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Distinct requirements for beta-catenin in pancreatic epithelial growth and patterning

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ABSTRACT

Pancreatic exocrine and endocrine lineages arise from multipotent pancreatic progenitor cells (MPCs). Exploiting the mechanisms that govern expansion and differentiation of these cells could enhance efforts to generate β-cells from stem cells. Although our prior work indicates that the canonical Wnt signaling component β-catenin is required qualitatively for exocrine acinar but not endocrine development, precisely how this requirement plays out at the level of MPCs and their lineage-restricted progeny is unknown. In addition, the contribution of β-catenin function to β-cell development remains controversial. To resolve the potential roles of β-catenin in development of MPCs and β-cells, we generated pancreas- and pre-endocrine-specific β-catenin knockout mice. Pancreas-specific loss of β-catenin produced not only a dramatic reduction in acinar cell numbers, but also a significant reduction in β-cell mass. The loss of β-cells is due not to a defect in the differentiation of endocrine precursors, but instead correlates with an early and specific loss of MPCs. In turn, this reflects a novel role for β-catenin in maintaining proximal–distal patterning of the early epithelium, such that distal MPCs resort to a proximal, endocrine-competent "trunk" fate when β-catenin is deleted. Moreover, β-catenin maintains proximal–distal patterning, in part, by inhibiting Notch signaling. Subsequently, β-catenin is required for proliferation of both distal and proximal cells, driving overall organ growth. In distinguishing two distinct roles for β-catenin along the route of β-cell development, we suggest that temporally appropriate positive and negative manipulation of this molecule could enhance expansion and differentiation of stem cell-derived MPCs.

Introduction

The adult pancreas can be divided into two functionally distinct domains, the exocrine function constituted by enzyme-secreting acinar cells and ducts, and the endocrine function performed by the islets of Langerhans. The division between the two domains begins shortly after specification where the pancreatic epithelium is separated into a distal "tip" domain at the periphery of the organ, which contains multipotent pancreatic progenitors (MPCs), and a proximal trunk domain, which contains bipotent progenitors that give rise to islet and duct cells. The capability of MPCs to produce all pancreatic lineages lasts roughly until the secondary transition at E13.5, after which distal tips are restricted to an acinar fate (Pan and Wright, 2011; Zhou et al., 2007). The early specification, maintenance and self-renewal of embryonic progenitors is critical to the development of the adult organ, as evidenced by the generation of a dysfunctional and smaller pancreas when early progenitors are partially ablated (Stanger et al., 2007).

With an ultimate goal of generating β-cells from stem cells, much effort has gone into understanding the transcription factors and intercellular signals that control pancreas progenitor patterning, expansion and differentiation (Pan and Wright, 2011). As signal transduction pathways mediate the response of progenitors to their microenvironment, they might serve as facile targets for manipulating stem cell differentiation. Nonetheless, while metazoan development requires only a few core signaling pathways, these can be deployed in complex and changing ways during organogenesis. This is exemplified, in the pancreas, by recent studies of the Notch signaling pathway. Originally implicated specifically as a negative regulator of endocrine differentiation (Apelqvist et al., 1999; Jensen et al., 2000), Notch has since been shown to inhibit acinar development as well (Esni et al., 2004; Hald et al., 2003; Murtaugh et al., 2003), potentially through modulating proximal–distal patterning of the early organ (Afelik et al., 2012; Magenheim et al., 2011; Schaffer et al., 2010). Although much has been gleaned from studying Notch and other pathways individually, relatively little is known about these signals coalesce to pattern and specify progenitors in the developing pancreas.

The goal of the present study is to elucidate the cellular mechanisms by which β-catenin, a component of the Wnt signaling pathway, controls the allocation of endocrine and exocrine lineages from pancreatic progenitor cells. In previous studies using

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two different "*Pdx1-Cre*^{early}" transgenic mice, both driving recombination in the early embryonic pancreas, conditional β-catenin knockout pancreata were found to be drastically smaller than wild-type and largely devoid of exocrine acinar cells, while containing a qualitatively normal complement of endocrine α- and β-cells (Murtaugh et al., 2005; Wells et al., 2007). However, deleting β-catenin using a different "*Pdx1-Cre*^{late}" transgene, beginning several days later in development (Heiser et al., 2006), produced little effect on acinar cell development but caused a mild decrease in islet cell mass (Dessimoz et al., 2005). It was hypothesized in that study that β-catenin was required separately for proliferation or survival of endocrine cells and exocrine progenitors. The discordant results of these studies may reflect the efficiency and timing of the different *Pdx1-Cre* deletor strains used (Murtaugh, 2008).The requirement for β-catenin in acinar cells persists through adulthood, when it is necessary for steady-state turnover and acinar cell regeneration following injury (Keefe et al., 2012). The role of Wnt/β-catenin signaling in differentiated endocrine cells has been an area of debate, with some studies suggesting it promotes β-cell proliferation and function (Dabernat et al., 2009; Rulifson et al., 2007), and another indicating that it is dispensable for adult mouse β-cell proliferation (Keefe et al., 2012). Together, these studies suggest that the contribution of β-catenin to endocrine islet development remains to be unraveled. Using time- and lineage-specific deletion experiments, we sought to investigate the roles of β-catenin during embryonic pancreas development, particularly in establishing endocrine β-cell mass.

Materials and methods

Mice

All experiments were performed according to protocols approved by the University of Utah IACUC. We obtained several mouse strains from the Jackson Laboratory: floxed and germline β-catenin loss-of-function mice (*Ctnnb1*$^{tm2Kem/J}$ and *Ctnnb1*$^{tm2.1Kem}$, henceforth *Ctnnb1*^{lox} and *Ctnnb1*$^{\Delta}$, respectively) (Brault et al., 2001); *Ngn3-Cre* BAC transgenic mice (Schonhoff et al., 2004) and the Cre-dependent EYFP reporter strain *Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$ (Srinivas et al., 2001), henceforth *R26R*^{EYFP}. *Pdx1-Cre* and *Pdx1-CreERT* transgenic mice (Gu et al., 2002) were provided by Doug Melton (Harvard University). To induce recombination with the *Pdx1-CreERT* transgene, we administered tamoxifen (Sigma) suspended in corn oil (Sigma) to pregnant dams, typically 8–16 weeks of age, by oral gavage. Embryos were genotyped by PCR, using primer sets described previously (Gu et al., 2002; Murtaugh et al., 2005).

Tissue processing and staining

Pregnant dams were euthanized with isoflurane followed by cervical dislocation. Whole embryos (E13.5 and younger) and pancreata (E14.5 and later) were dissected in ice-cold PBS for processing. Tissues were fixed overnight at room-temperature with zinc-buffered formalin (Polysciences) for paraffin sections or with 4% paraformaldehyde/PBS (2 h-overnight at 4 °C for frozen sections, and further processed as previously (Keefe et al., 2012; Kopinke and Murtaugh, 2010; Murtaugh et al., 2005)). A series of duplicate paraffin sections (6 μm) were collected sequentially across multiple slides, spaced with skipping to span the entire pancreas in the following age-dependent manner: for E17.5, 10 slides with 180 μm between sections; E12.5–E14.5, 8 slides with 96 μm between sections; E11.5, 7 slides with 84 μm between sections on slides. In this way, the entire volume of each pancreas is sample on multiple individual slides. Similarly, frozen sections (8 μm) were collected serially over 6–10 slides such that the each slide contained representative sections throughout the organ. For labeling S-phase nuclei, mice were injected with BrdU (50 μg/g body weight) one hour prior to sacrifice.

Antibodies used for immunostaining are listed in Table S1, and all secondary antibodies (raised in donkey) were purchased from Jackson Immunoresearch. Immunostaining was performed as previously (Keefe et al., 2012; Kopinke and Murtaugh, 2010; Murtaugh et al., 2005), including high-temperature antigen retrieval for paraffin sections. For anti-BrdU staining, frozen sections were pre-treated with DNase I (700 u/μl, in 40 mM Tris–HCl pH 7.4, 10 mM NaCl, 6 mM $MgCl_2$, and 10 mM $CaCl_2$) at room temperature for 30 min (Ye et al., 2007). Bright field images were obtained using an Olympus CX41 microscope and MicroSuite software. For immunofluorescence, Fluoromount-G (Southern Biotech) was used as a mounting substrate and images were obtained using an Olympus IX71 microscope and MicroSuite software. Identical exposure times and post-processing adjustments performed in Adobe Photoshop were used across control and experimental genotypes.

Quantification and analysis

To measure β-cell mass, pancreas size, and volume or number of cells expressing various markers, serial sections were stained by immunohistochemistry, and all sections on a single slide were photographed individually (4× original magnification at E17.5, and 10–20× for all other time points), to provide a representation of the entire pancreas. ImageJ (NIH) software was used to measure the surface area occupied by stained tissue. For Ptf1a, c-Myc, and Ngn3 at E11.5 and E12.5, stained nuclei were counted in Adobe Photoshop. The total number of cells per pancreas (E11.5–E13.5) was estimated by multiplying the number of cells counted per slide by the number of slides in the series. Calculations, graphs and *P*-values (two-tailed, unpaired *t*-test) were generated in Microsoft Excel, and results are presented as mean ± s.e.m.

For lineage tracing experiments using *Pdx1-CreERT* and *R26R*^{EYFP}, we photographed several independent fields (20× original magnification), per embryo, across multiple pancreatic sections per slide. For acinar cell labeling indices, we used the additive image overlay feature of ImageJ (NIH) software to identify the overlap of DAPI, amylase (acinar cells) and EYFP, and counted cells using the Analyze Particles function (Kopinke and Murtaugh, 2010). For all other differentiation markers, cells were counted using the Count Tool in Adobe Photoshop.

Explant cultures and wholemount immunostaining

For ex vivo explant cultures, the dorsal buds of E11.5 pancreata were dissected in ice-cold sterile PBS, and cultured at the air-media interface on 0.4 μm pore size PTFE cell culture inserts (Millipore), in DMEM with 10% fetal bovine serum and antibiotics. A small piece of tissue was collected for genotyping purposes at the time of dissection. Explants were treated with 100 nM of the γ-secretase inhibitor DBZ (Millipore 565789) for 3 days, while controls received no treatment. Media was changed daily.

Wholemount immunofluorescence was performed as previously described (Kopinke and Murtaugh, 2010). Briefly, explants were fixed overnight in 4% PFA, washed and stored in methanol until staining. For staining, explants were rehydrated to PBS, permeabilized for 1 h with 1% Triton-X100 in PBS, and then placed in blocking solution (5% donkey serum and 0.3% Triton X-100) for 2 h. Primary and secondary antibody incubations were performed overnight at room temperature. Explants were cleared in BABB (2:1 benzyl alcohol:benzyl benzoate) prior to imaging. Confocal images were obtained using an Olympus FV-1000 microscope.