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Sano M, Driscoll DR, De Jesus-Monge WE, Quattrochi BJ, Appleman VA, Ou J, Zhu L(, Lewis BC. (2016). Activation of WNT/beta-Catenin Signaling Enhances Pancreatic Cancer Development and the Malignant Potential Via Up-regulation of Cyr61. Open Access Articles. https://doi.org/10.1016/j.neo.2016.11.004. Retrieved from https://escholarship.umassmed.edu/oapubs/2978

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NEOPLASIA
www.neoplasia.com

Volume 18 Number 12 December 2016 pp. 785–794 785

Activation of WNT/β-Catenin Signaling Enhances Pancreatic Cancer Development and the Malignant Potential Via Up-regulation of Cyr61

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Abstract

Pancreatic ductal adenocarcinoma (PDAC), a poor prognostic cancer, commonly develops following activating mutations in the *KRAS* oncogene. Activation of WNT signaling is also commonly observed in PDAC. To ascertain the impact of postnatal activation of WNT-stimulated signaling pathways in PDAC development, we combined the *Elastase-tva*-based RCAS-TVA pancreatic cancer model with the established *LSL-Kras*^{G12D}, *Ptf1a-cre* model. Delivery of RCAS viruses encoding β-catenin^{S37A} and WNT1 stimulated the progression of premalignant pancreatic intraepithelial neoplasias (PanIN) and PDAC development. Moreover, mice injected with RCAS-*β-catenin*^{S37A} or RCAS-*Wnt1* had reduced survival relative to RCAS-*GFP*-injected controls ($P < .05$). Ectopic expression of active β-catenin, or its DNA-binding partner TCF4, enhanced transformation associated phenotypes in PDAC cells. In contrast, these phenotypes were significantly impaired by the introduction of ICAT, an inhibitor of the β-catenin/TCF4 interaction. By gene expression profiling, we identified Cyr61 as a target molecule of the WNT/β-catenin signaling pathway in pancreatic cancer cells. Nuclear β-catenin and CYR61 expression were predominantly detected in moderately to poorly differentiated murine and human PDAC. Indeed, nuclear β-catenin- and CYR61-positive PDAC patients demonstrated poor prognosis ($P < .01$). Knockdown of CYR61 in a β-catenin-activated pancreatic cancer cell line reduced soft agar, migration and invasion activity. Together, these data suggest that the WNT/β-catenin signaling pathway enhances pancreatic cancer development and malignancy in part via up-regulation of CYR61.

Neoplasia (2016) 18, 785–794

Introduction

Pancreatic cancer remains a leading cause of cancer-related deaths in the United States [1]. Approximately 53,000 people will be diagnosed with pancreatic cancer in 2016 and roughly 42,000 will die from this disease. The 5-year overall survival is only 7.7% according to data from the SEER database [2]. Only 9% of pancreatic cancer patients are diagnosed with localized disease and the 5-year relative survival for this patient group is 29% [2]. However, 52% of PDAC patients

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Received 7 October 2016; Revised 1 November 2016; Accepted 7 November 2016

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1476-5586
http://dx.doi.org/10.1016/j.neo.2016.11.004

already have distant metastases at diagnosis, and the 5-year relative survival rate for this group is a paltry 2.6%. These data highlight the early dissemination of pancreatic cancers and the resistance of this tumor type to currently employed therapeutic approaches.

Pancreatic ductal adenocarcinoma (PDAC), the predominant pancreatic cancer type, commonly develops through the progression of precursor lesions known as pancreatic intraepithelial neoplasia (PanIN) [3]. Recently, acinar-to-ductal metaplasia (ADM), particularly in the context of pancreatic injury, has been recognized as an important phenomenon in the initiation of PanIN [4,5]. Genetically, mutation of the *KRAS* oncogene is detected in >95% of PDAC cases and activation of KRAS signaling is sufficient for development of PanIN and PDAC [6–8]. Meanwhile, inactivation of the tumor suppressor genes *INK4A*, *TP53* and *DPC4* plays a pivotal role in PanIN progression and PDAC development [9]. Deletion of these tumor suppressor genes in genetically engineered mouse models confirmed the importance of these factors in constraining PanIN progression and the onset of invasive PDAC [10]. In addition to the above-mentioned genetic changes, activation of key developmentally regulated signaling pathways, including the Hedgehog, Notch and WNT pathways, is commonly observed in PDAC [11–13].

WNT ligands activate signaling through the “canonical” WNT/β-catenin pathway as well as “non-canonical” planar cell polarity (PCP) and WNT/Ca^{2+} pathways [14,15]. These precise modulations are essential for normal embryogenesis, organogenesis and homeostasis. In addition, activation of the WNT/β-catenin signaling axis, as a result of activating mutations in *CTNNB1* or inactivating mutations in the negative regulators *AXIN* and *APC*, is commonly observed in tumors of the colon, stomach and liver [16–24]. Mutations in *APC* and *CTNNB1* are found in uncommon pancreatic cancer types including acinar cell carcinomas, pancreatoblastoma and solid pseudopapillary neoplasm (SPN) [24–27]. However, despite common nuclear and cytoplasmic localization of β-catenin, indicative of pathway activation, in PDAC, mutation of pathway components is uncommon [28–31]. These findings suggest that other mechanisms, including ligand-mediated pathway activation, result in the stimulation of this signaling axis. Indeed, elevated expression of the protein ATDC has been shown to stabilize β-catenin resulting in pathway activation in PDAC [32,33]. Inactivation of the negative regulator RNF43 has been proposed as another mechanism [34]. Further, published findings suggest that WNT ligand mediated activation of the non-canonical WNT signaling pathways may play a role in PDAC pathogenesis. In agreement, WNT5A, which potently activates the non-canonical signaling pathways, has been demonstrated to enhance transformation in pancreatic cancer cells [35,36]. Yet, whether WNT ligands promote PDAC development in vivo remains unknown.

We therefore determined the ability of postnatal and sporadic expression of WNT1 and an activated β-catenin^{S37A} mutant protein to promote PDAC development and progression. We have previously reported that postnatal WNT1 expression promotes the development of mucinous cystic neoplasms through the paracrine activation of signaling in stromal cells [37]. Here, we demonstrate that WNT1 and β-catenin promote the progression of PanIN lesions and the development of PDAC. In addition, we show that activation of the canonical β-catenin signaling axis enhances the transformation of pancreatic cancer cells and is required for their transformation-associated phenotypes. Through gene expression profiling, we identify *Cyr61* as a β-catenin stimulated gene in pancreatic cancer cells, demonstrate that CYR61 inhibition impairs pancreatic cancer cell transformation, and show that β-catenin and CYR61 expression correlate with higher tumor grade and reduced survival in PDAC patients. Together, these findings confirm an important role for WNT signaling during pancreatic tumorigenesis and identify a mechanism that contributes to this phenotype.

Materials and Methods

Cell Lines

The murine pancreatic cancer cell lines 170#3 and 218#1 were derived from orthotopic tumors induced following the implantation of pancreatic ductal epithelial cells expressing KRAS^{G12D} and additionally null for the *Trp53* and *Ink4a/Arf* tumor suppressor loci [38]. 170#3 cells were transfected with pcDNA6-β-catenin^{S37A}, –TCF4, –ICAT or empty vector control by Superfect Transfection Reagent (QIAGEN, Carlsbad, CA). Knockdown of Cyr61 was performed using specific targeting shRNAs (m; sc-39,332-SH, Santa Cruz). Control shRNA plasmid-A (sc-108,060) was used as a negative control.

Cell Proliferation Assay

Cells (10^3) were seeded onto collagen-coated 96-well plate and incubated at 37°C under 5% CO_2 [39]. Cell metabolic activity was measured with CellTiter 96 Aqueous One Solution Cell proliferation assay (Promega) according to the manufacturer's instructions. Experiments were performed in triplicate and repeated at least twice.

Soft Agar Assay

Cells (10^5) were seeded onto soft agar and were incubated for 3.5 weeks at 37°C under 5% CO_2 [40]. The number of colonies in 15 microscopic fields (100×) was counted, while diameter of the colonies was measured with SPOT software. Experiments were performed in triplicate and repeated at least twice.

Migration and Invasion Assay

Migration and invasion assays were performed as previously described [41]. Cells (2.5×10^4) in 0.5 ml of serum-free DMEM were plated into either control or Matrigel-coated invasion chamber inserts (Becton Dickinson). Inserts were then placed in wells with 0.75 ml of DMEM containing 10% FBS as a chemoattractant. Experiments were performed in triplicate and repeated at least twice.

Spheroid Formation in Three Dimensional (3D) Cultures

Cells (4×10^3) were seeded onto a 3D culture plate coated with ultra-hydrophilic polymer (PrimeSurface® 96 U plate, Sumitomo Bakelite Co. Ltd., Tokyo, Japan), and incubated for 3 days at 37°C under 5% CO_2. Spheroid size was measured using ImageJ. Experiments were performed in triplicate and repeated at least twice.

TdT-Mediated dUTP–Biotin Labeling (TUNEL)

Apoptotic cells were detected in formalin-fixed and paraffin-embedded sections by TUNEL staining as previously described [42].

Gene Expression Profiling Microarray Analysis

RNA was isolated from cell lines using Trizol reagent (Life Technologies, 10,296,010). The RNA samples were labeled using the 3′ IVT Express Kit (Affymetrix, 901,228) and gene expression profiling conducted using GeneChip Mouse Genome 430 2.0 arrays (Affymetrix, 900,495).

The RMA method in the Affymetrix package from Bioconductor [43] was used in R to summarize the probe level data and normalize the dataset to remove across-array-variation. Log transformed data were used in subsequent analyses. Moderated T statistics in the Limma package from Bioconductor [44] was used to determine whether a

gene's expression level differs between treatments. Genes with an adjusted $P < .01$ using the B-H method [45] and at least 1.5-fold difference in expression were considered significantly changed.

The raw data files in addition to normalized expression data have been deposited in the NCBI GEO Archive under accession number GSE83196. Data can be accessed via the GEO website http://www.ncbi.nlm.nih.gov/geo/.

Real-Time Reverse-Transcription PCR (RT-PCR)

cDNAs were synthesized from total RNA using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time RT-PCR analysis was performed using QuantiTect™ SYBR Green PCR Kit (QIAGEN Inc., Valencia, CA) for ABI PRISM 7700 Sequence Detection Systems (PE Applied Biosystems Inc., Foster City, CA). The primers used for Cyr61 were (5′-CCCTGAACTTGTGGATGT CATTG-3′ and 5′-GTCATGATGATCCAGTCCTGCAAA-3′); for Axin2, (5′-TCAGTCGATCCTCTCCACTTTGC-3′ and 5′-GCAGTTTTTGAGGAGATCTGGGAC-3′); and for ß-actin, (5′-TGACAGGATGCAGAAGGAGA-3′ and 5′-CTGGAAGGT GGACAGTGAGG-3′).

DNA Extraction and Polymerase Chain Reaction (PCR)

DNA extraction from formalin-fixed and paraffin-embedded tissues as well as procedure of PCR were described previously [37].

Genetically Engineered Mice

The *Elastase-tva*, *LSL-Kras^{G12D}*, and *Ptf1a-cre* mouse strains have been previously described [7,46,47]. All animals were kept in specific pathogen-free housing under guidelines approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee. DF1 chicken fibroblasts (2×10^7 cells) transfected with RCAS-β-catenin^{S37A}, RCAS-Wnt1 or RCAS-GFP vectors were delivered via intraperitoneal injection into 3-day-old pups. Mouse tissue samples were fixed and processed as previously described [46]. The University of Massachusetts Medical School Institutional Animal Care and Use Committee approved all procedures.

Patient Materials

80 human pancreatic cancer samples, obtained from pancreatectomy at Department of Digestive Surgery, Nihon University Itabashi Hospital, Tokyo, Japan, were used under guidelines approved by the Nihon University Itabashi Hospital, Clinical Research Judging Committee (approval No. PK-150310-1). Formalin-fixed and paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and were diagnosed by licensed pathologists according to the classification of pancreatic carcinoma in Japan Pancreas Society under WHO histological classification of tumors of the exocrine pancreas [48]. Clinical data for the patients were collected from medical records by surgeons.

Immunohistochemistry (IHC)

Immunostaining for formalin-fixed and paraffin-embedded sections was performed as previously described [37,46]. Primary antibodies, except Cyr61 (1:250; H-78, sc-13,100, Santa Cruz, CA) and Ki67 antibodies (rabbit monoclonal, SP-6; Nichirei, Tokyo, Japan), were used as described previously [37,46]. The frequency of the positive PDAC cells in human specimens was scored as follows: 0, under detectable level; 1+, less than 25%; 2+, 25–50%; and 3+, more than 50% positivity of PDAC area. In Cyr61 staining, positive PDAC was identified as staining stronger than normal pancreatic ducts in the same specimen. However, faint intensity of Cyr61 was classified to 1+ regardless of the positive percentage.

PanIN Analysis Using SPOT Software

Four microscopic imaging pictures (100×) including i) one most progressive PanIN lesion, ii) one minimal progression lesion, and iii) two average progression lesions were analyzed the area population (%) with SPOT microscopy software (SPOT Imaging Solutions, Inc., Sterling Heights, MI).

Statistical Analyses

Statcel software version 2 (OMS Ltd., Saitama, Japan) was used for the statistical calculations. Survival curves of mice and patients were analyzed by Kaplan–Meier method and log-rank hazard ratio. Mann–Whitney's U test was used to determine the significant difference in soft agar, migration and invasion assay. Correlation between immunohistochemical score of nuclear β-catenin and cytoplasmic Cyr61 was analyzed by Spearman's correlation coefficient by rank test. Clinicophathological phenotypes of nuclear β-catenin and Cyr61 were analyzed by Chi-square (χ^2) for independence test or Mann–Whitney *U* test.

Results

To ascertain the effect of postnatal activation of WNT signaling on pancreatic tumorigenesis in vivo, we generated compound *elastase-tva;LSL-Kras^{G12D};Ptf1a-cre* mice and injected DF1 fibroblasts producing RCAS-β-catenin^{S37A}, RCAS-Wnt1 or RCAS-GFP (as a control) as previously described [37]. We first assessed the effect of ectopic WNT1 and β-catenin^{S37A} expression on PanIN development and progression in 6- and 9-month old mice using SPOT analysis (Supplementary Figure 1, *A* and *B*). At both time points, we observed that ectopic WNT1 and β-catenin^{S37A} expression led to an increase in PanIN lesions relative to GFP-expressing controls and that the lesions present in the WNT1- and β-catenin^{S37A}-expressing pancreata were of a higher grade than in the GFP-expressing controls (Supplementary Figure 1, *C* and *D*). These results suggest that activation of the WNT/β-catenin pathway promotes KRAS^{G12D}-induced acinar-to-ductal metaplasia (ADM) and PanIN development and progression.

We next determined whether delivery of RCAS viruses encoding WNT1 and β-catenin^{S37A} promoted progression to invasive carcinoma and reduced survival relative to RCAS-GFP controls. Kaplan–Meier survival curves indicated that mice infected with RCAS-Wnt1 and RCAS-β-catenin^{S37A} had reduced pancreatic cancer-specific survival relative to GFP controls (Figure 1*A*). Upon necropsy, large tumors were observed in the pancreas of RCAS-Wnt1 and RCAS-β-catenin^{S37A} injected animals (Figure 1, *B–D*; Supplementary Table 1). As we reported previously, female mice injected with DF1 cells producing RCAS-Wnt1 commonly developed cystic lesions resembling mucinous cystic neoplasms (MCN) [37]. However, other solid pancreatic tumors were also observed in mice of both genders. Microscopically, these pancreatic tumor masses were invasive ductal adenocarcinomas displaying varying degrees of differentiation (Figure 1*F*). The carcinoma cells were immunohistochemically positive for Ki67 indicating a high rate of proliferation (Figure 1*G*). They were also positive for cytokeratin and PDX1 indicative of their derivation from pancreas epithelial cells (Figure 1*H*, I), and phosphorylated ERK1/2 illustrating the presence of active KRAS signaling (Figure 1*J*). Meanwhile, sarcomatoid tumors composed of spindle cells with occasional nuclear atypia were occasionally observed with poorly differentiated PDAC (Supplementary Figure 2*A*). The sarcomatoid tumor cells were highly Ki67 positive

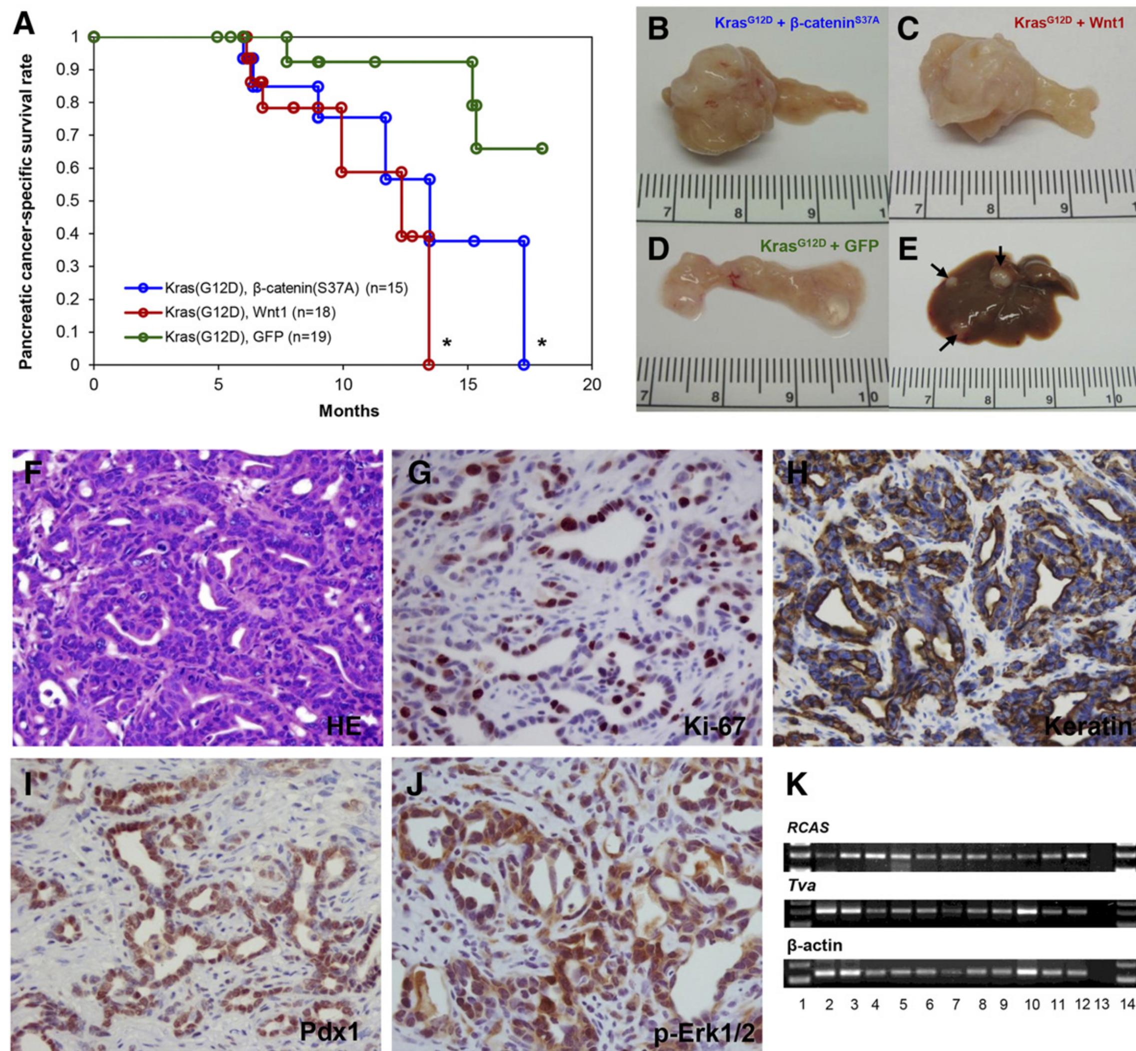

Figure 1. Overexpression of β-catenin or WNT1 accelerates pancreatic carcinogenesis. (A) Kaplan–Meier survival curves indicate that RCAS-Wnt1 and RCAS-β-catenin^{S37A} injection into *Elastase-tva*, *LSL-Kras^{G12D}*, *Ptf1a-Cre* mice reduced survival compared to RCAS-GFP injection. * $P < .05$ for RCAS-Wnt1 versus RCAS-GFP and RCAS-β-catenin^{S37A} versus RCAS-GFP. (B and C) Examples of large pancreatic tumor masses observed in 6-month old mice injected with (B) RCAS-β-catenin^{S37A} or (C) RCAS-Wnt1. (D) Pancreas from an age-matched mouse injected with RCAS-GFP. (E) Example of liver metastases observed in a mouse injected with RCAS-β-catenin^{S37A}. (F) H&E staining of a moderately differentiated PDAC observed in a mouse injected with RCAS-β-catenin^{S37A}. Immunostaining of induced PDAC for (G) Ki67, (H) wide cytokeratin, (I) PDX1 and (J) p-ERK1/2. (K) PCR amplification of RCAS and TVA from pancreatic tumors induced in *Elastase-tva*, *LSL-Kras^{G12D}*, *Ptf1a-Cre* mice following RCAS-β-catenin^{S37A} (lanes 2–7) or RCAS-Wnt1 (lanes 8–12). No template sample is shown in lane 13. 100-bp marker is shown in lanes 1 and 14.

(Supplementary Figure 2*B*) and were partially reactive to antibodies directed against PDX1, wide cytokeratin and vimentin (Supplementary Figure 2, *C–E*). Direct invasion into the duodenum, bile duct, spleen or retroperitoneum was seen in limited cases (Supplementary Table 1). Tumor metastases were observed in para-pancreatic lymph nodes and liver (Figure 1*E*; Supplementary Figure 2, *F* and *G*), but not the lungs.

To confirm that the pancreatic tumors were derived from RCAS infected cells, we purified genomic DNA from tumor tissue sections and confirmed the presence of RCAS DNA by PCR with specific primers (Figure 1*K*) [37]. Since infection of pancreas epithelial cells following systemic delivery of RCAS viruses is very inefficient, the presence of RCAS DNA in these lesions is indicative of their derivation from RCAS infected cells. We next sought to confirm the expression of the RCAS-encoded oncoproteins. Immunostaining of tumor sections of tumors identified in RCAS-Wnt1 and RCAS-β-catenin^{S37A} infected mice demonstrated the universal presence of WNT1 protein in the tumor epithelium of RCAS-Wnt1-induced, but not RCAS-β-catenin^{S37A}-induced tumors (Supplementary Figure 3 and Supplementary Table 2). Nuclear and cytoplasmic localized β-catenin was detected in tumors induced by both viruses as expected (Supplementary Figure 3 and Supplementary Table 2). While the β-catenin IHC does not allow distinction between exogenous β-catenin and endogenous protein, together with the WNT1 immunostaining and the genomic PCR data, these findings indicate that the identified tumors are derived from RCAS-infected cells.

To begin to elucidate the mechanisms underlying WNT1 and β-catenin^{S37A}-induced pancreatic tumor progression, we developed cell culture models. We ectopically expressed stabilized β-catenin^{S37A} or its DNA binding partner TCF4 in the murine pancreatic cancer cell line 170#3 [38]. We found that β-catenin^{S37A} and TCF4 expression

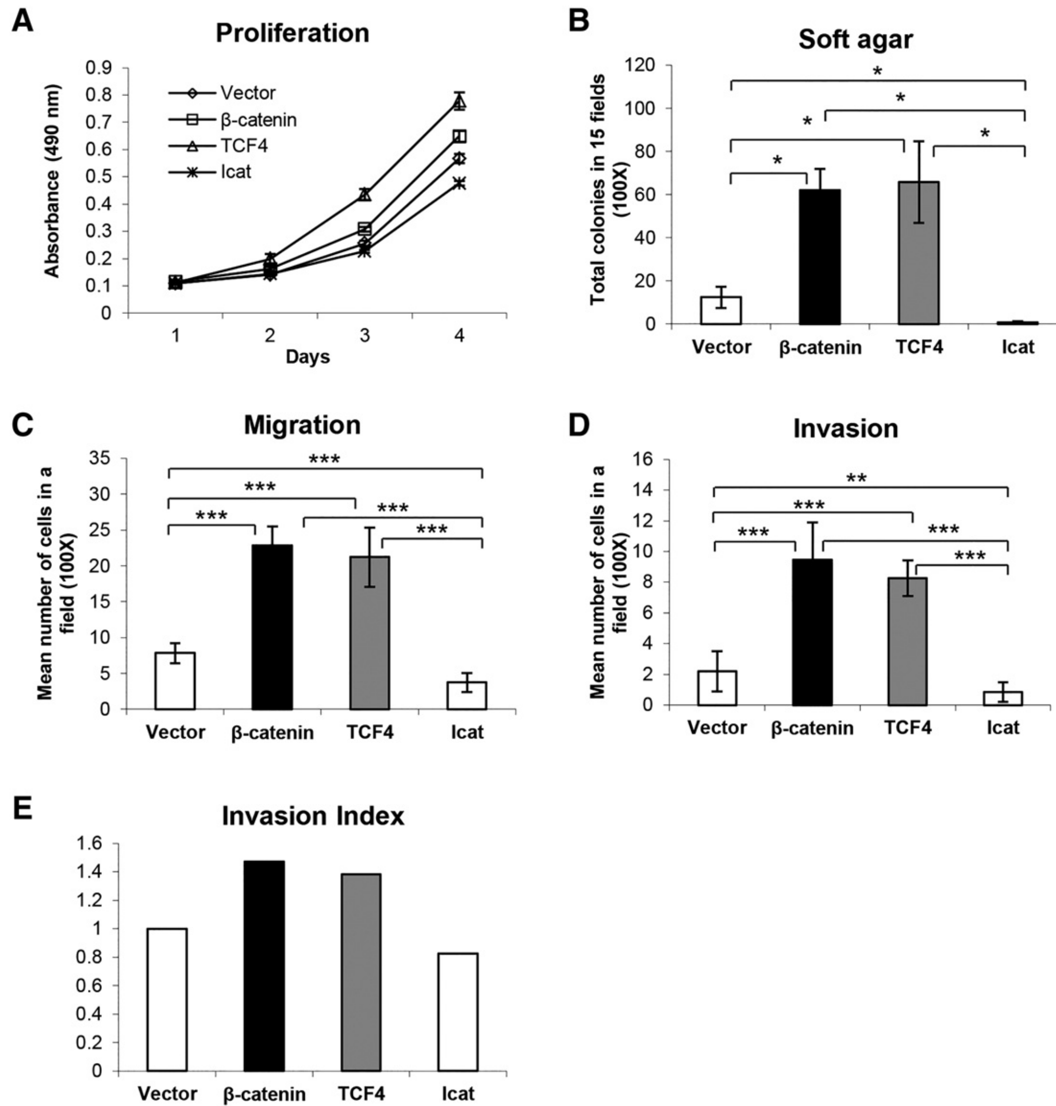

Figure 2. Activation of the WNT/β-catenin pathway enhances transformation phenotypes in pancreatic cancer cells. (A) Measurement of cell number, by MTS assay, in 170#3 cells expressing β-catenin^{S37A}, TCF4, ICAT or empty vector control. (B) Quantification of soft agar colony formation in 170#3 cells expressing β-catenin^{S37A}, TCF4, ICAT or empty vector control. (C) Migration and (D) invasion activity in 170#3 cells expressing β-catenin^{S37A}, TCF4, ICAT or empty vector control. (E) Invasion index (ratio of invading cells to migrating cells) in 170#3 cells expressing β-catenin^{S37A}, TCF4, ICAT or empty vector control. The invasion index for the vector control cells is set to 1. The invasion index for the other cells is shown relative to that for the controls. * $P < .05$, ** $P < .01$, *** $P < .001$ by Mann–Whitney's U test. Data shown are from representative experiments.

promoted pancreatic cancer cell proliferation as assessed using an MTS assay (Figure 2*A*). Moreover, β-catenin^{S37A} and TCF4 expression enhanced several phenotypes associated with cellular transformation, including anchorage independent growth (Figure 2*B*), cell migration (Figure 2*C*) and cell invasion (Figure 2, *D* and *E*). In contrast, expression of ICAT, an inhibitor of the β-catenin/TCF4 interaction impaired all of these cellular phenotypes (Figure 2, *A–E*). Similar results were obtained in a second murine pancreatic cancer cell line demonstrating that this was not a cell line specific phenomenon (Supplementary Figure 4). Together, these data demonstrate that WNT/β-catenin signaling is a potent regulator of the transformed phenotype in pancreatic cancer cells.

β-catenin acts as a transcriptional regulator. Therefore, to identify potential target genes regulated by the WNT/β-catenin signaling pathway in PDAC, we performed gene expression microarray analysis on 170#3 cells expressing β-catenin^{S37A} or ICAT using Affymetrix® Mouse Genome 430 2.0 arrays. We identified 756 genes with an adjusted p-value of 0.05 and greater than 2-fold change in expression between the groups (Supplementary Tables 3 and 4). We identified cysteine-rich angiogenic inducer 61 (Cyr61; as also known as CCN1) as significantly up-regulated by ectopic β-catenin^{S37A} expression. Up-regulation of Cyr61 mRNA and protein levels was induced by overexpression of β-catenin^{S37A} as determined by real-time RT-PCR (Figure 3*A*) and western blotting (Figure 3*B*). In contrast, Cyr61 levels were reduced following transfection of ICAT into 170#3 cells (Figure 3, *A* and *B*).

CYR61 has been previously linked to tumor progression in PDAC, as well as several other tumor types including breast, gastric and colorectal cancers [49–53]. Moreover, prior work suggested that *CYR61* expression

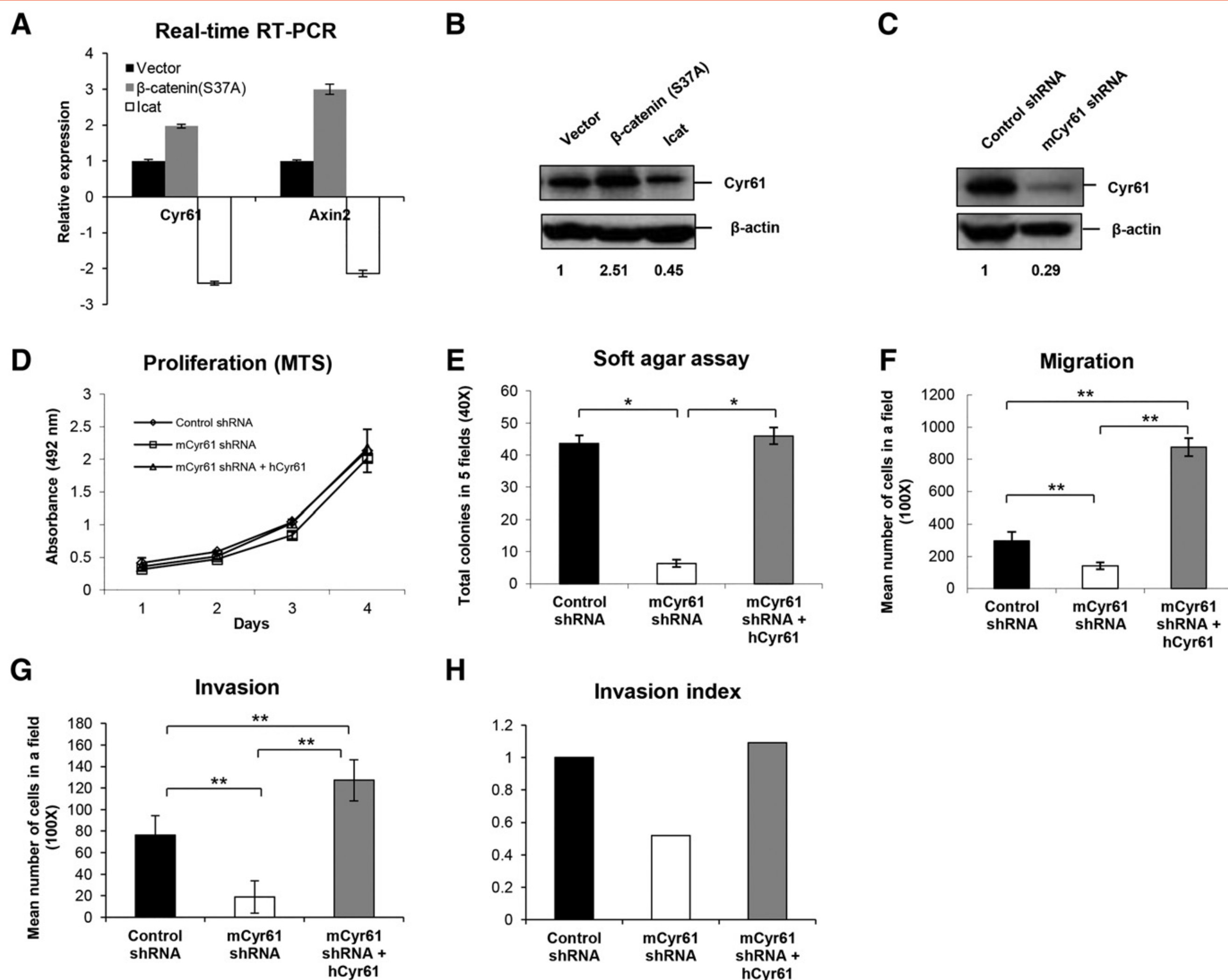

Figure 3. Cyr61 is a target of the WNT/β-catenin signaling pathway in pancreatic cancer cells. (A) Relative expression level of Cyr61 and the validated WNT-regulated gene Axin2 in 170#3 cells expressing β-catenin^{S37A}, ICAT or vector control as determined by qRT-PCR. (B) Determination of CYR61 protein levels by immunoblot in 170#3 cells expressing β-catenin^{S37A}, ICAT or vector control. β-actin is shown as a loading control. Numbers beneath the blot indicate relative ratio of CYR61/β-actin with the ratio in the vector control set to 1. (C) Immunoblot confirming shRNA-mediated Cyr61 knockdown in 170#3 cells expressing β-catenin^{S37A}. β-Actin is shown as a loading control. Numbers beneath the blot indicate relative ratio of CYR61/β-actin with the ratio in the non-silencing control set to 1. (D) Measurement of cell number, by MTS assay, in 170#3 cells expressing β-catenin^{S37A} and additionally expressing a Cyr61-targeting shRNA or a non-silencing control. Cyr61 knockdown cells expressing a human CYR61 cDNA that is resistant to the targeting shRNA are also shown. (E) Soft agar colony formation by 170#3 cells expressing β-catenin^{S37A} and additionally expressing a Cyr61-targeting shRNA or a non-silencing control. Expression of a human CYR61 cDNA that is resistant to the targeting shRNA rescues the soft agar phenotype. (F) Cell migration by 170#3 cells expressing β-catenin^{S37A} and additionally expressing a Cyr61-targeting shRNA or a non-silencing control. Expression of a human CYR61 cDNA that is resistant to the targeting shRNA rescues the migration phenotype. (G) Invasion activity by 170#3 cells expressing β-catenin^{S37A} and additionally expressing a Cyr61-targeting shRNA or a non-silencing control. Expression of a human CYR61 cDNA that is resistant to the targeting shRNA rescues the invasion phenotype. (H) Invasion index (ratio of invading cells to migrating cells) calculated for 170#3 cells expressing β-catenin^{S37A} and additionally expressing a Cyr61-targeting shRNA or a non-silencing control. The index of the cells infected with the non-silencing control shRNA is set to 1. Expression of a human CYR61 cDNA that is resistant to the targeting shRNA rescues the invasion phenotype. * P < .05, ** P < .01 by Mann–Whitney's U test. Data shown are from representative experiments.

is regulated by WNT/β-catenin signaling in hepatocellular carcinoma cells [54]. These findings suggested that CYR61 might play an important role of activated WNT signaling in pancreatic tumorigenesis.

To determine if CYR61 plays an important role in β-catenin-enhanced transformation, we knocked down CYR61 levels in 170#3 cells transfected with β-catenin^{S37A} (Figure 3*C*). We observed that while CYR61 knockdown did not impact cell proliferation as assessed by MTS assay (Figure 3*D*), it strongly abrogated β-catenin-stimulated transformation phenotypes including anchorage independent growth, cell migration and cell invasion (Figure 3, *E–H*). CYR61 knockdown also impaired spheroid formation by pancreatic cancer cells (Supplementary Figure 5, *A–F*). Moreover, the transformation phenotypes were rescued by the introduction of human CYR61 cDNA that is resistant to the shRNA (Figure 3, *E–H*). Together,

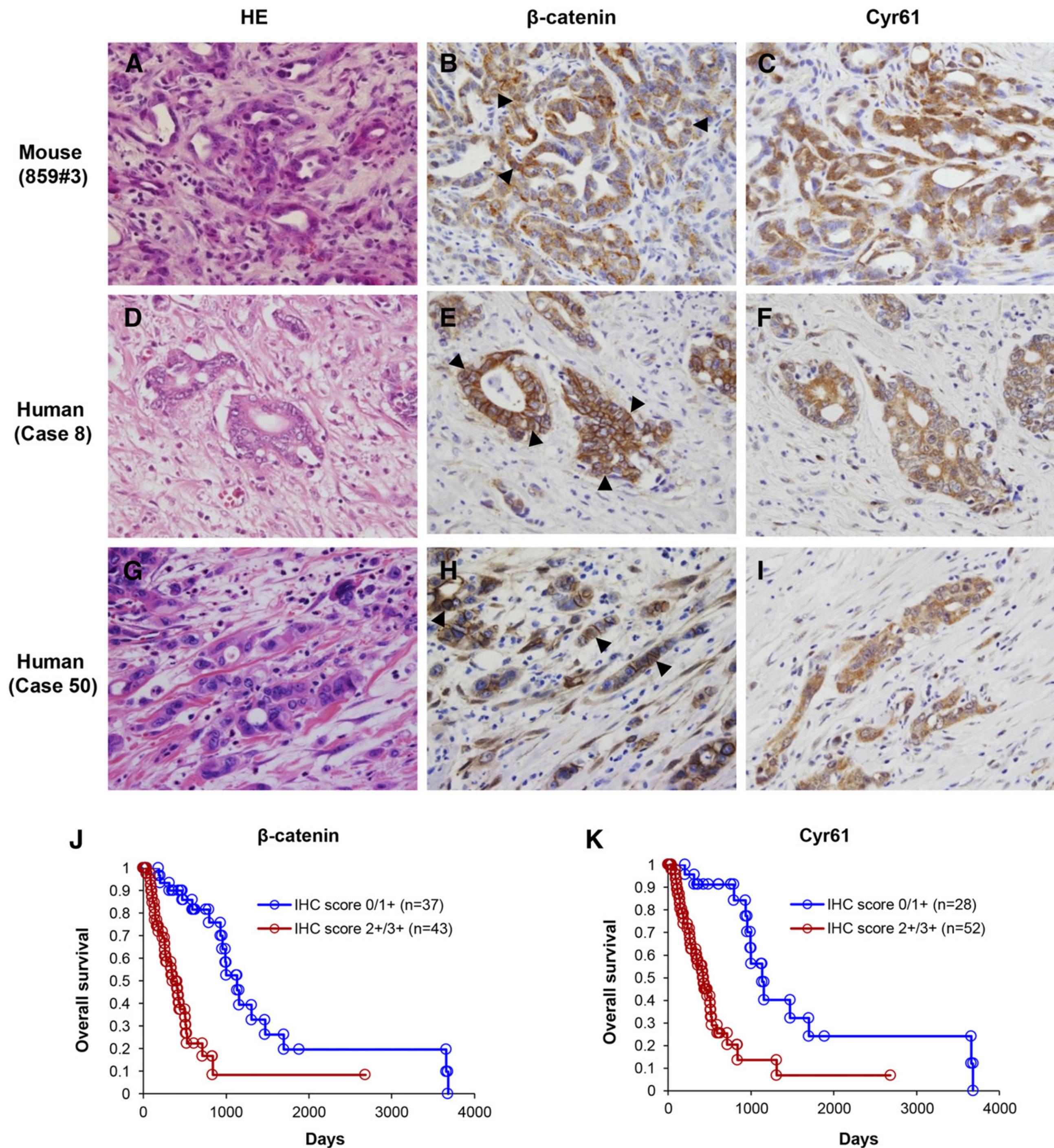

Figure 4. Activation of β-catenin and CYR61 expression correlates with poor prognosis in PDAC. (A) H&E stained tissue section of a PDAC induced by RCAS-Wnt1 in an *Elastase-tva*, *LSL-Kras*^{G12D}, *Ptf1a-Cre* mouse. (B) Immunostaining for β-catenin expression in this specimen. Arrowheads denote tumor cells with nuclear localized β-catenin. (C) Immunostaining for CYR61 in this tumor specimen. (D and G) H&E stained tissue sections from representative human PDAC samples. (E and H) Immunostaining for β-catenin expression in these specimens. Arrowheads denote tumor cells with nuclear localized β-catenin. (F and I) Immunostaining for CYR61 in these tumor specimens. Survival outcome data for PDAC patients stratified by β-catenin (J) or CYR61 (K) IHC score. $P < .01$ by log-rank test for high versus low expressing tumors for both β-catenin and CYR61.

these data suggest that CYR61, a target of the WNT/β-catenin signaling pathway, plays a pivotal role in the enhancement of malignant potential in PDAC cells.

Next, we determined the expression patterns of β-catenin and CYR61 in murine pancreatic tumors (Supplementary Table 4) as well as human PDAC (Supplementary Table 5). In the *LSL-Kras*^{G12D}, *Ptf1a-cre* mouse model, β-catenin was mainly localized at the plasma membrane in well differentiated PDAC, while aberrant cytoplasmic localization and nuclear accumulation of β-catenin were detected in moderately to poorly differentiated PDAC (Figure 4, *A* and *B*, Supplementary Table 2). Likewise, increased CYR61 staining was observed in moderately to poorly differentiated PDAC (Figure 4*C*,

Supplementary Table 2). These expression patterns of β-catenin and CYR61 were similarly present in human PDAC (Figure 4, *D–I*, Supplementary Table 5). In statistical analysis using immunohistochemical score, there was a positive correlation between nuclear β-catenin and cytoplasmic CYR61 expression (Spearman's correlation coefficient by rank test, P = .027). Kaplan–Meier curves of PDAC patients stratified by IHC score for β-catenin and CYR61 demonstrated that high IHC score for β-catenin and CYR61 independently correlated with poor prognosis (log-rank test, $P < .01$) (Figure 4, *J* and *K*). Therefore, these results indicate that nuclear accumulation of β-catenin and up-regulation of its target gene CYR61 are poor prognostic markers in PDAC patients.

Discussion

Activation of WNT signaling is a common feature of multiple types of pancreatic cancer. Mutation of components of the canonical WNT/β-catenin signaling axis is commonly observed in uncommon tumor types such as acinar cell carcinoma, pancreatoblastoma and solid pseudopapillary neoplasm [24–27]. Despite the absence of common occurrence of mutations in pathway components, WNT signaling is also frequently activated in pancreatic ductal adenocarcinoma (PDAC) the most common pancreatic tumor type. More commonly, the pathway is activated by stochastic mechanisms including elevated expression of WNT ligands and mutation or altered expression of regulators of the pathway including ATDC and RNF43 [32–34,55–58]. Yet, despite the common activation of WNT signaling in PDAC, few studies have evaluated whether activation of the pathway enhances pancreatic tumorigenesis in vivo.

To ascertain the postnatal function of canonical and non-canonical WNT signaling pathways in KRAS^{G12D}-induced PDAC development, we activated β-catenin or WNT1 in acinar cells using the RCAS retrovirus gene delivery system [38,46,59,60]. We previously showed that postnatal expression of WNT1 in sporadic pancreatic epithelial cells promoted the development of tumors with the features of mucinous cystic neoplasms (MCN) [37]. This phenotype is driven by paracrine WNT ligand activity on stromal cells. Here, we report that postnatal activation of WNT signaling – either by expression of WNT1 or the downstream transcriptional regulator β-catenin – additionally accelerates the progression of precursor PanIN lesions to invasive PDAC. The ability of β-catenin to accelerate tumorigenesis, coupled with the absence of activated WNT signaling in the stroma of PDAC lesions supports an autocrine mode of action. This finding is in agreement with the recent findings of Simeone and colleagues who demonstrated that expression of ATDC, a positive regulator of cell autonomous WNT signaling, in the pancreas epithelium cooperates with activated KRAS to drive pancreatic tumorigenesis [33]. They are also consistent with the findings of Pasca di Magliano and colleagues who observed that inhibition of WNT signaling by DKK1 or the chemical inhibitor OMP-18R5 impairs KRAS^{G12D}-driven PanIN formation [61].

However, our findings are in contrast to those of Hebrok and colleagues who found that expression of an activated β-catenin in the pancreas epithelium led to the formation of lesions resembling solid pseudopapillary neoplasms [62]. Moreover, co-expression of active β-catenin and activated KRAS in the pancreas inhibited the formation of PanIN lesions; indeed these pancreata had reduced mass compared to the pancreata in mice with activated KRAS expression alone [62]. This reduced mass appeared to be due to a rapid loss of acinar cells and their replacement with dilated ductal structures [62]. Nonetheless, these mice eventually develop pancreatic tumors with distinct cribiform histology. Together, these findings suggested that the combined activation of β-catenin and KRAS during pancreas development profoundly impacts the proper development of the organ. Indeed, prior work by Heiser et al. and Murtaugh and colleagues demonstrated that early activation of β-catenin, or deletion of β-catenin, impaired acinar cell differentiation and survival [63–65]. Taken together, the previously published findings and our data presented here indicate that the timing of β-catenin activation is critical to the resulting pancreatic tumor phenotype. Embryonic activation of β-catenin results in pancreatoblastoma and SPN, whereas postnatal (and sporadic) activation of β-catenin results in PanIN progression and PDAC formation.

Interestingly, our data indicate that WNT ligand-mediated activation of the pathway and activation of β-catenin by mutation promotes PDAC development and progression with similar kinetics. This observation suggests that modest activation of the canonical pathway, such as that achieved downstream of elevated ligand levels, may be sufficient to cooperate with activated KRAS in PDAC development. Alternatively, this observation may indicate that activation of the non-canonical signaling pathways downstream of WNT1 contributes to tumor development and progression. The roles of the non-canonical signaling pathways in pancreatic tumorigenesis are unclear. While WNT5A, which predominantly activates the non-canonical signaling pathways, promotes apoptosis resistance and cell migration in pancreatic cancer cells, other studies indicate that KRAS suppresses WNT/Ca^{2+} signaling [35,36,66]. Studies in which the non-canonical signaling pathways are inhibited downstream of WNT ligand engagement will be required to clarify this issue.

The phenotypes induced following the ectopic expression of activated β-catenin or its DNA binding partner TCF4 in pancreatic cancer cells support our in vivo observations. This cell culture system also provided an opportunity to elucidate some of the mechanisms underlying β-catenin-induced transformation in pancreatic cancer cells. By gene expression profiling, we identified a collection of differentially expressed genes that respond to the presence of β-catenin and its negative regulator ICAT. The dataset of differentially expressed genes includes several genes that have been previously linked to tumorigenesis and cellular transformation in pancreatic cancer or other tumor types, including *Gli2*, *Id2* and *Vegfc* [67–70]. Among the genes induced by β-catenin is *Cyr61*. We show that the canonical WNT/β-catenin pathway enhances malignant potential in PDAC, in part, via up-regulation *Cyr61* as knockdown of CYR61 reduced anchorage independent growth, cell migration and cell invasion. Prior studies have indicated that WNT signaling induced CYR61 expression in hepatocellular carcinoma [54,71]. However, our study is the first to demonstrate regulation of *Cyr61* by WNT signaling in pancreatic cancer cells and to functionally link it to β-catenin-induced transformation in pancreatic cancer cells.

CYR61 expression is frequently detected in human PDAC (~85% cases) [53] and up-regulation of CYR61 expression is associated with peritoneal metastases [52]. Likewise, in our study, CYR61 expression was observed in 76/80 (95%) of human PDAC and was predominantly detected in moderately to poorly differentiated PDAC. Nuclear accumulation of β-catenin and overexpression of CYR61 paralleled each other and were independent poor prognostic markers for PDAC patients. Interestingly, CYR61 can modulate WNT/β-catenin signaling by binding to the WNT co-receptor LRP6 [72]. Therefore, activation of the WNT/β-catenin pathway induces CYR61 expression, and CYR61 in turn activates WNT/β-catenin signaling resulting in a positive feedback loop, thereby leading to a more aggressive phenotype. Interrupting this circuit may represent an opportunity for therapeutic intervention.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2016.11.004.

Funding

This work was supported by National Institutes of Health research grants CA113896 and CA113896-S1 (to BCL), the Verville Foundation (to BCL), and the Pancreas Research Foundation of Japan (to MS). The funders had no role in the design or conduct of the study.

Conflict of Interest Statement

The authors declare that they have no financial conflicts to disclose.

Author Contributions

MS, DRD, DSK, and BCL designed experiments; MS, DRD, WEDJ-M, BQ and VAA acquired data; MS, DRD, WEDJ-M, DSK, JO, LJZ, and BCL analyzed data; NY, SY and TT analyzed correlation between Cyr61 expression and patients' data; MS (Masahiko Sugitani) and NN provided pathological samples; BCL supervised the study; and MS and BCL wrote the paper.

Acknowledgments

The authors thank Drs. Junhao Mao, Karl Simin, Leslie Shaw, and members of the Lewis laboratory for useful comments and discussion; Mr. Victor Adelanwa, Ms. Jiu-Feng Cai and Ms. Sharon A Magnusson for animal care and technical assistance; Ms. Yukari Hirotani, Department of Pathology and Microbiology, Nihon University for excellent sectioning of spheroids.

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