented with 10% FBS, 1% penicillin/streptomycin, 100 nmol/L l-glutamine, 1× essential amino acid solution, and 1× MEM Vitamins (all from Gibco/Life Technologies; ref. 17). Cell lines (AsPC1 and L3.6pl) were authenticated within 6 months prior to use at the MDACC Cancer Center Support Grant Characterized Cell Line Core (funded by NCI # CA 16672) by short tandem repeat fingerprinting.

For further in vitro experiments, tumor xenografts were mechanically minced and then incubated at 37°C with an enzyme blend (Matrase; InGeneron) for 30 minutes, washed with full medium, and seeded into cell culture dishes. Medium was changed after 48 hours.

CSC spheroid culture and assay

As described elsewhere (4), cells were trypsinized, washed with PBS, and seeded into low-attachment plates (Corning) at clonal density (1,000 cells/mL) in CSC sphere-medium [MEM-α medium supplemented with 1× B27 (Gibco), 100 nmol/L L-glutamine, 1% penicillin/streptomycin, and EGF (10 ng/mL)/FGF (20 ng/mL; both from Sigma)]. For sphere-formation ability (SFA) experiments, 500 or 1,000 cells were added to SFA experiments as indicated in the text. Spheres were counted after 10 to 12 days of culture. Otherwise, diameters are indicated in the text.

Holoclone culture

Holoclone assays were performed as described by Qin and colleagues (18) with minor modifications. In summary, 500 single cells were seeded in 3 mL of complete culture medium into 6-well plates and incubated at 37°C for approximately 10 days. Colonies with approximately 50 cells or more were described as holoclines and then counted with a fluorescent microscope in the case of 7TGC-transduced cell cultures and after staining with crystal violet. In experiments where we compared different morphologic growth patterns of GFP<sup>high</sup> and GFP<sup>low</sup> cultures, holoclines with tightly packed colonies were called "epithelial," those with loosely accumulated colonies were "mesenchymal," and colonies that did not fit either of the above-described morphologies were designated as mixed (see also Supplementary Fig. S2F).

Recombinant proteins and small-molecule inhibitors

Recombinant human proteins were purchased from the indicated suppliers: Wnt3a and TRAIL (R&D Systems), and RSPO2 (Peprotech). The small-molecule inhibitors IWR1-endo and IWR2 were purchased from ReagentsDirect, and UO126 from EMD Millipore Chemicals. Gemcitabine was provided by the general institutional pharmacy and supplied by Eli Lilly.

Reverse-phase protein analysis

Reverse-phase protein analysis (RPMA) was carried out as described before by the Functional Proteomics RPPA Core Facility of MD Anderson Cancer Center (19). Samples subjected to the assay were sorted into GFP<sup>high</sup>/low populations, subsequently seeded into 6-well plates, and harvested 24 hours later according to the core facility's instructions. See full datasets in Supplementary Table S1.

Lentiviral and luciferase reporter assays

Lentiviral production was carried out following the AddGene online protocol. Briefly, 1.5 × 10<sup>5</sup> HEK293T helper cells were transfected at a ratio of 1:4:5 with envelope plasmid pCMV-VSVG (AddGene Plasmid no. 8545), packaging plasmid pCMV-dR8.2 (AddGene Plasmid no. 8455), and 7TGC (AddGene Plasmid #24304) with FuGeneHD (Roche) in a 60-mm dish. After overnight incubation, medium was changed and lentiviral soup was generated for 48 hours. Before use, the lentivirus-containing supernatant was filtered through a 0.45-μm filter and added to the cell cultures or stored at −80°C until further use. Cells were then washed with PBS, expanded, and sorted for equal mCherry intensities to guarantee comparable integration levels of the plasmid.

For transient transfections in reporter assays, 5.0 × 10<sup>4</sup> cells were transfected with 500 ng inducible firefly luciferase expressing either SuperTOP or SuperFOP vector (AddGene Plasmids #12456 and #12457) and cotransfected with constitutively Renilla luciferase expressing normalization control vector pRL-TK (Promega) at a ratio of 50:1. Cells were then cultured or stimulated for 24 hours, lysed with passive lysis buffer, and firefly and Renilla luciferase activity was monitored with the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. All experiments were performed in triplicates.

In vivo xenograft experiments

Mouse experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) at MD Anderson. Immunocompromised age-matched male 5- to 6-week-old, athymic, nu/nu, Balb/c mice were purchased from an institutional breeder colony and kept at 24°C and in sterile conditions with water and food ad libitum. As indicated, different dilutions of cells in 50 μL PBS were subcutaneously injected in the flank via a 27G-needle. For orthotopic tumor implantations, the mice were anesthetized with continuous sevofoflurane (2.5 L/min) and oxygen inhalation; after opening the abdominal cavity with a lateral incision over the spleen, the tail of the pancreas was mobilized, injected with 50 μL tumor cell suspension in PBS,
relocated, and the wound was closed with single suture (Vicryl) of the peritoneum and wound clips on the skin. Tumor growth was controlled and measured regularly (subcutaneous model), and mice were checked for their vital status and weight. Tumor volume was calculated by the following formula: \( V = \frac{4}{3} \pi r^3 \) (\( r \) is the radius of the sphere). All mice were sacrificed when their tumor volumes reached high tumor burdens according to the IACUC protocol or at the end of the experiment. Subsequently, tumor parts that were selected for further in vitro cell culture experiments were processed as described above. For further experiments, fragments of the tumors were either fixed in 4% paraformaldehyde for histologic analyses or frozen in liquid nitrogen for molecular analyses.

Mouse models and assessment of primary murine pancreatic cancer cells

Murine tumor cells were isolated from primary pancreatic tumors by dissection, mincing, and subsequent collagenase type V digestion (1 mg/mL Sigma Aldrich) for 30 minutes at 37°C. Cells were filtered through a 100 μm nylon mesh, washed twice with PBS, and cultured in DMEM with 10% FBS and 1% PS (penicillin/streptomycin) all reagents from Gibco Invitrogen. Isolated pancreatic tumor cells from a p48Cre;LSL-Kras \((G12D)\) were assessed for CSC characteristics. Cells were trypsinized, washed with flow buffer, and blocked for 15 minutes on ice with Fcblock (Anti-Mouse CD16/CD32 Purified; eBioscience). After washing, cells were stained with CD24/CD44 \((g/mL, #11-0242; eBioscience) and appropriate anti-rat IgG-FITC/APC controls (eBioscience) for 40 minutes on ice in the dark. Cells were washed twice with flow buffer and sorted in a CD24/CD44™ and a CD24/CD44high cell population (FACSaria III; BD Biosciences) and collected in ice-cold flow buffer for RNA isolation.

RNA isolation was performed according to manufacturer's instructions with the RNaseasy Plus Mini Kit from Qiagen. Afterwards, 1 μg RNA was reverse-transcribed with the RevertAid First Strand cDNA Synthesis Kit from Thermo Scientific.

Gene expression was analyzed with quantitative real-time PCR (qRT-PCR) in the LightCycler LC480 (Roche) and carried out with 40 ng complementary DNA, 500 nmol/L forward and reverse primer, and SYBR Green Master Mix (Roche) in a final volume of 20 μL. The following primer pairs (5′ → 3′orientation) were used according to standard PCR protocol (Supplementary Table S2).

Immunohistochemistry and immunofluorescence

PIA-fixed and paraffin-embedded tissue sections underwent antigen retrieval in sodium citrate buffer (pH 6.0) at boiling temperature for 20 minutes. After cooling down, sections were washed in TBS plus 0.025% Triton X-100, blocked in 10% goat serum, and incubated with primary antibody solutions at 4°C overnight. After washing, slides were directly incubated with Alexa-Fluor–conjugated secondary antibody solution at room temperature for 1.5 hours in the dark. Immunofluorescent slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and then mounted in Fluorescence Mounting Medium (Dako). IHC sections were stained using the VECTASTAIN Elite ABC Kit (Universal) according to the manufacturer’s instructions (Vector Laboratories). For primary goat antibodies, we used a horseradish peroxidase–conjugated anti-goat secondary antibody solution.

mRNA expression analysis

For total RNA extraction, cells were homogenized with TRIzol (Invitrogen). Phase separation was performed by the addition of chloroform and subsequent centrifugation steps. Aqueous phase of samples was collected and RNA precipitated by isopropyl alcohol. After washing, RNA was redissolved in DEPC-treated water, and RNA quality and quantity were measured with a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific). For cDNA synthesis, the iScript Reverse Transcription Supermix (Bio-Rad) was used according to the manufacturer’s protocol, and the reaction mix was incubated in a thermal cycler (Bio-Rad MyIQ Single-Color RT-PCR Detection System iCycler) with the following protocol: priming (5 minutes at 25°C), reverse transcription (30 minutes at 42°C), and reverse transcription inactivation (5 minutes at 85°C). qRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad) according to the following protocol: initial denaturation and enzyme activation (1 cycle at 95°C for 3 minutes), denaturing (40 cycles at 95°C for 15 seconds) with annealing and extension (40 cycles at 55°C for 30 seconds), and melting curve (1 cycle at 55–95°C in 5°C increments for 30 seconds). Ct was measured in absolute quantification and compared with GAPDH as housekeeping gene. Human-specific primers were used (Supplementary Table S3).

Flow cytometry

For Wnt activity, flow cytometry was performed on trypsin-detached 7TGC cultures or freshly digested tumor tissues from in vivo studies. After trypsinization/digestion, cells were washed with and analyzed in flow buffer (1x PBS with 2% FBS, 4°C). Cells were stringently gated on viable cells with the same background intensity of mCherry. Then, Wnt activity correlating with GFP levels was monitored on the LSRFortessa cell analyzer (BD Biosciences). Nontransduced cells were used as negative controls for both mCherry and GFP levels. FACS sorting of GFP\textsuperscript{high} or GFP\textsuperscript{low} subpopulations was carried out accordingly on the Influx cell sorter (BD Biosciences).

Flow cytometric analysis of cell surface markers was carried out on cells or spheroids cultures after dissociation with Versene Solution (Invitrogen). Cells were dissociated, washed, counted, and resuspended at a density of 1 to 2 × 10^5 cells/100 μL in flow buffer. Then, cells were stained with the respective antibody concentrations according to the manufacturer’s recommendations for 30 minutes on ice. After washing, cells were analyzed
on the LSRFortesa cell analyzer. For cell cycle experiments, cells were trypsinized, washed, and stored in 70% EtOH at 4°C for at least 24 hours. Cells were then washed and stained with Propidium Iodide or DAPI for 30 minutes at 37°C, washed again, and analyzed; data were evaluated with FlowJo 7.6 software.

Statistical analyses

Results are expressed as the mean ± SEM. All statistical comparisons were made with a standard t test, using bioinformatics software from GraphPad Prism. The criteria for significance were $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) for all comparisons.

Results

Canonical Wnt signaling is heterogeneously activated in PDAC

To understand baseline canonical Wnt signaling activity in pancreatic cancer, we transfected various PDAC cell lines with the well-established Super TOP/FOP (STF) dual-luciferase system. As expected and previously reported (20), Wnt signaling activity levels were rather low, especially relative to β-catenin-mutated cell line HepG2 (Fig. 1A). Interestingly, STF levels of the different pancreatic cancer cell lines varied significantly. Heterogeneous expression of the Wnt transcription factor β-catenin was observed in human pancreatic cancer tissue (The Human Protein Atlas; refs. 21, 22) as well as in xenografts from our own study, with most of the β-catenin being membrane bound and occurring infrequently in cytoplasmic/nuclear locations (Fig. 1B). To further investigate such intratumoral heterogeneities and because the STF reporter system does not account for individual cell variability of Wnt activation, we used a lentiviral GFP-coupled Wnt reporter construct (23) that yields expression of mCherry constitutively but expression of GFP only in cells with activated Wnt/β-catenin signaling (Fig. 1C and Supplementary Fig. S1A). In accordance with the earlier STF results, more than 85% of the HepG2 cells displayed high levels of Wnt activity, whereas in untreated pancreatic cancer

![Image of Figure 1](https://cancerres.aacrjournals.org/content/cancerres/75/9/1866/F1.large.jpg)

**Figure 1.** Heterogeneous activation of canonical Wnt in PDAC can be detected by lentiviral Tcf/LEF reporter A, luciferase reporter assay for β-catenin transcriptional activity. Different PDAC cell lines and HepG2 were transduced with SuperTOP or SuperFOP Flash Reporter (with mutated binding sites) and normalized to pRL-TK transfection control. Shown are the normalized ratios of TOP/FOP. n = 3. FF, firefly luciferase; RL, Renilla luciferase; RLU, relative light units. B, β-catenin staining of primary human pancreatic cancer (courtesy of The Human Protein Atlas; left) and an orthotopic PDAC xenograft (right). Black arrows, cytosolic/nuclear β-catenin; white arrows, membrane-bound β-catenin localization. C, typical flow cytometry plot depicting unsorted L3.6pl cells after transduction with the lentiviral reporter 7TGC-eGFP/SV40-mCherry (7TGC). For subsequent enrichment, the 5% of cells with the highest (green box) and lowest (black box) GFP levels were sorted. D, quantitative evaluation of GFP<sup>hi</sup> cells in different cell lines (as in A) after 7TGC transduction. n = 3. E, graphical illustration of 7TGC cell pool enrichment and analysis. F, PDAC cells were serially enriched for 5% GFP<sup>hi</sup> and 5% GFP<sup>lo</sup> cells, respectively. Shown is the flow cytometric reanalysis of the respective subgroups: after several rounds of enrichment (GFP<sup>hi</sup>, green; GFP<sup>lo</sup>, black), G, immunofluorescent staining for β-catenin in GFP<sup>hi</sup> (top) and GFP<sup>lo</sup> (bottom) subsets. White arrowheads, nuclear β-catenin. After sorting for GFP<sup>hi</sup> and GFP<sup>lo</sup>, cells were seeded onto 8-well chamber slides and staining was carried out 24 hours later. H, immunofluorescent staining for cytokeratin 19 (CK19) in same panel as in G. Bar, 50 μm. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. 

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cell lines, 0.5% to 3.5% of cells were GFP/Wnt\textsuperscript{high} at baseline (Fig. 1D).

For further detailed analysis, we serially enriched for the 5% of pancreatic cancer cells with the highest (GFP\textsuperscript{high}) and lowest (GFP\textsuperscript{low}) expression of GFP, respectively (Fig. 1E and Supplementary Fig. S1B). These two subpopulations differed significantly in their GFP expression (Fig. 1F). To rule out false-positive background activity in our experimental system, we performed immunofluorescent staining for β-catenin (Fig. 1G). Active Wnt signaling in the GFP\textsuperscript{high} population was confirmed by a high percentage of nuclear β-catenin (white arrowheads), whereas GFP\textsuperscript{low} pancreatic cancer cells were mostly negative for nuclear β-catenin and high in membrane-bound β-catenin. Also, comparative Western blotting in these subsets corroborated Wnt activation in GFP\textsuperscript{high} cells with activating phosphorylation of the Wnt target genes/enhancers (Supplementary Fig. S1D). In contrast, the pancreatic ductal differentiation marker cytokeratin 19 was highly present in GFP\textsuperscript{low} cells and almost absent in GFP\textsuperscript{high} cells (Fig. 1H and Supplementary Fig. S1E), suggesting distinctive differentiation states of the two subpopulations. Immunofluorescent stains of the xenograft used in Fig. 1B could corroborate this finding. GFP\textsuperscript{high} cells (green arrows) expressed high levels of cytosolic/nuclear β-catenin and little membrane-bound CK19 (red arrows, Supplementary Fig. S1G, right).

**Wnt/GFP\textsuperscript{high} and Wnt/GFP\textsuperscript{low} cell populations display distinct biologic properties**

Consistent with the divergent Wnt/GFP status results, SFA—a functional in vitro surrogate assay for cancer stemness—was higher in GFP\textsuperscript{high} than in GFP\textsuperscript{low} cells (Fig. 2A and Supplementary Fig. S2A). Strikingly, quantitative RT-PCR analysis revealed that stemness-associated genes (e.g., SNAI1, NANOG, ZEB1) and Wnt target genes/enhancers (AXIN2, LGR5, RSPO2) had higher expression in GFP\textsuperscript{high} compared with GFP\textsuperscript{low} cells (Fig. 2B). Furthermore, we observed dramatic morphologic dissimilarities between L3.6pl-GFP\textsuperscript{high} and -GFP\textsuperscript{low} subsets, respectively, in two-
dimensional (2D) and three-dimensional (3D) cultures (Fig. 2C). As expected, the GFP<sup>low</sup> cells revealed epithelial cell clusters and tight sphere structures resembling the parental L3.6pl cell line (top; Supplementary Fig. S2B). In contrast, the GFP<sup>high</sup> cells formed elongated shapes (2D) with less cell–cell contact as well as grape-like features (3D), which are characteristic of mesenchymal PDAC cell types (Fig. 2C, Supplementary Figs. S2C and S1F). These effects were partially reversed when Wnt signaling was blocked by IWR1-endo, a small-molecule inhibitor of Wnt response, in pancreatic cancer cells in sphere culture (Supplementary Fig. S2D; ref. 24).

Western blot analysis revealed the upregulation of mesenchymal marker vimentin and transcription factors (SNAIL and Zeb1) in GFP<sup>high</sup> cells compared with GFP<sup>low</sup> cells (Fig. 2D) and the downregulation of epithelial markers E-cadherin (Supplementary Fig. S1E). Because EMT is reported to be linked to cancer stemness (7), we sought to verify these results in a primary PDAC cell line (Patx1) and also observed that typical EMT markers were differentially expressed. Appreciably higher expression of mesenchymal markers (Snail, vimentin, Zeb1) in GFP<sup>high</sup> cells and simultaneous slightly lower expression of epithelial markers (α-catenin, total β-catenin) hinted toward partially EMT-transformed, metastatic states of these cell populations (Fig. 2E; ref. 25). When these cell subsets were seeded at clonal density, holocline formation differed in number (Fig. 2F), as did their morphology, to an even more convincing degree (Fig. 2G, Supplementary Fig. S2E and S2F). Interestingly, GFP<sup>low</sup>-derived holoclines essentially consisted of GFP<sup>flow</sup>-epithelial cell colonies, whereas GFP<sup>high</sup>-derived holoclines consisted of 46% to 60% epithelial GFP<sup>flow</sup> colonies, 19% to 23% mesenchymal GFP<sup>high</sup> colonies, and 21% to 29% mixed colonies with intermittent GFP<sup>high</sup> cells. These results further corroborated the observation that different intrinsic Wnt activation levels lead to distinct biologic properties in pancreatic cancer cells.

Last, we determined whether GFP<sup>high</sup> and GFP<sup>low</sup> cells differed in their respective proliferation capacities. Here, we found that the GFP<sup>low</sup> subpopulation was slightly enriched for cells in G2 phase (Supplementary Fig. S2G); moreover, immunofluorescent costing for GFP and the proliferation marker Ki67 indicated that GFP<sup>high</sup> cells had greater sphere-forming ability than primary tumor cells, consistent with higher proliferative potential (Fig. 2C, Supplementary Figs. S2C and S1F). Interestingly, GFP<sup>high</sup>-derived holoclines consistently yielded smaller spheres (2D) with less cell–cell contact than the respective subpopulations might react in an orthotopic context. For that purpose, we again enriched for L3.6plGFP<sup>low</sup>- or L3.6plGFP<sup>high</sup>-derived cells and implanted 100,000 cells into the tail of the pancreas of male athymic nu/nu mice. All of the implanted cells formed tumors; however, the volumes were significantly larger in the GFP<sup>low</sup> population (Fig. 3D). From this first generation of orthotopic tumors, we isolated cells and sorted for the GFP<sup>high</sup> cells from GFP<sup>high</sup>-derived tumors (Nos. 126-128) and GFP<sup>low</sup> cells from GFP<sup>low</sup>-derived tumors (Nos. 132-134) and subsequently implanted them into second-generation recipients (Fig. 3E). Here, again, tumors arose from all injections, but earlier tumor onset (Supplementary Fig. S3A) and markedly greater tumor volumes were seen in GFP<sup>high</sup>-derived tumors (Fig. 3D and F). Histologically, the arising tumors resembled solid, nodular, and undifferentiated PDACs (Fig. 3G, left).

IHC GFP staining revealed a substantially higher number of GFP<sup>high</sup> cells in the GFP<sup>low</sup>-derived tumors than in the GFP<sup>low</sup>-derived tumors; nevertheless, we also detected a small number of GFP<sup>high</sup> cells in GFP<sup>low</sup> tumors, which suggests that intrinsic stimuli or microenvironmental signals can reactivate Wnt signaling in these cells. The difference in GFP<sup>high</sup> cells between the two groups became even more pronounced in the second-generation tumors, which might imply that GFP<sup>high</sup> cells are more likely to derive from GFP<sup>high</sup> precursors (Fig. 3G, right). Intriguingly, we also detected isolated metastases in GFP<sup>low</sup> tumors (e.g., in the spleen and peritoneum; Fig. S1H and Supplementary Fig. S3B). Moreover, metastasis-derived cells yielded higher numbers of GFP<sup>high</sup> cells and spheres with mesenchymal morphology in SFA experiments than did primary tumor-derived cells (Supplementary Fig. S3C-S3E). Finally, metastasis-derived PDAC cells usually had greater sphere-forming ability than primary tumor cells, confirming that SFA in our model system is a valid in vitro surrogate marker for anoikis-resistant growth, tumorigenesis, and metastatic ability.

Highly tumorigenic murine pancreatic CSCs overexpress Wnt enhancer genes

To determine the significance of Wnt enhancer genes in primary tumor cells, we used two different genetic mouse models, low tumorigenic KC-428 and high tumorigenic KPC-1050. Both lines were sorted by FACS differing in their CSC marker profile into CD24<sup>−</sup>CD44<sup>−</sup>CD133<sup>−</sup> and CD24<sup>+</sup>CD44<sup>+</sup>CD133<sup>−</sup> tumor cells (Fig. 4A). Although Rspo2 was slightly higher expressed in KC-428 CSCs, the expression pattern of further Wnt and CSC markers was not consistent (Fig. 4B). However, in the highly tumorigenic KPC-1050, we found significantly higher levels of Rspo2, Lgr5, Axin2, and Snail, suggesting an active role of Rspo2-enhanced Wnt signaling in KPC-1050-derived CSCs (Fig. 4C).

GFP<sup>high</sup> PDAC cells are chemoresistant and can be selectively enriched by chemotherapy

A clinically critical hallmark of cancer, especially CSCs, is intrinsic or acquired drug resistance (27). To explore this with regard to Wnt activation in PDAC, we implanted randomized L3.6pl...
orthotopic xenografts into two groups with one control group (Co1 and Co2) and one gemcitabine-treated group (GEM1 and GEM2), respectively (Fig. 4D). The resultant tumors were excised and subsequently either stained for GFP or digested and subjected to flow cytometric analysis (Fig. 4E). Gemcitabine treatment drastically increased the numbers of GFP<sup>high</sup> cells 3.4- to 15.5-fold <i>in vitro</i> (Supplementary Fig. S4A). Next, we tested whether gemcitabine had similar effects <i>in vivo</i> (Supplementary Fig. S4B). As proof of principle, we additionally treated the primary cell line PaTu1 with IWR1-endo alone or in combination with gemcitabine. IWR1-endo reduced baseline GFP levels and prevented gemcitabine-induced enrichment of GFP<sup>high</sup> PaTu1 cells, which could imply that the observed effect might not only be an enrichment of intrinsically Wnt<sup>high</sup> cells, but also an escape mechanism from drug-induced cell death by autocrine or paracrine Wnt induction (Supplementary Fig. S4C). Moreover, the observations that gemcitabine treatment <i>in vitro</i> increases gene expression levels of the Wnt-enhancing ligand RSPO2, the EMT-transcription factor SNAI1, and the Wnt target gene TCF4, especially in CSC-containing spheres, support this idea (Supplementary Fig. S4D).

Tumor spheres seemed to display an intrinsically higher Wnt activation as evidenced by elevated Wnt target gene expression such as SNAI1 (Supplementary Fig. S4E and S4F). Furthermore, freshly sorted GFP<sup>high</sup> cells displayed significantly higher resistance to gemcitabine (Fig. 4F) and, hence, a higher IC<sub>50</sub> value (15.12 ng/mL vs. 6.15 ng/mL) in the chemosensitive cell line L3.6pl (Supplementary Fig. S4G). PDAC cells derived from GFP<sup>high</sup> first- or second-generation orthotopic xenografts displayed an inherently greater drug resistance to gemcitabine (Fig. 4G and Supplementary Fig. S4H) as well as to the novel antitumor and chemopreventive drug TRAIL ( Supplementary Fig. S4H; ref. 28). Finally, TRAIL resistance could also be seen in freshly sorted GFP<sup>high</sup> PDAC cells compared with a more sensitive GFP<sup>low</sup> counterpart from the same cell pool (Fig. 4H).

Figure 3.

Certain Wnt/GFP<sup>high</sup> populations possess increased tumorigenic potential. A, tumor-free survival in subcutaneous tumor models with two different cell lines. Shown are the respective lowest dilutions that formed tumors (50,000 L3.6pl cells, n = 9; 10,000 AsPC1 cells, n = 5). B, growth of tumors formed in A, C, TIC frequency was determined by limited dilution in a subcutaneous mouse model and calculated using ELDA. D, graphical illustration of how first- and second-generation GFP<sup>high</sup> versus GFP<sup>low</sup> tumors in an orthotopic model were generated. E, weights of tumors described in D, n = 3. F, imaging of second-generation tumors by the IVIS system shortly before the mice were killed and the tumors extracted. Bar, 1 cm. G, examples of hematoxylin and eosin and GFP staining of the first- (top) and second-generation (bottom) GFP<sup>low</sup> (left side; 133 & 80) versus GFP<sup>high</sup> (right side; 127 & 78) tumors. H, metastasis formation of second-generation tumors; table indicates no metastasis in GFP<sup>low</sup> tumors, whereas GFP<sup>high</sup> tumors formed metastases in two of three cases. Black arrowheads, spleen metastasis; white arrow, the primary pancreatic tumor.
Wnt-responsive PDAC cells are susceptible to RSPO2-induced signaling enhancement

To gain a better understanding of the underlying responses of PDAC cells to extrinsic Wnt ligands and modifiers such as members of the R-Spondin family, we subjected different cell lines to recombinant human (rh) Wnt3a, rhRSPO2, or a combination thereof. As reported before (29), PDAC cells can be grouped into low responders (e.g., AsPC1, Patx1) or high responders (e.g., BxPC3, L3.6pl); however, that study only focused on induction of Wnt signaling by treatments with CHIR990221 or Wnt3-conditioned media. According to recent TCGA data, the RSPO2 gene copy number was the only Wnt enhancer upregulated in PDAC compared with normal pancreas (Supplementary Fig. S5A). Although not statistically significant, RSPO2 copy numbers in the same dataset tended to be higher in advanced tumor and nodal stages (Supplementary Fig. S5B). N1 stages also tended to express higher RSPO2 protein levels in tissue microarray (TMA) studies compared with N0 stages (Supplementary Fig. S5C and Supplementary Table S5). Conversely, it was recently reported that the RSPO2–LGR5 axis suppresses tumor growth in colorectal cancer, a surprising finding given the highly Wnt-dependent nature of this cancer type (30).

We observed that treatment with RSPO2 alone or in combination with Wnt3a had very small effects on Wnt stimuli in low-responder cells, whereas high-responders reacted rapidly to Wnt3a, RSPO2, and boldly to their combination (Fig. 5A and Supplementary Fig. S6A). To further decipher this responsiveness, we sorted GFP low, GFPmiddle, and GFP high fractions and subsequently exposed them to either rhWnt3a or rhRSPO2 (Fig. 5B). Overall, GFP middle PDAC cells seemed prone to each of the two stimuli, whereas GFP low cells reacted slightly more to rhRSPO2 and GFP high cells showed only limited increases in GFP levels, presumably due to their high baseline GFP levels (Fig. 5B and C).

As expected, sphere-formation capacity correlated with GFP levels (Fig. 5D). Similar to our observations in GFP high-derived tumor xenografts, spheres of GFP low PDAC cells also rarely harbored GFP high cells (Fig. 5D, white arrowhead). This led to the
assumption that these cells themselves or bystander PDAC cells might produce Wnt ligands. It also indicated a certain grade of Wnt plasticity for high responders with plastic stemness potential.

A closer examination of this behavior in high versus low responders uncovered that high responders rapidly react to RSPO2/Wnt3a stimuli and can be serially enriched, but revert back to a mostly GFPlow phenotype in regular 2D cell culture. Sphere culture or passaging in vivo preserves higher GFP expression, suggesting that these conditions offer a more adequate microenvironment for Wnt-positivity and that Wnthigh cells are more likely to produce Wnthigh offspring (Fig. 5E, top). In contrast, low responders only show modest responses to RSPO2/Wnt3a stimuli, but consistently maintain high GFP levels in adherent and sphere conditions as well as in vivo (Fig. 5E, bottom). Single GFPhigh-derived spheres revealed then that high responders often harbored a small subset of GFPhigh cells, leading to the notion that Wnt levels might vary in these tumorspheres, whereas low-responder tumorspheres remained GFPhigh throughout representing less Wnt plasticity here (Fig. 5E). These results led us to hypothesize that additional pathway regulations might be necessary to explain this phenomenon.

**Wnt activation stimulates downstream ERK1/2 and EMT**

The interplay/crosstalk of canonical Wnt and ERK1/2 pathways has been described as diverse in different organ systems, e.g., increased ERK1/2 signaling seems to inhibit Wnt in melanoma, whereas aberrant activation of Wnt/β-catenin in colorectal cancer stimulates downstream ERK1/2 (31–33). Recent data show concomitant activation of Wnt and ERK1/2 during pancreatic tumorigenesis (34). Accordingly, we wanted to investigate whether such a connection existed in our system. In Western blot analysis, we detected a positive correlation between Wnt and ERK activation in high-responder PDAC cell lines (Fig. 6A and Supplementary Fig. S6B). Moreover, murine CD24/CD44high CSCs tended to overexpress nuclear β-catenin and (p)-ERK corroborating their positive correlation (Supplementary Fig. S6C). To further understand these complex pathway interactions, we carried out RPPA, which confirmed distinctive ERK1/2 (MAPK; blue frame) activities as...
well as EMT phenotype in GFP<sup>high</sup> versus GFP<sup>low</sup> PDAC cells (Fig. 6B). Interestingly, additional pathways that can crosstalk with Wnt, such as AKT or p38, did not have concurrent regulation of GFP<sup>high</sup> or GFP<sup>low</sup> subsets in the different cell lines.

Next, we sought to comprehensively investigate longer-term effects of Wnt/ERK manipulation on GFP<sup>low</sup> as well as GFP<sup>high</sup> cells in high and low Wnt-responder PDAC cells. For that purpose, we treated GFP<sup>low</sup> subsets with RSPO2/Wnt3a and GFP<sup>high</sup> subsets (in which we predicted inherently higher Wnt and ERK levels) with RSPO2/Wnt3a, ERK inhibitor UO126, or Wnt inhibitor IWP2 for 3 days. As expected, RSPO2/Wnt3a had increased E-cadherin levels, suggesting that ERK is indeed subordinated to canonical Wnt and Zeb1 downstream to its induction, and p-ERK activation upon RSpo2/Wnt3a stimulation, regardless of GFP status (Fig. 6D). Remarkably, UO126 as well as IWP2 treatment reduced p-ERK and Zeb1 levels and increased E-cadherin levels, suggesting that ERK is indeed subordinated to canonical Wnt and Zeb1 downstream to its control (Fig. 6D). UO126 led to increased Wnt-activating effects of RSPO2 in a dose-dependent manner and hence hints toward a negative-feedback loop of p-ERK on LGR6 in PDAC (Supplementary Fig. S6D and S6E).

In vivo, p-ERK was predominantly expressed in areas of the invasive front and coincided there with a higher number of GFP<sup>high</sup> cells (Fig. 6E). Although Western blot analysis of low-responder PDAC cells did not reveal the changes seen in high responders, GFP<sup>low</sup> cells altered their phenotypic appearance

**Figure 6.** Molecular downstream mechanisms of intrinsic and extrinsic Wnt activation in PDAC cells. A, Western blot of GFP and p-ERK/ERK after treatment with Wnt3a, RSPO2, or the combination thereof for 24 hours. B, RPPA of highly enriched GFP<sup>low</sup> and GFP<sup>high</sup> PDAC cells. Shown are the ratios of low/high in L3.6pl (left) and Patx1 (right) cells. Red, upregulation and green downregulation of the indicated proteins. C and D, Western blot analysis of Wnt, ERK, and EMT proteins in GFP<sup>low</sup> subsets and SNAIL upregulation upon RSPO2/Wnt3a stimulation. UO126 treatment showed only modest responses, most apparent in Zeb1 downregulation. IWP2 in contrast reduced robustly p-LRP6, LRP5/6, Dvl2, SNAIL, and p-ERK levels (Fig. 6C). The high-responder cell line L3.6pl reacted with elevated levels of p-LRP6/LRP6 ratios, Dvl2 phosphorylation, SNAIL induction, and p-ERK activation upon RSpo2/Wnt3a stimulation, regardless of GFP status (Fig. 6D).
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drastically upon constant Wnt or ERK inhibition (Fig. 6F). In line with the downregulation of EMT transcription factors Zeb1 (by UO126 treatment) or SNAIL (by IWP2 treatment), Patx1 became more epithelial-like in morphology whereas RSPO2/Wnt3a stimulation increased the number of sphere-like shapes in accordance with the loss of epithelial-typical cell–cell adhesions (Fig. 6F). Notably, most mesenchymal PDAC cell lines also seemed to possess higher p-ERK levels compared with epithelial lines (Supplementary Fig. S7A).

Lastly, we knocked down ERK or β-catenin (the transcription factor of canonical Wnt signaling) by siRNA. The latter reduced GFP levels (Fig. 6G) and both, especially in combination, significantly reduced sphere formation in primary PDAC. Patx1 cells (Fig. 6H and I). Of note, concomitant knockdown of β-catenin and ERK only partially reduced GFP levels, as evidenced by flow cytometric analysis, suggesting a bilateral feedback mechanism (Fig. 6G). In conclusion, these data suggest an important interplay between canonical Wnt and ERK with greater effects in high-responding and susceptible cells. Interference with these pathways decreased EMT- and CSC-like abilities.

RSPO2 is expressed by GFP<sup>high</sup> tumors and enhances sphere-forming ability

As stated above, RSPO2 turned out to be the only gene with higher gene expression in PDAC compared with normal pancreas (Oncomine database, www.oncomine.org). In our model system, we found, as expected, that GFP<sup>high</sup> PDAC cell–derived tumors had less membrane-bound and more cytoplasmic β-catenin expression than GFP<sup>low</sup> PDAC cell–derived tumors and that this difference was greater in second-generation tumors (Fig. 7A). Correspondingly, we found a significantly higher expression of RSPO2 in these tumors. RSPO2 expression was also detectable in sections of human pancreatic cancers (Supplementary Fig. S7B) and seemed to correlate with Zeb1 expression in TMA datasets (Supplementary Fig. S7C and Supplementary Table S5). Stimulation with extrinsically added RSPO2 significantly increased sphere-forming ability in different PDAC cell lines (Fig. 7B), suggesting that CSCs especially might be susceptible to RSPO2 stimulation. This effect could be impeded by the use of a blocking antibody against RSPO2 in a similar assay, but blockage of RSPO2 had only minimal effects on cell viability (Supplementary Fig. S7D and S7E). Encouraged by this finding, we stimulated the high-responder cell line L3.6pl with RSPO2 overnight, sorted the 5% GFP<sup>high</sup> and 5% GFP<sup>low</sup> subpopulations with the aim to enrich for the best and worst RSPO2 responders, respectively, and then determined their sphere-forming ability. Intriguingly, high RSPO2 responsiveness conferred significantly improved sphere-forming capacity in the same cell line (Fig. 7C). This confirms that RSPO2 plays a major role in regulating cancer stemness events in susceptible PDAC cells. We further analyzed RSPO2 expression <i>in vivo</i> and found in IHC sections high expressions in certain areas of the pancreas and the invasive front. The latter correlated with a low frequency of membrane-bound

![Figure 7](https://www.aacrjournals.org)
Discussion

Metastasis formation and drug resistance are cardinal tumor traits that negatively impact the prognosis, morbidity, and mortality of patients. The CSC model has been proposed to account for the observation that in many tumors, a relatively rare neoplastic cell subpopulation seems to drive tumor propagation and survival (4, 5, 35). CSC behavior is in part regulated by archetypical self-renewal pathways, such as Sonic Hedgehog, Notch, and Wnt (6, 36, 37), which suggests that cancer stemness is dynamic and orchestrated by microenvironmental cues (7, 38, 39). In this work, we approached the question of whether canonical Wnt signaling plays a role in regulating stem cell qualities in pancreatic cancer.

To explore different Wnt activation levels, we used a lentiviral reporter system (23) and found that, first, highly intrinsic Wnt correlates with EMT phenotype, resulting in CSC appearance, as evidenced in vitro by sphere-forming assays. Second, the same cells define a more tumorigenic subpopulation of pancreatic cancer cells, especially in highly Wnt/Rspo2-responsive cells. Here, 7 of 9 Wnt\textasciitilde{high} L3.6pl tumors developed early and rapidly subcutaneously compared with 1 of 9 Wnt\textasciitilde{low} tumors, whereas Wnt\textasciitilde{high} and Wnt\textasciitilde{low} tumor burdens were only marginally different in Wnt\textasciitilde{low} H-sorted AsPC1 cells (Fig. 3B). Importantly, orthotopic implants confirmed this behavior with earlier and faster tumor growth in Wnt\textasciitilde{high} populations, although Wnt\textasciitilde{high} cells were slower (Fig. 2H and Supplementary Fig. S2G).

Recent reports explain this phenomenon with the presence of quiescent EMT-like CSCs and mesenchymal-epithelial transition (MET)-like more differentiated and proliferating cells (40). Here, we speculate that the higher Wnt activity in PDAC CSCs, the slower they cycle, but with high propensity to differentiate into fast-cycling cells in spheres or in vivo. In addition, our experiments showed that Wnt\textasciitilde{high} cells preferentially produced Wnt\textasciitilde{high} progeny in vitro that were more likely to metastasize and produce Rspo2, whereas Wnt\textasciitilde{low} cells failed to become Wnt\textasciitilde{high} in second-generation transplants and never metastasized (Figs. 3 and 7; Supplementary Fig. S3). Third, in primary murine pancreatic cancer cells, we found that CSCs of highly tumorigenic PDAC overexpressed Wnt enhancer molecules compared with their more differentiated counterparts. In less aggressive murine PDAC, this effect was only marginable. Fourth, Wnt\textasciitilde{high}-sorted cells and -unsorted cells derived from Wnt\textasciitilde{high} tumors proved to be more drug resistant to the standard regimen gemcitabine (a deoxycytidine analogue) as well as to TRAIL. These results show evidence that intrinsically high activation of canonical Wnt signaling in PDAC could define a population of (aggressive) cells with cancer stemness properties.

Finally, it was shown that pancreatic cancer cells can be dichotomized as high and low responders to extrinsic Wnt stimuli (29). The present study aligns with these data and extends our knowledge regarding many biologic aspects of these subpopulations both in vitro and in vivo. Even within the same pancreatic cancer line, cells of different Wnt susceptibilities can be identified after extrinsic addition of not only Wnt but also Rspo ligands (Fig. 5). The latter factor is of importance, because it was shown previously that the Wnt-Lgr5–Rspo axis is crucial to activate adult pancreatic progenitor cells for long-term in vitro expansion and, hence, stem cell self-renewal by Rspo1 (41, 42). Varying intercellular susceptibilities might depend on different baseline Wnt activity levels that could, in turn, depend on deviations in regulatory elements of the pathway. Secondly, if certain subsets of PDAC cells are the source of production of activating Wnt ligands in a paracrine or juxtacrine manner, Wnt\textasciitilde{high} cells would then lack these factors after experimental enrichment and would need prerequisite "differentiation" into these cells to regenerate the source. This mechanism might explain why highly susceptible cells in the present study lost their Wnt\textasciitilde{high} status after enrichment.

A third explanation might suggest that the cells are able to self-stimulate in an autocrine manner, receptors of Wnt\textasciitilde{high} cells might be mostly saturated, which would impede further stimulatory effects. Intriguingly, Arensman and colleagues (29) discovered that pancreatic tumors with high Wnt/β-catenin gene expression signature in pancreatic tumors strikingly correlated with significant lymphovascular invasion patterns, increased tumor size, and worse disease-specific survival. Our work explains these discoveries with extensive in vitro and in vivo data (e.g., Wnt-mediated mesenchymal transformation before invasive processes) and adds Rspo2 as an important contextual signal.

We found that Rspo2 was overexpressed in susceptible Wnt\textasciitilde{high} pancreatic cancer cells and tumors, CSC-enriched tumor spheres, and gemcitabine-treated cells, which indicates a high level of importance to this Wnt signaling enhancer. Moreover, Rspo2 alone was able to increase CSC behavior in vitro. Database analysis revealed mutual Rspo2 amplification in pancreatic cancer in a fair number of cases (~8% TCGA data on www.cbioportal.org), and as the only member of the R-spondin family, Rspo2 showed significantly higher gene expression in PDAC than in normal pancreas. We discovered marginally more Rspo2\textasciitilde{high} H-scores in N1 versus N0 in TMA datasets and positive correlations with Rspo2\textasciitilde{high} and Zeb1\textasciitilde{high} H-scores, adding further evidence to the role of Rspo2 in pancreatic CSCs.

Last, we found that intrinsic or extrinsic Wnt stimuli result in downstream ERK activation by increased phosphorylation levels. Both pathways seemed to independently control EMT transcription factors Snail (by Wnt) and Zeb1 (by ERK), which could be blocked by specific inhibitors of the pathways. ERK activation downstream of canonical Wnt, in this case, could therefore reinforce cancer stemness in PDAC cells through Zeb1, a well-known EMT- and CSC regulator in PDAC (5) and other cancers (43, 44), especially in intrinsically Wnt\textasciitilde{high} populations. On the other hand, activation of p-ERK might also be a negative regulator in a finely tuned feedback mechanism of pancreatic CSCs.

In conclusion, this work offers insights into intercellular Wnt heterogeneity in pancreatic cancer cells of the same tumor as well as different tumors. We also show for the first time that Rspo2-enhanced canonical Wnt signaling contributes to cancer stemness traits in a subset of susceptible pancreatic cancers and that Rspo2 production can stem from the pancreatic cancer cell itself or from other vicinal cells of the tumor microenvironment. Subsequent ERK activation amplifies and/or regulates Wnt (summarized in Supplementary Fig. S8). Therefore, testing for Rspo2 overexpression or amplification might identify pancreatic cancer patients who could benefit from a triple combination therapy, e.g., with gemcitabine and alternating ERK and canonical Wnt signaling inhibition. Further studies are needed to shed light on the detailed role of Rspo2 during tumor progression and metastasis, for
instance in syngeneic mouse models or RSPO2-inducible cell systems. Deeper insight into a highly complex and variable response system to the growing canonical Wnt modulation family is hence necessary, especially with contrary findings in different systems (15, 30, 45). Importantly, whole sections of primary human pancreatic tumors of different origins as well as their metastasis will improve the current knowledge of the exact location (invasive front vs. tumor center) and colocalization of RSPO2 with EMT and CSC markers.

The concept of phenotypic EMT/MET plasticity versus genetic predisposition (steady EMT/CSC state) in the context of metastasis was recently discussed in a seminal opinion article (1). It was suggested that in the case of EMT/MET-capable cancer cells environmental triggers modify epigenetically epithelial primary tumor cells, which then circulate as EMT-transformed CSCs and seed differentiated epithelial metastasis after undergoing MET. In the case of genetic predisposition, primarily undifferentiated tumors give rise to equally undifferentiated metastasis due to their intrinsic genetic endowment that fixes the cancer cells in an EMT-CSC state. In the figurative sense, in our model, RSPO2-responsive cells might be representative of the phenotypic plasticity inhibiting the "steady-state" type cells, especially because these cells seemed to naturally be much more mesenchymal. Ultimately, we believe that not the baseline level of Wnt, but the functional response and adaptability to contextual Wnt signals is the determinant for pancreatic cancer stemness and should be pursued as a CSC readout.

**References**


**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: M. Ilmer, E. Alt, J. Vykoukal

Development of methodology: M. Ilmer, A.R. Boiles, I. Wistuba

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Ilmer, A.R. Boiles, I. Regel, K. Yokoi, I. Wistuba, J. Rodriguez, J. Vykoukal

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Ilmer, A.R. Boiles, I. Regel, K. Yokoi, J. Rodriguez, E. Alt, J. Vykoukal

Writing, review, and/or revision of the manuscript: M. Ilmer, A.R. Boiles, C.W. Michalski, I. Wistuba, J. Vykoukal

Study supervision: M. Ilmer, E. Alt, J. Vykoukal

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RSPO2 Enhances Canonical Wnt Signaling to Confer Stemness-Associated Traits to Susceptible Pancreatic Cancer Cells

Matthias Ilmer, Alejandro Recio Boiles, Ivonne Regel, et al.


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