Abstract

Wnt ligand expression and activation of the Wnt/β-catenin pathway have been associated with pancreatic ductal adenocarcinoma, but whether Wnt activity is required for the development of pancreatic cancer has remained unclear. Here, we report the results of three different approaches to inhibit the Wnt/β-catenin pathway in a established transgenic mouse model of pancreatic cancer. First, we found that β-catenin null cells were incapable of undergoing acinar to ductal metaplasia, a process associated with development of premalignant pancreatic intraepithelial neoplasia lesions. Second, we addressed the specific role of ligand-mediated Wnt signaling through inducible expression of Dkk1, an endogenous secreted inhibitor of the canonical Wnt pathway. Finally, we targeted the Wnt pathway with OMP-18R5, a therapeutic antibody that interacts with multiple Frizzled receptors. Together, these approaches showed that ligand-mediated activation of the Wnt/β-catenin pathway is required to initiate pancreatic cancer. Moreover, they establish that Wnt signaling is also critical for progression of pancreatic cancer, a finding with potential therapeutic implications.

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Introduction

Pancreatic ductal adenocarcinoma (PDA), the most common form of pancreatic cancer, is one of the deadliest human malignancies with an overall 5-year survival rate of less than 5% (1). There is a dire need for improving our understanding of the biology of pancreatic cancer to develop effective therapeutic options.

PDA is almost universally associated with the presence of a mutant, constitutively active form of Kras – most frequently the glycine to aspartic acid substitution KrasG12D – a small GTPase protein that is involved in signal transduction downstream of receptor tyrosine kinases (for review see ref. 2). Progression to PDA seems to occur through ductal precursor lesions, most commonly pancreatic intraepithelial neoplasia (PanIN), that accrue morphologic and molecular atypia. PDA is characterized by genomic instability, and a large number of mutations and chromosomal abnormalities are found in each individual tumor (3). Notwithstanding the complexity of their genome, most human PDAs display mutations in 12 core signaling pathways including Wnt and other embryonic signaling pathways (3). Genomic studies support previous work, indicating inappropriate activation of Hedgehog, Notch, and Wnt signaling in PDA (for review see ref. 4). Although the functional role of Hedgehog and Notch signaling has been studied in depth, less is known about the function of Wnt signaling during disease progression.

The Wnt signaling pathway is complex and includes canonical and noncanonical branches (for review see ref. 5). For the purpose of this study, we focused our analysis to canonical Wnt signaling. The canonical pathway is activated by Wnt family ligands binding to Frizzled/LRP receptor complexes. The cascade of events that ensues prevents β-catenin degradation within the cytoplasm and allows its stabilization and nuclear translocation. In the nucleus, β-catenin binds to transcriptional factors of the Tcf/Lef family to form a transcriptional activator (6). Both inhibitory components of the pathway, such as Axin 2, and transcriptional coactivators, such as Tcf1 and Lef1, are also target genes, contributing to the intricate feedback mechanisms that control pathway activity.

Mice with Cre-dependent, conditional expression of oncogenic Kras^{G12D} in the pancreas (hereby referred to as KC mice) develop PanIN lesions that progress to PDA, closely mimicking the human disease (7). Because these animals develop tumors in a stepwise manner in the context of an intact microenvironment, they have emerged as a relevant model for basic discovery as well as for preclinical studies (for review see ref. 8). KC mice also recapitulate the aberrant reactivation
of embryonic signaling pathways, including Wnt, observed in the human disease (9). Thus, they constitute a valid model to study the functional requirement for Wnt signaling during pancreatic carcinogenesis.

Previous functional studies addressing Wnt signaling in PDA have been mainly based on enforced aberrant β-catenin activity using a dominant-active, degradation-resistant allele. When combined with mutant Kras, stabilized β-catenin prevents the formation of PanINs and PDA and instead leads to the development of tumors reminiscent of human intraductal tubular tumors (ITT; ref. 10), a rare form of pancreatic cancer unrelated to PDA. Thus, high-level activation of Wnt signaling is incompatible with PDA formation. On the other hand, accumulation of β-catenin and activation of Wnt target genes are observed in PanINs and PDA (4, 9, 11, 12), and β-catenin functionally supports maintenance of PDA cell proliferation and tumor-forming capacity in xenograft models (9, 13). This paradox compelled us to explore whether activation of the Wnt/β-catenin pathway is required during pancreatic carcinogenesis, and what role it plays during different stages of disease progression.

We used three complementary approaches to block Wnt/β-catenin activation in the KC mouse model. First, we genetically inactivated β-catenin, a key pathway component. Second, we used a tetracycline-regulated system to overexpress Dkk1, a secreted protein that has been shown to specifically and effectively inhibit canonical-Wnt pathway activation (14, 15). Third, we took a therapeutically relevant approach by using OMP-18R5, a monoclonal antibody that inhibits Wnt signaling by binding Frizzled (Fzd) receptors (16).

Materials and Methods

Mouse strains

The mice were housed in specific pathogen-free facilities at the Comprehensive Cancer Center, University of Michigan (Ann Arbor, MI), or at the University of California, San Francisco (San Francisco, CA). All the studies were conducted in compliance with the respective Institutional Committees on Use and Care of Animals guidelines. Detailed strain information is included in the Supplementary Methods.

Cell lines

Primary mouse pancreatic cancer cell lines 65671, 4292, and 9805 and primary human pancreatic cancer cell lines UM2, 1319, UM18, and UM19 were used in this study. The cell line 65671 was derived from the tumor of a KPC mouse (8) in pure FVBN genetic background. In addition, an E2F-luciferase reporter allele was carried by that mouse, thus the tumor cells were labeled with luciferase. The cell line was derived in 2008 and the percentage of duct-like structures in 5 wells for each group. Each cluster counted was classified as normal (nl), tubular (IT), ductal (D), tubular ductal (TIT), ductal tubular (DT), and tubular tumors (ITT; ref. 10), a rare form of pancreatic cancer unrelated to PDA. Thus, high-level activation of Wnt signaling is incompatible with PDA formation. On the other hand, accumulation of β-catenin and activation of Wnt target genes are observed in PanINs and PDA (4, 9, 11, 12), and β-catenin functionally supports maintenance of PDA cell proliferation and tumor-forming capacity in xenograft models (9, 13). This paradox compelled us to explore whether activation of the Wnt/β-catenin pathway is required during pancreatic carcinogenesis, and what role it plays during different stages of disease progression.

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Three-dimensional acinar cell culture

The three-dimensional (3D) culture of Elastase-Cre; β-catenin<sup>−/−</sup> pancreatic acinar cells was prepared in a collagen matrix, as previously described (19); the culture media was supplemented with 100 ng/mL TGF-α to induce acinar-ductal metaplasia. KC or KDC mice were treated with doxycycline 3 days before harvest for 3D culture (also see Supplementary Data). The percentage of duct-like structures in 5 wells for each group was counted at day 3 of 3D culture. A two-tailed unpaired t test was used for statistical analysis.

OMP-18R5 treatment

Monoclonal antibody OMP-18R5 was provided by OncoMed Pharmaceuticals. OMP-18R5 was isolated from the MorphoSys HuCAL GOLD library. KC mice were treated with OMP-18R5 (10 mg/kg, twice/wk) or PBS by intraperitoneal injection for 2 months before sacrificed for study. Primary mouse pancreatic cancer cell line 65671 and human pancreatic cancer cell line UM2 were treated with OMP-18R5 at 10 μg/mL and 20 μg/mL, respectively, in culture.

Recombinant Dkk1 treatment

The mouse pancreatic cancer cell line 65671 and the human pancreatic cancer cell line UM2 were treated with recombinant mouse or human Dkk-1, respectively (500 ng/mL).

Histopathologic analysis

Histopathologic analysis was conducted by a pathologist (W. Yan) on deidentified slides. Five images (×20 objective) were taken in standardized positions (as to cover the whole section) for each slide. A minimum of 50 total acinar or ductal clusters were counted from at least 3 independent animals for each group. Each cluster counted was classified as normal (nl), ADM, PanIN1A, 1B, 2, or 3 based on the classification consensus (20). The data were expressed as percentage of total counted clusters. Error bars represent SEM.

Immunohistochemistry and immunofluorescence

Immunohistochemistry and immunofluorescence were conducted as previously described (21). A list of antibodies is included in the Supplementary Table S1. Images were taken with an Olympus BX-51 microscope, Olympus DP71 digital camera, and DP Controller software. The immunofluorescent images were acquired using an Olympus IX-71 confocal microscope and FluoView FV500/IX software.
Proliferation analysis
The proliferation index was calculated as percentage of Ki67-positive cells. Error bars represent SEM. A two-tailed unpaired t test was used for statistical analysis.

TUNEL staining
For apoptosis detection, the ApopTag Red In Situ Apoptosis Detection Kit (S7165; Millipore) was used in accordance with the manufacturer’s protocol.

Western blotting
Western blotting was conducted as previously described (21). Detailed antibody information is included in Supplementary Table S1.

Quantitative RT-PCR
Quantitative real-time PCR (qRT-PCR) was conducted as previously described (21). The primers are listed in Supplementary Table S2. Values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene expression control, and expressed as ratio over E-cadherin/ Cdh1 expression, to account for the different proportion of epithelium across different samples. A two-tailed unpaired t test was used for statistical analysis.

Detailed protocols and standard procedures are described in the Supplementary Methods.

Results
β-Catenin–negative cells do not contribute to Kras-driven PanIN lesions
To determine if β-catenin is required for PanIN formation, we generated Ptfla-Cre; LSL-KrasG12D; β-cateninfl/fl (KBC) mice where both alleles of β-catenin are floxed in the context of mutant Kras (Supplementary Fig. S1A). Recombination of the floxed β-catenin allele results in lack of functional protein (22). KBC mice survived at a slightly lower than Mendelian ratio, but then reached adulthood in apparent normal health. Pancreata dissected from 2-months-old KC mice had frequent PanIN lesions, surrounded by desmoplastic stroma (Supplementary Fig. S1B). Further analysis revealed that β-catenin knockout clusters in KBC pancreata neither expressed CK19 and p-ERK1/2 nor displayed an increase in proliferation, unlike β-catenin–positive clusters within the same KBC tissues and in control KC pancreata (Supplementary Fig. S1E). Thus, β-catenin null cells did not seem to contribute to PanIN lesions.

Mutant Kras can promote PanIN formation by driving ADM, a process by which acinar cells dedifferentiate to a duct-like state capable of giving rise to PanIN lesions (10, 26). To determine if β-catenin–negative acini were able to undergo ductal reprogramming, we used a 3D culture system. We isolated acinar clusters from adult, tamoxifen-treated Elastase-CreERT2; β-cateninfl/fl or Elastase-CreERT2; β-cateninfl/fl animals to study cells with specific deletion of β-catenin in the adult acinar compartment. The clusters were cultured in a collagen matrix and stimulated to undergo ADM with TGF-α treatment, as previously described (19). In control, Elastase-CreERT2; β-cateninfl/fl ADM was widespread, with few remaining acinar clusters after 5 days of TGF-α treatment (Supplementary Fig. S1F). In contrast, cultures derived from Elastase-CreERT2; β-cateninfl/fl mice displayed a mixture of ductal structures and persistent acinar clusters (Supplementary Fig. S1F). By coimmunofluorescence, we observed that the ductal structures (expressing the ductal marker CK19) were nearly universally positive for β-catenin, whereas the remaining acinar clusters (identified by morphology and amylase expression) were predominantly formed by β-catenin null cells (Supplementary Fig. S1G). Quantification of these results identified a striking change in cell fate between the β-catenin–positive and -negative cell population: cells expressing β-catenin were overwhelmingly found in the CK19+ (ductal) population, whereas virtually all the β-catenin null cells were CK19-negative (Supplementary Fig. S1G). Therefore, β-catenin is required for acinar cells to undergo ADM. Taken together, these data provide, to our knowledge, the first functional evidence of the cell-autonomous requirement for intact β-catenin function during the onset of pancreatic carcinogenesis.

Inhibition of ligand-dependent Wnt signaling inhibits PanIN formation
Because β-catenin is not only a key component of Wnt signaling, but also a structural component of the cadherin-based adherens junctions (27), we sought a different approach to block Wnt signaling during the onset of pancreatic carcinogenesis. Therefore, we used a different genetic approach to target canonical-Wnt signaling. We generated Ptfla-Cre; Rosa26rtTA/+; TetO-Dkk1 mice (referred to as DKK1 mice; Supplementary Fig. S2A) that allow inducible, doxycycline-dependent expression of the secreted Wnt inhibitor Dkk1 in the pancreas. Dkk1 specifically inhibits canonical Wnt signaling by binding to the Kremen family of receptors, a process that results in endocytosis, and thus elimination of the obligate canonical Wnt LRPs/6 coreceptors from the cell surface (28). First, we validated our system by administering doxycycline to 1-month-old DKK1 animals to induce Dkk1 expression (Supplementary Fig. S2B). By activating Dkk1 in postweaning mice, we were able to bypass the potential developmental
effects of inhibiting Wnt signaling during pancreas development. Two months later, we observed widespread, but mosaic Dkk1 expression by immunohistochemistry (Supplementary Fig. S2D). Histologic analysis showed normal pancreatic histology, indicating that Dkk1 expression did not affect homeostasis of the adult pancreas (Supplementary Fig. S2C).

To address the role of ligand-dependent Wnt signaling during PDA initiation, KC mice were crossed with Rosa26\textsuperscript{ERT2/ERT2} (29) and TetO-Dkk1 (14) mice to generate KC:Rosa26\textsuperscript{ERT2/ERT2}:TetO-Dkk1 quadruple transgenics, hereby referred to as KDC (Fig. 1A). In absence of doxycycline, the disease progression in KDC mice was histologically indistinguishable from that of KC mice (Supplementary Fig. S2E and S2F). Then, we expressed Dkk1 in the adult pancreatic epithelium of KDC mice, starting at 1 month of age (Fig. 1B) when KC and KDC mice only have rare PanINs (7,30); thus, in this experiment, Dkk1 expression preceded PanIN formation. Doxycycline water was continuously administered to KC and KDC littermates until the pancreatic tissue was harvested. On the basis of the expected disease progression in KC mice, we harvested tissue after 1, 2, 5, and 8 months (Fig. 1B) with a minimum of 4 animals per genotype per time point. One month following doxycycline administration, the pancreatic parenchyma in KC mice was almost completely replaced by PanIN lesions, ranging from PanIN1A to PanIN2, with occasional PanIN3 lesions. In contrast, in age-matched KDC mice, most of the pancreas was composed of normal acini, with rare PanINs, mainly PanIN1A (Fig. 1C). Both KC and KDC mice showed areas of ADM. The analysis of tissues 2 months after doxycycline administration showed more and higher grade PanINs in KC animals, but still mostly normal acini and ADM in KDC littermates. At later time points (5 and 8 months after doxycycline administration), however, PanINs ranging from PanIN1A to PanIN2, interspersed between normal acini and ADM were also observed in KDC mice (Fig. 1C). At these stages, KDC littermates had a higher percentage of PanIN2 and PanIN3 with almost no normal acini and much fewer ADM lesions. Histopathologic quantification confirmed that KDC mice had fewer PanINs overall and lower grade lesions than KC littermates at all of the time points (Fig. 1D). Summarily, KDC mice displayed inhibited PanIN development and progression.

The inhibitory effect on PanIN formation observed in KDC mice, however, diminished over time (Fig. 1C), as revealed by the progressive increase in several PanIN markers (Supplementary Figs. S3A–S3C and S4A and S4B). Of note, similarly low levels of apoptosis was observed in PanIN lesions in KC and KDC mice (Supplementary Fig. 4C).

We then determined the ability of Dkk1 to inhibit Wnt/β-catenin signaling in the pancreas harvested at the indicated time points (Fig. 2A). Expression of Dkk1 was confirmed in KDC, but not KC mice by Western blot analysis (Fig. 2B). We then measured the levels of total β-catenin, which accumulates in the presence of active Wnt signaling, and phospho-β-catenin, present when the Wnt pathway is not active. In KC animals, PanINs showed high levels of total β-catenin and very little to no phospho-β-catenin; in contrast, KDC tissues had lower levels of total β-catenin but increased levels of phospho-β-catenin at all the time points (Fig. 2B and C). However, at 8 months, expression of Dkk1 was lower in KDC mice, and correspondingly we observed less phospho-β-catenin. Finally, we analyzed the expression of Wnt target genes and pathway components in KC and KDC mice, comparing cohorts of 3-month-old animals that had been on doxycycline for 2 months. As expected, Dkk1 expression was higher in KDC pancreata; the Wnt target genes Axin2, Left1, MMP7, and the Wnt pathway components Wnt2, Fzd1, and Fzd2 had lower expression in KDC tissues, consistent with reduced Wnt signaling. The effect was not due to overall reduced transcription, as we observed no change in other Wnt pathway components not regulated by canonical pathway activity, such as LRPS, LRPS6, and Wnt3a, among others (Fig. 2D and data not shown). In addition, the Hedgehog pathway ligands sonic hedgehog (Shh) and Indian hedgehog (Ihh) and the Notch target gene Hes1 were also significantly reduced in KDC tissues compared with KC (Fig. 2D), consistent with the lower PanIN load in these animals. Thus, our data show that Dkk1 expression efficiently blocks activation of the Wnt signaling pathway and inhibits PanIN formation, therefore providing evidence that activation of the Wnt/β-catenin pathway is required to initiate pancreatic carcinogenesis. To determine whether the inhibition of the Wnt pathway was maintained over time, we analyzed the expression of the Wnt target gene Axin2 (Fig. 2D). Initially, Axin2 expression was lower in KDC than in KC animals, but its expression later rebounded in KDC mice. In fact, Axin2 expression was significantly higher in KDC than KC mice at the 8 month on doxy time point, a finding that might reflect the different cellular composition of the tissues in each cohort at this time point and possibly activation of the pathway in nonepithelial cell types, or positive selection for cells expressing higher levels of Wnt components.

To determine whether inhibition of Wnt signaling prolonged overall survival, we aged a cohort of KC (n = 46) and KDC (n = 24) mice on doxycycline from 1 month of age. Although only about one third of the KC mice developed frank adenocarcinoma, most animals were sacrificed because of declining body condition within 1 year of age and presented at autopsy with PanIN3 and severe exocrine insufficiency due to loss of the pancreas parenchyma. KDC mice had significantly longer survival (Supplementary Fig. S5A). We observed invasive tumors with liver metastases both in KC and KDC mice and lung metastases only in the KC cohort. Tumors from KC and KDC mice were highly proliferative, expressed phospho-ERK1/2, Muc1, and MMP7, and displayed accumulation of desmoplastic stroma (Supplementary Fig. S5B and S5C). We also observed accumulation of β-catenin, indicating active Wnt pathway. Furthermore, immunostaining for Dkk1 in the KDC tumor revealed that its expression was lost, in contrast with the surrounding tissue (Supplementary Fig. S5C). These findings suggest that the invasive tumor either originated from cells that had never recombined the rtTA transgene, thus never expressing Dkk1, or from cells with inactivated Dkk1 expression possibly due to transgene silencing or insufficient doxycycline exposure.

**Treatment with the OMP-18R5 therapeutic monoclonal antibody recapitulates Dkk1 overexpression**

Given the genetic evidence of the ability of Wnt inhibition to prevent PanIN formation, as shown earlier, we sought to...
complement our studies by using a therapeutically relevant approach. Namely, we used the monoclonal antibody OMP-18R5 that binds to multiple Frizzled receptors and blocks their activity (16). One-month-old KC mice were treated with OMP-18R5 (10 mg/kg, twice/wk) or isotype control by intraperitoneal injection for 2 months, and then sacrificed (Fig. 3A). As a
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biodmarkor of the OMP-18R5 activity, we used hair growth, as Wnt/β-catenin signaling in hair follicle precursor cells serves as a crucial signal for the telogen–anagen transition in mice (31). Treatment with OMP-18R5 prevented hair growth in KC mice after depilation, indicating effective Wnt inhibition (Supplementary Fig. S6B). Two months after initiating the study, histologic analysis of the pancreas revealed extensive PanINs, including high-grade lesions, in control KC animals, whereas KC mice treated with OMP-18R5 had a prevalence of normal acini and ADM, and fewer lesions (Fig. 3B and quantification in Fig. 3C). Importantly, we observed an increase in phospho-β-catenin, indicating effective inhibition of Wnt signaling (Fig. 3B). In dissected tissues, pathway inhibition was confirmed by qRT-PCR for the Wnt target genes Axin2, Lef1, and MMP7 (Fig. 3D). Moreover, we detected lower phospho-ERK1/2 in OMP-18R5−treated samples, and a significant decrease in Ki67-positive cells (Fig. 3B). Thus, treatment with OMP-18R5 recapitulated the effect of Dkk1 overexpression, and validated the role of ligand-driven Wnt signaling in PanIN formation.

OMP-18R5 binds multiple Frizzled receptors and thus potentially inhibits both canonical and noncanonical Wnt signaling. In addition, inhibition of canonical Wnt signaling might lead to redirection of the signaling toward the noncanonical pathway through a feedback mechanism (32). Therefore, we examined if OMP-18R5 treatment also affected noncanonical Wnt signaling. We measured the expression of the core component of the planar cell polarity (PCP) pathway, Vangl2 (33) and the core component of Wnt/Ca2+ pathway Cdc42 (34), as well as the noncanonical ligand Wnt5a and found no significant change in KDC mice or KC mice treated with OMP-18R5 compared with control (Fig. 3E).

Cross-talk of Wnt/β-catenin and MAPK/ERK signaling during PanIN formation

Activation of the mitogen-activated protein kinase/extracellular signal–regulated kinase (MAPK/ERK) pathway accompanies, and is necessary for, PanIN formation (35, 36); even in presence of oncogenic Kras, activation of the MAPK/ERK pathway requires additional stimuli, such as inflammation or EGF signaling (4, 37–42). We measured phospho-ERK1/2 as readout of the MAPK/ERK pathway activation in KC and KDC mice at the time points indicated in Fig. 4A. Phospho-ERK1/2 was highly expressed in PanINs in KC pancreata (Fig. 4B and C). In contrast, KDC pancreata had lower levels of phospho-ERK1/2 as measured by immunohistochemistry and Western blot analysis. Intriguingly, even the levels of total ERK1/2 protein were slightly lower in KDC pancreata than in KC animals, possibly indicating additional levels of regulation besides phosphorylation. However, when PanIN lesions were observed in KDC tissues, they had high expression of phospho-ERK1/2, comparable with that observed in KC tissues (Fig. 4C; see 5 and 8 months time points). To further investigate this finding, we conducted coimmunofluorescence for CK19 and Dkk1 (a ductal marker expressed in ADM and PanINs), Dkk1, and phospho-ERK1/2. In 2-month-old KDC animals, cells expressing Dkk1 were mostly CK19-negative (Fig. 4D). In 9-month-old KC mice, we frequently observed high-grade PanIN lesions with high phospho-ERK1/2 expression. High-grade PanIN lesions were rare in age-matched KDC animals, and those lesions that were present were largely negative for Dkk1 expression (Fig. 4D). In 9-month-old KDC mice, Dkk1 was expressed in ADM and low-grade PanINs, but like the 2-month time point, cells expressing Dkk1 were largely CK19-negative, and cells coexpressing Dkk1 and CK19 generally displayed low or near undetectable levels of phospho-ERK1/2 expression. Taken together, our data indicate that Dkk1-expressing cells inefficiently undergo Kras-driven ADM; moreover, even if they undergo ADM they rarely upregulate phospho-ERK1/2 correlated with impaired PanIN specification and progression to higher lesion grade. Thus, the increase in Wnt target gene expression and phospho-ERK1/2−positive lesions in older KDC mice might be explained by a progressive loss of Dkk1-expressing cells−possibly due to proliferation disadvantage.

We then investigated the Wnt/MAPK cross-talk in established pancreatic cancer cells. Primary mouse pancreatic cancer cells (65671) were derived from tumor-bearing KPC mice (Ptf1a-Cre; LSL-KrasG12D, p53fl/fl) inbred in the FVB/NJ strain; they are a pure epithelial and highly tumorigenic population. The 65671 cells expressed Wnt ligands and had active Wnt signaling. Treatment of 65671 cells in culture with recombinant rDkk1 or OMP-18R5 led to inhibition of Wnt activity, as determined by decrease in expression of the Wnt target gene Axin2 (Fig. 4E). Interestingly, rDkk1 or OMP-18R5 treatment also lead to downregulation of the MAPK signaling pathway (as indicated by reduced phospho-ERK1/2 levels), but had no effect on phospho-Akt (Fig. 4F). We then investigated noncanonical Wnt signaling in response to OMP-18R5 treatment in this panel of primary tumor cells by measuring the level of activation of two kinases, c-Jun-NH2 kinase (JNK) and protein kinase C (PKC), which mediate the Wnt/PCP and Wnt/Ca2+ signaling branches, respectively (for review see ref. 43). We observed no changes in p-JNK levels, and correspondingly no change in the expression level of Vangl2 (Supplementary Fig. S6C and S6E). In contrast, p-PKC levels transiently decreased upon treatment with OMP-18R5 (Supplementary Fig. S6C), a finding that might indicate changes in the Wnt/Ca2+ signaling pathway.

To determine whether the cross-talk between the Wnt and MAPK pathway exists in human tumors, we used the primary human PDA cell lines UM2, 1319, UM18, and UM19 (18). Wnt inhibition resulted in downregulation of phospho-ERK1/2 and phospho-Akt (Supplementary Fig. S6C). Thus, even in primary human tumor cells, Wnt signaling is a positive regulator of the MAPK pathway. Using RT-PCR of canonical Wnt-targets to verify pathway inhibition, treatment with rhDkk1 only inhibited Axin2 expression in 2 of 4 cell lines tested and OMP-18R5 was effective in 3 of 4 cell lines (Supplementary Fig. S6F). Similarly, among 3 primary
Figure 3. Inhibition of the Wnt/β-catenin signaling by OMP-18R5 prevents PanIN formation. A, experimental design for OMP-18R5 treatment; n = 5 mice per cohort. B, H&E staining and immunohistochemical analysis for β-catenin, phospho-β-catenin, phospho-ERK1/2, and Ki67. Scale bar, 50 μm. C, quantification of the lesions. D, qRT-PCR analysis of Wnt/β-catenin target genes. E, qRT-PCR analysis of noncanonical Wnt signaling components. Each dot represents one mouse. Data represent mean ± SEM. The statistical difference was determined by two-sided Student t test. ns, not significant.
mouse pancreatic cancer cell lines, both agents inhibited Axin2 expression in 2 lines, 65671 and 4292, whereas only OMP-18R5 inhibited Axin2 expression in the third line 9805 (Supplementary Fig. S6D). The different response of individual cell lines to Dkk1 and OMP-18R5 might reflect biologic differences such as mutations in signaling components that affect the functionality of each inhibitor differently, based on their different mechanisms of action.

**Wnt/β-catenin signaling regulates pancreatic cell proliferation and Kras-driven acinar cell reprogramming**

The adult pancreas has a very low frequency of Ki67-positive cells, as most cells are not dividing (Supplementary Fig. S7C). Proliferation was similarly low in Dkk1-expressing pancreata (Supplementary Fig. S7C). In KC animals, significantly increased proliferation—both in the epithelial compartment and in the surrounding stroma—accompanies PanIN formation (Supplementary Fig. S7B and S7C). In contrast, we observed a decrease in proliferation in KDC tissues, specifically in the epithelial compartment (Supplementary Fig. S7B and S7C), both in ductal structures (ADM and PanINs) and acinar cells (Supplementary Fig. S7D). Therefore, our data suggest that active Wnt signaling supports Kras-dependent epithelial proliferation during the early steps of pancreatic carcinogenesis.

We then investigated the effect of Wnt signaling inhibition on the onset of ADM. To follow a specific cell cluster over time, we used 3D cultures of acinar clusters in Matrigel. WT and KC acinar clusters spontaneously underwent ADM in Matrigel; however, the formation of duct-like structures was significantly faster and more penetrant in KC acini (Fig. 5A and B). Moreover, KC-derived ductal structures accumulated more intraluminal debris and more intracellular mucin over time (as shown by PAS staining; Fig. 5C). Formation of ductal structures in KDC-derived cultures was equivalent to WT, with slower and less penetrant duct formation compared with KC clusters (Fig. 5A and quantification in Fig. 5B). Moreover, mucin accumulation—an indication of progression form ADM to PanIN—was reduced in KDC, compared with KC clusters (Fig. 5C). Thus, Dkk1 expression inhibited the capacity of Kras to drive acinar to ductal reprogramming and enforce an aberrant mucinous program in metaplastic ducts. This set of data was consistent with our in vivo observation that both Kras-driven ADM formation and progression to PanINs are inhibited in KDC pancreata.

**Inhibition of Wnt signaling in established PanINs**

The inducible Dkk1 expression in KDC mice gave us the opportunity to investigate the effect of Wnt inhibition in established PanINs. In this set of experiments, doxycycline was administered to 2-month-old KC and KDC animals (n = 4), when a discrete number of PanINs lesions can be observed in the majority of human pancreatic tumors (3), as well as in a mouse screen for mutations synergizing with Kras in driving pancreatic carcinogenesis (51). The exact biologic significance of each of the mutations identified is in need of further exploration. In addition to canonical Wnt signaling, noncanonical signaling may also play an important role in this disease (52–56). The relative importance of the different modalities of Wnt signaling requires further investigation.

Here, we show that inhibition of Wnt signaling significantly delayed PanIN formation. Taken together, our results suggest that there is a minimal threshold of ligand-mediated, canonical Wnt signaling required for PanIN formation (Fig. 6). In contrast, high levels of Wnt activation prevent specification of the PanIN-PDA lineage (Fig. 6; refs. 10, 48). In the future, genetic models that permit controllable, graded increases in β-catenin activity, or inducible expression of Wnt ligands will elucidate the threshold at which canonical Wnt signaling is no longer compatible with Kras-driven PanIN development.

An essential step for the initiation of pancreatic carcinogenesis is activation of the MAPK/ERK signaling pathway. In KC mice, activation of MAPK signaling is a key step for PanIN formation (35). Mutant Kras and Wnt signaling pathway cooperate to drive tumorigenesis in other tissues, for example...
Figure 4. Cross-talk of Wnt and MAPK signaling pathways. A, experimental design; n = 3–5 mice per time point. B, Western blot analysis for total ERK1/2 and phospho-ERK1/2; quantification of the fold change of phospho-ERK1/2/total ERK1/2. Data represent mean ± SEM, n = 3. C, immunohistochemistry for ERK1/2 and p-ERK1/2.
in lung and colon adenocarcinoma (57, 58). Epistatic interactions between MAPK and Wnt signaling have been recently described both in melanoma and in colon cancer (59, 60). Intriguingly, these interactions are clearly tissue-specific: in melanoma Wnt signaling inhibits MAPK signaling (60), whereas in colon cancer Wnt signaling stabilizes Ras and thus promotes MAPK activity (59). Here, we show that Wnt signaling in the pancreas promotes MAPK signaling and has a tumor-promoting effect. Thus, the identity of β-catenin targets that antagonize or promote Kras-dependent transformation may

Figure 5. Wnt signaling is required for Kras-driven acinar-ductal metaplasia and PanIN proliferation. A, transmitted light images, H&E staining, and amylase immunofluorescent staining of KC and KDC pancreatic cell clusters in 3D culture. Arrows, duct structures; asterisks, acinar clusters. Scale bar, 20 μm. B, quantification of duct-like structures at day 3. Data represent mean ± SEM; n = 5. The statistical difference was determined by two-sided Student t test. C, PAS staining. Arrows, PAS-positive cells. Scale bar, 20 μm. D, experimental design; n = 4 mice. E, pancreas size (mean ± SEM; n = 4). The statistical difference was determined by two-sided Student t test. F, proliferation index in epithelial and stromal compartments. Data represent mean ± SEM; n = 4. The statistical difference was determined by two-sided Student t test. G, gross morphology of KC and KDC pancreata (scale bar, 2 mm); H&E and Ki67 staining (scale bar, 50 μm).
be tissue specific, and thus may provide unique cancer-specific therapeutic targets.

Studying the role of Wnt/β-catenin during the initiation of PDA highlighted the potential role of targeting Wnt/β-catenin signaling in disease initiation. This finding has implications for the potential use of Wnt inhibitors in patients with pancreatic cancer, at a time when several Wnt inhibitors, including OMP-18R5 have entered the clinical pipeline for treatment of a variety of cancers, including PDA (for review see refs. 61, 62). OMP-18R5 has been found to be active in combination with a variety of chemotherapeutic agents in patient-derived xenografts, including gemcitabine in PDA xenografts (16). Our data indicate that the mechanism of action might involve a reduction in the activation of the MAPK/ERK signaling, and inhibition of proliferation. Further studies might elucidate whether interactions between the tumor cells and the surrounding stroma are also regulated through Wnt signaling.

Disclosure of Potential Conflicts of Interest

M. Hebrok is a consultant/advisory board member of Merck. M.P. di Magliano has a commercial research grant from OncoMed. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

The study sponsors had no role in the design of this study or in the collection, analysis, and interpretation of the data.

References


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