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A dosage-dependent requirement for *Sox9* in pancreatic endocrine cell formation

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ABSTRACT

We have previously shown the transcription factor SOX9 to be required for the maintenance of multipotential pancreatic progenitor cells in the early embryonic pancreas. However, the association of pancreatic endocrine defects with the *Sox9*-haploinsufficiency syndrome campomelic dysplasia (CD) implies additional later roles for *Sox9* in endocrine development. Using short-term lineage tracing in mice, we demonstrate here that SOX9 marks a pool of multipotential pancreatic progenitors throughout the window of major cell differentiation. During mid-pancreogenesis, both endocrine and exocrine cells simultaneously arise from the SOX9$^+$ epithelial cords. Our analysis of mice with 50%-reduced *Sox9* gene dosage in pancreatic progenitors reveals endocrine-specific defects phenocopying CD. By birth, these mice display a specific reduction in endocrine cell mass, while their exocrine compartment and total organ size is normal. The decrease in endocrine cells is caused by reduced generation of endocrine progenitors from the SOX9$^+$ epithelium. Conversely, formation of exocrine progenitors is insensitive to reduced *Sox9* gene dosage, thus explaining the normal organ size at birth. Our results show that not only is SOX9 required for the maintenance of early pancreatic progenitors, but also governs their adoption of an endocrine fate. Our findings therefore suggest that defective endocrine specification might underlie the pancreatic phenotype of individuals with CD.

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Introduction

During embryonic development, all differentiated cells in the vertebrate pancreas are generated from a common pool of multipotential pancreatic progenitor cells. Lineage-tracing studies in mice have shown that progenitors marked by the transcription factors PDX1 and PTF1a give rise to the acinar and ductal cells of the exocrine pancreas as well as to the endocrine lineages of the islets of Langerhans, including the insulin-producing β-cells, the glucagon-producing α-cells, the somatostatin-producing δ-cells, and the pancreatic polypeptide (PP)-producing cells (Gu et al., 2002; Kawaguchi et al., 2002). With the initiation of the secondary transition, which marks the onset of major endocrine and exocrine cell differentiation around embryonic day (e) 13.5, PDX1 and PTF1a become markers of mature pancreatic cell types. PDX1 expression becomes restricted to insulin$^+$ cells and newly-differentiated acinar cells (Guz et al., 1995), while PTF1a becomes compartmentalized within the acini (Krapp et al., 1996). Because neither PDX1 nor PTF1a are exclusive progenitor cell markers throughout the duration of pancreas development, it is still unclear whether multipotential progenitor cells persist in the pancreas throughout its development.

Endocrine cell differentiation is initiated by the transcription factor NGN3, which marks a transient population of specified endocrine progenitors throughout embryogenesis. Studies in mice have shown that *Ngn3* is both required and sufficient to induce endocrine differentiation (Gradwohl et al., 2000; Gu et al., 2002; Jensen et al., 2000; Schwitzgebel et al., 2000). While endocrine-committed progenitors can be found as early as e10.5, exocrine commitment does not occur until around e14. PTF1a- and carboxypeptidase A- (CPA-) expressing cells are initially multipotential, but later in development exclusively mark committed exocrine progenitors that reside in the tips of the branching pancreatic epithelium (Zhou et al., 2007).

We have recently shown that the HMG box transcription factor SOX9 is co-expressed with PDX1 in multipotential progenitors of the undifferentiated pancreatic epithelium between e9 and e12.5. However, in contrast to PDX1, SOX9 is excluded from lineage-committed progenitors and differentiated cells at the beginning of the secondary transition (Seymour et al., 2007). During this time, SOX9 becomes exclusively localized to the epithelial cords, which have been suggested to harbor uncommitted progenitor cells (Fujitani et al., 2006). Notably, in the epithelial cords, NGN3$^+$ cells exist in an intercalated arrangement amongst the SOX9$^+$ cells, suggesting that endocrine progenitors may arise from the SOX9$^+$ cell population. Postnatally, SOX9 expression becomes restricted to the ductal and centroacinar cell compartment (Seymour et al., 2007), which has been suggested to harbor endocrine-differentiation-competent progenitors

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(Bonner-Weir et al., 2000; Sharma et al., 1999; Xu et al., 2008). This raises the question of how long SOX9$^+$ cells normally contribute to cell neogenesis during pancreas development.

Through pancreas-specific *Pdx1-Cre*-mediated inactivation of *Sox9*, we demonstrated that SOX9 controls the maintenance of pancreatic progenitors by stimulating their proliferation, survival, and persistence in an undifferentiated state (Seymour et al., 2007). However, because the *Pdx1-Cre* transgene results in efficient early deletion of *Sox9* and severe pancreatic hypoplasia by e11.5, our analysis of SOX9-deficient pancreata precluded the dissection of possible additional roles of SOX9 during pancreatic cell differentiation at later stages of development. Several pancreatic transcription factors have been found to play distinct early and later roles in pancreogenesis. For example, PDX1 initially promotes outgrowth of the pancreatic anlagen (Jonsson et al., 1994), but subsequently plays a role in maintaining proper β-cell function (Ahlgren et al., 1998). Furthermore, *Pdx1*-haploinsufficiency is associated with increased islet apoptosis in adult mice (Johnson et al., 2003), suggesting that correct *Pdx1* gene dosage is required for endocrine cell maintenance. Likewise, *Sox9* is a known haploinsufficient gene: in humans, heterozygosity for loss-of-function mutations in *Sox9* is associated with the semi-lethal skeletal malformation syndrome campomelic dysplasia (CD) (Foster et al., 1994; Wagner et al., 1994). Neonates with CD have recently been shown to display pancreatic islet defects, including islet hypoplasia and decreased expression of hormones and β-cell maturity markers (Piper et al., 2002). Though the findings in individuals with CD strongly suggest a role for *Sox9* in islet cell development, the mechanism underlying islet hypoplasia as a result of *Sox9*-haploinsufficiency has yet to be identified.

In this study, we have examined whether the SOX9$^+$ epithelial cords serve as a reservoir of multipotential progenitors throughout pancreatic development and whether *Sox9* function is required to initiate cell differentiation from this epithelium. Utilizing *Sox9-eGFP* mice for short-term *in vivo* lineage tracing of SOX9$^+$ cells, we show that during mid-pancreogenesis the SOX9$^+$ epithelium simultaneously gives rise to NGN3$^+$ endocrine progenitors, the different endocrine cell types and exocrine acinar cells. Our analysis is the first to demonstrate that a compartment of multipotential progenitors persists after the secondary transition of pancreas development. To further test whether *Sox9* plays a role in pancreatic cell differentiation, we analyzed pancreatic development in mice in which *Pdx1-Cre* mediates deletion of a single *Sox9^{flox}* allele. Similar to individuals with CD, such *Sox9$^{+/\Delta pan}$* mice display a 50% reduction in islet cell mass, while their pancreatic exocrine compartment is unaffected. We show that the reduction in endocrine cells results from a defect in the generation of sufficient endocrine progenitors, thus revealing a dosage-sensitive requirement for SOX9 in initiating endocrine fate.

Materials and methods

Mouse strains

Sox9-eGFP (*Tg(Sox9-eGFP)209Gsat/Mmcd*) mice (Gong et al., 2003) were obtained from the MMRRC and were maintained on a CD1 genetic background. *Sox9$^{+/\Delta pan}$* embryos and Cre$^-$ littermate controls were generated by crossing heterozygous males of the *Pdx1-Cre* line (Gu et al., 2002) with homozygous females of the *Sox9-flox* line (Kist et al., 2002). The *Sox9-flox* line was maintained on a mixed FVB/N×C57Bl/6J genetic background. Mice were maintained on a 12 h light–dark cycle and all protocols were approved by the UC Irvine Institutional Animal Care and Use Committee. Embryos were harvested from timed matings in which noon of the day of vaginal plug appearance was considered as e0.5. For BrdU labeling, pregnant females were injected i.p. with 50 μg/g body weight of BrdU (Sigma) and embryos were harvested 1 h after injection.

Histological analysis

Staining was performed on sections of whole embryos (e12.5), dissected guts (e15.5), or isolated pancreata (e18.5). Length of fixation in 4% paraformaldehyde in PBS at 4 °C was determined empirically. For frozen sections, tissues were cryoprotected in 30% sucrose in PBS, embedded in O.C.T. (Sakura Finetek) and sections cut at 10 μm. For paraffin sections, tissue was dehydrated in an increasing ethanol series, cleared in xylene and embedded in Paraplast (Kendall): sections were cut at 7 μm. H and E staining and immunofluorescence analysis of proteins was performed as described previously (Sander et al., 1997). When required, antigen retrieval was performed in pH 6.0 citrate buffer followed by additional permeabilization in 0.15% Triton X-100 in PBS. For detection of BrdU, DNA was denatured with 2 M HCl at 37 °C for 1 h and a M.O.M. Kit (Vector Labs) was used. For triple staining, the M.O.M. Kit was used in conjunction with AMCA Avidin D (Vector Labs). DBA was stained for using biotinylated DBA (1:200) and visualized with Texas Red Avidin (Vector Labs). TUNEL staining was performed using the ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon). When necessary, nuclei were counterstained with DAPI (Sigma) at 0.1 μg/ml.

The following primary antibodies were used at the given dilutions: rabbit anti-SOX9 (Stolt et al., 2003), 1:2000; rabbit anti-SOX9 (Chemicon), 1:1000; guinea-pig anti-PDX1 (kindly provided by C. V. E. Wright, Vanderbilt University, Nashville, TN), 1:10,000; rabbit anti-PDX1, (kindly provided by H. Edlund, Umeå University, Sweden), 1:3000; rabbit anti-PTF1a (kindly provided by H. Edlund), 1:1000; guinea-pig anti-NGN3 (Henseleit et al., 2005), 1:1000; guinea-pig anti-NKX6.1 (Henseleit et al., 2005), 1:1000; rabbit anti-NKX6.1 (kindly provided by P. Serup, Hagedorn Research Institute, Gentofte, Denmark), 1:10,000; rabbit anti-MAFA (Bethyl Labs), 1:1500; guinea-pig anti-ISL1 (kindly provided by J. Ericson, Karolinska Institute, Stockholm, Sweden), 1:5000; rabbit anti-HB9 (kindly provided by J. H. Kehrl, NIH), 1:8000; rat anti-E-cadherin/uvomorulin (Sigma), 1:1000; guinea-pig anti-insulin (DAKO), 1:5000; mouse anti-insulin (Sigma), 1:5000; mouse anti-glucagon (Sigma), 1:5000; goat anti-ghrelin (Santa Cruz), 1:1000; mouse anti-somatostatin (P. Serup), 1:2000; rabbit anti-pancreatic polypeptide (DAKO), 1:2000; rabbit anti-amylase (Sigma), 1:500; rabbit anti-carboxypeptidase A (Biotrend), 1:1000; rabbit anti-GLUT2 (Alpha Diagnostics), 1:200; rabbit anti-PC1/3 (kindly provided by D. F. Steiner, University of Chicago, IL), 1:2000; rabbit anti-IAPP/amylin (Peninsula), 1:2000; mouse anti-GAPDH (Ambion), 1:100,000; and mouse anti-BrdU (Chemicon), 1:50.

For immunofluorescence detection, the following goat-raised secondary antibodies were used (all at 1:2000 dilution): Cy3-conjugated anti-rabbit, anti-mouse, anti-guinea-pig or anti-goat, Cy5-conjugated anti-rabbit or anti-mouse (all Jackson Labs, raised in donkey); Alexa- (488 nm) conjugated anti-rabbit, anti-mouse, or anti-guinea-pig (all Molecular Probes) and Alexa- (555 nm) conjugated anti-mouse (Molecular Probes).

Specimens were viewed on a Zeiss Axioplan 2 microscope and 24-bit TIFF images were acquired with a Zeiss AxioCam digital camera driven by Zeiss AxioVision v. 3.1 software. Co-localization studies for GFP, SOX9 and NGN3 in *Sox9-eGFP* mice were performed with a Zeiss LSM510META laser scanning confocal microscope. Images were processed with Adobe Photoshop 6.0. Cell counting and/or morphometry was performed on every fifth section through the early embryonic pancreas from a minimum of three mutants and three wild-type somite-matched littermates. Morphometry was conducted using Image-Pro Plus v. 5.0.1 (Media Cybernetics). Relative pancreatic areas of β-cells, α-cells and acini were calculated and cell mass determined as described previously (Garofano et al., 1998).