β-Catenin is essential for pancreatic acinar but not islet development

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Summary

Despite our increasingly sophisticated understanding of transcriptional regulation in pancreas development, we know relatively little about the extrinsic signaling pathways involved in this process. We show here that the early pancreatic epithelium exhibits a specific enrichment in unphosphorylated β-catenin protein, a hallmark of activation of the canonical Wnt signaling pathway. To determine if this pathway is functionally required for normal pancreas development, we have specifically deleted the β-catenin gene in these cells. Pancreata developing without β-catenin are hypoplastic, although their early progenitors appear normal and exhibit no premature differentiation or death. Surprisingly, and in marked contrast to its role in the intestine, loss of β-catenin does not significantly perturb islet endocrine cell mass or function. The major defect of the β-catenin-deficient pancreas is an almost complete lack of acinar cells, which normally comprise the majority of the organ. β-Catenin appears to be cell-autonomously required for the specification of acinar cells, rather than for their survival or maintenance, as deletion of β-catenin specifically in differentiated acinar cells has no effect. Thus, our data are consistent with a crucial role for canonical Wnt signals in acinar lineage specification and differentiation.

Key words: Pancreas, Exocrine, Acinar, β-catenin, Wnt

Introduction

The mammalian pancreas begins as a pair of undifferentiated epithelial buds expressing the transcription factor *Pdx1* (*Ipf1* – Mouse Genome Informatics) (Ohlsson et al., 1993). As embryogenesis proceeds, these buds proliferate, elongate and branch to form a dense ductal network. Simultaneously, subsets of cells within this population begin to assume differentiated fates with a stereotypical temporal and spatial arrangement. Thus, glucagon-producing alpha (α) cells appear quite early after bud formation (approximately E10.5 in mouse), while insulin-secreting beta (β) cells do not appear in large numbers until the onset of the 'secondary transition', at approximately E13.5. It is also at this time that peripherally located epithelial cells begin to differentiate into exocrine acini (Jensen, 2004; Pictet and Rutter, 1972). Newly-differentiated acinar cells are highly proliferative, and quickly come to comprise the majority of the mass of the organ (Jensen, 2004) (and see below), whereas islet cells divide rarely but sufficiently to maintain their numbers throughout adult life (Dor et al., 2004).

Formation of the pancreas, as of other organs, requires the coordination of proliferation, differentiation and morphogenesis. For decades, it has been known that neighboring tissues, including the adjacent mesenchyme, provide some of this coordination. When cultured alone, pancreatic bud epithelium exhibits minimal growth and branching in vitro, and fails to generate acini, whereas it undergoes robust growth and acinar differentiation following recombination with mesenchyme (Golosow and Grobstein, 1962; Wessells and Cohen, 1967). The mesenchyme may also produce distinct signals that promote endocrine differentiation (Li et al., 2004). Understanding the morphogenetic signals active in the pancreas is of practical interest, as well as academic, in that the ability to mimic endogenous regulatory mechanisms will be necessary to convert stem cells into β-cells in vitro. To this end, it is particularly important to identify those signaling pathways that affect cell fate specification and differentiation from pancreatic progenitors.

Among the few signaling pathways implicated in pancreatic development by knockout studies are the Notch (Apelqvist et al., 1999; Jensen et al., 2000) and Fgf (Bhushan et al., 2001) pathways, both of which appear to negatively regulate progenitor differentiation (Esni et al., 2004a; Hald et al., 2003; Hart et al., 2003; Murtaugh et al., 2003; Norgaard et al., 2003). Positively acting signals, such as the pro-acinar signal provided by the mesenchyme, remain undefined by genetic experiments. In vitro culture and transgene misexpression experiments have identified additional signals that can affect pancreas development, although their genetic relevance remains unclear. These include Egf family members, which appear to inhibit both exocrine and endocrine differentiation (Cras-Meneur et al., 2001; Esni et al., 2004b), and Tgfβ family members, which can selectively promote endocrine differentiation under some

circumstances (Miralles et al., 1998; Sanvito et al., 1994). In addition, the expression of several Wnt genes has been detected in the developing pancreatic mesenchyme and epithelium; misexpression of Wnt1 and Wnt5a in the early foregut results in agenesis or hypoplasia of the pancreas, respectively (Heller et al., 2002; Lin et al., 2001). How these and other Wnts act during normal pancreas development, however, remains unknown.

Wnt proteins play crucial roles in the development of multiple tissues. In the intestine, for instance, Wnt signaling is required both for stem cell maintenance and for endocrine development (Ireland et al., 2004; Korinek et al., 1998; Pinto et al., 2003). In these and many other instances, Wnts act via the so-called canonical pathway, in which receptor activation leads to the stabilization of cytosolic β-catenin protein (Logan and Nusse, 2004). Cytosolic β-catenin is normally phosphorylated and targeted for proteolysis by a complex of proteins, including Apc, axin and the serine/threonine kinase Gsk3β. Wnt activation of the Frizzled and Lrp co-receptors results in dissociation of this complex, so that newly synthesized β-catenin is no longer phosphorylated and degraded. Stabilized β-catenin then enters the nucleus to activate target genes in collaboration with Lef/Tcf family transcription factors, and possibly other partners (Logan and Nusse, 2004).

We show here that the early pancreatic epithelium exhibits a specific accumulation of unphosphorylated β-catenin, a hallmark of active Wnt signaling. Furthermore, by tissue-specific knockout, we find that β-catenin is absolutely required for the formation of exocrine acini, but is dispensable for endocrine differentiation and function. Moreover, β-catenin protein is dispensable for the survival of mature acinar cells, and for the maintenance of their cell type-specific gene expression. Although β-catenin is also required for the robust proliferation of early pancreatic progenitor cells, these cells do not prematurely differentiate or apoptose in the absence of β-catenin. Our results suggest a requirement for canonical Wnt signaling in the specification or differentiation of acinar cells.

Materials and methods

Transgenic mouse generation and maintenance

Mice carrying a floxed allele of the β-catenin gene (Brault et al., 2001) (here referred to as *Catnb^{lox}*) were obtained from the Jackson Laboratory (Catnb^{tm2Kem}/J) as homozygotes, and maintained by inbreeding. To generate mice carrying a germline-deleted allele of *Catnb* (*Catnb$^{\Delta}$*), these mice were interbred with *CMV-Cre* mice (Schwenk et al., 1995), also from Jackson [Tg(CMV-cre)1Cgn/J]. Mice carrying combinations of wild-type, floxed and deleted *Catnb* alleles were genotyped by PCR, with the following combination of oligos, to yield bands of 221 (wild type), 324 (*Catnb^{lox}*) or 500 (*Catnb$^{\Delta}$*) base pairs: 5′-AAGGTAGAGTGATGAAAGTTGTT-3′, 5′-CACCATGTCCTCTGTCTATTC-3′, 5′-TACACTATTGAATCACA-GGGACTT-3′. *Pdx1-Cre* transgenic mice [Tg(Pdx1-cre)1Dam/Ucd] have been described previously (Gu et al., 2002). *R26R* indicator mice (Soriano, 1999) were obtained from the Jackson Laboratory [Gt(ROSA)26Sor^{tm1Sor}] as homozygotes, and maintained by inbreeding.

The *Ela-CreERT* construct was generated by fusing the acinar-specific enhancer of the elastase gene [a gift from G. Swift and R. MacDonald (Swift et al., 1989)] to a minimal hsp68 promoter (a gift from M. Gannon), and placing the chimeric promoter upstream of *CreERT* [a gift from A. McMahon (Danielian et al., 1998)]. Two founders were identified that, when crossed with Z/AP mice (Lobe et al., 1999), showed acinar-specific expression of human placental alkaline phosphatase upon tamoxifen injection, performed as described (Dor et al., 2004). One of these lines exhibited more robust tamoxifen-induced recombination than the other, and was maintained and expanded; all of the experiments described here made use of this line.

For timed pregnancies, it is assumed that the morning of plug detection corresponds to E0.5. Embryo ages are rounded to the nearest half day.

Tissue processing

Mice were euthanized with isoflurane, and adult or embryonic tissues dissected in ice-cold PBS. To label S-phase nuclei, some mice were injected with BrdU (50 μg/g body weight) one hour prior to sacrifice. For paraffin embedding, tissues were fixed for 1-2 hours at 4°C in zinc-buffered formalin (Polysciences). Paraffin sections (6 μm) were collected in ribbons, and subsequently subdivided serially across a series of 4-16 slides (depending on age and tissue size), such that each

Table 1. Antibodies used in this study

Antigen	Species	Source	Dilution	Frozen	Paraffin
β-catenin, dephospho-specific	Mouse	Upstate Biotechnology, 05-665	1:400	N	Y
β-catenin, pan (amino acids 571-781)	Mouse	BD Transduction Laboratories, 610153	1:400	N	Y
β-catenin, pan (amino acids 768-781)	Rabbit	Sigma, C-2206	1:2000	Y	N
β-catenin, pan (full length)	Goat	R&D Systems, AF1329	1:2000	Y	Y
Insulin (C-peptide)	Guinea pig	Linco, 4021-01	1:2500	Y	Y
Glucagon	Guinea pig	Linco, 4031-01F	1:2500	Y	Y
Glucagon	Rabbit	Zymed, 18-0064	1:200	Y	N
Amylase	Rabbit	Sigma, A-8273	1:500	Y	Y
Amylase	Sheep	Abcam, ab8943	1:2000	Y	N
Pdx1	Guinea pig	Gift of Chris Wright, Vanderbilt University	1:1000	Y	Y
Ptf1a/p48	Rabbit	Gift of Helena Edlund, University of Umea	1:1000	Y	N
BrdU	Rat	Abcam, ab6326	1:2000	Y	Y
E-cadherin	Rat	Zymed, 13-1900	1:2000	Y	N
Plakoglobin	Goat	Santa Cruz, sc-1497	1:50	Y	N
Cleaved caspase-3	Rabbit	Cell Signaling, 9661	1:100	Y	Y

Antibodies were used at the indicated dilutions, on frozen and/or paraffin sections. (In all cases, paraffin sections were subjected to high-temperature antigen retrieval – see Materials and methods for details.) Of the three pan-specific β-catenin antibodies used, two (Transduction Laboratories and Sigma) were raised specifically against epitopes outside of the floxed region of *Catnb^{lox}* [amino acid 1-312 (see Brault et al., 2001)]. Neither of these antibodies, nor a polyclonal antiserum against full-length β-catenin (R&D Systems), stained pancreatic epithelial cells following Cre-mediated deletion of *Catnb^{lox}*, confirming the loss of β-catenin expression. In most cases, where antibodies are marked as not being used for frozen or paraffin wax sections (N), they were tested and failed to yield specific staining.

N, no; Y, yes.

slide would contain semi-adjacent sections across the entire tissue of interest.

For frozen sections, tissues were fixed for 1-2 hours at 4°C in fresh 4% paraformaldehyde, washed in PBS and cryoprotected overnight in 30% sucrose (w/v) in PBS, before embedding and freezing in Tissue-Tek OCT compound. Cryosections (12-14 μm) were collected serially across 6-16 slides (depending on age and tissue size), as above.

Immunostaining

Primary antibodies used in this study are listed in Table 1. Secondary antibodies, raised in donkey and biotinylated or conjugated to fluorophores, were purchased from Jackson ImmunoResearch. A biotin conjugate of the duct-binding lectin *Dolichos biflorus* agglutinin (Kobayashi et al., 2002) was purchased from Vector Laboratories, and detected with streptavidin-conjugated fluorophores (Jackson ImmunoResearch). Unless otherwise noted, all photomicrographs shown are representative of at least three samples of the indicated genotype or condition.

After dewaxing and rehydrating paraffin sections, endogenous peroxidase activity was eliminated by treatment with hydrogen peroxide (1% v/v in methanol, 20 minutes). Sections were then subjected to high-temperature antigen retrieval, using the automated Retriever instrument (PickCell Laboratories) and antigen unmasking solution (Vector Laboratories). Primary and secondary (biotinylated) antibody incubations were followed by detection with VectaStain reagents, and diaminobenzidene or Vector Blue substrates, all from Vector Laboratories.

For immunofluorescence staining, frozen sections were rehydrated and washed in PBS, permeabilized with 0.25% Triton X-100 in PBS (15 minutes, room temperature), and subjected to primary and secondary antibody (rhodamine or fluorescein-conjugated) incubations. For triple labeling, one antigen was detected with a biotinylated secondary antibody and subsequently reacted with Alexa 647-coupled streptavidin (Molecular Probes). Stained sections were mounted with Mowiol/DABCO and analyzed on a Zeiss LSM 510 Meta confocal microscope, in 2-3 μm optical sections.

In situ hybridization

Non-isotopic in situ hybridization was performed with DIG-labeled cRNA probes, transcribed from full-length IMAGE consortium EST clones. In situ hybridization on frozen or paraffin sections followed the protocol of Brent et al. (Brent et al., 2003). For quantitation of endocrine and exocrine volumes at E15.5, individual sections were photographed, along with a stage micrometer to normalize area, and the area occupied by stained cells was measured using ImageJ software (Wayne Rasband, NIH; http://rsb.info.nih.gov/ij/). The relative volume for each sample was determined by multiplying the total measured area across a single slide (‘area’) by the total number of slides used to collect and analyze the sample (‘depth’). Statistical significance was assessed by two-tailed *t*-test, with Bonferroni

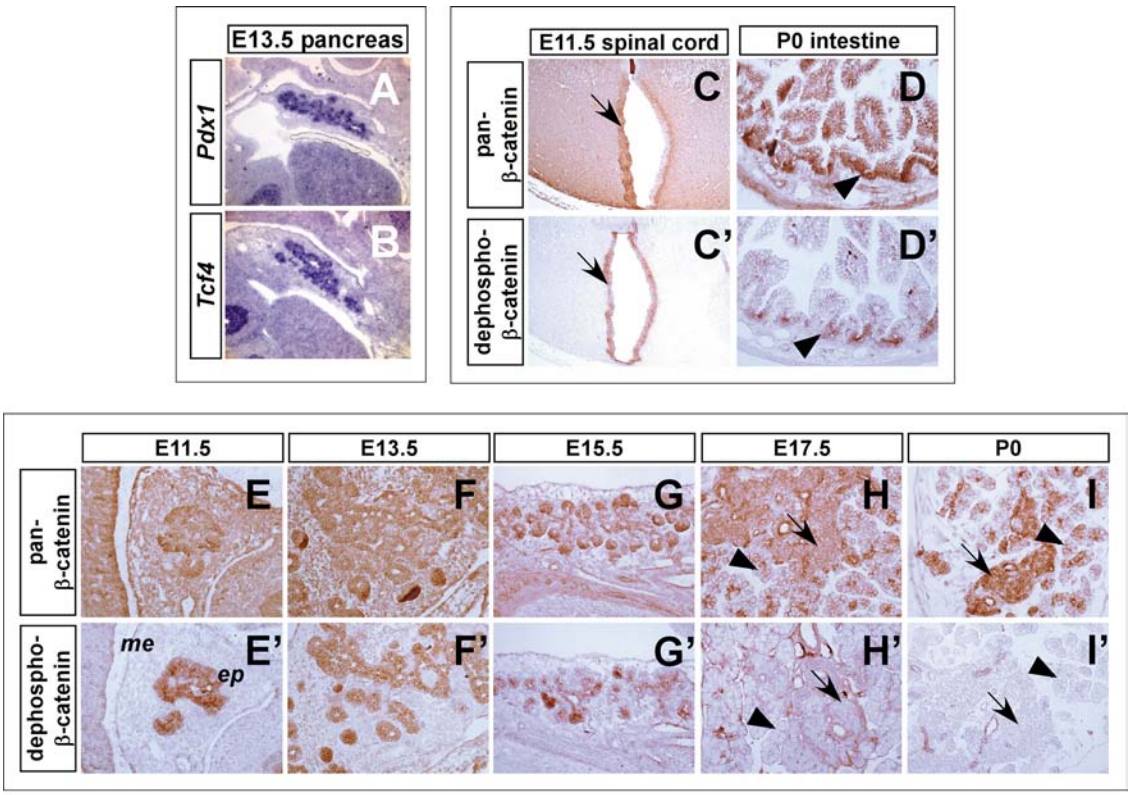

Fig. 1. Potential mediators of Wnt signaling in the developing pancreas. (A,B) In situ hybridization (purple) to E13.5 pancreatic primordia detects co-expression of *Pdx1* (A) and the β-catenin-binding transcription factor *Tcf4* (B), while the related factor *Lef1* is not expressed (data not shown). (C-D′)To confirm the specificity of pan-specific (C,D) and dephospho-specific (C′,D′) anti-β-catenin monoclonal antibodies, we immunostained (brown) near-adjacent sections of embryonic spinal cord and neonatal (P0) intestine. As expected, anti-dephospho-β-catenin recognizes regions where Wnt signaling is known to be active, such as the ventricular zone of the spinal cord (arrow) and the intestinal crypts (arrowhead). (E-I′) Pan-β-catenin (E-I) and dephospho-β-catenin (E′-I′) staining was similarly performed on various stages of developing pancreas. Arrowheads and arrows indicate morphologically recognizable islet and acinar tissue, respectively. Dephospho-β-catenin is specifically detected in the early pancreatic epithelium, and declines as the organ matures. me, pancreatic mesenchyme; ep, pancreatic epithelium.

correction for multiple testing (comparing insulin$^+$, proglucagon$^+$ and *Cpa1*$^+$ volumes between wild type and knockout).

Results

Mapping sites of potential Wnt/β-catenin signaling in the developing pancreas

Several Wnt genes, and their Frizzled receptors, have been shown to be expressed in and around the developing pancreas (Heller et al., 2002). Moreover, the undifferentiated pancreatic epithelium expresses the Lef/Tcf transcription factor *Tcf4*, a mediator of canonical Wnt signaling (Fig. 1A,B). To determine whether this expression correlates with canonical pathway activation, we examined the spatial distribution of unphosphorylated β-catenin (dephospho-β-catenin), which normally accumulates in response to canonical Wnt signaling. Detection of this protein species, using a specific monoclonal antibody, marks cells in which the pathway is active in vivo (Mohamed et al., 2004; van Noort et al., 2002).

We compared staining with the dephospho-specific monoclonal antibody to that of a monoclonal recognizing all β-catenin species, and confirmed enrichment of dephospho-β-catenin in known sites of canonical Wnt signaling, such as the ventricular zone of the E11.5 spinal cord (Fig. 1C,C′) and the crypts of the neonatal intestine (Fig. 1D,D′). We then stained sections of the pancreas, at various developmental stages, with each antibody. Total β-catenin protein is seen throughout the pancreatic epithelium and mesenchyme at all stages (Fig. 1E-I). However, dephospho-β-catenin staining is considerably more specific; at E11.5-E13.5 it is robustly detected in the pancreatic epithelium and excluded from the mesenchyme (Fig. 1E′-I′). Dephospho-β-catenin levels in the pancreas decline between E15.5-E17.5, and by birth are undetectable in differentiated endocrine and exocrine cells (Fig. 1I′). Dephospho-β-catenin is similarly undetectable in the adult pancreas (data not shown).

This staining pattern is consistent with canonical Wnt signaling being active in the early pancreas, during the transition from undifferentiated progenitors to committed exocrine and endocrine cells. It should be noted that we could not unambiguously detect nuclear β-catenin in any tissue, with any antibody, including the monoclonal anti-dephospho β-catenin antibody, the monoclonal pan-specific antibody, and two independent polyclonal antisera (see Materials and methods). We assume this is because endogenous Wnt signaling generates only low levels of nuclear β-catenin, relative to the high levels of β-catenin constitutively localized to the cell membrane. Indeed, previous studies have reported difficulty in detecting endogenous nuclear β-catenin protein in wild-type (as opposed to *Apc* mutant) tissue sections (Anderson et al., 2002), suggesting that unconventional sensitivity is required.

Pancreas-specific deletion of β-catenin results in dramatic organ hypoplasia

To establish its function in the developing pancreas, we took advantage of a conditional allele of β-catenin (*Catnb*^{lox}) generated by Brault et al., in which the first five coding exons are flanked by *loxP* sites. Deletion of these exons removes codons 1-312 of β-catenin, resulting in an absence of functional protein (Brault et al., 2001). We achieved deletion of the β-catenin gene by breeding *Catnb*^{lox} mice with mice in which Cre is expressed under control of the *Pdx1* promoter (*Pdx1-Cre*), thus driving recombination in all endodermal lineages of the developing pancreas (Gu et al., 2002).

We examined the guts of neonatal offspring from experimental crosses to look for a gross pancreatic phenotype (Fig. 2A). In all cases where the pancreas had at least one

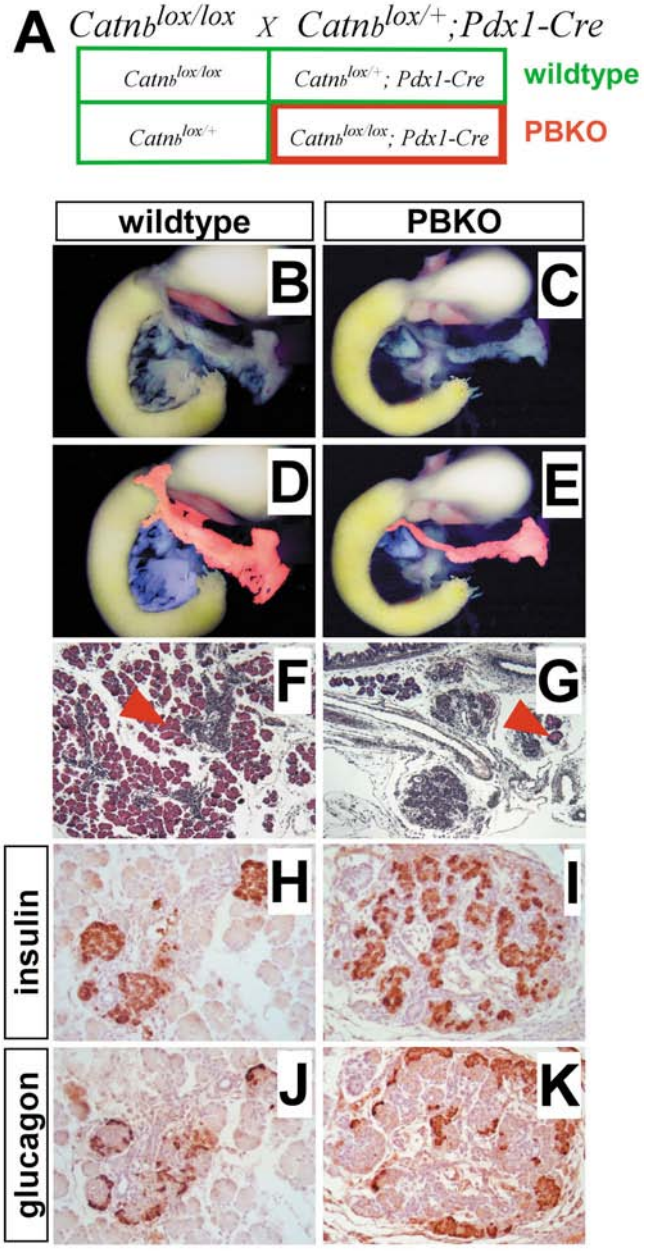

Fig. 2. Pancreas-specific β-catenin knockout (PBKO) mice. (A) Breeding scheme to generate wild-type (green) and PBKO (red) genotypes. (B,C) Gross appearance of neonatal wild-type and PBKO guts. (D,E) Dorsal (red) and ventral (blue) pancreata highlighted in above genotypes. (F,G) Hematoxylin and Eosin stained sections of wild-type and PBKO pancreata reveals the abnormal morphology of latter genotype, and the scarcity of acini (arrowheads). (H-K) Immunostaining (brown) for insulin (H,I) and glucagon (J,K) in wild-type and PBKO pancreata reveals large clumps of islet tissue in the latter genotype; Hematoxylin counterstain is blue.

undeleted allele of *Catnb* (*Catnb*$^{lox/+}$; *Pdx1-Cre*, *Catnb*$^{+/+}$; *Pdx1-Cre*, or lacking *Pdx1-Cre*), the organ was completely normal; for sake of simplicity, we refer to these pancreata as 'wild type'. By contrast, the majority of mice that inherited the *Pdx1-Cre* transgene and were homozygous for the floxed allele (*Catnb*$^{lox/lox}$; *Pdx1-Cre*) had considerably smaller pancreata than their wild-type littermates, with particularly severe reduction of the ventral lobe (Fig. 2B-E). Variability in this genotype results from a stochastic inefficiency in Cre recombination (see below) (c.f. Murtaugh et al., 2003); as such, it was reduced by pre-deleting one allele of *Catnb* in the germline (*Catnb*$^{\Delta}$; see Materials and methods). Below, we designate both sets of genotypes (*Catnb*$^{lox/lox}$; *Pdx1-Cre* and *Catnb*$^{\Delta/lox}$; *Pdx1-Cre*) as 'PBKO' (pancreatic beta-catenin knockout), unless otherwise specified.

At birth, the wild-type pancreas comprises islets scattered among a dense field of acinar clusters (Fig. 2F,H,J). In addition to the smaller overall size, PBKO pancreata exhibited a marked paucity of histologically recognizable acinar tissue (Fig. 2G), as well as large clumps of islet cells (Fig. 2I,K). Thus, removing β-catenin from the developing pancreas produces a smaller, morphogenetically abnormal organ at birth. It should be noted that the *Pdx1-Cre* transgene used here is relatively inactive outside the pancreas, therefore we do not observe β-catenin-deficient cells in the stomach or duodena of these mice.

Maintenance of hypoplastic early progenitors in the absence of β-catenin

Pancreatic hypoplasia is observed in several other mouse mutants, notably those lacking components of the Notch signaling pathway, including *Dll1*, *RBPJk* (*Rbpsuh* – Mouse Genome Informatics) and *Hes1* (Apelqvist et al., 1999; Jensen et al., 2000), as well as in *Fgf10* mutants (Bhushan et al., 2001). In all of these cases, the hypoplasia has been attributed to premature depletion of *Pdx1*$^{+}$ progenitor cells, which normally persist through approximately E13.5 (Guz et al., 1995). To ascertain whether progenitors were similarly lost in the absence of β-catenin, we analyzed marker expression in wild-type and PBKO pancreatic buds at E11.5, when the majority of the epithelium consists of undifferentiated progenitor cells. Confocal immunofluorescence confirmed that nearly all cells (>95%) of the PBKO pancreatic epithelium lacked detectable β-catenin (Fig. 3A,A′; in this and in all subsequent figures, β-catenin was detected with pan-specific antibodies). The expression of Pdx1 in β-catenin-deficient cells was indistinguishable from that of wild type (Fig. 3B,B′), and the overall size and morphology of the early bud appeared similarly unaffected by loss of β-catenin. At these early stages, the only mature pancreatic marker detectable in wild type was glucagon, expressed by a few scattered cells in the E11.5 bud (Fig. 3C). A similar number and distribution of glucagon^{+} cells was seen in PBKO pancreata (Fig. 3C′), and no other endocrine or exocrine markers were ectopically expressed (data not shown). In further contrast to Notch component and *Fgf10* mutants, PBKO pancreata retain normal Pdx1^{+} progenitors through E13.5 (Fig. 3E-F′).

To directly assess progenitor cell proliferation in the absence of β-catenin, we performed a BrdU-labeling experiment on E11.5 embryos, administering BrdU to a pregnant female one hour prior to sacrifice. We stained near-adjacent sections of the dorsal pancreas (two per embryo, two embryos per genotype) with BrdU and counted positive and negative cells (Fig. 4A,A′). There is a moderate but statistically significant decrease in the proliferation rate of PBKO cells (35.4%, 324/916), when compared with wild type (44.5%, 321/722; $P<0.001$ by χ^2 test).

Several Wnt/β-catenin target genes, likely to regulate proliferation, are expressed in the wild-type pancreas at this stage, including cyclin D1 (Shtutman et al., 1999; Tetsu and McCormick, 1999), *Id2* (Rockman et al., 2001) and *Myc* (He et al., 1998) (Fig. 4B-D). Although cyclin D1 and *Id2* expression appears to be unaffected in PBKO littermates (Fig. 4B′-C′), expression of the proto-oncogene *Myc* appears to be downregulated in the absence of β-catenin (Fig. 4D′). As *Myc* mutants die before E10.5 (Davis et al., 1993), their pancreatic phenotype cannot be evaluated, but it is possible that *Myc* mediates the effects of β-catenin on proliferation. Finally, although Wnt and Notch signaling appear to cooperate in several tissues (Radtke and Clevers, 2005), we find that expression of the Notch target gene *Hes1* is maintained in

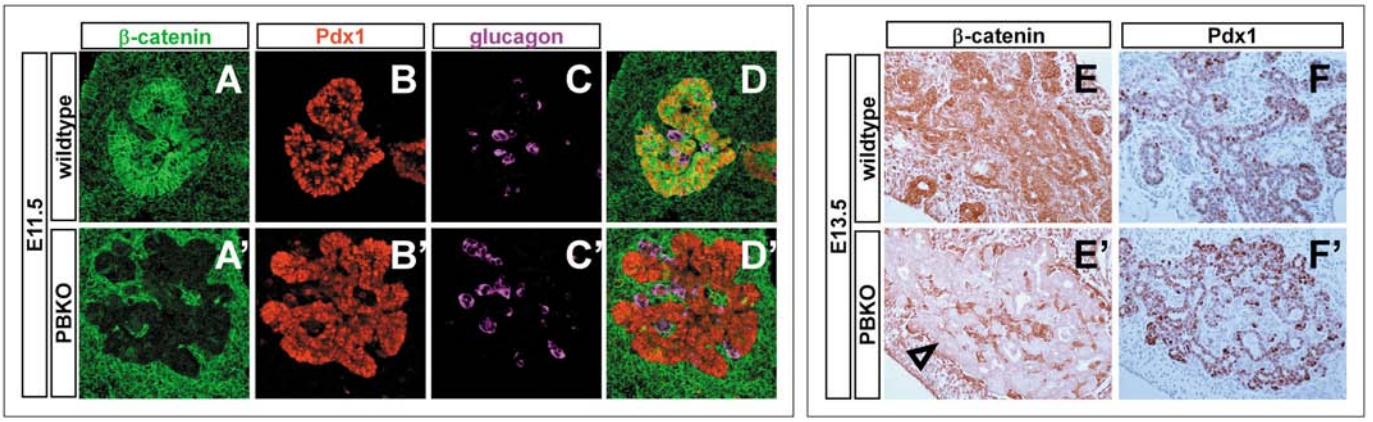

Fig. 3. Maintenance of early pancreatic progenitors in the absence of β-catenin. Three-color confocal immunofluorescence for β-catenin (A,A′), Pdx1 (B,B′) and glucagon (C,C′) in E11.5 dorsal pancreatic buds. The relative distribution of Pdx1^{+} and glucagon^{+} cells is normal in regions of the PBKO pancreas lacking β-catenin (A′-D′). Immunohistochemical staining for pan-β-catenin or Pdx1 in near-adjacent sections of E13.5 wild-type (E,F) or PBKO (E′,F′) pancreata reveals normal expression of Pdx1 in β-catenin-deficient epithelia. Pancreata are counterstained with Hematoxylin (blue) in F,F′. Arrowhead in E′ indicates background staining that outlines β-catenin-deficient epithelia.

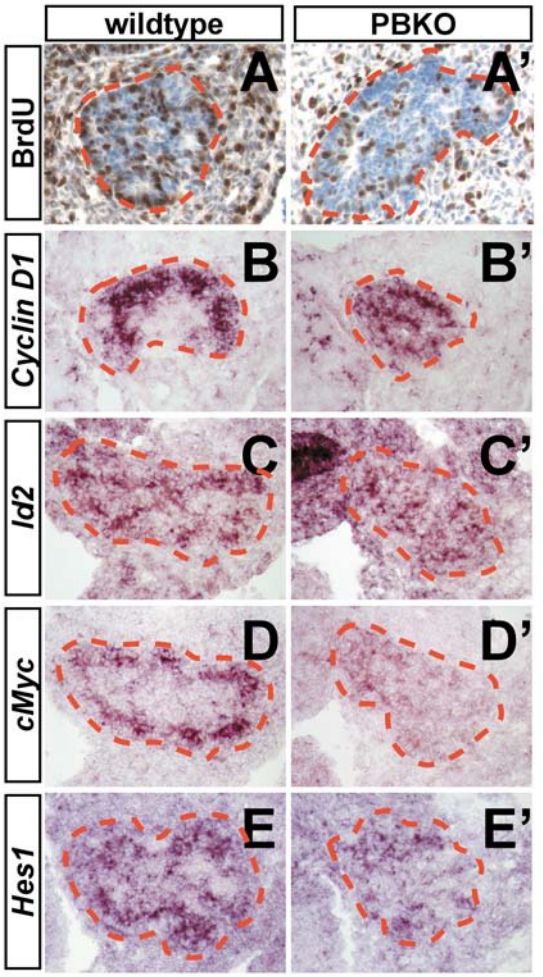

Fig. 4. Impaired proliferation in β-catenin knockout pancreata. (A-E′) E11.5 wild-type (A-E) or PBKO (A′-E′) dorsal pancreata were analyzed by immunohistochemistry or in situ hybridization. Anti-BrdU staining (brown, A,A′) labels proliferating cells; pregnant females were injected 1 hour prior to harvest (50 μg/kg). PBKO pancreata show a moderate but significant decrease in proliferation (35.4% $BrdU^+$) compared with wild type (44.5% $BrdU^+$). In situ hybridization (purple) reveals little or no change in expression of the potential Wnt/β-catenin target genes cyclin D1 (B,B′) and *Id2* (C,C′) between wild type and PBKO, whereas that of *Myc* (D,D′) is strongly downregulated in PBKO. Expression of the Notch target gene *Hes1* (E,E′) is similar in the two genotypes. Red dashed lines outline pancreatic epithelia.

PBKO pancreata (Fig. 4E,E′), consistent with the observed maintenance of early progenitor cells.

Given that β-catenin was discovered as a potential link between cadherin receptors and the cytoskeleton (Ozawa et al., 1989), its loss might be expected to affect epithelial organization and compromise cellular viability. However, neither of these effects was observed. Using an antibody specific to cleaved capsase-3, an early marker of apoptosis, we detected no increased cell death in PBKO pancreata at any stage examined (E11.5-E17.5). In addition, we found that E-cadherin staining was indistinguishable between β-catenin-positive and -negative cells, at all stages (see Fig. S1 in the supplementary material; and data not shown). This likely reflects upregulation of the related protein plakoglobin: while plakoglobin expression was barely detectable in the wild-type pancreas, it was dramatically upregulated in β-catenin-deficient PBKO cells (see Fig. S1 in the supplementary material), as seen in β-catenin-deficient blastocysts (Haegel et al., 1995).

In summary, loss of β-catenin does not impair the survival or epithelial organization of early pancreatic progenitors, nor does it appear to perturb the maintenance of their undifferentiated state. Cell proliferation in the early PBKO pancreas is moderately decreased when compared with wild type, an effect that probably contributes to the overall smaller organ size at birth. However, the massive wave of cell differentiation between E13.5 and birth is itself likely to affect organ size: confirming classic observations made in cell culture (Pictet et al., 1972), we observe that newly differentiated acinar cells are highly proliferative in vivo (see Fig. S2 in the supplementary material). We therefore turned our attention to the process of differentiation in the embryonic PBKO pancreas.

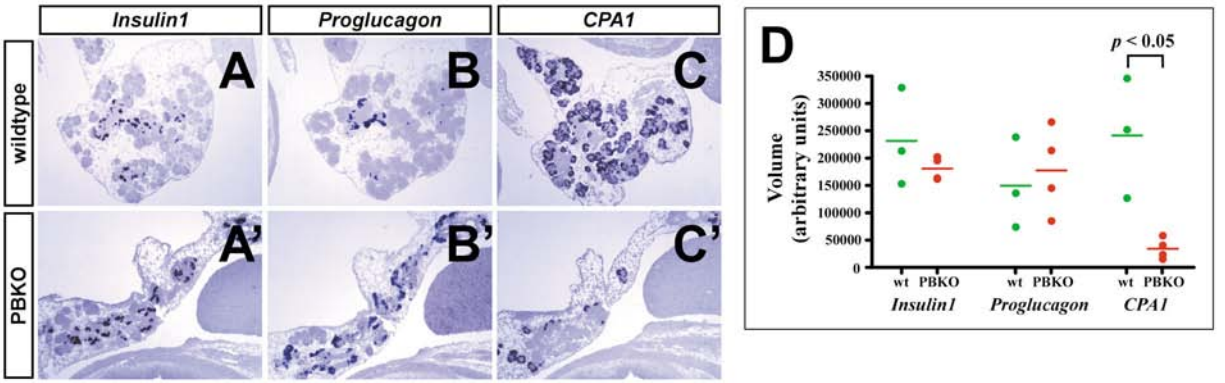

Fig. 5. Relative impact of β-catenin deletion on the endocrine and exocrine compartments. In situ hybridization (purple) to detect insulin1 (A,A′), proglucagon (B,B′) and carboxypeptidaseA1 (*Cpa1*) (C,C′) on near-adjacent sections of E15.5 wild-type (A-C) or PBKO (A′-C′) pancreata. (D) Morphometric quantitation of in situ hybridization to calculate endocrine and exocrine volumes in wild-type (n=3) and PBKO ($Catnb^{\Delta/lox}$;*Pdx1-Cre*; n=4) pancreata. Shown are individual measurements (points) and means (bars); the difference between wild type (green) and PBKO (red) is statistically significant only for *Cpa1* (P<0.05 by two-tailed t-test).

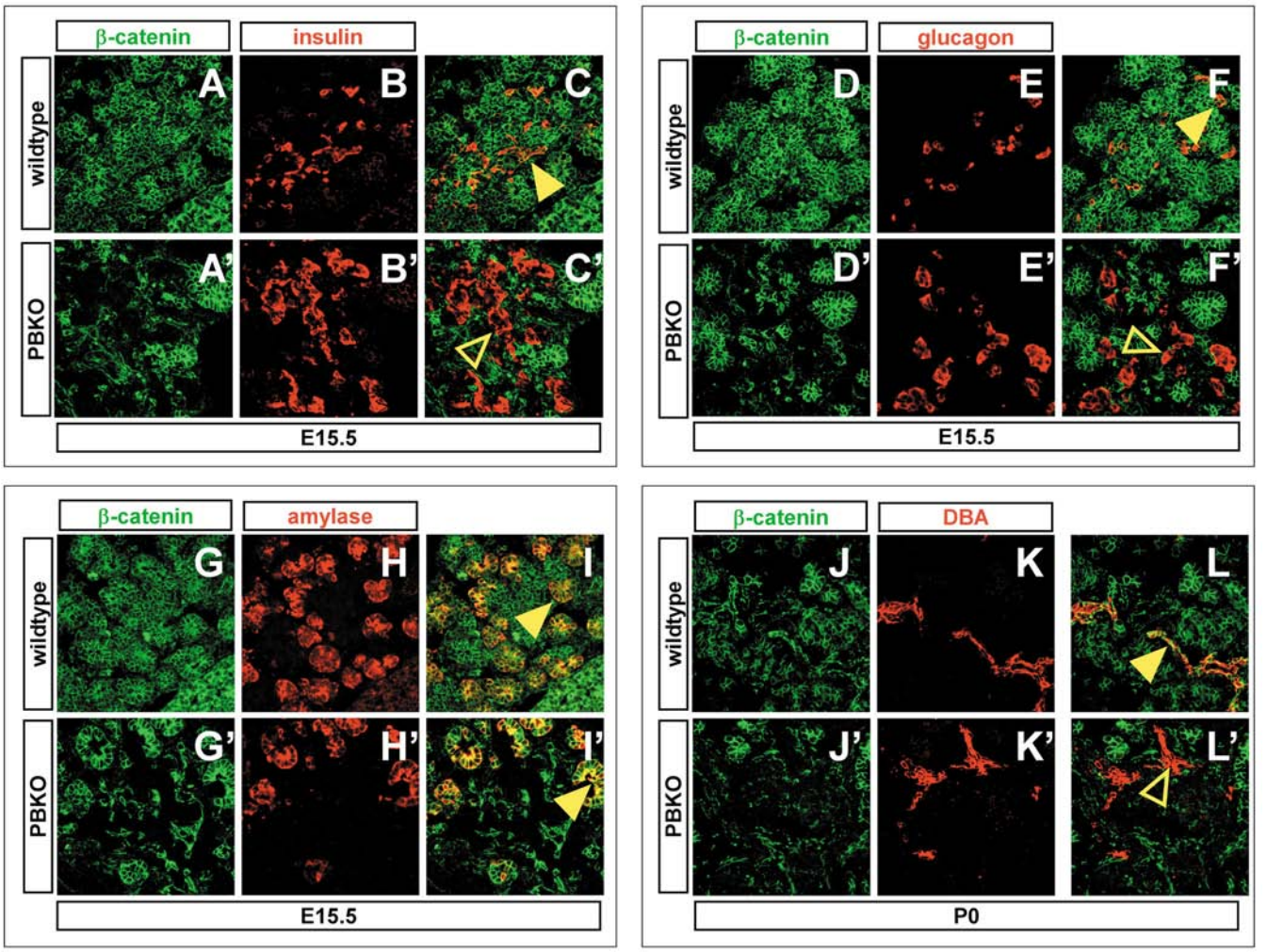

Fig. 6. Relative requirement for β-catenin in individual endocrine and exocrine cells. Confocal immunofluorescence on E15.5 (A-I′) or neonatal (J-L′) wild-type versus PBKO pancreata, to detect co-expression of β-catenin (A,A′,D,D′,G,G′,J,J′) with insulin (B,B′), glucagon (E,E′), amylase (H,H′) or the duct marker DBA lectin (K,K′). Filled arrowheads indicate examples of cells co-expressing the markers; open arrowheads indicate a lack of co-expression. Note that all insulin$^+$ and glucagon$^+$ cells are β-catenin$^-$ in PBKO sections (C′,F′), as are the majority of DBA$^+$ cells (L′). By contrast, all residual amylase$^+$ cells in these pancreata are β-catenin$^+$ (I′), indicating that they derive from cells in which the *Catnb* gene did not undergo Cre-mediated excision.

β-Catenin is required for acinar but not islet cell development

The wave of endocrine and exocrine differentiation termed the secondary transition begins at E13.5-E14.5 and continues approximately through birth (Jensen, 2004; Pictet and Rutter, 1972). To minimize complications introduced by differences in proliferation between differentiated cell types, we focused our analysis on E15.5 embryos, in which the pancreas comprises a mixture of progenitors and recently differentiated acinar and islet cells. As expected, in situ hybridization on wild-type pancreata for the endocrine markers insulin1 and proglucagon, and the exocrine marker carboxypeptidase A1 (*Cpa1*), revealed a core of endocrine cells surrounded by acinar clusters (Fig. 5A-C). In PBKO pancreata, however, the relative area occupied by insulin1$^+$ and proglucagon$^+$ cells appeared to be expanded at the expense of the *Cpa1*$^+$ population (Fig. 5A′-C′). Quantitative morphometry on these sections, spaced evenly across the entire extent of wild-type (n=3) or mutant (n=4) pancreata, revealed that while there was little or no change in the absolute volume of endocrine cells between wild-type and PBKO, PBKO pancreata contained approximately sevenfold less acinar tissue (P<0.05; Fig. 5D). Thus, β-catenin is apparently dispensable for early endocrine cell formation, but is required for acinar development.

To address the possibility that deletion of the *Catnb* gene occurs more robustly in one lineage than another, we performed confocal immunofluorescence to detect β-catenin co-expression with islet and acinar markers. Whereas both insulin$^+$ β-cells and glucagon$^+$ α-cells of wild-type E15.5 pancreata co-expressed β-catenin (Fig. 6A-F), in PBKO pancreata, nearly all (>95%) of both cell types lacked detectable β-catenin (Fig. 6A-F′). Nevertheless, numerous β-catenin$^+$ cells were detected in PBKO pancreata, typically organized in epithelial clusters separate from the β-catenin$^-$ endocrine cells. These primarily represented the residual acinar cells, which were uniformly (>99%) β-catenin$^+$ (Fig. 6G′-I′).

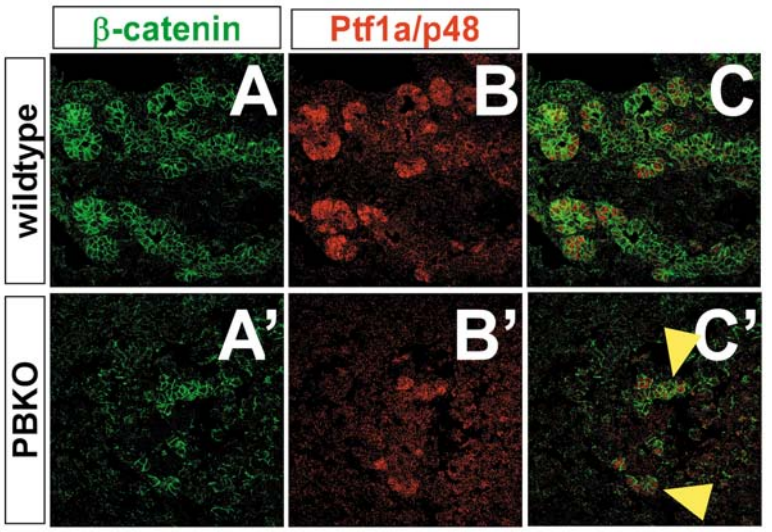

Fig. 7. Loss of Ptf1a/p48$^+$ acinar precursors in the absence of β-catenin. Confocal immunofluorescence at E13.5 detects peripheral clusters of Ptf1a$^+$ cells in wild-type pancreata (A-C), presumably representing acinar precursor cells. Ptf1a$^+$ cells are much more infrequent in PBKO pancreata (A′-C′), and are restricted to residual β-catenin$^+$ regions, indicating that they derive from cells in which *Catnb* remains undeleted.

Thus, there is a specific and nearly absolute requirement for β-catenin in acinar development.

To determine whether loss of β-catenin affects the entire exocrine compartment, i.e. ducts as well as acini, we stained newborn pancreata with the lectin DBA, which marks differentiated ducts at this stage (Kobayashi et al., 2002) (Fig. 6J-L). In contrast to acinar cells at this and other timepoints, abundant normal DBA$^+$ ducts were found lacking β-catenin in the PBKO pancreas (Fig. 6J′-L′).

The PBKO phenotype, with respect to differentiation, appears to be selective for acinar cells. To further understand this phenotype, we examined expression of the transcription factor Ptf1a/p48. Ptf1a directly regulates many acinar-specific genes, and its expression in the mature pancreas is restricted to acinar cells (Krapp et al., 1996). Although Ptf1a is expressed broadly in the early pancreatic primordium, in progenitors of all mature cell types (Kawaguchi et al., 2002), it becomes specifically upregulated in peripherally epithelial clusters 1-2 days prior to acinar differentiation, likely marking the nascent acini (Esni et al., 2004a). We observe this upregulation in the wild-type E13.5 pancreas (Fig. 7A-C), as expected, but these Ptf1a$^+$ clusters are rarer in PBKO pancreata, and, more importantly, are restricted to residual β-catenin$^+$ regions (Fig. 7D-F). Thus, β-catenin appears to be required for the upregulation of Ptf1a prior to acinar differentiation, potentially providing a mechanism for the PBKO acinar phenotype.

Our immunofluorescence analysis suggests that the sevenfold decrease in acinar volume observed by morphometry (Fig. 5D) almost certainly understates the biological requirement for β-catenin: complete deletion of *Catnb* would likely result in no acini whatsoever. Because acini comprise the majority of the pancreas by birth, it is likely that their depletion contributes to the overall hypoplasia of the late-stage PBKO pancreas; moreover, variability in this hypoplasia is likely attributable to uneven *Catnb* deletion in the acinar lineage.

β-Catenin is dispensable for the function and survival of differentiated islet and acinar cells

To determine whether β-catenin-deficient islet cells survive and function normally, we examined adult PBKO mice. The islets of adult PBKO mice exhibited an almost complete lack of detectable β-catenin protein (Fig. 8A,A′), similar to embryos, but their architecture was essentially normal. In

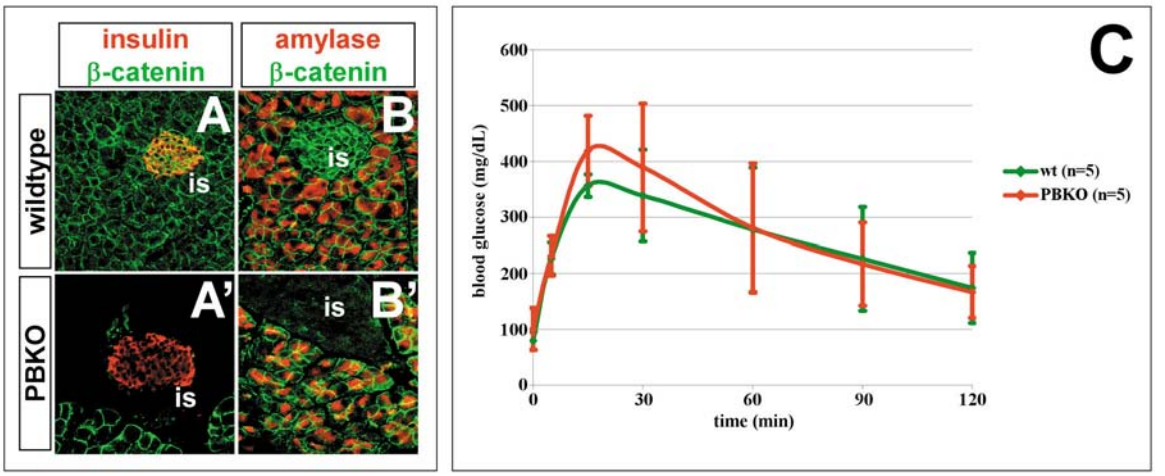

Fig. 8. β-catenin is dispensable for maintenance and function of adult endocrine cells. Confocal immunofluorescence reveals that nearly all insulin$^+$ islet cells are β-catenin-deficient in PBKO adult pancreata (A,A′), whereas all amylase$^+$ acinar cells retain β-catenin expression (B,B′), just as in the developing organ. (C) Glucose tolerance tests of wild-type (*n*=5) and PBKO (*n*=5) adult mice. Points represent mean blood glucose for each timepoint following intraperitoneal glucose injection (2 mg/g body weight), error bars indicate standard deviation. There are no statistically significant differences between genotypes at any timepoint. is, islet.

addition, we performed glucose tolerance tests on adult PBKO mice (age 12-15 weeks) to test this cardinal function of β-cells. As shown in Fig. 8C, PBKO mice exhibited no significant impairment in glucose clearance when compared with wild types, indicating that β-catenin is not required for glucose-stimulated insulin release by β-cells. This contrasts with the phenotype of mice hemizygous or null for the canonical Wnt receptor *Lrp5*, which exhibit impaired blood sugar homeostasis (Fujino et al., 2003). That we do not observe such a defect in adult PBKO mice suggests that Lrp5 may mediate β-catenin-independent aspects of Wnt signaling.

Most PBKO mice survive to adulthood due to incomplete deletion of β-catenin in the acinar lineage; their remaining acinar cells are invariably β-catenin$^+$ (>99%), as in embryos (Fig. 8B,B′). Although we have attributed the lack of β-catenin-deficient acinar cells to an impairment of differentiation, it is hard to definitively rule out a role for β-catenin in acinar survival, as dying cells may be so rapidly cleared that we cannot observe them. To directly examine the acute requirement for β-catenin in acini, we made use of a deletor mouse (*Ela-CreERT*; see Materials and methods) in which tamoxifen-dependent CreERT (Danielian et al., 1998) is expressed under the control of an acinar-specific promoter derived from the elastase gene (Swift et al., 1989).

To test transgene function, we bred *Ela-CreERT* mice with *R26R* indicator mice that carry a Cre-activateable *lacZ* reporter (Soriano, 1999). As expected, in the absence of Cre there is no detectable β-galactosidase (β-gal) activity in the adult *R26R* pancreas (data not shown). In the presence of *Ela-CreERT*, but without injected tamoxifen (TM), a small proportion of acinar cells (⩽1%) are β-gal$^+$ (Fig. 9A), indicating a low level of TM-independent recombination. When TM is administered to double-transgenic mice (5 mg daily, 3×), robust β-gal activity is seen throughout the acini, with ⩾50% being labeled two weeks after the first injection (Fig. 9B). Although no labeling is seen in the ducts (Fig. 9B), a low level does occur in islet cells (not shown), presumably reflecting inappropriate transgene expression. Although this ectopic labeling, and the low level of TM-independent activity, limit the utility of this transgenic line for long-term lineage-tracing experiments (Dor et al., 2004), it remains useful for acute manipulation of adult acinar cells.

To delete β-catenin in adult acinar cells, we crossed *Ela-CreERT* onto a *Catnb$^{lox/lox}$* background, administered tamoxifen as above, and examined the mutant pancreata (n=3) two weeks after the initial injection. These pancreata were histologically unremarkable, and exhibited no increased apoptosis (data not shown). In the absence of either tamoxifen or the deletor transgene, expression of β-catenin protein was observed in all acinar cells as expected (Fig. 9C, data not shown). By contrast, two weeks after tamoxifen administration to *Catnb$^{lox/lox}$; Ela-CreERT* mice, a widespread clearance of β-catenin protein is seen in the acinar cells (Fig. 9C′). Confocal immunofluorescence revealed that β-catenin-deficient acini retained normal expression of the digestive enzyme amylase (Fig. 9C-E′), indicating that their differentiation program remained intact. The scarcity of acinar cells in the PBKO pancreas is therefore unlikely to reflect an acute requirement for β-catenin in their survival, or in the maintenance of cell type-specific gene expression; instead, it is most readily explained by a requirement for β-catenin in the initial formation of acinar cells.

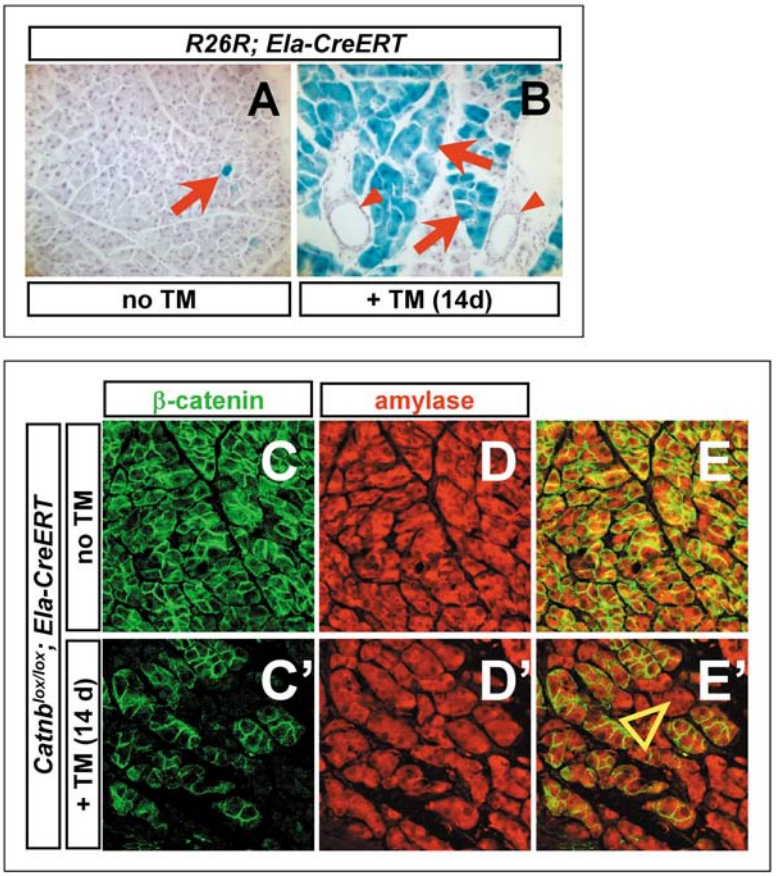

Fig. 9. β-catenin deletion in adult exocrine cells. (A,B) X-gal staining (blue) on sections of adult *R26R;Ela-CreERT* double transgenic pancreata. In the absence of tamoxifen (TM) administration (A), a low level of TM-independent acinar labeling is observed (arrow), whereas widespread acinar labeling is observed two weeks after TM administration (B). No labeling is observed in ductal elements (arrowheads). (C-E′) Confocal immunofluorescence to detect β-catenin and amylase expression. All amylase$^+$ cells in the TM-untreated pancreas retain β-catenin expression (E), whereas many β-catenin$^-$/amylase$^+$ cells can be found following TM treatment, including entire acini (arrowhead) comprising β-catenin-deficient cells but exhibiting normal architecture (E′).

Discussion

In a simple model for pancreatic development, cell fate determination would occur as cells stochastically escape from negative signals, such as Notch and Fgf, and go on to assume endocrine and exocrine fates due to pre-existing transcriptional biases. Yet actual pancreatic differentiation occurs with a remarkable morphogenetic precision, implying the existence of positive signals that act to parse out progenitor cell fates. Here, we have tested the possibility that Wnt signaling might be

involved in pancreatic development, by genetic inactivation of its canonical mediator β-catenin.

We find that pancreata lacking β-catenin are dramatically smaller than those of wild-type animals, and contain a striking paucity of acinar cells. In part, this may be attributed to a decrease in the proliferation rate of early progenitors, potentially reflecting loss of *Myc* expression. Nonetheless, there is no evidence for premature differentiation or depletion of the progenitor pool in PBKO pancreata. Indeed, the endocrine compartment appears relatively unaffected in these pancreata, with α- and β-cells differentiating in approximately normal numbers and with normal timing. The acinar lineage, by contrast, appears to cell autonomously require β-catenin for its differentiation, but not for its maintenance or survival.

What is the relationship between the hypoplasia and acinar depletion phenotypes in the PBKO pancreas? A comparison with the *Fgf10* mutant phenotype may be instructive. In *Fgf10*$^{-/-}$ pancreata, the Pdx1^{+} progenitor pool is lost to hypoplasia by E12.5-E13.5, and the final organ is even smaller than that of PBKO mice. Nonetheless, most of the remnant pancreas in the *Fgf10* mutant is composed of differentiated acini (Bhushan et al., 2001). If the lack of exocrine cells observed in PBKO pancreata reflected an indirect effect on progenitor proliferation, we would expect that the acinar phenotype of *Fgf10* mutants should be at least as severe as that of PBKO mice. The fact that it is actually less so implies that β-catenin function extends beyond simply promoting proliferation.

It is also possible that β-catenin functions solely in the expansion of differentiated exocrine cells. We regard this as unlikely: at E15.5, shortly after acinar differentiation has commenced, we observe very few amylase^{+}/β-catenin^{-} cells (<1% of all amylase^{+} cells), whereas at E13.5, prior to differentiation, we find that Ptf1a expression is restricted to the residual β-catenin^{+} cells. Therefore, we think that the PBKO exocrine phenotype is most readily explained by a direct requirement for β-catenin in the development of progenitor cells into acini, rather than by an indirect effect of decreased proliferation.

We find that β-catenin-deficient cells are capable of normal contribution to islet endocrine cells. This contrasts with the intestine, where canonical Wnt signaling is required for endocrine development (Pinto et al., 2003), possibly acting through a direct β-catenin/Tcf regulation of hormone genes such as proglucagon (Yi et al., 2005). Interestingly, and consistent with our results, β-catenin/Tcf-binding sites are dispensable for proglucagon expression in pancreatic α-cell lines (Yi et al., 2005). These results identify an important difference in the developmental program of the two classes of endocrine cells, which is in contrast to their shared requirement for upstream regulators such as *Ngn3* (Gradwohl et al., 2000; Jenny et al., 2002) and the Notch target *Hes1* (Jensen et al., 2000).

Our finding that unphosphorylated β-catenin is enriched in the early pancreatic epithelium, as in tissues where the Wnt pathway is known to be active, is strongly consistent with the possibility that Wnt proteins are acting on the pancreas at these stages. Several Wnt genes are expressed in the pancreatic mesenchyme (Heller et al., 2002; Lin et al., 2001), and the mesenchyme has long been known to be required for acinar differentiation in vitro (Golosow and Grobstein, 1962; Wessells and Cohen, 1967). Although transgenic expression of *Wnt1* under control of the *Pdx1* promoter leads to pancreatic agenesis (Heller et al., 2002), this may reflect an alternative role for Wnt signaling specifically at the early stages that *Pdx1* expression initiates, for instance in specifying intestinal fates (Okubo and Hogan, 2004). Future work will address whether or not Wnt signaling components upstream of β-catenin are similarly required for pancreas development, and with what downstream partners β-catenin might interact.

We are also keenly interested in determining the fate of those β-catenin-deficient cells that would normally contribute to acini: are they are diverted to a non-acinar fate, such as islets? Although we found no expansion of the endocrine population in PBKO pancreata, it is possible that any increase would have been masked by the decreased proliferation of early β-catenin-deficient progenitors. Resolving this issue in the future will require a Cre-deletor transgene specific to acinar precursor cells.

In summary, we have demonstrated that β-catenin is required both for the robust proliferation of pancreatic progenitor cells and for their subsequent differentiation into acinar cells, potentially acting upstream of the *Myc* and *Ptf1a* genes in these respective processes. As β-catenin is dispensable for islet endocrine development, it occupies a unique place in the genetic cascade of pancreas development (Murtaugh and Melton, 2003), and its crucial role in the Wnt cascade suggests that Wnt proteins may prove to be important intercellular signals in pancreatic organogenesis.

Note added in proof

Consistent with our finding that loss of Wnt/β-catenin signaling does not impair pancreatic endocrine development, a recent publication (Pedersen and Heller, 2005) indicates that hyperactivation of Wnt/β-catenin signalling actively antagonizes endocrine differentiation induced by Ngn3 misexpression.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/21/4663/DC1

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