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Oncogenic Function of ATDC in Pancreatic Cancer through Wnt Pathway Activation and β-Catenin Stabilization

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SUMMARY

Pancreatic cancer is a deadly disease characterized by late diagnosis and resistance to therapy. Much progress has been made in defining gene defects in pancreatic cancer, but a full accounting of its molecular pathogenesis remains to be provided. Here, we show that expression of the ataxia-telangiectasia group D complementing gene (*ATDC*), also called *TRIM29*, is elevated in most invasive pancreatic cancers and pancreatic cancer precursor lesions. ATDC promoted cancer cell proliferation in vitro and enhanced tumor growth and metastasis in vivo. ATDC expression correlated with elevated β-catenin levels in pancreatic cancer, and β-catenin function was required for ATDC's oncogenic effects. ATDC was found to stabilize β-catenin via ATDC-induced effects on the Disheveled-2 protein, a negative regulator of glycogen synthase kinase 3β in the Wnt/β-catenin signaling pathway.

INTRODUCTION

Pancreatic cancer is a highly lethal disease that is often diagnosed in an advanced state. In fact, though it is the fourth most common cause of cancer death in the United States, resulting in 37,000 deaths per year (Jemal et al., 2007), it has the worst prognosis of any major malignancy (<5% 5-year survival rate). Recent advances in surgical and medical therapy have had only a modest impact on pancreatic cancer mortality. A major hallmark of pancreatic cancer is extensive local tumor invasion and early systemic dissemination. Pancreatic cancer is also notoriously resistant to chemotherapy and ionizing radiation.

To further understand the molecular pathogenesis of pancreatic cancer, genomic and proteomic profiling has been performed to identify differentially expressed genes and proteins that might represent novel therapeutic targets (Cao et al., 2004; Chen et al., 2005; Logsdon et al., 2003; Lowe et al., 2007). Using Affymetrix gene expression profiling, we previously found that pancreatic cancer cells overexpress the ataxia-telangiectasia group D complementing gene (*ATDC*) at an average

SIGNIFICANCE

Pancreatic cancer is an aggressive malignancy, and an improved understanding of the molecular mechanisms governing its highly aggressive behavior is needed for more effective treatment, early detection, and prevention. Defects in Wnt/β-catenin signaling are common in certain cancers, such as colorectal carcinoma, and recent evidence suggests that Wnt/β-catenin signaling may contribute to pancreatic cancer. In this report, we show that the ataxia-telangiectasia group D complementing gene (*ATDC*) is overexpressed in the majority of invasive pancreatic cancers and pancreatic cancer precursor lesions. ATDC contributes to pancreatic cancer via its ability to interact with and stabilize expression of Disheveled-2, with resultant stabilization of β-catenin. Besides highlighting ATDC as a potential therapeutic target in pancreatic cancer, our studies have defined a mechanism for activating Wnt/β-catenin signaling in cancer.

level 20-fold higher than epithelial cells from normal pancreas or chronic pancreatitis-derived tissues (Logsdon et al., 2003). *ATDC* was initially described in the hunt for the gene responsible for the genetic disorder ataxia-telangiectasia (AT) (Kapp et al., 1992). Both alleles of the *ATDC* gene in an AT patient-derived cell line were found to contain early stop codon mutations leading to a truncated and nonfunctional ATDC protein (Tauchi et al., 2000). No germline *ATDC* mutations were found in AT patients, and subsequently the gene responsible for AT was identified as ataxia-telangiectasia mutated (*ATM*) (Savitsky et al., 1995).

The *ATDC* gene, located at chromosome 11q23, encodes a 588 amino acid protein with multiple zinc-finger motifs and an adjacent leucine-zipper motif that may allow the ATDC protein to form homo- or heterodimers (Kapp et al., 1992). Northern blot analysis revealed that *ATDC* is normally expressed in placenta, lung, thymus, prostate, testis, and colon, while no expression is observed in heart, brain, skeletal muscle, pancreas, spleen, ovary, or small intestine (Hosoi and Kapp, 1994). In addition to our observation of high *ATDC* levels in primary pancreatic cancers and pancreatic cancer cell lines, a search of the Oncomine database (http://www.oncomine.org/) revealed that *ATDC* has been reported to be overexpressed in lung (Hawthorn et al., 2006), bladder (Dyrskjot et al., 2004), colorectal (Glebov et al., 2006; Ohmachi et al., 2006), ovarian (Santin et al., 2004), and endometrial cancers (Mutter et al., 2001) and in multiple myeloma (Zhan et al., 2002), with apparent reduced expression in melanoma (Smith et al., 2005) and in breast (Nacht et al., 1999), head and neck (Zhang et al., 2006), and prostate cancers (LaTulippe et al., 2002; Luo et al., 2001; Yu et al., 2004). A recent report identified a correlation between *ATDC* expression in gastric cancer and poor histological grade, large tumor size, extent of tumor invasion, and lymph node metastasis (Kosaka et al., 2007).

The ATDC protein, also known as TRIM29, is a member of the tripartite motif (TRIM) family. TRIM proteins have a series of conserved domains, which include a RING (R), a B box type 1 (B1) and B box type 2 (B2), and a coiled-coil (CC) region. While some of the domains may be absent or present in the different TRIM proteins (ATDC contains the B1-B2-CC domains but lacks the R domain), their order is always maintained (R-B1-B2-CC) (Reymond et al., 2001). Proteins belonging to the TRIM family have been implicated in a variety of cellular processes, such as development and growth, and in several human diseases, including HIV infection (Stremlau et al., 2004) and leukemia (Goddard et al., 1991). The function of ATDC has not been studied previously in any physiologic or pathologic process, though the ATDC protein appears to be localized primarily to the cytoplasm (Reymond et al., 2001). In this report, we describe our data examining the expression and functional role of ATDC in pancreatic cancer.

RESULTS

ATDC Is Overexpressed in Human Pancreatic Adenocarcinoma

To identify genes with potential roles in the development and progression of pancreatic adenocarcinoma, we assessed gene expression in microdissected samples of human pancreatic carcinoma using Affymetrix arrays. We compared the expression patterns in cancer tissues to those seen in normal pancreas and chronic pancreatitis, the latter of which served as an important control for the extensive fibrosis typically observed with pancreatic cancer (Logsdon et al., 2003). One of the most highly upregulated genes was the *ATDC* gene, which showed elevated expression in 10 out of 10 samples of pancreatic cancers relative to normal pancreas or chronic pancreatitis samples (Figure 1A). On average, *ATDC* expression was roughly 20-fold higher in pancreatic adenocarcinomas. The gene expression data were further confirmed in an analysis of *ATDC* mRNA levels of pancreatic cancer using quantitative real-time RT-PCR (qRT-PCR) (Figure 1B). Immunohistochemical staining confirmed that ATDC protein expression was present in the neoplastic epithelium of pancreatic cancer (Figure 1C).

A pancreatic cancer progression model is now widely accepted in which normal pancreatic ductal epithelium progresses to infiltrating cancer through a series of morphologically defined pancreatic precursors called pancreatic intraepithelial neoplasias (PanINs) (Hruban et al., 2000). This progression is associated with accumulation of specific genetic changes, such as K-ras mutations and inactivation of p16, that are observed in invasive pancreatic cancer. We found that ATDC was not expressed in PanIN 1 lesions (0 of 4) but was occasionally expressed in PanIN 2 lesions (1 of 7) and was more often expressed in PanIN 3 lesions (3 of 6) (Figure 1D). These data suggest that upregulation of ATDC occurs prior to the development of invasive pancreatic cancer.

ATDC Promotes Cellular Proliferation In Vitro and Pancreatic Tumorigenesis In Vivo

To understand the function of ATDC in pancreatic cancer, we first explored the effect of ectopic *ATDC* expression on cellular growth in vitro in multiple cell lines with differing levels of endogenous ATDC expression. Following transfection with an *ATDC* cDNA expression construct, HEK293 cells, which normally do not express ATDC, and MiaPaCa2 pancreatic cancer cells, which express low endogenous levels of ATDC, demonstrated a significant increase in cellular proliferation (Figures 2A and 2B). Similar changes were observed in monoclonal and polyclonal HEK293 cells lines stably overexpressing ATDC (see Figure S1 available online). Conversely, cellular proliferation was attenuated when endogenous ATDC expression was silenced by stable transfection with two different shRNA vectors targeting distinct regions of *ATDC* in Panc1 and BxPC3 pancreatic cancer cell lines, both of which have high endogenous levels of ATDC (Figures 2C and 2D). Expression of ATDC shRNAs 1 and 2 did not alter basal cell proliferation rates in HEK293 cells (Figure S2), verifying the specificity of the inhibitory function of the ATDC shRNAs on ATDC's function.

To examine the effects of ATDC silencing on pancreatic tumor growth in vivo, we infected Panc1 cells expressing a control shRNA or ATDC shRNA1 with a luciferase-expressing lentivirus. Following injection of 5×10^5 cells into the tail of the pancreas, tumor growth was assessed using bioluminescent imaging (n = 8 animals per group). All of the animals injected with Panc1 cells expressing control shRNA demonstrated tumor formation 14 days postinjection, while tumors were not detected in the animals injected with Panc1 ATDC shRNA cells (Figure 2E, left panels). At 60 days postinjection, the control shRNA animals' tumors grew significantly larger, with evidence of metastatic

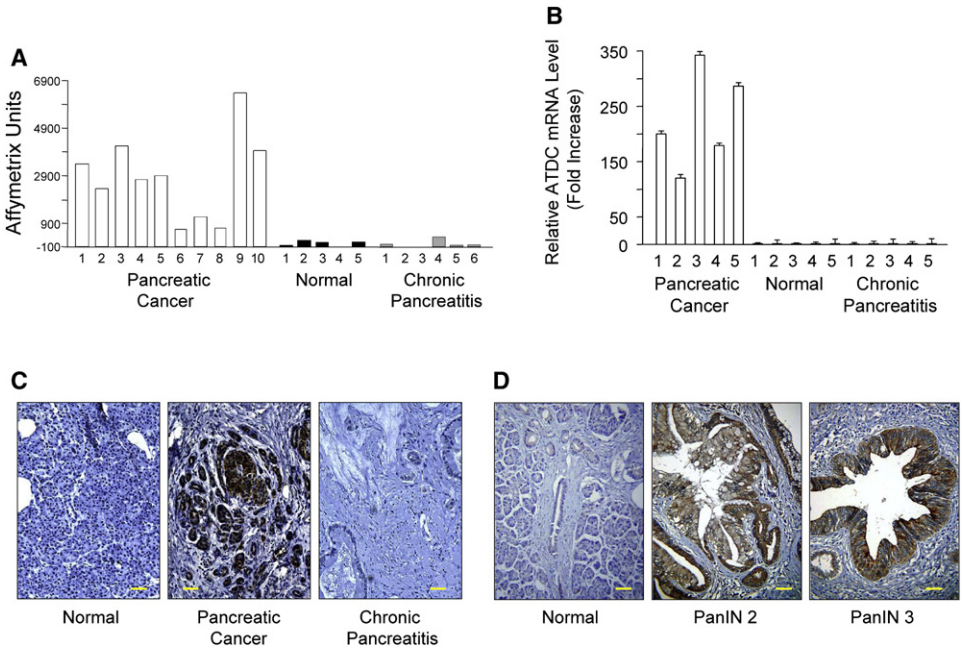

Figure 1. ATDC Is Highly Expressed in Human Pancreatic Cancer

(A) cDNA microarray analysis (Logsdon et al., 2003) was performed using Affymetrix HuGeneFL arrays containing 7129 probe sets. Microdissected samples of human pancreatic cancer (n = 10, white bars), normal pancreas (n = 5, black bars), and chronic pancreatitis (n = 5, gray bars) were analyzed. mRNA expression levels of *ATDC* were expressed as Affymetrix units.

(B) Validation of microarray results of *ATDC* mRNA levels using quantitative real-time RT-PCR analysis.

(C) ATDC immunostaining of representative samples of normal pancreas, pancreatic cancer, and chronic pancreatitis. Scale bars = 100 μm.

(D) Immunohistochemical examination of ATDC expression in normal pancreas or pancreatic intraepithelial neoplasia (PanIN) lesions. Scale bars = 100 μm.

spread, while only 25% (2 of 8) of the ATDC shRNA animals demonstrated evidence of macroscopic tumors (Figure 2E, right panels; Figure 2F). The mean tumor volume was significantly larger in the tumors grown in mice injected with Panc1 cells expressing control shRNA compared to mice injected with Panc1 cells expressing ATDC shRNA (203.2 ± 68.8 versus 2.01 ± 1.0 mm^3; $p < 0.05$) (Figure 2G). These data support the role of ATDC in promoting growth of pancreatic cancer cells.

ATDC Activates the Wnt/β-Catenin/TCF Signaling Cascade

In exploring possible downstream mediators of ATDC's growth-promoting effects, we noted that overexpression of ATDC resulted in a significant increase in β-catenin levels in HEK293 cells (Figure 3A). In addition, the overexpression of ATDC increased expression of the under- or nonphosphorylated forms of β-catenin ("active" β-catenin) presumed to be the forms of β-catenin responsible for mediating Wnt signaling in cells (van Noort et al., 2002) (Figure 3A). Similar results were obtained in MiaPaCa2 pancreatic cancer cells ectopically expressing ATDC (Figure 3B). Conversely, ATDC shRNA1- or 2-mediated silencing of endogenous ATDC expression significantly decreased total β-catenin and active β-catenin levels in Panc1 cells (Figures 3C and 3E) and BxPC3 cells (Figures 3D and 3F).

The active form of β-catenin exerts its growth-promoting effects by translocating from the cytoplasm to the nucleus, where it binds to transcription factors such as the T cell factor (TCF)/lymphoid enhancer-binding factor and thereby stimulates the transcription of Wnt target genes (Clevers, 2006). Overexpression of ATDC in HEK293 and MiaPaCa2 cells increased expression of the Wnt/β-catenin target genes c-*Myc* and Dickkopf homolog 1 (*DKK1*) (Figures 3A and 3B), while c-*Myc* and *DKK1* levels were significantly reduced in Panc1 and BxPC3 cells expressing ATDC-targeting shRNA1 or 2 (Figures 3C and 3D). Consistent with the ability of ATDC to increase β-catenin levels in HEK293 cells, we found that ATDC strongly activated the TCF-dependent TOPflash reporter construct in HEK293 cells (Figure 3G). Together, these results strongly suggest that ATDC enhances β-catenin levels and activates β-catenin/TCF target gene expression in pancreatic cancer cells.

ATDC Increases the Free Intracellular Pool of β-Catenin through Activation of the Canonical Wnt Signaling Pathway

To address in more detail the means by which ATDC increases β-catenin/TCF-regulated gene expression, we assessed the abundance of the free pool of β-catenin in ATDC-expressing cells compared to control cells. To measure the free pool of β-catenin, we utilized a recombinant glutathione S-transferase (GST) fusion protein containing the cytoplasmic tail of E-cadherin (GST-Ecad). It has been previously shown that GST-Ecad can readily be used to monitor the abundance of the free pool of β-catenin that is stabilized in response to activation of the Wnt signaling pathway (Winer et al., 2006). As shown in Figure 3H, following ectopic expression of *ATDC* or the S33Y mutant form of β-*catenin* in HEK293 cells, significant increases in the levels of the free β-catenin pool were seen as

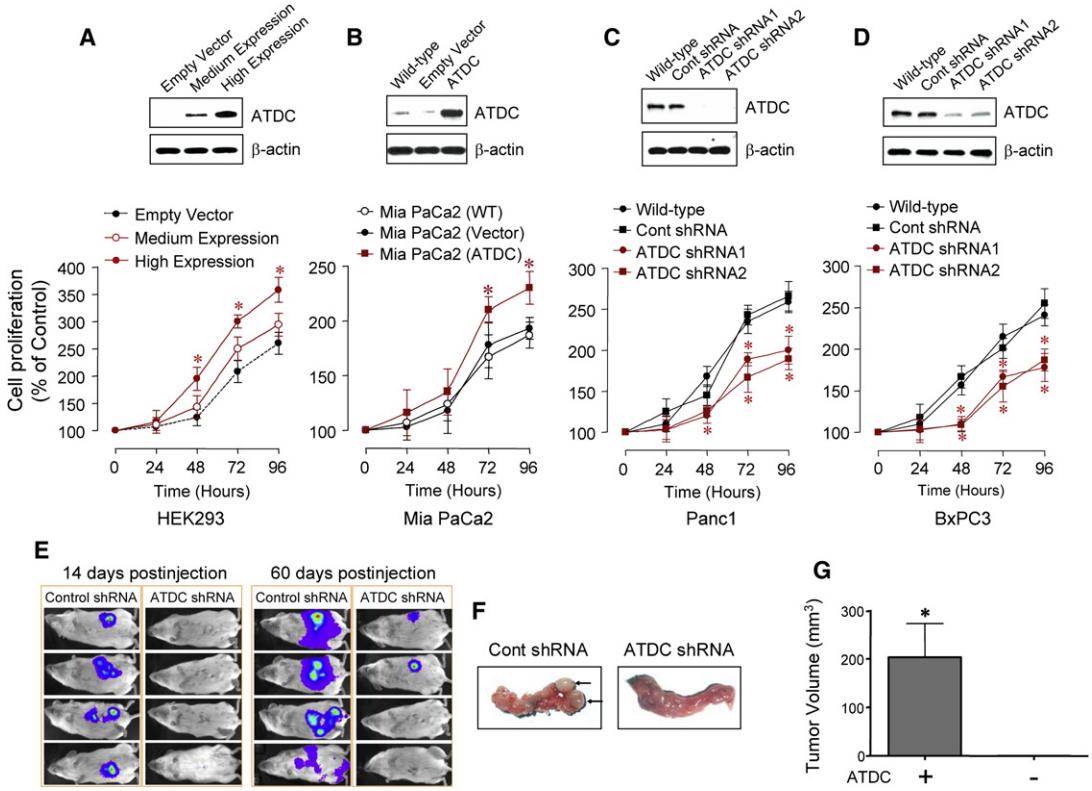

Figure 2. ATDC Promotes Cell Proliferation and Pancreatic Tumorigenesis

(A) Top: medium or high expression of ATDC in stably transfected HEK293 cells. Bottom: MTS proliferation assay in ATDC-transfected HEK293 cells (mean ± SEM; n = 4, *p < 0.05 versus empty vector-transfected cells).

(B) Top: ATDC expression in wild-type (WT), empty vector-transfected, or ATDC-transfected MiaPaCa2 cells. Bottom: MTS proliferation assay in ATDC-transfected MiaPaCa2 cells (mean ± SEM; n = 3, *p < 0.05 versus empty vector-transfected cells).

(C and D) Top: ATDC expression in wild-type, control shRNA-transfected, and ATDC shRNA1- or 2-transfected Panc1 cells (C) or BxPC3 cells (D). Bottom: MTS proliferation assays in ATDC shRNA-transfected Panc1 and BxPC3 cells (mean ± SEM; n = 4, *p < 0.05 versus wild-type cells).

(E) Representative bioluminescent images of half of the animals in the control or ATDC shRNA groups are shown at 14 (left panels) and 60 (right panels) days after injection, depicting the extent of tumor burden.

(F) Representative pictures 60 days after injection of mouse pancreata with control shRNA- or ATDC shRNA-transfected Panc1 cells. Only 25% of the mice (2 of 8) injected with Panc1 cells expressing ATDC shRNA displayed evidence of tumor formation.

(G) Average tumor volume 60 days after injection in animals injected with control shRNA- or ATDC shRNA-transfected Panc1 cells (mean ± SEM; n = 3, *p < 0.05).

demonstrated by the recovery of β-catenin with GST-Ecad. In contrast, no significant β-catenin was recovered from control cell lysates following incubation with GST-Ecad.

To confirm that the increase in the free pool of β-catenin seen with ATDC overexpression was associated with increased cytoplasmic and nuclear levels of β-catenin, extracts from control and *ATDC*-overexpressing HEK293 cells were separated into membrane, cytoplasmic, and nuclear fractions, and the relative abundance of β-catenin in these fractions was analyzed. ATDC overexpression increased both the nuclear and cytoplasmic fractions of β-catenin while having no effect on the membrane-bound fraction of ATDC (Figure 3I). Previous reports have demonstrated an important role for β-catenin in cell adhesion as a part of a protein complex that includes E-cadherin. The E-cadherin expression pattern in pancreatic cancer cells was unaltered by ATDC shRNA (Figure S3), suggesting that increased levels of ATDC affect β-catenin levels and β-catenin/TCF-dependent transcription in a fashion similar to that seen following activation of the canonical Wnt signaling pathway.

ATDC Stimulates Cell Proliferation and Tumor Growth via β-Catenin/TCF Activation

We next sought to determine whether the growth-promoting effects of ATDC are mediated by activation of the β-catenin signaling pathway. Constitutive activation of Wnt/β-catenin signaling by mutations in known Wnt pathway components, such as inactivating mutations in the *APC* (adenomatous polyposis coli) or *Axin1* tumor suppressor genes, or activating mutations in *β-catenin* are commonly seen in certain cancers, such as colorectal or hepatocellular carcinomas, but are rarely seen in pancreatic adenocarcinoma (Gregorieff and Clevers, 2005; Lustig and Behrens, 2003). However, constitutive activation of β-catenin/TCF-dependent transcription, independent of mutations, has been suggested to play an important role in the development of certain human breast and ovarian cancers (Bafico et al., 2004) and in mouse models of basal cell carcinoma (Hoseong Yang et al., 2008) and pancreatic cancer (Pasca di Magliano et al., 2007). To address the contribution of β-catenin/TCF transcription in the growth-promoting effects of ATDC,

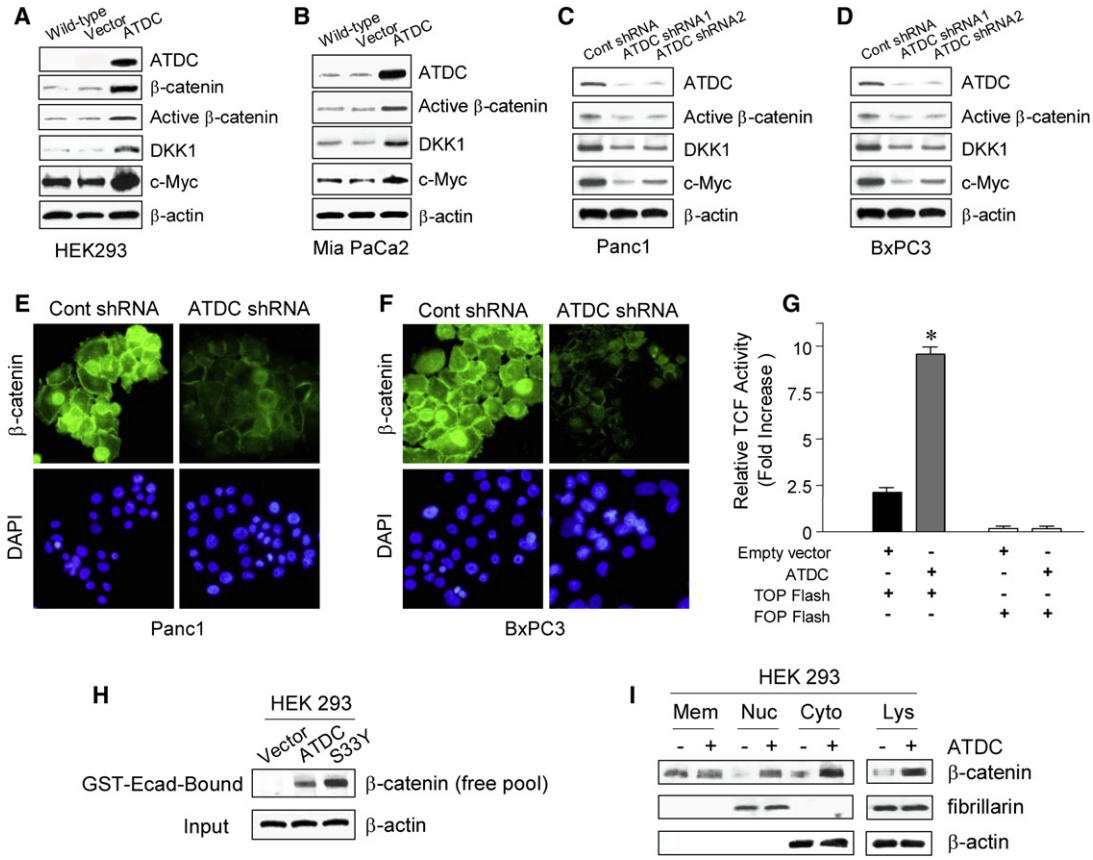

Figure 3. ATDC Upregulates β-Catenin Levels and TCF Transcriptional Activity

(A and B) Representative western blots of wild-type, empty vector-transfected, and ATDC-transfected HEK293 (A) and MiaPaCa2 cells (B). Overexpression of ATDC results in upregulation of β-catenin, active β-catenin, and the T cell factor (TCF) target genes *DKK1* and c-*Myc*. β-actin was used as a loading control.

(C and D) Representative western blots of Panc1 (C) and BxPC3 (D) cells expressing control shRNA, ATDC shRNA1 or 2. Silencing of ATDC in Panc1 and BxPC3 cells decreases levels of active β-catenin, DKK1 and c-Myc. β-actin was used as a loading control.

(E and F) Photomicrographs of control shRNA- and ATDC shRNA1-expressing Panc1 cells (E) and BxPC3 cells (F) immunostained with an anti-β-catenin antibody (green). Cell nuclei were counterstained with DAPI (blue).

(G) TCF reporter activity was assessed using the β-catenin-responsive TOPflash reporter and the mutant control FOPflash reporter in HEK293 cells stably transfected with empty vector or an ATDC expression vector (mean ± SEM; *$p < 0.05$ versus empty vector-transfected cells).

(H) The GST-E-cadherin (GST-Ecad) fusion protein detects increases in the free β-catenin pool. HEK293 cells expressing ATDC or S33Y β-catenin were harvested. Free β-catenin levels were assessed by western blotting of GST-Ecad-bound fractions of cell lysate using a specific anti-β-catenin antibody. β-actin (input) was used as a loading control.

(I) Representative blots of β-catenin levels in membrane (Mem), nuclear (Nuc), and cytoplasmic (Cyto) fractions and total lysates (Lys). Fibrillarin (nuclear expression) and β-actin (cytoplasmic expression) were used as loading controls.

we ectopically expressed a β-catenin shRNA or a dominant-negative TCF (dnTCF) protein in either control vector-transfected or ATDC-expressing HEK293 and MiaPaCa2 cells. Transfection of either the β-catenin shRNA or dnTCF constructs significantly inhibited ATDC-induced TOPflash reporter activity (Figures 4A and 4B, upper panels) and cell proliferation (Figures 4A and 4B, lower panels) in HEK293 and MiaPaCa2 cells. Similarly, transfection of β-catenin-targeting shRNA or dnTCF in Panc1 cells inhibited TOPflash reporter activity (Figure 4C, upper panel) and inhibited the enhanced cellular proliferation seen in Panc1 cells in the setting of elevated endogenous ATDC levels (Figure 4C, lower panel).

To examine the effects of β-catenin silencing on pancreatic tumor growth in vivo, we infected control or β-catenin shRNA-transduced Panc1 cells with a luciferase-expressing lentivirus. Following injection of 5×10^5 cells into the tail of the pancreas, tumor growth was assessed using bioluminescent imaging (n = 8 animals per group). All of the animals injected with Panc1 cells expressing control shRNA demonstrated tumor formation 14 days postinjection, while tumors were not detected in the animals injected with Panc1 cells expressing the β-catenin shRNA (Figure 4D, left panels; three representative animals are shown). At 60 days postinjection, the control tumors grew significantly larger, with evidence of metastatic spread, while the extent of primary tumor size and metastasis was markedly diminished in animals injected with Panc1 cells expressing the β-catenin shRNA (Figure 4D, right panels), similar to the effects observed with silencing ATDC in Panc1 cells (Figure 2E). Western blot analysis of tumors derived from β-catenin shRNA-transfected Panc1 cells harvested at 60 days after

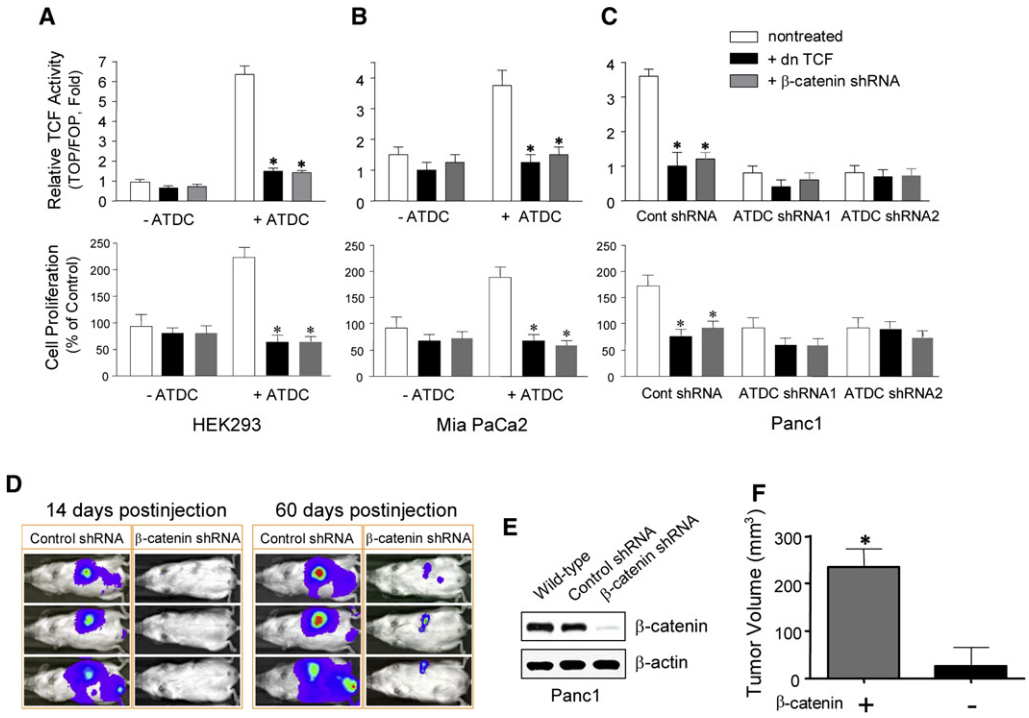

Figure 4. ATDC Stimulates Cell Proliferation and Tumor Growth via β-Catenin/TCF Activation

(A–C) In the upper panels, TCF reporter activity was measured using the β-catenin-responsive TOPflash reporter and the mutant control FOPflash reporter. The effects of stable transfection of cells with dominant-negative TCF (dnTCF, black bars) and β-catenin shRNA (gray bars) on relative TCF activity are shown in HEK293 (A) and MiaPaCa2 (B) cells (with empty or ATDC expression vector) or Panc1 cells (C) (with ATDC shRNA1 or 2 expression) (mean ± SEM; n = 3, *p < 0.05 versus control, nontreated cells [white bars]). In the lower panels, the effects of stable transfection of dnTCF (black bars) and β-catenin shRNA (gray bars) on cell proliferation are shown in HEK293 (A) and MiaPaCa2 (B) cells (with empty or ATDC expression vector) or Panc1 cells (C) (with ATDC shRNA1 or 2 expression) (mean ± SEM; n = 3, *p < 0.05 versus control, nontreated cells [white bars]).

(D) Representative bioluminescent images of animals in the control and ATDC shRNA groups are shown at 14 (left panels) and 60 (right panels) days after injection, depicting the extent of tumor burden.

(E) Western blotting verifies downregulation of β-catenin in tumors derived from β-catenin shRNA-transfected Panc1 cells harvested at 60 days.

(F) Average tumor volume 60 days after injection in animals injected with control shRNA- or β-catenin shRNA-transfected Panc1 cells (mean ± SEM; n = 3, *p < 0.05).

injection (Figure 4E) demonstrated effective silencing of β-catenin (Figure 4E). The mean tumor volume was significantly greater in the mice injected with Panc1-Luc cells expressing control shRNA compared to mice injected with Panc1-Luc cells expressing β-catenin shRNA (251.5 ± 79.2 versus 35.2 ± 7.8 mm^3, respectively; $p < 0.05$) (Figure 4F). Overall, these data indicate that ATDC plays a key role in cell proliferation and tumor growth and that ATDC's growth-promoting effects are dependent at least in part on the β-catenin/TCF signaling pathway.

Correlation between ATDC and β-Catenin Expression in Pancreatic Adenocarcinoma

Based on our findings that ATDC is highly expressed in human pancreatic cancers and that ATDC-mediated cancer cell growth in pancreatic cancer cells is dependent on β-catenin signaling, we predicted that ATDC and β-catenin expression levels would be well correlated in pancreatic cancer cell lines and primary pancreatic cancers. As shown in Figure 5A, the levels of ATDC and β-catenin in pancreatic cancer cell lines correlated well, with the highest levels of β-catenin being observed in BxPC3 cells, which have high levels of endogenous ATDC. Intermediate levels of β-catenin and ATDC proteins were found in Panc1 cells, and both β-catenin and ATDC were minimally expressed in MiaPaCa2 cells. To further study the relationship of ATDC and β-catenin expression in pancreatic cancer, immunohistochemical analysis of ATDC and β-catenin in human pancreatic normal and adenocarcinoma tissues was performed. We did not observe ATDC staining in normal pancreas but did observe expression of β-catenin localized to the cell membrane (Figure 5B, left panels), as has been described previously (Pasca di Magliano et al., 2007). It is commonly thought that both cytoplasmic and nuclear localization of β-catenin is an indicator of active β-catenin signaling in the Wnt pathway (Fodde and Brabletz, 2007). To assess the correlation between ATDC expression and β-catenin, we analyzed a pancreatic cancer tissue microarray containing 47 pancreatic carcinoma samples. We observed focal nuclear staining for β-catenin in 5 tumors (11%) and elevated cytoplasmic levels of β-catenin in 24 tumors (51%). These results correlate well with those previously published on β-catenin expression in pancreatic cancer (Pasca di Magliano et al., 2007). We observed a strong correlation between ATDC and β-catenin expression in the 47 pancreatic

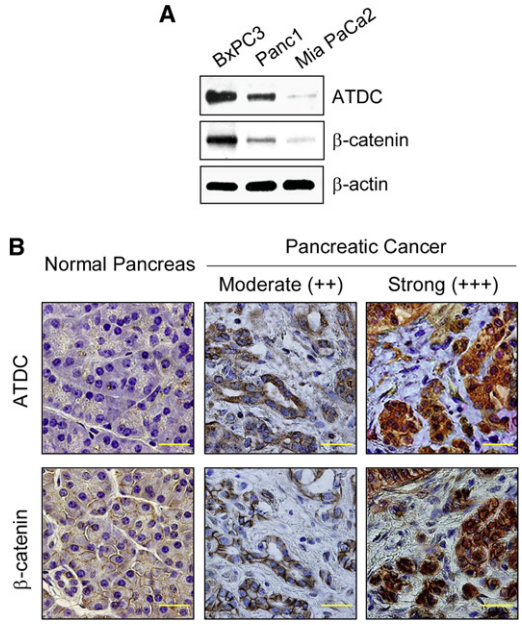

Figure 5. Correlation between ATDC and β-Catenin Expression in Pancreatic Cancer

(A) Western blot analysis of ATDC and β-catenin expression in BxPC3, Panc1, and MiaPaCa2 cells. β-actin was used as a loading control.

(B) Immunohistochemical (IHC) staining of samples of normal human pancreas (left panels) and human pancreatic adenocarcinomas (middle and right panels). A correlation between ATDC and β-catenin expression in pancreatic adenocarcinoma samples is evident. Scale bars = 50 μm. IHC scores are graded as moderate (++, intermediate staining) or strong (+++, intense staining).

carcinoma cases, with moderate to high expression of ATDC in the cancer samples that showed elevated cytoplasmic and/or nuclear β-catenin expression (Table S1). Representative samples of pancreatic cancers shown in Figure 5B (middle and right panels) demonstrate the correlation in ATDC and β-catenin expression in cancer samples with moderate or high levels of ATDC. Importantly, we did not find any evidence of pancreatic cancers expressing elevated levels of β-catenin without expressing high levels of ATDC, suggesting that elevated cytoplasmic and nuclear levels of β-catenin expression may in fact be dependent on elevated expression of ATDC.

ATDC Interacts with Disheveled-2 and Components of the β-Catenin Destruction Complex to Stabilize β-Catenin

We were interested in defining the mechanism by which elevated levels of ATDC result in increased β-catenin expression. In the absence of Wnt ligands, cytoplasmic levels of β-catenin are regulated by a multiprotein complex termed the destruction complex that contains axin, APC, and glycogen synthase kinase-3β (GSK-3β). Axin and APC are believed to facilitate efficient phosphorylation of β-catenin by GSK-3β at multiple serine and threonine residues in its N terminus. Phosphorylated β-catenin is then ubiquitinated, leading to its rapid proteasomal degradation (Gordon and Nusse, 2006). To determine whether ATDC increases β-catenin levels by stabilizing β-catenin, Panc1 cells (with or without ATDC silencing) were incubated with 10 μg/ml cycloheximide (CHX) to prevent new β-catenin synthesis, and β-catenin levels were then measured, with levels reflective of the rate of β-catenin protein degradation. Silencing of ATDC expression significantly increased the β-catenin degradation rate, resulting in a marked reduction in the levels of remaining β-catenin (Figures S4A and S4B). This effect of ATDC on β-catenin stability was validated by performing a [^{35}S]methionine pulse-chase assay. We found that β-catenin was rapidly degraded in Panc1 cells in which ATDC expression had been silenced compared to control Panc1 cells (Figures 6A and 6B).

To ascertain the mechanism by which ATDC stabilizes β-catenin, we first examined whether ATDC interacts with the components of the multiprotein complex that regulates β-catenin stability. In coimmunoprecipitation experiments using antibodies directed against axin, GSK-3β, and β-catenin, evidence of complexes containing ATDC and axin and complexes containing ATDC and GSK-3β was observed in HEK293 cells transfected with a FLAG-tagged *ATDC* construct (Figure 6C). To verify that this interaction was physiologically relevant, we tested the ability of endogenous ATDC in BxPC3 cells to interact with axin and GSK-3β, and similar results were obtained (Figure 6D). ATDC did not interact with β-catenin in either HEK293 cells overexpressing ATDC or in BxPC3 cells (Figures 6C and 6D). These results are consistent with the possibility that ATDC may interfere with GSK-3β-dependent phosphorylation of β-catenin by the destruction complex.

Based on the findings, we examined whether ATDC might activate Disheveled (Dvl), a cytoplasmic protein that is activated by binding of Wnt ligands to the Frizzled/low-density lipoprotein receptor-related protein (LRP) coreceptor at the cell surface. Activated Dvl binds to the axin/GSK-3β complex and antagonizes GSK-3β-dependent phosphorylation of β-catenin in a manner not dissimilar to what we observed with ATDC. Indeed, we found increased Dvl-2 levels (total and phosphorylated forms) in HEK293 cells overexpressing ATDC as well as in Panc1 and BXPC3 cells (Figures 6E–6G). Furthermore, silencing of endogenous ATDC expression in Panc1 and BxPC3 cells reduced the expression of Dvl-2 (Figures 6F and 6G). Increased levels of Dvl-2 were present in primary pancreatic cancer samples that had elevated expression of ATDC (Figure 6H). Finally, immunohistochemical analysis revealed colocalization of ATDC and Dvl-2 in ATDC-transfected HEK293 cells and Panc1 cells (Figure S5). Coimmunoprecipitation experiments in HEK293 cells and BxPC3 cells demonstrated that ATDC formed a complex with Dvl-2 (Figures 6I and 6J). Complex formation between ATDC and Dvl-1 or Dvl-3 in either ATDC-transfected HEK293 or BxPC3 cells was not observed (data not shown). To determine which region of the ATDC molecule interacts with Dvl-2, we created a series of FLAG-tagged *ATDC* truncation mutants and performed coimmunoprecipitation experiments. We found that the *ATDC*Δ220 and *ATDC*Δ260 deletion mutants were able to interact with Dvl-2 while the *ATDC*Δ348 mutant was not, suggesting that amino acids 260–348 of *ATDC*, in a region that contains a coiled-coil domain, interact with Dvl-2 (Figure S6).

We then tested whether modulating the levels of ATDC in cells affects the abundance of *Dvl-2* transcripts. *Dvl-2* gene

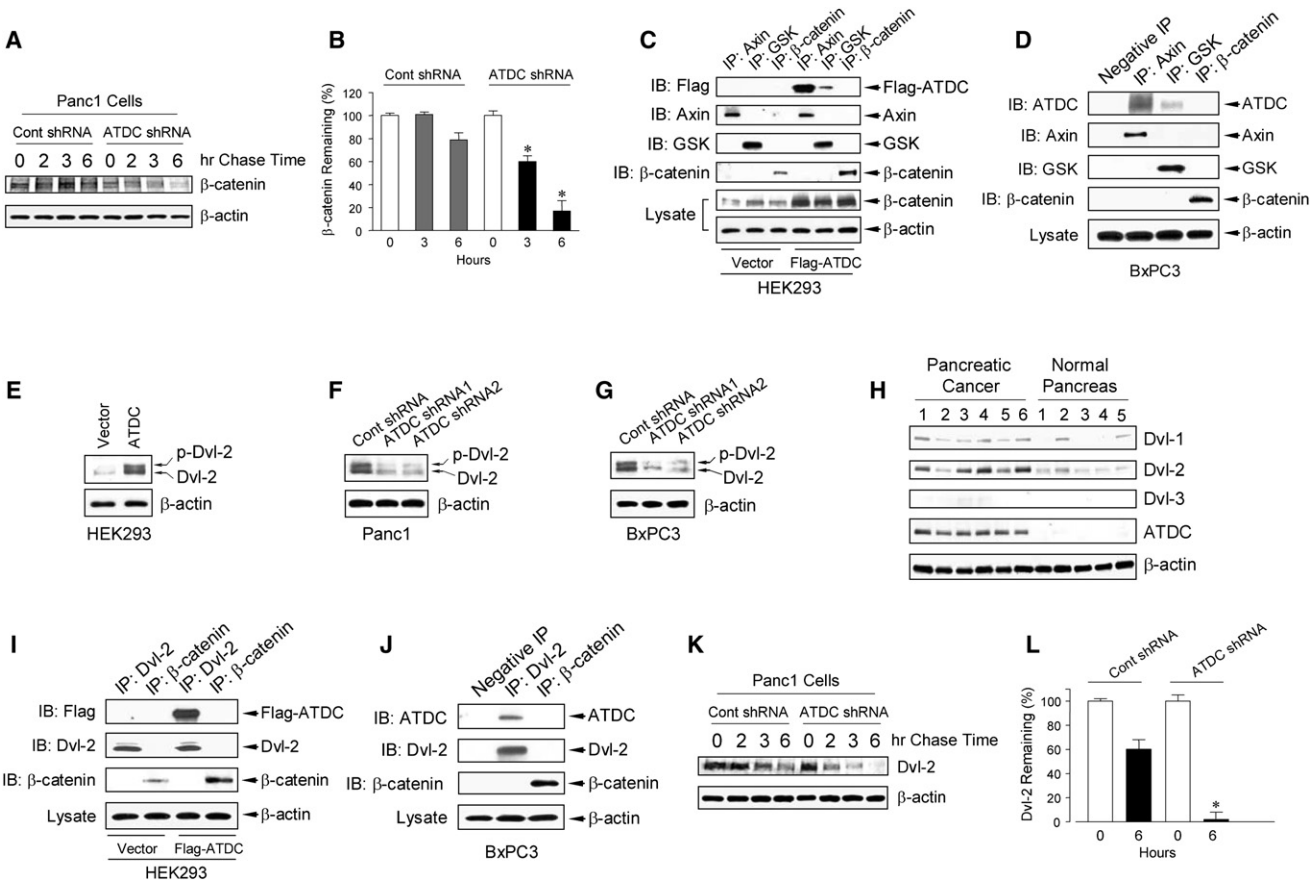

Figure 6. ATDC Stabilizes β-Catenin by Interacting with Disheveled-2 and the β-Catenin Destruction Complex

(A) Pulse-chase assays in Panc1 cells (with or without ATDC silencing) were performed to determine β-catenin stability.

(B) β-catenin remaining in (A) was quantitated by densitometry at 0, 3, and 6 hr and normalized relative to the 0 hr time point. Results are the mean ± SEM of three independent experiments (*$p < 0.05$ versus control shRNA cells at 6 hr).

(C and D) Cell lysates from HEK293 cells transfected with empty vector or FLAG-ATDC (C) and BxPC3 cells (D) were subjected to immunoprecipitation (IP) with axin, GSK-3β, or β-catenin antibodies. Immunocomplexes were resolved by SDS-PAGE and subjected to western analysis with anti-FLAG antibody for HEK293 cells (C) and ATDC antibody for BxPC3 cells (D). Blotting with an anti-β-actin antibody showed equal loading.

(E–G) Lysates of HEK293 cells transfected with empty vector or ATDC expression vector (E) and control shRNA- or ATDC shRNA1- or 2- expressing Panc1 (F) and BxPC3 cells (G) were subjected to western blotting with an anti-Dvl-2 antibody. The upper arrow indicates the phosphorylated form of Dvl-2, and the lower arrow indicates the nonphosphorylated form. Experiments were performed twice with similar results.

(H) Western blotting of five samples each of pancreatic adenocarcinoma and normal pancreas. Dvl-1, Dvl-2, Dvl-3, and ATDC expression in pancreatic tissue samples was measured. The experiments were repeated twice with similar results.

(I and J) Cell lysates from HEK293 cells transfected with empty vector (I) or FLAG-ATDC and BxPC3 cells (J) were subjected to IP with Dvl-2 or β-catenin antibodies. Immunocomplexes were resolved by SDS-PAGE and subjected to western analysis with an anti-FLAG antibody for HEK293 cells (I) and ATDC antibody for BxPC3 cells (J). Blotting with an anti-β-actin antibody showed equal loading.

(K) Pulse-chase assays were performed in Panc1 cells (with or without ATDC silencing) to determine Dvl-2 stability.

(L) Dvl-2 remaining in (K) was quantitated by densitometry at 0 and 6 hr and normalized relative to the 0 hr time point. Results are the mean ± SEM of three independent experiments (*$p < 0.05$ versus control shRNA cells at 6 hr).

expression, as measured by quantitative real-time RT-PCR, was not altered in either HEK293 cells overexpressing ATDC or in Panc1 cells with *ATDC* knockdown compared to control cells (Figure S7). To examine whether ATDC might alter Dvl-2 levels by affecting protein stability, [^{35}S]methionine pulse-chase assays were performed. Dvl-2 was rapidly degraded in Panc1 cells with *ATDC* knockdown compared to control Panc1 cells (Figures 6K and 6L), suggesting that ATDC forms a complex with Dvl-2 and increases Dvl-2 levels by regulating Dvl-2 posttranscriptionally, perhaps via direct effects on Dvl-2 protein stability.

The Oncogenic Effects of ATDC Are Mediated by Dvl-2

To verify that the ability of ATDC to increase TCF activity and cellular proliferation is mediated through Dvl-2, we transfected two Dvl-2 shRNA constructs targeting different regions of *Dvl-2* into HEK293 and MiaPaCa2 cells and examined the effects of *Dvl-2* knockdown on ATDC function. Both Dvl-2 shRNAs 1 and 2 were effective in knocking down levels of Dvl-2 in HEK293 and MiaPaCa2 cells (Figure S8). Knockdown of *Dvl-2* in both HEK293 and MiaPaCa2 cells overexpressing ATDC inhibited increases in β-catenin levels (Figures 7A and 7B), TCF activity

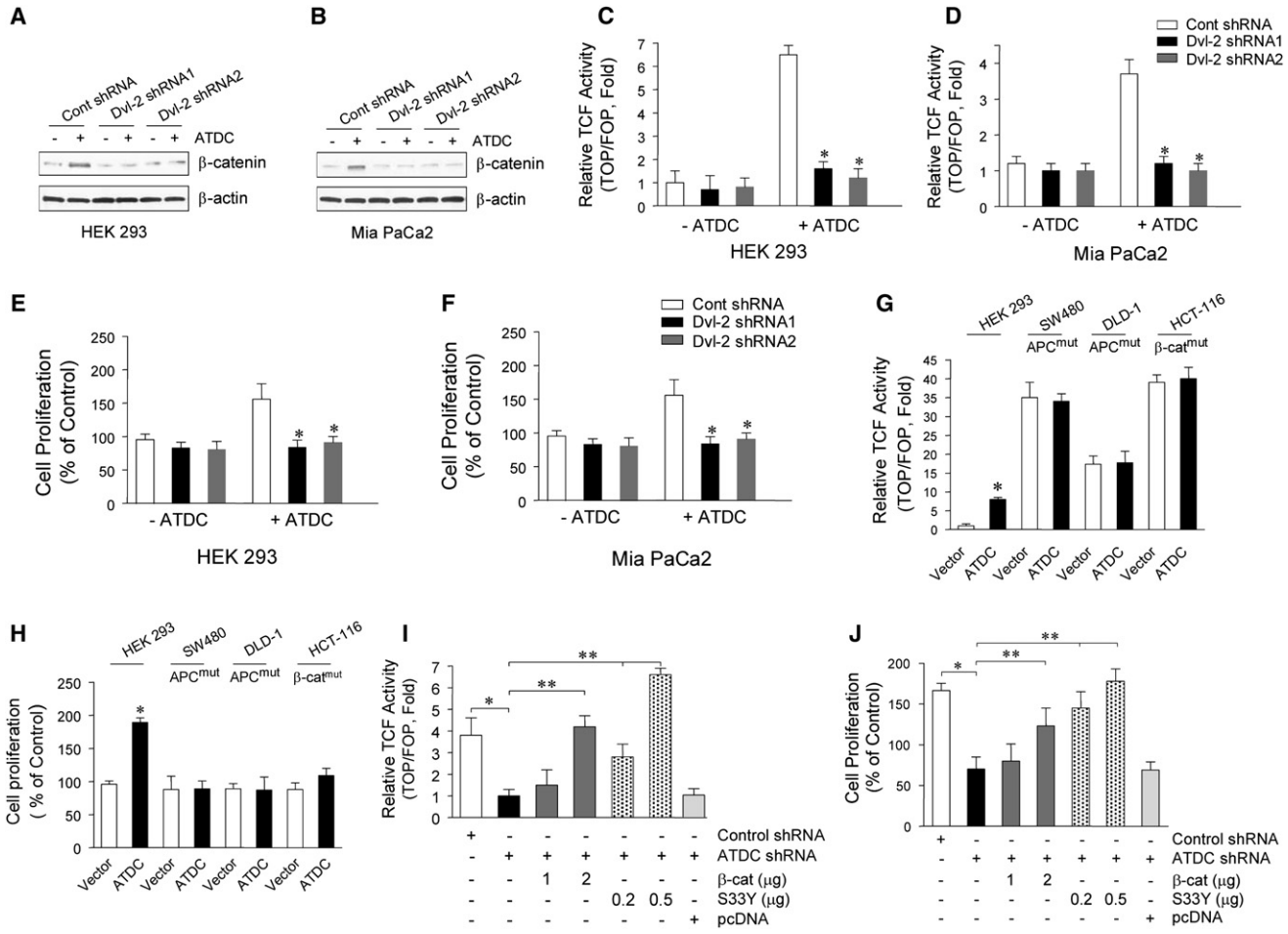

Figure 7. The Oncogenic Effects of ATDC Are Mediated by Dvl-2

(A and B) The effects of Dvl-2 shRNAs 1 and 2 on β-catenin expression is shown in representative western blots of HEK293 (A) and MiaPaCa2 cells (B) with or without ATDC overexpression.

(C and D) TCF reporter activity was assessed in Dvl-2 shRNA1- or 2-transfected HEK293 (C) or MiaPaCa2 cells (D) with (+) or without (−) ATDC overexpression (mean ± SEM; n = 3, *p < 0.05 versus empty vector-transfected cells).

(E and F) MTS proliferation assays in Dvl-2 shRNA1- or 2-transfected HEK293 (E) and MiaPaCa2 cells (F) with or without ATDC overexpression (mean ± SEM; n = 3, *p < 0.05 versus wild-type cells).

(G) TCF reporter activity was measured in HEK293, SW480, DLD-1, and HCT116 cells with vector (white bars) or ATDC (black bars) transfection using the β-catenin-responsive TOPflash reporter and the mutant control FOPflash reporter (mean ± SEM; n = 3, *p < 0.05).

(H) Cell proliferation of empty vector- and ATDC expression vector-transfected HEK293, SW480, DLD-1, and HCT116 cells is shown (mean ± SEM; n = 3, *p < 0.05 versus control, nontreated cells).

(I) Varying amounts of wild-type β-catenin (β-cat) or constitutively active mutant β-catenin (S33Y) constructs with TOPflash or FOPflash reporter constructs were cotransfected into Panc1 cells with control shRNA or ATDC shRNA expression. Forty-eight hours after transfection, TOPflash reporter assays were performed. Data are presented as mean ± SEM; n = 3. *p < 0.05, control shRNA versus ATDC shRNA; **p < 0.01, Panc1 cells (ATDC shRNA) with β-catenin versus without β-catenin or Panc1 cells (ATDC shRNA) with S33Y versus without S33Y.

(J) Varying amounts of wild-type β-catenin (β-cat) or constitutively active mutant β-catenin (S33Y) constructs were cotransfected into Panc1 cells with control shRNA or ATDC shRNA expression. Forty-eight hours after transfection, cell growth rates were assessed. The experiments were repeated three times, and data are expressed as mean ± SEM. *p < 0.05, control shRNA versus ATDC shRNA; **p < 0.01, Panc1 cells (ATDC shRNA) with β-catenin versus without β-catenin or Panc1 cells (ATDC shRNA) with S33Y versus without S33Y.

(Figures 7C and 7D), and cell proliferation (Figures 7E and 7F) induced by ATDC.

If ATDC stabilizes β-catenin levels by acting on upstream signaling events mediated by Dvl-2, then modulating ATDC levels should not influence β-catenin levels in cell lines with either *APC* or *β-catenin* mutations. Indeed, overexpression of ATDC in the *APC* mutant SW480 and DLD-1 cell lines or the *β-catenin* mutant HCT116 cell line did not alter TCF activity or cell proliferation as compared to control cells (Figures 7G and 7H). Furthermore, silencing of ATDC in Panc1 cells significantly inhibited the increase in TOPflash reporter activity seen in control Panc1 cells, and transfection with wild-type or a mutant oncogenic form of β-catenin (S33Y) was able to reverse the inhibitory effect of silencing of ATDC on TOPflash reporter activity (Figure 7I) and cell proliferation (Figure 7J) in a dose-dependent fashion.

DISCUSSION

Our studies have identified ATDC as a protein highly expressed in the majority of human pancreatic adenocarcinomas and pancreatic cancer precursor lesions. We also have demonstrated that expression of ATDC in pancreatic cancer cells promotes cellular proliferation and enhanced tumor growth and metastasis. Additionally, we have provided evidence that elevated levels of ATDC expression correlate with elevated β-catenin levels in pancreatic cancer cell lines and primary pancreatic cancers, and that silencing of ATDC via shRNA approaches antagonizes β-catenin/TCF-mediated reporter activation and activation of TCF target genes. β-catenin was implicated in the oncogenic effects of ATDC in vitro and in vivo, and the ability of ATDC to increase β-catenin levels appears to be attributable to ATDC's effects on Disheveled-2 protein expression. In summary, our findings implicate ATDC as an important positive regulator of β-catenin-dependent signaling in pancreatic cancer.

ATDC has been reported to be upregulated in a number of different cancer types, including lung, bladder, colorectal, ovarian, and endometrial cancers and multiple myeloma (Dyrskjot et al., 2004; Glebov et al., 2006; Hawthorn et al., 2006; Mutter et al., 2001; Ohmachi et al., 2006; Santin et al., 2004; Zhan et al., 2002). A recent report correlated *ATDC* expression in gastric cancer with poor histological grade, large tumor size, extent of tumor invasion, and lymph node metastasis (Kosaka et al., 2007). *ATDC* has also been reported to be downregulated in some cancer types (Ernst et al., 2002; Nacht et al., 1999; Smith et al., 2005; Zhang et al., 2006), suggesting that the function of ATDC may be dependent on cellular context. In none of these reports was the role of ATDC in tumorigenesis examined in functional studies.

We found that ATDC upregulated β-catenin levels in pancreatic cancer cell lines and primary pancreatic cancers. A large body of data supports the contribution of activation of the canonical (β-catenin-dependent) Wnt signaling pathway in the development of colorectal cancer. Sustained β-catenin pathway activation independent of *APC*, *Axin1*, or β-*catenin* mutations has been demonstrated in a subset of breast and ovarian cancers (Bafico et al., 2004). Mutations in *APC* or β-*catenin* appear to be rare in pancreatic adenocarcinoma (Zeng et al., 2006). While robust activation of the pathway due to signature mutations in components of the β-catenin signaling cascade that are commonly observed in other gastrointestinal cancers is not present in pancreatic adenocarcinoma, immunohistochemical analysis of β-catenin suggests a possible contribution of β-catenin signaling during PanIN progression and the development of invasive pancreatic cancer. Increased levels of both cytoplasmic and nuclear β-catenin, indicative of β-catenin signaling activity, have been reported in a substantial group of pancreatic adenocarcinomas and PanINs (Pasca di Magliano et al., 2007; Zeng et al., 2006). Pasca di Magliano and colleagues reported that 65% of pancreatic cancers exhibit an increase in either cytoplasmic and/or nuclear β-catenin. Similar results were obtained in *Pdx-Cre;Kras*^{G12D} and *Pdx-Cre;Kras*^{G12D}*;p53*$^{F/+}$ transgenic mice that developed PanIN lesions and subsequent invasive pancreatic cancers that were phenotypically indistinguishable from human pancreatic adenocarcinomas. Moreover, the authors showed that cancer cell survival and proliferation depend on β-catenin signaling activity in multiple human pancreatic cancer cell lines.

The mechanisms by which ATDC levels are upregulated in pancreatic adenocarcinomas remain unclear. Interestingly, Pasca di Magliano and colleagues (2007) demonstrated that increased Hedgehog signaling, one of the earliest changes in PanIN lesions, activates β-catenin signaling in transgenic mice and untransformed pancreatic ductal cells, suggesting that Hedgehog may play a role in upregulating β-catenin activity in some pancreatic adenocarcinomas. A possible connection between Hedgehog upregulation and ATDC expression in human pancreatic cancer cell lines or primary tumors remains to be explored.

The levels of the free cytoplasmic pool of β-catenin are known to be regulated by Wnt ligands. In the absence of an activating Wnt signal, mediated via Wnt binding to the Frizzled/LRP5/6 coreceptor complex, cytoplasmic β-catenin is destabilized by a multiprotein complex containing axin, GSK-3β, and APC. Axin acts as the scaffold of this complex and interacts with the other components—β-catenin, APC, and GSK-3β. Interaction of GSK-3β with axin in the complex facilitates efficient phosphorylation of β-catenin by GSK-3β. Phosphorylated β-catenin is then ubiquitinated, leading to its rapid proteasomal degradation. We found that ATDC bound to axin and GSK-3β in pancreatic cancer cells, suggesting that ATDC interacts with the destruction complex to prevent phosphorylation and subsequent ubiquitination of β-catenin.

When Wnt binds to the Frizzled/LRP coreceptors at the cell surface, a cytoplasmic protein, Dvl, antagonizes GSK-3β-dependent phosphorylation of β-catenin. Although it is not known whether Dvl binds directly to the Frizzled/LRP coreceptor or whether intermediary proteins are involved in the signal transduction between Frizzled and Dvl, Dvl appears to bind to axin and inhibit GSK-3β-dependent phosphorylation of β-catenin, APC, and axin. Once the phosphorylation of β-catenin is reduced, β-catenin dissociates from the axin complex, resulting in its accumulation in the cytoplasm. Once stabilized, a fraction of the β-catenin is translocated to the nucleus, where it binds to transcription factors such as the TCF/lymphoid enhancer-binding factor and thereby stimulates the transcription of β-catenin target genes. We noted that ATDC expression in HEK293 cells induces expression of Dvl-2 and demonstrated that ATDC forms a complex with Dvl-2 in pancreatic cancer cells. We also demonstrated that levels of ATDC in primary pancreatic cancers correlate well with Dvl-2 levels, suggesting that ATDC upregulates Dvl-2 levels in primary pancreatic cancers and increases β-catenin levels by this mechanism. We further showed that knockdown of *Dvl-2* in ATDC-expressing cells abrogates enhanced TCF activity and cell proliferation induced by ATDC, directly implicating Dvl-2 as an intermediary in this process.

The regulation of the Disheveled protein is still poorly understood, but recent data suggest that Disheveled, like β-catenin, may be controlled by ubiquitination and degradation by the proteasome (Hershko and Ciechanover, 1998; Miyazaki et al., 2004; Simons et al., 2005). Dvl-1 has been reported to interact with the neuronal homologous to E6AP carboxyl terminus (HECT)-type ubiquitin ligase NEDL1 (Miyazaki et al., 2004). The Wnt-induced antagonist naked cuticle has been shown to modulate the stability of Dvl-1 (Creyghton et al., 2005; Simons et al., 2005).

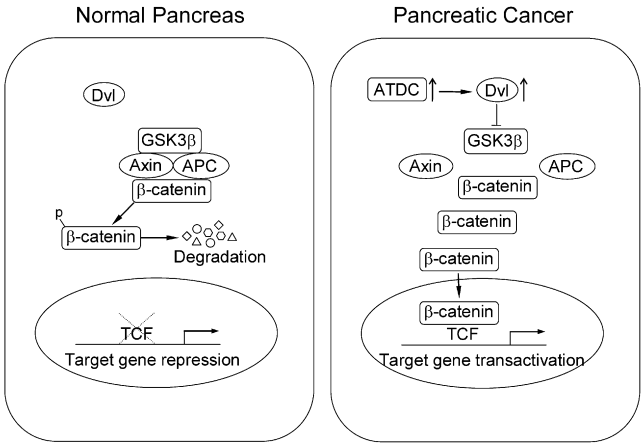

Figure 8. Model of How ATDC Mediates Activation of β-Catenin Signaling in Pancreatic Cancer Cells

Left: in unstimulated normal pancreatic cells lacking ATDC, Disheveled (Dvl) is in the cytoplasm and is not bound to the axin/GSK-3β/APC destruction complex. This allows the destruction complex to phosphorylate β-catenin and target it for ubiquitin-mediated degradation.

Right: in pancreatic cancer cells expressing high levels of ATDC, ATDC binds to stabilized Dvl, bringing it to the β-catenin destruction complex. Binding of ATDC and Dvl to the destruction complex inhibits destruction complex function, resulting in the release of β-catenin from the destruction complex, leading to increased β-catenin levels and subsequent activation of downstream target genes.

And in a recent study by Angers and colleagues, KLHL12-Cullin-3 ubiquitin ligase was shown to negatively regulate the Wnt/β-catenin pathway by targeting Disheveled for degradation (Angers et al., 2006). We found that ATDC increased Dvl-2 levels not by changes in *Dvl-2* gene expression but rather by enhancing the stability of the Dvl-2 protein, supporting that changes in Dvl stability serve as an important mechanism in regulating the Wnt/β-catenin signaling pathway.

The data in the present study support the following model for the mechanism by which ATDC functions to promote the oncogenesis of pancreatic cancer cells (Figure 8). In unstimulated, normal pancreatic cells lacking ATDC, Dvl-2 is in the cytoplasm and is not bound to the axin/GSK-3β/APC destruction complex. This allows the destruction complex to phosphorylate β-catenin and target it for ubiquitin-mediated degradation. In pancreatic cancer cells expressing high levels of ATDC, ATDC binds to and stabilizes Dvl-2, resulting in the release of β-catenin from the destruction complex, increased β-catenin levels, and subsequent activation of downstream β-catenin/TCF-regulated target genes These studies define a functional role of ATDC in human tumorigenesis and, besides highlighting ATDC as a potential therapeutic target in pancreatic cancer, define a mechanism for activating Wnt/β-catenin signaling in cancer.

EXPERIMENTAL PROCEDURES

Cell Lines and Human Samples

The human pancreatic ductal adenocarcinoma cell lines Panc1, MiaPaCa2, and BxPC3 and the human embryonic kidney cell line HEK293 were purchased from the American Type Culture Collection (ATCC). Fresh-frozen and paraffin-embedded human pancreatic tissues were obtained from patients undergoing surgical resection at the University of Michigan Medical Center. All human samples were obtained following approval by the Institutional Review Board of the University of Michigan Medical Center. A pancreatic cancer tissue microarray constructed by the University of Michigan Medical Center's Tissue Procurement Facility contained 5 samples of normal pancreas and 47 samples of pancreatic adenocarcinoma and was used for immunohistochemical analysis of ATDC expression. Twenty-five paraffin-embedded human pancreatic tissue samples containing pancreatic intraepithelial neoplasia (PanIn) lesions of various stages were analyzed for ATDC expression using an anti-ATDC antibody. ATDC expression in PanIn lesions was evaluated by an experienced pancreatic pathologist and graded as absent or present.

Constructs

The cDNA of human *ATDC* (kindly provided by J. Murnane, University of California, San Francisco) was subcloned into the pcDNA3.1 expression vector (Invitrogen). Sequence analysis after cloning showed 100% homology to the published sequence of *ATDC*. The methods used to create the control shRNA and ATDC shRNA constructs are listed in the Supplemental Experimental Procedures. Endogenous Dvl-2 was knocked down by transient transfection of specific Dvl-2 shRNA1 and 2 (OriGene Technologies) in HEK293 and MiaPaCa2 cells with or without ATDC overexpression. A reporter plasmid carrying three TCF binding sites upstream of a minimal c-*fos* promoter driving the firefly luciferase gene (TOPflash), a plasmid carrying the mutated TCF binding sites upstream of a minimal c-*fos* promoter driving luciferase expression (FOPflash), and the expression constructs of wild-type or constitutively active β-*catenin* containing a missense mutation of serine to tyrosine at codon 33 (S33Y) were used (Caca et al., 1999). The pGEX-2T vector containing C-terminal E-cadherin/glutathione S-transferase (GST) was generated as described previously (Winer et al., 2006).

Creation of Stable Cell Lines

Details regarding the creation of HEK293 and MiaPaCa2 cell lines with stable expression of *ATDC* and Panc1 and BxPC3 cells with silencing of ATDC using shRNA can be found in the Supplemental Experimental Procedures.

Quantitative Real-Time RT-PCR

Total RNA from the human pancreatic ductal adenocarcinoma, normal pancreas, and chronic pancreatitis specimens or Panc1 cells (with or without ATDC shRNA) were isolated using TRIzol reagent (Invitrogen). To avoid amplification of genomic DNA, RNA was pretreated with DNase (DNA-Free Kit from Applied Biosystems). cDNA synthesis was performed using a High-Capacity cDNA Archive Kit (Applied Biosystems). The cDNA served as a template in quantitative real-time RT-PCR utilizing TaqMan Fast Universal PCR Master Mix and TaqMan Gene Expression assay probes for ATDC (Hs00232590_m1), Dvl-2 (Hs01005253_ml), or ribosomal protein S6 (RPS6) (Hs02339423_g1) (Applied Biosystems) and an ABI 7500 Fast Sequence Detection System. All reactions were performed in triplicate. *ATDC* mRNA expression of different group specimens was normalized to endogenous ribosomal protein S6. Relative ATDC mRNA levels are presented as unit values of $2^{-\Delta Ct} = 2^{-(Ct\ (ribosomal\ protein\ S6)\ -\ Ct\ (ATDC))}$, where Ct is the threshold cycle value defined as the fractional cycle number at which the target fluorescent signal passes a fixed threshold above baseline.

Immunohistochemical Analysis

Paraffin-embedded pancreatic tissue sections (4 μm thick) were cut, deparaffinized, and subjected to a heat-induced epitope retrieval step. Endogenous peroxidase activity was blocked with 1% (v/v) hydrogen peroxide in distilled water. Details of the immunohistochemical analysis can be found in the Supplemental Experimental Procedures.

Luciferase Reporter Gene Assays

Pancreatic cancer cells (with or without β-catenin silencing) or HEK293 and MiaPaCa2 cells (with or without ATDC overexpression) were transfected using a Lipofectamine 2000 transfection kit (Invitrogen) according to the manufacturer's instructions with 0.2 μg of the TOPflash or FOPflash reporter constructs with or without varying amounts (0.2–2 μg) of constructs expressing either wild-type β-*catenin* or S33Y (constitutively activated) mutant β-*catenin* or 25 ng of a construct expressing β-*galactosidase* as an internal control. The total DNA for each transfection was kept constant by adding empty pcDNA vector.

Forty-eight hours after transfection, luciferase activity was measured in a luminometer and normalized to β-galactosidase expression. Endogenous β-catenin or Dvl-2 was silenced by stable expression of β-catenin shRNA or transient expression of Dvl-2 shRNAs (OriGene Technologies). TCF transcriptional activity was inhibited by transient transfection of dnTCF vector (Upstate).

Nuclear and Membrane Fractionation

Nuclear and cytoplasmic proteins were extracted using a NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce). Membrane proteins were extracted using a Mem-PER Eukaryotic Membrane Protein Extraction Kit (Pierce) according to the manufacturer's instructions.

C-Terminal E-Cadherin/GST Fusion-Mediated Precipitation of the Free β-Catenin Pool

The free β-catenin pool in ATDC-transfected and control HEK293 cells was measured using a C-terminal E-cadherin/glutathione S-transferase fusion protein as described previously (Winer et al., 2006).

Immunoblot Analysis

Immunoblot analysis was performed as described previously (Zhang et al., 2004). Details of the immunoblot analysis can be found in the Supplemental Experimental Procedures.

Proliferation Assay

Cell proliferation was measured using a CellTiter 96 AQ nonradioactive cell proliferation assay (Promega) as described previously (Zhang et al., 2004).

Coimmunoprecipitation Experiments

Cells were lysed by sonicating for 5 s in 1 ml of detergent-free lysis buffer (PBS, 5 mM EDTA, 0.02% sodium azide, 10 mM iodoacetamide, 1 mM PMSF, and 2 μg leupeptin) at 4°C. The lysates were cleared by microcentrifuging for 15 min at 16,000 × g at 4°C. Antibody-conjugated beads were prepared by combining 1 μg of polyclonal antibodies with 30 μl of a 50% protein A Sepharose bead slurry in 0.5 ml of ice-cold PBS for 1 hr at 4°C in a tube rotator and were then washed two times with 1 ml of lysis buffer. The antibodies used for coimmunoprecipitation were Dvl-2, β-catenin (Cell Signaling Technology), axin, and GSK-3β (Upstate). Cell lysates (500 μg) were incubated with the prepared beads and 10 μl of 10% BSA overnight at 4°C. The beads were washed four times with washing buffer (50 mM Tris-HCl [pH 7.4], 300 mM NaCl, 5 mM EDTA, 0.02% sodium azide, 0.1% Triton X-100) and once with ice-cold PBS. Proteins were revealed after SDS-PAGE and western blotting with antibodies against FLAG (Sigma), Dvl-2, β-catenin, axin, GSK-3β, and ATDC (Santa Cruz Biotechnology). Images were visualized using an ECL detection system.

Pulse-Chase Assays

Cells were cultured to 70% confluence in six-well plates and then starved in 2 ml methionine-deficient DMEM (Sigma) for 30 min at 37°C with 5% CO_2. Cells were pulse labeled with 100 μCi/ml [^{35}S]methionine (Amersham Pharmacia) for 30 min at 37°C. Labeled cells were chased in DMEM with a saturating amount of cold methionine (2 mM) for various times and lysed in RIPA buffer (10 mM Tris [pH 7.2], 158 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM PMSF, and 1× Complete Protease Inhibitor Cocktail [Roche Molecular Biochemicals]). Total cell lysates were then prepared and immunoprecipitated with a β-catenin antibody or Dvl-2 antibody and protein G Sepharose beads (Invitrogen) for 3 hr at 4°C. Immunocomplexes were separated on SDS-PAGE gels (4%–20% gradient) and transferred to nitrocellulose. The precipitates were subjected to autoradiography, and the densities of the labeled proteins were analyzed by Kodak Gel Documentation System (model 1D 3.6).

In Vivo Tumorigenicity Studies

Six-week-old male NOD/SCID mice (Taconic) were housed under pathogen-free conditions. Animal experiments were approved by the University of Michigan Animal Care and Use Committee and were performed in accordance with established guidelines. Mice were anesthetized with an intraperitoneal injection of xylazine (9 mg/kg) and ketamine (100 mg/kg). A median laparotomy was performed, and 30 μl of media containing 5 × 10^5 Panc1 cells (expressing control shRNA, ATDC shRNA1, or β-catenin shRNA stably transfected) infected with a lentivirus encoding luciferase was injected into the pancreatic tail using a 30G needle (n = 8 per group). To prevent leaking at the injection site, the needle was withdrawn slowly and a sterile cotton swab was applied to the injection site for 30 s. Bioluminescent imaging of mice was performed biweekly using an IVIS 200 imaging system (Xenogen Biosciences). Sixty days after cancer cell injection, mice were euthanized with carbon dioxide inhalation, and autopsies were performed to assess the extent of primary tumor growth and metastasis.

Statistical Analysis

Data are represented as mean ± SEM from at least three independent experiments. Significance of differences between groups was evaluated by Student's t test or ANOVA. $p < 0.05$ was considered significant.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures and eight figures and can be found with this article online at http://www.cancercell.org/supplemental/S1535-6108(09)00027-0.

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